EXPRESSION OF $GABA_A$ RECEPTOR SUBUNIT GENES IN THE AVIAN SONG SYSTEM AND THEIR ROLE IN LEARNING AND MEMORY

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ABSTRACT

γ-aminobutyric acid type A ($GABA_A$) receptors are the primary mediators of inhibitory neurotransmission in the brain. In avian systems, 14 $GABA_A$ receptor subunits (α1-6; β2-β4; γ1, 2 and 4, δ and π) have been identified. These assemble into pentameric transmembrane structures with an intrinsic chloride-selective pore and are involved in the modulation of learning and memory. Following imprinting training in the one-day old chicken, mRNA encoding the $GABA_A$ receptor γ4 subunit is significantly reduced in learning-relevant brain regions indicating a role for receptors comprising this subunit in learning and memory. The zebra finch (*Taenopygia guttata*) song system has long since been used as a paradigm for studying the underlying molecular mechanisms of learning and memory due to the discrete nature of song, the song system and established stages in song development. The avian brain displays many comparable structures and pathways to mammalian systems and there are striking parallels between birdsong and speech production in humans hence the fundamental neuronal mechanisms are similar. Despite major developments towards understanding the anatomical and electrophysiological properties of various song-system nuclei, the nature of the underlying molecular and biochemical/genetic architecture remains largely unknown. Electrophysiological and pharmacological techniques have localised $GABA_A$ receptors in the song system and more recently the spatial distribution of γ4-subunit mRNA has been mapped, producing striking results. This had inspired this study (the first of its kind in zebra finch) to isolate all complementary DNAs for zebra finch α1-6; β2-β4; γ1, γ2, γ4, δ and π subunits and characterise their expression in this learning and memory paradigm. Radioactive *in situ* hybridisation was employed to quantitatively map all $GABA_A$ receptor subunits within four important nuclei of the song system (LMAN, Area X, HVC and RA) and revealed region-specific transcription of genes; each subunit exhibiting an individual expression profile in brain, indicative of a variety of major and minor subtype assemblies. Real-time RT-PCR confirmed developmentally-associated subunit mRNA levels in all song-system nuclei. γ4-subunit mRNA exhibited the most robust expression and coordinated peaks of mRNA expression at specific developmental time points in the relevant nuclei, subsequently expression of the γ4-subunit mRNA was examined in a behavioural context. Corresponding mRNA was down-regulated in response to acquisition and production of song (not evident with γ2-subunit mRNA), indicative of a specialised role for these receptors in cognitive processes. Preliminary *in vitro* RNA interference experiments indicated that γ4-subunit gene expression could be reduced in chicken neurons, paving the way for *in vivo* gene silencing experiments in zebra finch.

-from the Edwin Smith Surgical Papyrus, (Egypt, 1700 BC), the oldest written record of the word “brain”

‘*After the first glass of you see things as you wish they were. After the second you see them as they are not. Finally, you see things as they really are, and that is the most horrible thing in the world*’ - Oscar Wilde on Absinthe (a modulator of $GABA_A$ receptors)

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ABBREVIATIONS AND SYMBOLS

α	Alpha
$\alpha^{35}S$	Deoxyadenosine 5'(alpha-thio) triphosphate
AA	Amino acid
AFP	Anterior forebrain pathway
ATP	Adenosine triphosphate
BLAST	Basic local alignment search tool
BZ	Benzodiazepine
bp	Base pair
β	Beta
CACA	*Cis*-4-aminocrotonic acid
cDNA	Complementary deoxyribonucleic acid
CNS	Central nervous system
δ	Delta
DNA	Deoxyribonucleic acid
dH_2O	Deionised water
DEPC	Diethylpyrocarbonate
DTT	Dithiothreitol
DLM	Medial nucleus of the dorsolateral thalamus
EDTA	Ethylenediaminetetraacetic acid
ε	Epsilon
γ	Gamma
g	Acceleration due to gravity
GABA	Gamma (γ)-aminobutyric acid
GABAR	Gamma (γ)-aminobutyric acid receptor
GAD	Glutamic acid decarboxylase
HA	Apical part of the hyperpallium
HD	Dorsal part of the hyperpallium
HVC	Formal name
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IEG	Immediate early gene
Kb	Kilobase
LGIC	Ligand-gated ion channel
LMAN	Lateral magnocellular nucleus of the anterior nidopallium
l	Litre
M	Molar
MBP	Maltose binding protein
m	Milli
μ	Micro
min	Minute
mRNA	Messenger ribonucleic acid
Mst	Medial striatum
ng	Nanogram
nm	Nanometer
NCBI	National centre for biotechnology information
NIf	Interfacial nucleus of the nidopallium
NXIIts	Tracheosyringeal part of the hypoglossal nucleus
PHD	Post hatch day
qPCR	Quantitative PCR
π	Pi
®	Registered trademark
RA	Robust nucleus of the arcopallium
ρ	Rho
RNA	Ribonucleic acid
RNAi	Ribonucleic acid interference
RPM	Revolutions per minute
rTdT	Terminal deoxynucleotidyl transferase, recombinant
RT-PCR	Reverse transcription polymerase chain reaction
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl suphate-polyacrylamide gel electrophoresis
shRNA	Short hairpin ribonucleic acid
siRNA	Short interfering ribonucleic acid
SSC	Standard sodium citrate
TEMED	*N*,*N*.*N*',*N*'-tetramethylethylenediamine
θ	Theta
™	Trademark
Tm	Melting temperature
TM	Transmembrane domain
UV	Ultraviolet
VMP	Vocal motor pathway
V/V	Volume per volume
W/V	Weight per volume
XGAL	5-bromo-4-chloro-3-indolyl-β-D-galactoside

1. GENERAL INTRODUCTION

1.1 γ-aminobutyric acid (GABA)

Successful functioning of a neuronal network relies upon efficient signaling between individual neurons. The central nervous system (CNS) is composed of a hugely intricate network of neurons whereby sensory information is interpreted, analysed, stored and responded to. Predominantly, chemical neurotransmitters carry nerve impulses across the majority of synapses (Eccles, 1982). In the vertebrate brain the two most prevalent neurotransmitters are the amino acids, L-glutamate and γ-aminobutyric acid (GABA); which upon binding to corresponding post-synaptic receptors elicit excitatory and inhibitory (except in the developing nervous system) responses respectively. This balance between excitatory and inhibitory neurotransmission results in a finely tuned neuronal network (Hablitz *et al*., 2009). Unlike the excitatory neurotransmitter glutamate, GABA is a non-protein amino acid; it is rarely used as a component of proteins, but is functional in its own right (Li and Xu, 2008). GABA was first reported in the brain in 1950 during a study of free amino acids (Roberts and Frankel, 1950). Further research concluded GABA to act at synapses eliciting an inhibitory effect (for a review see Brioni, 1993) and it is now known to be the most widely distributed inhibitory neurotransmitter in the CNS (Li and Xu, 2008). Due to localisation of GABAergic interneurons in hypothalamic and brain stem regions, GABA (via its receptors), also acts in other capacities. It is involved in regulation/modulation of blood pressure (Hayakawa *et al*., 2002), hormone release (Zemkova *et al*., 2008), respiration (Fujii *et al*., 2007), circadian rhythms (Liu and Reppert, 2000; Mignot *et al*., 2002), food/drink intake (Turenius *et al*., 2009), locomotion (Ménard *et al*., 2007), anxiety (Rudolph and Möhler, 2006) and learning and memory (Chapouthier and Venault, 2002; 2004; Kalueff, 2007; McNally *et al*., 2008). However, its predominant effects are within the brain, mediating inhibitory neurotransmission.

Biosynthesis of GABA occurs in GABAergic interneurons where it is packaged into synaptosomes in the nerve terminal ready for release (Hablitz *et al*., 2009). Rapid α-decarboxylation of L-glutamic acid by GAD (glutamic acid decarboxylase), with coenzyme pyridoxal phosphate (a form of vitamin B6) produces GABA. There are two isoforms of

GAD in vertebrate brain; GAD_{65} (65 kDa protein) and GAD_{67} (67 kDa protein). The larger form is found in its active state alongside cofactor pyridoxal phosphate distributed throughout the neuron, and is responsible for basal GABA levels. The smaller form is localised to axon terminals, where only a heightened demand for GABA will cause a transient activation (Kaufman *et al*., 1991). Breakdown of GABA is catalysed by a mitochondrial aminotransferase, GABA-transaminase (GABA-T). Along with α-ketoglutarate, reversible transanimation of GABA produces succinic semialdehyde and glutamic acid. Both GAD and GABA-T are found throughout inhibitory neurons; GAD is more highly concentrated at the pre-synaptic terminal and GABA-T is confined to the mitochondria. It is GAD that governs the steady state of endogenous GABA rather than GABA-T (Buddhala *et al*., 2009).

1.2 $GABA_A$ receptors

GABA is the endogenous agonist of GABA receptors which have been identified based on molecular, electrophysiological and receptor binding studies. There are three recognised types: $GABA_A$, $GABA_B$ and $GABA_C$. $GABA_A$ and $GABA_C$ receptors are ionotropic and possess intrinsic chloride-selective channels. $GABA_B$ receptors are metabotropic heterodimers that are coupled to G-proteins, through which they trigger second messenger pathways (Chebib and Johnston, 1999; Bormann, 2000). $GABA_A$ and $GABA_B$ receptors are located in the CNS, whereas the majority of $GABA_C$ receptors are confined to the retina (Albrecht and Darlison, 1995; McCall *et al*., 2002; Rozzo *et al*., 2002). Although mounting evidence has revealed some $GABA_C$ receptor expression in the rat brain; it is only at low levels (discussed further in section 1.3. Boue-Grabot *et al*., 1998; Ogurusu *et al*., 1999; Rozzo *et al*., 2002; Milligan *et al*., 2004; Alakuijala *et al*., 2005). Within the CNS, $GABA_A$ receptors far outnumber $GABA_{B/C}$ receptors in their distribution and so are universally regarded as the major mediators of fast inhibitory synaptic transmission (Bormann, 2000).

1.2.1 $GABA_A$ receptor-mediated neurotransmission

When a pre-synaptic GABAergic interneuron is activated, the ensuing action potential travels along the neuron until it reaches the nerve terminal, where GABA is stored in membrane-bound vesicles. Depolarisation of the nerve-terminal plasma membrane

transiently opens voltage-gated Ca^{2+} channels which causes an influx of Ca^{2+} into the terminal and increases cytosolic [Ca^{2+}]. This results in calcium-dependant exocytosis of GABA which rapidly diffuses across the synaptic cleft (< 1 ms). Two molecules of GABA simultaneously bind to post-synaptically located $GABA_A$ receptors, which become activated and undergo a conformational change, opening the Cl^- -specific pore. A Cl^- influx follows down the electrochemical gradient (~10^6 ions/sec), into the post-synaptic neuron, hyperpolarising the membrane. IPSP (inhibitory post-synaptic potential) is induced and the influx of Ca^{2+} becomes inhibited, resulting in a decrease neuronal excitability. Excess GABA in the synaptic cleft is taken up into the pre-synaptic neuron and surrounding glial cells by high affinity neuronal uptake systems.

In addition to the rapid (phasic) inhibitory action of $GABA_A$ receptors, they can also mediate a persistent tonic current, via receptors located extrasynaptically and perisynaptically (Semyanov *et al*., 2004). Tonic inhibition is a result of micromolar GABA levels which persist extracellularly and are contributed to by 'overspill' following fast phasic inhibitory transmission. Extrasynaptic receptors are constantly activated and exhibit a high GABA affinity (Mody and Pierce, 2004; Farrant and Nusser, 2005). More recent work has elucidated that the subunit composition of extrasynaptic $GABA_A$ receptors is also different to those located at the synapse (refer to section 1.2.4; Glykys *et al*., 2008).

1.2.2 $GABA_A$ receptor function during development

As previously outlined, excitatory neurotransmitters (e.g. glutamate) induce Ca^{2+} influx which increases the excitability of the post-synaptic neuron; inhibitory neurotransmitters (e.g. GABA) reduce Ca^{2+} influx into the neuron, thereby decreasing neuronal excitability. However, in early development of the mammalian CNS (in restricted brain regions), release of GABA *increases* the Ca^{2+} concentration in the post-synaptic neuron, eliciting an excitatory effect (Lin *et al*., 1994; Ganguly *et al*., 2001). During CNS development, $GABA_A$ receptors appear first (before glutamate receptors) and are widely distributed. Accordingly, they mediate the majority of signal transduction (Li and Xu, 2008), by acting in an excitatory capacity. The paradoxical excitatory action mediated by $GABA_A$ receptors early in development can be attributed to a high intracellular Cl^- concentration (Stein,

2004). It is purported that this temporal GABAergic excitatory signal transduction is assuming the role of the relatively undeveloped excitatory inputs, in order to provoke increased intracellular Ca^{2+} and stimulate neuronal growth (Laurie *et al*., 1992b; Li and Xu, 2008). When the $GABA_A$ receptors are activated, the central ion channel opens and Cl^- effluxes resulting in depolarization of the post-synaptic membrane. As development of the CNS ensues, intracellular Cl^- concentration progressively decreases until it is lower than that of the extracellular matrix and at this point 'GABA switching' terminates (Herlenius, 2004). Intracellular Cl^- concentration is primarily governed by cation-cotransporters: KCC1-4 (K^+-dependent Cl^- cotransporters); NCC (Na^+-dependent Cl^- cotransporters and NKCC1 and 2 (K^+- and Na^+-dependent Cl^- cotransporters). *In situ* hybridisation experiments have mapped mRNA expression of the two primary cotransporters found in the mammalian CNS, NKCC1 (Cl^- into cell) and KCC2 (Cl^- out of cell) (Kahle *et al*., 2005; Giménez, 2006). NKCC1 mRNA levels are high in the embryonic and early developing brain where KCC2 transcript levels are low; however, <1 week postnatal the mRNA levels for NKCC1 were decreased accompanied by an increase in the distribution of mRNA encoding of KCC2 (Clayton *et al*., 1998; Li *et al*., 2002; Yamada *et al*., 2004).

1.2.3 $GABA_A$ receptor structure

$GABA_A$ receptors are members of the Cys-loop pentameric ligand-gated ion channel (LGIC) superfamily. A group named according to the characteristic 15 AA conserved motif in the extracellular N-terminus with two terminal cysteines which form a disulphide bridge (Simon *et al*., 2004). Other members include, the nicotininc acetylcholine receptors (nAChRs), glycine receptors, 5-hydroxytryptamine type 3 ($5\text{-}HT_3$) receptors and more recently a Zn^{2+}-activated ion channel (Davies *et al*., 2003). These channel proteins form tiny hydrophilic pores in the membrane through which solutes can pass through by diffusion. They are selective and gated, the selectivity of the channel depends upon its diameter, shape and the distribution of charged amino acids in its lining (Jensen *et al*., 2002; Karlin, 2002). nAChRs, 5-HT3Rs, and Zn^{2+}-activated channels are selective for cations (thereby eliciting an excitatory response); $GABA_A$ and Glycine receptors are selective for anions, eliciting an inhibitory response. In keeping with the characteristics of the other group members, the $GABA_A$ receptor is composed of five membrane-spanning

subunits arranged around a central pore (Fig. 1.1b) into a heteropentameric glycoprotein of ~275 kDa. The absolute structure of LGICs cannot be defined due to limitations of the various techniques. For example, crystal structures may be contorted due to the process of purification and crystallisation, and the loss of information regarding interactions with other proteins and the lipid environment (Bogdanov *et al*., 2005). To date, no X-ray structure or nuclear magnetic resonance (NMR) data is available for LGICs in mammals, however recent studies have resolved the structure of bacterial LGIC homologues (of which there are more than 20: Hilf and Dutzler, 2008, Bocquet *et al*., 2009 . The determination of the crystal structure of acetylcholine–binding protein (AChBP) from *Lymnea stagnalis* has provided some comprehension pertaining to the structure of the extracellular domain of ACh receptor subunits and subsequently serves as a template for other LGICs (Brejc *et al*., 2001). *In silico* analysis has most often been employed for the determination of protein structure, modeled on the hydrophobic nature of the constituent amino acids, though these present only 60-70% accuracy in predicting protein topology (Taylor *et al*., 2003). Insights into TM segment orientation and structure of the pore have been further eluded by using site-directed mutagenesis and substituted-cysteine-accessibility-methods (SCAM; Karlin and Akabas, 1998; Bogdanov *et al*., 2005). In brief, protein analysis in this manner requires the removal of all native cysteine residues and subsequent systematic mutation of individual residues to cysteine where the thiol side-chains react with a range of charged and polar MTS (methanethiosulfonate) reagents developed by Karlin and colleagues (1998). By characterisation of the reaction and accessibility (i.e. buried or exposed) of the substituted cysteine with the thiol-specific reagents (which can be either membrane-permeable, or impermeable dependent on whether residues to be labelled are buried within the protein, exposed to the lipid bilayer or exposed to water within the channel), features such as channel-lining residues, secondary structure, physical size, electrostatic potential, location of selectivity filters, gates, binding sites of channel blockers can be identified (Karlin and Akabas, 1998).

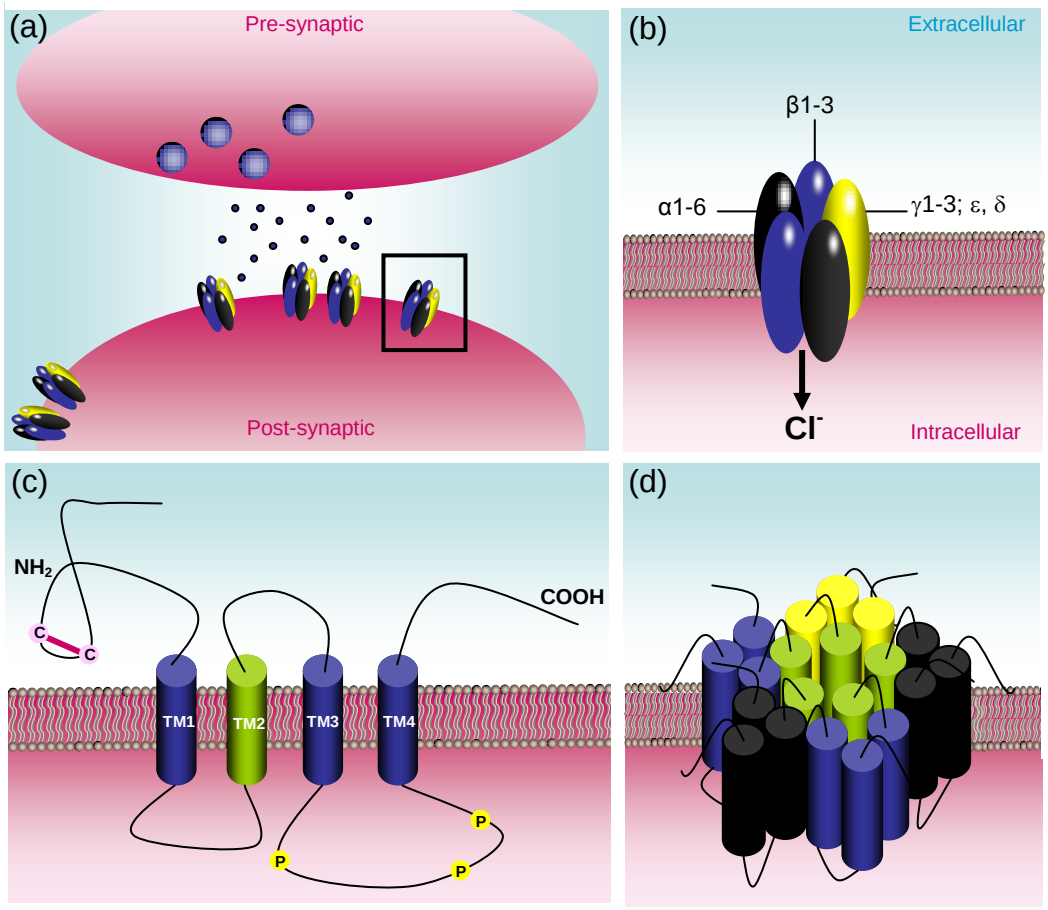


Figure 1.1 Schematic representation of the toplogy of the $GABA_A$ receptor. (a) The $GABA_A$ receptor is a heteromeric transmembrane structure, the five subunits most commonly consist of 2 α's, 2 β's and a single γ subunit (Pirker *et al*., 2000; Whiting, 2003a) with the stoichiometry α-β-α-β-γ (Baumann *et al*., 2002) assembled around a chloride pore. (b) $GABA_A$ receptor subunits are individual polypeptides of ~450 amino acids (AA) in length (50-60 kDa). They comprise a short signal peptide (7 AA) and a long amino-terminal (NH_2) extracellular domain (220 to 250 AA) which contains sites for glycosylation and the well conserved characteristic 15 AA di-cysteine loop (depicted in pink; Jones and Sattelle, 2008). There are four highly conserved hydrophobic transmembrane α-helices (TM1-4, ~20 AA each); it is TM2 (highlighted in green) which forms the lining of the central chloride anion pore in conjunction with TM1 (Sine and Engel, 2006). The third and fourth transmembrane domains are linked by a large variable intracellular loop which contains numerous consensus sites for phosphorylation (highlighted in yellow) and an α-helical domain within this loop which is thought to influence ion conduction (Peters *et al*., 2005), followed by a short extracellular carboxyl-terminal (COOH). All the subunits share a similar tertiary structure and are of a similar size (30 Å x 40 Å x 160 Å). (d) They assemble into pentamers with their long axes perpendicular to the lipid bilayer (Unwin, 2005).

A combination of the above described methods (alongside cryo-electron microscopy and site-directed mutagenesis studies) has resulted in resolution of the structure of the central ion-conducting channel of $GABA_A$ receptors. It is composed primarily of five α-helical TM2 regions which are slightly 'bent' at the centre with TM1, TM3 and TM4 separating the TM2 domains from the lipid bilayer with a surrounding ring of α-helices (Unwin, 2005). The TM2 domains form a funnel structure which narrows towards the intracellular side (Karlin, 2002). A selectivity filter (selecting ion charge and size) is located nearer to the intracellular end (Xu and Akabas, 1996; Karlin, 2002). Selectivity can be attributed to individual pore-lining residues; mutation of five amino acids of the $GABA_A$ receptor β3 subunit TM2 domain resulted in a cation-selective $GABA_A$ receptor. Interestingly, no such effect was observed for the α1 and γ2 subunits indicating the importance of β subunits in influencing ion-selectivity (Jensen *et al*., 2002). When the channel is closed or in a resting state, there is a gate which occludes ion passage; this gate is opened upon agonist binding to the extracellular ligand-binding domain (in N-terminus), allowing solutes to pass into the cell (Akabas, 2004). With prolonged exposure to agonist, the receptor becomes desensitised and conduction of ions is suspended. There is some considerable debate as to the location of the gate within TM2 or the TM1-TM2 intracellular loop (Peters *et al*., 2005; Bali and Akabas, 2007) and whether the gating method is the same for closed and desensitised receptors (Purohit and Grosman, 2006).

1.2.4 $GABA_A$ receptor subunits

Complexity within the $GABA_A$ receptor system can be attributed to a vast combination of available subunits which co-assemble into heteropentameric structures. In the mammalian CNS, 16 individual $GABA_A$ receptor subunit polypeptides have been identified (α1-6, β1-3, γ1-3, δ, ε, θ and π) each encoded for by a separate gene. Detailed analysis of the human genome database has revealed no further subunit sequences exist (Simon *et al*., 2004). Subunits are classified exclusively upon their sequence homology with other members of a group. Subunits within the same class share ~70% sequence identity, whereas with subunits from different classes share only ~30% sequence identity (Darlison *et al*., 2005). Despite the plethora of subunits, they all share a highly conserved structure (Fig. 1.1c).

In lower vertebrates and avian species, there are notable differences in the subunit repertoire. Most notably, two additional subunit polypeptides, termed β4 and γ4 (Lasham *et al*., 1991; Harvey *et al*., 1993). Coupled with the absence of θ and ε subunits in these animals, it has been subsequently concluded that the β4 and γ4 subunits are the lower vertebrate orthologues of θ and ε respectively, with the ε subunit sharing 50% sequence identity with the γ4 polypeptide (Harvey *et al*., 1998) and β4 and θ sharing 56% sequence similarity (Bonnert *et al*., 1999), suggesting evolution from a common ancestor. This degree of identity is slightly higher than between subunit classes but lower than between isoforms of the same class (Darlison *et al*., 2005). θ- and ε-subunit genes are located in cluster with α3 on the human X chromosome (Simon *et al*., 2004), occupying the same positions and transcriptional orientation as the β4α3γ4 subunit gene cluster on chromosome 4A of the zebra finch genome (Fig. 3.8 Chapter 3). *In silico* analysis of the human genome has revealed no sequences encoding β4 and γ4 subunits (Simon *et al*., 2004). γ4-subunit encoding mRNA had been detected in chicken (*Gallus gallus domesticus*), zebra finch (*Taenopygia guttata*), canary (*Serinus canaria*) and red-eared slider turtle (*Trachemys scripta elegans*), and the sequences were highly similar (C. Thode and M. G. Darlison, unpublished results). High sequence similarity was also observed for the β4 subunit transcript, which was detected chicken, zebra finch, clawed frog (*Xenopus borealis*), lizard (*Podarcis muralis*) and fish (species unspecified). Such high sequence homology points to conservation of physiological function (I. Pahal, C. Thode, and M. G. Darlison, unpublished results). Data suggests that the mammalian θ subunit is unable to replace the β-subunit polypeptide in functional receptors (Bonnert *et al*., 1999); however the ε subunit may substitute for a γ subunit. This confers different electrophysiological and pharmacological properties for the receptor, most notably these receptors are insensitive to benzodiazepines and open spontaneously (Davies *et al*., 1997; Whiting *et al*., 1997a). Some more recent evidence suggests the ε subunit may be able to substitute a β subunit (particularly β3). In this case, potentially two ε-subunits can assemble into a single receptor complex (Jones and Henderson, 2007) agreeing with previous data (Thompson *et al*., 2002), although this is contradicted by data suggesting an ε can replace an α or β subunit but only one ε subunit exists per complex (Bollan *et al*., 2008).

The heterogeneity of the $GABA_A$ receptor family is furthered by the existence of splice variants for many of the subunits (α2, α4, α5, α6, β2, β3, β4, γ2, γ3 and ε), usually resulting in a short and long form, further details and references in Chapter 3, section 3.1.3.

1.2.5 $GABA_A$ receptor subtypes

Despite a multitude of potential $GABA_A$ receptor subtypes (some 360,000 in mammals, although a maximum of 800 subtypes was postulated by Barnard *et al*. (1998)), preferred assemblies of subunit polypeptides are observed, with the majority of receptors comprising 2α's 2β's and a single γ, δ or ε subunit (Pirker *et al*., 2000; Sieghart and Sperk, 2002; Whiting, 2003a), typically with the stoichiometry α-β-α-β-γ (Baumann *et al*., 2002). Distinct $GABA_A$ receptor subtypes exist in different neuronal populations. Furthermore, individual neurons can express several different subtypes (for more details refer to Chapters 4 and 6). Techniques including: reverse-transcription polymerase-chain reaction (RT-PCR), real-time RT-PCR, *in situ* hybridisation, immunohistochemistry, immunocytochemistry, co-precipitation and recombinant expression systems assaying pharmacological and electrophysiological characteristics have all been applied to answering the elusive question of native $GABA_A$ receptor stoichiometry (for review see Sieghart and Sperk, 2002; Olsen and Sieghart, 2008). In fact, despite the vast volumes of experimental work completed on the $GABA_A$ receptors, due to the stringent criteria for confirming the stoichiometry of native $GABA_A$ receptors (described by Olsen and Sieghart, 2008), and the promiscuous nature of the subunits, to date only 11 receptor subtypes are formally classified as native $GABA_A$ receptor subtypes (Table 1).

Category A	Category B	Category C
Confirmed native $GABA_A$ receptor subtypes; *all criteria met*	High probability as native $GABA_A$ receptor subtypes; *most criteria met*	Tentative as $GABA_A$ receptor subtypes; *some criteria met*
α1β2γ2*	α1β3γ2	ρ1
α2βγ2	α1βδ	ρ2
α3βγ2	α5β3γ2	ρ3
α4βγ2	αβ1γ	αβγ1
α4β2δ	αβ1δ	αβγ3
α4β3δ	αβ	αβθ
α5βγ2	α1α6βγ	αβε
α6βγ2	α1α6βδ	αβπ
α6β2δ		αxαyβγ2
α6β3δ		
ρ		

Table 1. Classification of native mammalian $GABA_A$ receptor subtypes adhering to stringent experimental criteria as described by Olsen and Sieghart (2008), adapted from Olsen and Sieghart (2009). There are a total of 28 $GABA_A$ receptor subtypes of which only 11 are confirmed (category A). Text highlighted in red designates an unspecified subunit. (*) The most abundant $GABA_A$ receptor subtype in the mammalian CNS (Whiting, 2003a).

1.2.6 Clinical importance of $GABA_A$ receptors

$GABA_A$ receptors are of significant clinical importance due to their contribution to the etiology of a number of neurological and psychological diseases. These include anxiety disorders (Rudolph and Mohler, 2004), Epilepsy (Benarroch, 2007), Huntington's disease (Thompson-Vest *et al*., 2003), Fragile X syndrome (D'Hulst and Kooy, 2007), Angelman syndrome (DeLorey and Olsen, 1999), Autism (Fatemi *et al*., 2009), alcoholism (Krystal *et al*., 2006), stroke (Costa *et al*., 2004) and Alzheimer's disease (Marcade *et al*., 2008). In addition they are the site of action for a number of clinically important exogenous and endogenous compounds which are able to interfere with the delicate physiological balance of the nervous system (Reynolds, 2008). Substances can mimic the natural ligand (GABA) and bind in its place (at the αβ interface), bind to the receptor at some other modulatory site where they can elicit inhibition or over-stimulation of the natural activity, or bind to the ion channel. To this end, the pharmaceutical industry puts much effort into the development of substances that can exert a precisely defined effect by specific binding. $GABA_A$ receptors

are of clinical interest due to their sensitivity to benzodiazepines (BZs), barbiturates, volatile anaesthetics, neurosteroids and ethanol (Reynolds, 2008). Potentiation of $GABA_A$ receptors produces inhibition of neuronal firing, dependant on the compound and the dose, the result of inhibition can range from anxiolysis to complete sedation of a patient. Attenuation of GABAergic transmission elicits anxiety, insomnia, arousal, restlessness, exaggerated reactivity and seizures (Sieghart, 1995). The stoichiometry of the receptor which determines the precise pharmacological profile. The primary problem is that many $GABA_A$ receptor-targeting compounds were developed before the stoichiometry and varying pharmacological properties of the receptors were elucidated, leading to many unwanted side effects (Nemeroff, 2003) such as dependence, cognitive impairments, unwanted sedation, tolerance and ataxia (loss of muscle coordination; Reynolds, 2008). A further caveat is the widespread distribution of $GABA_A$ receptors in the CNS, particularly the brain, which results in generalised effects of non-selective agonists and antagonists on CNS function. Complicated further by the high degree of similarity between subunits (Wingrove *et al*., 2002), and the fact that any subunit can be present in more than one subtype combination (refer to Table 1). However, with increasing understanding of native $GABA_A$ receptor stoichiometry and localisation of individual subtypes within the brain, the heterogeneity and wide-spread expression can become advantageous in subtype-specific drug design.

Ligands of the GABA site include the agonist muscimol and the antagonists bicuculline and gabazine; $GABA_A$ receptors are un-competitively blocked by picrotoxin. Benzodiazepines (BZs) e.g. diazepam (Vallium) and alprazolam (Xanax) are amongst the most commonly prescribed classes of drugs, producing anxiolytic, anticonvulsant, sedative, myorelaxant and antipsychotic effects by allosterically modulating GABA-activated currents by binding at an extracellular surface at the α/γ subunit interface (Smith and Olsen, 1995). Therefore sensitivity of the receptor to BZs is governed by these subunits (Pritchett *et al*., 1989; Günther *et al*., 1995). Conventional BZs are primarily active at receptors comprising $\alpha1$, $\alpha2$, $\alpha3$ or $\alpha5$ subunits in partnership with a $\gamma2$ subunit (Pritchett *et al*., 1989; Sieghart, 1995). They are unable to modulate receptors containing $\alpha4$ and $\alpha6$ subunits, and demonstrate low affinity for receptors with $\gamma1/\gamma3$, or α/β pentamers (Sieghart, 1995). In

contrast, $GABA_A$ receptors comprising the α4 and α6 subunits are highly sensitive to modulation by imidazobenzodiazepines. For example Ro15-4513, flumazenil and bretazenil which posses an imidazo ring and lack the 5-phenyl substituent common to classic BZs (Korpi *et al*., 2002), and thus bind slightly differently to BZs, but within the same binding pocket (Kucken *et al*., 2003).

Different BZs also demonstrate variable affinities for $GABA_A$ receptors dependant upon the subtype (Atack, 2003). Subtype-specific receptor activity by BZs is further complicated by subtype-specific effects, for example, diazepam (Vallium), which is a common drug prescribed primarily for anxiety, insomnia, muscle spasm, seizures (although not long term) and symptoms of acute alcohol withdrawal. A single residue mutation (Arg → His) within the α-subunit sequence can render the receptor diazepam-insensitive (Wieland *et al*., 1992; Kleingoor *et a*l., 1993; Benson *et al*., 1998). Sedative, anticonvulsant and amnesic effects of diazepam are mediated by receptors containing the α1 subunit (Rudolph *et al*., 1999), whereas anxiolytic effects are mediated by α2-subunit-containing receptors (Löw *et al*., 2000) and myorelaxant effects are mediated by α2-, α3- and/or α5-subunit containing receptors (Rudolph *et al*., 1999).

The BZ site also binds a large selection of non-BZ compounds, such as the hypnotic compound zolpidem (an imidazopyridine), which has a high affinity for α1-subunit-containing receptors, low affinity for α2- and α3-subunit-containing $GABA_A$ receptors and virtually no sensitivity at receptors assembled with the α5 subunit (Pritchett and Seeburg, 1990). As with classical BZs, zolpidem sensitivity is only exhibited when a γ2 subunit is present, regardless of α subunits (Sanna *et al*, 2002). Although they share the same binding pocket, different residues are involved in BZ and zolpidem binding within the γ2 subunit (Sancar *et al*., 2007), thus there is differentiation between two types of ligands.

It is important to elucidate the structural determinants of ligand binding in order to develop more pharmacologically (and thus behaviourally) sensitive ligands. Anaesthetics induce reversible unconsciousness at low concentrations. They are structurally diverse and include volatile agents such as isoflurane and high dose ethanol, and intravenous compounds such

as neuroactive steroids, etomidate, propofol and barbiturates, all of which allosterically bind and enhance $GABA_A$ receptor currents (there are different binding sites, not all of which have been resolved; for a review see Franks, 2006). Barbiturates such as pentobarbital, phenobarbital and thiopental are positive allosteric modulators of $GABA_A$ receptors. At high concentrations they can directly activate the receptor and at extremely high concentrations they can completely block Cl^- conductance (Jackson *et al*., 1982). Such compounds bind at a site located ~50 Å below the GABA binding site (also a similar site to general anaesthetics; Mercado and Czajkowski, 2008). They potentiate $GABA_A$ receptor-mediated inhibition by inducing conformational changes in the GABA binding site, increasing the receptors affinity for GABA and stabilising an open state structure; subsequently increasing the duration of channel opening, eliciting sedative, hypnotic, anaesthetic and anticonvulsant effects (Olsen, 1982; Mercado and Czajkowski, 2008). However, the prolonged use of barbiturates results in physical dependency and so BZs are generally preferred (Reynolds, 2008). Ethanol has long been known to induce sedative effects via $GABA_A$ receptors (Wafford *et al*., 1991), but the precise mode of action and particular subtypes involved was less clear. More recently it has been resolved that extrasynaptic δ-subunit-containing receptors (in combination with β3 and α1, α4 or α6 subunits), display greatest sensitivity to ethanol at low concentrations (10 mM). Only at higher concentrations (100 mM), do synaptic γ2-subunit-containing receptors exhibit sensitivity (reviewed by Olsen, 2007; Santhakumar *et al*., 2007).

1.2.7 Endogenous modulators of $GABA_A$ receptors

There are several endogenous modulators of $GABA_A$ receptors including protein kinases, Zn^{2+} and neurosteroids. Zn^{2+} ions serve to allosterically inhibit $GABA_A$ receptor function. Three individual binding sites have been identified; the first within the lining of the ion channel and the other two at the α/β interfaces (Hosie *et al*., 2003). Interestingly, $GABA_A$ receptors can also be modulated by protons and these appear to bind at the same position as Zn^{2+} ions (H267 on the TM2 channel lining of β subunits). In contrast to Zn^{2+}, H^+ potentiates $GABA_A$ receptor function. It is postulated that these paradoxical effects are attributed to the different way in which cations interact with imidazole side chains of the histidine residue (Wilkins *et al*., 2002). Redox reagents such as glutathione and ascorbic

acid can modulate $GABA_A$ receptor function; reducing agents potentiate GABA-evoked currents and oxidising agents inhibit these currents (Amato *et al*., 1999; Pan *et al*., 2000). There is some speculation as to whether or not they act at the disulphide bridged region within the N-terminus, as homomeric ρ1 $GABA_C$ receptors (which have this Cys-loop) are not modulated by redox reagents, although there is new evidence to the contrary (Calero and Calvo, 2008). Instead, a cysteine residue within TM3 of β subunits was proposed as a potential binding site (Pan *et al*., 2000; Wilkins and Smart, 2002). In part, the potentiation of GABA-evoked currents by reducing agents at a low concentration is likely to be accredited to chelation of inhibitory Zn^{2+} from H267 (Wilkins and Smart, 2002). The most important endogenous $GABA_A$ receptor modulators are neurosteroids (Belelli and Lambert, 2005). Until recently the position of a neurosteroid binding site remained unclear, with studies indicating a binding site located at TM domains with residues in TM1 (Q241) and TM4 (N407 and/or Y410) playing a role, although in which subunits remained unclear (Hosie *et al*., 2006, 2007). More recently further confirmation of a well conserved binding site within the TM1 domain of α-subunits was reported (Q241; Hosie *et al*., 2009), and the two previously reported TM4 residues were found not to be involved in the formation of hydrogen bonds between the steroid and the receptor but instead are involved in potentiation of the channel (Li *et al*., 2009). At nanomolar concentrations neurosteroids allosterically increase GABA-induced chloride flux by increasing the open state of the receptor. However, at micromolar concentrations they are able to directly activate the receptor in the absence of GABA, similar to barbiturates (Shu *et al*., 2004). Synthetic neurosteroid analogues (e.g. alphaxolone, hydroxydione) are generally used for sedation, however, currently under development is ganaxolone (a synthetic analogue of allopregnanolone) which is currently being tested in clinical trials for the treatment of epilepsy (D'Hulst *et al*., 2009).

Determining the native stoichiometry of $GABA_A$ receptors and their relative distribution within brain would allow the development of more specialised therapeutic strategies, which could be highly effective without the undesirable side effects.

1.2.8 $GABA_A$ receptor trafficking

Regulation of neuronal excitability in the brain is partly dependent on synaptic inhibition (Hablitz *et al*., 2009). Strength of synaptic inhibition is attributable to the number of synaptic $GABA_A$ receptors. Surface $GABA_A$ receptor numbers are in turn determined by a variety of mechanisms such as rate of assembly, trafficking between the synapse and intracellular structures (either for degradation or recycling), and their stability at the membrane (Arancibia-Cárcamo and Kittler, 2009). $GABA_A$ receptor subunits are assembled into their pentameric structure in the endoplasmic reticulum (ER) via interactions of subunit N-terminal assembly domains (Connolly and Wafford, 2004). Assembly occurs within five minutes of translation, but only at 25% efficiency (Gorrie *et al*., 1997). Chaperone molecules including calnexin and immunoglobulin heavy chain binding protein (BiP), which ensure correct folding. Plic-1 also interacts with intracellular domains of all α- and β-subunit polypeptides where it aids receptor stability in the ER, primarily by preventing proteosomal degradation (Bedford *et al*., 2001). Single subunits, homomers and αγ or βγ heteromers are generally retained within the ER and subsequently degraded (Kittler *et al*., 2002), thus it is in the ER where subunits compete for their preferential assembly (Jacob *et al*., 2008). Within the golgi apparatus, $GABA_A$ receptor associated protein (GABARAP) and golgi-specific DHHC zinc finger domain protein (GODZ) interact with γ subunits and aid trafficking of assembled $GABA_A$ receptors to the plasma membrane (Kittler *et al*., 2001; Keller *et al*., 2004). In addition to their insertion and stability at the synapse, $GABA_A$ receptor number is dictated by internalisation processes. Generally these are clathrin- and dynamin-dependent (Kittler *et al*., 2000). Intracellular domains within β- and γ-subunit polypeptides interact with clathrin adapter protein (AP2) resulting in receptor internalisation, where they can be degraded or recycled back to the surface. Retention and clustering of $GABA_A$ receptors at the synapse is purported to be partly due to their association with gephyrin. Gephyrin is essential for clustering of glycine receptors, mediated by a direct interaction between the β-subunit loop and gephyrin which has been demonstrated *in vivo* (Meyer *et al*., 1995). Although data suggests that gephyrin accumulates at GABAergic synapses and there is some evidence of interaction with the γ2 subunit (Alldred *et al*., 2005) and α2 subunit *in vitro* (Tretter *et al*., 2008), there is no concrete *in vivo* data of such an association. α5-subunit receptors, which are extrasynpatic,

are anchored by radixin to the actin cytoskeleton (Loebrich *et al*., 2006). Receptors are able to laterally diffuse within the plasma membrane forming discrete populations which may be synaptic or extrasynaptic. For a full review concerning $GABA_A$ receptor trafficking consult Arancibia-Cárcamo and Kittler (2009).

To summarise, GABAergic neurotransmission is highly-tuned at many different levels i.e. two types of GAD, many different conformational states of GABA, Ca^{2+}-dependant and independent release, re-uptake in neurons and glial cells, the presence numerous receptor subtypes which can be located pre-, post- and extra-synaptically, which are activated by a range of endogenous ligands. All of these factors contribute to a multifaceted system operating at a molecular, cellular and systemic level.

1.3 $GABA_C$ Receptors

Like the $GABA_A$ receptors, $GABA_C$ receptors are a subfamily of the Cys-loop LGIC superfamily. Where $GABA_A$ receptors are the most complicated members, $GABA_C$ receptors are the simplest, with only three identified subunits in mammals, ρ1, ρ2 and ρ3, and only ρ1 exhibiting alternative splicing (Martínez-Torres *et al*., 1998; Simon *et al*., 2004). There is considerable debate pertaining to the classification of these receptors as to whether they are a simply a sub-class of the $GABA_A$ receptors, or an individual sub-family of the LGIC superfamily (Olsen and Sieghart, 2008). The International Union of Pharmacology Committee on Receptor Nomenclature and Drug Classification (NC-IUPHAR) have recently recommended that the former $GABA_C$ receptor ρ1-ρ3 subunits be designated as part of the $GABA_A$ receptor family but still retain the Greek subunit letters as designated by Barnard *et al*. (1998), and the term $GABA_C$ receptor should not be used (Collingridge *et al*., 2009). The primary reasons for this controversy are manifold. Firstly, $GABA_C$ receptor subunits have an almost identical structure to $GABA_A$ subunits, sharing 30-38% sequence identity at an amino-acid level with $GABA_A$ receptor subunits (Bormann, 2000) although, in the TM2 region they share greatest homology with the glycine receptor α subunits (Feigenspan *et al*., 1993). They have an identical pentameric arrangement forming a chloride-specific channel of a similar diameter (~5.1 Å; Bormann and Feigenspan, 1995; Bormann, 2000). However, $GABA_C$ receptor subunits are thought to

commonly exist in homomers (unlike the majority of $GABA_A$ receptor subunits; Enz and Cutting 1998, 1999), although there is some evidence demonstrating the assembly of ρ1- and ρ2-subunit polypeptides into functional heteropentamers (Enz and Cutting, 1999), although this may not represent native receptor stoichiometry. This is in stark contrast to the apparent promiscuity of $GABA_A$ receptor subunits. Furthermore, there is no firm evidence for existence for native receptors comprising both $GABA_A$ and $GABA_C$ receptor subunits, despite reported assembly of ρ and γ2 subunits in functional recombinant receptors (Ekema *et al*., 2002; Milligan *et al*., 2004; Pan and Qian, 2005).

The prevailing differences between $GABA_A$ and $GABA_C$ receptors are their pharmacological profiles and electrophysiological responses (Bormann, 2000). Unlike $GABA_A$ receptors, $GABA_C$ receptors are sensitive to the GABA analog CACA (*cis*-4-aminocrotonic acid; Johnston, 1996) and (except for δ-containing $GABA_A$ receptors; Brown *et al*., 2002) have a significantly higher affinity (~10 x) for GABA. Upon binding, the response is slower and more sustained, with a weak desensitisation even at high GABA concentrations (Bormann and Feigenspan, 1995; Johnston, 1996; Qian & Pan, 2002).

Relative to $GABA_A$ receptors, $GABA_C$ receptors show much lower single-channel conductance only ~7 pS (picosiemens) and a pore opening time that is 6 x longer (Bormann and Feigenspan, 1995; Johnston, 1996). Further distinctions between receptor types include a comparable insensitivity to bicuculline and baclofen (4-amino-3-(4-chlorophenyl) butanoic acid, a $GABA_B$ receptor agonist; Curtis *et al*., 1970; Johnston, 1996; Chebib and Johnston, 2000) and high sensitivity to TPMPA [(1, 2, 5, 6-tetrahydropyridine-4-yl) methylphosphinic acid] (Murata *et al*., 1996). In addition, $GABA_C$ receptors are generally not modulated by benzodiazepines, barbiturates, neuroactive steroids and general anaesthetics at appropriate concentrations (Shimada *et al*., 1992; Bormann and Feigenspan, 1995; Qian and Pan, 2002). Akin to $GABA_A$ receptors, the subunit stoichiometry of $GABA_C$ subtypes dictates the pharmacological (Chebib *et al*., 1998; Enz and Cutting, 1999; Bormann, 2000; Chebib *et al*., 2007; Xie *et al*., 2008) and biophysical properties (Bormann, 2000; Zhu *et al*., 2007). For example, $GABA_A$ receptors are sensitive to cyclothiazide (as are $GABA_C$ receptor ρ2 homomers), yet ρ1 homomers display no such sensitivity. This

sensitivity is attributed to a single residue at the 2' position of TM2 (Xie *et al*., 2008), the same residue which regulates channel conductance (Zhu *et al*., 2007). Picrotoxin exerts a similar effect by potently antagonising $GABA_A$ and ρ1 homomeric $GABA_C$ receptors but ρ2 homomers and ρ1ρ2 heteromeric receptors are insensitive (Borman and Feigenspan, 1995; Enz and Cutting, 1999).

Moreover, (and maybe most significantly), $GABA_C$ receptor subunits are predominately expressed in the bipolar and horizontal cells of the retina, where they mediate synaptic inhibition (Albrecht and Darlison, 1995; Chebib and Johnston, 1999; Rozzo *et al*., 2002) although expression has been documented in some regions of mammalian brain (Boue-Grabot *et al*., 1998; Ogurusu *et al*., 1999; Rozzo *et al*., 2002; Milligan *et al*., 2004; Alakuijala *et al*., 2006), chicken brain (Albrecht *et al*., 1997) mammalian ovary and testes (Rozzo *et al*., 2002) and gut (Jansen *et al*., 2000).

Another disparity concerns the chromosomal location of the subunit genes. Within the human genome, $GABA_C$ receptor subunit genes are not closely associated with $GABA_A$ receptor genes. ρ1- and ρ2-subunit genes are clustered on chromosome 6, at position 6q13–q16.3 (Bailey *et al*., 1999; Simon *et al*., 2004), perhaps positioned for their possible co-assembly in receptors (Enz and Cutting, 1999); and the ρ3-subunit gene is localised to chromosome 3 in the human genome at position 3q11.2 (Bailey *et al*., 1999; Simon *et al*., 2004), along with no other $GABA_A$ or $GABA_C$ receptor genes. Previously, it has been suggested that native $GABA_A$ receptors may be composed of their chromosomal partners (Barnard *et al*., 1998), thus the isolated genomic positioning of the ρ-subunit genes would therefore point to homomeric assemblies. However, there are numerous exceptions to this rule (especially in $GABA_A$ receptors), suggesting chromosomal partnering is not an important determinant in receptor assembly (Olsen and Sieghart, 2008).

1.4 The avian song system

It may be assumed that studies on mammalian brain would be most appropriate for understanding the mechanisms of human cognitive functions, from an evolutionary perspective. However, humans and songbirds are members of a small group of animals

which have to learn from a conspecific tutor to produce their complex vocal signals (Thorpe, 1958; Konishi, 1965; Marler, 1970). Other members include cetaceans, such as whales and dolphins, and some species of bat. Unlike primates, there are many species of vocal-learning songbirds (~4000). Birdsong is a learned vocal behaviour ranging in complexity and repertoire between different species (DeVoogd, 2004; DeVoogd *et al*., 1993; Brenowitz *et al*., 1997). Songbirds, as a term encompasses primarily passerine (perching) birds of which the sub-order oscines are able to produce learned vocalisations. There are also some non-songbirds which are able to mimic vocalisations but are not classified as songbirds, such as parrots and hummingbirds (Marler, 1997).

1.4.1 History of songbird research

Observations of songbird behaviour, such as their requirement for a conspecific (same species) tutor during early development, were noted as early as Charles Darwin (1871). However, experimental research on birdsong commenced with the pioneering work of British ethologist William Thorpe some 80 years later (1958, 1961). He observed that juvenile chaffinches (*Fringilla coelebs*) reared in isolation produced abnormal songs, but if they were exposed to song of wild chaffinches they eventually produced songs reminiscent of these, confirming the need for a tutor. However, adult chaffinches exposed to the same songs did not imitate these songs, which was indicative of a critical period of learning. Thorpe's work was continued by Marler, who proposed the notion that songbirds possessed an innate auditory template, that was speculated to be genetically encoded. This was attributed to the observation that two different species of sparrows, when isolated from tutors, produced abnormal and completely different songs, but the individual songs still retained some characteristic species-specific elements (Marler, 1970, 1976). Work was continued by students of Marler; Masakazu Konishi who demonstrated the need for auditory feedback during the sensorimotor phase for normal song learning (Konishi, 1965) and Fernando Nottebohm who identified the neuronal circuits controlling the production of song (i.e. the song system; Nottebohm *et al*., 1976). Fifty years after Thorpe's original research, work continues in the field with earnest, providing us with numerous, well characterised avian models to understand motor function and learning and memory processes. Many of these early observations are still upheld today.

1. Birds require a tutor during sensory acquisition

Akin to speech acquisition in humans, the learning of song is a process of imitation whereby a period of perceptual learning is followed (or in some cases, overlapped) by a rehearsal period requiring auditory feedback (Konishi 1965, 2004; Marler, 1997; Mooney, 1999). Birds reared in isolation from tutors produce highly abnormal songs (Thorpe, 1958; Immelmann, 1969; Marler and Sherman, 1983; Eales, 1987; Searcy and Marler, 1987; Tchernichovski and Nottebohm, 1998; Kojima and Doupe, 2007; Fehér *et al*., 2009) as do humans (Fromkin *et al*., 1974; Lane, 1976). If juveniles are reared in the presence of heterospecific tutors (different species), they will learn/attempt to learn the song of the foster tutor (Immelmann, 1969). However, if presented with a choice of hetero- or conspecific (same species) tutors, juveniles will always preferentially learn the song of the conspecific tutor, suggesting an innate pre-disposition for conspecific song (Immelmann, 1969; Marler and Peters, 1988; Riebel *et al*., 2002). Furthermore, isolate songs still retain some species-specific characteristic features (Marler and Sherman, 1985; Zann, 1996; Konishi, 2004; Kojima and Doupe, 2007), which has been attributed (at least in part) to an innate auditory template (Marler, 1970, 1976).

2. Birds require auditory feedback during song learning

Birds must be able to hear themselves sing in order to produce normal song (Konishi 1965; 2004). Birds deafened as juveniles who cannot hear their own vocalisations produce highly unstructured songs (Konishi, 1965, 1985, 2004; Price, 1979). Despite harboring a species-specific innate auditory template, they cannot match their vocal output to it, thus producing random noises (Konishi, 2004). This is also apparent in the human population, whereby deaf children cannot vocalise normally (Kuhl, 2004). If birds are deafened after sensory acquisition (listening to tutor sing), but before sensorimotor learning (the rehearsal stage), they also develop highly abnormal songs, even though the tutor song no longer needs to be heard at this stage (Konishi, 1965). Birds raised in isolation and then deafened before the sensorimotor phase, produce songs that are the same as birds which have been deafened from the start with no identifiable features at all (Konishi, 1965, 1985).

3. Innate template

One of the main features of birdsong is that it is characteristic to each species. Both humans and birds posses versatile vocal apparatus, so motor constraints are not considered a limiting factor of vocal learning. Marler was the first to propose the notion of an innate auditory template (which is present from hatching), pre-disposing birds to sing a song characteristic of their own species (1970). Evidence supporting this arose from several behavioural studies. Firstly, isolated birds produced songs with characteristic elements despite no auditory input from a tutor (Marler and Sherman, 1985; Searcy and Marler, 1987; Zann, 1996; Konishi, 2004; Kojima and Doupe, 2007). Physiological responses (such as begging calls and increased heart rate) of young birds are much greater in response to conspecific song (Dooling and Searcy, 1980; Nelson and Marler, 1993) and when presented with a choice of tutor, juveniles preferentially learn the song of a conspecific (Immelmann, 1969; Marler and Peters, 1988; Riebel *et al*., 2002). Thus songbirds posses an innate (possibly genetically encoded) auditory template coupled with a pre-disposition for perceptual learning which precludes song production. However, in addition to these, normal song production also relies on both sensory input (tutor song) and auditory feedback (birds own song).

4. Neural substrate for song learning

Perception of song, acquisition of an auditory template, motor production of song and modification of vocal output via auditory feedback are processes that all require specialised neuronal circuits. The neural substrates for vocal learning are referred to as the song system (Nottebohm *et al*., 1976), further details given in section 1.4.2. Birds which do not sing (learned song), are able to discriminate between different sounds but are unable to mimic what they hear and comparative anatomical analysis has shown the nuclei responsible for motor control to be absent in non-learners (Gahr, 2000). However, in avian species where only the males sing (e.g. zebra finch, *Taenopygia guttata*) females posses some of the song-system nuclei but they are significantly smaller than their male counterparts (Nottebohm and Arnold, 1976; MacDougall-Shackleton and Ball, 1999).

One of the most frequently studied songbirds is the zebra finch. These are small birds native to the semi-arid regions of Australia and Timor (Zann, 1996). They are social animals nesting in groups of 20-1000 birds, living up to 3 years in their natural environment and 5-7 years in captivity. Zebra finches are close-ended learners (they can only learn as juveniles and after sexual maturity cannot change their song, discussed in section 1.4.4) and their development process and songs have been extensively characterised. Thus they provide an excellent model for studying the underlying molecular mechanisms involved in the acquisition and production of song. This study exclusively utilises male zebra finches, as female zebra finches do not sing (Nottebohm and Arnold, 1976).

1.4.2 Structure of the song system

A discrete group of interconnected nuclei within in the telencephalon (in both hemispheres) are responsible for memorisation and production of learned song; referred to as the song system (Nottebohm *et al*., 1976). The avian song system is broadly divided into two functionally and anatomically distinct pathways, the anterior forebrain pathway (AFP; primarily involved during sensory acquisition) and vocal motor pathway (VMP; control of the motor production of song) although more recent evidence indicates the two circuits are far more interconnected than originally postulated (Fig. 1.2).

HVC (formal name, formally higher vocal centre) is the site of auditory input into the song system. Auditory information originates from Uva (nucleus uvaeformis, a thalamic nucleus) via NIf (interfacial nucleus of the nidopallium) and Field L (auditory area in nidopallium). In turn the HVC projects to nuclei located in both pathways of the song system (RA and Area X). HVC is involved in the timing of song syllables (both their duration and the length of the interval between them; Long and Fee, 2008). HVC composes three different types of neurons; projection neurons which innervate the pre-motor area (HVC_{RA}), projection neurons which innervate the basal ganglia circuit (HVC_X) and interneurons (Wild *et al*., 2005; Mooney and Prather, 2008), of which there are two classes (Scott and Lois, 2007). Area X is the first nuclei of the AFP. Located within the striatum, it is composed of a mixture of striatal and pallidal cells, with large GABAergic interneurons sparsely

distributed throughout (Luo and Perkel, 1999; Farries and Perkel, 2002). Area X demonstrates the greatest levels of neurogenesis in post-natal songbird brains in the form of medium spiny neurons (Scott and Lois, 2007). Medium spiny neurons contribute a large portion of the mammalian basal ganglia, where they are under much scrutiny due to their loss in Huntington's disease (Mitchell *et al*., 1999). Area X is the equivalent of the mammalian basal ganglia whereby inputs (from HVC and LMAN) are processed in a similar way and it comprises many equivalent cell types (Luo and Perkel, 1999; Farries and Perkel, 2002). From Area X, the AFP pathway descends to the medial nucleus of the dorsolateral thalamus (DLM) and then to the lateral portion of the magnocellular nucleus of the anterior nidopallium (LMAN), which is the output of the AFP pathway (Nottebohm *et al*., 1976). LMAN is the one song system nuclei which is sexually monomorphic in zebra finch brains (Nixdorf-Bergweiler, 2001), although mRNA expression is generally lower in females than that of males (Thode *et al*., 2008; Tomaszycki *et al*., 2009). Projections from the LMAN connect to Area X and RA, thus the LMAN in the final stage where auditory information can be processed before being transmitted to the VMP or back into the AFP (potential feedback mechanisms; Fig. 1.2b), these projection neurons are innervated by GABAergic interneurons (Rosen and Mooney, 2000).

(a) (b)

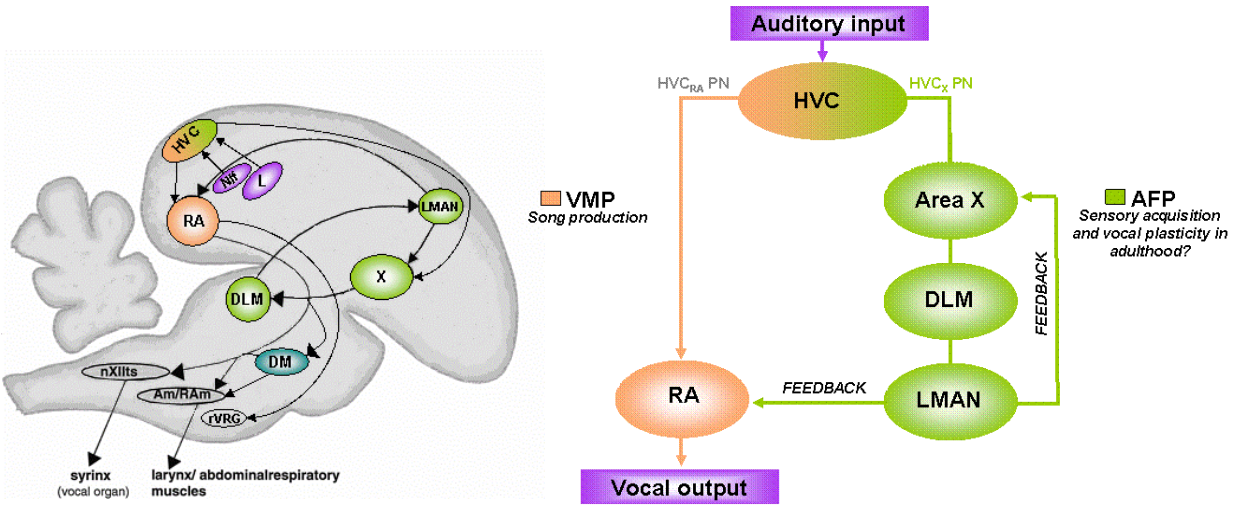


Figure 1.2. (a) Schematic representation of the song system nuclei within zebra finch brain. (b) Flow diagram illustrating the flow of information through the song system. HVC (formal name) projects to both the AFP

and VMP, but its connections within the VMP are far more robust; projecting neurons from HVC to RA are more prevalent over those projecting to Area X (4:1; Wild *et al*., 2005). RA is the output of the song system and there are considerably more HVC synapses at RA in comparison to the number of LMAN synapses (20:1; Canady *et al*., 1988). Despite being classified as separate pathways there is a link between LMAN and RA and circuits within the AFP enabling performance-based feedback (Brainard and Doupe, 2002). Abbreviations: Am, ambigualis; DLM, medial nucleus of the dorsolateral thalamus; DM, dorsal medial nucleus; HVC, formal name; L, field L; LMAN, lateral magnocellular nucleus of the nidopallium; NIf, interfacial nucleus of the nidopallium: nXIIts, tracheosyringeal part of the hypoglossal nucleus; PN, projection neuron; RA, robust nucleus of the arcopallium; Ram, retroambigualis; rVRG, rostro–ventral respiratory group; X, area X

The VMP is more direct (Fig. 1.2). Projection neurons originating exclusively in the ventricular region, dorsal to the HVC, descend to the RA during song learning in juvenile animals (Scott and Lois, 2007). The sexually dimorphic RA represents the output of the song system, projecting to hyperglossal motor neurons which innervate the syrinx (nXIIts) and Ram (nucleus retroambigualis), which is the site of vocal and respiratory muscle coordination (Nottebohm *et al*., 1976). Ram is comprised of five types of projection neurons and is the functional equivalent of the mammalian NRA (nucleus retroambigualis; Wild *et al*., 2009). The VMP is important for song production. Ablation of RA or HVC effectively halts normal song production (Nottebohm *et al*., 1976; Simpson and Vicario, 1990), resulting in highly abnormal song or muteness. Although, it is now recognised that the AFP also plays a role in adult song production, as both Area X and LMAN are active during singing (Hessler and Doupe, 1999) and microlesions in HVC can be recovered by ablation of LMAN (Scott *et al*., 2007; Thompson *et al*., 2007b). This may be because inactivation of LMAN disrupts the feedback mechanisms involved in the control of song (Fig. 1.2b; Brainard and Doupe, 2000)

Disruption of the AFP pathway does not completely impair the bird's ability to produce learned vocalisations, however, it stops further advancement of vocal learning and new sounds can no longer be imitated. Lesions of LMAN in juvenile birds impact on song development, producing over-simplified, highly repetitive song with premature crystallisation, as soon as one day post-surgery (Bottjer *et al*., 1984; Scharff and Nottebohm, 1991). Conversely, in adults, LMAN lesions have little effect on crystallised

song (Bottjer *et al*., 1984). Lesions of Area X (rendering it non-functional) in juveniles produce plastic (changeable) songs which appear not to crystallise (become stable), a sharp contrast to the premature crystallisation induced by LMAN lesions (Scharff and Nottebohm, 1991). Accordingly, it was deduced that the AFP was not necessary for song production, but was important in song acquisition. However, further work demonstrated that previously crystallised song of adult birds is subject to degradation if birds are deafened or auditory feedback is perturbed (Nordeen and Nordeen, 1992; Woolley *et al*., 1997; Leonardo and Konishi, 1999; Funabiki and Konishi, 2003; Zevin *et al*., 2004), indicating the potential for plasticity even after crystallisation. Degradation of stable adult zebra finch song is proportional to the age of deafening in that the song of younger adults deteriorates faster than that of older animals following deafening (Lombardino and Nottebohm, 2000). Furthermore, if perturbations are discontinued, some recovery of normal song is observed (Leonardo and Konishi, 1999; Woolley and Rubel, 2002; Funabiki and Konishi, 2003) although, to varying degrees (Zevin *et al*., 2004). It is therefore proposed that there is ongoing comparison of vocal output with the sensory template (whether this template be the tutor song or a memory of their own crystallised vocalisations remains unknown), beyond the critical period of learning (Woolley, 2004). However, these experiments do not allow for modification of song by the bird in response to perturbation of auditory feedback. Recent work by Sober and Brainard (2009) has revealed that by mechanically changing the pitch of song during auditory feedback (by use of headphones) resulted in adult Bengalese finches readjusting the pitch of their song in attempts to bring it in line with their original sensory template. In addition, after temporary deafening, when hearing was restored, birds were able to learn the songs of cage-mates (Woolley and Rubel, 2002). It can therefore be postulated that song remains plastic into adulthood, perhaps by similar mechanisms utilised by juvenile songbirds prior to crystallisation, indicating a potential role for the AFP after sensory acquisition, but the exact mechanism remains unknown (Thompson *et al*., 2007b). It is only the integration of both pathways (AFP and VMP) which can result in the normal production of learned song.

1.4.3 Sexual dimorphism

Zebra finch behaviour is highly dimorphic, with only male birds learning to sing. This is reflected in the song system where females exhibit significantly smaller nuclei and less interconnected circuitry (Nottebohm and Arnold, 1976; Konishi and Akutagawa, 1985; Fig. 1.3). Female zebra finches treated with estradiol as juveniles, then with testosterone as adults can produce basic song (Arnold, 1997); although the growth of the HVC appears to be independent of steroid hormones (Gahr and Metzdorf, 1999), perhaps an attributing reason as to why hormonally-treated female zebra finches can never produce male-quality songs. It has been speculated by Bolhius and Macphail (2001) that the sex differences (i.e. different nuclei volumes) are actually related to song production and not song acquisition. This is of interest as, female zebra finches, which do not sing, appear to undergo a critical learning period at a similar developmental stage to male birds (Bailey and Wade, 2005), although the data is controversial. It is agreed that females show preference for conspecific song; this is also true of female zebra finches isolated from adult males (Clayton, 1988; Laulay *et al*., 2004). However it was originally postulated that this preference is not apparent in females isolated before PHD 25 (Clayton, 1988), but isolation at a later stage (PHD 35) resulted in females favouring their fathers song (Miller, 1979). In contrast, recent data shows auditory isolation as early as PHD 18 still resulted in females which preferred conspecific song (Laulay *et al*., 2004). However, females isolated at PHD 18 showed no preference for tutored (normal quality) or untutored (poor quality) song (Laulay *et al*., 2004). Furthermore, during the period of sensory acquisition, there are significant differences in gene expression between male and female zebra finches with many sex-linked, differentially expressed genes within song system nuclei (Tomazycki *et al*., 2009).

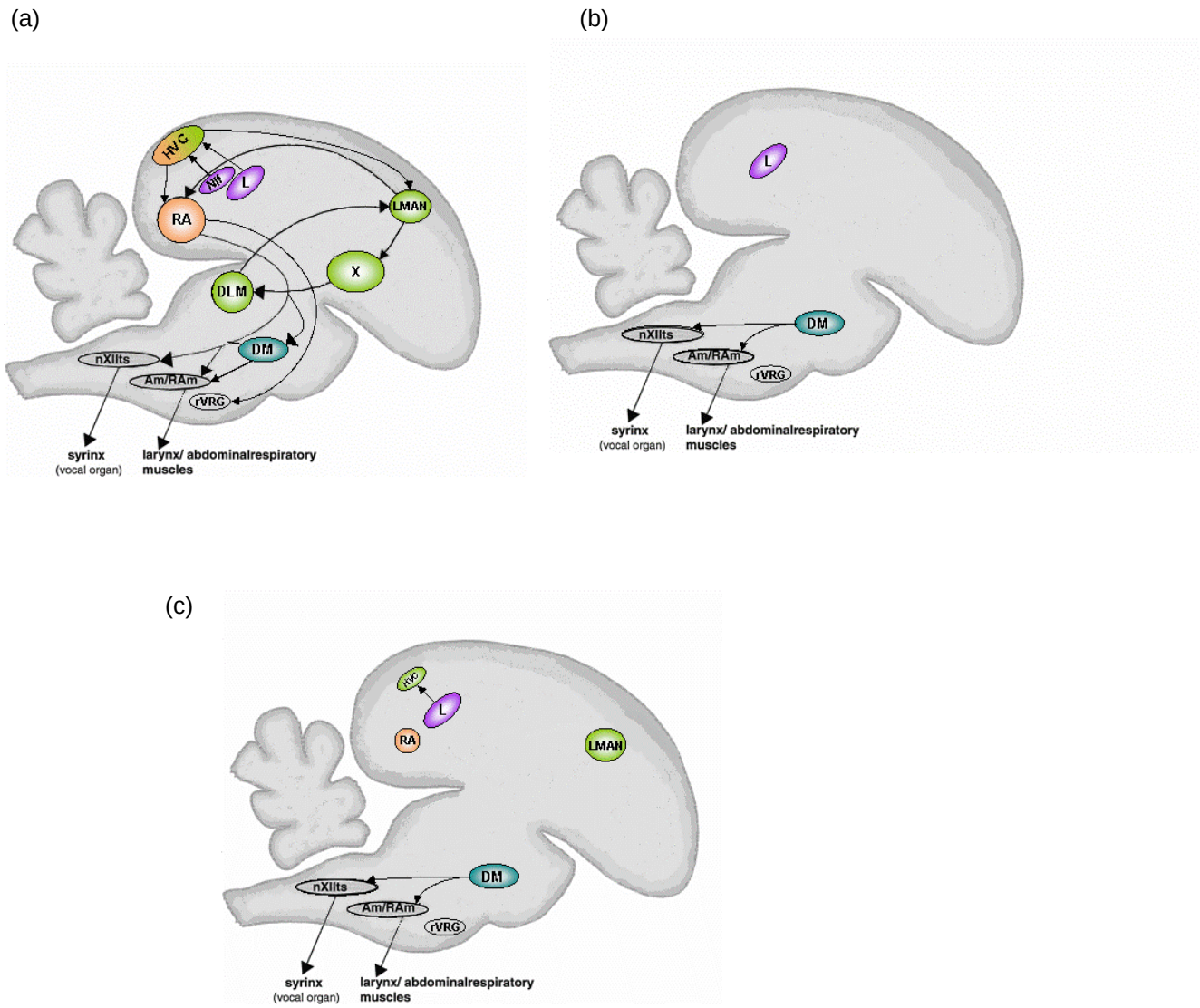


Figure 1.3. Sagittal schematics of male zebra finch (a), non-song bird, e.g. pigeon (b) and female zebra finch (c) brains. The zebra finch song system is sexually dimorphic; females typically have smaller nuclei (except for LMAN) and Area X is completely absent. Furthermore, connections between HVC→RA cannot be detected. All the major nuclei of the song system are absent in non-songbirds, but they are able to produce non-learned vocalisations, this is also the case with female zebra finches (Zann, 1996; Price, 1979; Bolhius and Gahr, 2006). Both females and non-songbirds have field L which is the primary auditory area which in males projects to the song system. Data obtained from Bolhius and Gahr, 2006 and Tomazycki *et al*., 2009. **Abbreviations**: Am, ambigualis; DLM, medial nucleus of the dorsolateral thalamus; DM, dorsal medial nucleus; HVC, formal name; L, field L; LMAN, lateral magnocellular nucleus of the nidopallium; NIf, interfacial nucleus of the nidopallium: nXIIts, tracheosyringeal part of the hypoglossal nucleus; RA, robust nucleus of the arcopallium; Ram, retroambigualis; rVRG, rostro–ventral respiratory group; X, area X

1.4.4 Stages of song development

Song learning is a process of imitation where a tutor model is required (Thorpe, 1958, Marler, 1970). The song learning process has been compared to imprinting, in that information is learned very quickly and then retained for long periods of time. Zebra finches can learn songs as juveniles with as little as 1 min tutor exposure per day (Tchnernichovski *et al*., 1999), the tutor template is stored as a long-term memory (Funabiki and Konishi, 2003). In the production of song there are two types of learners, the age-limited (or close-ended) learners (e.g. the zebra finch (*Taeniopygia guttata*) and white-crowned sparrow (*Zonotrichia leucophrys*) and the open-ended learners (e.g. the canary (*Serinus canaria*) and the European starling (*Sturnus vulgaris*); Brainard and Doupe, 2002). With birds in the former category, song memorisation is restricted to the early stages of life, with no new songs being learned in adulthood (Brenowitz, *et al*., 1997). In contrast, open ended learners are able to develop new songs into adulthood. Often this occurs on a seasonal basis, usually during the spring (Mooney, 1999). In both open- and close-ended learners there are 'critical' periods for song development.

Despite the presence of an innate auditory template, to produce normal song, juvenile birds must be exposed to conspecific tutor song during the critical phase of song learning (~PHD 20-40). This is essential to retain a more precise representation of the tutor song (Konishi, 1965, 2004; Immelmann, 1969; Marler, 1970). Similar to humans, long term memories are formed of the tutor song (Funabiki and Konishi, 2003), which are thought to be stored within the auditory system (Phan *et al*., 2006). Once equipped with an auditory template, the bird enters the sensorimotor phase where it begins to sing (Fig. 1.4; Marler, 1997). The first utterances of song are relatively unstructured and do not really resemble the tutor song (termed subsong), much like the ramblings of a human infant (Thorpe, 1958). As the bird continues matching its own vocalisations with the sensory template, the song output becomes more structured and refined and is known as plastic song (Catchpole and Slater, 1995). With further practice the song becomes crystallized, and is a close replica to the song they were exposed to during sensory acquisition (Fig. 1.4). Song crystallisation coincides with sexual maturity at ~PHD 90 (Immelman, 1969; Mooney, 1999; Harding, 2004), where there is a large surge of circulating testosterone (Pröve, 1983). Close-ended

learners such as zebra finches generally do not modify their songs, which remain stable and stereotyped following crystallisation (Marler, 1997). However, there are circumstances when close-ended learners display adult-song plasticity. These include deafening (Nordeen and Nordeen, 1992), syrngeal denervation (Williams and McKibben), muting (Pytte and Suthers, 2000), delayed auditory feedback (Leonardo and Konishi, 1999) and perturbation of auditory feedback (Funabiki and Konishi, 2003; Sober and Brainard, 2009). In cases such as these, adult zebra finches may modify their crystallised song, although the effects are not immediate (Williams *et al*., 2003). These changes are mediated by the AFP, also known as the basal ganglia circuit, which is primarily involved in the learning of song. Experimentally induced song plasticity does not occur in birds with LMAN lesions (Brainard and Doupe, 2000). Thus these 'critical' periods may be more flexible than originally postulated (Funabiki and Konishi, 2003).

The song system is not fully developed in males immediately after hatching (Kirn, 2009). Many of the nuclei involved in the acquisition/production of song are small in juvenile birds and increase in size in correlation with the song learning process (Fig. 1.4). HVC is the entry point of auditory information into the song system and is involved in the motor production of song. HVC projections to Area X develop at ~PHD 20 (onset of sensory acquisition) and HVC projections to RA develop at ~PHD 35 (onset of sensorimotor phase; Fig. 1.4); these projections are not detected in female zebra finches (Konishi and Akutagawa, 1985; Mooney and Rao, 1994; Scott and Lois, 2007). HVC reaches its full size in males at ~PHD 60 (Ward *et al*., 2001), but in females at ~PHD 30 neurons begin to die and the HVC reduces in size (Nixdorf-Bergweiler, 2001). The RA is also involved in the production of song by projecting to the respiratory tract and syrinx (Wild, 2004). In male zebra finches, RA is the first song system nuclei to begin increasing in size (~PHD 6), and decreasing in females at ~PHD 30 due to cell death (Tomaszycki *et al*., 2009). Area X is not visible in female zebra finches (Nottebohm and Arnold, 1976), but interestingly LMAN is sexually monomorphic, being of a similar size in males and females (Nixdorf-Bergweiler, 2001). Gene expression is also sexually dimorphic in zebra finches, with enhanced expression in nuclei of the song system in male birds relative to females (Thode *et al*., 2008; Tomaszycki *et al*., 2009).

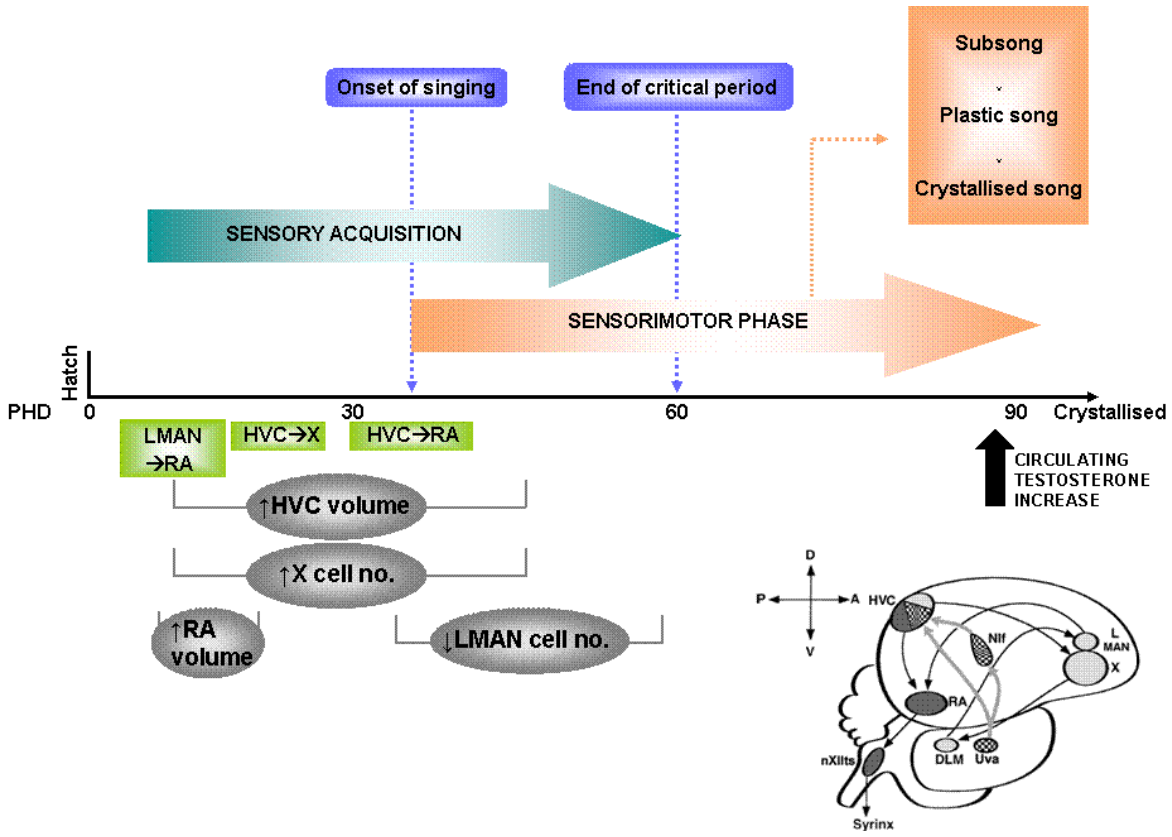


Figure 1.4. Timeline detailing stages of song learning in the zebra finch, the entire process of song development takes ~90-120 days. There are distinct stages to song development (Marler, 1970), but in the zebra finch these stages overlap (Brainard and Doupe, 2002). The juvenile chick first listens to and memorises the song of a tutor (sensory acquisition) and then begins vigorous singing (sensorimotor phase) whereby the song output is gradually matched to the template acquired during sensory acquisition, at this stage the tutor no longer needs to be heard (Konishi 1965, 1985). As song learning progresses the nuclei increase in size and connections are formed between them (green boxes). A large increase in circulating testosterone at ~PHD (post-hatch day) 90 (Pröve, 1983) is thought to increase song stereotypy (Williams *et al*., 2003). Data modified from Tomaszycki *et al*. 2009. Abbreviations: DLM, dorsolateral thalamus; HVC (formal name); LMAN, Lateral magnocellular nucleus of the anterior nidopallium; NIf, interfacial nucleus of the nidopallium: nXIIts, tracheosyringeal part of the hypoglossal nucleus; RA, Robust nucleus of the arcopallium; X, Area X; Uva, nucleus uvaeformis.

1.4.5 Song structure

Song is easily quantified using an audiospectrogram (which measures vibrations per sec at individual frequencies (Hz)) and as aforementioned, is characteristic to each species, some

having a repertoire of thousands of songs (e.g. mocking bird, *Mimus polyglottis*) and some having only one (e.g. the zebra finch, *Taenopygia guttata*; DeVoogd *et al*., 1993; Brenowitz *et al*., 1997; Brainard and Doupe, 2002). Song has a well defined structure which can be broken down into clear structural components. The most basic sound produced by a bird is termed an element, several elements together are a syllable and multiple repeated syllables become a phrase or motif. If the phrases are broken up by variable length silent intervals, the sequence becomes a song 'bout' (Brenowitz *et al*., 1997). Each species of song bird has its own characteristic core elements which are relatively well conserved, with the exception of species such as the Mockingbird which is able to mimic the songs and calls of other species (Marler, 1997). Zebra finch song is acoustically complex with syllables produced during inspiration and expiration (Goller and Daley, 2001; Leadbeater *et al*., 2005), but shows remarkable stereotypy across the species.

1.4.6 Functions of song

Song is not exactly like spoken language, which conveys complex meanings (Fitch, 2009); it is simple and is used to communicate defensive (although not zebra finches; Zann, 1996), attractive and warning signals. Zebra finches which live in large groups use song to identify individual members (Zann, 1996). Faithful reproduction of tutor song is therefore necessary to ensure a successful life for the bird. Strength of song can be an indication of fitness, whereby birds with a large repertoire or more complex songs attract females more easily (Catchpole and Slater, 1995) and juveniles will preferentially learn from the tutor with the largest repertoire, if given a choice, even if this is not the father (Soma *et al*., 2009). Male zebra finches have the smallest repertoire, with just one song (although there are directed and non-directed versions), so more emphasis is placed upon the singing rate (Houtman, 1992) and complexity of the song (Clayton and Pröve, 1989). The song itself is a repeated motif lasting 0.5-1.5 sec with a consistent number of syllables; of which the average is 6.7 (range 3-10 syllables; Spencer *et al*., 2003), preceded by a few introductory notes. Factors such as nutritional stress during development can significantly reduce the number of syllables in a song (Spencer *et al*., 2003) and the accuracy of learning, which is representative of a lower level of fitness during development (Brumm *et al*., 2009). Furthermore, increased levels of corticosterone (released under stressful conditions) can

facilitate or detrimentally affect learning and memory (Coburn-Litvak *et al*., 2003; Martin *et al*., 2009; Quirarte *et al*., 2009). Corticosterone administration during development results in adult zebra finches producing shorter and less complex songs than their untreated counterparts (Spencer *et al*, 2003).

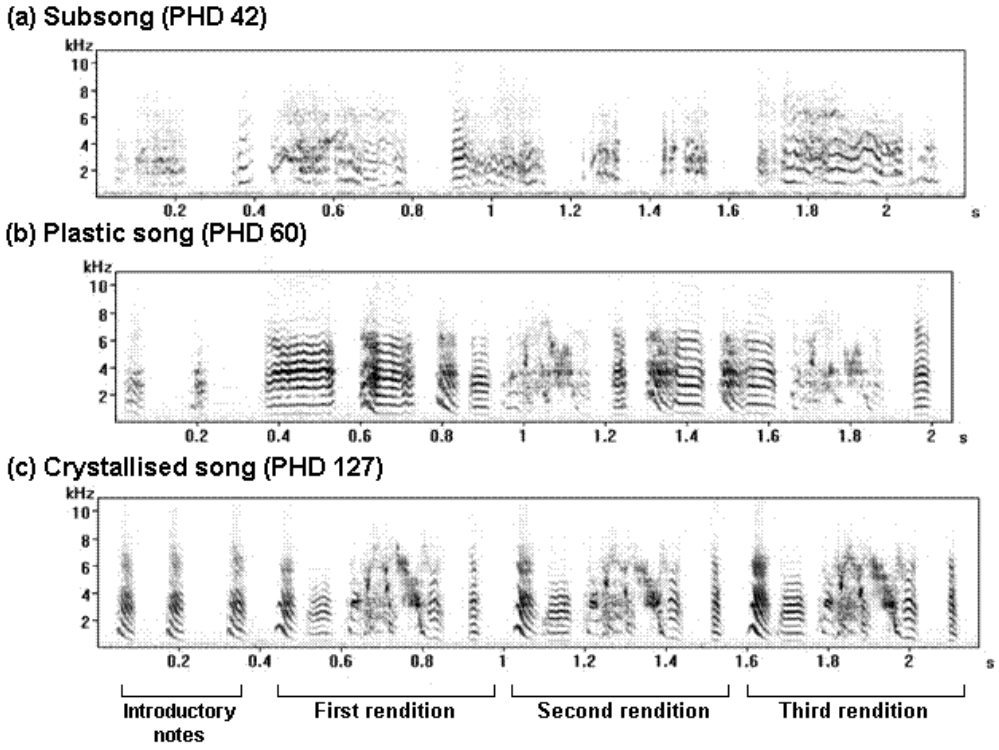


Figure 1.5. Audiospectrograms showing zebra finch song at different developmental stages, displayed as frequency vs. time. When zebra finches enter the sensorimotor phase their first vocalisations are rambling and soft, akin to the babbling of an infant (a). As the bird continues to practice and match its vocalisations to the sensory template, the song becomes more structured and syllables emerge (plastic song), although some sequencing errors still occur (b). Following sexual maturity, song has clear structure and is very similar to that memorised during sensory acquisition, once the song is crystallised it remains relatively unchanged throughout adulthood (c); shown here are a few introductory syllables followed by three renditions of the song. Audiospectrograms adapted from Johnson laboratory at Florida State University (www.psy.fsu.edu/~johnson/johnsonlab/johnson.htm).

The structure of song is unique to each species, although there is slight variation amongst different members (akin to regional dialects observed in the human population; Kuhl, 2004; Fehér *et al*., 2009), during cultural transmission (Marler and Tamura, 1964; Marler and Slabbekoorn, 2004; Fehér *et al*., 2009). The characteristic elements are retained throughout,

with females preferring the song of their local dialect (Clayton, 1989). These small diversities are thought to be advantageous in discriminating several individuals from the same clutch which have had an identical tutor (Böhner, 1983). The younger siblings tended to produce a crystallised song most reminiscent to that of their fathers (Tchernichovski and Nottebohm, 1998). An interesting finding was that if non-tutored zebra finches (i.e. produce abnormal song) are allowed to breed, the subsequent generations faithfully produce the isolate song, but with each generation, songs converged closer to that of a normal zebra finch, until the fifth generation when the song could not be easily distinguished from a normal zebra finch song (Féher *et al*., 2009). These examples further support the idea of an innate auditory template, which may be in part genetically encoded (inducing species-typical constraints), but is subject to environmental influences (Marler, 1970).

1.4.7 Seasonal plasticity

The song(s) of some birds exhibits seasonal plasticity which is accompanied by volumetric changes in the nuclei, most notably in the HVC, which increases during the spring (breeding season) and improved connections between the nuclei (DeGroof *et al.,* 2008). Changes in nuclei size may be attributable to an increase/decrease in neuronal number, or alterations in their relative size or spacing, these changes are not uniform across the nuclei and may be triggered by different environmental cues such as photoperiod, which causes an increase in circulating testosterone (Smith *et al*., 1997), or social cues such as the presence of females (Tramontin *et al*., 1999). Zebra finches are opportunistic breeders and are generally not considered to be photoperiodic (Zann, 1996), although there is some evidence to the contrary (Bently *et al*., 2000). Age-limited learners such as zebra finches, which do not modify their song on a seasonal basis, still undergo seasonal changes in circulating hormones and nuclei size but their songs remain unaffected (Brenowitz *et al*., 1991). Conversely, in the white crowned sparrow (close-ended learner), no seasonal fluctuations in nuclei volumes are observed (Baker *et al*., 1984)

Song-stereotypy of both open- and close-ended learners is purported to be linked to circulating testosterone levels (Pröve, 1983). In canaries, testosterone levels are at their highest during the breeding season in the spring, and this is when the songs are most

stereotyped (Nottebohm, 1981). In contrast, in zebra finches (which are close-ended learners), there is a surge of testosterone towards the end of the sensorimotor phase which coincides with song crystallisation (Pröve 1983). Testosterone is detected in juvenile zebra finches during development (Nottebohm, 1989), but if levels are artificially raised, it induces premature song crystallisation (Korsia and Bottjer, 1991). Correspondingly, if testosterone is blocked, song development is also disrupted (Bottjer and Hewer, 1992), indicating that testosterone plays a role in song stereotypy. Furthermore, female zebra finches treated with estradiol during development, followed with testosterone as adults can produce basic song (Arnold, 1997). This is also seen in testosterone-treated female canaries (Nottebohm, 1980). In adult male zebra finches, testosterone administration results in a change in the frequency of song (Cynx *et al*., 2005). All this evidence points to an important role for testosterone in crystallization. However, a rise in circulating testosterone may not be the only trigger for crystallisation. Testosterone (androgen) induces singing, but it may be that the motor act of singing itself which serves to close the critical period (Eales, 1987; Brainard and Doupe, 2002). Evidence supporting this theory arises from the fact that adult zebra finches which had been raised isolation or with females are able to incorporate some new zebra finch syllables into already crystallised isolate song (Slater *et al*., 1988; Livingston *et al*., 2000); suggesting a plasticity of adult song and inferring that crystallisation may be guided by experience and not only by hormones (Eales, 1987; Morrison and Nottebohm, 1993; Jones *et al*., 1996). Furthermore, zebra finches raised by Bengalese finches and then re-exposed to conspecifics as adults were able to add characteristic zebra finch song syllables into their songs after the end of the critical period. However, zebra finches raised normally with conspecifics were not able to modify their songs once they had crystallised (Immelmann, 1969; Slater *et al*., 1988). It can therefore be speculated that during the process of learning, singing, in addition to testosterone, may play a role in the closure of the critical period (reviewed by Doupe and Kuhl, 1999).

1.4.8 Song production and human speech

Phylogenetically, mammals and birds separated some 300 million years ago (Bolhius and Macphail, 2001) and although not identical, there are some striking parallels between birdsong and speech production, consequently the underlying neuronal mechanisms are

similar (Doupe and Kuhl, 1999; Kuhl, 2004; Bolhius and Gahr, 2006). Both birdsong and human speech are complex vocalisations, requiring coordination of both vocal and respiratory apparatus and the presence of a suitable tutor early in life to imitate. Darwin (1871) actually speculated that singing came before speech, and such things as singing apes may have arisen, communicating in a way similar to birds, bats and whales today. Similar cell types are found in avian brain as are present in mammalian/human brain circuitry (Farries and Perkel, 2000; Mooney and Prather, 2005; Scott and Lois, 2007), and although the overall morphology of the telencephalic systems may somewhat vary, the core neuronal circuitry remains remarkably similar (Jarvis *et al*., 2005; Bolhuis and Gahr, 2006). The VMP is homologous to a mammalian motor pathway which originates in the cerebral cortex and descends through the brainstem (Nottebhom *et al*., 1976) and the mammalian equivalent of the AFP is a cortical loop pathway which traverses the basal ganglia and thalamus (Luo and Perkel, 1999). The striatal Area X is homologous to mammalian striatum, in that both comprise nearly identical physiological cell types (Farries and Perkel, 2002). Johnson and Whitney (2005) have shown that the telencephalon is the origin of a large majority of the processes involved in human speech and the production of song in zebra finches. When compared proportionally to a mammal (rat) and a non-singing bird (pigeon) they noted that a notably larger proportion of the human and zebra finch brain was occupied by the telencephalon (>50%) while other species only showed 35% occupancy. This is not necessarily a discrimination between vocal learners and non-learners, but it is nonetheless noteworthy. Both human speech and birdsong production occur during critical periods, and upon termination of these periods, learning becomes increasingly difficult (generally after sexual maturity; Marler, 1970; Konishi, 1985; Doupe and Kuhl, 1999). In the human population, cases of extreme social and auditory deprivation have a negative impact to the point where normal speech can never be learned (Lane, 1976), e.g. in the case of Genie (Fromkin *et al*., 1974). This phenomenon is also observed in close-end learners such as the zebra finch whereby isolation during the song-learning phase (PHD 35-90) results in production of abnormal song (Thorpe, 1958; Immelmann, 1969; Marler and Sherman, 1983; Eales, 1987; Searcy and Marler, 1987; Tchernichovski and Nottebohm, 1998; Kojima and Doupe, 2007; Fehér *et al*., 2009). Both birdsong and human speech require auditory feedback during the learning period and are subject to degradation if

auditory feedback is disturbed later in life (Cowie and Douglas-Cowie, 1992; Nordeen and Nordeen, 1992; Leonardo and Konishi, 1999; Woolley, 2004; Zevin *et al*., 2004; Funabiki and Funabiki, 2007; Sober and Brainard, 2009). All these factors contribute to the recognition of the songbird as a suitable paradigm for understanding the neuronal mechanisms of cognitive processes which may be extrapolated to humans, giving insights into common sensory and motor learning, as well as the acquisition of speech (Brainard and Doupe, 2002; Kuhl, 2004).

1.4.9 $GABA_A$ receptors in the avian song system

As detailed earlier, there are 16 $GABA_A$ receptor subunits identified in mammals, each encoded for by an individual gene; no further genes of this family exist in the human genome (Simon *et al*., 2004). In avian species only 14 $GABA_A$ receptor subunits have been detected (α1-α6, β2-β4, γ1, γ2, γ4, δ and π); where the γ4 and β4 subunits are orthologues of the mammalian ε and θ subunits respectively (Darlison *et al*., 2005). Despite a multitude of studies investigating the expression of $GABA_A$ receptor subunit genes within rat (Laurie *et al*, 1992a, b; Wisden *et al*, 1992; Pirker *et al*., 2000; Pöltl *et al*., 2003; Wafford *et al*., 2004) and monkey (*Macaca mulatta*) brain (Kultas-Ilinsky *et al*., 1998; Huntsman *et al*., 1999), no such studies have been completed within the zebra finch brain. This may be considered unusual, due to its suitability as a model of cognitive function; although it may be attributable to the paucity of available zebra finch $GABA_A$ receptor isolated cDNAs (only the γ4-subunit sequence was published at the time of this work). GABA is well distributed within the zebra finch song system (Pinaud and Mello, 2007). $GABA_A$ receptors have been detected electrophysiologically in song system nuclei (Bottjer *et al*., 1998; Dutar *et al*., 1998; Spiro *et al*., 1999; Farries *et al*., 2005; Mooney and Prather, 2005; Prather *et al*., 2008, for further details refer to Chapter four) and pharmacologically (for example, Carlisle *et al*., 1998; Vicario and Raksin, 2000; Farries *et al*., 2005; Mooney and Prather, 2005; Ölveczky *et al*., 2005), but this has yielded little information pertaining to the stoichiometry of receptor subtypes present, or indeed their individual physiological roles. Only the $GABA_A$ receptor γ4-subunit has been identified and the corresponding mRNA mapped throughout the entire zebra finch song system (Thode *et al*., 2008). Besides this, no

other studies have thoroughly investigated the distribution of the $GABA_A$ or $GABA_C$ receptor subunit genes within the song system.

Song is a learned behaviour which can be quantified by use of a sound spectrogram (Mooney, 1999). Due to the discrete nature of song, the song system and the distinct stages in song development, the zebra finch song system provides an excellent neuronal model for studying the underlying mechanisms of learning and memory. As $GABA_A$ receptor-mediated neurotransmission is involved in modulation of learning and memory processes, work herein aimed to fully characterise the expression of $GABA_A$ and $GABA_C$ receptor subunit genes within nuclei of the male zebra finch song system.

1.5 Project Aims

- Isolation of partial cDNAs encoding $GABA_A$ and $GABA_C$ receptor subunit genes from zebra finch (α1-α6, β2-β4, γ1, γ2, γ4, δ, π and ρ1-ρ3), which had not been previously sequenced
- Real-time reverse-transcription polymerase chain reaction (RT RT-PCR) and radioactive *in situ* hybridisation were employed to quantitatively ascertain the spatial and temporal expression profiles of $GABA_A$ receptor subunit genes in brain.
- Behavioural experiments using male zebra finches raised in auditory isolation were then completed in an effort to confirm the role of γ4-subunit-containing $GABA_A$ receptors.
- Subsequently, generation of γ4-subunit-targetting shRNA constructs were developed for RNA interference experiments to further confirm the role of these receptors in cognitive processing in zebra finch, these were tested at an *in vitro* level.
- Finally, a γ4-subunit-fusion protein was constructed and purified ready for synthesis into a γ4-subunit-specific antibody for future protein analyses.

2. MATERIALS AND METHODS

2.1 Animals

Animals were raised and treated in accordance with Home Office guidelines. Male zebra finches (*Taeniopygia guttata*) at different post hatch days (PHDs) were obtained from a variety of locations according to availability and different experimental criteria; details of which are further specified where relevant. Fertilised chicken eggs (Leghorn; *Gallus gallus domesticus*) were obtained from Henry Stewart Ltd., Lincolnshire, UK.

Zebra finches and chicks were sacrificed according to the Code of Practice for Humane Killing of Animals under Schedule 1 of the Animals Act (1986), by dislocation of the neck. Brains and any other tissues were quickly removed and frozen over dry ice before being wrapped in foil and parafilm and stored at -80^{o}C until further use.

2.2 Isolation of total RNA from tissues

2.2.1 Isolation of total RNA

Tissue was weighed and chopped prior to homogenisation in a glass-Teflon homogeniser. RNA was extracted with cold Tri® reagent (Ambion®, USA), in a 100µl per 10mg tissue ratio. On complete homogenisation, tissue was kept at 4^{o}C for 5 min and then centrifuged at 12,000*g* at 4^{o}C for 20 min (Micro 22R; Hettich, Germany). 200µl chloroform was added per ml of original Tri® reagent. The sample was vortexed briefly, maintained at 4^{o}C for a further 5 min and centrifuged at 12,000*g* for 5 min at 4^{o}C. The aqueous layer containing RNA was recovered and transferred to a sterile 1.5ml microfuge tube where 500µl isopropanol per ml original Tri® reagent was added. The sample was briefly vortexed and kept at 4^{o}C for 10 min. RNA was precipitated with ethanol as described in section 2.3.3 and the pellet resuspended in an appropriate volume of 0.1% (v/v) diethylpyrocarbonate (DEPC)-treated H_2O (DNase- and RNase-free H_2O). Unless specified, all following experiments utilised water treated in this way.

2.2.2 Determining concentration of nucleic acids

The yield of nucleic acids (5µl sample) was determined spectrophotometrically at 260nm and the purity verified by determining the 260/280 ratio (DU® 530 UV Spectrophotometer; Beckman Coulter, USA). Concentration of nucleic acids (ng/µl) was calculated by the following equation (where A is absorbance):

$$\text{Concentration (ng/µl)} = A_{260} \times \text{dilution factor} \times \begin{matrix} 20\text{ng/µl (single-stranded DNA)} \\ 40\text{ng/µl (single-stranded RNA)} \\ 50\text{ng/µl (double-stranded RNA)} \end{matrix}$$

During an RNA extraction, RNA concentration was adjusted to 80µg/108µl with H_2O. Any samples not used immediately were suspended in a 2.5x volume of absolute ethanol and stored at -80^{o}C until required.

2.3 Synthesis of complementary DNA (cDNA) from isolated RNA

2.3.1 Removal of genomic DNA

Extracted total RNA suspension (80µg) was added to the following reaction mix: 32µl 5x transcription optimised buffer (40mM Tris-HCl, pH 7.9; 6mM $MgCl_2$; 2mM spermidine and 10mM NaCl), RNasin (RNase inhibitor; 80U), 10µl dithiothreitol (DTT; 100mM), RQ1 DNase (6U; all from Promega, UK), and incubated at 37^{o}C for 15 min.

2.3.2 Phenol/chloroform extraction

Isolated RNA (this method also applies to DNA) was then subjected to a phenol/chloroform extraction to eradicate any remaining protein contamination from the nucleic acid sample. An equal volume of cold PCI solution (phenol: chloroform: isoamyl alcohol 25:24:1; pH 4.5) was added to the sample and mixed thoroughly, then centrifuged at 12,000*g* at 4^{o}C for 5 min creating a biphasic mixture. The upper aqueous phase (containing suspended RNA) was aspirated avoiding the interface and lower organic phase and transferred to a sterile 1.5ml microfuge tube. An equal volume of cold CI solution (chloroform: isoamyl

alcohol, 24:1) was added to the aqueous phase to remove any traces of phenol. The sample was mixed thoroughly and centrifuged at 12,000*g* at 4°C for 5 min. Again, the top aqueous phase was removed and added to a sterile 1.5ml microfuge tube. RNA was then recovered from the aqueous phase by precipitation with ethanol (section 2.3.3).

2.3.3 Ethanol precipitation

When DNA/RNA was required to be further concentrated, washed or used after storage in absolute ethanol, the following protocol was observed. A 2.5x sample volume of absolute ethanol and 1/10th sample volume of 3M sodium acetate pH 5.2 (300mM final) were added to the sample. Contents were gently mixed and precipitated at -80°C (RNA) or -20°C (DNA) for a minimum of 30 min. Following precipitation, the sample was centrifuged at 12,000*g* (RNA) or 20,000*g* (DNA) for 20 min at 4°C. The supernatant was aspirated then discarded and 150µl 75% (v/v) ethanol was added. Contents were mixed gently to free the pellet from the wall of the tube and remove any salts bound to the DNA/RNA. The mixture was centrifuged at 12,000*g* (RNA) or 20,000*g* (DNA) for 5 min at 4°C and all residual ethanol was removed. The pellet was left to air dry for 5-7 min and resuspended in 108µl H_2O.

2.3.4 cDNA synthesis: reverse transcription reaction

80µg RNA solubilised in 108µl H_2O was heated at 65°C for 5 min to denature RNA secondary structures before being added to the following reaction mix: RNasin (80U; Promega), 20µl 5x M-MLV buffer (Molony murine leukaemia virus; 50mM Tris-HCl, pH 8.3; 75mM KCl; 3mM $MgCl_2$ and 10mM dithiothreitol (DTT); Promega), 40µl 5mM deoxynucleotide mix (dNTPs; 5mM each of : dATP, dCTP, dGTP and dTTP; Sigma-Aldrich®, UK), random primers (9mer) (2.16µg; Promega), M-MLV reverse transcriptase (800U; Promega) and incubated at 42°C for 90 min.

2.3.5 Precipitation and washing of cDNA

cDNA was precipitated with an equal volume of 100% (v/v) isopropanol and 1/10th volume of 3M sodium acetate (pH 5.2) at -20°C for 1 h. cDNA was

subsequently centrifuged at 20,000*g* for 20 min at 4^{o}C. Isopropanol was decanted and the pellet was subjected to two subsequent ethanol precipitations (section 2.3.3) with 80% and 75% (v/v) ethanol. cDNA was then solubilised in 50µl H_2O and stored at -20^{o}C ready for use.

2.4 Amplification of $GABA_A$ and $GABA_C$ receptor subunit cDNAs

For this section of work, adult male zebra finches were obtained from a breeding colony at Newcastle University, UK. All birds were PHD >100 and brains were extracted as described in section 2.1.

2.4.1 Reverse transcription-polymerase chain reaction (RT-PCR) primer design

Degenerate oligonucleotide primers were designed to amplify specific partial cDNAs of $GABA_A$ and $GABA_C$ receptor subunit genes of interest (i.e. α3, α4, α6, γ1, δ, π and ρ1-3) from zebra finch. Sequences were already available for the remainder of the $GABA_A$ receptor subunits (i.e. α1, α2, α5, β2, β3, β4, γ2 and γ4 (GenBank accession number AM086933); as these had been previously cloned in the laboratory (data unpublished). Primers were designed adhering to standard criteria: 50% G+C (guanine and cytosine) content, 18-25mer in length and no more than three identical consecutive bases. All forward primers were in the region of sequence encoding the N-terminus and the reverse primers corresponded to a sequence that encodes part of the large intracellular loop (between transmembrane domains (TM) 3 and 4) of the subunit gene (Fig. 1.1, Chapter 1). The primers were degenerate and based on highly conserved subunit sequence domains between species and non-homologous regions between different subunits. They were designed primarily with chicken and rat sequences which were the most relevant sequences available at the time (for accession numbers refer to Appendix). For this, sequences were all obtained from the National Centre for Biotechnology Information (NCBI) gene database (www.ncbi.nlm.nih.gov/) and aligned using an online DNA alignment tool available at http://www.justbio.com/aligner/index.php, details of primers are given in Tables 2.1 and 2.2.

Target $GABA_A$ receptor gene	Forward: 5'→3'	Reverse: 5'→3'	Anneal. temp. (°C)	Cycle No.	Amplicon (bp)
α_3	ATGGA**R**TA**Y**ACTCTAGA**Y**GT**N**TT**Y**TT	GG**R**TATGT**N**GT**N**CCAAC**K**ATGTT**R**TA**R**G	53	35	882
α_4	TTGATGGTTCGAAGGTATGGACTCC	GCAGAAGA**Y**AA**D**GT**Y**TCTGTGCATT	60	40	980
α_6	TGATGAGAGGTTGAGTTTGGTGGG	CGGAGTTTACTCGTTTCTTCAGGT	62	30	878
γ_1	TTCCGGAATCGAGGAAATCTGATGC	CCTTCTAGGCACTCATATCCATATC	62	30	788
δ	**N**GCCTG**R**TT**Y**CA**Y**GA**Y**GTGAC**N**GTGG	G**S**AC**D**ATGGC**R**TTC**Y**T**N**AC**R**TCCAT**Y**T	55	40	715
π	TGGTCTTTCATGGCAACAAGAGC	TGACTTTTCATGGTCAAGTCACTGC	59	35	820

Table 2.1. RT-PCR primer combinations, detailing conditions required for successful $GABA_A$ receptor partial subunit cDNA amplification from zebra finch brain and expected amplicon sizes. Nucleotides at degenerate positions are represented by a single letter code: (**N**) A,C,G,T; (**D**) G, A, T; (**H**) A, T, C; (**W**) A, T; (**M**) A,C; (**R**) A,G; (**K**) G,T; (**S**) G, C; (**Y**) C, T.

Target $GABA_C$ receptor gene	Forward: 5'→3'	Reverse: 5'→3'	Anneal. temp. (°C)	Cycle No.	Amplicon (bp)
ρ1	TGGA**Y**GT**S**CA**R**GT**K**GA**R**AG**YY**TGG	GC**M**AGGCT**S**CACGCGC**W**GGGGA**K**CTT**M**T	57	30	822
ρ2 (outer)	TGGA**Y**GT**S**CA**R**GT**K**GA**R**AG**YY**TGG	TTCTTGGGTA**R**CC**W**GCCAGG	62	40	940
ρ2 (inner)	GAAGATCTGGGTCCC**X**GA**Y**G	GAATGA**R**G**Y**AT**Y**CCACACGTGCA	65	35	722
ρ3	TGG**R**TTTGGAGG**N**TC**D**CCA**R**T**R**CC	**Y**G**Y**TT**Y**C**D**CTCTTC**M**A**Y**TGTGG	61	40	1118

Table 2.2. RT-PCR primer combinations, detailing conditions required for successful $GABA_C$ receptor partial subunit cDNA amplification from zebra finch brain and expected amplicon sizes. Denoted lettering as in Table 2.1. *In the case of the ρ2 subunit, a nested RT-PCR was carried out (section 2.4.3).

2.4.2 Reverse-transcription polymerase chain reaction (RT-PCR)

Amplification of $GABA_A$ and $GABA_C$ receptor subunit cDNAs was carried out by RT-PCR. All 50µl reactions contained: 10µl 5x buffer (Promega); 3µl $MgCl_2$

(1.5mM final concentration; Promega); 8µl dNTPs (1.25mM; Sigma-Aldrich®), zebra finch cDNA (160ng), 200ng each of forward and reverse primer (Eurofins MWG Operon, Germany), Go*Taq*® flexi DNA polymerase (1.5U; Promega) in storage buffer B (20mM Tris-HCl, pH8.0; 100mM KCl; 0.1mM ethylenediaminetetraacetic acid (EDTA), 1mM DTT; 50% (v/v) glycerol; 0.5% (v/v) Tween®20 and 0.5% (v/v) Nonidet®-P40; Promega), made up to 50µl with H_2O. A negative control reaction was always completed concurrently, with the cDNA template omitted, to ensure products were amplified from the cDNA template and not any contamination.

Cycling conditions were as follows; 5 min *initial denaturation* (94°C) followed by, 30-40 cycles at: 1 min *denaturation* (94°C), 1 min *annealing* (Tables 2.1 and 2.2), 2 min *extension* (72°C) and a final 10 min extension at 72°C. RT-PCR reactions were carried out in thin-walled 0.5ml microfuge tubes in a thermocycler (Genius or TC-512; both Techne).

2.4.3 Nested RT-PCR

A nested RT-PCR strategy was employed for amplification of the $GABA_C$ receptor ρ2-subunit gene as there were problems with the specificity of the original primers and low levels of mRNA expression. In this case, two pairs of RT-PCR primers were used for a single locus (Table 2.2). The first pair (outer) amplified various products in a standard RT-PCR reaction as described above. In a second RT-PCR the second set of primers (inner) bind within the first PCR product, producing a shorter and more specific amplicon. This ensured that un-wanted products amplified in the first reaction were not be amplified a second time, by a second set of primers. This strategy is essential in cases like this where the expression of the gene of interest is very low, and the sequence of the gene is unknown.

The first round of RT-PCR was completed as detailed in section 2.4.2. From the 50µl reaction mix after the RT-PCR, 10µl was removed and added to 10µl 5x buffer (Promega), 3µl $MgCl_2$ (1.5mM; Promega), 8µl dNTPs (1.25mM; Sigma-Aldrich®), 200ng each (inner) forward and reverse primer (Table 2.2), Go*Taq*®

flexi DNA polymerase (1.5U; Promega) and made up to 50µl with H_2O. Cycling conditions as above.

2.4.4 Agarose gel electrophoresis

5µl of 6x loading dye (0.2% (w/v) bromophenol blue; 28% (w/v) sucrose; 26% (v/v) TE buffer (protects DNA/RNA from degradation); 10mM Tris-HCl, 1mM EDTA; pH 8) and 72% (v/v) H_2O) was added to the 50µl RT-PCR product. Separation of PCR products was performed on 1.5-2.5% (w/v) agarose (Bioline, UK) gels (depending on the expected fragment size), which contained ethidium bromide (10µg/100ml; Sigma-Aldrich®), in 1x TAE buffer (Tris-acetate EDTA; 40mM Tris-base; Sigma-Aldrich®, 20mM glacial acetic acid; Fisher Scientific, UK, 5mM EDTA; pH 8). Gels were electrophoresed between 80-100V for 45-90 min depending on the expected amplicon size. Samples were run alongside molecular weight markers; Hyperladder IV (Bioline) and 1kb DNA ladder (stock: 1.0µg/µl ladder in 10mM Tris-HCl, pH 7.5; 50mM NaCl; 0.1mM EDTA; Invitrogen™, UK). PCR products were visualised using a UV (ultraviolet) transilluminator (Syngene™, UK) at a 80 ms exposure and the images captured and analysed using Syngene™ tools software.

2.4.5 RT-PCR product purification

Products of expected size were excised from gels with a sterile scalpel and stored on ice in pre-weighed 1.5ml sterile microfuge tubes. Each DNA gel slice was purified according to the standard GFX™ PCR DNA and gel band purification kit protocol (GE Healthcare, UK). The kit utilises a chaotropic agent in acetate to dissolve agarose and proteins, DNA binds selectively to the silica membrane within the spin-column where it is washed of salts and other contaminants by an ethanolic buffer (80% (v/v) ethanol; Tris-EDTA 10mM; Tris-HCl, pH 8.0; 1mM EDTA). DNA was then eluted with 50µl H_2O and used immediately or stored at -20°C.

2.5 Cloning of partial cDNAs

2.5.1 Ligation

PCR products were ligated into the pGEM®-T Easy vector (Promega; for full vector map see Appendix). The linearised vector has single thymine (T) base overhangs which covalently bind with complementary adenine (A) overhangs on the amplified product added during *Taq* polymerase RT-PCR. Each ligation reaction contained: 7.5µl 2x buffer T4 (60mM Tris-HCl, pH 7.8; 20mM $MgCl_2$; 20mM DTT, 2mM ATP and 10% (v/v) polyethylene glycol), pGEM®-T Easy vector (50ng), T4 DNA ligase (3U; all Promega) and 5.5µl purified RT-PCR product. This was ligated overnight at 4°C.

2.5.2 Synthesis of competent Escherichia coli XL1-Blue and TB1 cells

Competent cells are able to take up naked exogenous DNA from the environment; *E. coli* XL1-Blue and TB1 cells were made artificially competent by chemical treatment with calcium chloride. Both types of competent cells used for different experiments were synthesised in the same way. A 100µl aliquot of a previous *E. coli* XL1-blue/TB1 culture was taken from storage at -80°C, defrosted on ice and added to 2ml LB (Luria Bertani) medium (1% (w/v) peptone from casein; 0.5% (w/v) yeast extract; Merck, UK; 1% (v/w) NaCl; Sigma-Aldrich®; pH 7.0) supplemented with 0.1% (v/v) tetracycline (100µg/ml; Sigma-Aldrich®). The culture was incubated at 37°C, 200 rpm (revolutions per minute) overnight in an orbital shaker (S150 Orbital Incubator, Stuart Scientific, UK). 2ml of the overnight culture was then added to 100ml pre-warmed LB medium (100µg/ml tetracycline). Immediately, a 1ml aliquot of the inoculated culture was transferred to a cuvette and stored on ice to be used as a blank. The inoculated culture was then incubated as before. After an initial 2 h incubation, absorbance (A_{660}) was measured every 30 min until it reached an optical density (OD) of ~0.5. At this stage the culture was divided between two 50ml tubes and centrifuged at 900*g* (Allegra® X-15R: Beckman Coulter) at 4°C for 15 min. The supernatant was discarded and each pellet resuspended in 20ml filter sterilised (0.2µm), cold TFB I buffer (transformation buffer 1; 30mM potassium acetate; 50mM $MnCl_2$; 100mM KCl; 10mM $CaCl_2$; 15% (v/v) glycerol; pH 5.8), and incubated on ice for 30 min.

Cells were centrifuged again at 900*g* for 20 min at 4°C, the supernatant discarded and each pellet gently resuspended in 2ml filter sterilised (0.2µm) cold TFB II buffer (10mM 3-(N-Morpholino) propanesulfonic acid (MOPS); 76mM $CaCl_2$; 10mM KCl; 15% (v/v) glycerol, pH 6.5; all Sigma-Aldrich®). Ensuring the cells were kept chilled at all time, 100µl aliquots were carefully prepared and stored at -80°C until use.

2.5.3 Transformation of competent E.coli XL1-Blue cells

The pGEM®-T Easy vector contains the *lacZ* gene that encodes for the protein β-galactosidase, which metabolises X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) to produce bright blue colonies. If the open reading frame of this gene is interrupted by the presence of an inserted sequence, β-galactosidase is non-functional. By adding IPTG (isopropyl β-D-1-thiogalactopyranoside the *lacZ* gene inducer) and X-Gal (the substrate) to the agar plate, this chromogenic assay visually indicates the presence or absence of an insert. 15µl ligation mix was added to 100µl XL1-Blue cells and incubated on ice for 45 min (chilling in the presence of divalent cations prepared the cell walls to become permeable to plasmid DNA). Cells were then thermal shocked at 42°C for 1 min to incorporate the plasmid into the cells and immediately placed back on ice. 150µl cold SOC (formal name) medium (2% (w/v) bacto-tryptone; 0.5% (w/v) bacto-yeast extract; 10mM NaCl; 10mM KCl; 20mM Mg^{2+} and 20mM glucose) was added and cells were incubated at 37°C whilst shaking at 200 rpm for 30-45 min (sufficient time for expression of the antibiotic resistance gene prior to plating). Culture was then divided equally between three replicate LB agar plates (1% (w/v) peptone from casein; 0.5% (w/v) yeast extract; 1% (v/w) NaCl; 1.2% (w/v) agar-agar, pH 7.0; Merck) supplemented with 0.1% (v/v) ampicillin (100µg/ml; Sigma-Aldrich®), which were previously spread with 100µl IPTG (200mg/ml; Sigma-Aldrich®) and 40µl X-Gal (20mg/ml: Fluka Chemicals, Germany) and incubated overnight at 37°C in a humid environment.

2.5.4 Overnight cell culture

Recombinant cells were differentiated by blue/white colony screening. Selected white colonies were sub-cultured using a sterile toothpick to gently transfer an

individual colony into 2ml LB medium supplemented with 0.1% (v/v) ampicillin (100µg/ml; Sigma-Aldrich®). Cultures were incubated overnight at 37°C with agitation at 200 rpm, to ensure good aeration.

2.5.5 Isolation of plasmid DNA

pGEM®-T Easy vectors containing cloned inserts were isolated from recombinant *E. coli* cells using a GenElute™ plasmid miniprep kit (Sigma-Aldrich®), according to the manufacturer's protocol. The kit contains a series of solutions whereby cells are lysed with a sodium dodecyl sulfate **(**SDS) mixture, the reaction is neutralised, DNA is adsorped onto silica, impurities are washed away with an ethanolic buffer and bound DNA is eluted with 50µl H_2O.

2.5.6 Restriction digestion

Each cloned PCR fragment was digested from the pGEM®-T Easy vector with an *Eco*RI restriction digest, the vector contains recognition sites for this enzyme either side of the insert in the multiple-cloning sites (see Appendix). Reaction mix contained; 1µl eluted plasmid DNA, 1.5µl 10x Buffer H (90mM Tris-HCl, pH 7.4; 10mM $MgCl_2$; 50mM NaCl), *Eco*RI (6U; Promega), made up to 15µl with H_2O. This was incubated at 37°C for 90 min. Gel electrophoresis was performed with digested clones (section 2.4.4), alongside an undigested plasmid DNA sample. Clones containing an insert of the anticipated size were sent for automated DNA sequencing (Eurofins MWG Operon, UK). Multiple clones were sequenced and aligned using online alignment software (section 2.4.1) to identify any errors brought about by the *Taq* DNA polymerase or sequencing inaccuracies.

2.6 Radioactive *in situ* hybridisation

Radioactive *in situ* hybridisation utilises a radiolabelled nucleic acid probe to localise specific RNA transcripts within a tissue. Tissues are fixed and the radiolabelled complementary DNA probe is able to hybridise at an elevated temperature with target mRNA in the tissue. Excess probe is then washed away and the target mRNA bound to the radiolabelled probe in the tissue can be localised by autoradiography.

2.6.1 Oligonucleotide probe design

Synthetic oligonucleotide probes were designed which were complementary to mRNA encoding the zebra finch $GABA_A$ and $GABA_C$ receptor subunit genes as detailed in Table 2.3. All probes adhered to the following criteria where possible: 45mer in length, between 45-55% G+C content, no more than 3 consecutive bases, bridging of an exon boundary (to ensure high specificity) and non-homologous (i.e. no sequence similarity) with other $GABA_{A/C}$ receptor subunit mRNAs. Two probes were designed to target each subunit gene to ensure an accurate mRNA distribution was observed. All sequences were analysed using BLAST (Basic local alignment search tool) as per section 2.4.1. Oligonucleotides were produced commercially at Eurofins MWG Operon (Germany), and delivered in a lyophilised form.

Target gene	Probe sequence (5'→3')	mRNA sequence location	%GC
$GABA_A$ receptor subunit genes			
α1*	TGCTGGTTCCCGTGTCCACTCATACACAACCTCTGCTCTGGTATA	N-terminus	51
α1	TCTCACAGTCAATCTCATTGTGTATAGCAACGTCCCATCCTCTGT	N-terminus	44
α2	GCCTAAAAGATCATACTGGTTCAGTCTTGAGCCATCTGGTGCCAC	N-terminus	49
α2*	GAGCCGTCATTACTGTATATTCACCTGTGCTTGACTTAACTGTCT	N-terminus	42
α3	GTATTCGCCTGTGCTGGATCGAACCATCTCTGTCCCAACCACGTG	N-terminus	56
α3*	TTTTCTTCATCTCCTGGGCCTCCAGCACCTTCTTGCCATCCCAGG	Loop	56
α4*	ACACTCTGCACTTATTGTAAGCCTCATTGTGTACAGGATGGTGCC	N-terminus	47
α4	CCTTTGTACTGGAGCAACAGGAAATTCAAGAAGGGACTTGACCGT	Loop	47
α5*	TTCACCTGTACTGGTACTGATGTTTTCCGTGCCCACGGTTTGTCC	N-terminus	51
α5	CGTGAGCCATCTTCGGCCACCACCACAGACGTTGTGGTGCTGTTA	N-terminus	58
α6*	TTGGAGTCAGAATGCGAGACAATCTCGTCCTCTGCCGGTACAGTT	Loop	51
α6	TGGAGAAGACTGGAAGACTCCTGTGGTACTTCTACTGAATGCAAT	N-terminus	44
β2	GAGATGTCCACGGATGGGTTCCGACAGAGAGTCGAAATTTGAATT	N-terminus	47
β2*	TTGAAGAAGGAAAAAACAACGGGGAAGAATATGCGAGACCATCGA	Loop	42
β3*	TTCCACCTGAAGCTCAGTGAAAGTCTTGGATAGGCACCTGTGGCA	N-terminus	51
β3	AATGTGAATGGAAACACCATTCTTGACCATCTGTCGATGGGATTC	Loop	42

β4	CTTGTCAATGGTGCTGACGTCTGTCAGGTCTGGGATTTTGAGCTT	Loop	49
β4*	ACAGCAGAGTCATTCCCTTGCCAGAAGAAGACAATGTCATCCACC	N-terminus	49
γ1*	TTGGAATTAGTGTTGATCCAGGTCGAACAGCAATTGCAGGTGGTT	Loop	44
γ1	TTCTGGGATATCCATAGCTTGAAAACTCCAGAGGACAGGAGTGTT	N-terminus	44
γ2*	CATTTGAATGGTAGCTGATCGGGGGCGGATGTCAATAGTGGGTGC	Loop	53
γ2	TCTCCTGAAGTAGTCTTCACCACCTCAGTTGTGTTTCTTAGTCCT	N-terminus	44
γ4	TGCATGATGTGGTTGATGTTGATGGTGGTGAAGGTTGGCATCACC	Loop	49
γ4*	AGAGTGTAGAGCACTTTTCCATCGTTCCAGATGCGGAGGAGCTGG	N-terminus	53
δ*	AAGGTGGAAGTGGAGACTGAGCCTGGGAAACTGACCAGATTTGAA	N-terminus	49
δ	TCCTCTGAAGAGTAGCCATAGCTCTCCAAATCCAACATGCATTCC	N-terminus	47
$GABA_C$ receptor subunit genes			
ρ1*	TACCACCCTGTGCTGCTGTAAAAGGCAAGCTTTGTGGTAGTGTGA	N-terminus	49
ρ1	CAGCCAGATGGGAAGGTACTTTATAGCCTCAGAGTTACAGTAACA	N-terminus	44
ρ2*	CACTGTGATCCTCATGCTGTAGAGGACATGACCATCAGGGAACAC	N-terminus	51
ρ2	GTCTCAATTCTTGATACAGAAATTCCACACTACATCAAGACTGGC	N-terminus	40
ρ3	GCAAGTCCACTGGAAGCACTGAATTCCTCAATGAAGAACTGGGAG	N-terminus	49
ρ3*	AGCAGAAACAGTTATTCTCAGACTGAAGAGGACATTGCCATCGGG	N-terminus	47

Table 2.3. Sequences encoding 45mer synthetic oligonucleotide probes complementary to the subunit-sequences of the $GABA_A$ and $GABA_C$ receptors. Sequences shown are the reverse complement to the sense strand. Two probes targeting each subunit mRNA were designed and tested, and in all cases gave identical binding patterns with all genes. Probes denoted with an (*) were utilised for all subsequent experiments.

2.6.2 Silanisation of microscope slides

The majority of animal tissues carry a negative charge. To ensure optimal adherence of the tissues to microscope slides, slides were pre-treated with (3-Aminopropyl) triethoxysilane (Sigma-Aldrich®) which creates a positively charged surface and allows covalent bonding of tissue sections. Slides (twin-frost 0.8-1.0mm thick; VWR™, UK) were initially sterilised at 180°C for 3 h and were then passed through a series of baths: 30 sec in 100% (v/v) acetone; 5 min in 2% (v/v) (3-aminopropyl)-triethoxysilane in acetone; 2x 2 min rinses in 100% (v/v) acetone (to remove (3-aminopropyl) triethoxysilane) and 3x 2 min rinses in H_2O

(to remove acetone). Once slides were completely dry, they were sterilised for a further 3 h at 180^{o}C.

2.6.3 Rearing of zebra finches and sectioning of brains

For initial *in situ* hybridisation experiments (localisation of $GABA_{A/C}$ receptor subunit transcripts within the song system), adult male zebra finches (all PHD >100) were obtained from a breeding colony at The University of Sheffield and sacrificed as in section 2.1.

A second set of *in situ* hybridisation studies were undertaken to investigate whether $GABA_A$ receptor γ4-subunit mRNA expression in zebra finches was affected by song learning, whereby birds were able to sing properly (aviary-reared, with tutor) or unable to produce 'normal song' (cage-isolated, without tutor). All birds were reared at sacrificed at Max-Plank-Institut für Ornithologie in Seewiesen, Germany. The work formed two separate parts. For the primary experiments there were two groups of zebra finches (all male; n = 5 per group); the first group were 'normally' reared birds in that they were raised in an aviary with their parents and other conspecifics; the second group were raised in individual isolation cages where they remained with their parents until PHD 5 and then were raised in the cage alone, in physical and auditory isolation. In the second set of experiments there were three groups of zebra finches (all male; n=5 per group). The first group were raised in an aviary with tutors and conspecifics, as before. A second group were raised in individual isolation cages with their parents (tutor present). A third group were raised in individual isolation cages, but instead of being alone (as in the preliminary work), they were accompanied by their parents until PHD 7, their mother until PHD 30 and then reared by two unrelated females (which do not sing) until PHD 80. Songs produced by birds in the experimental groups were recorded with a sonogram at PHD 80 in the presence of an unfamiliar female bird (directed song). All birds were sacrificed at PHD 80, their brains removed, immediately frozen over dry ice and shipped to the laboratory in the fastest possible time, where upon receipt, they were immediately stored at -80^{o}C until use. Brains were sectioned on a cryostat (CM 1900; Leica, Germany) where 10µm coronal sections were cut and freeze-thaw mounted onto

pre-silanised slides (section 2.6.2) at -12^{o}C. Mounted cryo-sections were then stored at -80^{o}C until use.

2.6.4 Fixation of tissue sections

Tissue fixation ensures the preservation of tissue morphology and protects the integrity of RNA in cells. Paraformaldehyde is a cross-linking fixative; the aldehyde group binds primarily with nitrogen groups on proteins in the tissue where it forms a methylene bridge. Nucleic acids are retained in the matrix of the cross-linked protein molecules and they themselves remain chemically unaffected. Prior to hybridisation, the sections were fixed for 5 min in 2% (w/v) paraformaldehyde (Sigma-Aldrich®) in phosphate buffered saline (PBS; 130mM sodium chloride, 7mM disodium hydrogen orthophosphate, 3mM sodium dihydrogen orthophosphate, pH 7.4) at 4˚C. Sections were then washed twice (5 min each) with PBS at room temperature, dehydrated in 70% (v/v) ethanol followed by 100% (v/v) ethanol, and left to air dry.

2.6.5 Labelling of the oligonucleotide probe

Each oligonucleotide probe was radioactively labelled at the 3' end with ^{35}S (deoxyadenosine 5' (alpha-thio) triphosphate). Terminal deoxynucleotidyl transferase, recombinant (rTdT) catalyses the repetitive addition of mononucleotides (radiolabelled adenosine) to the terminal 3'-OH of a DNA initiator, in this case the ssDNA probe. The mix was as follows: 5µl 5x TdT buffer (100mM cacodylate buffer pH 6.8; 1mM $CoCl_2$ and 1mM DTT; Promega); 5ng oligonucleotide probe (resuspended in H_2O); α^{35}S-dATP (15.625µCi; Perkin-Elmer® Life Sciences Ltd., USA); rTdT (30U; Promega) made up to 25µl with H_2O. The mix was incubated at 37^{o}C for 75 min. Following this, 1µl of the reaction was added to 5ml scintillation fluid (Ultima Gold™; Perkin Elmer® Life Sciences Ltd.) to verify activity before purification of the probe.

Any unincorporated nucleotides from the labelling reaction were removed using commercially prepared spin columns (Sephadex G25 suspended in 1x sodium chloride–Tris–EDTA buffer; Roche Applied Science, UK) according to the manufacturer's protocol; the columns use gel-filtration chromatography to

separate molecules according to their size whereby smaller molecules are retained (unincorporated nucleotides) and larger molecules such as oligonucleotides can pass through. Activity of the purified radiolabelled probe was quantitated by adding 1µl purified probe to 5ml scintillation fluid in a scintillation vial (Packard®, USA) and measuring on a liquid scintillation analyser (Tri-Carb® 2250CA: Packard®), along with the sample taken prior to purification. This determined the counts per minute (cpm) of 1µl purified probe and thus the volume of probe needed to hybridise slides at 100,000 cpm/slide could be calculated.

2.6.6 Hybridisation of the oligonucleotide probe

Hybridisation (200µl of buffer per slide under parafilm cover slips) was performed in: 50% (v/v) deionised formamide (Fluka Chemicals), 4x standard saline citrate (SSC – the high ionic strength solution maximises the rate of probe annealing, 600mM NaCl (60mM Tri-sodium citrate dehydrate pH 7; Sigma-Aldrich®), 5x Denhardt's solution (this contains high molecular weight polymers capable of saturating non-specific binding sites), 25mM sodium phosphate (pH 7), 1mM sodium pyrophosphate, 20mM DTT (this is used in all solutions concerning *in situ* hybridisation as ^{35}S can give high background signals), 100µg/ml polyadenylic acid, 120µg/ml heparin (this increases specificity of the *in situ* hybridisation), 200µg/ml denatured, fragmented salmon sperm DNA (competitive blocking agent; all Sigma-Aldrich®), 10% (w/v) dextran sulphate (this increases probe binding to target mRNA by increasing the rate of re-association of nucleic acids; Fluka Chemicals) and 100,000 cpm/µl radiolabelled probe. Sections were hybridised in a humid atmosphere at 42°C overnight. Control slides for each experiment contained, in addition, a 200-fold excess of the same unlabelled oligonucleotide, this competitive hybridisation verified the hybridisation of the probe was specific.

2.6.7 Washing of sections

Hybridised sections were initially rinsed in 1x SSC (150mM NaCl; 15mM Tri-sodium citrate dehydrate, pH 7.0) at room temperature, to remove the cover slips and the majority of the hybridisation buffer. Slides were then subjected to two high stringency washes for 30 min each at 55^{o}C in a shaking water bath (Clifton,

UK). The washes were followed by rinsing in 1x SSC then 0.1x SSC (30 sec each) at room temperature. Hybridised sections were then dehydrated in 70% (v/v) ethanol and 100% (v/v) ethanol (30 sec each), loosely covered and left to air dry for 2-3 h. Slides were secured (arranged randomly) in a standard X-ray film cassette where they were apposed to Kodak Biomax MR X-ray film (Perkin-Elmer® Life Sciences Ltd). β-electrons from the ^{35}S are completely absorbed by the film to produce a global image. Exposure time was usually one month unless otherwise stated. Two films per experiment were developed after two different exposure times to ensure that any densitometric measurements were taken in the linear range of the film. All exposures were completed at room temperature.

2.6.8 Densitometric analysis of autoradiographs

Autoradiographs were developed (Compact X4 developing machine; Xograph Healthcare Ltd., UK) and individual images of sections were visualised with a light box and CCD camera (TM-6CN PULNiX Europe Ltd.) then captured using Easygrab Picolo™ software (Euresys™, Belgium). mRNA expression was quantified by densitometric analysis of individual brain sections using Scion image software (Scion Corporation, USA). Brain regions (more specifically, the nuclei of the song system), were identified by use of a stereotaxic atlas of the zebra finch brain (available at www.ncbi.nlm.nih.gov/books/bookres.fcgi/atlas/atlaspdf) in combination with a recent publication of a three-dimensional magnetic resonance imaging stereotaxic atlas of the zebra finch brain (Poirier *et al*., 2008). Autoradiographic signal in areas of interest was calculated as pixel density per brain area measured. Three consecutive brain sections containing the nucleus of interest were measured to eliminate the possibility of section-specific labelling. Densitometric readings of the background were also taken to remove any fluctuations in signal intensity which were not a result of probe binding. Furthermore, internal control measurements were taken from the Nidopallium which is a brain area not associated with learning and memory. This was verified by comparison of gene expression in this region which remained unaffected by the different parameters of the experiment (Figs. 5.3, 5.6 and 5.10, Chapter 5). These readings were considered as baseline (100% signal), and thus the autoradiographic signal (mRNA expression) from other brain regions could be expressed as a

percentage of the baseline. Data is expressed as mean ±SE and the significance of any differences between mRNA expression in any of the nuclei of the three groups of birds were analysed by one way analysis of variance (ANOVA).

2.7 Real-time quantitative RT-PCR

Real-time RT-PCR was employed to fully quantify expression of mRNA encoding selected $GABA_A$ receptor subunit genes (α1, α2, α3, α4, β2, β3 and γ4) within Area X, HVC and RA of the song system and any changes of mRNA expression during important developmental stages. Facilities for the micro-dissection of the individual song-system were not available to us and so the work was carried out by our collaborative group at the Max-Plank-Institut für Ornithologie in Germany.

2.7.1 Animals and tissue dissection

Male zebra finches were used at PHD 30, 55, 80 and 100 and were reared in breeding aviaries (6 m^3; 7 breeding couples) at the Max-Planck-Institut für Ornithologie, Seewiesen. The following light regime was used: 0.5 h dawn, 14 h light, 0.5 h dusk, 9 h darkness. Experimental birds were collected in the same season of the same year; they were sacrificed and their brains quickly removed and shock-frozen. Constant areas of HVC, Area X and RA tissue were micro-dissected from cryostat sections of both hemispheres by means of a laser interfaced into a research microscope. All experiments were performed with permission of the government of Oberbayern.

2.7.2 Isolation of RNA

In brief, RNA was purified from the micro-dissected nuclei of zebra finches following the manufacturer's instructions of the RNeasy® Micro Kit (Qiagen, UK) and any remaining DNA was digested in the RNeasy MinElute spin column with RNase-free DNase I, the RNA was eluted with H_2O. The quality and yield of RNA was digitally determined on a 2100 Bioanalyzer using the pico-chip (Agilent Technologies, USA). First-strand cDNA synthesis was carried out with the SuperScript™ III First-strand synthesis system for RT-PCR (Invitrogen) following the standard protocol with random hexamers and equal amounts of RNA (10ng).

2.7.3 Real-time RT-PCR primer design

Specific primers were designed against a selection of $GABA_A$ receptor subunit genes (α1, α2, α3, α4, β2, β3 and γ4), β-actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and hypoxanthine-guanine-phosphoribosyl transferase 1 (HPRT1) gene sequences (Table 2.4) of zebra finch using the Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). All primers adhered to the standard stringent real-time RT-PCR primer criteria: amplicon between 80-250bp and should span intron/exon boundaries, primer length between 15-25bp, no more than three consecutive bases. All primer pairs require the same melting temperature (T_m) and typically the same annealing temperature to avoid differing reaction efficiencies. The efficiency (E) was 95 – 100 % for all primer pairs used.

Target gene	Forward: 5'→3'	Reverse: 5'→3'	Amplicon size (bp)
$GABA_A$ R α_1	ACTGTGAGAGCAGAATGTCCAA	TTCCCGTGTCCACTCATACA	123
$GABA_A$ R α_2	CCAATGCACTTGGAGGACTT	CAGTCTTGAGCCATCTGGTG	147
$GABA_A$ R α_3	CAGTCTTGAGCCATCTGGTG	ACCCTTGGCTACTTCCACAGATTT	155
$GABA_A$ R α_4	TGGTCATGCTTGTCCTCTCA	ATGCCCAAGCAGGTCATACT	142
$GABA_A$ R β_2	GTGGGGGTGATAATGCAGTC	AAGCTGAGAGACAGCCTTGG	130
$GABA_A$ R β_3	GTCACTGGCGTGGAGAGAAT	CCACCTGAAGCTCAGTGAAAG	120
$GABA_A$ R γ_4	GGAACGATGGAAAAGTGCTC	CCTCCCGAGGGTATCCATAA	132
β-actin	AACCGGACTGTTTCCAACAC	CACCTTCACCGTTCCAGTTT	143
GAPDH	CCAACCCCTAACGTGTCTGT	TGTCATCGTACTTGGCTGGT	68
HPRT 1	ACGACCTGGATTTGTTCTGC	TTTCTCGTGCCAGTCTCTCC	108

Table 2.4. Real-time RT-PCR primers and amplicon sizes for various $GABA_A$ receptor subunits and the house-keeping genes β-actin (GenBank accession number XM_002192780), glycerinaldehyd-3-phosphat-dehydrogenase (GAPDH; GenBank accession number AF255390) and Hypoxanthine-guanine-phosphoribosyl-transferase 1 (HPRT 1) of the zebra finch. All $GABA_A$ receptor subunit-specific primers were designed from sequences cloned within our laboratory and sent to our collaborators in Germany where the RT-PCR reactions were performed.

2.7.3 Real-time RT-PCR

Reverse transcriptase (RT) reactions were performed in duplicate with a non-RT control for each RNA sample. For qPCR, 12.5µl *Power* SYBR® Green PCR master mix (Applied Biosystems, USA) and 2µl of each RT reaction (1:5 dilution) were used. qPCRs were run on separate 96-well plates for each song nucleus with RT and non-RT reactions in a final volume of 25µl, and a final primer concentration of 100 nM. Assays were performed on an Mx300 5P™QPCR System (Stratagene). Cycling conditions were as follows; 10 min *initial denaturation* (95 ºC), 45 cycles at: 30 sec denaturation (95 ºC), 1 min *annealing* (60 ºC) and 30 sec *extension* (72 ºC). A melting curve was generated at the end of every run to ensure product uniformity. Product sizes were confirmed with electrophoresis on 3.0% (w/v) agarose gels stained with ethidium bromide (section 2.4.4).

2.7.4 Relative quantification of mRNA levels

The mean number of cycles of threshold (CT) determined for the reference genes (ref), β-actin, GAPDH and HPRT1 and primer pair efficiencies (E) were used to calculate normalised mRNA expression levels of $GABA_A$ receptor subunits following the equation for MNE (mean normalised expression) (Muller *et al*., 2002 and Simon, 2003)

$$MNE = (E_{ref,\ mean})^{CT\ ref,\ mean} / (E_{GABAAR})^{CT\ GABAAR}$$

To visualise expression of $GABA_A$ receptor subunit mRNAs at different developmental stages during song learning, MNE levels were expressed in relation to the level at PHD 30 (100%), this was the earliest developmental stage analysed. Comparisons of relative expression levels were conducted using two-way analyses of variance (ANOVA) followed by LSMeans Tukey's post hoc tests. For considering the problem of multiple comparisons (seven genes per song control nucleus) the sequential Bonferroni correction procedure was used.

2.8 Generation of γ4-subunit-specific antibodies

Although real-time RT-PCR and *in situ* hybridisation were useful tools in providing a qualitative and quantitative insight into expression levels and spatial

distribution of the $GABA_A$ receptor subunit mRNAs, it was important to consider if this corresponded at a protein level, with particular reference to the $GABA_A$ receptor γ4 subunit. Two different strategies were employed in order to generate specific antibodies (as none were commercially available), the first involved synthesis of a protein antibody using fusion-protein technology and the second was generation of a peptide antibody which was out-sourced commercially.

2.8.1 pMAL™ protein fusion and purification system

A fusion protein was generated using the pMAL™ protein fusion and purification system (New England Biolabs® Inc., USA). In this system a cloned sequence, encoding a region of the $GABA_A$ receptor γ4-subunit sequence was inserted into the pMAL™-c2X vector in the same translational reading frame, downstream from the *malE* gene, which encodes maltose-binding protein (MBP); this results in the expression of a MBP-fusion protein. The fusion protein was then purified by amylose-affinity chromatography (specific for MBP) and the target protein could then be cleaved from the fusion protein by a specific protease (factor Xa).

2.8.2 Design and construction of the fusion plasmid

A 120bp region of the target $GABA_A$ receptor γ4-subunit nucleotide sequence (GenBank accession number: AM086993) was carefully selected, adhering to the following criteria:

1) Sequence must have no similarity with other $GABA_A$ receptor subunits
2) Sequence must have no internal restriction sites that are of relevance
3) Sequence must not start with an arginine residue
4) Sequence must contain a stop codon (TAG/TGA/TAA)
5) Sequence must not show homology to the factor Xa cleavage site (ATC GAG GGA AGG ATT)
6) Sequence must ligate into polylinker site in the vector, i.e. have appropriate restriction sites (the *Eco*R1 restriction site was selected as this ensured that there was minimal vector derived sequence remaining after cleavage with factor Xa)
7) Sequence must be in the same reading frame as the *malE* sequence

A region of the $GABA_A$ receptor γ4-subunit nucleotide sequence was identified in the large intracellular loop (between TM3 and TM4), as this is the part of the subunit sequence which shares the lowest homology with other $GABA_A$ receptor genes. Once the region of sequence was selected, specific primers were designed to amplify this section with RT-PCR. Some single-base alterations were engineered into the primer design to ensure that a translational stop codon (TAG – highlighted in grey) and the desired recognition sites for the *Eco*R1 restriction enzyme (underlined and modified bases highlighted in bold) were present in the correct positions. Primer sequences were: 5'-CTGGTG**GAATTC**AAGAAACCA CTG-3' and 5'-GGGC**GAATTC**ATCTAGTGGTT GAT-3' (both primers had 46% G+C and T_m 60°C). Primers were subjected to a BLAST search to verify there was no homology to the other $GABA_A$ receptor subunit mRNAs.

2.8.3 Amplification and cloning of the fusion protein

RT-PCR was performed with single stranded cDNA synthesised from adult male zebra finch brain as described in section 2.4.2. Cycling conditions were as follows; 5 min *initial denaturation* (94°C) followed by 30 cycles at: 1 min *denaturation* (94°C), 30 sec *annealing* (56°C) and a 30 sec *extension* (72°C). RT-PCR reactions were carried out in thin-walled microfuge tubes in a thermocycler (TC-3000; Techne). The RT-PCR product was run on a 2.5% (w/v) agarose gel and visualised as per section 2.4.4. The amplicon of expected size (120bp) was excised and purified as per section 2.4.5.

The DNA fragment and pMAL™-c2X vector (New England Biolabs® Inc.) were then subjected to *Eco*R1 restriction digestions to create complementary 'sticky ends' for ligation. Reaction mixes were as follows: 5µl purified RT-PCR product, 1µl 10x buffer H, *Eco*RI (6U; Promega), made up to 10µl with H_2O which was incubated at 37°C for 90 min. For linearisation of the plasmid: 500ng vector, 2µl buffer H, *Eco*RI (6U; Promega) made up to 20µl with H_2O. Both samples were digested at 37°C for 90 min. Digested DNA/plasmid was then immediately purified by use of the GFX™ PCR DNA and gel band purification kit (GE Healthcare) protocol as per section 2.4.5. The linearised pMAL™-c2X vector (40ng) and digested RT-PCR product (1µl) were then combined in a ligation

reaction with T4 DNA ligase (6U; Promega), 2µl 10x buffer (as per section 2.5.1; Promega) made up to 20µl with H_2O and ligated at 4^{o}C overnight.

The vector containing the insert was transformed into chemically competent *E. coli* TB1 cells (section 2.5.2-2.5.5). However, unlike with previous cloning, there was no X-Gal or IPTG added to the LB agar plates as the manufacturers (New England Biolabs® Inc.) suggested they would cause loss of part of the fusion gene or dramatically reduce the expression levels of the gene due to the strength of the P_{tac} promoter. Therefore, the plates were incubated overnight on standard LB agar plates supplemented with 0.1% (v/v) ampicillin (100µg/ml; Sigma-Aldrich®) at 37^{o}C and the resulting colonies picked with a sterile toothpick and stabbed onto a master LB agar plate (0.1% (v/v) ampicillin) and then onto an LB agar plate (0.1% (v/v) ampicillin) which was layered with 100µl IPTG (200mg/ml) and 40µl X-Gal (20mg/ml). These were incubated at 37^{o}C overnight and any positive 'white' colonies on the X-Gal/IPTG plate were recovered from the corresponding patch on the master plate. Colonies were subcultured as per section 2.5.4 and then screened for the presence of inserts by minipreparation (section 2.5.5) and subsequent *Eco*R1 digestion (section 2.5.6). The digested plasmid clones were electrophoresed on a 2% (w/v) agarose gel (section 2.4.4) and any samples containing the insert of expected size were sent for automated sequencing (Eurofins MWG Operon, UK) with the *Mal*E sequencing primer (5´ GGTCGTCAGACTGTCGATGAAGCC 3'; New England Biolabs® Inc.), which generates sequence data from inserts within the polylinker region of the pMAL™-c2X vector. The sequence was verified for any inaccuracies against the original sequence design and $GABA_A$ receptor γ4-subunit mRNA sequence. The successful plasmid containing the insert was stored at -20^{o}C ready for use.

2.8.4 Pilot experiment

It was first necessary to determine the behaviour of the MBP-fusion protein before any larger-scale experiments were undertaken.

2.8.4.1 Transformation

A 10ng sample of suspended plasmid DNA (from the minipreparation containing the successful fusion plasmid) was added to 50µl of competent *E. coli* TB1 cells and placed on ice for 2 min. Following a 30 sec, 42^{o}C thermal shock, the mixture was placed back on ice for 2 min, where 150µl room temperature SOC medium was added. 100µl of the cells were spread on an LB agar plate (0.1% (v/v) ampicillin) and incubated overnight at 37^{o}C.

2.8.4.2 Subculturing

Two colonies were subcultured into 2ml LB medium (0.1% (v/v) ampicillin) and incubated at 37^{o}C overnight at 200 rpm in an orbital shaker with good aeration. The following day, 80ml of rich broth (1% (w/v) bacto-tryptone; 0.5% (w/v) bacto-yeast extract; 0.2% (w/v) glucose; 90mM NaCl) was inoculated with 1ml of the overnight culture. Cells were grown at 37^{o}C with agitation at 200 rpm to 2x 10^{8} cells/ml (A_{600} ~0.5); this took approximately 3 h, (1ml samples were taken every hour and the absorbance read against a blank taken immediately after inoculation and stored on ice). A 0.5ml sample was withdrawn and microcentrifuged at 20,000*g* for 2 min, the supernatant was discarded and the cells were resuspended in 50µl 2x sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (Laemmli; 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol, 0.004% (w/v) bromophenol blue and 0.125 M Tris HCl, pH 6.8; Sigma-Aldrich®), vortexed to mix and stored at -20^{o}C. These were the uninduced cells (sample 1).

2.8.4.3 IPTG induction

IPTG was added to the remaining culture to a final concentration of 0.3mM and incubation was continued as before for 2 h. A 0.5ml sample was withdrawn and treated as above (Sample 2, induced cells). Remaining culture was divided between two 50ml centrifuge tubes and to each tube, 0.5ml protease inhibitor cocktail was added (215mg cocktail: 1ml DMSO: 5ml dH_2O). Protease cocktail is a mixture of protease inhibitors with broad specificity for the inhibition of serine, cysteine, aspartic and metallo-proteases, and aminopeptidases; containing 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF), pepstatin A, E-64, bestatin, and

sodium EDTA; Sigma-Aldrich®). Cells were harvested by centrifugation at 4000*g* for 10 min (AvantiTM J-30; Beckman Coulter). Supernatants were discarded and each pellet was resuspended in 2.5ml column buffer (1M Tris-HCl, pH 7.4; 0.5M EDTA; 11.7% (w/v) NaCl and 0.154% (w/v) DTT; all Sigma-Aldrich®). The two samples were pooled and 2ml protease cocktail was added, this was frozen overnight at -20^{o}C.

2.8.4.4 Protein extraction from cells

Cells were defrosted slowly in cold water and were transferred to a beaker sat in a bath of ice. The suspension was sonicated (Soniprep 150 ultrasonic disintegrator; MSE UK Ltd.) at an amplitude of 9 microns, to release cellular proteins, in 15 sec pulses for two minutes. Optimum sonication duration and amplitude for maximum protein release was assessed by the bicinchoninic acid (BCA) assay (section 2.8.7.3). Cells were transferred back to a 50ml centrifuge tube and centrifuged at 9000*g* for 20 min at 4^{o}C. The supernatant was decanted (crude extract) and stored on ice and the pellet resuspended in 5ml column buffer (insoluble matter). 10µl samples were taken from both the crude extract and insoluble matter and vortexed with 10µl 2x SDS-PAGE sample buffer and stored at -20^{o}C (Samples 3 and 4 respectively).

200µl amylose resin (a composite of amylose/agarose beads in 20% (v/v) ethanol; New England Biolabs® Inc.) was added to a microfuge tube and centrifuged briefly, the supernatant was aspirated and the resin was resuspended in 1.5ml column buffer. 50µl of amylase-resin slurry was mixed with 50µl of crude extract and kept for 15 min on ice. The sample was centrifuged at 20,000*g* for 1 min, the supernatant was removed and the pellet was washed with 1ml column buffer. After a further 1 min centrifugation at 20,000*g*, the supernatant was aspirated and the resin resuspended in 50µl SDS-PAGE sample buffer (Sample 5; protein bound to amylose).

1µl of each sample was diluted 1:10 with SDS-PAGE sample buffer (in preparation for a Western blot) and 10µl of each of the samples (in preparation for SDS-PAGE) were boiled for 5 min.

2.8.5 Discontinuous SDS-PAGE

SDS-PAGE enables the separation of an assortment of proteins within a single sample by their size and molecular weight. Samples are first mixed with sodium dodecyl sulfate (SDS). SDS is an anionic detergent which, when heated to 100^{o}C in the presence of protein, denatures the protein into individual polypeptides and binds to them, conferring a negative charge to the polypeptide in proportion to its length. The buffer system is discontinuous, whereby a large pore polyacrylamide gel (stacking gel), which is about 2 pH units lower than the electrophoresis buffer, is cast over a small pore acrylamide gel (resolving gel). The stacking gel serves to concentrate the SDS-coated proteins into a small area ready to migrate into the resolving gel where they separate according to their size.

2.8.5.1 Preparation of the resolving gel

Throughout the experiments vertical mini gels (10cm) were cast in the omniPAGE gel casting system (VS10CBS; Geneflow, UK) using 2mm spacers and a 2mm, 12-well comb.

10% (w/v) acrylamide resolving gels were routinely used as these have a separating resolution of ~20-75kDa. This was selected as the molecular weight of MBP was ~42.4kDa and the $GABA_A$ receptor γ4 peptide was ~4kDa, so the expected size of the fusion protein was ~46kDa. It was a high enough resolution to distinguish a 4kDa difference in product size.

10% Resolving gel	2x10cm gels	1x10cm gel
dH_20	6.3ml	3.15ml
30% (w/v) Stock acrylamide	5.0ml	2.5ml
4x Resolving tris solution pH8.8*	3.75ml	1.875ml
10% (w/v) Ammonium persulphate (APS)	150µl	75µl

Table 2.5. Composition of the 10% (w/v) resolving gel for SDS-PAGE. All components were added in the order stated.* (1.5M Tris-HCl; 0.4% (w/v) SDS). As APS is an initiator for gel formation, it was added last.

The solution was made as per Table 2.5. The final addition was 7.5µl or 15µl of the polymerising agent *N,N,N',N'*-tetramethylethyenediamine (TEMED; Sigma-

Aldrich®), for one or two gels respectively. APS is a strong oxidising agent and works in conjunction with TEMED to catalyse the polymersisation of acrylamide. The mixture was carefully mixed to avoid creating bubbles and loaded between two glass plates of the casting module, leaving a 3cm gap from the top. A 1ml overlay of isopropanol was layered on the top of the resolving gel to create an even surface and minimise contact with the air. After 30 min polymerisation, the isopropanol was carefully decanted, the top of the gel rinsed with dH_2O and blotted dry with filter paper.

2.8.5.2 Preparation of the stacking gel

Stacking gel	
dH_20	4.2ml
30% (w/v) Stock acrylamide	0.65ml
4x Stacking tris solution pH6.8*	1.6ml
10% (w/v) APS	67µl

Table 2.6. Composition of the stacking gel (enough for two gels), detailing components, the APS was always added last. * (0.5M Tris-HCl; 0.4% (w/v) SDS; pH 6.8).

After addition of 6.7µl TEMED and gentle mixing, the stacking gel solution was added directly on top of the resolving gel, a comb was inserted and the gel left to polymerise for a further 30 min at room temperature. When the gel was completely set, the comb was removed and any un-polymerised material was removed from the wells by rinsing them with Tris-glycine electrophoresis buffer (5mM Tris-base; 50mM glycine, pH 8.3; 0.02% (w/v) SDS; all Sigma-Aldrich®). The gel was removed from the casting chamber and placed in to the electrophoresis tank where it was submerged entirely in Tris-glycine electrophoresis buffer. As aforementioned, samples, including the ladder and marker, were heated at 100^{o}C for 5 min before loading, and a maximum of 25µl was loaded per well. Samples were run against a pre-stained broad range protein marker (2-212kDa) (0.1-0.2mg/ml of each protein in 70mM Tris-HCl pH 6.8, 33mM NaCl, 1mM Na_2 EDTA, 2% (w/v) SDS, 40mM DTT, 0.01% (w/v) bromophenol blue and 10% glycerol; New England Biolabs® Inc) and a MBP2 standard which is a protein encoded for by the pMAL-c2X vector, with a stop codon inserted into the *Xmn*I site and a molecular weight of 42,482Da (6mg/ml

protein; 20mM Tris-HCl; 0.2 M NaCl; 1mM EDTA; 50% (v/v) Glycerol, pH 7.2; New England Biolabs® Inc.). The gel was run at 50V for the first 15 min (to allow the molecules to concentrate in the stacking gel) then at 100V for a further 50 min.

2.8.5.3 Staining of the protein gel

The gel was gently removed from the glass plates after the dye line had run the full length of the gel and was immediately washed twice (10 min each) with dH_2O on a slowly rotating platform (50 rpm) to remove any traces of the electrophoresis buffer. The gel was then immersed in 5x volume of the protein stain, Coomassie blue (0.25% (w/v) coomassie brilliant blue R250; National Diagnostics, USA; 45% (v/v) methanol; 45% (v/v) dH_2O and 10% (v/v) glacial acetic acid; Sigma-Aldrich®) and placed on a slowly rotating platform (50 rpm) for 1 h. The stain was decanted and the gel was de-stained (45% (v/v) methanol; 45% (v/v) dH_2O and 10% (v/v) glacial acetic acid) for 6-8 h; the de-staining solution was changed 3-4 times during this period. The gel was visualised under white light and the image captured using Syngene™ software.

2.8.6 Western Blotting

2.8.6.1 Assembly of the blot sandwich

Nitrocellulose membrane (0.2µm, Optiran BA-S 83) and 2mm thick filter paper (both Whatman International Ltd., UK) were cut to the size of the gel and then pre-soaked, along with two fibre pads, in 1x continuous transfer buffer (CTB; 25mM Tris; 192mM glycine; 20% (v/v) methanol; pH 8.3) for 15 min. Using forceps throughout, the blot sandwich was assembled, with the gel facing the negative pole. The tank was part filled with pre-chilled transfer buffer along with a magnetic stirrer. The module containing the blot sandwich was then inserted, followed by a cooling pack. The electrophoresis tank was then filled with the remainder of the chilled CTB and the tank placed on ice. The blot was run at 75V for 100 min.

2.8.6.2 Blocking of the membrane (reduction of non-specific binding)

Unbound sites in the nitrocellulose membrane filter were saturated by incubating the blot in 50ml 3% (w/v) non-fat dry milk (Bio-Rad Laboratories) in 1x Tris-

buffered saline (TBS; 50mM Tris-HCl pH 7.4; 0.9% (w/v) NaCl) for 1.5 h at 50 rpm at room temperature.

2.8.6.3 Immunoprobing with primary antibody

5µl of the primary antibody (anti-MBP antiserum (rabbit); New England Biolabs® Inc.) was added directly into the blocking solution (1:10,000 dilution). This was left at room temperature for 30 min to 1 h at 50 rpm to allow the antibody to mix, and then overnight at 4°C to bind. Any unbound primary antibody was removed by washing the membrane in 1x TBS/0.1% (v/v) Tween®20 (Sigma-Aldrich®) for 6x 10 min washes.

2.8.6.4 Immunoprobing with secondary antibody

25µl of the secondary antibody (goat anti-rabbit IgG, AP-linked; New England Biolabs® Inc.) was added into 50ml 3% (w/v) non-fat dry milk in 1x TBS, at a 1:2000 dilution and gently agitated (50 rpm) for 2 h at room temperature. Any unbound secondary antibody was washed off as described in section 2.8.6.3.

2.8.6.5. Immunodetection of proteins

Identification of proteins on the membrane was completed using the BCIP (5-bromo-4-cholo-3-indoyl-phosphate)/NBT (nitro blue tetrazolium) chromogenic assay. This is a three component system whereby combination of BCIP, NBT and an alkaline buffer result in a substrate solution for alkaline phosphatase. When incubated with this enzyme, an insoluble NBT diformazan product is formed which is identified as a purple precipitate, thus allowing colorimetric detection of alkaline phosphate-labelled molecules. Firstly, the membrane was rinsed in 50ml alkaline phosphatase buffer (100mM Tris HCl, pH 9; 1mM $MgCl_2$; 150mM NaCl) to equilibrate the pH of the blot. 330µl NBT (50mg/ml; 70% (v/v) dimethylformamide; Promega) and 165µl BCIP (50mg/ml; 100% (v/v) dimethylformamide; Promega) were added to a further 50ml of the alkaline phosphatase buffer, mixed and added to the membrane, this was left in the dark at room temperature. Colour development occurred in 3-6 h. The membrane was rinsed with dH_2O to stop the colour reaction and was blotted dry with filter paper.

The membrane was then visualised under white light and the image captured using Syngene™ software.

2.8.7 Affinity chromatography

The fusion protein was purified from the cell lysate by affinity chromatography. A chromatographic method of separating individual proteins on the basis of reversible biological interactions between a protein (in this case MPB) and a specific ligand (amylose) which is immobilised within a solid matrix. Using a biorecognition technique such as this enables high specificity and recovery of target protein.

2.8.7.1 Subculturing

12 individual colonies were subcultured into 12ml rich broth and incubated at 37^{o}C overnight at 200 rpm on an orbital shaker. Following this, 1l of pre-warmed rich broth was inoculated with all the overnight culture. Cells were grown at 37^{o}C, 200 rpm for a further 3 h. IPTG was added to a final concentration of 0.375mM to induce protein expression within the cells and incubation was continued for 3.5 h. Culture was divided equally between two 750ml centrifuge tubes and 2ml protease inhibitor cocktail was added to each tube. Cells were harvested by centrifugation at 4000*g* for 20 min at 4^{o}C (Allegra® X-15R; Beckman Coulter). Each pellet was resuspended in 24ml ice-cold column buffer and the two samples were pooled. 2ml protease inhibitor cocktail was added and cells were frozen overnight at -20^{o}C

2.8.7.2 Protein extraction

Cells were thawed in cold water (30 min) and transferred to a sterile beaker. Cells were lysed by sonication in 15 sec pulses for 3 min. Cells were then transferred to tubes and centrifuged at 9000*g* for 30 min at 4^{o}C (Avanti™ J-30I; Beckman Coulter). The supernatant was aspirated and kept on ice and the pellet discarded. A bicinchoninic acid (BCA) assay was completed to estimate the concentration of extracted protein in the crude extract.

2.8.7.3 Protein estimation assay

The bicinchoninic acid (BCA) assay was employed to estimate the protein concentration after sonication. The assay is based on the principle that, under alkaline conditions proteins form complexes with Cu^{2+} which is then reduced to Cu^{+}. BCA itself is a highly specific chromogenic agent which forms a purple-blue complex with the reduced Cu^{+}, thus the proteins reduce the Cu^{2+} in a concentration-dependant manner and the more Cu^{2+} is reduced, the more Cu^{+} complexes with the BCA and the greater the colour change. Absorbance was read at 562nm and a standard curve created from bovine serum albumin (BSA) resuspended in TBS calibration standards of a known concentration. By taking readings from this, the deduced protein concentration in the original sample was obtained.

The assay was completed in a 96-well plate (Starstedt, Germany). 25µl of each BSA standard (in 1x TBS) were added to the plate in triplicate (concentrations of 0µg/ml, 200µg/ml, 400µg/ml, 600µg/ml, 800µg/ml and 1000µg/ml). The crude extract was diluted 2x, 4x, 8x, 32x and 64x in 1x TBS, 25µl of each of these was added to the plate in triplicate. In a separate beaker, 8ml of bicinchoninic acid was added to 160µl copper (II) sulphate solution (4% (w/v) copper (II) sulphate pentahydrate; both Sigma-Aldrich®) and mixed. Immediately, 200µl of this was added to each well containing either standards or samples. The plate was covered and incubated at 37°C for 30 min. Absorbance was read at 570nm (Expert 96; ASYS Hitech GmbH, Austria) and analysed with DigiRead software; Liberté, South Africa). A standard curve of absorbance vs. µg/ml protein was prepared and the equation of the curve was rearranged to calculate the concentration of protein in the unknown sample, taking into account the dilution factors (Fig. 6.7, Chapter 6). The crude extract was then diluted with ice-cold column buffer to approximately 2.5mg/ml ready for the affinity purification.

2.8.7.4 Affinity chromatography

A $30cm^3$ column (20 x 1.5cm; Biosepra, USA) was packed with 8ml amylose resin slurry. Care was taken to ensure the resin was kept wet at all times and no air bubbles became trapped (this causes the resin to split and decreases binding efficiency). When the column was secure, it was attached to a peristaltic pump

and ddH_2O was run through the column at a rate of 2ml/min to ensure the equipment worked properly and there were no leaks. When satisfied, the ddH_2O was exchanged for column buffer; 8 column washes were passed through (64ml total). Approximately half-way through the first set of column washes, the pH of the flow-through was measured; if it was similar to that of the original column buffer then the column was deemed calibrated. Following the washes, the crude extract was added, this was pumped through the column at a rate of 1ml/min. A 0.5ml sample of the crude extract was withdrawn before purification and stored at -20^{o}C for diagnostic purposes later on. Flow-though from the crude extract contained unbound proteins, so this was collected and stored at -20^{o}C in case the target protein did not bind properly. A 0.5ml sample of this was taken and stored at -20^{o}C. The column was then washed with 12 volumes of column buffer (94ml total) at 1ml/min. Fusion protein (bound to the amylose) was then eluted with 30ml elution buffer (column buffer +10mM maltose) and collected in 20x 1.5ml fractions (2110 fraction collector; Bio-Rad Laboratories). Which were stored on ice ready to be analysed.

A BCA assay was performed as in section 2.8.7.3, however it was noted that the maltose in the elution buffer interfered with the assay giving erroneous results. Therefore BSA standards were made up in undiluted, 10- and 20-fold diluted elution buffer and analysed. Results indicated that the 10- and the 20-fold dilutions of the elution buffer gave identical results (thus at these concentrations maltose no longer interefered with the assay) and so the BSA standards in the 10-fold diluted elution buffer could be used (10mM maltose final concentration). This was also fortuitous as all eluted samples were diluted 10-fold before analysis. Samples were loaded on a SDS-PAGE gel and subjected to western blot analysis to determine the purity of the eluted fusion protein.

2.8.7.5 Dialysis

In order to remove the residual maltose from the eluent, dialysis was employed. Utilising a cellulose semi-permeable membrane where larger protein molecules are retained inside the bag and the smaller maltose molecules diffuse out, down the osmotic gradient.

Following western blot analysis, only eluent fractions giving a single product of the expected size were combined and dialysed. Handling only with gloves, a 12cm length of dialysis tubing (MWCO 12-14 kDa; Medicall International Ltd., USA) was cut and placed into a beaker with dH_2O. This was boiled, the dH_2O replaced, and boiled again (to prepare the tubing). dH_2O was replaced again and the tubing was left at 4°C to cool. Once suitably chilled, dH_2O was discarded and one end of the tube clamped. Sample was poured into the bag and the other end was firmly sealed. The dialysis tube was then submerged in 500ml cold Tris buffer (23mM, pH 7.5) and kept at 4°C. Buffer was replaced every 20-30 min for 4 h. After this time the buffer was discarded and dialysed protein was poured into a sterile 15ml tube. The sample was analysed by BCA assay (section 2.8.7.3) to determine the concentration and the purity was confirmed by SDS-PAGE. The fusion construct was then ready for commercial production into an antibody.

2.8.8 Generation of a peptide anti-GABA$_A$ receptor γ4-subunit antibody

In addition to the fusion protein being produced, a peptide antibody was also generated. In this case, a region of nucleotide sequence encoding a portion of the large variable intracellular loop between TM3 and TM4 of the $GABA_A$ receptor γ4 subunit gene was selected and all the subsequent production was outsourced commercially to Fisher Scientific. The sequence was homologous between the zebra finch and chicken to enable production of an antibody which could target either species. Due to mis-matches between the species, only an 11AA sequence could be found which was suitable (Fig. 2.1).

(a) Nucleotide alignment

```
                          TM 3
Zebra Finch γ4     GCTCTCATGGAGTACGCCACGCTCAACTACCTGGTGGGAAACAAGAAACCACTGGAGCAC
Chicken γ4         GCTCTCATGGAGTATGCTACACTCAACTACCTGGTGGGAAACAAGAAGCCACTGGAGCAC
                   ************** ** ** ************************** ************

Zebra Finch γ4     AGCCACAGGAGAGCCAGGCTGCCACCTGCCGGTGCTCAGGTGATGCCAACCTTCACCACC
Chicken γ4         AGCAGCAGGAAAGCCAGACTGCCACCCGCTGGTGCACAGGTGATGCCATCCTTTACTGCT
                   ***  ***** ****** ******** ** ***** ************ **** **  *

Zebra Finch γ4     ATCAACAT------CAACCACATCATGCACTGGCCCCCGGAGAACCTTCACCACCATCAA
Chicken γ4         ATCAACATTAACATCAACAACATCATGCACTGGCCTCCAGAGA---TAGAGGAGGATGAA
                   ********      **** *************** ** ****   *  *  *  ** **

Zebra Finch γ4     CATCAACCACATCATGCACTGGCCCCCGGAGGGGAAGGAGTGTGAGAGG
Chicken γ4         GAT-GACGACCCTGGCTCTCCGTGCCTGGAAGGAAAGGAATGTGAGAGG
                    **  ** **           *  ** *** ** ***** *********
```

(b) Peptide alignment

```
                  TM2                    TM3
Zebra finch γ4    LSTISRKHLPRVSYITAMDLFVSVCFIFVFAALMEYATLNYLVGNKKPLEHSHRRARLPP
Chicken γ4        LSTISRKHLPRVSYITAMDLFVSVCFIFVFAALMEYATLNYLVGNKKPLEHSSRKARLPP
                  ************************************************** *:*****

Zebra finch γ4    AGAQVMPTFTTININ--HIMHWPPENLHHHQHQPHHALAPGGEGVE
Chicken γ4        AGAQVMPSFTAINININNIMHWPPEIEEDEDDDPGSPCLEGKECER
                  *******:**:****  :*******  ...:.:*  .   * *  .
```

Figure 2.1. Nucleotide (a) and peptide (b) alignments of partial zebra finch $GABA_A$ receptor γ4-subunit intracellular loop sequence (GenBank accession number: AM086993) between transmembrane domains (TM) 3 and 4 with the analogous chicken sequence (GenBank accession number X73533). (-) denotes gaps in the sequence which have been introduced to maintain the alignment and (*) indicates perfect alignment. (.) and (:) below the peptide sequence indicate partial codon homology despite a difference of amino acids. Highlighted in grey are single-base mis-matches between the nucleotide sequences of the two species. Highlighted in blue are those single base mis-matches that result in a change to the peptide sequence. In order to create a sequence which was specific to both species the sequence shown in orange was selected as the most appropriate.

The selected sequence was sent to Fisher Scientific and the BioPerformancePlus™ antibody production plan used. Initially, the sequence was analysed to verify it was suitable. The check included domain exclusion analysis, hydrophobicity and antigenicity analysis. The peptide was then synthesised and purified. The purified peptide was coupled to a carrier protein and this conjugate was used to immunise the rabbit.

2.9 RNA interference (RNAi)

In order to verify the potential functional role of γ4-subunit-containing $GABA_A$ receptors in learning and memory in the song system of the male zebra finch, RNAi was employed to silence the expression of this gene. Constructs designed for this could be injected into specific nuclei of the song system and any effect on song acquisition or production could then be observed *in vivo*. This method involves introducing exogenous short-hairpin (sh)RNA into a cellular system whereby it enters the natural RNAi pathway within the cell and RNA expression is suppressed. A more detailed introduction to the technique is included in Chapter 6.

2.9.1 Short-hairpin (sh)RNA construct design

The system utilised in this study was the p*Silencer*™ 4.1-CMV neo vector kit (New England Biolabs® Inc.), which enables the ligation of a shRNA construct into an expression vector. The plasmid offers a powerful promoter in the form of a modified cytomegalovirus (CMV) promoter, which enables constitutive expression of a cloned shRNA template, and a modified simian virus-40 (SV40) polyadenylation signal which terminates transcription. The vector also encodes an ampicillin and neomycin resistance gene to enable antibiotic screening (for full vector map, refer to Appendix).

Prior to silencing the $GABA_A$ receptor γ4-subunit gene in the zebra finch *in vivo*, it was necessary to determine that the RNAi reactions would work efficiently. Therefore constructs were initially transfected into primary neurons and the degree of corresponding mRNA 'knock-down' was assayed by real-time RT-PCR. As zebra finches were not readily accessible and no cell lines expressing the $GABA_A$ receptor γ4-subunit gene were available, the work was completed with one-day-old chicks (*Gallus gallus domesticus*), which, phylogenetically, are very similar. Thus three sets of 55bp-constructs were designed, the first to silence the $GABA_A$ receptor γ4-subunit gene in the chicken, the second set to silence the same gene in the zebra finch and the third set as controls (Fig. 2.2). It was critical to include positive and negative control constructs. Non-targeting shRNA is a useful negative control that activates RISC and the RNAi pathway, but does not target any chicken/zebra finch genes. This allowed for examination of any potential effects of shRNA transduction on gene expression. Cells transfected with the non-target shRNA also provide a useful reference for interpretation of knockdown. A positive control served to measure the transduction efficiency and aids in optimising shRNA delivery.

Initially, 21-base mRNA targets were selected within the $GABA_A$ receptor γ4-subunit nucleotide sequence: in regions encoding the extracellular amino-terminus and large intracellular loop between TM3 and TM4 of the chicken (GenBank accession number X73533) and the zebra finch (GenBank accession number AM086993). These regions were selected as they exhibit the least similarity to

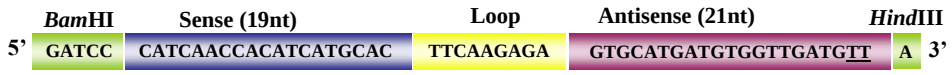
other $GABA_A$ receptor subunit sequences (i.e. to avoid cross-silencing). siRNA targets were chosen by use of a target finder on the Ambion™ Website (www.ambion.com/techlib/misc/siRNA_finder.html), which selects suitable targets from an mRNA sequence based on guidelines first specified by Tuschl and colleagues (Elbashir *et al*., 2001a, b); the guidelines are only available at www.rockefeller.edu/labh eads/tuschl/sirna.htm. In addition to this, each 21-base sequence fulfilled the recommended criteria (Chalk *et al*., 2004; Reynolds *et al*., 2004), namely:

1. At least 100-200bp downstream of the AUG codon and upstream of termination codon
2. G+C content between 20% and 50%
3. No more than 3 consecutive bases (especially G and C)
4. Asymmetric (A/T at the 5'end and G/C at the 3' of the sense strand), if possible
5. 2 nt overhang

The 21-base target then served as the basis for the design of the 55bp construct (Fig. 2.2). Specifically, a 19-base sense stem is joined to a 21-base antisense version of this stem by a non-coding loop sequence (9 bases) that had been previously proved successful (Yu *et al*., 2002). At each end of the construct there were single-stranded overhangs encoding the *Bam*HI and *Hind*III restriction sites for ligation into the p*Silencer*™ 4.1-CMV neo vector.

Zebra finch ($GABA_A$ R γ4 gene):
Original target mRNA Sequence (sense) – AACATCAACCACATCATGCAC – **Intracellular loop**

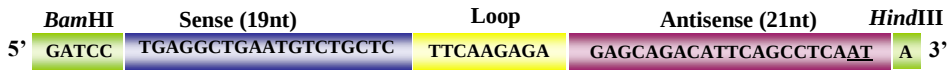
Top strand oligonucleotide



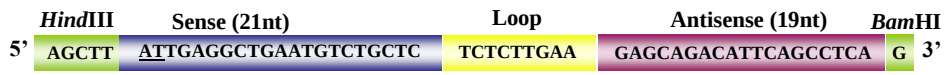
Bottom strand oligonucleotide

Zebra finch/Chick ($GABA_A$ R γ4 gene):
Original target mRNA Sequence (sense) – ATTGAGGCTGAATGTCTGCTC – **Amino-terminus_**

Top strand oligonucleotide

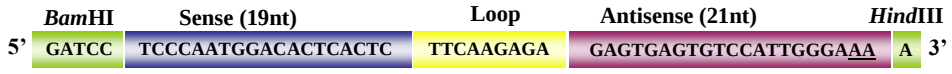


Bottom strand oligonucleotide



Zebra finch/chick ($GABA_A$ R γ4 gene):
Original target mRNA Sequence (sense) – TTTCCCAATGGACACTCACTC – **Amino-terminus**

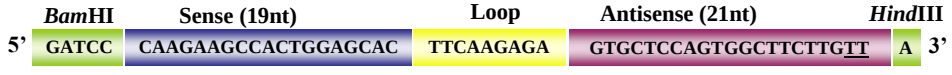
Top strand oligonucleotide



Bottom strand oligonucleotide

Chick ($GABA_A$ R γ4 gene):
Original target mRNA Sequence (sense) – AACAAGAAGCCACTGGAGCAC – **Intracellular loop**

Top strand oligonucleotide



Bottom strand oligonucleotide

Chick (GAPDH gene):

Original target mRNA Sequence (sense) – AAATGGGCAGATGCAGGTGCT – **Intracellular loop**

Top strand oligonucleotide

	*Bam*HI	Sense (19nt)	Loop	Antisense (21nt)	*Hind*III	
5'	GATCC	ATGGGCAGATGCAGGTGCT	TTCAAGAGA	AGCACCTGCATCTGCCCATTT	A	3'

Bottom strand oligonucleotide

	*Hind*III	Sense (21nt)	Loop	Antisense (19nt)	*Bam*HI	
5'	AGCTT	AAATGGGCAGATGCAGGTGCT	TCTCTTGAA	AGCACCTGCATCTGCCCAT	G	3'

Chick (non-coding negative control):

Top strand oligonucleotide

	*Bam*HI	Sense (18nt)	Loop	Antisense (21nt)	*Hind*III	
5'	GATCC	ACTACCGTTGTTAAGGTG	TTCAAGAGA	CACCTATAACAACGGTAGTTT	A	3'

Bottom strand oligonucleotide

	*Hind*III	Sense (21nt)	Loop	Antisense (18nt)	*Bam*HI	
5'	AGCTT	AAACTCAAGTTGTTATAGGTG	TCTCTTGAA	CACCTTAACAACGGTAGT	G	3'

Figure 2.2. The 55bp shRNA constructs designed for ligation into the p*Silencer*™ 4.1-CMV neo vector. Several constructs were designed to target the chicken/zebra finch $GABA_A$ receptor γ4-subunit gene to ensure selection of the optimal sequence. Negative control sequence was modified from Rumi *et al*. (2006) and the positive-control chick glyceraldehyde-3-phosphate dehydrogenase (GAPDH) construct was based on the sequence (GenBank accession number NM_204305). The first two bases of the oligonucleotide sequence that are discarded in the sense strand when using a top strand oligonucleotide template, but they are included in the antisense strand where they form the 2-nucleotide overhang (underlined). The non-coding loop sequence is highlighted in yellow, *Bam*HI and *Hind*III restriction sites were included for ligation into the p*Silencer*™ 4.1 CMV-neo vector. Top and bottom strands were annealed together to form a double stranded construct which was ligated into the p*Silencer*™ 4.1-CMV neo vector.

2.9.2 Ligation of shRNAs into pSilencer™4.1-CMV neo vector

Single-stranded shRNA constructs (Fig. 2.2) were synthesized by Eurofins MWG operon (Germany) and upon arrival were diluted to 1μg/μl in H_2O. The annealing mix was assembled as follows: sense oligonucleotide (top strand; 2μg); antisense oligonucleotide (bottom strand; 2μg) and 46μl 1x DNA annealing solution (Ambion™). The mixture was heated for 3 min at 90°C and then incubated for 1 h

at 37°C. 5µl of the annealed shRNA construct was added to 45µl H_2O to a final concentration of 8ng/µl.

Annealed oligonucleotides were ligated into the vector in the following mix: annealed shRNA insert (8ng), 1µl 10x T4 DNA ligase buffer (Promega), 0.1µg linearised p*Silencer*™ 4.1-CMV neo vector (Ambion™), T4 DNA ligase (5U; Promega) and 6µl H_2O. The ligation reaction was left overnight at 4°C. XL1-Blue cells were then transformed with the ligation reaction (section 2.8.4.1) and shRNA-containing plasmids were extracted as per sections 2.5.4-2.5.5. Due to problems with the efficiency of a double restriction digest with *Hind*III and *Bam*HI, insert-containing plasmids were sent for automated DNA sequencing immediately after the minipreparation, to confirm presence of an insert with no prior diagnostic digest. Suitable constructs were 'bulked up' with a plasmid maxi kit (Qiagen®), which is a replica of the minipreparation kit used in section 2.5.5 only on a larger scale. Yield was determined by UV photospectrometry as per section 2.2.2; plasmids were stored at -20°C ready for use.

2.9.3 Primary neuronal culture

2.9.3.1 Animals

Fertilised chicken eggs (Leghorn) were obtained from Henry Stewart & Co. Ltd., Lincolnshire, UK. Eggs were initially incubated at 37°C at 20% relative humidity (INCA 50 incubator; Fiem, Italy). After two days the humidity was increased to 50-60% where it was maintained until embryonic day (E) 20, at this point the humidity was increased to 70-80% to encourage hatching. Eggs were subjected to a 12:12 h light: dark cycle and were constantly turned to avoid the embryo 'sticking' to the side of the shell, affecting development. Eggs were candled at regular intervals to ensure healthy development and any embryos showing no signs of growth were removed from the incubator.

2.9.3.2 Establishment of primary neuronal culture

Once hatched, at one day old, chicks were sacrificed by dislocation of the neck, their brains quickly excised and the cerebellum dissected out and stored in cold 1x Hank's balanced salt solution (HBSS; 400mg/l KCl; 60mg/l KH_2PO_4; 8g/l

NaCl; 350mg/l $NaHCO_3$; 90mg/l $NaH_2PO•7H_2O$; 1g/l glucose; 20mg/l phenol red; Lonza, Switzerland). Cerebellums were minced with a McIllwain automatic tissue chopper to $1mm^3$ pieces (Vibratome™, USA) and then trypsinised with 0.04% (v/v) trypsin (Sigma-Aldrich®) in 5ml HBSS at 37°C for 30 min with gentle agitation. The reaction was terminated by the addition of 20ml growth medium (Neurobasal medium supplemented with 20% (v/v) fetal bovine serum; FBS and 1% (v/v) penicillin/streptomycin and 0.5mM L-glutamine; all Lonza). Tissue was triturated by pipetting up and down ~10 times with a 10ml pipette and poured through a 70µl mesh into a sterile 50ml tube. A further 10ml medium was washed through the mesh (2x 5ml rinses) and the mesh discarded. Cells were left to settle for a few minutes between each rinse of the mesh. Filtrate was centrifuged at 200*g* for 5 min at 4°C (5702R; Eppendorf, UK) and the supernatant aspirated and discarded. Cells were resuspended in 5ml fresh growth medium (as above) and were ready for seeding; 20µl of cells were removed and reserved for counting.

20µl of cell suspension was aliquotted into a 0.5ml sterile microfuge tube with 20µl Trypan blue (0.4% w/v in 0.81% (w/v) sodium chloride; 0.06% (w/v) potassium phosphate; Sigma-Aldrich®). Prior to counting, cell aggregates were broken up with a 0.6mm syringe, the cells were loaded onto a haemocytometer and cells which excluded the trypan blue were counted.

Cells were seeded at 200,000 cells/well in a 24-well plate with 1ml growth medium. They were gently mixed and then left for 24 h without disruption to allow them to establish in a humidified 37°C/5% CO_2 incubator (Sanyo, Japan). After 24 h, half (500µl) of the medium was changed and after 48 h, all the medium was replaced with fresh medium. This was left for 24 h and then replaced with fresh medium, on average the neuronal cultures survived 11-14 days. The day prior to transfection the growth medium was removed and replaced with fresh medium containing no antibiotics

2.9.3.3 Transfection of cells

Individual shRNA constructs were co-transfected with pRK5-Clomeleon vector (kindly provided by Thomas Kuner (Kuner and Augustine, 2000) in order to verify the transfection efficiency. Each construct was transfected in six

samples/wells (γ4-targetting shRNA, GAPDH-targetting shRNA, non-specific targeting shRNA and the Clomeleon vector alone). Per sample (well), 200ng of shRNA construct and 200ng of pRK5-Clomeleon vector were diluted in 50µl OptiMEM® I reduced serum medium (Gibco™, Invitrogen) and gently mixed. Concurrently, 2µl Lipofectamine™ 2000 was diluted in 50µl OptiMEM® I reduced serum medium (final concentration 1µg/25µl), mixed and incubated for 5 min at room temperature. Following this, the diluted DNA and Lipofectamine™ 2000 were combined (total volume 100µl) and incubated at room temperature for 20 min to allow DNA-Lipofectamine™ 2000 complexes to form; these were then added to the wells containing cells and medium and very gently mixed. Cells were incubated at 37°C/5% CO_2 for 24 h, after which the transfection medium was removed and fresh growth medium was added, 72 h after this the cells were harvested.

2.9.4 RNA extraction from cells

Medium was aspirated from each well and discarded, 500µl PBS was added per well and gently agitated to wash the cells of any remaining medium. PBS was aspirated and 25µl Tri® (Ambion™) reagent was added to each well. Cells were harvested with a cell scraper and the replicates of each treatment group were pooled into 0.5ml sterile microfuge tubes. 30µl chloroform was added to each tube and vigorously mixed. Following 10 min incubation on ice, cells were centrifuged at 12,000*g* for 15 min at 4°C. The top aqueous layer was aspirated and combined with 150µl isopropanol, mixed and centrifuged at 12,000*g* for 10 min at 4°C. The supernatant was removed and discarded and remaining RNA was ethanol precipitated (section 2.3.3) and solubilised in 300µl H_2O. Subsequently, first-strand cDNA was synthesised as per section 2.3.4

2.9.5 Real-time RT-PCR

This was a slightly different method to that described in section 2.7 as these experiments were completed in our laboratory. Real-time RT-PCR primers were designed to amplify partial regions of the chicken $GABA_A$ receptor γ4-subunit and GAPDH genes in order to quantify the silencing efficiency of the shRNA

constructs. In addition, primers were designed for various chicken-specific house-keeping genes in order to validate the integrity of the RT-PCR reaction (detailed in Table 2.6) and allow normalisation of the data during analysis. All primers were designed adhering to the criteria outlined in section 2.4.1.

Target gene	Forward: 5'→3'	Reverse: 5'→3'	Amplicon (bp)
$GABA_A$ receptor γ4 subunit	GGTGTGAGAGCACAGAGGAG	GACAACAAACTGAGGCCTGA	142
GAPDH	AGTCCAAGTGGTGGCCATC	AGTCCAAGTGGTGGCCATC	154
β-actin	GAGAAATTGTGCGTGACATCA	TGGCAATGAGAGGTTCAGG	149
18S Ribosomal RNA	TCAGATACCGTCGTAGTTCC	AACTTAAAGGAATTGACGGAA	154
β-2-Microglobulin	AAGGAGCCGCAGGTCTAC	GTATGACGGCAAAGAGCAAG	151

Table 2.6. Sequences and amplicon sizes of real-time RT-PCR primers targeting the chicken $GABA_A$ receptor γ4-subunit and GAPDH genes to validate the effects of RNAi. House-keeping primer sequences were taken from Li *et al*., (2005) and designed from published chicken sequences. GenBank accession numbers as follows; β-actin (L08165); 18S Ribosomal RNA (AF173612); β-2-Microglobulin (Z48922).

Real-time RT-PCR was performed in a Rotor-gene 6000 (Qiagen, formerly Corbett Life Science) using the QuantiTect® SYBR® Green PCR system (Qiagen). Master mix comprised of (per 25µl reaction) 12.5µl 2x QuantiTect® SYBR® Green PCR Master Mix (which contained: HotStarTaq® DNA polymerase, QuantiTect® SYBR® Green PCR buffer (Tris-Cl, KCl, $(NH_4)_2SO_4$, 5mM $MgCl_2$, pH 8.7), dNTP mix, SYBR® Green I and ROX and RNase-free H_2O), 100ng each of forward and reverse primer and 0.5µl cDNA template, made up to 25µl with H_2O. Cycling details were as follows; an initial incubation step of 15 min at 95°C, which activated the HotStarTaq® DNA polymerase thereby reducing the formation of primer-dimers and mis-primed products and then 35 cycles of: 94°C *denaturation* (30 sec), 59.5°C *annealing* (30 sec), 72°C *extension* (30 sec).

During all preliminary real-time RT-PCR reactions a DNA dissociation (melt) analysis was completed in order to optimise the cycling conditions and identify any dimers or non-specific products. All primers gave a single product and following 1% (w/v) agarose gel electrophoresis (section 2.4.4), were deemed suitable for use.

Data was normalised and quantified with the Rotor-gene analysis software (Qiagen, formerly Corbett Life Science), no statistical analysis was completed as the full experiment was only completed twice.

3. SEQUENCES ENCODING ZEBRA FINCH $GABA_A$ AND $GABA_C$ RECEPTOR SUBUNITS

3.1 Introduction

3.1.1 Isolation of $GABA_A$ receptor subunit sequences

Pentameric $GABA_A$ receptors are the primary mediators of rapid phasic inhibitory-synaptic neurotransmission and extrasynaptic tonic inhibition in the mammalian brain; this is also true of lower vertebrates such as the zebra finch. Isolation of sequences encoding the individual mammalian $GABA_A$ receptor subunits began in earnest in the mid-late 1980s after the first subunit cDNAs (encoding α1 and β1 subunits) were isolated from bovine brain (Schofield *et al*., 1987). A plethora of mammalian subunits have been uncovered over the last 20 years: α1-α6, β1-β3, γ1-γ3, δ, ε, θ and π; and they are classified solely by their sequence similarity (for reviews see Burt and Kamatchi, 1991; Barnard *et al*., 1998; Olsen and Sieghart, 2008, 2009). Members of the same class typically share 60-80% identity (at a nucleotide level) and subunits from different classes share 30-40% similarity. More recently a complete analysis of the human genome has revealed no further $GABA_A$ receptor subunit sequences exist and confirmed that each subunit polypeptide is encoded for by a separate gene (Simon *et al*., 2004).

3.1.2 $GABA_A$ receptor subunit sequences

Each subunit gene encodes for a large extracellular hydrophilic N-terminus of ~230 AA which contains the putative 15 AA Cys-loop domain, characteristic of the LGIC superfamily. The amino-terminal is also the site of ligand binding and houses two highly conserved residues, Ser^{171} and Tyr^{172}, which are positioned close to the Cys-loop sequence and are purported to be involved in receptor association and cell-surface expression (Jin *et al*., 2004), but not ligand binding (Whiting *et al*., 1997b). Following this, there are four highly conserved hydrophobic transmembrane (TM) α-helices of ~20 AA, the second of which forms the lining of the intrinsic chloride pore and contains a highly conserved octapeptide sequence presumably critical to pore formation (discussed in further detail in section 3.4; Imoto *et al*., 1986; Xu and Akabas, 1996; Horenstein *et al*., 2001). It is also purported that TM1 may also contribute to the channel formation (Sine and Engel, 2006). The small intracellular loop connecting TM1 and TM2 is relatively conserved within mammalian $GABA_A$ receptor subunit sequences

and mutations of this pentapeptide sequence of the β3 (not α2 or γ2) subunit revealed that it plays a role in ion selectivity (where mutations resulted in a cation-selective channel), but the pharmacology remained mostly unchanged (Jensen *et al*., 2002). The long intracellular loop linking TM3 and TM4 is the most variable part between $GABA_A$ receptor subunit sequences and is speculated to be involved in receptor trafficking (Chen *et al*., 2005) and influence ion conduction (Peters *et al*., 2005); this is by the far the largest loop within the subunit complex (discussed later in further detail). Each subunit terminates in a short extracellular carboxyl domain.

3.1.3 $GABA_A$ receptor subunit splice variants

Following the isolation of cDNAs encoding 16 mammalian $GABA_A$ receptor subunits, heterogeneity of this already complex family was furthered by the detection of splice variants of many $GABA_A$ receptor subunits. The first of which was reported for the chicken β4-subunit gene where there was a presence/absence of 4 AA within the large intracellular loop between TM3 and TM4; the variant arose due to the use of a different 5'-donor site (Bateson *et al*., 1991). Two polypeptides have also been identified for the chicken β2-subunit gene, which differ by a 17 AA presence (β2L) or absence (β2S) in the large variable intracellular loop. The 17 AA insert/deletion encodes a potential PKC phosphorylation site and was reported to be encoded for by a separate, additional exon (Harvey *et al*., 1994) which, via alternative splicing is present in β2L. Although a slightly longer insertion (38AA) was found for the human version of the gene, the splice site remained the same (McKinley *et al*., 1995; Simon *et al*., 2004). Rat and human β3-subunit isoforms exist due to alterative splicing of exon 1 or 1a, resulting in two alternative signal peptides which are the same length but differ substantially in sequence (Kirkness and Fraser, 1993).

Within the class of α subunits, the α2-subunit gene demonstrates the greatest number of isoforms (in rat), six in total due to three 5'-UTR (untranslated region) exons which can be variably spliced (Fuchs and Celepirovic, 2002). In the human genome 12 exons have been identified for the α2-subunit gene, resulting six potential isoforms (Simon *et al*., 2004). Alternative splicing of human and mouse α4-subunit pre-mRNA results in a truncated mature mRNA and an inactive protein (Mu *et al*., 2002) due to deletion of exons 3-8 (Simon *et al*., 2004). In humans and rats, the α5-subunit gene has the largest

number of exons (13) with four 5'-UTR exons which can be variably deleted, resulting in three mRNA isoforms (Kim *et al*., 1997; Simon *et al*., 2004). Furthermore, alternative splicing is evident in the α6-subunit gene in rat where there is 10 AA deletion in the large intracellular loop (between TM3 and TM4); resulting in a non-functional polypeptide, attributed to incorrect splicing at a 3'-acceptor site within the exon (Korpi *et al*., 1994).

It is the γ2-subunit splice variants which have attracted the most attention. They differ by the presence (γ2L) or absence (γ2S) of an 8 AA insertion in the large variable intracellular loop (between TM3 and TM4) bearing a putative PKC site (Ser^{343}) in bovine, human, rat (Whiting *et al*., 1990) and mouse brain (Kofuji *et al*., 1991). γ2L accumulates at post-synaptic sites with the highest efficiency. Efficiency is further increased after short-term PKC activation, indicative of a role for phosphorylation of the Ser^{343} site in the accumulation of $GABA_A$ receptors at inhibitory sites (Meier and Grantyn, 2004). More recently a third γ2-subunit splice variant was uncovered (γ2XL) which contains a 40 AA insert in the N-terminus created by a weak splice site within intron 5 by an *Alu*-J sequence (Jin *et al*., 2004). This primate-specific family of short interspersed elements has also been identified in intronic sequences of human α1-, α3-, α5-, β1-, γ2-, ρ1- and π-subunit genes, whereby alternative subunit isoforms may be generated that are not detectable by examination of the exon content (Simon *et al*., 2004). γ3-subunit splice variants have been identified in human. There is a presence (γ3L) or absence (γ3S) of 6 AA in the large intracellular loop (between TM3 and TM4) of the γ3-subunit gene which encodes an additional PKC phosphorylation site (Poulsen *et al*., 2000). The ε-subunit gene is composed of 9 coding exons (Sinkonnen *et al*., 2000; Simon *et al*., 2004). Deletions in the first 3 exons and part of the fourth exon give rise to four expressed ε-subunit variants, including the full length transcript (Davies *et al*., 1997; Garret *et al*., 1997; Whiting *et al*., 1997; Wilke *et al*., 1997).

3.1.4 $GABA_C$ receptor subunit splice variants

The human ρ1 subunit of the $GABA_C$ receptors also has two additional smaller, alternatively spliced variants. The first (named $GABA_{\rho 1}\Delta 450$) has a 450 nt deletion within the N-terminus and the second (named $GABA_{\rho 1}\Delta 51$) has a 51 nt deletion from

the same 5' position (Martínez-Torres *et al*., 1998). $GABA_{\rho 1}\Delta 51$ has lost exon 2 and was found to be functional whereas $GABA_{\rho 1}\Delta 450$ is missing exons 2-5 (Simon *et al*., 2004) and is rendered non-functional due to loss of the GABA-binding site motif (Martínez-Torres *et al*., 1998). Furthermore, a 300 bp *Alu* sequence was also detected within intronic sequence of the human ρ1-subunit gene whereby a splice variant could potentially arise, these were not apparent in the human ρ2- and ρ3-subunit genes (Simon *et al*., 2004), and no alternative splicing has been documented in any species for the ρ2 and ρ3 subunit polypeptides.

3.1.5 $GABA_C$ receptors

Ionotropic $GABA_C$ receptors are less well characterised, their structure is identical to that of $GABA_A$ receptors, with the subunits sharing all the same structural features. Where $GABA_A$ receptors are the most complicated member of the cys-loop LGIC superfamily, $GABA_C$ receptors are the simplest, with only three documented subunits (ρ1-ρ3) which are primarily localised to bipolar and horizontal cells of the retina (Qian and Dowling, 1993; Albrecht and Darlison, 1995; Enz *et al*., 1995; Chebib *et al*., 1998) and tend to form homomeric Cl^- channels (Enz and Cutting, 1998; Borman, 2000). They are not well expressed within the mammalian brain (in comparison to the majority of $GABA_A$ receptor subunit genes) and are insensitive to classical $GABA_A$ receptor modulators such as barbiturates and benzodiazepines (Qian and Pan, 2002), so have avoided much scrutiny. Consequently there is no evidence of their role within the zebra finch song system. However, some data has reported expression of $GABA_C$ receptor subunits in mammalian brain (Delaney and Sah, 1999; Ogurusu *et al*., 1999; Rozzo *et al*., 2002; Milligan *et al*., 2004; Alakuijala *et al*., 2005), which may be able to co-localise with $GABA_A$ receptor subunits to form functional receptors (Ekema *et al*., 2002; Qian and Pan, 2002; Milligan *et al*., 2004). Moreover, relatively high levels of ρ1- and ρ2-subunit mRNAs have been detected in the chicken brain (Albrecht *et al*., 1997), where it was postulated they may be implicated in short-term memory formation (Gibbs and Johnston, 2005). Therefore, it was deemed important for these subunit cDNAs to be amplified and included in the expression studies.

To date 14 $GABA_A$ receptor subunit polypeptides have been identified in lower vertebrates α1-α6, β2-β4, γ1, γ2 and γ4, δ and π; where it is accepted that the γ4 and β4

subunits are orthologues of the mammalian ε and θ subunits respectively (Darlison *et al*., 2005), and three $GABA_C$ receptor subunit polypeptides (ρ1-ρ3). However, not all of these have been formally identified (i.e. cloned) from zebra finch. Evidence for functional $GABA_A$ receptors within the song system is purely electrophysiological (Bottjer *et al*., 1998; Dutar *et al*., 1998; Luo and Perkel, 1999; Spiro *et al*., 1999; Farries *et al*., 2005; Prather *et al*., 2008) and pharmacological (Carlisle *et al.*, 1998; Luo and Perkel, 1999; Vicario and Raksin, 2000; Farries *et al*., 2005; Mooney and Prather 2005; Ölveczky *et al*., 2005). A recent study by Thode *et al*. (2008) isolated γ4-subunit cDNA from zebra finch and fully documented the expression of the transcript within the song system of the zebra finch brain. Findings demonstrated a robust expression pattern encompassing nearly all the nuclei of the male song system. This was the first time that a $GABA_A$ receptor subunit transcript had been spatially mapped within the zebra finch brain and results indicated that $GABA_A$ receptors containing the γ4-subunit polypeptide may play an important role in song acquisition and production in the zebra finch (further details in Chapters 1 and 5).

Due to the vast repertoire of polypeptide subunits which can assemble into a large variety of receptor subtypes (each which has an individual pharmacological, biochemical and electrophysiological profile), and to understand which particular subtypes exist within zebra finch brain, all subunits must be considered. Work outlined in this chapter aimed to isolate partial cDNAs encoding all $GABA_A$ and $GABA_C$ receptor subunits from zebra finch so subsequent expression studies could be undertaken. At the time this work was completed, no zebra finch $GABA_A$ receptor subunit sequences, aside from the γ4 subunit (Thode *et al*., 2008) were published. Some partial cDNAs (α1, α2, α5, β2, β3, β4, γ2, γ4) had already been isolated in the laboratory (data unpublished), but the remainder (e.g. α3, α4, α6, γ1, δ and π) required cloning. Therefore, degenerate primers were based on human, rat and chicken sequences (where available). All $GABA_A$ and $GABA_C$ receptor subunit sequences demonstrate high inter-species conservation and high sequence homology between classes of subunits in the regions encoding the four transmembrane domains, but show some variability within the areas encoding the N-terminus and large intracellular loop situated between TM3 and TM4; it was within these regions that degenerate primers were designed and partial cDNAs amplified.

3.1.6 Sequencing of the zebra finch genome

The zebra finch song system is a valuable research tool for understanding the mechanisms underlying vocal learning and behaviour, which can be extrapolated to humans. Recognition of this has lead to its selection for whole genome sequencing. It is the second avian genome to be sequenced, after the chicken. Sequencing and assembly were carried out at Washington University Genome Sequencing Centre (http://genome.wustl.edu/genomes/view/taeniopygia_guttata/) and was released in 2008 (following completion of this section of the study), revealing a genome of 1.2 Gb in length. Although many of the genes have been assigned and annotated they still remain predicted sequences and, as discussed later on, should be validated by experimental evidence. To this end, sequences which were cloned from the zebra finch (prior to the sequencing of the genome), were compared in the analyses to the corresponding, recently released sequences.

3.2 Results

3.2.1 Sequences encoding zebra finch $GABA_A$ receptor subunits

Degenerate primers based on chicken and rat sequences were used to amplify partial sequences of $GABA_{A/C}$ receptor subunits from zebra finch first strand cDNA (list of primer details in Tables 2.1 and 2.2; Chapter 2). Amplified RT-PCR products of expected size were purified, ligated into the pGEM®-T Easy vector and transformed into *E.coli* XL1 blue cells. Plasmids were extracted with minipreparations and digested with *Eco*R1 endonuclease to determine presence of inserts; positive clones were sent for automated sequencing. The process was repeated several times for each subunit clone to ensure consistency. Sequences were subjected to BLAST analyses for identification and were aligned using software available on the internet alongside recently published predicted sequences.

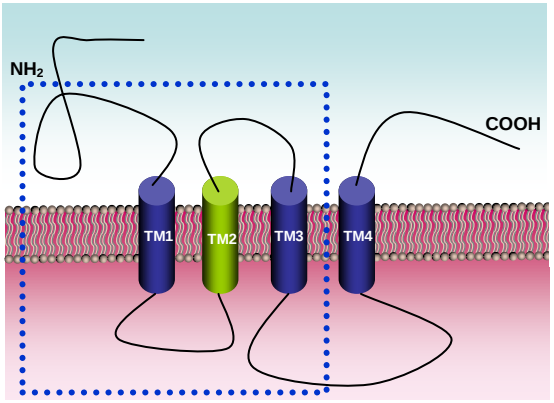


Figure 3.1. Schematic diagram depicting $GABA_A/GABA_C$ receptor subunit topology, highlighting the region amplified by RT-PCR (----). Isolated cDNAs encompassed a portion of the amino-terminus, including the Cys-loop, putative α-helical transmembrane domains (TM) 1-3 and a section of the large intracellular loop linking TM3 and TM4.

(a) Sequence of zebra finch $GABA_A$ receptor α3 subunit

```
Zebra finch α3      RQSWRDERLKFDGPMKILPLNNLLASKIWTPDTFFHNGKKSVAHNMTTPNKLLRLVDNGT
Chicken α3          RQSWRDERLKFDGPMKILPLNNLLASKIWTPDTFFHNGKKSVAHNMTTPNKLLRLVDNGT
Zebra finch α3(P)   RQSWRDERLKFDGPMKILPLNNLLASKIWTPDTFFHNGKKSVAHNMTTPNKLLRLVDNGT
                    ************************************************************

Zebra finch α3      LLYTMRLTIHAECPMHLEDFPMDVHACPLKFGSYAYTKTEVIYTWTLGKDKSVEVAKGGS
Chicken α3          LLYTMRLTIHAECPMHLEDFPMDVHACPLKFGSYAYTKTEVIYTWTLGKDKSVEVAKGGS
Zebra finch α3(P)   LLYTMR-TCSAKSPLQLEKTPKDSNVCPLTAGSYAYTKTEVIYTWTLGKDKSVEVAKGGS
                    ****** *  *:.*::**. * * :.***. ******************************
                                                          _________TM 1_________
Zebra finch α3      RLNQYDLLGHVVGTEMVRSSTGEYVVMTTHFHLKRKIGYFVIQTYLPCIMTVILSQVSFW
Chicken α3          RLNQYDLLGHVVGTEMVRSSTGEYVVMTTHFHLKRKIGYFVIQTYLPCIMTVILSQVSFW
Zebra finch α3(P)   RLNQYDLLGHVVGTEMVRSSTGEYVVMTTHFHLKRKIGYFVIQTYLPCIMTVILSQVSFW
                    ************************************************************
                              _________TM 2_________          _________TM 3_________
Zebra finch α3      LNRESVPARTVFGVTTVLTMTTLSISARNSLPKVAYATAMDWFMAVCYAFVFSALIEFAT
Chicken α3          LNRESVPARTVFGVTTVLTMTTLSISARNSLPKVAYATAMDWFIAVCYAFVFSALIEFAT
Zebra finch α3(P)   LNRESVPARTVFGVTTVLTMTTLSISARNSLPKVAYATAMDWFIAVCYAFVFSALIEFAT
                    ******************************************:*****************

Zebra finch α3      VNYFTKRSWAWDGKKVLEAQEMKKKEPVALAKKTNN
Chicken α3          VNYFTKRSWAWDGKKVLEAQEMKKKEPVALVKKTNN
Zebra finch α3(P)   VNYFTKRSWAWDGKKVLEAQEMKKKEPVALAKKTNN
                    ****************************.*****
```

Figure 3.2a. Alignment of the partial amino-acid sequence (in single letter code) encoding the zebra finch $GABA_A$ receptor α3 subunit with the corresponding chicken sequence (GenBank accession number XP_420268) and predicted zebra finch sequence (as no peptide sequence was available, it was translated from the nucleotide sequence; GenBank accession number XR_053905). **Abbreviations** for all figure 3.2a-f: (P) denotes predicted sequence, asterisks (*) denote complete homology between sequences and dots represent low (.) or partial (:) homology, in some cases gaps (-) were introduced to maintain the alignment. Differences between sequences are highlighted in black. Also shown are transmembrane spanning domains (TM); putative Cys-loop consensus sequence (red) and conserved serine (S) and tyrosine (Y) residues (grey).

(b) Sequence of zebra finch GABA$_A$ receptor α4 subunit

```
Chicken α4          DTFFRNGKKSVAHNMTAPNKLFRIMRNGTILYTMRLTISAECPMRLVDFPMDGHACPLKF
Zebra finch α4      DTFFRNGKKSVAHNMTAPNKLFRIMRNGTILYTMRLTISAECPMRLVDFPMDGHACPLKF
                    ************************************************************

Chicken α4          GSYAYPKSEMIYTWTKGPEKSVEVPEESSSLVQYDLLGHTVSSETIKSITGEYIVMTVYF
Zebra finch α4      GSYAYPKSEMIYTWTKGPEKSVEVPEESSSLVQYDLLGHTVSSETIKSITGEYIVMTVYF
                    ************************************************************
                              TM 1                      TM 2
Chicken α4          HLRRKMGYFMIQTYIPCIMTVILSQVSFWINKESVPARTVFGITTVLTMTTLSISARHSL
Zebra finch α4      HLRRKMGYFMIQTYIPCITTVILSQVSFWINKESVPARTVFGITTVLTMTTLSISARHSL
                    ****************** ****************************************
                              TM 3
Chicken α4          PKVSYATAMDWFIAVCFAFVFSALIEFAAVNYFTNIQMERAKRKTVKSLLEFPVAPIQRE
Zebra finch α4      PKVSYATAMDWFIAVCFAFVFSALIEFAAVNYFTNIQMEKAKRKTVKSLLEFPVAPVQRK
                    ****************************************:***************:**:

Chicken α4          KCTEETHTSTDANSNVRKRTNATVQSEADGGSRIDTRHSSIQPPSVAQGSSDVTPHSLSA
Zebra finch α4      RSTEETFTSTDANSNVRKRTNATVQSEADGGSRIDTRHSSVQPPSVAHGSSDLTPHSLSA
                    :.****.**********************************:******:****:*******

Chicken α4          SSPNPFTRI
Zebra finch α4      SSPNPFTRI
                    *********
```

Figure 3.2b. Alignment of the partial amino-acid sequence (in single letter code) encoding the zebra finch GABA$_A$ receptor α4 subunit with the corresponding chicken sequence (GenBank accession number XP_420724). No predicted zebra finch sequence encoding α4 was available

(c) Sequence of zebra finch GABA$_A$ receptor α6 subunit

```
Zebra finch α6(P)   PTEILRLNNLMVSKIWTPDTFFRNGKKSIAHNMTTPNKLFRIMQNGTILYTMRLTINADC
Zebra finch α6      PTEILRLNNLMVSKIWTPDTFFRNGKKSIAHNMTTPNKLFRIMQNGTILYTMRLTINADC
Chicken α6          PTEILRLNNLMVSKIWTPDTFFRNGKKSIAHNMTTPNKLFRIMQNGTILYTMRLTINADC
                    ************************************************************

Zebra finch α6(P)   PMRLVNFPMDGHACPLKFGSYAYPKSEIIYTWKKGPLHSVEVPQESSSLLQYDLIGQTVS
Zebra finch α6      PMRLVNFPMDGHACPLKFGSYAYPKSEIIYTWKKGPLHSVEVPQESSSLLQYDLIGQTVS
Chicken α6          PMRLVNFPMDGHACPLKFGSYAYPKSEIIYTWKKGPLHSVEVPQESSSLLQYDLIGQTVS
                    ************************************************************
                                                TM 1                 
Zebra finch α6(P)   SETIKSNTGEYVIMTVYFHLQRKMGYFMIQIYTPCIMTVILSQVSFWINKESVPARTVFG
Zebra finch α6      SETIKSNTGEYVIMTVYFHLQRKMGYFMIQIYTPCIMTVILSQVSFWINKESVPARTVFG
Chicken α6          SETIKSNTGEYVIMTVYFHLQRKMGYFMIQIYTPCIMTVILSQVSFWINKESVPARTVFG
                    ************************************************************
                      TM 2                      TM 3
Zebra finch α6(P)   ITTVLTMTTLSISARHSLPKVSYATAMDWFIAVCFAFVFSALIEFAAVNYFTNLQTQRAM
Zebra finch α6      ITTVLTMTTLSISARHSLPKVSYATAMDWFIAVCFAFVFSALIEFAAVNYFTNLQTQRAM
Chicken α6          ITTVLTMTTLSISARHSLPKVSYATAMDWFIAVCFAFVFSALIEFAAVNYFTNLQTQRAM
                    ************************************************************

Zebra finch α6(P)   RKAARAAALAAALSAATVPAEDEIVSHSDSNS
Zebra finch α6      RKAARAAALAAALSAATVPAEDEIVSHSDSNS
Chicken α6          RKAARAAALAAALSAATVPAEDEIVSHSDSNC
                    ********************************.
```

Figure 3.2c. Alignment of the partial amino-acid sequence (in single letter code) encoding the zebra finch GABA$_A$ receptor α6 subunit with the corresponding chicken sequence (GenBank accession number NP_990389) and predicted zebra finch sequence (GenBank accession number XP_002193341).

(d) Sequence of zebra finch GABA$_A$ receptor γ1 subunit

```
Zebra finch γ1(P)   HWITTPNRLLRIWSDGRVLYTLRLTINAECYLQLHNFPMDEHSCPLEFSSYGYPRNEIEY
Zebra finch γ1      HWITTPNRLLRIWSDGRVLYTLRLTINAECYLQLHNFPMDEHSCPLEFSSYGYPRNEIEY
Chicken γ1          HWITTPNRLLRIWSDGRVLYTLRLTINAECYLQLHNFPMDEHSCPLEFSSYGYPRNEIEY
                    ************************************************************

                                                                        _______
Zebra finch γ1(P)   KWKKTSVEVADPKYWRLYQFAFVGLRNTTEISHTLSGDYIIMTIFFDLSRRMGYFTIQTY
Zebra finch γ1      KWKKTSVEVADPKYWRLYQFAFVGLRNTTEISHTLSGDYIIMTIFFDLSRRMGYFTIQTY
Chicken γ1          KWKKTSVEVADPKYWRLYQFAFVGLRNTTEISHTLSGDYIIMTIFFDLSRRMGYFTIQTY
                    ************************************************************
                           _______TM 1_____     _________TM 2___________      ________
Zebra finch γ1(P)   IPCILTVVLSWVSFWINKDAVPARTSLGITTVLTMTTLSTIARKSLPKVSYVTAMDLFVS
Zebra finch γ1      IPCILTVVLSWVSFWINKDAVPARTSLGITTVLTMTTLSTIARKSLPKVSYVTAMDLFVS
Chicken γ1          IPCILTVVLSWVSFWINKDAVPARTSLGITTVLTMTTLSTIARKSLPKVSYVTAMDLFVS
                    ************************************************************
                           _____TM 3_____
Zebra finch γ1(P)   VCFIFVFAALMEYGTLHYFTSNRKGDKGKEKKAKSKPSKPPAIAVRPGSTLIPINNINHL
Zebra finch γ1      VCFIFVFAALMEYGTLHYFTSNRKGDKGKEKKAKSKPSKPPAIAVRPGSTLIPINNINHL
Chicken γ1          VCFIFVFAALMEYGTLHYFTSNRKGDKGKDKKAKPKPSKPSAIAVRPGSTLIPINNINNL
                    ****************************:****.*****.*****************:*

Zebra finch γ1(P)   PERDD
Zebra finch γ1      PERDD
Chicken γ1          PERDD
                    *****
```

Figure 3.2d. Alignment of the partial amino-acid sequence (in single letter code) encoding the zebra finch GABA$_A$ receptor γ1 subunit with the corresponding chicken sequence (GenBank accession number XP_420725) and predicted zebra finch sequence (GenBank accession number XP_002194407).

(e) Sequence of zebra finch GABA$_A$ receptor δ subunit

```
Zebra finch δ       NKLIRLQPDGVILYSIRITSTVACDMDLSKYPMDEQECMLDLESYGYSSEDIVYHWSENQ
Zebra finch δ(P)    NKLIRLQPDGVILYSIRITSTVACDMDLSKYPMDEQECMLDLESYGYSSEDIVYHWSENQ
Chicken δ           NKLIRLQPDGVILYSIRITSTVACDMDLSKYPMDEQECMLDLESYGYSSEDIVYHWSENQ
                    ************************************************************
                                                                  ___TM 1___
Zebra finch δ       EEIHGLDKLQLAQFTITNYQFTTEIMNFKS-GQFPRLSLHFHLRRNRGVYIIQSYVPSIL
Zebra finch δ(P)    EEIHGLDKLQLAQFTITNYQFTTEIMNFKS-GQFPRLSLHFHLRRNRGVYIIQSYVPSIL
Chicken δ           DEIHGLDKLQLAQFTITNYQFTTELMNFKSAGQFPRLSLHFHLRRNRGVYIIQSYVPSIL
                    :***********************:***** ****************************
                    __________      _____TM 2_____                ___TM 3____
Zebra finch δ       LVAMSWVSFWISQSAVPARVSLGITTVLTMTTLMVSARSSLPRASAIKALDVYFWICYVF
Zebra finch δ(P)    LVAMSWVSFWISQSAVPARVSLGITTVLTMTTLMVSARSSLPRASAIKALDVYFWICYVF
Chicken δ           LVAMSWVSFWISQSAVPARVSLGITTVLTMTTLMVSARSSLPRASAIKALDVYFWICYVF
                    ************************************************************
                    __________
Zebra finch δ       VFAALVEYAFAHFNADYMKKQKNKIKARRQSA
Zebra finch δ(P)    VFAALVEYAFAHFNADYMKKQKNKIKARRQSA
Chicken δ           VFAALVEYAFAHFNADYMKKQKNKIKARRQSG
                    ********************************.
```

Figure 3.2e. Alignment of the partial amino-acid sequence (in single letter code) encoding the zebra finch GABA$_A$ receptor δ subunit with the corresponding chicken sequence (GenBank accession number XP_001234041) and predicted zebra finch sequence (GenBank accession number XP_002197205).

At a peptide level the cloned zebra finch α3-subunit sequence shared 95.7% and 99.3% homology with the predicted zebra finch and chicken reference sequences respectively (Fig. 3.2a). Interestingly, the region where there was greatest variability between the

cloned and predicted zebra finch sequences was in the N-terminus within the highly conserved Cys-loop domain. In all other cloned sequences the Cys-loop sequence was identical between the predicted and cloned zebra finch sequences and the chicken reference sequence. The single mis-match in TM3 is potentially a single-nucleotide polymorphism (as it was present in all cloned sequences) whereby a guanine residue was substituted at the final base of the codon changing the amino acid from isoleucine (I) to methionine (M). A BLAST search using the cloned α3-subunit sequence as a probe against the chicken and zebra finch genomes revealed the gene to be localised to chromosome 4A in the zebra finch genome (GenBank accession number XR_053905) and chromosome 4 in the chicken genome (GenBank accession number XM_420268; Fig. 3.8). Available information on the NCBI database indicates that the zebra finch α3-subunit gene comprises 9 exons yielding a polypeptide of 48.6 kDa.

The deduced zebra finch α4-subunit amino acid sequence demonstrated 97.7% similarity with the chicken reference sequence (Fig. 3.2b). There was no documented or predicted zebra finch α4-subunit sequence available on relevant databases. Following sequencing of the α4-subunit gene, the cloned sequence was subjected to a BLAST search against the zebra finch genome which yielded no corresponding annotated sequence in the zebra finch, however a region on chromosome 4 was homologous (100% identity, genomic position 45,548,719-45,548,979) and as the α4 gene is localised to chromosome 4 in the chicken genome (GenBank accession number XM_420724), it was assumed that this was the corresponding zebra finch sequence. As expected, greatest variation between the chicken and zebra finch sequences occurred in the intracellular loop region between TM3 and TM4. The complete zebra finch α4-subunit gene comprises 10 exons (as opposed to the normal coding pattern of 9 exons) yielding a polypeptide of 61.3 kDa (considerably larger than the α3-subunit protein).

Highest sequence identity observed for any of the isolated subunit sequences was for that encoding the zebra finch α6-subunit. It presented 100% and 99.6% homology with the predicted zebra finch and chicken reference sequences respectively (Fig. 3.2c). The only difference was the final residue, which lies within the intracellular loop between TM3 and TM4, a naturally variable region. The same residue was present in all the clones from two different RT-PCR products and so could not be attributed to *Taq*

polymerase or sequencing errors and was present in the predicted zebra finch sequence. BLAST analysis showed the α6-subunit gene to be localised to chromosome 13 in the zebra finch (GenBank accession number XM_002193305) and chicken genome (GenBank accession number NM_205058), where it encodes 9 exons, yielding a polypeptide of 50.5 kDa.

The isolated zebra finch γ1-subunit amino acid sequence exhibited 100% and 98.4% homology with the predicted zebra finch and chicken reference sequences respectively at a peptide level (Fig. 3.2d). As observed with the α4 sequence (Fig. 3.2b), the greatest variability between the sequences occurred within the large intracellular loop connecting TM3 and TM4. The $GABA_A$ receptor γ1-subunit gene (GenBank accession number XM_002194366) is localised to chromosome 4 of the zebra finch and chicken genome (GenBank accession number XM_420725; Fig. 3.8).

There was 100% and 98.1% homology between the isolated δ-subunit sequence and predicted zebra finch and chicken reference sequences respectively (Fig. 3.2e). The two zebra finch sequences were homologous, with some residues different to the chicken sequence within the N-terminal region, but not within any of the highly conserved regions. The $GABA_A$ receptor δ-subunit gene (GenBank accession number XM_002197169) is localised to chromosome 21 in the zebra finch and the chicken genome (GenBank accession number XM_001234040; Fig. 3.2e). The complete zebra finch δ-subunit gene comprises 9 exons yielding a polypeptide of 48.7 kDa.

A complete alignment of all the $GABA_A$ receptor subunit genes is presented in Figure 3.5, where regions of interest are highlighted.

(f) Sequence of the zebra finch GABA$_A$ receptor π subunit

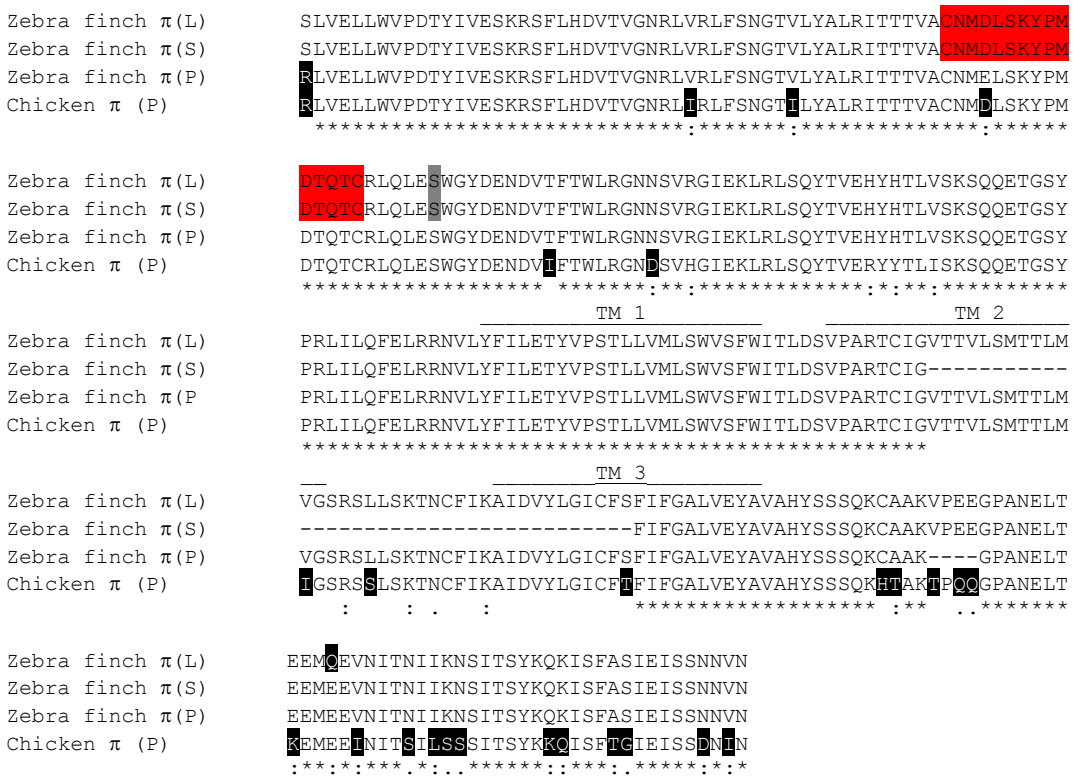


Figure 3.2f. Alignment of the partial amino-acid sequence (in single letter code) encoding the zebra finch GABA$_A$ receptor π subunit with the corresponding chicken sequence (GenBank accession number XP_414507) and predicted zebra finch sequence (GenBank accession number XP_002188723). Abbreviations as before. (L) long, (S) short.

Isolation of partial cDNAs encoding the π-subunit yielded two similar size but distinct products, of apparent equal intensity (data not shown). Both products were purified and sent for automated sequencing and BLAST analysis confirmed that they were both DNA sequences encoding the GABA$_A$ receptor π subunit, but the smaller sequence had a 37 AA deletion; the missing region of sequence encodes part of TM2 and TM3; the reading frame remained unchanged with the deletion (Fig. 3.2f). The predicted zebra finch GABA$_A$ receptor π-subunit gene (GenBank accession number XM_002188687) is localised to chromosome 13, as is the predicted corresponding chicken gene (GenBank accession number XM_414507; Fig. 3.8). The full length predicted zebra finch π-subunit gene comprises 8 exons yielding a polypeptide of 48.6 kDa (Fig. 3.3).

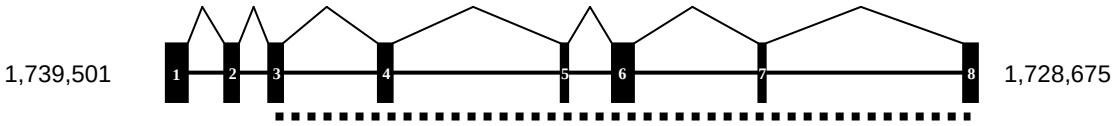


Figure 3.3. Exonic arrangement of zebra finch $GABA_A$ receptor π-subunit gene adapted from the Ensembl genome browser (www.ensembl.org). Genomic coordinates (chromosome 13) corresponding to the amplified cDNA sequences are given; there are 8 coding exons within the gene. Exon 7 is partly deleted in the shorter version of the π-subunit mature mRNA. The dashed line denotes the exons within the region amplified by RT-PCR.

Discounting the deletion, there was 99.6% similarity between the two deduced $GABA_A$ receptor π subunit amino acid sequences cloned from the zebra finch at a peptide level. (Fig. 3.2f) Furthermore, there was 99.6% identity for both the long and short sequences with the predicted zebra finch reference sequence (excluding the deletion). The short sequence demonstrated 90.3% sequence similarity with the chicken reference sequence and the long sequence shared 90.2% identity with the corresponding chicken gene. A Spidey analysis (www.ncbi.nlm.nih.gov/IEB/Research/Ostell/Spidey /index.html) was performed to compare the cloned mRNA sequences to the genomic sequence of the zebra finch (GenBank accession number ABQF01055946) and this revealed that the shorter version was missing an exonic section (7) of the gene (Fig. 3.4). Recognition of splice sites in pre-mRNAs (precursor mRNAs) to remove intronic sequence by spliceosomes is achieved by consensus sequences located at polar termini of the introns (Berget, 1995). There are matrices available for the consensus recognition sequences, for U2 dependent introns (U2 is a small nuclear ribonucleoprotein (snRNP), which along with U1, U4, U5 and U6 compose spliceosomes); these are the major class of introns, and splice sites conform as follows (Zhang *et al*., 1999):

Intron Exon
3' splice sites: C<u>AG</u> | G

Intron Exon
5' splice sites: MAG | <u>GT</u>RAGT (where M is A or C and R is A or G)

These sites can vary slightly dependent on the bases used, but the underlined bases have a 100% usage frequency. By comparison of the cloned sequences with the genomic sequences it was possible to determine the location of the splice sites and proffer an explanation as to the 37 AA deletion in the short version (Fig. 3.4).

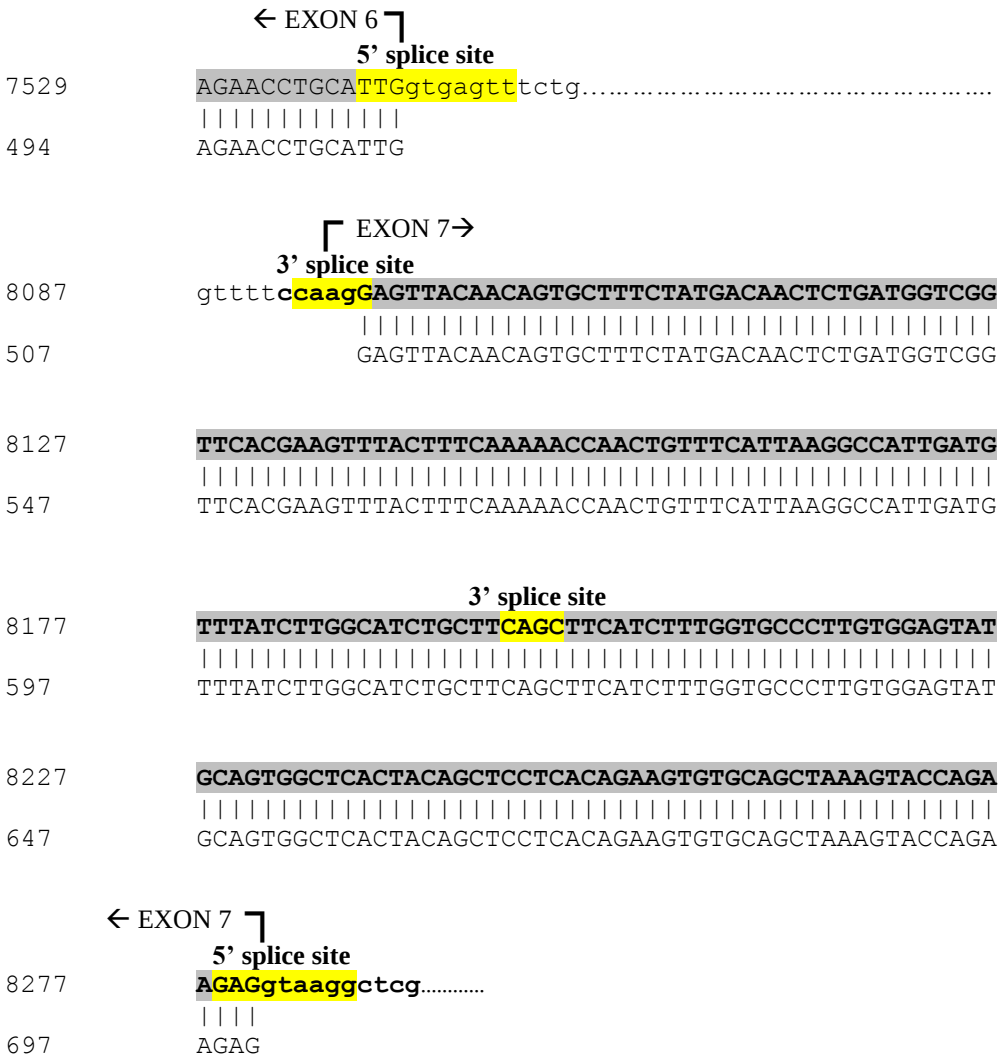
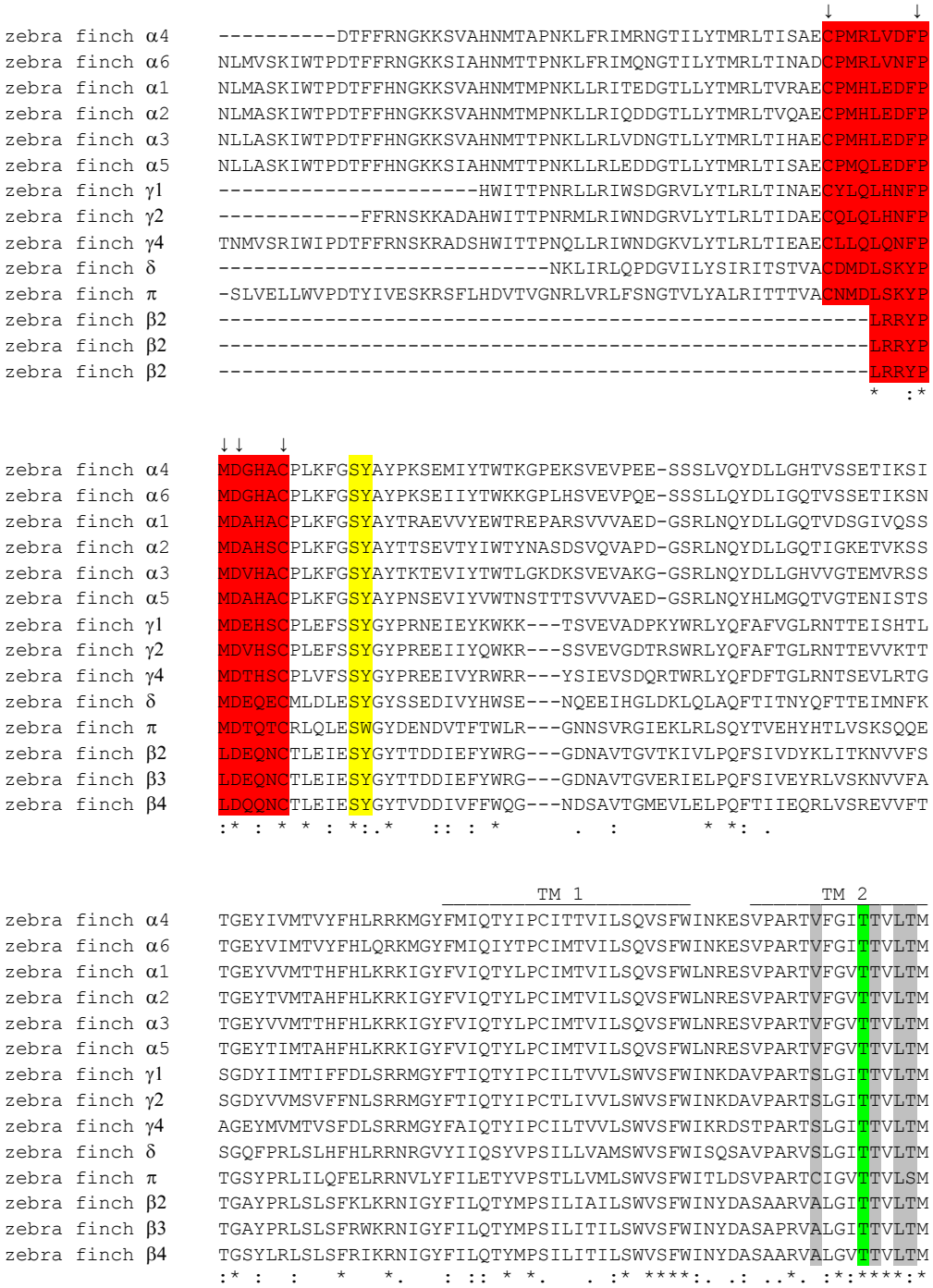


Figure 3.4. Spidey alignment of amplified partial cDNA encoding the $GABA_A$ receptor π subunit with the corresponding region of chromosome 13 of zebra finch genomic sequence (GenBank accession number ABQF01055946). Highlighted in grey are exons (which are also numbered), lowercase lettering denotes intronic sequence and splice junctions occurring in wild type long-form pre-mRNA are highlighted in yellow. Genomic and cloned mRNA coordinates are given. Splicing at genomic coordinates 7538 and 8096 removes intronic sequence between exon 6 and exon 7 and the long version of the cDNA is spliced in this way, thereby preserving all of exon 7. The short cDNA is spliced at the 5' splice site at the terminus of exon 4 but the splice junction at the beginning of exon 5 is skipped and a second splice junction is used which is located within exon 7 (position 8199, genomic coordinates), thereby removing a large portion of exon 7 from the mature mRNA. Both sequences were spliced at the junction located at the end of exon 7 and the beginning of exon 8 as normal.

The shorter sequence occurs due to a 37 AA deletion removing part of TM2 and TM3 domains and the extracellular loop in between (Fig. 3.4). This is a result of a deletion in exon 7, caused by the skipping of the putative 3' splice junction at the beginning of

exon 7 (genomic position 8097 and cloned mRNA position 507), and instead splicing at an alternative acceptor site located at coordinates 8199 of the genomic sequence (Fig. 3.4). The following 5' splice junction remained the same in the short sequence as in the long sequence; this resulted in a truncated mature mRNA.



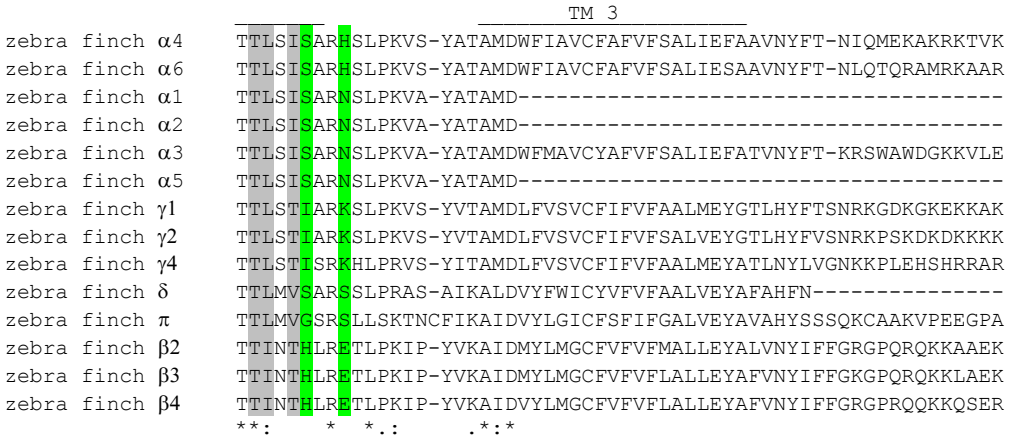


Figure 3.5. Alignment of all cloned zebra finch $GABA_A$ receptor subunit amino acid sequences. TM domains are annotated, (*) denote complete homology between sequences and dots represent low (.) or partial (:) homology, (-) gaps were introduced to maintain the alignment. The 15AA Cys-loop domain is well conserved between subunit classes (red) with the two peripheral cysteines and central PMD/PLD (single-letter code) sequence identical in all sequences (↓) where cloned. Conserved Ser^{171} and Tyr^{172} residues shortly after the Cys-loop consensus sequence are indicated in yellow. Within the TM2 region, channel lining residues are highlighted in grey and positions at which disulphide bonds are formed are indicated in green (Horenstein *et al*., 2001)

Figure 3.5 illustrates the high homology existing between all zebra finch $GABA_A$ receptor subunit genes. Regions of strong sequence conservation include the Cys-loop domain, serine and tyrosine residues within the N-terminus and hydrophobic transmembrane domains, suggestive of important physiological roles. Moreover, the intracellular TM1-TM2 loop is well conserved within subunit classes, indicative of a subunit specific function; this is also observed in the extracellular TM2-TM3 linker domain. It is only the π-subunit sequence which does not have the conserved hydrophilic Tyr^{172} residue and has a hydrophobic tryptophan residue in its place.

3.2.1 Sequences encoding zebra finch $GABA_C$ receptor subunits

The three subunits of the $GABA_C$ receptor were isolated from the zebra finch brain. The cloned zebra finch ρ1-subunit peptide sequence exhibited 98.4% homology with the chicken reference sequence (Fig. 3.6a). No annotated sequence exists for the zebra finch $GABA_C$ receptor ρ1-subunit gene, a BLAST analysis of the cloned transcript against the zebra finch genome revealed homology with a region on chromosome 3 slightly upstream of the gene encoding the $GABA_C$ receptor ρ2-subunit within the zebra finch

genome. The corresponding gene in the chicken is localised to chromosome 3 (GenBank accession number XM_426190).

The cloned zebra finch ρ2-subunit peptide sequence shared 96.9% and 99.6% identity with the predicted zebra finch and chicken reference sequences respectively (Fig. 3.6b). The zebra finch $GABA_C$ receptor ρ2-subunit gene (GenBank accession number XR_054611) is localised to chromosome 3 as in the chicken genome (GenBank accession number XM_419839) close to the ρ1-subunit gene in the same transcriptional orientation. Both the ρ1- and ρ2-subunit genes comprise 9 exons yielding polypeptides of 48.6 and 54.2 kDa respectively.

(a) Sequence of the zebra finch $GABA_C$ receptor ρ1 subunit

```
Chicken ρ1(P)     SMTFDGRLVKKIWVPDMFFVHSKRSFIHDTTTDNVMLRVQPDGKVLYSLRVTVTAMCNMD
Zebra finch ρ1    SMTFDGRLVKKIWVPDMFFVHSKRSFIHDTTTDNVMLRVQPDGKVLYSLRVTVTAMCNMD
                  ************************************************************

Chicken ρ1(P)     FSRFPLDTQTCSLEIESYAYTEDDLMLYWKNGNDSLKTDERISLSQFLIQEFHTTTKLAF
Zebra finch ρ1    FSRFPLDTQTCSLEIESYAYTEDDLMLYWKNGNDSLKTDERISLSQFLIQEFHTTTKLAF
                  ************************************************************
                                  _____TM 1_________          _____TM 2____
Chicken ρ1(P)     YSSTGWYNRLYINFTLRRHIFFFLLQTYFPATLMVMLSWVSFWIDRRAVPARVPLGITTV
Zebra finch ρ1    YSSTGWYNRLYINFTLRRHIFFFLLQTYFPATLMVMLSWVSFWIDRRAVPARVPLGITTV
                  ************************************************************
                  __________          ________TM 3_______
Chicken ρ1(P)     LTMSTIITGVNASMPRVSYIKAVDIYLWVSFVFVFLSVLEYAAVNYLTTVQERKPRKLRD
Zebra finch ρ1    LTMSTIITGVNASMPRVSYIKAVDIYLWVSFVFVFLSVLEYAAVNYLTTVQERKXRKLRD
                  ************************************************** *****

Chicken ρ1(P)     KLPCACSLPQPRPMMMDGSY
Zebra finch ρ1    KPACACSLPQPRPMMVDGSY
                  * .************:****
```

Figure 3.6a. Alignment of the partial amino-acid sequence (in single letter code) encoding the zebra finch $GABA_C$ receptor ρ1 subunit with the corresponding chicken sequence (GenBank accession number XP_426190). **Abbreviations** for all figure 3.6a-c: (P) denotes predicted sequence, asterisks (*) denote complete homology between the two sequences and the dots (.), (:), represent partial homology Differences between the sequences are highlighted in black. Also shown are transmembrane spanning domains (TM); putative Cys-loop consensus sequence (red) and the putative Serine (S) and Tyrosine (Y) residues (grey).

(b) Sequence of the zebra finch GABA$_C$ receptor ρ2 subunit

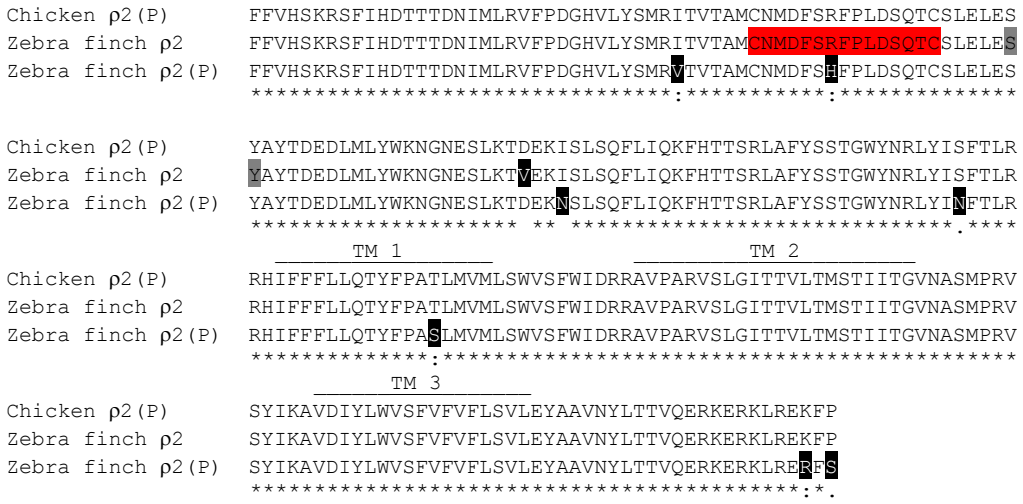


Figure 3.6b. Alignment of the partial amino-acid sequence (in single letter code) encoding the zebra finch GABA$_C$ receptor ρ2 subunit with the corresponding chicken sequence (GenBank accession number XP_419839) and the predicted zebra finch sequence (as no peptide sequence was available it was translated from the nucleotide sequence, GenBank accession number XM_054611).

(c) Sequence of the zebra finch GABA$_C$ receptor ρ3 subunit

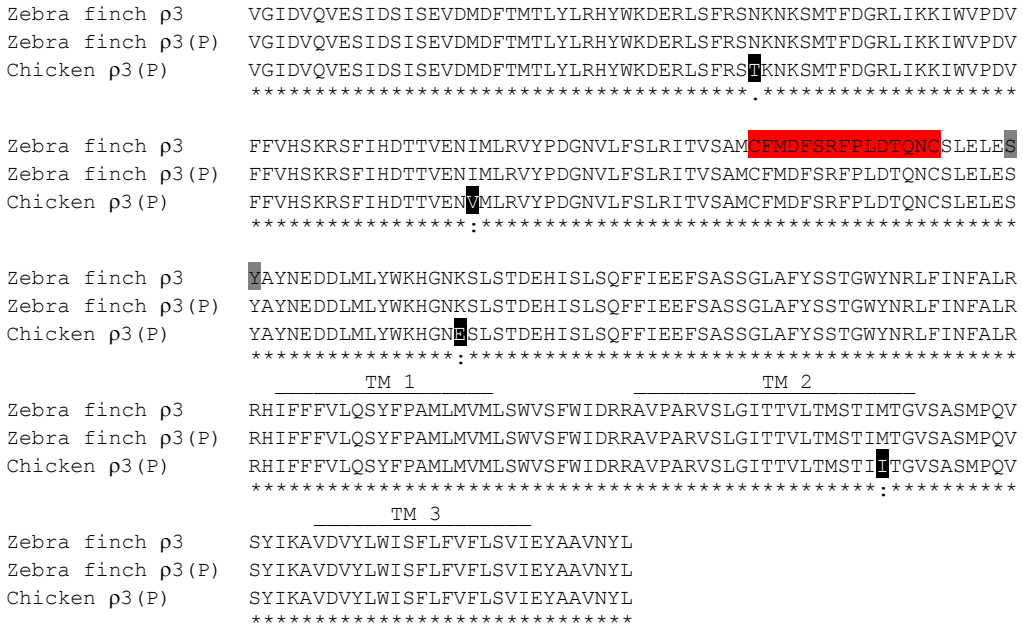


Figure 3.6c. Alignment of the partial amino-acid sequence (in single letter code) encoding the cloned zebra finch GABA$_C$ receptor ρ3 subunit with the corresponding chicken sequence (GenBank accession number XP_428531) and the predicted zebra finch sequence (Genbank accession number XP_002190668).

Zebra finch amino-acid ρ3-subunit sequence exhibited 100% and 98.5% homology with the predicted zebra finch and chicken reference sequences respectively at a peptide level; any mis-matches between zebra finch and chicken sequences were found within the region encoding the N-terminus, although not within any well conserved domains such as the Cys-loop (Fig. 3.6c). The zebra finch $GABA_C$ receptor ρ3-subunit gene (GenBank accession number XM_00219068) is localised to chromosome 1 as is the corresponding gene in the chicken genome (GenBank accession number XM_428531; Fig. 3.8), although this is some distance from the $GABA_A$ receptor gene cluster γ3α5β3 on the same chromosome. On the NCBI database the ρ3-subunit gene is annotated with 9 exons translating into a polypeptide of 49.5kDa.

```
                                                          ↓       ↓↓↓   ↓
zebra finch ρ1      FFVHSKRSFIHDTTTDNVMLRVQPDGKVLYSLRVTVTAMCNMDFSRFPLDTQTCSLEIES
zebra finch ρ2      FFVHSKRSFIHDTTTDNIMLRVFPDGHVLYSMRITVTAMCNMDFSRFPLDSQTCSLELES
zebra finch ρ3      FFVHSKRSFIHDTTVENIMLRVYPDGNVLFSLRITVSAMCFMDFSRFPLDTQNCSLELES
                    **************.:*:**** ***:**:*:*:**:*** *********:*.****:**

zebra finch ρ1      YAYTEDDLMLYWKNGNDSLKTDERISLSQFLIQEFHTTTKLAFYSSTGWYNRLYINFTLR
zebra finch ρ2      YAYTDEDLMLYWKNGNESLKTDEKISLSQFLIQKFHTTSRLAFYSSTGWYNRLYISFTLR
zebra finch ρ3      YAYNEDDLMLYWKHGNKSLSTDEHISLSQFFIEEFSASSGLAFYSSTGWYNRLFINFALR
                    ***.::********:**.**.***:******:*::* ::: *************:*.*:**
                      _______ TM 1 _______           ________ TM 2 ___ _
zebra finch ρ1      RHIFFFLLQTYFPATLMVMLSWVSFWIDRRAVPARVPLGITTVLTMSTIITGVNASMPRV
zebra finch ρ2      RHIFFFLLQTYFPATLMVMLSWVSFWIDRRAVPARVSLGITTVLTMSTIITGVNASMPRV
zebra finch ρ3      RHIFFFVLQSYFPAMLMVMLSWVSFWIDRRAVPARVSLGITTVLTMSTIMTGVSASMPQV
                    ******:**:**** ********************.************:***.****:*
                        _______TM 3_______
zebra finch ρ1      SYIKAVDIYLWVSFVFVFLSVLEYAAVNYLTTVQERKXRKLRDKPACACSLPQPRPMMVD
zebra finch ρ2      SYIKAVDIYLWVSFVFVFLSVLEYAAVNYLTTVQERKERKLREK------FP--------
zebra finch ρ3      SYIKAVDVYLWISFLFVFLSVIEYAAVNYL-------------------------------
                    *******:***:**:******:********
```

Figure 3.7. Alignment of all cloned zebra finch $GABA_C$ receptor subunit amino acid sequences. TM domains are annotated, (*) denote complete homology between sequences and dots represent low (.) or partial (:) homology, (-) gaps were introduced to maintain the alignment. The 15AA Cys-loop domain is well conserved (red) with the two peripheral cysteines and central PMD/PLD sequence identical in all sequences (↓). Conserved Ser^{171} and Tyr^{172} residues shortly after the Cys-loop consensus sequence are indicated in yellow. Within the TM2 region, potential channel lining residues are highlighted in grey.

The high sequence conservation that exists between all the $GABA_C$ receptor subunits is illustrated in Figure 3.7. As with the $GABA_A$ receptor subunits, regions of strong sequence conservation include the Cys-loop domain, serine and tyrosine residues within the N-terminus and hydrophobic transmembrane domains. There is high similarity between all the zebra finch and chicken $GABA_A$ and $GABA_C$ receptor subunit sequences. When compared to mammalian sequences the identity remains high at ~90%

(data not shown), indicative of common ancestry and an evolutionary conservation of function. This is further validated by the chromosomal arrangement of the genes within the zebra finch genome, which is almost identical to that of humans (Fig. 3.8).

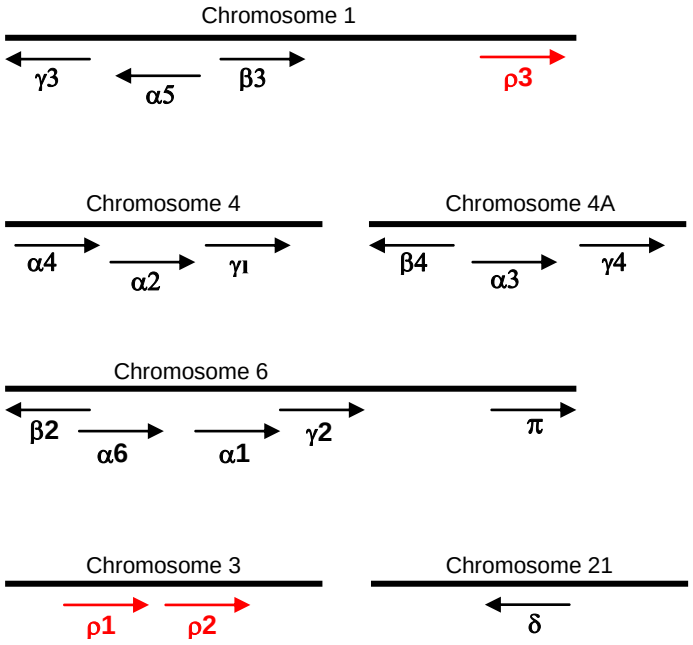


Figure 3.8. Schematic depicting chromosomal arrangements and transcriptional orientation of isolated $GABA_A$ and $GABA_C$ receptor subunit genes within the zebra finch genome. Data has been adapted from information available on Ensembl and NCBI databases following BLAST analyses and data obtained from Tsang *et al*. (2007). Arrows denote forward (→) and reverse (←) transcriptional orientation. $GABA_A$ receptor subunit genes are labelled in black and $GABA_C$ receptor subunit genes are labelled in red.

Genes encoding the $GABA_A$ receptor subunits within the zebra finch genome are localised in clusters similar to those of the human genome (Fig. 3.8). There are four primary gene clusters, three of which comprise αβγ-subunit encoding genes; in all cases the β-subunit gene is in an opposing transcriptional orientation to the α– and γ-subunit genes. Subunit-encoding genes outside the αβγ class (π, δ and ρ1-3) are not found in clusters, although ρ1- and ρ2-subunit genes are in close proximity on chromosome 3. The zebra finch δ-subunit gene is the only gene located on a separate chromosome from other $GABA_A$ receptor genes. Although the zebra finch π-subunit gene is located on chromosome 13 with genes encoding α1, α6, β2, γ2 subunits it is some distance from the cluster, as in the human genome (Simon *et al*., 2004).

3.4 Discussion

Partial cDNAs encoding $GABA_A$ receptor α3, α4, α6, γ1, δ and π subunits and $GABA_C$ receptor ρ1-ρ3 subunits were amplified from zebra finch. Subsequently, discriminative real-time RT-PCR primers and *in situ* hybridisation probes could be designed in order to systematically map and quantify expression of all subunit genes within the zebra finch song system. Peptide alignments with corresponding chicken sequences (which are phylogenetically similar) presented high sequence similarity (> 97%, except for π-subunit sequences which exhibited only 90% similarity), thus confirming their identity (Fig. 3.2a-f) and indicating a strong conservation of function. At least five clones were sequenced from two independent RT-PCR experiments for each subunit; ensuring any *Taq* DNA polymerase and sequencing errors could be easily identified. All RT-PCRs gave multiple products (due to the degeneracy of the primers), but amplicons of expected size were excised, purified, cloned and sequenced. It was only after all the subunit cDNAs (apart from the π subunit) had been amplified that predicted zebra finch $GABA_A$ receptor subunit sequences were released on NCBI, following the sequencing of the zebra finch genome. These were published as predicted sequences but when aligned with the sequences obtained from the zebra finch clones there was > 95% homology in all cases.

All isolated sequences encoded the extracellular N-terminus (encompassing the Cys-loop and conserved serine and tyrosine residues), characteristic putative transmembrane domains TM1-TM3 and their linking intracellular/extracellular loops. All $GABA_A$ receptor subunit sequences contained the highly conserved octapeptide TTVLTMTT (single-letter code) within the TM2 domain (Fig. 3.5). In $GABA_C$ receptor subunit sequences there was an amino acid substitution giving the sequence TTVLTSTT, which was identical in all three subunit encoding sequences (Fig. 3.7). This sequence (towards the extracellular end of TM2) incorporates residues which line the anion pore (detailed on Figs. 3.5 and 3.7) and is the region in which disulphide bonds are formed (Horenstein *et al*., 2001), and is therefore highly conserved. Characterisation of this region in cation-selective members of the LGIC superfamily has deduced that the ion channel gate is located to TM2 or the TM1-TM2 linker, although the precise location remains an issue of some debate (Peters *et al*., 2005).

The Cys-loop consensus sequence is located within the region encoding the extracellular N-terminus (ligand binding) domain and first transmembrane pore-forming domain. It is a 15AA disulphide-bridged region which is highly conserved across all known vertebrate $GABA_A$ receptor sequences (and other members of the LGIC superfamily, Simon *et al*., 2004). Results indicated that two peripheral cysteine residues and the PMD/PLD residues at positions 9-11 were conserved in all cloned $GABA_A$ and $GABA_C$ receptor sequences (Figs. 3.2a-f and 3.6a-c). Due to the location, it is proposed that in nAChRs (and hence other LGICs) the Cys-loop functions as a pivot which effectively stabilises the receptor conformation during the gating process (Unwin *et al*., 2002; Grutter *et al*., 2005; Unwin, 2005). Data provided by mutations of the motif indicates the same function in $GABA_A$ receptors (Tierney *et al*., 2008). The Cys-loop disulphide bond is essential for receptor assembly (Blount and Merlie, 1990; Fu and Sine, 1996). The highly conserved hydrophobic PMD/PLD domain (at the tip of the Cys-loop) is not required for normal protein folding and expression, but rather is involved in impedance of drug-potentiated channel gating and direct activation of the receptor in a subunit-dependant manner (Tierney *et al*., 2008). Poor homology between cloned and predicted zebra finch α3-subunit sequences within the Cys-loop domain was unanticipated. The isolated zebra finch sequence presents a characteristic Cys-loop domain sequence (Fig. 3.2a), however in the predicted α3-subunit sequence (annotated from the published zebra finch genome (http://genome.wustl.edu/genomes/view/taeniopygia_guttata/), the initial cysteine residue was missing and the highly conserved central motif was changed. Predicted/annotated sequences are not always accurate (as inferred by the name) and are generated by computational algorithms, leaving much room for error as was the case here. This highlights the requirement for experimental evidence to confirm the integrity of theoretical/predicted sequences.

Six residues downstream from the Cys-loop consensus sequence are located conserved serine (S) and tyrosine (Y) residues (Figs. 3.5 and 3.7). These are both highly conserved within the $GABA_A$ and $GABA_C$ receptor subunit sequences with the exception of the π-subunit gene, whereby the hydrophilic tyrosine (Y) is replaced with hydrophobic tryptophan (W). Mutagenesis studies with the γ2 subunit have demonstrated that substitution of Ser^{171} and Tyr^{172} with glycine and threonine respectively, prevented receptor association and expression at the cell surface (Jin *et al*., 2004). Yet this effect

was not evident when the same mutations were introduced to the α2 and β1 subunits. Furthermore, previous work whereby Tyr^{172} was substituted with a serine residue in the γ2-subunit sequence showed no discernable effects on binding or association (Whiting *et al*., 1997), thus it may be only the Ser^{171} residue which is critical for receptor association.

Another observation was the high sequence homology of the TM1-TM2 loop within different subunit classes; this was indicative of a conserved physiological function that may be specific to each class of subunits (Fig. 3.5). Previous studies have shown mutations of residues within this region of the β3 subunit alter ion selectivity of the pore from anion to cation without affecting the pharmacology. This was only apparent with the β3 subunit as the same mutations in the α2- and γ2-subunit polypeptides elicited no such changes (Jensen *et al*., 2002), further substantiating the theory of subunit-class specific effects. Greatest variability between the cloned sequences and the chicken/zebra finch reference sequences was within regions encoding the large intracellular loop between TM3 and TM4 (Fig. 3.5). This is the most variable region within, otherwise well conserved sequences, and serves to differentiate the subunits. This linker region is well situated for interactions with the intracellular environment, the primary function appears to be trafficking of the receptors via interactions of the loop and associated proteins such as $GABA_A$ receptor-associated protein (GABARAP; Chen *et al*., 2005); golgi-specific zinc-finger protein (Keller *et al*., 2004); $GABA_A$ receptor interacting factor-1 (Kittler *et al*., 2004); phospholipase C-related inactive protein type 1 (Terunuma *et al*., 2004) and ubiquitin-like protein Plic-1 (Bedford *et al*., 2001), all of which are subunit specific (due to variability within the loop sequences) and demonstrate an involvement in receptor trafficking (for further details refer to section 1.2.8). Thus variations between subunit sequences are necessary to provide different binding sites for a range of associated proteins fulfilling diverse physiological functions. It is also postulated that the intracellular loop may influence ion conduction (Peters *et al*., 2005).

Isolation of $GABA_A$ receptor π-subunit partial cDNA was challenging due to contradictory information in databases (NCBI and Ensembl), which resulted in unreliable sequences with which to design even degenerate RT-PCR primers. Two chicken $GABA_A$ receptor π-subunit sequences were available (GenBank accession

numbers: XM_414507 and XM_426524). The former was reported to be located on chromosome 13 and the latter on chromosome 6. Both were generated from predictive automated computational analysis sharing only 55% identity to each other and, 49% and 56% identity, (at amino-acid level) to corresponding rat and human sequences respectively. This degree of similarity was low compared to other subunits, as $GABA_A$ receptor subunit sequence identity between vertebrate species is close to 90% (Fig. 3.2a-f). Degenerate primers based on these sequences did not successfully amplify the π-subunit cDNA. However, the published zebra finch genome predicted a π-subunit sequence localised to chromosome 13 sharing 77% homology, at a nucleotide level, with the corresponding gene in rat (GenBank accession numbers: rat, NM_031029, zebra finch XM_002188687) and this was the sequence used to design the final set of primers. Long and short forms of the π-subunit cDNA were isolated from the zebra finch brain and both exhibited 90% sequence identity with the chicken reference sequence (Fig. 3.2f). Levels of heterogeneity within the $GABA_A$ receptor family have been furthered by the identification of splice variants; however, these have not been described for all subunits. A novel splice variant of the $GABA_A$ receptor π-subunit gene has been identified in this study, which has not been previously reported (Figs. 3.2f and 3.4). Analysis of the human genome has revealed the π-subunit gene to compose 10 exons (9 coding), conforming to normal $GABA_A$ receptor coding patterns (Simon *et al*., 2004), and in the zebra finch and chicken genomes there are 8 and 9 coding exons respectively. Interestingly, no splice variants have been reported for the $GABA_A$ receptor π subunit, in any species. *In silico* examination of the human genome revealed the presence of an *Alu* sequence within the intronic sequence which, should a favourable intronic splice site be created, would result in a well expressed splice variant (Simon *et al*., 2004). However, these short interspersed elements are exclusive to primates, thus a splice variant (similar to that seen with the γ2-subunit gene) would not be observed within the zebra finch gene. The variant reported here is likely to be a non-functional protein due to the deletion of a large portion (37AA) of exon 7 corresponding to part of TM2 (which forms intrinsic chloride pore lining) and TM3 and the extracellular loop lying between them. Analysis of the deleted sequence revealed no loss of phosphorylation sites.

Another observation was that during amplification of π-subunit cDNA from zebra finch brain; agarose gel bands representing both amplicons (full length and splice variant) were of equal intensity (data not shown). It could therefore, tentatively be speculated that the truncated form appeared at a frequency of ~50%. However, it must be considered that this may have been an artefact of the RT-PCR due to the increased efficiency of amplification of a shorter product. RT-PCRs and subsequent sequencing reactions were performed in triplicate (with cDNA from different zebra finch brains) to ensure the data were consistent and the variant was not an anomaly, all reactions gave the same result.

Furthermore, initial attempts were made to isolate the π-subunit utilised zebra finch cDNA from ovarian, gastrointestinal and lung tissue due to its primarily peripheral localisation (Symmans *et al*., 2005; Xiang *et al*., 2007). Following poor amplification of expected products, the primers were tested with brain-derived cDNA and two products were immediately apparent. This was in conflict with published data which suggests that π-subunit mRNA is rarely expressed in the brain (Hedblom and Kirkness, 1997; Jin *et al*., 2005) rather, it is predominantly expressed in alveolar tissues of the lungs (Jin *et al*., 2005; Xiang *et al*., 2007) where receptors containing this subunit are implicated in alveolar fluid homeostasis (Jin *et al*., 2006). In addition, the π-subunit transcript has also been detected in ovaries and smooth muscle cells of the urinary bladder and uterus in rat, where it has been suggested that π-subunit-containing $GABA_A$ receptors play a role in the regulation of muscle contraction (Hedblom and Kirkness, 1997; Fujii and Mellon, 2001). Moreover, it has been defined as a potential marker for detection of breast lymph node metastasis (Backus *et al*., 2005; Symmans *et al*., 2005), and is over-expressed in pancreatic adenocarcinomas (malignant tumour originating in glandular tissue; Johnson and Haun, 2005). Robust expression in zebra finch brain may be indicative of a physiological role more relevant to avian species than mammalian systems. However, no such differences are documented for any of the other $GABA_A$ receptor subunit transcripts. Low expression in mammalian CNS may also explain why no splice variants have been previously detected, or the variant may be exclusive to lower vertebrates. It is conceivable that the alternatively-spliced variant is restricted to the brain/CNS and not found in peripheral tissues, akin to the ε-subunit transcript variants, which are differentially expressed. The full-length ε transcript is well

expressed in heart and lung tissue, and at low levels in the rat brain (Davies *et al*., 1997; Whiting *et al*., 1997; Wilke *et al*., 1997), and two further variants are expressed in peripheral tissues (Wilke *et al*., 1997). Although no specific function has been assigned to particular isoforms of the $GABA_A$ receptor subunits; there is evidence of regional- (Kirkness and Fraser, 1993; Harvey *et al*., 1994; Korpi *et al*., 1994; Paulson *et al*., 2000; Fuchs and Celepirovic, 2002; Mu *et al*., 2002), and developmental-specific (Kirkness and Fraser, 1993; Fuchs and Celepirovic, 2002; Mu *et al*., 2002) regulation of expression within tissues (most notably the brain), which is suggestive of functionality. Splice variants are important as they have been implicated in disease states (Huntsman *et al*., 1998; Dredge *et al*., 2001; Volk *et al*., 2002; Zhao *et al*., 2009) and severely truncated polypeptides (such as α4 and π subunits) may perform a regulatory function in the trafficking and assembly of certain $GABA_A$ receptors (Mu *et al*., 2002).

Zebra finch was the second avian species to have its genome sequenced, after that of the chicken. There is almost identical similarity between the chicken and zebra finch genome with the exception of chromosome 4 of which in passerine species there are two chromosomes, denoted 4 and 4A (Derjusheva *et al*., 2004). Therefore some of the genes present on chromosome 4 and 4A of the zebra finch are present on chromosome 4 in the chicken. Within the NCBI gene database, predicted sequences encoding the zebra finch α1- and α2-subunit polypeptides have been localised to chromosomes 4 and 13 of the genome respectively. In contrast to the chicken genome, where the α1-subunit gene is on chromosome 13 and the α2-subunit gene is on chromosome 4. As the remainder of the $GABA_A$ receptor subunit chromosomal locations are identical to that of the chicken, it was assumed this may be an error in the annotation of the zebra finch sequences. Preliminary BLAST searches probing the chicken genome with the predicted zebra finch α1-subunit sequence produced a match within chromosome 4 (location of α2-subunit gene), furthermore, a second analysis with the zebra finch α2-subunit sequence produced a match within chromosome 13 in the chicken genome (location of α1-subunit gene). Nucleotide alignments have demonstrated that the zebra finch α2-subunit sequence was 93% identical to the α1-subunit sequence of chicken, indeed alignments of chicken and zebra finch α2-subunit sequences showed only 65% homology and with the α1-subunit sequence showed 68% homology, but alignment of zebra finch α1-subunit and chicken α2-subunit sequences gave a sequence identity of 90%. It was

therefore concluded that the predicted zebra finch α1- and α2-subunit sequences (GenBank accession number XM_002194306 and XM_002193396 respectively) have been mis-labelled, but the chromosomal locations are correct. This would then be in accordance with the clustering of the same genes within the human genome. Again this highlights the requirement for experimental evidence to confirm the integrity of theoretical/predicted sequences.

Isolated zebra finch sequences were aligned with the whole zebra finch genome and in agreement with the clustering of the $GABA_C$ receptor subunit genes within the human genome (Fig. 3.8), the zebra finch ρ1- and ρ2-subunit genes are located together on chromosome 3 (chromosome 6 in humans; Cutting *et al*., 1992) and the ρ3-subunit gene is located on chromosome 1 (chromosome 3 in humans; Bailey *et al*., 1999; Simon *et al*., 2004). Isolated sequences encoding the $GABA_C$ receptor ρ1- and ρ2-subunits exhibit the greatest similarity (84%), with the ρ3-subunit sequence sharing 74% and 67% homology with sequences encoding ρ1 and ρ2 subunits respectively. High identity between the three different subunits is indicative of a common ancestor, ρ1- and ρ2-subunit genes are closely located on the same chromosome, and so are likely to be a consequence of local duplication during evolution (Bailey *et al*., 1999), whereas it is purported that the ρ3-subunit gene may be a product of a duplication of a ρ1-/ρ2-subunit gene ancestor (Bailey *et al*., 1999). $GABA_C$ receptor subunits display relatively low sequence identity with their $GABA_A$ counterparts (28-42% in human homologues); suggesting an early evolutionary divergence from the $GABA_A$ receptor subunit genes (Bailey *et al*., 1999). Native $GABA_A$ receptors may be composed of their chromosomal partners (Barnard et al 1998). They are not thought to commonly form homomers and subunit genes are found in αβγ clusters on chromosomes (Simon *et al*., 2004; Darlison *et al*., 2005; Olsen and Sieghart, 2008), with the β-subunit gene in an opposite transcriptional orientation to α- and γ-subunit genes (Tsang *et al*., 2007). For example the most prevalent subtype in the mammalian CNS is α1β2γ2 (Whiting, 2003) and the respective genes are located in a single cluster on chromosome 5 (Simon *et al*., 2004). Chromosomal positions of the $GABA_C$ receptor subunit genes both in the human and zebra finch genome do not easily lend themselves to the possibility of co-expression, however there is a small amount of evidence to the contrary (Qian and Pan, 2002; Milligan *et al*., 2004). Furthermore, confirmed native $GABA_A$ receptor subtypes such as

α3βγ2, α4βγ2, α4β3δ, α6β3,δ (Olsen and Sieghart, 2008), to name but a few, all compose of polypeptides from up to three different chromosomes, in light of this, $GABA_A$ and $GABA_C$ receptor subunit association cannot be completely discounted due to chromosomal positioning. However, the $GABA_C$ receptor subunits more readily form homomeric assemblies (Bormann, 2000), and do so frequently (Olsen and Sieghart, 2008), so there is less requirement for them to be close chromosomal partners.

It has been generally accepted that in avian species, β1- or γ3-subunit polypeptides have been lost (Darlison and Albrecht, 1995) or have evolved into non-functioning pseudogenes (Darlison *et al*., 2005). However, in the recent sequencing of the zebra finch genome, sequences encoding these subunits have been identified, the β1-subunit gene is localised to chromosome 4A (GenBank accession number XM_002186787) and the γ3-subunit gene is on chromosome 1 (GenBank accession number XM_002197375). Furthermore, predicted sequences encoding these polypeptides are present in the chicken genome where the γ3-subunit gene is reported to be located on chromosome 1 (GenBank accession number XM_001233420) and the β1-subunit gene (GenBank accession number XM_420267) on chromosome 4, in accordance with the chromosomal assignment of the zebra finch genes. Predicted exonic arrangements show 9 coding exons for both chicken and zebra finch γ3-subunit genes and 9 and 7 exons for the chicken and zebra finch β1-subunit genes respectively. However, it was observed that the chicken and zebra finch β1 genes were localised on chromosome 4/4A in a cluster with genes encoding α3 and γ4 subunits. In the human genome (on the X chromosome) the gene cluster is ε, α3 and θ, where ε is the orthologue of γ4 and θ is the orthologue of β4 (Darlison *et al*., 2005), therefore it would be expected that in the zebra finch and chicken genome it would be the β4-subunit gene occupying this position, not the β1-subunit gene. Within the predicted zebra finch $GABA_A$ receptor subunit sequences, there was no gene identified which encodes the β4-subunit. Therefore, alignments were prepared of the predicted zebra finch β1-subunit and β4-subunit genes (cloned within the laboratory, data not published) which demonstrated almost perfect identity. High similarity would be anticipated between sequences of two genes from the same class within the same organism, approximately 60-80% identity (Darlison *et al*., 2005), but not as high as 99% (see Appendix for alignment). Subsequently it appears that the databases have annotated the sequences incorrectly and the β1-subunit gene is

essentially identical to the previously cloned β4-subunit gene. This would also mean that the chromosomal allocation of the genes within the zebra finch would be identical to that of the human genome with a cluster of the β4-, α3- and γ4-subunit encoding genes. In the chicken genome, there is the verified β4-subunit gene (Bateson *et al*., 1991) and a predicted β1-subunit gene, alignment of these two genes displayed > 99% homology, indicating that the predicted β1-subunit gene was in fact the previously cloned β4-subunit gene, and now the β4-subunit gene can be localised to chromosome 4 (see Appendix for alignment). Again, this highlights the need to verify the integrity of predicted sequences and their annotations.

Sequences encoding all zebra finch $GABA_A$ and $GABA_C$ receptor subunits have been isolated and their identity verified. A novel splice variant has been described for the π-subunit gene, which is expressed along with the full-length transcript in the avian brain. Further expression studies involving this subunit would be interesting as no splice variants have been previously reported and data pertaining to π-subunit gene expression in brain is scarce. Analysis of the cloned zebra finch $GABA_A$ and $GABA_C$ receptor subunit sequences with predicted sequences from the zebra finch genome have revealed some discrepancies, demonstrating that caution must be taken when using such data and sequences should all be validated with experimental evidence. All cloned sequences were subsequently used to design real-time RT-PCR primers and *in situ* hybridisation probes for expression studies as detailed in the following chapter.

4. EXPRESSION OF $GABA_A$ AND $GABA_C$ RECEPTOR SUBUNIT GENES IN THE SONG SYSTEM

4.1 Introduction

Excitatory action elicited by glutamate in the brain is counterbalanced by the inhibitory activity of γ-aminobutyric acid (GABA) acting at post-synaptic receptors, resulting in a finely tuned neuronal network (Hablitz *et al*., 2009). Heteromeric $GABA_A$ receptors are by far the most abundantly distributed of the GABA receptors in the central nervous system CNS (Bormann, 2000). As previously mentioned, their arrangement is pentameric and it is the stoichiometry of an individual receptor that dictates its electrophysiological, pharmacological and functional properties (Whiting 2003a, b; Wafford, 2004; Rudolph and Möhler, 2006; Olsen and Sieghart, 2008). Over the past 20 years 16 $GABA_A$ receptor subunits have been identified in the mammalian CNS: α1-α6; β1-β3; γ1-γ3; π, θ, δ and ε, each encoded for by a separate gene (Simon *et al*., 2004). However in avian species there are only 14 documented $GABA_A$ receptor subunits: α1-α6; β2-β4; γ1, γ2, γ4, δ and π; where the γ4 and β4 subunits are orthologues of mammalian ε and θ subunits respectively (Darlison *et al*., 2005). The most frequent combination of subunits is 2α's, 2β's and a single γ; with α1β2γ2 as the predominant subtype in mammals (Whiting, 2003a). The promiscuous nature of the subunits in their associations provides potential for a multitude of receptor subtypes, although preferred assemblies do exist, but after two decades of research only 11 confirmed native subtypes have been identified in mammals (Table 1, Chapter One; Olsen and Sieghart, 2008, 2009). $GABA_A$ receptors are of considerable clinical interest due to their sensitivity to benzodiazepines, barbiturates, ethanol, volatile anaesthetics and steroids; thus the elucidation of specific receptor subtypes would enable design of more specific therapeutic agents (Sieghart and Ernst, 2005). One of the first steps in identifying $GABA_A$ receptor subtypes is determining which subunits are co-expressed *in vivo*. Several previous studies have spatially mapped expression of $GABA_A$ receptor subunit genes in mammalian brain, both in embryonic and post-natal rat (Laurie *et al*, 1992a, b; Wisden *et al*, 1992; Sperk *et al*., 1997; Pirker *et al*., 2000; Pöltl *et al*., 2003) and monkey (*Macaca mulatta*) brain (Kultas-Ilinsky *et al*., 1998; Huntsman *et al*., 1999). Although, none have documented *all* the $GABA_A$ receptor subunit genes, or considered

the ρ-subunit genes of the $GABA_C$ receptor in parallel, despite considerable speculation that these should be classified as part of the $GABA_A$ family (Olsen and Sieghart, 2008; Collingridge *et al*., 2009). All studies have demonstrated discrete, yet overlapping cellular and regional distributions of $GABA_A$ receptor subunit transcripts in brain which provides a preliminary insight into which subunits may be co-expressed (reviewed by Sieghart and Sperk, 2002). Furthermore, expression studies have indicated differences in $GABA_A$ receptor subunit gene expression during different developmental stages, demonstrating that subtype composition is highly variable depending on brain region and is also subject to temporal regulation (Laurie *et al*., 1992a; Huntsman *et al*., 1999), further details in section 4.3.

4.1.1 Heteromerisation of $GABA_A$ and $GABA_C$ receptors

Recent speculation has suggested potential heteromerisation of $GABA_A$ and $GABA_C$ receptor subunits (Milligan *et al*., 2004). All three subunit genes (ρ1-ρ3) are transcribed in rat brain; though ρ1 and ρ2 at significantly higher levels than the ρ3 subunit (Ogurusu & Shingai, 1996; Milligan *et al*., 2004). Furthermore, Qian and Pan (2002), Ekema *et al*. (2002) and Milligan *et al*. (2004) revealed that the $GABA_A$ receptor γ2-subunit polypeptide can associate with the $GABA_C$ receptor ρ1 and ρ2 subunits *in vitro* to form functional recombinant receptors, indicating the potential for new 'hybrid' receptor subtypes in brain. Recombinant receptors comprising both $GABA_A$ and $GABA_C$ receptor subunit polypeptides do not always exhibit classical $GABA_C$ or $GABA_A$ receptor activity (Qian and Pan, 2002), inferring that the combination of subunits from these two receptor families may yield a novel receptor subtype (Milligan *et al*., 2004). For this reason, expression of $GABA_C$ receptor subunits has been included in the *in situ* hybridisation experiments (further information concerning the properties of $GABA_C$ receptors in section 1.3).

4.1.2 Zebra finch song system

The zebra finch (*Taenopygia guttata*) song system has long since been used as a paradigm for studying the underlying molecular mechanisms of learning and memory; due to the discrete nature of song, the song system and the finite period of song development (Brainard and Doupe, 2002). It comprises a set of interconnected telencephalic nuclei which can be subdivided into two relatively distinct pathways

which control the acquisition and production of song (Nottebohm *et al*., 1976). Firstly, the anterior forebrain pathway (AFP); this is important for the development of normal song in juveniles and adult song plasticity (Mooney, 1999; Woolley, 2004; Funabiki and Funabiki, 2007; Sober and Brainard, 2009). Notable nuclei within this pathway include: Area X, the lateral magnocellular nucleus of the anterior nidopallium (LMAN) and the medial nucleus of the dorsolateral thalamus (DLM). Secondly, the vocal motor pathway (VMP), which is primarily responsible for song production; this comprises the NIf (nucleus interfacialis of the nidoapllium), HVC (formal name) and RA (robust nucleus of the arcopallium; Nottebohm *et al*., 1976). This pathway links to the syrinx via projections from the RA to the tracheosyringeal portion of the hypoglossal nucleus (nXllts). These forebrain systems which control motor systems are exclusive to vocal learners e.g. humans, songbirds and some cetaceans (Brainard and Doupe, 2002; Bolhius and Gahr, 2006). The avian brain displays many comparable structures and pathways to mammalian systems (Farries and Perkel, 2000; Jarvis *et al*., 2005; Mooney and Prather, 2005; Bolhuis and Gahr, 2006; Scott and Lois, 2007) and there are striking parallels between birdsong and speech production in humans, most notably in infants; hence the fundamental neuronal mechanisms appear to be similar (Doupe and Kuhl, 1999; Kuhl, 2004); discussed further in section 1.4.8.

4.1.3 $GABA_A$ receptors in zebra finch song system

GABA is widely distributed in the song system (Pinaud and Mello, 2007), but less work has involved the identification and functionality of $GABA_A$ receptors within the telencephalon. Pharmacological evidence confirms the presence of $GABA_A$ receptors within all the major nuclei of the song system (for example, Carlisle *et al*., 1998; Vicario and Raksin, 2000; Farries *et al*., 2005; Mooney and Prather, 2005; Ölveczky *et al*., 2005). In adult zebra finches, $GABA_A$ receptors have been identified by electrophysiological studies to occur on HVC neurons projecting to Area X as well as on HVC interneurons (Dutar *et al*., 1998), on projecting neurons of the RA (Spiro *et al*., 1999) and on small spiny Area X neurons (Farries *et al*., 2005). However, the role of $GABA_A$ receptors expressed in the song control system is not well understood. It has been postulated that in HVC, $GABA_A$ receptors might be involved in the establishment of the auditory-vocal mirror cell function of neurons projecting to Area X neurons (Prather *et al*., 2008). In RA $GABA_A$ receptors appear to be involved in the coordinated firing of multiple projection neurons (Spiro *et al*., 1999) and so contribute to the

initiation and temporal pattern of vocalisation (Vicario and Raksin, 2000). In Area X, $GABA_A$ receptors affect the activity of indirect but not direct innervations of pallidal Area X neurons projecting to DLM (Farries *et al*., 2005). More recently, *in situ* hybridisation studies have localised γ4-subunit mRNA in many of the major song system nuclei, suggesting that $GABA_A$ receptors containing this polypeptide play a role in the regulation of the acquisition and production of song (Thode *et al*., 2008). Besides this, no other studies have thoroughly investigated the distribution of the $GABA_A$ or $GABA_C$ receptor subunit genes within the song system. Here is provided the first complete study documenting spatially and quantitatively, the expression of all the avian $GABA_A$ and $GABA_C$ receptor subunit genes (with the exception of the π subunit; which has a primarily peripheral localisation; Symmans *et al*., 2005; Xiang *et al*., 2007) in four nuclei of the adult male zebra finch song system (LMAN, Area X, HVC and RA). In addition, real-time RT-PCR was employed to determine the temporal expression profile of selected $GABA_A$ receptor subunit genes (α1-α4, β2, β3 and γ4) in the song system (Area X, HVC and RA) throughout different stages of song development (PHD 30, 55, 80 and 100). This data provides a basis for further investigations into subtype composition and the functional role of $GABA_{A/C}$ receptors in this learning and memory model.

4.2 Results

Transcript-specific oligonucleotide probes were utilised to spatially and quantitatively investigate the distribution of all documented avian $GABA_A$ receptor subunit transcripts (namely α1-α6, β2-β4, γ1, γ2 and γ4 and δ) in selected nuclei of the adult (PHD >100) male zebra finch song system. The π subunit was not included due to its' primarily peripheral localisation (Symmans *et al*., 2005; Xiang *et al*., 2007). Initially, two probes targeting the corresponding mRNA of each subunit were used for hybridisation studies (Table 2.5), and in all cases the spatial distribution of the corresponding mRNA was identical for both probes (data not shown). Subsequent, grouped *in situ* hybridisation experiments for all probes were carried out on parallel 10μm coronal sections which were hybridised, then washed together and opposed for 4 weeks to the same X-ray film to reduce procedural variability. Anatomical and densitometric analysis enabled qualitative and quantitative analysis of the expression of each subunit gene respectively within the four nuclei examined (LMAN, Area X, HVC and RA). Expression of each gene could be quantitatively compared between the four nuclei, due to normalisation with an unrelated internal control region (nidopallium; refer to section 5.2.1). mRNA levels in each nucleus were calculated as a percentage of the internal control signal. One-way ANOVA tests were employed to determine any significant deviations in levels of mRNA from baseline levels (those measured in nidopallium). Cross-analysis of different probes within a single nucleus could not be completed quantitatively as the probes were not normalised to each other, due to different binding affinities.

Spatial distribution patterns of individual $GABA_A$ receptor subunit mRNAs are shown in Figures 4.1, 4.3 and 4.5. No two $GABA_A$ receptor subunit genes exhibited identical spatial expression patterns throughout the whole zebra finch brain. Furthermore, although mRNA levels were quantitatively variable, there was no complete absence of mRNA encoding any of the $GABA_A$ receptor subunits within the four nuclei of the song system examined. Taken together, this implicated that a variety of potential major and minor $GABA_A$ receptor subtype assemblies may exist within the zebra finch song system.

4.2.1 Expression of $GABA_A$ receptor α-subunit genes in the song system

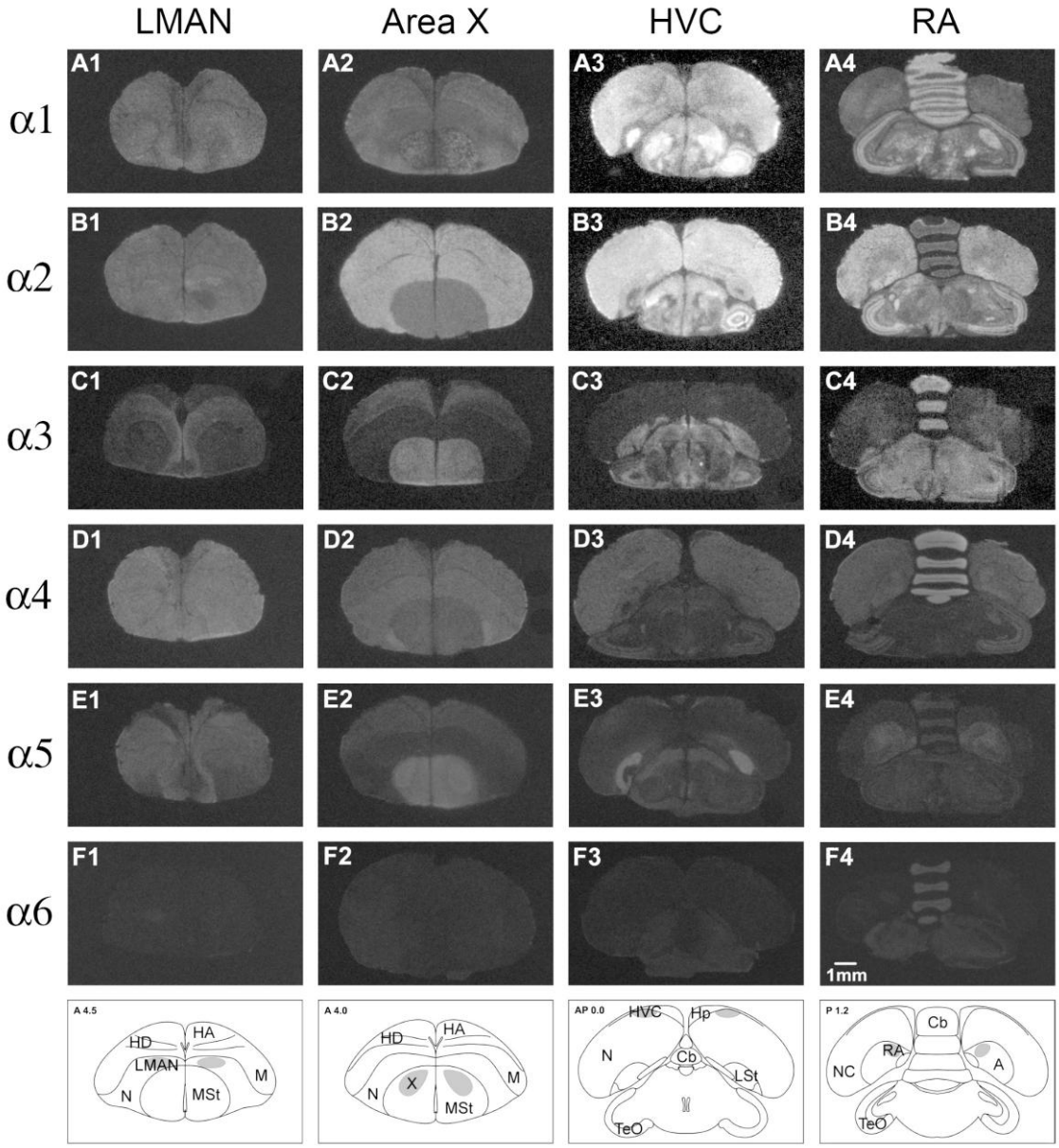


Figure 4.1. Inverse autoradiographs highlighting the expression of $GABA_A$ receptor α-subunit genes in four nuclei of the adult male zebra finch song system (LMAN, Area X, HVC and RA). **Abbreviations:** A, arcopallium; Cb, cerebellum; HA, apical part of the hyperpallium; HD, densocellular part of the hyperpallium; Hp, hippocampus; HVC (formal name); LSt, lateral striatum; M, mesopallium; MSt, medial striatum; N, nidopallium; NC, caudal nidopallium; LMAN, lateral magnocellular nucleus of the anterior nidopallium; RA, robust nucleus of the arcopallium; TeO, optic tectum; X, Area X.

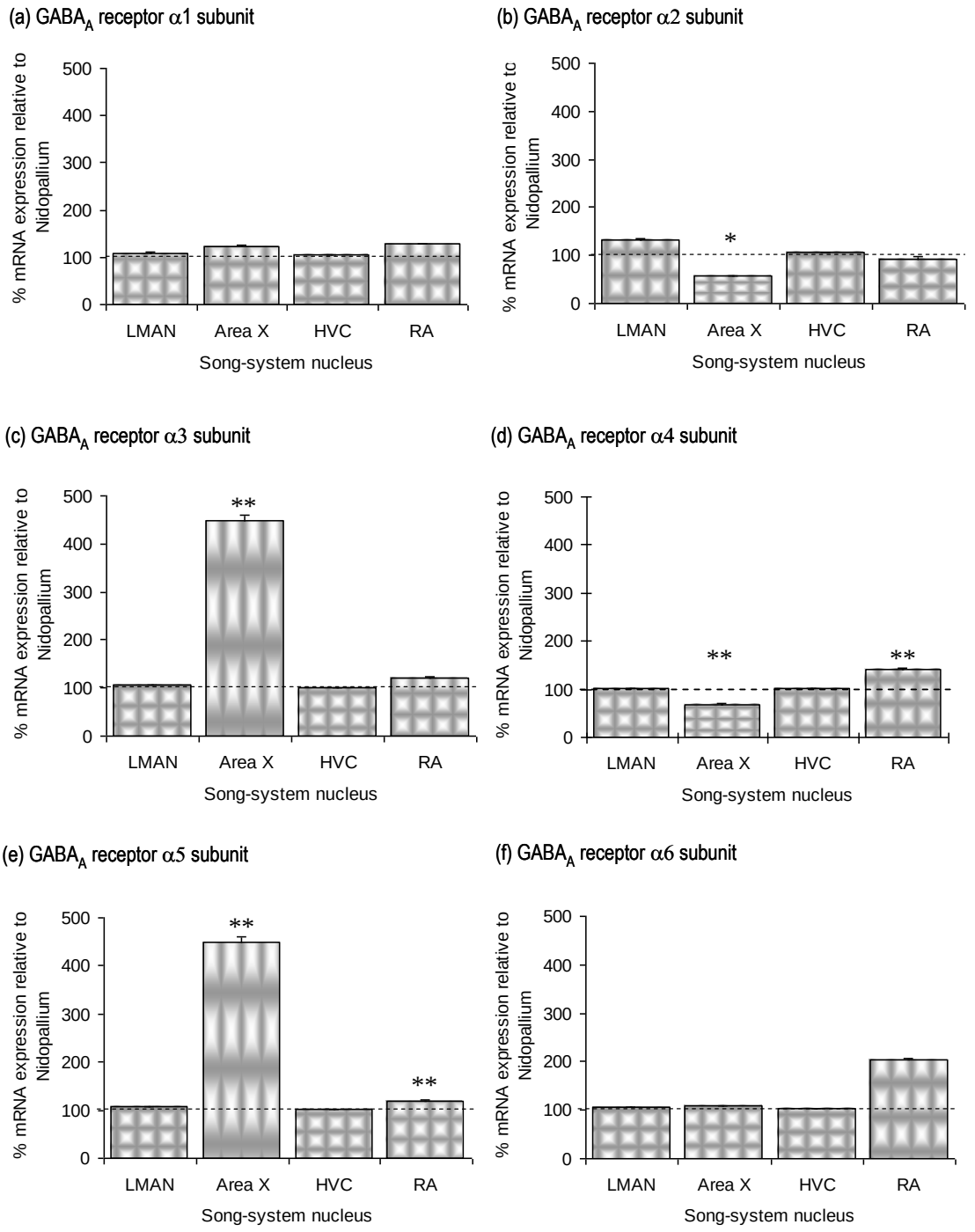


Figure 4.2. Densitometric quantification of $GABA_A$ receptor α-subunit mRNAs (a-f) in four selected nuclei (LMAN, Area X, HVC and RA) of the adult male zebra finch song system, relative to the nidopallium. Data is presented as mean ± SE (n=5 per group, * $p \leq 0.05$; ** $p \leq 0.01$). The dashed line indicates gene expression in the nidopallium; these readings were considered as baseline mRNA expression, at 100%. **Abbreviations:** Area X (formal name); HVC (formal name); LMAN, lateral magnocellular nucleus of the anterior nidopallium; RA, robust nucleus of the arcopallium.

Within LMAN, the most predominant gene expression for the $GABA_A$ receptor α-subunit class was that encoding the α2 subunit (131%, Figs. 4.1 (B1) and 4.2b). All

other α-subunit transcripts demonstrated a relatively homogenous distributions, whereby the amount of detectable mRNA was similar to that of their established individual baseline levels (Fig. 4.2). In Area X, α-subunit gene expression levels were much more variable. α1- and α6-subunit mRNAs were both detected at baseline levels; α3- and α5-subunit mRNAs were abundantly distributed ($p \leq 0.01$ for both). α2- and α4-subunit mRNAs showed markedly low signal intensities (56%; $p \leq 0.05$ and 68%; $p \leq 0.01$ respectively) relative to their individual baseline mRNA levels. Interestingly akin to LMAN, the HVC illustrated a fairly homogenous distribution of the transcripts encoding the α-subunit genes (Fig. 4.2), all at basal levels. All α-subunit (except for α2) genes were well expressed throughout the RA (Fig. 1 and Fig. 4); with particular reference to the α4- and α5-subunit mRNAs where signal intensity was especially high (143% and 119% respectively; $p \leq 0.01$) and α6-subunit mRNA levels were twice that of nidopallium.

4.2.2 Expression of $GABA_A$ receptor β-subunit genes in the song system

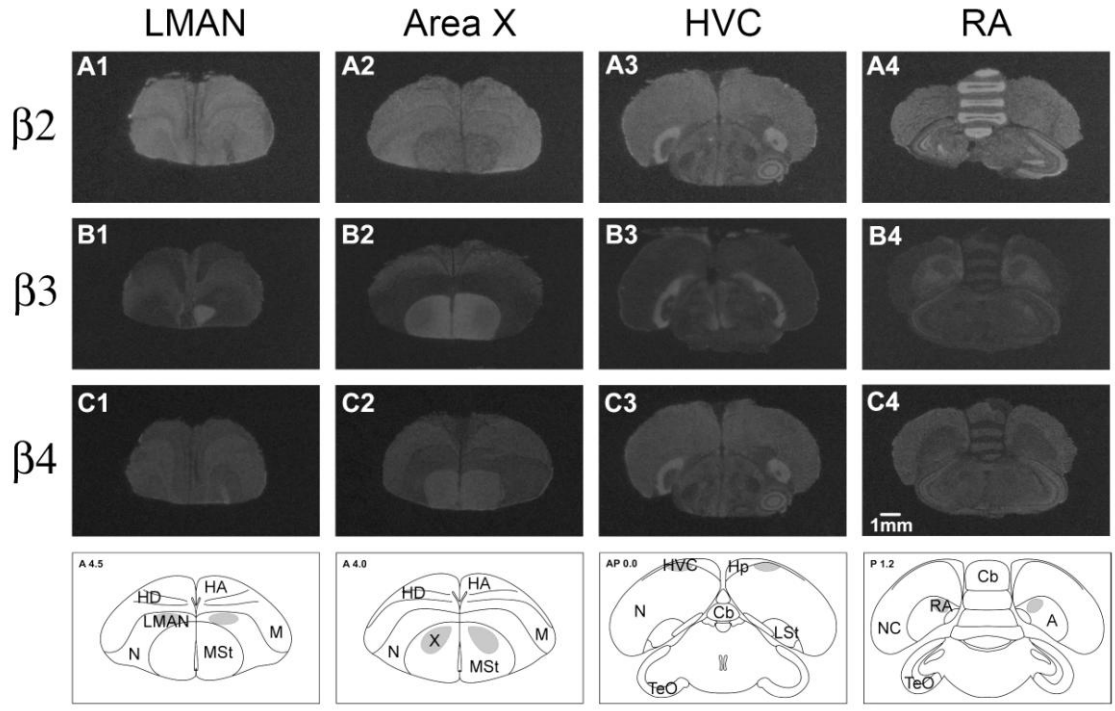


Figure 4.3. Inverse autoradiographs highlighting the expression of $GABA_A$ receptor β-subunit genes in four nuclei of the adult male zebra finch song system (LMAN, Area X, HVC and RA). **Abbreviations:** A, arcopallium; Cb, cerebellum; HA, apical part of the hyperpallium; HD, densocellular part of the hyperpallium; Hp, hippocampus; HVC (formal name); LSt, lateral striatum; M, mesopallium; MSt,

medial striatum; N, nidopallium; NC, caudal nidopallium; LMAN, lateral magnocellular nucleus of the anterior nidopallium; RA, robust nucleus of the arcopallium; TeO, optic tectum; X, Area X.

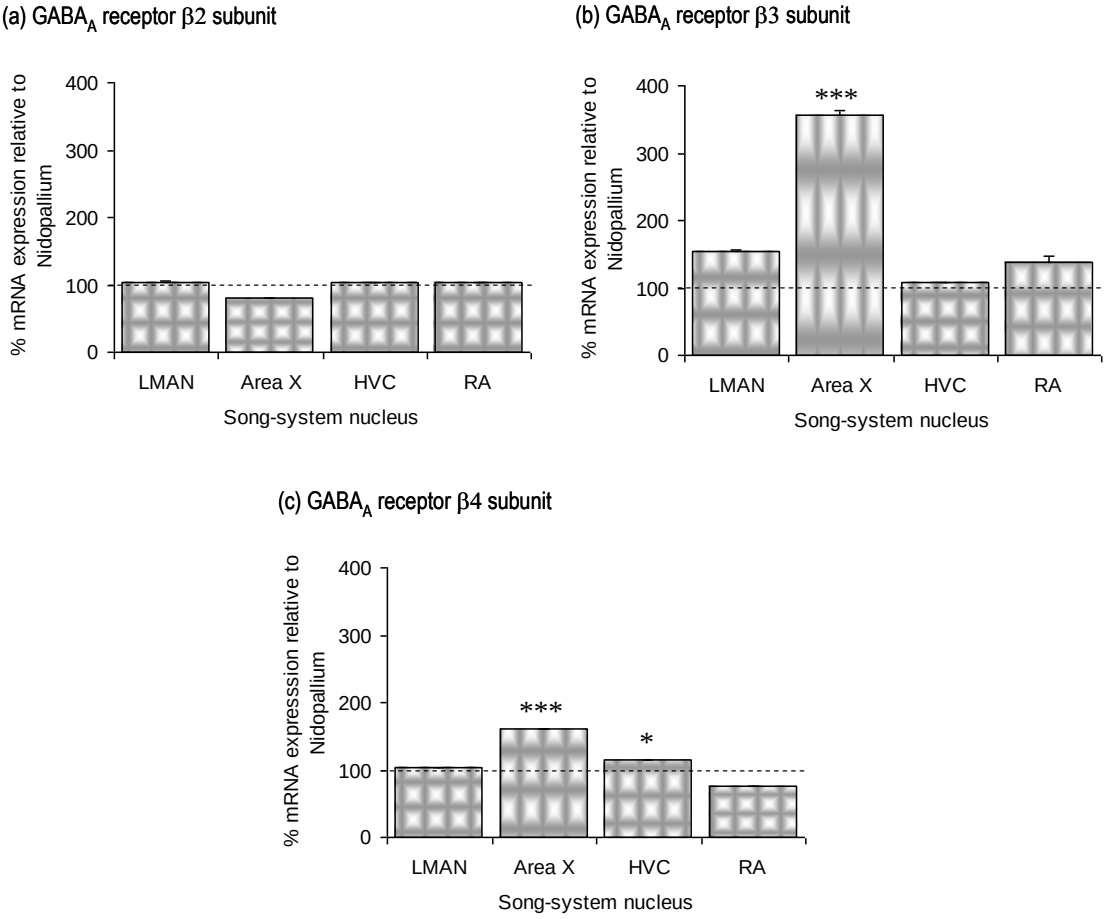


Figure 4.4. Densitometric quantification of $GABA_A$ receptor β-subunit mRNAs (a-c) in four selected nuclei (LMAN, Area X, HVC and RA) of the adult male zebra finch song system, relative to the nidopallium. Data is presented as mean ± SE (n=5 per group, * $p \leq 0.05$; *** $p \leq 0.001$). The dashed line indicates gene expression in the nidopallium; these readings were considered as baseline mRNA expression, at 100%. **Abbreviations:** Area X (formal name); HVC (formal name); LMAN, lateral magnocellular nucleus of the anterior nidopallium; RA, robust nucleus of the arcopallium.

The primary β-subunit transcript detected in the LMAN was that encoding the β3 subunit; this showed reasonably high signal intensity within the nucleus (154%) relative to baseline levels (Fig. 4.3 (B1) and Fig. 4.4b), whereas β2- and β4-subunit transcripts were both present in LMAN at a basal level (Fig. 4.3 (A1 and C1) and Fig. 4.4a, c). Predominant β-subunit genes identified in Area X were β3 and β4 (Fig. 4.3 (B2 and C2) and Fig. 4.4b, c). The corresponding mRNAs displayed robust hybridisation signals in Area X (355% and 160% respectively) relative to baseline ($p \leq 0.001$ for both); whereas

the level of the β2-subunit transcript was considerably less (81%; Fig. 4.4a). It was only the β4 subunit which exhibited pronounced gene expression in HVC (114% $p \leq 0.05$). All β-subunit transcripts were detected in the RA, but the relative abundance of each subunit was quite different (Figs. 4.3 and 4.4). β2-subunit mRNA was observed at a basal level, similar to that of the nidopallium (Fig. 4.4a). β3-subunit transcript was present at higher levels than in the nidopallium (137%; Fig. 4.4b), whereas mRNA encoding the β4 subunit was considerably lower than its counterparts (75%; Fig. 4.4c).

4.2.3 Expression of γ- and δ-subunit genes in the song system

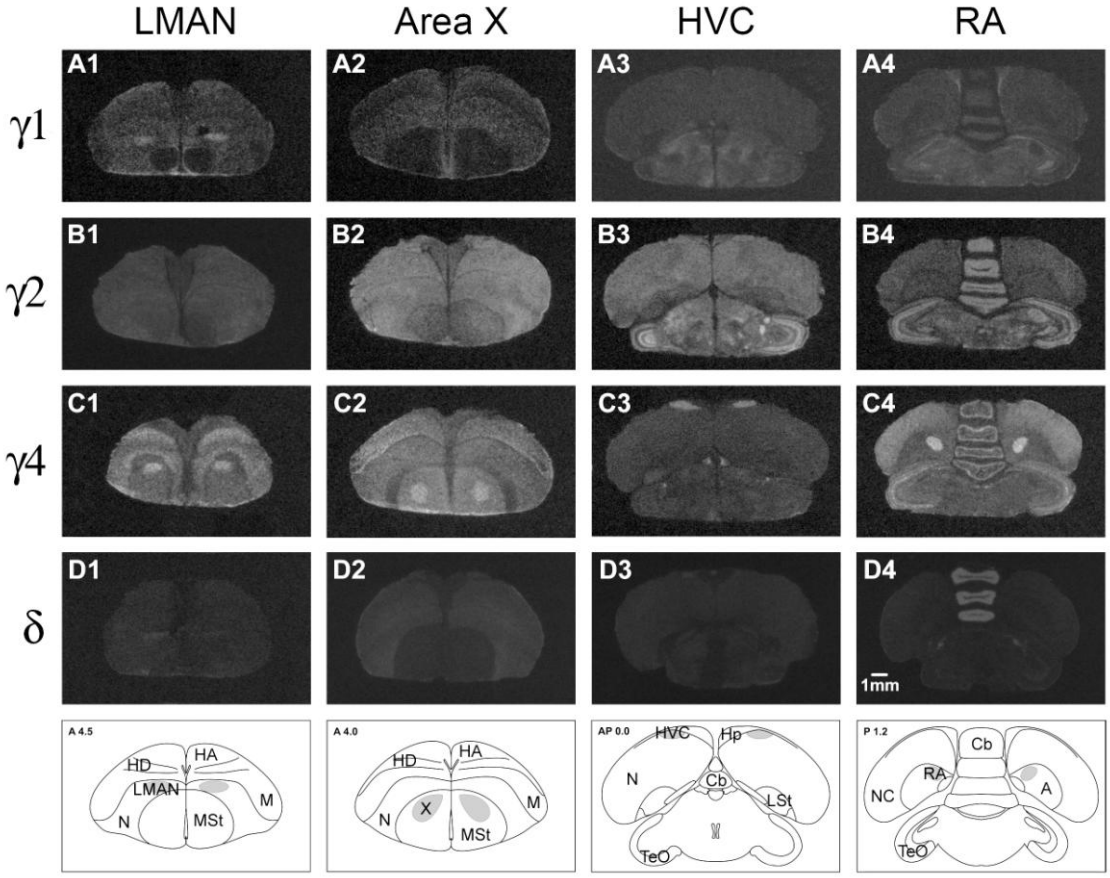


Figure 4.5. Inverse autoradiographs highlighting the expression of $GABA_A$ receptor γ- and δ-subunit genes in four nuclei of the adult male zebra finch song system (LMAN, Area X, HVC and RA). **Abbreviations:** A, arcopallium; Cb, cerebellum; HA, apical part of the hyperpallium; HD, densocellular part of the hyperpallium; Hp, hippocampus; HVC (formal name); LSt, lateral striatum; M, mesopallium; MSt, medial striatum; N, nidopallium; NC, caudal nidopallium; LMAN, lateral magnocellular nucleus of the anterior nidopallium; RA, robust nucleus of the arcopallium; TeO, optic tectum; X, Area X.

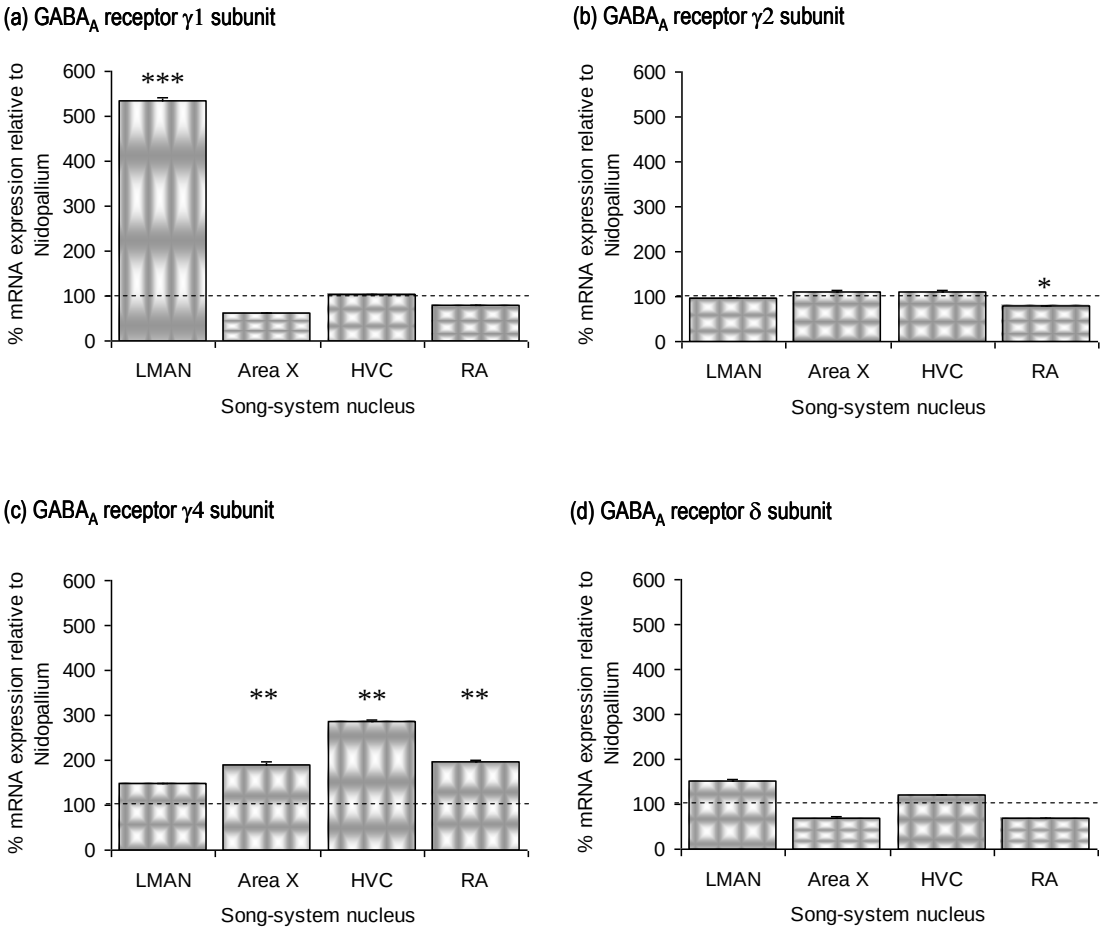


Figure 4.6. Densitometric quantification of $GABA_A$ receptor γ- and δ-subunit mRNAs (a-d) in four selected nuclei (LMAN, Area X, HVC and RA) of the adult male zebra finch song system, relative to the nidopallium. Data is presented as mean ± SE (n=5 per group, * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$). The dashed line indicates gene expression in the nidopallium; these readings were considered as baseline mRNA expression, at 100%. **Abbreviations:** Area X (formal name); HVC (formal name); LMAN, lateral magnocellular nucleus of the anterior nidopallium; RA, robust nucleus of the arcopallium.

The most striking $GABA_A$ receptor subunit gene expression in LMAN was that of the γ1 subunit (540%; $p \leq 0.001$; Fig. 4.6a). This was a considerably higher increase from baseline than that of any of the other subunit genes in any of the song-system nuclei under observation. Abundant expression was also observed for γ4- and δ-subunit genes (148% and 151% respectively; Figs 4.6c, d) within LMAN; only γ2-subunit mRNA was detected at basal levels (Fig. 4.6b). γ4-subunit gene expression was robust in Area X (Fig. 4.5 (C2) and Fig. 4.6c), with corresponding mRNA levels significantly higher than baseline (190%; $p \leq 0.01$). γ1- and δ-subunit genes displayed nominal expression (61% and 70% respectively; Fig. 4.6a, d) whereas, similar to LMAN, mRNA encoding the γ2

subunit in Area X was at a level close to baseline (Fig. 4.5 (B2) and Fig. 4.6b). Within HVC, γ1- and γ2-subunit transcripts were present at levels similar to that of their individual baselines (Figs 4.5 (A4 and B4) and 4.6a, b). δ-subunit transcript was detected at a level marginally higher (120%; Fig. 4.6d); but it was the γ4-subunit gene which exhibited the most pronounced expression in this nucleus, nearly 3-fold greater than baseline (285%; $p \leq 0.01$; Fig. 4.6c).

	mRNA levels relative to nidopallium		
Nucleus	**Higher**	**Equivalent**	**Lower**
LMAN	α2, β3, γ1***, γ4, δ	α1, α3-6, β2, β4, γ2	-
Area X	α1, α3**, α5**, β3***, β4***, γ4**	α6, γ2	α2*, α4**, β2, γ1, δ
HVC	β4*, γ4**, δ	α1-6, β2, β3, γ1, γ2	-
RA	α1, α3, α4**, α5**, α6, β3, γ4**	β2	α2, β4, γ1, γ2*, δ

Table 4.1. Summary data illustrating the quantitative distribution of all the $GABA_A$ receptor subunit mRNAs within selected nuclei of the adult zebra finch song system. Data is grouped according to whether the hybridisation signal was higher, equal to, or below that detected in the nidopallium. ($p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$). **Abbreviations:** Area X (formal name); HVC (formal name); LMAN, lateral magnocellular nucleus of the anterior nidopallium; RA, robust nucleus of the arcopallium.

α1-, α3- and α5-subunit genes demonstrated fairly homogeneous distribution profiles across the four nuclei examined. All were detected at a basal level in LMAN and HVC and at high levels within Area X and RA (Table 4.1). Diametric expression patterns were observed for the α2-subunit gene in the song system. Corresponding mRNA was not well represented in Area X or RA (where α1-, α3- and α5-subunit mRNA levels were high), but it was elevated in LMAN where α1-, α3- and α5-subunit mRNAs were detected at low levels. α4-subunit mRNA exhibited a variable quantitative distribution pattern with levels most pronounced within the RA, at a basal level within the LMAN and HVC and lowest in Area X. An interesting observation was that α6-subunit mRNA was detected at a basal level in LMAN, Area X and HVC but corresponding hybridisation signals were quite prominent in the RA, relative to baseline. Maximum levels of mRNA for this gene was observed in cerebellum (Fig. 4.1, F4), but this was not quantified. Transcripts encoding β2 and γ2 subunits were detected at baseline levels throughout song system nuclei (Table 4.1); demonstrating a rather ubiquitous and

diffuse mRNA distribution. In contrast β3- and γ4-subunit mRNAs gave strong signals in all nuclei, neither one exhibiting a low level in any of the nuclei under examination. β4 and γ1 subunits displayed the greatest variability in mRNA levels, ranging from relatively meagre (e.g. RA) to very robust (e.g. LMAN and Area X). This was also evident with δ-subunit mRNA, which was at appreciable levels in LMAN and HVC, but was barely detectable in Area X and RA (Fig. 4.6d).

Anatomical and densitometric analysis clearly demonstrated that the γ4-subunit gene was strongly expressed in all the nuclei examined; a feature which was not apparent with any of the other subunit genes. Where the γ4-subunit transcript is abundant (Area X, HVC and RA; Table 4.1) the other γ-subunit transcripts are at lower levels, but in LMAN, the region in which the γ4-subunit transcript levels were weaker relative to the other areas, γ1-subunit mRNA distribution pronounced (Fig. 4.6). In all selected nuclei (with the exception of the HVC) there was at least 1α-, 1β- and 1γ- subunit transcript detected at a high level, indicative of complex receptor populations.

Although not quantified, $GABA_A$ subunit gene expression was also documented in other regions of the zebra finch brain. All $GABA_A$ receptor subunit genes gave strong signals in cerebellum, except for β3- and γ1-subunits (Figs. 4.1, 4.3 and 4.5). In the optic tectum (TeO), α1-α4, α6-, β2-, γ1-, γ2- and γ4-subunit genes exhibited strong expression and α5-, β3-, β4- and δ-subunit genes were transcribed at basal levels. In the lateral striatum (LSt), all subunit mRNAs were well expressed, except for those encoding the α6, γ2 and δ subunits. Prominent expression was also observed in zebra finch medial striatum (MSt, region surrounding Area X) for α1-, α3-, α5-, β3-, β4- and γ4-subunit transcripts, at a lower level α6 was expressed and expression for the remaining subunits was virtually absent. Within the arcopallium (area surrounding RA) transcript levels were more variable with α1-, α5- and β3-subunit genes being well expressed; α3-, α4-, α6- and β2-subunit genes expressed at lower levels, and α2-, β4-, γ1-, γ2-, γ4- and δ-subunit genes poorly expressed. In the apical part of the hyperpallium (HA) only mRNAs encoding α2, α4, α5, β2, β3, γ1 and δ subunits were well distributed, which differed from the densocellular part of the hyperpallium (HD) where α1-, α3-, α4-, β2- and γ4-subunit mRNAs were most prominent.

Furthermore, it should be noted that there was no difference in mRNA expression levels of any subunit within any nucleus between the left and right hemispheres of the zebra finch brain (data not shown).

4.2.4 $GABA_A$ receptor π subunit

π-subunit gene expression was not included in the *in situ* analysis, as it was not considered as important as the other subunit cDNAs due to its peripheral location (Symmans *et al*., 2005; Xiang *et al*., 2007). However, RT-PCR experiments indicated that π-subunit mRNA was expressed in zebra finch brain, potentially even at higher levels than in the gastro-intestinal tract and testes (Fig. 4.7).

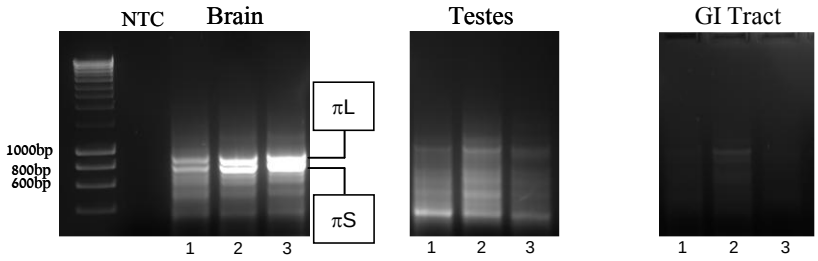


Figure 4.7. Gel electrophoresis (1.5% (w/v) agarose gels) of RT-PCRs detailing amplification of π-subunit partial cDNAs from different tissues of the zebra finch. Each reaction was completed in triplicate with tissues from three different adult male zebra finches and run alongside a molecular weight marker Hyperladder IV (Bioline). Two products were amplified, purified and sequenced revealing a long (831bp) and short (718bp) version of the π-subunit cDNA. **Abbreviations:** GI, gastro-intestinal tract; NTC, no template control; πL, $GABA_A$ receptor π-subunit long version; πS, $GABA_A$ receptor subunit short version. Numbers represent replicates at 58°C, 59°C and 60°C annealing temperature.

Although the RT-PCR data was not quantitative, it clearly suggests π-subunit gene expression in zebra finch brain, in addition to testes and GI tract, where the products appeared somewhat weaker.

4.2.5 Expression of $GABA_C$ receptor subunit mRNAs in song system

Expression of $GABA_C$ receptor subunit genes (ρ1, ρ2 and ρ3) was also assessed by *in situ* hybridisation (Fig. 4.8). Two probes targeting each subunit mRNA were designed and tested and in all cases gave identical binding patterns.

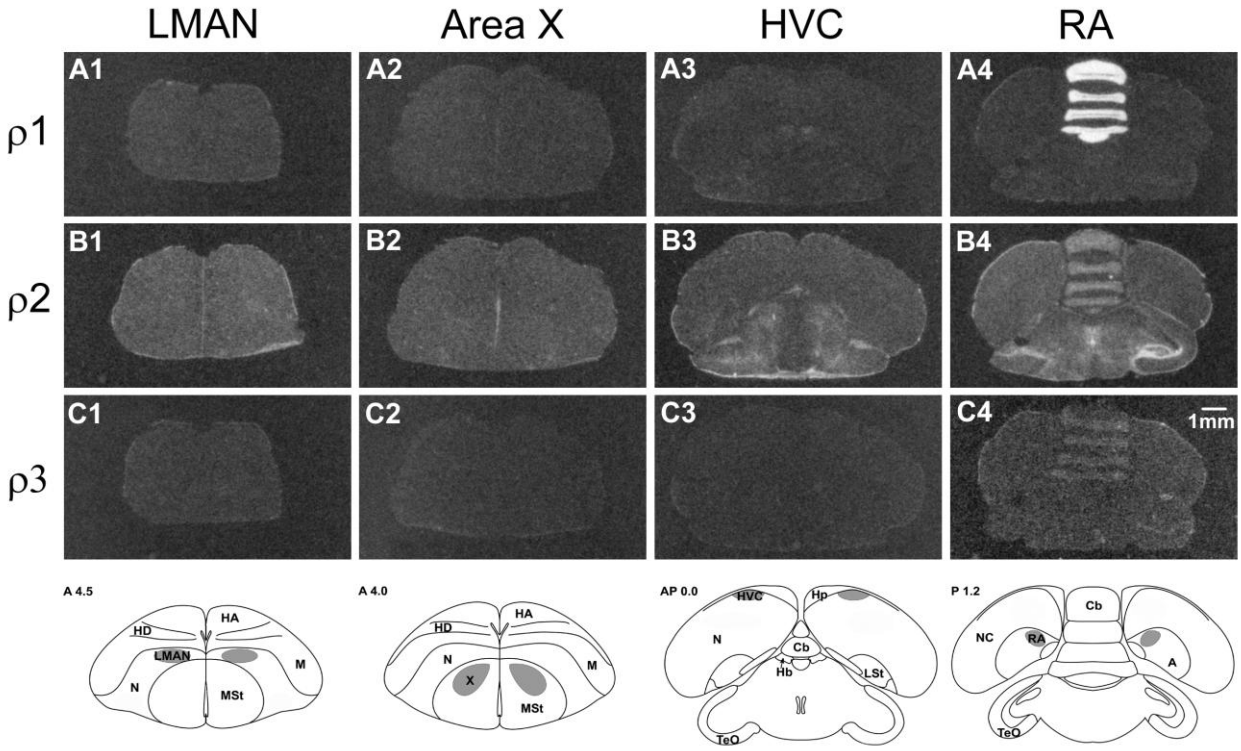


Figure 4.8. Inverse autoradiographs highlighting the expression of $GABA_c$ receptor ρ1-, ρ2-, and ρ3-subunit genes in four nuclei of the adult male zebra finch song system, (LMAN, Area X, HVC and RA). **Abbreviations:** A, arcopallium; Cb, cerebellum; HA, apical part of the hyperpallium; HD, densocellular part of the hyperpallium; Hp, hippocampus; HVC (formal name); LSt, lateral striatum; M, mesopallium; MSt, medial striatum; N, nidopallium; NC, caudal nidopallium; LMAN, lateral magnocellular nucleus of the anterior nidopallium; RA, robust nucleus of the arcopallium; TeO, optic tectum; X, Area X.

$GABA_C$ receptor ρ1-subunit gene expression was low and diffuse throughout the entire zebra finch brain, with the striking exception of the cerebellum where there was prominent expression (Fig. 4.8, A4). This was similar to the ρ3-subunit gene, which demonstrated very weak expression throughout the whole zebra finch brain of adult males (an identical pattern of expression was observed with juvenile zebra finches < PHD 90, data not shown), and a marginal elevation in expression in cerebellum (Fig 4.8, C4). Although the expression was nominal, data indicated that both ρ1- and ρ3-subunit genes were expressed in zebra finch brain, and thus potentially in the song system. The $GABA_C$ receptor ρ2-subunit gene showed the greatest variability in

expression (Fig. 4.8, row B). Again, corresponding ρ2-subunit mRNA was present in the cerebellum, but it was also identified in the optic tectum (TeO; Fig. 4.8, B4). None of the subunits were particularly well transcribed in zebra finch brain or specifically in any of the nuclei of the song system, but nonetheless, their corresponding mRNAs were all present.

4.2.6 Expression of $GABA_A$ receptor subunit genes in the zebra finch song system during development

Developmental expression patterns of $GABA_A$ receptor subunit genes in the male zebra finch song system were investigated. Levels of α1-α4, β2-, β3- and γ4-subunit mRNAs were assessed in the HVC, RA and Area X during the phase of song learning by real-time RT-PCR. Zebra finch $GABA_A$ receptor subunit-specific PCR-primer sets were designed (Table 2.4, Chapter Two) and mRNA was isolated from laser-microdissected HVC, RA and Area X tissues obtained at PHD 30, 55, 80 and 100 from male zebra finches. mRNA expression levels of the $GABA_A$ receptor subunits in these age groups were quantified relative to the mRNA expression levels of β-actin, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and hypoxanthine phosphoribosyltransferase (HPRT) that were constantly expressed throughout the phase of song learning in RA (β-actin) and HVC/Area X (all three genes). During all preliminary qPCR reactions, a DNA dissociation (melt) analysis was completed in order to optimise the cycling conditions and identify any dimers or non-specific products, all primers gave a single product (data not shown).

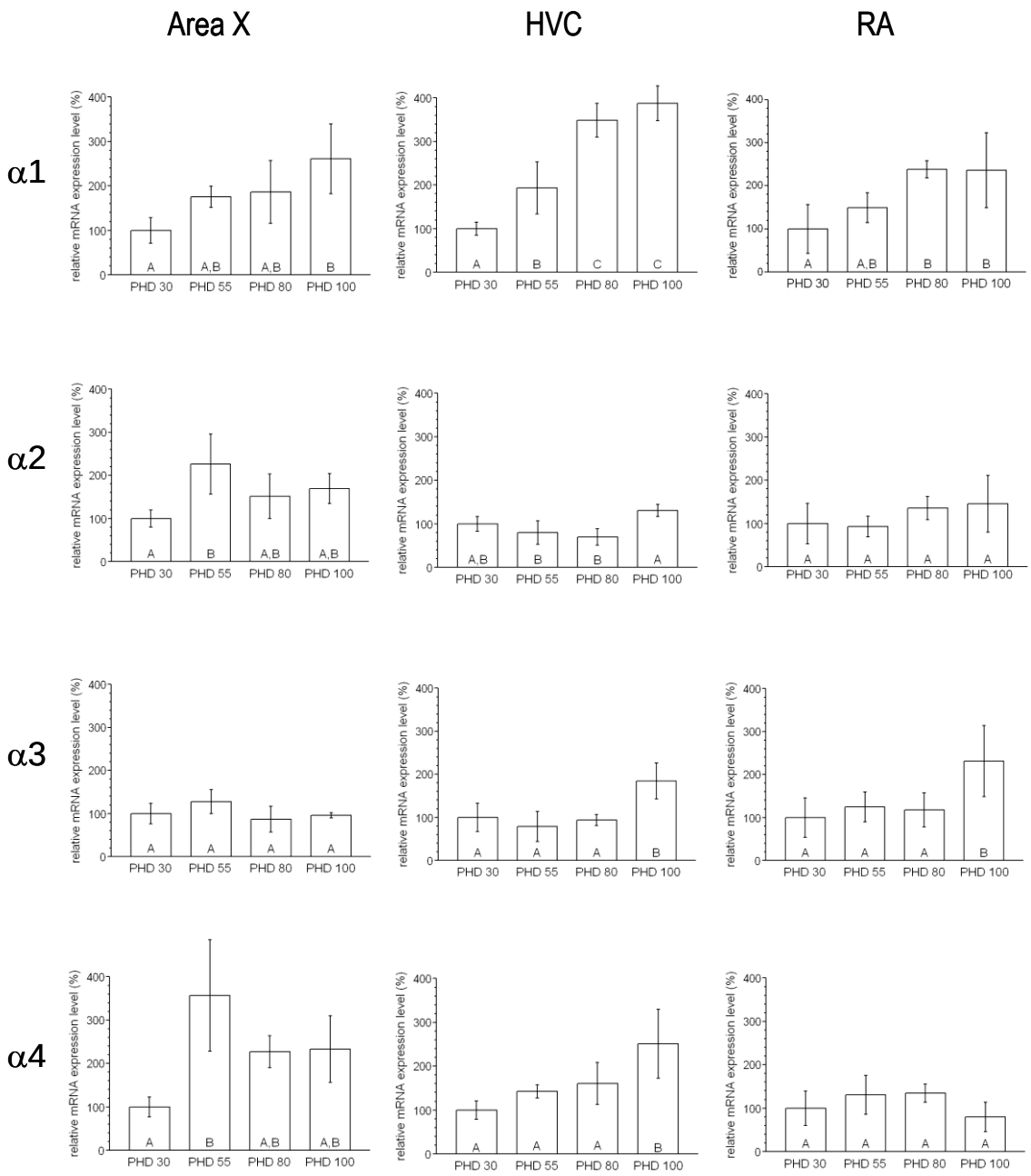


Figure 4.9. Developmental expression of $GABA_A$ receptor α-subunit genes in song control nuclei of male zebra finches during the phase of song learning. Mean normalised expression (MNE) was determined for $GABA_A$ receptor subunits α1, α2, α3 and α4 in HVC, Area X and RA at post-hatching days (PHD) 30, 55, 80 and 100, respectively. For each song control nucleus and receptor subunit MNE values (n=5) were taken within each age group and means and standard deviations are shown as percentage of the results at PHD 30. Expression levels not connected by same letter within the bars (A, B, C) were significantly different (Tukey's post hoc test; $p \leq 0.05$).

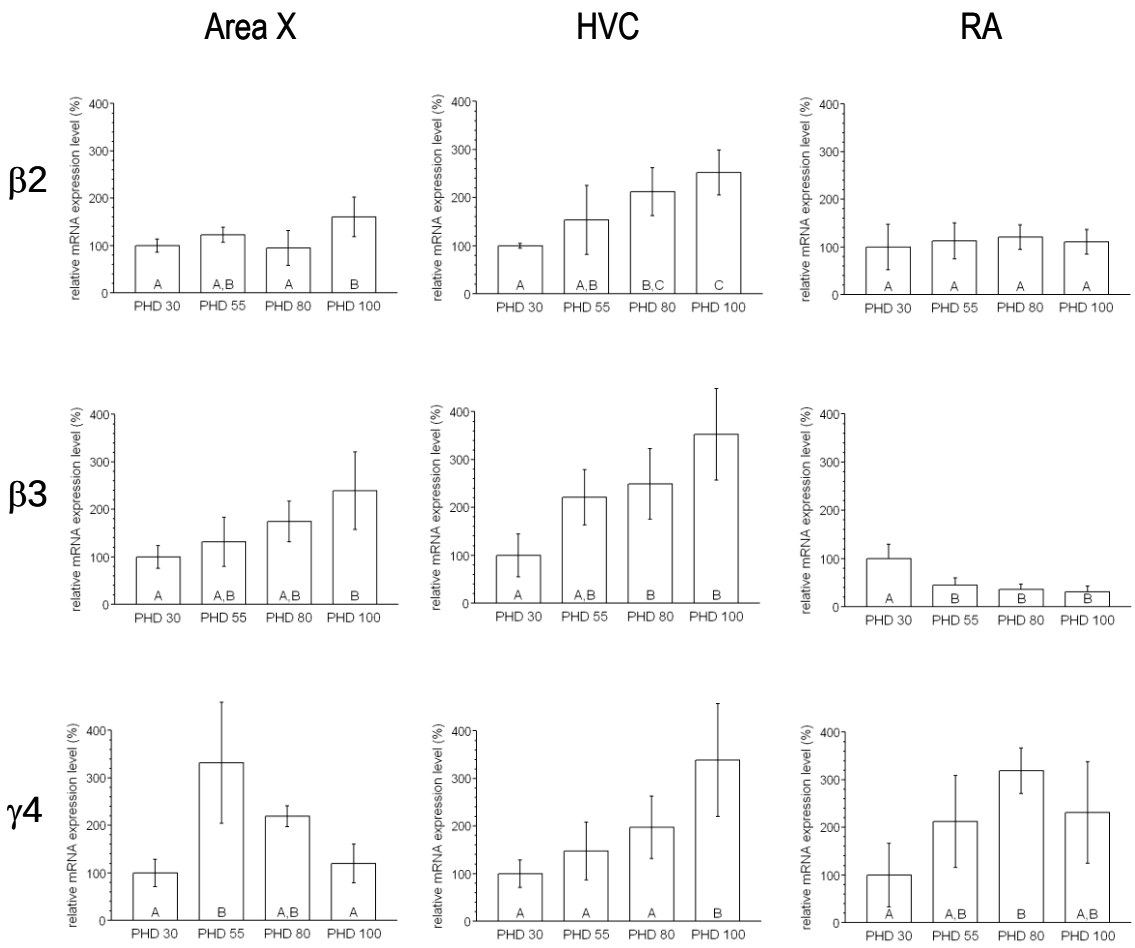


Figure 4.10. Developmental expression of $GABA_A$ receptor β2-, β3- and γ4-subunit genes in song control nuclei of male zebra finches during the phase of song learning. Mean normalised expression (MNE) was determined for $GABA_A$ receptor subunits β2, β3 and γ4 in HVC, Area X and RA at post-hatching days (PHD) 30, 55, 80 and 100, respectively. For each song control nucleus and receptor subunit MNE values (n=5) were taken within each age group and means and standard deviations are shown as percentage of the results at PHD 30. Expression levels not connected by same letter within the bars (A, B, C) were significantly different (Tukey's post hoc test; $p \leq 0.05$).

Developmental expression patterns of $GABA_A$ receptor subunit genes: α1-α4, β2, β3 and γ4 determined for HVC, Area X and RA during the phase of song learning are presented in Figs. 4.9 and 4.10. All subunit transcripts were detectable in all examined nuclei at each developmental time point, concurring with the *in situ* hybridisation data. The relative level of individual subunit mRNAs however, varied considerably according to developmental stage and nuclei observed. In both HVC and Area X the expression of all subunit genes (except α3 in Area X) exhibited developmental regulation (ANOVA; $p < 0.005$ for all subunits in HVC, $p < 0.05$ for α1, α4, β3 and γ4 in Area X and $p < 0.1$

for α2 and β2 in Area X. For almost all $GABA_A$ receptor subunit genes, there was a significant peak in expression level in HVC (except α2-subunit) and Area X (except α3-subunit) during and/or at the end of the phase of song learning (PHD 100). However the exact timing of developmental changes in gene expression varied between subunits. In HVC, early increases in expression levels of α1-, α3-, α4-, β2-, β3- and γ4-subunit genes occurred between PHD 30-55 and all subunit genes shared the same expression pattern of increasing levels as development progressed. However, at PHD 55 only increases in α1-subunit gene expression were significant; increases in α3-, α4-, and γ4-subunit gene expression were not significant until PHD 100 (post-crystallisation), and increases in β2- and β3-subunit gene expression were significant by PHD 80 (relative to expression at PHD 30). Similarly in Area X, $GABA_A$ receptor subunits: α2-, α4- and γ4-subunit genes exhibited an early peak in expression (at PHD 55, sensory acquisition), which then declined thereafter; whereas the expression of α1-, β2- and β3-subunit genes increased significantly towards the end of the song learning phase. Within RA, not all subunit genes displayed developmentally regulated expression, α2-, α4- and β2-subunit mRNA levels remained relatively unchanged. In RA, α1- and γ4-subunit mRNAs were significantly increased by PHD 80 (p <0.05) which was maintained at a similar level post-crystallisation and α3-subunit mRNA was significantly increased slightly later at PHD 100 (p <0.05). The β3-subunit was the only example of a developmentally-associated decrease in corresponding mRNA level, occurring at an early time point of song learning (between PHD 30 and 55). The temporal expression profile of the γ4-subunit gene was unique. There was a peak in mRNA levels at PHD 55 (sensory acquisition) in Area X and a peak at PHD 80 (sensorimotor phase) in RA, no other subunit gene exhibited such developmentally-specific expression.

The α1-subunit was the only transcript which demonstrated the same temporal expression pattern in each of the three nuclei. α4- and γ4-subunit mRNAs displayed similar temporal patterns in Area X and HVC, but differed in RA. In fact, no two subunits shared the same temporal-expression profiles in RA. α3-subunit mRNA was only increased in HVC and RA post-crystallisation, suggesting no involvement in the song-learning process. In conclusion, during the phase of song learning, gene expression patterns of seven $GABA_A$ receptor subunits were song-control-nucleus specific. Nevertheless, developmental changes that occurred in the expression levels of

these $GABA_A$ receptor subunit mRNAs during the phase of song learning were more often similar in HVC and Area X compared to RA.

4.3 Discussion

Literature indicates a role for GABAergic neuronal inhibition in the zebra finch song system and although there is data indicating the presence of $GABA_A$ receptors within this system, the main body of work is focused exclusively on electrophysiological and pharmacological approaches, which gives us little insight into receptor subtypes and their distribution (Livinston and Mooney, 1997; Bottjer *et al*., 1998; Dutar *et al*., 1998; Luo and Perkel, 1999; Spiro *et al*., 1999; Vicario and Raksin, 2000; Farries *et al*., 2005; Prather *et al*., 2008). This study was the first to examine the complete spatial distribution of all the $GABA_A$ receptor subunit mRNAs (with the exception of π; which has a primarily peripheral distribution, Symmans *et al*., 2005; Xiang *et al*., 2007) in four nuclei of the adult male zebra finch song system; as it is only when *all* relevant subunits are considered that meaningful conclusions can be drawn. Expression of each subunit gene within each nucleus was quantified relative to an internal control region (nidopallium), enabling individual expression profiles for each subunit to be established. Furthermore, although levels of subunit mRNAs cannot be directly compared against each other (Briode *et al*., 2004), tentative conclusions may be drawn concerning the emergence of specific $GABA_A$ receptor subtypes within each nucleus. In addition, developmental expression of selected $GABA_A$ receptor subunit genes was also quantified in selected nuclei of the zebra finch song system at important developmental time points (PHD 30, 55, 80 and 100) during the phase of song learning, enabling regional- and temporal-specific gene expression profiles to be generated.

4.3.1 Spatial expression of $GABA_A$ receptor subunit genes in the song system

The molecular heterogeneity of the $GABA_A$ receptor gene family is much larger than any other members of the ligand-gated ion channel superfamily, so a vast number of potential subtypes are possible. Despite this, it appears that there are preferred assemblies (Olsen and Sieghart, 2008, 2009). All $GABA_A$ receptor subunit transcripts (α1-α6, β2-β4, γ1, γ2, γ4 and δ) were detectable in all nuclei observed (LMAN, Area X,

HVC and RA), and the spatial distribution profile of each subunit mRNA across the zebra finch brain was unique to each individual transcript. Therefore it can be assumed that numerous receptor populations comprising varying subunit assemblies may exist within different brain regions. This concurs with previous data completed in rat (Laurie *et al*., 1992a, b; Wisden *et al*., 1992; Sperk *et al*., 1997; Pirker *et al*., 2000) and monkey (Kultas-Ilinsky *et al*., 1998; Huntsman *et al*., 1999) brain indicating spatial regulation of individual $GABA_A$ receptor subunit transcription. Only γ4-subunit mRNA demonstrated robust signalling within all the major nuclei of the song system (Fig. 4.5), hence its consideration as a suitable molecular marker for this system (Thode *et al*., 2008).

The α1-subunit gene is the most abundantly expressed of its class in the mammalian brain (Sieghart and Sperk, 2002). If this is knocked out then the total $GABA_A$ receptor content of the brain is reduced by ~50% (Sur *et al*., 2001). *In situ* hybridisation experiments showed the α1-subunit gene to be well expressed throughout the brain with higher expression in Area X and RA (Figs 4.1, row A and 4.2a), this was confirmed by real-time RT-PCR, which also showed this subunit to be well expressed within these regions. This data indicated a steady increase in α1-subunit mRNA levels as development progressed, with highest levels (in Area X, HVC and RA) at the time of song crystallisation (~PHD 90) (Fig. 4.9). The α1-subunit polypeptide most commonly associates with β2 and γ2 subunits *in vivo* to comprise the most prevalent mammalian $GABA_A$ receptor subtype (Whiting *et al*., 2003a). This was apparent in HVC and LMAN, where all three subunit mRNAs gave hybridisation signals similar to those in nidopallium (Table 4.1) and increases in β2-subunit mRNA levels in HVC during development correlated with those encoding the α1 subunit (Fig. 4.10). The generally low, but diffuse mRNA distribution pattern of these three subunits was indicative of a generalised function within the brain, not specific to the song system and thus potentially not playing a pivotal role in learning and memory (refer to Chapter 5). When considering temporal expression, mRNA distribution patterns of α2 and β2 subunits in Area X and RA were similar, both remaining relatively unchanged in the RA during song development and showing small peaks at PHD 55 and PHD 100 in Area X (Figs. 4.9 and 4.10), in contrast to the steadily-increasing expression of α1-subunit gene. A complementary distribution was also observed in *in situ* hybridisation studies whereby in Area X and RA, when the α1-subunit gene was highly expressed, the α2-subunit

gene was poorly expressed and in LMAN, when the α2-subunit gene was highly expressed, α1-subunit mRNA was at level equivalent to baseline (Table 4.1). This data indicated the presence of the well documented α2βγ2 subtype in addition to the α1β2γ2 complex (both are present in mammalian systems; Olsen and Sieghart, 2008). Generally, α1-subunit transcripts are more abundant in post-natal mammalian brain than those encoding the α2 subunit (in embryonic brain α2-subunits prevail; Laurie *et al*., 1992a; Huntsman *et al*., 1999; Sieghart and Sperk, 2002); this was reflected in the steady increase in α1-subunit mRNA levels as the song system underwent maturity (Fig. 4.9).

α1-, α3- and α5-subunit genes demonstrated overlapping expression profiles which indicated a variety of $GABA_A$ receptor subtypes may be present within an individual nuclei (Fig. 4.1). On the other hand, it is certainly possible that more than one type of α-subunit polypeptide may comprise a single $GABA_A$ receptor (Nusser *et al*., 1998; Pöltl *et al*., 2003; Benke *et al*., 2004; Minier and Sigel, 2004), this has most commonly been investigated with the α1 and α6 subunits (Benke *et al*., 2004; Minier and Siegel, 2004). Olsen and Sieghart (2008) have concluded there is sufficient evidence for the α1α6βγ subtype to exist in brain. Elevated expression of α1- and α6-subunit genes in RA indicated this receptor species may indeed be present in the avian song system. Interestingly, previous data has shown the α5-subunit gene to be poorly expressed in brain with the exception of the hippocampus in rat (Laurie *et al*., 1992; Sperk *et al*., 1997; Pirker *et al*., 2000), where α5-subunit-containing receptors constitute ~20% of the $GABA_A$ receptor population (Sur *et al*., 1998, 1999). Conversely, *in situ* hybridisation studies herein have demonstrated high levels of α5-subunit mRNA in a variety of brain regions, most notably in Area X and RA, relative to baseline levels (Fig. 4.1 and 4.2). Strong expression was also detected in the hippocampus, cerebellum and arcopallium (although this was not quantified), which correlates with data found in rats. α5-subtype receptors play an important role in spatial learning in rats (Cavallaro *et al*., 2002; Collinson *et al*., 2002; Crestani *et al*., 2002; Chambers *et al*., 2004), due to their specific location in the hippocampus (a region associated with spatial learning; Wanatabe and Bischof, 2004), thus the same theory may be extrapolated to explain the robust expression of the γ4-subunit gene within the song system.

α4-subunit-encoding mRNA was abundant in the cerebellum (Fig. 4.1), which disagrees with previous studies (Laurie *et al*., 1992a, b; Wisden *et al*., 1992). Although natural variation amongst different species is to be expected, for example, Pöltl *et al*. (2003) demonstrated differences in $GABA_A$ receptor subtype assemblies in the cerebellum of mice and rats. The α4-subunit gene was also highly expressed in RA exhibiting a distribution somewhat reciprocal to that of the α6 subunit (Fig. 4.1), although a subtype containing these two subunits has not been previously postulated, it is recognised that ~50% of α4-subunit-containing $GABA_A$ receptors do not posses a γ or δ subunit and are α/β pentamers (Bencsits *et al*., 1999); these are localised extrasynapically on rat hippocampal pyramidal neurons (Mortensen and Smart, 2006). Similar to the α4 subunit, the distribution of α6-subunit transcript found in this study disagrees somewhat with previous literature which reports expression of this gene to be restricted to the cerebellum (Laurie *et al*., 1992a, b; Persohn *et al*., 1992; Pirker *et al*., 2000). Although signal intensity was highest in the cerebellum (Fig. 4.1), there was also evidence that the α6-subunit mRNA was present at low levels in all nuclei of the adult song system and at marginally increased levels in RA (the most dorsally examined nucleus; Fig. 4.2, Table 4.1). Thus it appeared that α6-subunit mRNA levels increase towards the posterior regions of the brain. Although it must be acknowledged that as the levels of mRNA were relative to baseline; therefore, as the expression of α6-subunit gene was fairly weak in the nidopallium (internal control area) and any small increase in a nucleus would be appreciable.

There is no concrete evidence to indicate that two different β subunit isoforms co-assemble into a heteromeric receptor although it has been postulated (Li and De Blas, 1997; Jechlinger *et al*., 1998), thus presence of high levels of mRNA encoding β3 and β4 subunits in Area X points at two different receptor species. Although β2-subunit mRNA was well distributed, it was lower and more diffuse (relative to baseline) than observed for other β subunits (Fig. 4.3). β3-subunit transcript was the most abundant in the song system relative to the nidopallium, with the exception of the HVC, where β4-subunit mRNA was well represented (Table 4.1). The β4-subunit gene is localised in a cluster with γ4- and α3-subunit genes on chromosome 4A of the zebra finch genome (Fig. 3.8), analogous to the θεα3 cluster on the X chromosome of the human genome

(Simon *et al*., 2004). Coordinate chromosomal expression would suggest an α3β4γ4 subtype in the zebra finch brain, but although expression was overlapping, only in Area X, were all three subunit genes expressed at a high level relative to their individual baselines. Thus this combination was not emerging as a strong candidate for a major receptor subtype in the song system.

Levels of γ1-subunit mRNA in LMAN were striking, suggesting that there were receptors containing this subunit in this nucleus; such a robust distribution is not seen anywhere within the mammalian brain (Laurie *et al*., 1992a, b; Wisden *et al*., 1992; Sperk *et al*., 1997). There is no definitive evidence suggesting that γ1-subunit polypeptides assemble into receptors in mammalian systems (Olsen and Sieghart, 2008), although this can primarily be attributed to a paucity of studies. Immunoprecipitaion studies have indicated that all three mammalian γ subunits are able to associate with α1, α2, α5 (not γ1), β2/3 subunits, and in addition, the γ2 subunit was found to co-precipitate with γ3 (Benke *et al*., 1996). This evidence suggested γ1 may well be able to associate with any of the subunits also detected within LMAN, and the highly confined distribution (γ1-subunit mRNA was low in all other song system nuclei), may be indicative of a specialised role within the song system, particularly as LMAN was the only nucleus where γ4-subunit mRNA was not significantly higher than baseline. The most intriguing expression pattern of all was that of the γ4-subunit gene (Fig. 4.5). Robust expression of the γ4-subunit gene in the song system of the zebra finch has been recently demonstrated by Thode *et al*. (2008), and accordingly, its role as a molecular song system marker has been proposed. In light of this, it is interesting to speculate that the γ4-subunit polypeptide is able to co-assemble with a variety of other $GABA_A$ receptor subunits (as with other γ subunits) as no other subunit mRNA profile was similar to that generated for the γ4 subunit, both spatially or developmentally. Thus it may form the basis of a variety of receptor subtypes which would account for the relative abundance of the transcript.

The δ-subunit gene was well expressed in the telencephalic HVC and LMAN nuclei, and in accordance with previous data, it was most abundant in the cerebellum (Fig. 4.5 (D4); Laurie *et al*., 1992a, b). δ-subunit containing receptors are exclusively extra-synaptic where they mediate tonic inhibition (Kaur *et al*., 2009). They are generally

thought to co-assemble with α4 or α6 subunits (Jones *et al*., 1997; Sur *et al*., 1999), but have more recently been shown to assemble with the α1 subunit in hippocampus (Glykys *et al*., 2007). There was no obvious indication of this partnership in the song system (but both were in cerebellum). Moreover, upon knockout of α5-subunit containing receptors (which are also extrasynaptic) in mice, those comprising the δ subunit were upregulated in compensation in hippocampal regions (Glykys and Mody, 2006). This agrees with data presented here (Table 4.1) which showed the α5-subunit gene to be highly expressed in Area X and RA (where δ was poorly expressed) and low expression in LMAN and HVC where the δ-subunit gene was highly expressed. However, when δ-subunit-containing receptors are knocked out, a small residual tonic current is still detected (not attributed to by α5-subunit containing receptors) in regions of the hippocampus (Glykkys *et al*., 2007), and this is speculated to be due to $GABA_A$ receptors containing the ε subunit. As afore mentioned the ε subunit is the mammalian orthologue of the avian γ4 subunit and *in situ* hybridisation studies (including this one) have shown γ4-subunit mRNA to be well expressed in the hippocampus (Thode *et al*., 2008). This suggests that the γ4 subunit, like its' mammalian counterpart, may play a role in tonic inhibition within the zebra finch brain.

4.3.2 Expression of $GABA_A$ receptor π-subunit gene in avian brain

Although it is regarded as a bona-fide $GABA_A$ receptor subunit, the π-subunit is different in that it is generally expressed in peripheral tissues such as lung (Jin *et al*., 2005; Xiang *et al*., 2007), ovaries and uterus (Hedblom & Kirkness, 1997; Fujii and Mellon, 2001) in rat. It has been suggested that π-subunit-containing $GABA_A$ receptors play a role in the regulation of muscle contraction and alveolar fluid homeostasis in the lungs (Jin *et al*., 2006), where they may act in an excitatory capacity (Xiang *et al*., 2007). π-subunit-containing receptors are significantly more sensitive to the potent progesterone metabolite allopregnanolone (which is at high circulating levels during pregnancy; Concas *et al*., 1999) and there is a drastic decrease in the level of π-subunit mRNA in uterine tissues just prior to labour, hence these receptors could help regulate uterine contractions (Fujii and Mellon, 2001). More recently, the π-subunit has been established as a molecular marker for detecting breast cancer metastasis (Backus *et al*., 2005; Symmans *et al*., 2005) and tumorigensis in pancreatic cancer (Johnson and Haun,

2005) but there is little data documenting its expression within brain. However, some recent data has shown that not only is the π-subunit transcript present in rat brain, but it demonstrates a learning-associated increase in expression in hippocampus following training with the Morris water maze (Cavallaro *et al*., 2002). Coupled with the seemingly robust product amplification from brain cDNA (Fig. 4.7), it would appear that π-subunit mRNA is present in the avian brain and this merits further investigation.

4.3.3 Spatial expression of $GABA_C$ receptor subunit genes in the song system

Although not quantified densitometrically, spatial expression of $GABA_C$ subunit genes was investigated within the male zebra finch brain. $GABA_C$ receptor subunit genes are predominately expressed in the bipolar and horizontal cells of the retina, where they mediate synaptic inhibition (Albrecht and Darlison, 1995; Chebib and Johnston, 1999; Rozzo *et al*., 2002). Thus, unlike the majority of $GABA_A$ receptor-subunit genes, expression of $GABA_C$ receptor subunit genes was low and diffuse; with no notable regions of discrete expression except in cerebellum. This was in agreement with previous data which initially suggested ρ-subunits were not transcribed in brain, but now purports expression to be present at low levels in mammalian brain (Ogurusu and Shingai, 1996, Boue-Grabot *et al*., 1998; Rozzo *et al*., 2002; Milligan *et al*., 2004; Alakuijala *et al*., 2006) and chicken brain (Albrecht *et al*., 1997). They are also expressed in mammalian ovary, testes (Rozzo *et al*., 2002) and gut (Jansen *et al*., 2000). Data indicated that $GABA_C$ receptor ρ3-subunit mRNA was present at very low levels in zebra finch brain compared to ρ1 and ρ2 (Fig. 4.8), concurring with northern blotting analysis which initially illustrated that the $GABA_C$ receptor ρ3-subunit gene is expressed at lower levels in the retina compared to ρ1- and ρ2-subunit genes (Ogurusu and Shingai, 1996). Real-time RT-PCR has confirmed these findings and further revealed that although mRNA encoding the ρ3 subunit is present in the cerebellum and hippocampus, it is at significantly lower levels than the other two $GABA_C$ receptor subunit mRNAs (Milligan *et al*., 2004). All subunits were well expressed in cerebellum, where they could potentially associate into heteromers (Enz and Cutting, 1999), but generally $GABA_C$ receptors tend to form homooligomeric complexes (Enz and Cutting 1998, 1999). In recombinant receptor systems, ρ-subunits have been reported to

associate with the γ2 subunit of the $GABA_A$ receptor into functional receptors (Ekema *et al*., 2002; Milligan *et al*., 2004; Pan and Qian, 2005). Coupled with the more recent identification of $GABA_C$ subunits in brain and the suggestion that $GABA_C$ receptors are indeed a sub-class of $GABA_A$ receptors (Olsen and Sieghart, 2008; Collingridge *et al*., 2009), it was of interest to observe if there were any regions of co-expression. Their expression in cerebellum was overlapping with that of $GABA_A$ receptor subunit genes; so this could be a site for potential co-assembly between the two receptor classes, however due to the variety of cell types that exist within cerebellum, it is equally likely that the $GABA_C$ receptors could be localised to different cells (or even on the same cell in different receptor populations) as there is no documented evidence of native $GABA_{A/C}$ complexes existing in brain. None of the $GABA_C$ receptor subunit genes were well expressed in the song system of zebra finches (not quantified) so analysis was not taken any further.

4.3.4 Developmental expression of $GABA_A$ receptor subunit genes in the song system

All $GABA_A$ receptor subunit mRNAs (α1, α2, α3, α4, β2, β3 and γ4) were detected in each nuclei studied (Area X, HVC and RA) of the zebra finch song system, at each developmental stage (Figs. 4.9 and 4.10), which concurred with findings from the *in situ* hybridisation experiments. In HVC, transcription of all $GABA_A$ receptor subunits appeared to be developmentally regulated. All subunits exhibited the highest level of gene expression in HVC at PHD 100 (post-crystallisation) after the song-learning phase was finished. α1, α4, β2, β3 and γ4 subunits all demonstrated steady increases in gene expression as development progressed (perhaps correlating with the growing HVC which does not reach full size until ~PHD 60; Ward *et al*., 1999), only α2- and α3-subunit genes exhibited lower expression during the sensorimotor phase, but following crystallisation, levels increased once more (Figs. 4.9 and 4.10). This was in agreement with previous data which showed that α2-, α3-, α5- and β3-subunit genes were well expressed in embryonic cortex and thalamus of rats and decreased in adult brain and the reverse was true for α1-, α4-, β2- and δ-subunit genes (Laurie *et al*., 1992a). Overall, α1- and α4-subunit genes demonstrated increased expression in the developing song system whereas α2- and α3-subunit genes remained at relatively low and unchanging levels. The HVC is involved in both the anterior forebrain pathway (AFP) and vocal

motor pathway (VMP) and thus is important in both sensory acquisition and the sensorimotor phase (Nottebohm *et al*., 1976; Wild *et al*., 2005; Mooney and Prather, 2008). This steady increase during song development was also observed for α1- and β3-subunit mRNAs in Area X, whereas α2, α3, α4 and γ4 subunits all displayed discontinuous temporal mRNA distributions with the highest levels during sensory acquisition (~PHD 35-60) and a decrease by the end of the sensorimotor phase (PHD 80). Area X forms part of the AFP and thus is primarily involved in sensory acquisition during song learning (Bottjer *et al*., 1984; Scharff and Nottebohm, 1991) and so would be most active during this stage (up to PHD 60) and cell number within Area X increases between ~PHD 10-50 (Tomaszycki *et al*., 2009). RA housed the smallest number of developmentally-regulated subunit genes with levels α2-, α4- and β4-subunit mRNAs all remaining relatively unchanged over time. Interestingly, in previous studies, RA displays the lowest number of genes regulated by song production (Wada *et al*., 2006). β3 was the only subunit to show a decrease in transcript expression following the onset of song learning and γ4-subunit mRNA again showed a discontinuous profile with highest levels towards the end of song learning, indicating a rapid increase in gene transcription during the whole period of song learning, followed by a small decrease post-crystallisation. Previous findings using *in situ* hybridisation suggested that γ4-subunit mRNA only appears in RA at ~PHD 35 (coinciding with establishment of the VMP; Nordeen and Nordeen, 1988; Wang *et al*., 1999; Scott and Lois, 2007; Thode *et al*., 2008). This was echoed in the real-time RT-PCR data which indicated a large increase of γ4-subunit mRNA in RA occurred after PHD 30 (Fig. 4.10). The RA forms part of the VMP and is the output of the song system, thus it only becomes fully active during the sensorimotor phase of song learning. Thus γ4-subunit mRNA was highest in Area X during sensory acquisition, highest in RA during the sensorimotor phase and highest in HVC following crystallisation. Correlation of gene expression with specific developmental time points is highly indicative of a functional role for receptors containing this subunit in the learning of song; no other subunit displayed such a defined temporal profile. The level of γ4-subunit mRNA is developmentally regulated in the chicken (Harvey *et al*., 1993; Enomoto *et al*., 2001) and is down-regulated in response during imprinting training in the one-day-old chicken (Harvey *et al*., 1998). Coupled with the robust expression observed here and previously (Thode *et al*., 2008), and coordinated expression during song development in the zebra finch, it can be

concluded that $GABA_A$ receptors containing the γ4-subunit may play an important role in learning and memory systems.

4.3.5 Concluding remarks

The heterogeneity of the $GABA_A$ receptor family is both its redeeming feature and greatest encumbrance. $GABA_A$ receptors mediate the majority of inhibitory neurotransmission within the brain and the plethora of subunits enables temporal- and regional-specific expression of many different subtypes (demonstrated herein), each with its own individual pharmacological and electrophysiological signature, which potentially fulfil different physiological functions (Wafford *et al*., 2004; Olsen and Sieghart, 2008). However, the extreme promiscuity of the subunits causes problems in elucidating defined native $GABA_A$ receptor subtypes, this is frequently observed in co-precipitation studies (Sieghart and Sperk, 2002) and expression studies such as this are notoriously hard to decipher as all subunits are present and may be assembling into many combinations. As $GABA_A$ receptors are targets of many clinically important drugs (benzodiazepines, barbiturates, steroids and volatile anaesthetics; Reynolds, 2008), it is important to resolve specific receptor subtypes involved in mediating different physiological functions in an effort to generate novel subtype-specific therapeutic strategies (such as those being developed for α5-subunit containing receptors; Chambers *et al*., 2004). $GABA_A$ receptors have long been implicated in modulation of learning and memory (for review, see Chapouthier and Venault, 2002; Maubach, 2003; McNally *et al*., 2008). In the chicken (*Gallus gallus domesticus*), which is phylogenetically similar to the zebra finch, augmentation of $GABA_A$-receptor-mediated inhibition with muscimol produced amnesia and bicuculline improved memory formation (Clements and Bourne, 1996). A later study specifically identified a significant decrease in levels γ4-subunit mRNA (but not γ2-subunit mRNA) in learning-relevant regions of chicken brain 10 h following imprinting training (further details in Chapter Five; Harvey *et al*., 1998). Following this, the γ4-subunit gene was mapped in the zebra finch brain and a unique, robust expression profile was revealed (Thode *et al*., 2008). The avian song system is a well established paradigm for studying neuronal mechanisms of cognitive processes and development of sophisticated motor skills. Due to the numerous parallels with human speech production (Doupe and Kuhl, 1999; Brainard and Doupe, 2002; Kuhl, 2004; Bolhius and Gahr, 2006), defining $GABA_A$

subtypes and their functional roles in this system may extrapolate to human behaviour and drugs can be designed to target cognitive defects and give insight into diseases which affect motor coordination. Robust expression of the γ4-subunit gene and developmental regulation of transcription in the song system points to an important role for $GABA_A$ receptors containing this subunit. Electrophysiological evidence shows that γ4-subunit-containing receptors demonstrate some different pharmacological characteristics to those comprising the other γ subunits, including a novel benzodiazepine pharmacology and potent blocking of currents by Zn^{2+} ions (Forster *et al*., 2001), thus creating an attractive therapeutic target.

5. THE EFFECT OF AUDITORY LEARNING ON THE EXPRESSION OF THE $GABA_A$ RECEPTOR γ4-SUBUNIT GENE IN THE SONG SYSTEM

5.1 Introduction

Previous data (Chapter Three) illustrated a robust, region-specific distribution of mRNA encoding the $GABA_A$ receptor γ4 subunit, within the zebra finch song system. The spatial gene expression and the strength of hybridisation signals was exclusive to the γ4 subunit and was indicative of an important role for $GABA_A$ receptors containing this subunit in song acquisition and production in the zebra finch. This was further compounded by the correlation of gene expression with specific developmental time points, which again was exclusive to the γ4-subunit gene. Such findings warranted further investigation.

The zebra finch song system is an important paradigm for investing the underlying mechanisms of cognitive function, which can be extrapolated to humans due to the numerous parallels between the two systems (Brainard and Doupe, 2002; Kuhl, 2004; Bolhuis and Gahr, 2006; refer to section 1.4.8). This is exemplified by studies of *FOXP2* (which encodes the forkhead box transcription factor). It is documented that genetic aberrations can cause developmental verbal dyspraxia in humans. Studies using twins have eluded that despite equal intelligence and opportunity, developmental speech impairments can occur (Bishop, 1995). This can be attributed to, at least in part, an inherited genotype. Further work by Lai *et al*. (2001, 2003) and MacDermot *et al.* (2005), identified a mutation in *FOXP2* in human subjects, which caused speech and language impairments; this was the first gene to be implicated in such a disorder. RNA interference (RNAi) studies with the same gene within the zebra finch song system have revealed that when *FOXP2* gene expression was 'knocked-down', vocalisations were affected in the manner of poor imitation of tutor song and production of song with abnormally variable syllables (Haesler *et al*., 2007). Due to the complexity of neuronal networks, these effects cannot be attributed to a single gene and thus the search for other genetic links is paramount. As previously mentioned, songbirds are one of the closest accessible models for neuronal circuitry controlling learned vocalisations similar to that of humans (Brainard and Doupe, 2002;

Kuhl, 2004; Bolhuis and Gahr, 2006); this was therefore a relevant system to observe γ4-subunit gene expression and how it is implicated in the acquisition and production of song.

Birds live in heterogeneous populations where they are exposed to a variety of songs from different species. It is a matter of survival that they learn a song specific to their species as song is principally used for attraction of a mate, individual identification (which is especially relevant in zebra finches which live in groups of up to 1000 members) and defence of territory (Zann, 1996; Brenowitz *et al.*, 1997) and thus needs to be interpreted by conspecifics. The auditory template model of song learning has been proposed Marler, 1970). It suggests that song birds, from birth, have a genetically-encoded, innate template which pre-disposes them to sing a song representative of their own species. It is the songs that they hear from their conspecifics (tutors) during the sensory acquisition phase that 'trigger' this song learning process (Konishi, 1965, 1985, 2004; Marler, 1970, 1976). Evidence supporting this arises from the fact that young birds and female birds respond more to conspecific song than heterospecific song both at a behavioural (Dooling and Searcy, 1980; Clayton, 1989; Nelson and Marler, 1993) and molecular level (Nick and Konishi, 2005; Tomaszycki *et al*., 2006); and will learn conspecific song in preference to heterospecific song (Marler and Peters, 1988). Although a tutor is required during sensory acquisition, when the bird enters the sensorimotor phase of song learning, the tutor is no longer necessary. At this stage the bird must be able to hear its own vocal output to compare to the acquired template (Konishi, 2004) and this need for auditory feedback extends into adulthood, even after crystallisation (Nordeen and Nordeen, 1992; Leonardo and Konishi, 1999; Woolley, 2004; Funabiki and Funabiki, 2007; Sober and Brainard, 2009). Deprivation experiments have highlighted that birds raised in isolation are able to produce songs that include some species-specific features, which are enough to evoke a reaction from other adult male or female conspecifics (Marler and Sherman, 1985; Searcy and Marler, 1987; Zann, 1996; Konishi, 2004; Kojima and Doupe, 2007). Deafened birds produce a song which is more abnormal than isolate song (Konishi, 2004), yet even these songs retain some basic features of natural song, but not enough to elicit a reaction from conspecifics (Marler and Sherman, 1983, 1985; Searcy and Marler, 1987). Disruption of auditory feedback in adult birds results in a gradual deterioration of song quality (Nordeen

and Nordeen, 1992; Woolley *et al*., 1997; Leonardo and Konishi, 1999; Funabiki and Konishi, 2003; Zevin *et al*., 2004). However, despite all of the previous behavioural work regarding the isolation and deafening of song birds, the underlying molecular mechanisms are still not properly understood. With regards to gene expression and song behaviour, the majority of work has concentrated on mapping expression of genes within the song system in response to song production. Some of the first genes to be studied were immediate early genes (IEG)s, which are characterised by their rapid and transient response to a stimulus and consequently are often used as endogenous markers of neuronal activity (Chaudhuri, 1997; Tischmeyer and Grimm, 1999). *ZENK* (a.k.a. *zif-268, NGFI-A, egr-1* or *Krox-24*; Jarvis and Nottebohm, 1997), *c-fos* (Kimpo and Doupe, 1997), *BDNF* (brain-derived neurotrophic factor; Li *et al*., 2000), *Arc/Arg3.1* (activity-regulated cytoskeleton-associated protein/activity-regulated gene; Tarcisco *et al*., 2005), *SytIV* (synaptotagmin IV; Poopatanapong *et al*., 2006) and *UCHL1* (ubiquitin carboxyl-terminal hydrolase; Lombardino *et al*., 2005) all show a rapid increase in gene expression in nuclei of the zebra finch song system at the onset of song production. Continuing along this theme, more recently, Wada *et al*. (2006) considered 33 of the many genes expressed within four nuclei of the song system; these genes encode for proteins such as transcription factors (e.g. *c-fos*, cellular-fos; *c-jun*, cellular-jun; *Atf4*, activating transcription factor 4), actin-binding proteins (e.g. *β-actin*; *Tagln2*, transgelin2), cell signalling molecules (e.g. *Penk*, Proenkephalin; *Stard7*, START domain containing 7) and many more. All observed genes were regulated by the onset of singing. Of the four regions, Area X showed the greatest variability of gene expression with 94% of genes regulated by singing; the majority of which were up-regulated in response to song production. In contrast, within the RA, only 33% of the genes examined demonstrated song-driven expression (Wada *et al*., 2006), which is interesting as RA is heavily involved in the motor production of song. In addition to regional-specific gene expression, the zebra finch song system also displays contextual gene expression. During undirected singing (in the absence of females) *ZENK* expression is high in HVC, Area X and elevated in RA, whereas when song is directed at females, *ZENK* expression is only high in HVC and is virtually absent in Area X and RA (Jarvis *et al*., 1998). Conversely, c-*fos*, another immediate early gene is better expressed in RA during directed singing (Kimbo and Doupe, 1997). Despite numerous gene expression studies in

zebra finch, there still remains little data available documenting gene expression in relation to the learning of song (with the exception of *FOXP2*). Studies such as these are necessary for understanding the complex molecular mechanisms underlying the acquisition and production of learned song.

In addition to the zebra finch song system, imprinting in the one-day-old chicken is another well established learning paradigm (Horn, 1998). Gene expression levels of various IEGs, which are involved in the formation of long-term memories, have been measured in response to auditory imprinting. Results revealed significantly increased expression of *ZENK* and *Arc/Arg3.1* within regions involved in visual and auditory imprinting (Bock *et al*., 2005; Thode *et al*., 2005). Similar experiments utilising naive one-day-old chicks trained on auditory and visual stimuli, indicated a regional-specific down-regulation of γ4-subunit transcript within numerous brain areas associated with auditory and visual imprinting (Harvey *et al*., 1998), which suggested a role for receptors containing this subunit in learning and memory. Decreases between trained and untrained chicks were apparent 5 h post-training but only became significant 10 h post-training. Brain regions significantly affected were the entopallium (E) and the intermediate medial mesopallium (IMM) - an area involved in recognition memory during imprinting (Horn, 1998). The authors postulated that a down-regulation of GABAergic transmission ameliorated learning and memory processes (Harvey *et al*., 1998). Furthermore, when compared to the embryonic (E18) chicken brain (Harvey *et al*., 1993), γ4-subunit gene expression in the optic tectum of the one-day-old chicken was very high. This area is involved in processing visual information which is necessary to some extent during embryogenesis (where light passing through the shell modulates brain development; Rogers, 1982), but is critically important following hatching; the increase in γ4-subunit transcript correlated with this.

As aforementioned, GABA is widely distributed in the avian song system (Pinaud and Mello, 2007). Although electrophysiological and pharmacological evidence confirms the presence of $GABA_A$ receptors (Livingston and Mooney, 1997; Bottjer *et al*., 1998; Dutar *et al*., 1998; Luo and Perkel, 1999; Spiro *et al*., 1999; Vicario and Raksin, 2000; Farries *et al*., 2005; Prather *et al*., 2008), these studies give no insight as to the subtype composition. A

recent study by Thode *et al*. (2008) highlighted the wide-spread distribution of the γ4-subunit transcript in the majority of song-system nuclei and the interesting observation that the γ4-subunit mRNA only appears in the RA in birds ~PHD 35 or older (coinciding with the onset of singing), suggesting a role for receptors containing this subunit in control of motor production of song, indicative of developmentally-regulated transcription. Developmental expression of the γ4-subunit gene was also observed in the embryonic chicken brain (*Gallus gallus domesticus* - where cDNA encoding the $GABA_A$ receptor γ4 subunit was initially identified). Northern blot analysis revealed a developmentally-regulated pattern of gene expression in the brain whereby the γ4-subunit transcript was first expressed at embryonic day (E) 13 and continued to increase in expression during embryogenesis (Harvey *et al*., 1993), correlating with expression in the brainstem which is not detected until E8, some time after many other $GABA_A$ receptor subunit mRNAs are detected (Enomoto *et al*., 2001). *In situ* hybridisation studies of chicken brain at E18 demonstrated discrete, robust expression of the γ4-subunit transcript in many areas of the brain most notably the entopallium, nucleus rotundus (both of which are involved in the processing of visual information), mesopallium and low levels of transcript were also detected in the optic tectum (Harvey *et al*., 1993).

Down-regulation of GABAergic transmission appears to play a role in learning and memory processes (Harvey *et al*., 1998; Cavallaro *et al*., 2002; Collinson *et al*., 2002; Maubach, 2003). $GABA_A$ receptors are the major mediators of inhibition within the brain and much evidence suggests modulation of $GABA_A$ receptors significantly affects learning and memory behaviour (reviewed by Chapouthier and Venault, 2002; McNally *et al*., 2008). This concurs with evidence that augmentation of GABAergic function via $GABA_A$ receptors with muscimol results in amnesia and antagonism with bicuculline improves memory formation in the chick (Clements and Bourne, 1996), rat (Zarrindast *et al*., 2006) and mouse (Brioni and McGaugh, 1988). Due to the unusually strong expression of the γ4-subunit transcript the zebra finch song-system (Thode *et al*., 2008), another learning and memory paradigm; it was interesting to characterise the expression of receptors containing this subunit at a behavioural level and determine how γ4-subunit gene expression is affected by auditory input (e.g. from a tutor). As birds that are raised in auditory isolation

from a tutor are unable to produce normal song (Immelmann, 1969; Marler and Sherman, 1983; Eales, 1987; Searcy and Marler, 1987; Tchernichovski and Nottebohm, 1998), it can be assumed that the molecular mechanisms involved in song acquisition and production would be affected. Exploitation of this paradigm was used to highlight the importance of the $GABA_A$ receptors containing the γ4-subunit in four nuclei of the zebra finch song system, namely, LMAN, Area X, HVC and RA. Male zebra finches were raised in the presence of a tutor or in auditory isolation until PHD 80 where their songs were recorded (in the presence of a female) and analysed and the expression of the γ4-subunit mRNA was quantified densitometrically within the four nuclei and compared between the experimental groups. This assisted in determining the role of the γ4-subunit-containing $GABA_A$ receptors in learning and memory processes (i.e. acquisition and production of song).

5.2 Results

Two rounds of behavioural experiments were completed. In preliminary experiments, male zebra finches were reared in the presence of a tutor and conspecifics (i.e. in an aviary) or in tutor-isolation (i.e. in an individual, sound-proof isolation cage) to determine if tutor-isolation and thus a disruption to song acquisition and production would affect the expression of the $GABA_A$ receptor γ4-subunit gene within selected nuclei of the song system (LMAN, Area X, HVC and RA). Following *in situ* hybridisation for the γ4-subunit transcript, experiments were repeated with a γ2-subunit selective probe to ascertain if effects were subunit specific. To ensure that environmental and social factors were not affecting gene expression, in the subsequent set of experiments, three sets of male zebra finches were raised. The first group were raised in an aviary with tutors and conspecifics, as before. A second group were raised in individual isolation cages with their parents (so a tutor was present), a third group were raised in individual isolation cages, but instead of being alone (as in the preliminary work), they were accompanied by a female zebra finch (which does not sing). Again, γ4-subunit mRNA levels were measured in LMAN, Area X, HVC and RA and compared between groups. At PHD 80, the song of each bird was recorded in the presence of an unfamiliar female (directed song), then sacrificed, their brains excised and immediately shock-frozen. Sequential 10μm coronal sections were taken

(through the entire brain) and hybridised with a radiolabelled γ4-specific oligonucleotide probe; sections were washed and exposed to the same film together to reduce procedural variability. Autoradiographic images were digitally acquired with a light box and CCD camera and captured using Easygrab Picolo™ software (Euresys™). Gene expression in individual regions (nuclei) and internal control regions (nidopallium), was quantified by densitometric analysis of three consecutive sections using Scion image software (Scion Corporation, USA). Autoradiographic signal in areas of interest was calculated as pixel density per brain area measured. Gene expression was determined by subtracting background hybridisation signals from both the nuclei and internal control readings and then mRNA expression in each nucleus was calculated as a percentage of the internal control. One-way ANOVA tests were employed to determine any significant deviations in gene expression from baseline levels (nidopallium).

5.2.1 Internal control region (Nidopallium)

To quantify the autoradiographic signal (of γ4- or γ2-subunit transcript), an internal control region was required. It was essential to be able to normalise the results, to enable quantitative comparison between levels of mRNA in different nuclei of the brain. The internal control area selected was the nidopallium, which has no documented involvement with learning and memory processes and is a continuous structure throughout the brain (Fig. 5.1). Furthermore, it was ascertained that the genes were expressed in this region and that the nidopallium remained unaffected by the parameters of the experiment, i.e. gene expression was unchanged in nidopallium of tutor-isolated and non-isolated birds.

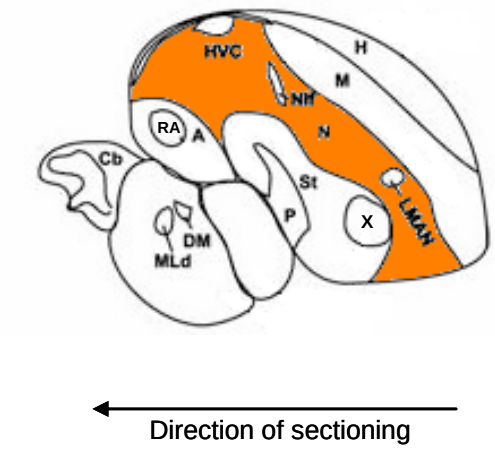


Figure 5.1. Sagittal schematic illustrating layers within the zebra finch brain. Nidopallium (N) is highlighted in orange and encompasses HVC (formal name) and lateral magnocellular nucleus of anterior nidopallium (LMAN); it forms a continual structure throughout the brain. Area X (X) lies within the striatum (St) and robust nucleus of the arcopallium (RA) lies within the arcopallium (A). **Abbreviations:** Cb, cerebellum; DM, dorsal medial nucleus of the midbrain; H, hyperpallium; M, mesopallium; MLd, Mesencephalic lateral dorsal nucleus; NIf, interfacial nucleus of the nidopallium; P, pallidum; St, striatum; X, Area X.

When the measurement of mRNA expression was taken in each nucleus, a simultaneous reading was also taken from the corresponding region of the nidopallium. Figure 5.2 illustrates the areas of the nidopallium selected for internal controls at each observed stereotactic level of the zebra finch brain (i.e. in parallel to each nucleus of interest). Each stereotactic level is denoted by a letter (Fig. 5.2):

- Stereotactic level A corresponds to nidopallium at level of LMAN
- Stereotactic level B corresponds to nidopallium at level of area X
- Stereotactic level C corresponds to nidopallium at level of HVC
- Stereotactic level D corresponds to nidopallium at level of RA

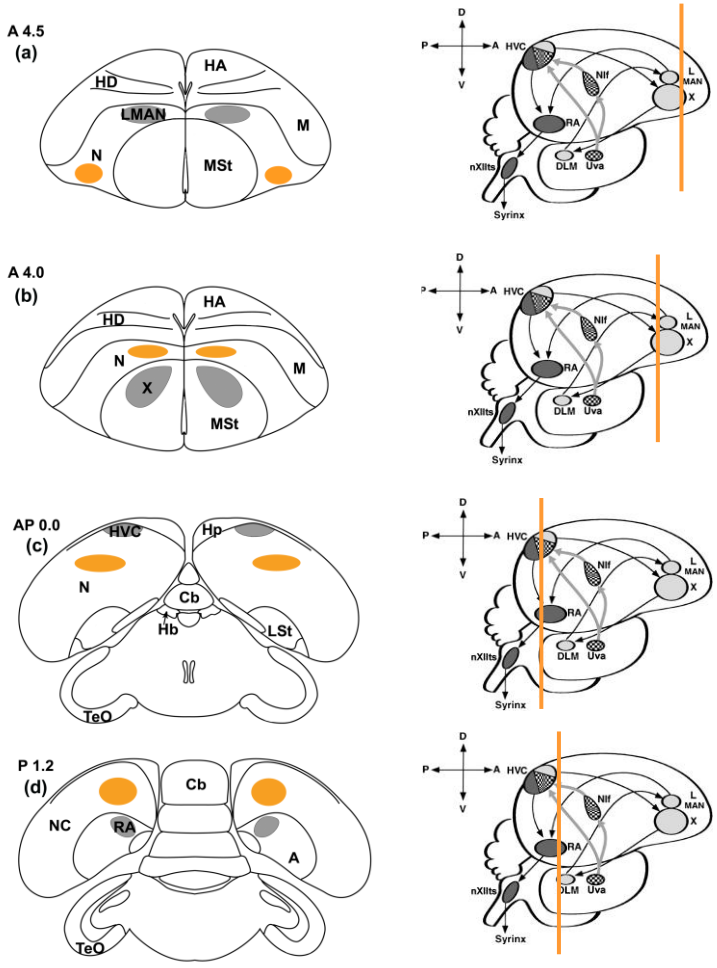


Figure 5.2. Coronal (column 1) and sagittal (column 2) schematics detailing the location of the selected nidopallial internal control areas (orange), which lie in parallel to each song-system nucleus (highlighted in grey). Stereotactic levels are hereafter termed a, b, c and d corresponding to each level sampled. Abbreviations: A, arcopallium; Cb, cerebellum; Hb, habenula; HA, apical part of the hyperpallium; HD,

densocellular part of the hyperpallium; Hp, hippocampus; HVC (formal name); LSt, lateral striatum; M, mesopallium; MSt, medial striatum; N, nidopallium; NC, caudal nidopallium; LMAN, lateral magnocellular nucleus of the anterior nidopallium; RA, robust nucleus of the arcopallium; TeO, optic tectum; X, Area X. Images have been modified from Thode *et al*. 2008.

Nidopallial regions immediately surrounding the nuclei were not measured to avoid any accessory regions being included in the analyses, such as the HVC shelf (which is present in all birds; Jarvis *et al*., 2002); LMAN shell and RA cup (Bottjer, 1989; Iyengar and Bottjer, 2002). Software enabled coordinates of the internal control regions to be mapped to ensure consistent sampling of regions throughout the experiments.

5.2.2 Spatial expression of $GABA_A$ receptor γ4-subunit gene in aviary-reared and tutor-isolated male zebra finch brains

Preliminary results showed discrete expression of the $GABA_A$ receptor γ4-subunit gene was apparent in all four selected nuclei within the song system (LMAN, Area X, HVC and RA), both in the aviary-reared birds and those that were isolated from a tutor (data not shown). Spatial gene expression in both groups of birds was identical to that demonstrated in Chapter Four (Fig. 4.5) with the γ4-subunit gene well transcribed in many important structures of the avian brain. In addition to those mentioned above, strong hybridisation signals were detected in dorsolateral thalamus (DLM) which forms part of the AFP; field L (formal name), a primary auditory area; optic tectum (TeO) which is involved in visual processing; pallial regions including medial magnocellular nucleus of the nidopallium (MMAN), densocellular part of the hyperpallium (HD) and mesopallium (M); medial and lateral striatum (MSt and LSt); habenula (Hb) which lies adjacent to the LSt; hippocampus (Hp) and cerebellum (Cb) in the brains of both aviary-reared and tutor-isolated zebra finches (refer to Fig. 5.2 for anatomical locations). Signal intensity was analysed densitometrically to clarify and quantify the mRNA levels in each song-system nucleus between the two groups of birds.

5.2.3 Quantitative expression of $GABA_A$ receptor γ4-subunit gene in aviary-reared and tutor-isolated male zebra finch brains

5.2.3.1 Internal control (γ4-subunit gene expression in nidopallium)

mRNA levels encoding the $GABA_A$ receptor γ4 subunit remained largely unchanged by the parameters of the experiment in the nidopallium of aviary-reared and tutor-isolated zebra finches at each stereotactic level (Fig. 5.3). There were elevated mRNA levels in the nidopallium of aviary-reared birds at the equivalent levels for LMAN (A), HVC (C) and RA (D), but these differences were marginal. There was no relationship between the acquisition and production of structured song and $GABA_A$ receptor γ4-subunit mRNA expression in the nidopallium; it was therefore acceptable to use this area as an internal control to normalise further results (i.e. expression of γ4- and γ2-subunit genes in song nuclei).

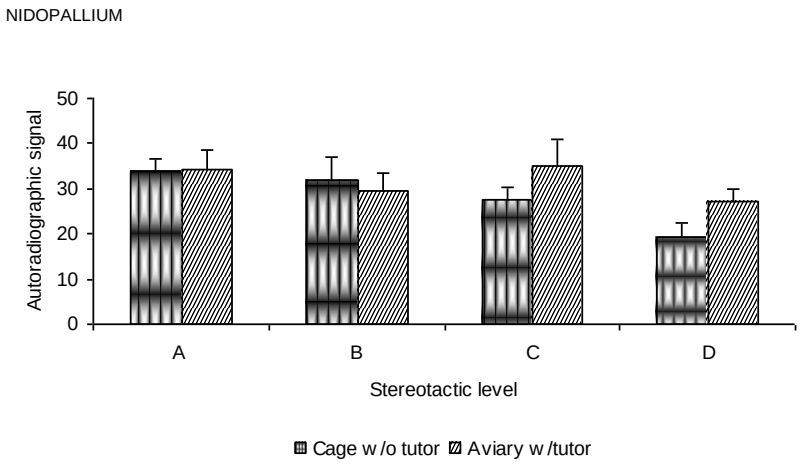


Figure 5.3. Densitometric quantification of $GABA_A$ receptor γ4-subunit mRNA in the nidopallium at the stereotactic levels of each song-system nucleus (A, B, C and D in parallel to LMAN, Area X, HVC and RA respectively; refer to Fig. 5.2). Units are arbitrary, and were quantified by subtraction of any background (i.e. non-specific hybridisation signals from the film). Gene expression was compared between birds which were raised in an aviary with a tutor (birds produced normal song) and those raised in individual isolation cages, without a tutor (birds produced abnormal song); all birds were the same age (PHD 80).

$GABA_A$ receptor γ4-subunit mRNA was expressed throughout the nidopallium and in all examined nuclei of the song system. Transcript levels remained unchanged in the nidopallium between the aviary-reared and tutor-isolated birds (Fig. 5.3).

5.2.3.2 GABA$_A$ receptor γ4-subunit mRNA expression in song-system nuclei

Expression of the GABA$_A$ receptor γ4-subunit mRNA in LMAN, Area X, HVC and RA was quantified as per section 5.2, the results are shown in Figure 5.4.

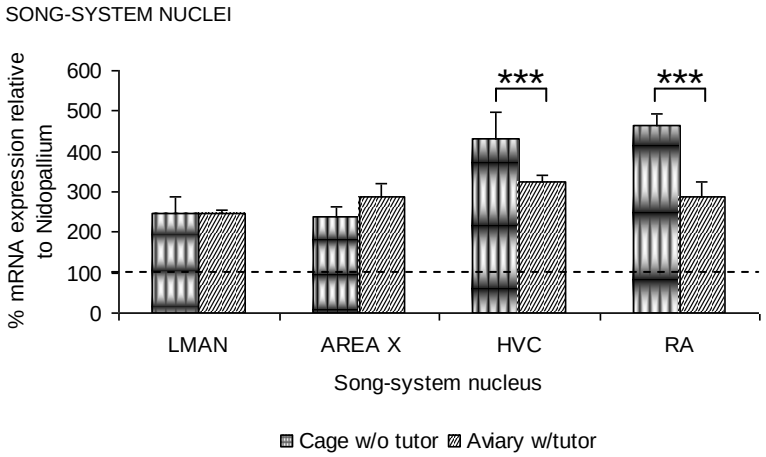


Figure 5.4. Densitometric quantification of GABA$_A$ receptor γ4-subunit mRNA levels within four nuclei of the zebra finch song system relative to the nidopallium. The dashed line indicates mRNA expression in the nidopallium; these readings were considered as baseline, at 100%. Data is presented as mean ±SE, (n=5 per group; *** $p \leq 0.001$). Transcript expression was compared between birds which were raised in an aviary with a tutor (birds produced normal song) and those raised in individual isolation cages, without a tutor (birds produced abnormal song); all birds were the same age (PHD 80). Abbreviations: Area X (formal name); LMAN, lateral magnocellular nucleus of the anterior nidopallium; HVC (formal name); RA, robust nucleus of the arcopallium.

Detailed densitometric analysis of the results demonstrated a highly significant reduction in levels of GABA$_A$ receptor γ4-subunit mRNA in HVC (24%) and RA (38%; both $p \leq 0.001$), in brains of aviary-reared birds relative to tutor-isolated birds (Fig. 5.4). Interestingly HVC and RA form part of the VMP (vocal motor pathway), which is responsible for the production of song. However, there was no significant difference in the level of the γ4-subunit transcript, between tutor-isolated and aviary-reared birds, in LMAN and Area X, which form part of the AFP (anterior forebrain pathway); which is involved in the memorisation of song. In fact, in Area X, gene expression was marginally higher in brains of tutor-isolated animals compared to their aviary-reared counterparts. In brains of tutor-isolated zebra finches, the highest levels of γ4-subunit gene expression were detected

in the RA followed closely by the HVC (Fig. 5.4) – also the sites of the greatest differences in expression following song-learning. $GABA_A$ receptor γ4-subunit gene expression levels across nuclei varied between tutor isolated birds (where RA > HVC > LMAN > Area X) and aviary-reared animals (where HVC > Area X > RA > LMAN). Area X was the only nucleus where γ4-subunit gene expression was higher in the aviary-reared birds. In summary, data illustrated a significant, regional-specific down-regulation of $GABA_A$ receptor γ4-subunit gene expression within the nuclei of the VMP pathway (HVC and RA) in response to acquisition and production of normal song.

Levels of γ4-subunit mRNA was also measured in left and right hemispheres of the zebra finch brain to determine lateralisation of expression. Data revealed (Fig. 5.5a and b) that there were no significant differences between the γ4-subunit gene expression in nuclei located in the left and right side of the brain. In tutor-isolated birds, the nuclei of the right hemisphere showed slightly higher expression (Fig. 5.5b). Aviary-reared birds mirrored this pattern with the exception of the RA where the nucleus located within the left hemisphere had marginally higher expression (Fig. 5.5a).

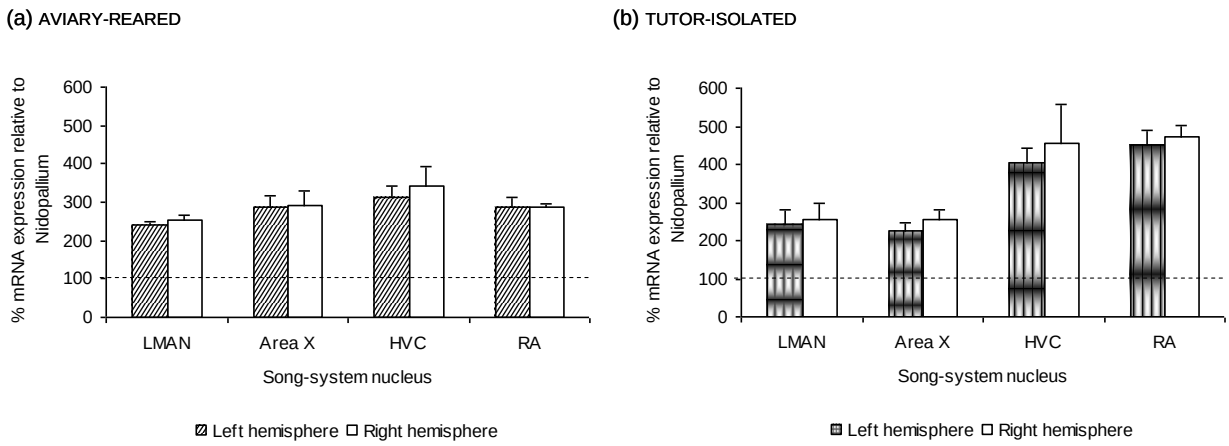


Figure 5.5. Densitometric quantification of $GABA_A$ receptor γ4-subunit mRNA levels in four selected nuclei of the song system relative to the nidopallium. Gene expression was compared between nuclei within the left and right hemispheres in the brains of aviary-reared (a) and cage-reared, tutor-isolated (b) zebra finches. Data is presented as mean ± SE (n=5 per group). The dashed line indicates mRNA levels in the nidopallium; these readings were considered as baseline, at 100%. Abbreviations: Area X (formal name); LMAN, lateral

magnocellular nucleus of the anterior nidopallium; HVC (formal name); RA, robust nucleus of the arcopallium.

5.2.4 Spatial expression of $GABA_A$ receptor γ2-subunit gene in aviary-reared and tutor-isolated male zebra finch brains

It was speculated that the observed decreases in expression within the HVC and RA following normal song learning may not have been exclusive to the $GABA_A$ receptor γ4 subunit so the experiment was repeated, with sections from the same brains as before with a probe selective to the $GABA_A$ receptor γ2-subunit transcript (same probe as in Chapter 3). This subunit was selected as previous work involving imprinting in the one-day-old chicken showed no increase/decrease of the corresponding mRNA following training (Harvey *et al.*, 1998) and it forms the basis of the most prevalent subtype in the mammalian brain (Whiting *et al*., 2003a).

Distribution of mRNA encoding the γ2 subunit was mapped within the zebra finch brain (Fig. 5.6) and autoradiographs illustrated that the spatial distribution of mRNA was identical within brains of aviary-reared and tutor-isolated birds. This concurred with previous data found (for a full description of the γ2-subunit distribution consult Chapter 3). No song-system nucleus demonstrated particularly robust gene expression (i.e. above basal), with the exception of Area X where γ2-subunit mRNA levels were higher than the surrounding striatum (Fig. 5.6C and D). Due to the diffuse distribution of γ2-subunit mRNA throughout the brain, there existed regions where both the γ2- and γ4-subunit transcripts were co-expressed, most notably in Area X. It was only the RA where expression of the γ2-subunit gene was nominal (Fig. 5.6G and H). Visually there appeared to be no differences in γ2-subunit gene expression between tutor-isolated and aviary-reared zebra finches.

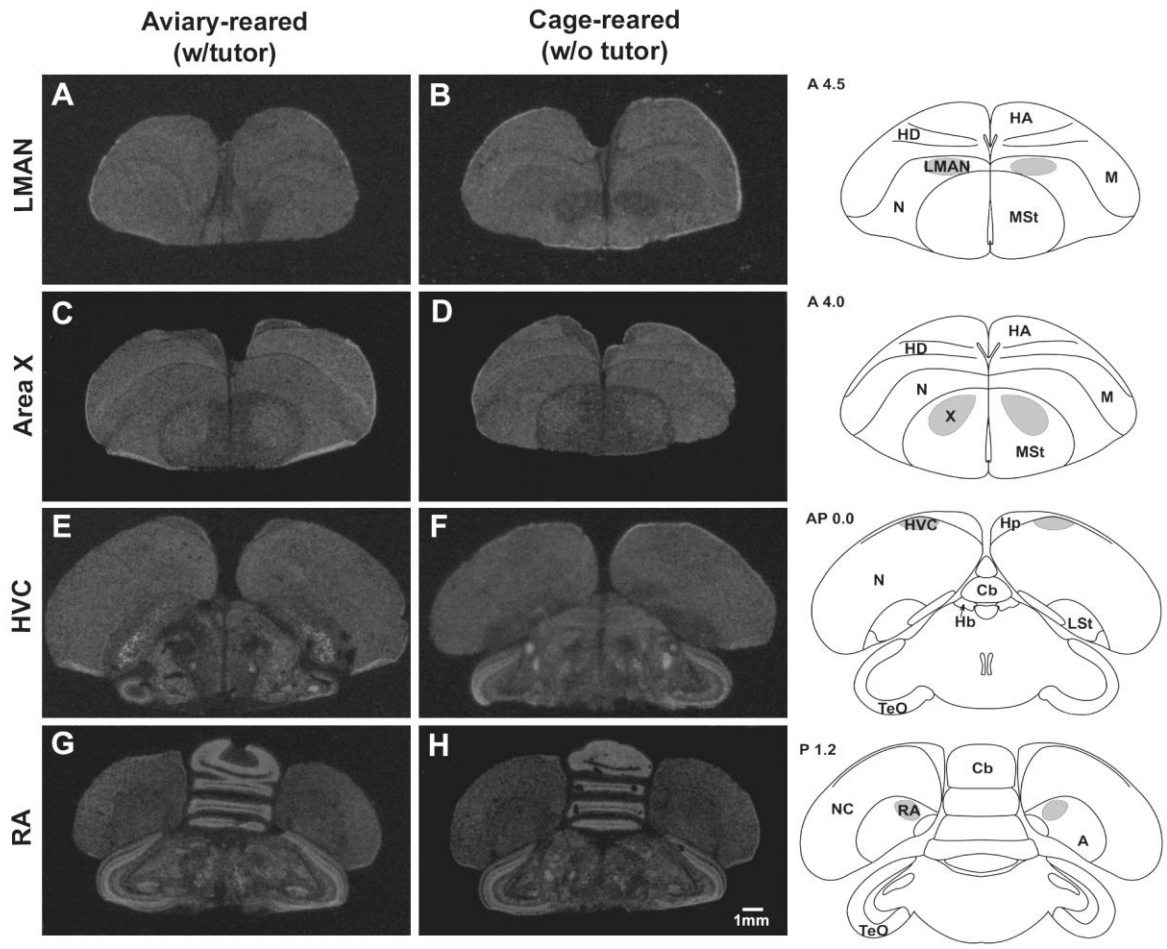


Figure 5.6. Inverse autoradiographs highlighting the distribution of the $GABA_A$ receptor γ2-subunit transcript in four nuclei of the male zebra finch song system, (LMAN, Area X, HVC and RA). Gene expression was compared between birds reared in an aviary with their parents and other conspecifics (column one; A, C, E and G) and birds reared in individual isolation cages without a tutor (column two; B, D, F and H. All birds were sacrificed at PHD 80. **Abbreviations:** A, arcopallium; Cb, cerebellum; Hb, habenula; HA, apical part of the hyperpallium; HD, densocellular part of the hyperpallium; Hp, hippocampus; HVC (formal name); LSt, lateral striatum; M, mesopallium; MSt, medial striatum; N, nidopallium; NC, caudal nidopallium; LMAN, lateral magnocellular nucleus of the anterior nidopallium; RA, robust nucleus of the arcopallium; TeO, optic tectum; X, Area X.

5.2.5 Quantitative expression of $GABA_A$ receptor γ2-subunit gene in tutor isolated and non-tutor isolated male zebra finch brains

5.2.5.1 Internal control (γ2-subunit gene expression in nidopallium)

Hybridisation signals were quantified in the same way as for the γ4-subunit transcript (section 5.2). Initially levels of γ2-subunit mRNA were measured at the four stereotactic levels of the nidopallium (A, B, C, and D, refer to Fig. 5.2) the results are given in Fig. 5.7, below.

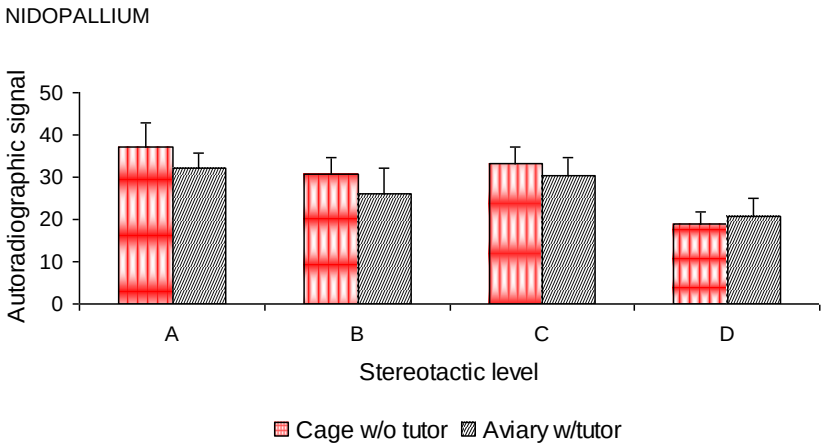


Figure 5.7. Densitometric quantification of $GABA_A$ receptor γ2-subunit mRNA levels in the nidopallium at the stereotactic levels of each song-system nucleus (A, B, C and D in parallel to LMAN, Area X, HVC and RA respectively; refer to Fig. 5.2). Units are arbitrary, and were quantified by subtraction of any background (i.e. non-specific hybridisation signals from the film). Gene expression was compared between birds which were raised in an aviary with a tutor (birds produced normal song) and those raised in individual isolation cages, without a tutor (birds produced abnormal song); all birds were the same age (PHD 80).

Data demonstrated no significant differences in $GABA_A$ receptor γ2-subunit mRNA levels in the nidopallium of aviary-reared and tutor-isolated birds at any stereotactic level (Fig. 5.7). In contrast to the results obtained for the γ4-subunit transcript, there were slightly higher mRNA levels in the nidopallium of tutor-isolated birds compared to aviary-reared birds, with the exception of the RA, but these differences were negligible and in no way significant. Again, the nidopallium was resolved to be a suitable internal control region.

5.2.5.2 $GABA_A$ receptor γ2-subunit gene expression in song-system nuclei

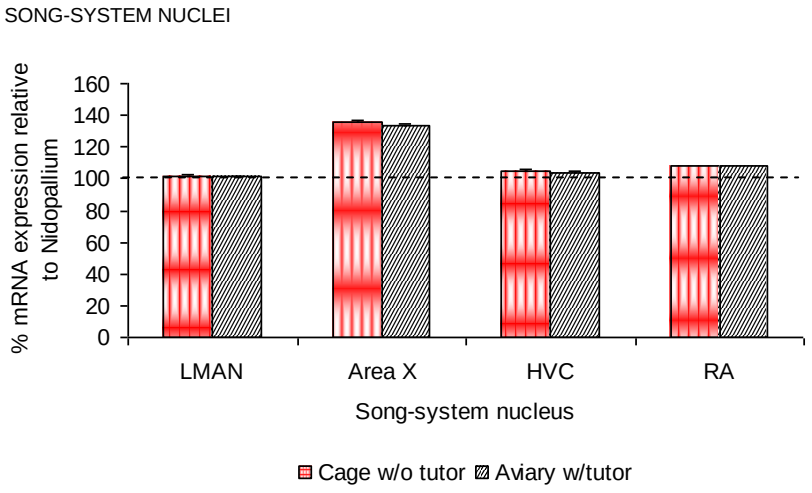


Figure 5.8. Densitometric quantification of $GABA_A$ receptor γ2-subunit mRNA within four nuclei of the zebra finch song system relative to the nidopallium. The dashed line indicates the mRNA level in the nidopallium; these readings were considered as baseline, at 100%. Data is presented as mean ±SE, (n=5 per group). Gene expression was compared between birds which were raised in an aviary with a tutor (birds produced normal song) and those raised in individual isolation cages, without a tutor (birds produced abnormal song); all birds were the same age (PHD 80). **Abbreviations:** Area X (formal name); LMAN, lateral magnocellular nucleus of the anterior nidopallium; HVC (formal name); RA, robust nucleus of the arcopallium.

Data clearly showed that there were no discernable differences between the expression level of the $GABA_A$ receptor γ2-subunit gene in any of the nuclei between aviary-reared and tutor-isolated birds (Fig. 5.8). Highest levels of mRNA expression were detected in Area X; this was also visually apparent in the autoradiographs (Fig. 5.6C and D), but generally the distribution was relatively low and diffuse throughout the song system (and the remainder of the brain; data not shown), of both groups of birds. mRNA levels within LMAN, HVC and RA were similar to that of the nidopallium. Results therefore confirmed that the differences observed in γ4-subunit gene expression were likely to be exclusive to that subunit. As before, γ2-subunit mRNA distribution was analysed within the nuclei of the left and right hemispheres (Fig. 5.9a and b).

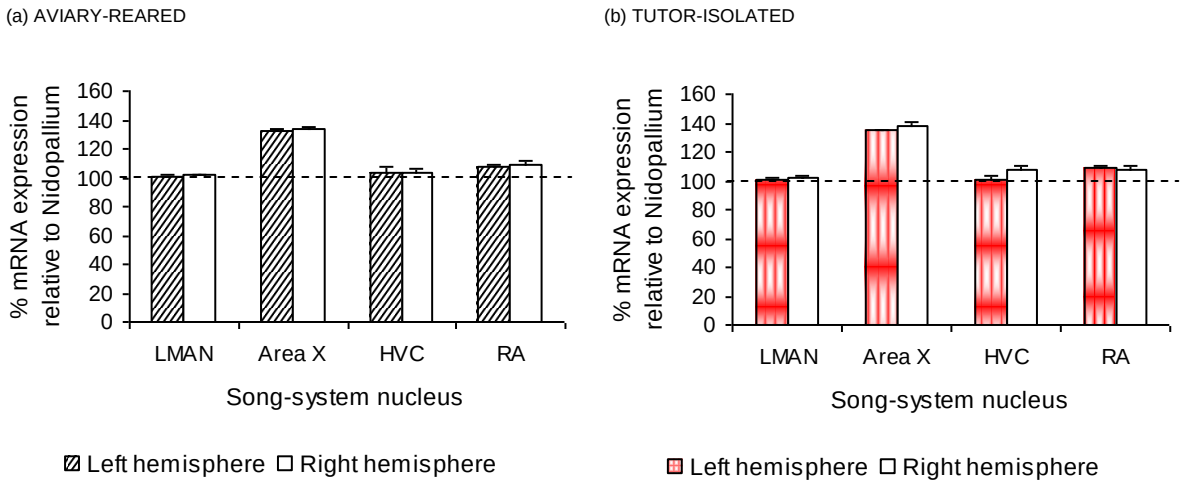


Figure 5.9. Densitometric analysis of $GABA_A$ receptor γ2-subunit gene expression in the left and right hemispheres of four selected nuclei of the zebra finch song system within the brains of aviary-reared (a) and cage-reared, tutor-isolated (b) zebra finches. Signal was quantified by subtracting background signals and expressed as an increase compared to the internal control area. Baseline is defined as the signal intensity in the nidopallium (internal control) minus any background signal (100%). Data is expressed as mean ±SE, n=5 per group. **Abbreviations:** Area X (formal name); LMAN, lateral magnocellular nucleus of the anterior nidopallium; HVC (formal name); RA, robust nucleus of the arcopallium.

Similar to γ4-subunit mRNA, there were no significant differences in levels of γ2-subunit encoding transcript between nuclei located within the two sides of the avian brain. γ2-subunit mRNA was generally, marginally higher within the nuclei of the right hemisphere with the exception of the RA in the tutor-isolated birds (Fig. 5.9b).

5.2.6 Effects of tutor-isolation on song production

Following the results of the preliminary experiments, the hypothesis that γ4-subunit containing $GABA_A$ receptors play a role in song acquisition and production in zebra finch needed to be further validated. It was so far ascertained that the γ4-subunit mRNA was reduced in the song system of birds (with the exception of Area X) which learned normal song and that this was specific to the γ4 subunit, as no such changes in distribution were visible for γ2-subunit mRNA. It was considered that the effects on gene expression were not entirely due song learning but may have been an artefact of the experimental

conditions. To combat this, three further sets of male zebra finches were raised; the first group were raised with their parents in an aviary with other conspecifics (as in the preliminary experiments), the second group were raised with their parents in an individual cage (this ensured the difference in surroundings was not affecting the results) and the third group were raised in individual cages by their parents until PHD 5, then by their mother until PHD 30 and then by >2 unrelated females for the remainder of the experiment. Presence of females (who do not sing) ensured that any effects of gene expression could not be attributed to social isolation, only tutor-song isolation. Furthermore, the songs of each bird were recorded at PHD 80 in the presence of an unfamiliar female and analysed with a sonograph. All these precautions were in place to ensure that the effects observed in song learning and gene expression by tutor-isolation could be exclusively linked.

Song produced by the two groups of zebra finches raised in individual cages either with or without a tutor was recorded, in the presence of an unfamiliar female at PHD 80. Data revealed that syllable length was much longer in birds that were reared in isolation. There were no differences in the pitch, quality of the pitch, the frequency or entropy between the two groups of birds. Data confirmed that an adult male (tutor) is necessary during the period of song learning for the production of normal song in zebra finches (data not shown). The next step was to determine any differences in the expression of the γ4-subunit transcript in response to birds which learned song and those which did not.

5.2.7 Spatial expression of the $GABA_A$ receptor γ4-subunit gene in aviary-reared, cage-reared and tutor-isolated zebra finches

Autoradiographs demonstrated that $GABA_A$ receptor γ4-subunit transcript was detected in all four nuclei examined (LMAN, Area X, HVC and RA), in all three sets of brains (Fig. 5.10). Unlike in the preliminary experiments, it was apparent from visually comparing the images that there was an increase of transcript in LMAN and Area X in the brains of tutor-isolated birds (Fig. 5.10, column three, C and F). If differences existed between HVC and RA these were not immediately apparent by eye. Spatial mRNA distribution patterns were identical in the brains of all three groups of birds and were comparable to previously obtained data.

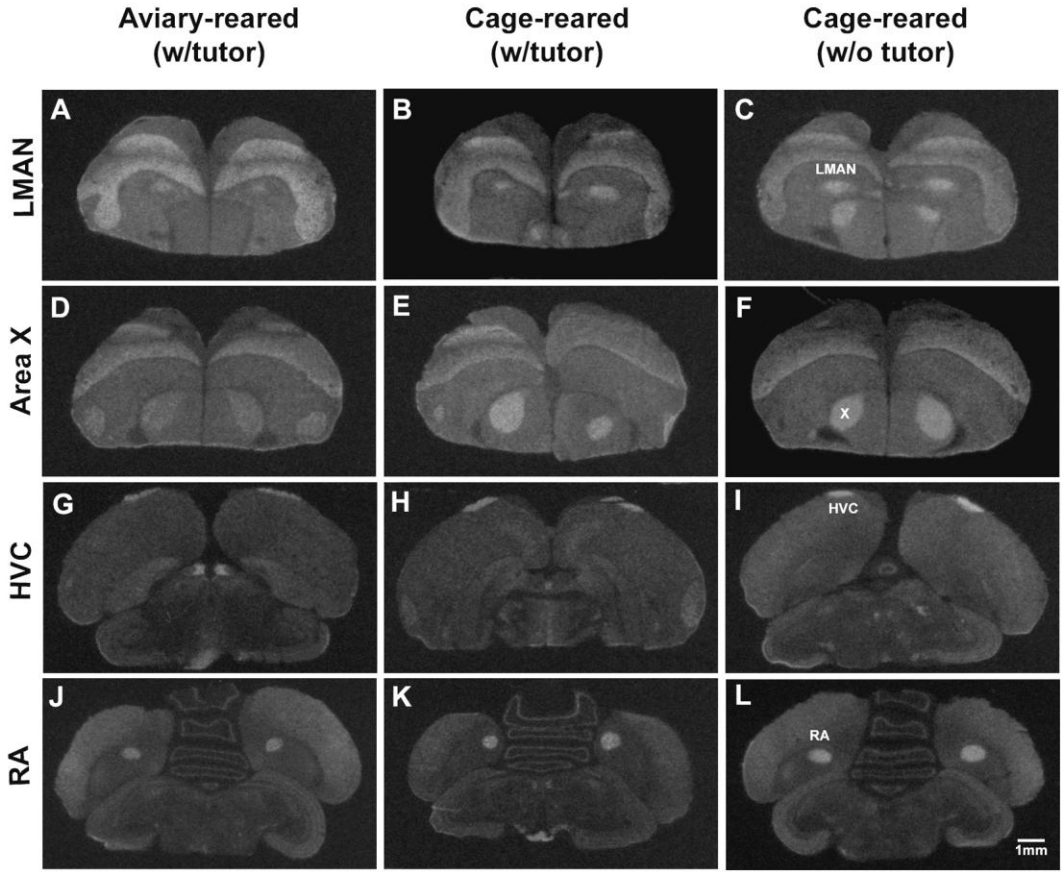


Figure 5.10. Inverse autoradiographs highlighting the distribution of the $GABA_A$ receptor γ4-subunit transcript in four nuclei of the male zebra finch song system (LMAN, Area X, HVC and RA). Gene expression was compared between birds reared in an aviary with their parents and other conspecifics (column one; A, D, G and J); reared in individual cages with their parents (column two; B, E, H and K); or reared in individual isolation cages in the presence of the mother until PHD 30 and then > 2 unrelated females (column three; C, F, I and L). All birds were sacrificed at PHD 80. **Abbreviations:** LMAN, lateral magnocellular nucleus of the anterior nidopallium; X, Area X; HVC, formal name; RA, robust nucleus of the arcopallium.

5.2.8 Quantitative expression of the GABA$_A$ receptor γ4-subunit gene in aviary-reared, cage-reared and tutor-isolated zebra finches

5.2.8.1 Internal control (γ4-subunit gene expression in nidopallium)

Distribution of GABA$_A$ receptor γ4-subunit transcript within the zebra finch song system was quantified to verify any differences between groups. Firstly the levels of transcript were determined within the internal control regions (nidopallium); the results are shown in Figure 5.11.

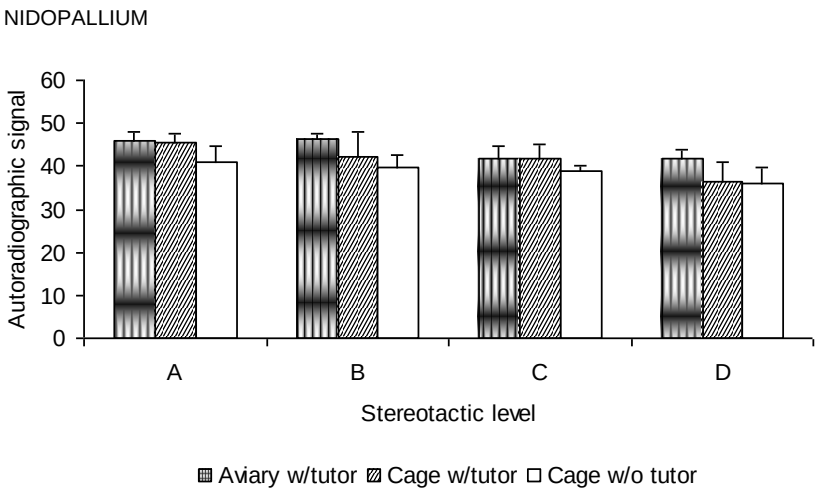


Figure 5.11. Densitometric quantification of GABA$_A$ receptor γ4-subunit mRNA levels in the nidopallium at the stereotactic levels of each song-system nucleus (A, B, C and D in parallel to LMAN, Area X, HVC and RA respectively; refer to Fig. 5.2). Units are arbitrary, and were quantified by subtraction of any background (i.e. non-specific) hybridisation signal. Transcript expression was compared between birds which were raised in an aviary with a tutor and other conspecifics (produced normal song), those raised in individual isolation cages with their parents (produced normal song) and birds raised in individual isolation cages with only females (produced abnormal song). All birds were the same age (PHD 80).

Analysis of the nidopallium again demonstrated that this area was not significantly affected by the parameters of the experiment (Fig. 5.11). There was a nominal decrease in the level of GABA$_A$ receptor γ4-subunit mRNA in cage-reared, tutor-isolated birds but was not significant. Therefore cage-rearing (as opposed to in an aviary), had little effect on the gene expression in the internal control region.

5.2.8.2 $GABA_A$ receptor γ4-subunit mRNA expression in song-system nuclei

In accordance with results obtained from the preliminary experiments, there were higher levels of mRNA encoding the $GABA_A$ receptor γ4 subunit in tutor-isolated zebra finches in all four examined nuclei of the song system, compared to aviary-reared birds (Fig. 5.12; $p \leq 0.001$), potentially demonstrating a decrease of γ4-subunit mRNA in response to acquisition and production of structured song. Strength of response was far greater relative to preliminary data (Fig. 5.4). Decreases in γ4-subunit encoding mRNA following song learning were highly significant for all four nuclei; LMAN (40%), Area X (30%), HVC (70%) and RA (100%), whereas in preliminary experiments increases were only observed in RA and HVC (Fig. 5.4). Although it was within these nuclei where the most pronounced effects were measured (Fig. 5.12).

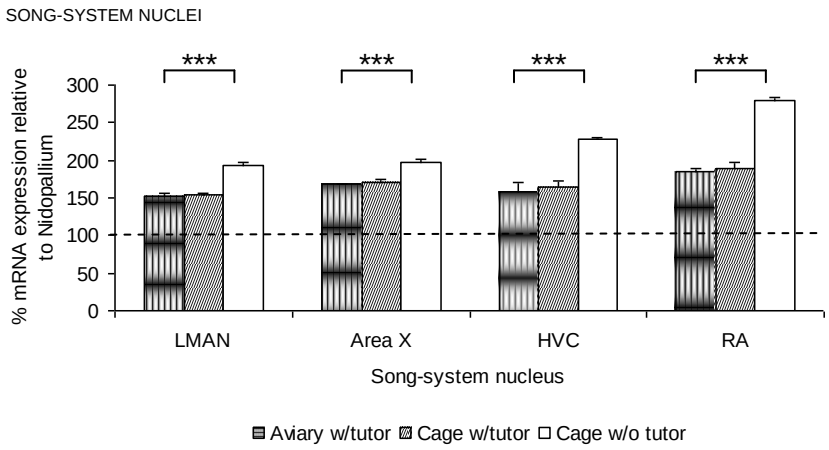


Figure 5.12. Densitometric quantification of $GABA_A$ receptor γ4-subunit mRNA within four nuclei of the zebra finch song system relative to the nidopallium. The dashed line indicates gene expression in the nidopallium; these readings were considered as baseline, at 100%. Data is presented as mean ±SE, (n=5 per group; *** $p \leq 0.001$). Expression was compared between birds which were raised in an aviary with a tutor and other conspecifics (produced normal song), those raised in individual isolation cages with their parents (produced normal song) and birds raised in individual isolation cages with only females (produced abnormal song). All birds were the same age (PHD 80). **Abbreviations:** Area X (formal name); LMAN, lateral magnocellular nucleus of the anterior nidopallium; HVC (formal name); RA, robust nucleus of the arcopallium.

There were no significant differences in gene expression in brains of birds which were reared normally in an aviary (produced normal song) and those reared in isolation cages

with their parents (produced normal song; Fig. 5.12), thereby demonstrating that rearing in a cage environment or absence of conspecifics had no effect on the outcome of the experiment. Presence of female birds (which do not sing) in cages of tutor-isolated birds ensured that none of the effects could be attributed to the stress of social isolation, thus the decrease of γ4-subunit expression in all four observed nuclei was more likely linked to the acquisition and production of song. In these results, gene expression in nuclei within both the AFP and VMP pathway were significantly affected (although HVC and RA of the VMP pathway were the regions exhibiting the greatest decreases in gene expression; Fig. 5.12), thereby demonstrating significant decreases in $GABA_A$ receptor γ4-subunit mRNA in response to both song-acquisition and production in zebra finch.

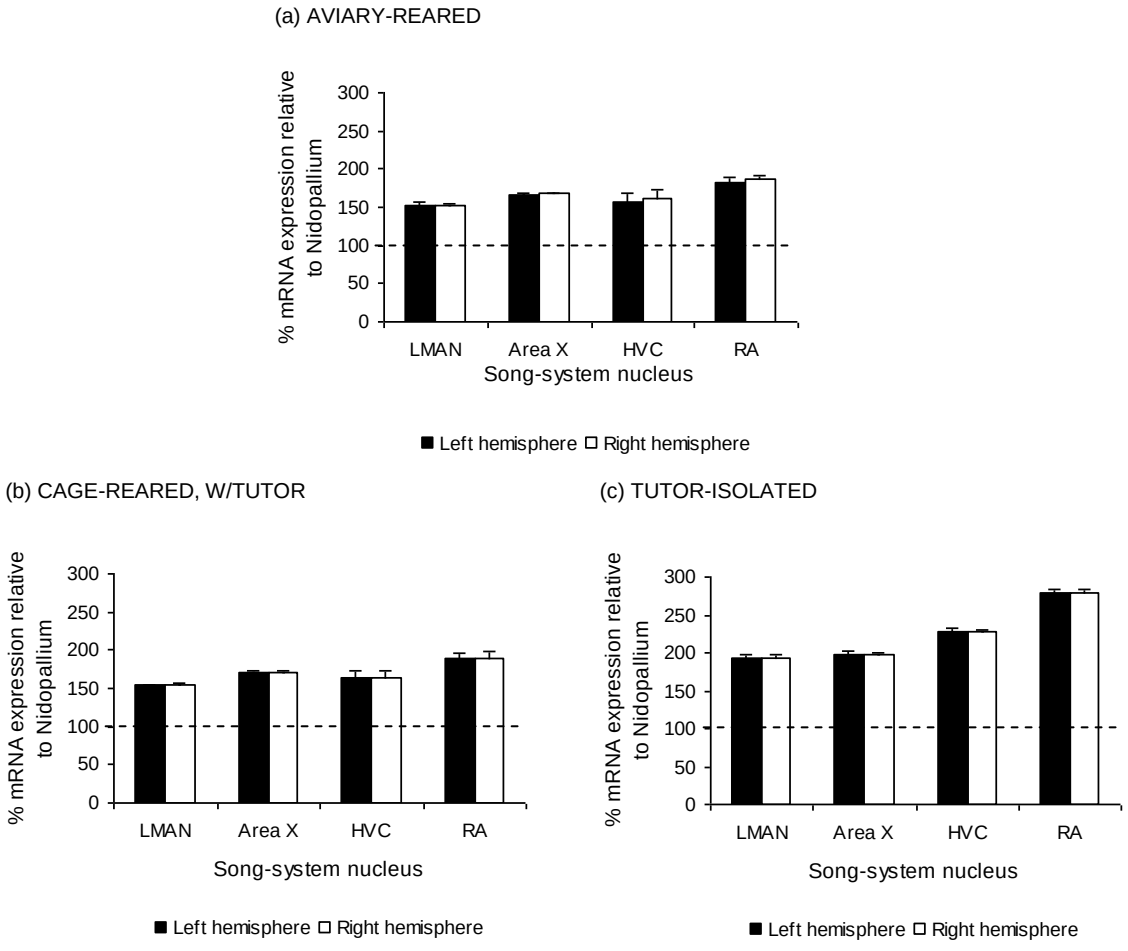


Figure 5.13. Densitometric analysis of $GABA_A$ receptor γ4-subunit gene expression in the left and right hemispheres of four selected nuclei of the zebra finch song system within the brains of aviary-reared (a) cage-reared with parents (b) and cage-reared, tutor-isolated (c) zebra finches. The signal was quantified by

subtracting background signal and expressed as an increase compared to the internal control area. Baseline is defined as the signal intensity in the nidopallium (internal control) minus any background signal (100%). Data is expressed as mean ±SE, n=5 per group. **Abbreviations:** Area X (formal name); LMAN, lateral magnocellular nucleus of the anterior nidopallium; HVC (formal name); RA, robust nucleus of the arcopallium.

As seen previously (Fig. 5.5) there were no significant differences in γ4-subunit transcript expression between nuclei occupying the left or right hemispheres of the brain (Fig. 5.13) showing no evidence of lateralisation of gene expression.

5.3 Discussion

It has been recently documented that the $GABA_A$ receptor γ4-subunit is well transcribed in virtually all nuclei of the zebra finch song system (Thode *et al*., 2008). Gene expression was so robust and specific that is was proposed as a potential molecular marker for the song system. Following these observations, the work outlined in this chapter quantitatively mapped expression of the γ4-subunit gene and determined the effect of song learning on transcript levels. The data revealed several key findings. Firstly, birds isolated from a conspecific tutor during song development (up to PHD 80) produced abnormal song. Birds raised both in an aviary or separate cages, with a tutor, produced normal vocalisations. Secondly, *in situ* hybridisation revealed that adult male zebra finches which learned song and those that did not, both shared identical spatial gene expression profiles where the corresponding transcript was detected within all the major nuclei of the song system involved in the acquisition and production of song. Thirdly, although spatially similar, levels of $GABA_A$ receptor γ4-subunit mRNA were significantly different between normally-, (aviary)-reared and tutor-isolated zebra finches. Acquisition and production of normal song was associated with a down-regulation of $GABA_A$ receptor γ4-subunit gene expression in four relevant nuclei of the song system. LMAN and Area X which form part of the AFP and HVC and RA which form part of the VMP. Finally, the decrease in expression in response to normal song learning was specific to the γ4-subunit gene as no learning-associated decreases were observed for the γ2 subunit transcript.

There is a widespread distribution of GABAergic interneurons within the zebra finch song system (Pinaud and Mello, 2007). $GABA_A$ receptors have been detected electrophysiologically (for example, Bottjer *et al*., 1998; Dutar *et al*., 1998; Spiro *et al*., 1999; Farries *et al*., 2005; Mooney and Prather, 2005; Prather *et al*., 2008) and pharmacologically (for example, Carlisle *et al*., 1998; Vicario and Raksin, 2000; Farries *et al*., 2005; Mooney and Prather, 2005; Ölveczky *et al*., 2005), but little information concerning the subtypes present or their physiological roles is available. Only the $GABA_A$ receptor γ4-subunit has been unequivocally identified (Thode *et al*., 2008). The $GABA_A$ receptor family is highly diverse with a large number of promiscuous subunits associating to form a currently unknown number of receptor subtypes. Each receptor subtype exhibits slightly different biophysical, electrophysiological and pharmacological properties (conferred by the particular combination of subunits; Olsen and Sieghart, 2008; 2009). Furthermore, in mammalian brain, there is regional-specific expression of all the subunit genes indicative of different $GABA_A$ receptor subtypes fulfilling specific functions (Laurie *et al*, 1992; Wisden *et al*, 1992; Pirker *et al*., 2000; Pöltl *et al*., 2003; Wafford *et al*., 2004). This diversity of expression has now been observed in zebra finch brain (refer to Chapter 4) and so the stoichiometry and functionality of different $GABA_A$ receptor subtypes can begin to be uncovered in this well established learning and memory paradigm.

5.3.1 Effects of tutor-isolation on song

Songs of all birds were recorded at PHD 80 in the presence of an unfamiliar female (directed song). Data from the resulting sonographs illustrated that zebra finches raised in tutor isolation produced abnormal song, which concurs with much previous data (Thorpe, 1958; Immelmann, 1969; Marler and Sherman, 1983; Eales, 1987; Searcy and Marler, 1987; Tchernichovski and Nottebohm, 1998; Kojima and Doupe, 2007; Fehér *et al*., 2009). Most notably, the syllable duration was considerably longer in songs of tutor-isolated birds relative to their aviary-reared counterparts. There were no discernable differences in the pitch, quality of the pitch, the frequency or entropy between the two groups of birds. An increase in syllable length is a feature of the song of birds raised in the absence of a tutor (Kojima and Doupe, 2007), potentially associated with a disruption of neuronal processing in the HVC (Long and Fee, 2008). As this nucleus projects to both pathways of the song

system and is the entry point of auditory information into the song system, it is of critical importance to both the acquisition and production of learned song. Slowing of neuronal processing in HVC results in songs with longer syllable durations and longer periods between syllables (but the song remains stable; Long and Fee, 2008). Generally untutored song is less complicated with reduced syllable variety and longer pauses between syllables (Price, 1979; Laulay *et al*., 2004). However, interestingly, isolate song also incorporates some species-specific features (Marler and Sherman, 1985; Zann, 1996; Konishi, 2004; Kojima and Doupe, 2007), perhaps attributable to the proposed intrinsic auditory template, present form birth which pre-disposes birds to sing a song selective to their species (Marler, 1970; Konishi, 2004).

5.3.2 Effects of environment on song production

Aviary- and cage-reared (both with tutor) zebra finches produced normal song. With regards to gene expression, levels of γ4-subunit mRNA were slightly higher in all four nuclei of cage-reared birds relative to aviary-reared, although the differences were not significant (Fig. 5.12). Birds raised in a richer auditory environment with much more complex stimuli (i.e. in an aviary with many other conspecifics and tutors) produce much more complex songs (Adar *et al*., 2008). Therefore, learning of structured song was associated with a decrease in $GABA_A$ receptor γ4-subunit gene expression and correspondingly, exposure to a richer auditory stimuli during song learning (and perhaps learning of a more complex song – although this was not tested) was associated with a further decrease in γ4-subunit mRNA within relevant regions of the song system. Pertaining to this, only songs of zebra finches raised in cages (with parents) were compared with those of tutor-isolated birds.

Zebra finches are social birds living in large groups (up to 1000 members in the wild). To ensure the stress associated with social-isolation was not a factor in the experiments, tutor-isolated animals were raised in the presence of conspecific females. Female zebra finches do not sing (Nottebohm and Arnold, 1977); both males and females produce vocalisations after hatching, but these are begging calls (Zann, 1996) and are not learned (Price, 1979). Females cannot produce structured song; they can make whistling noises and calls and thus

cannot create a sufficient auditory template for juveniles to learn (Nottebohm and Arnold, 1977; Price 1979; Zann, 1996; Reibel, 2003). However, tutor-isolated zebra finches can incorporate some female call notes when permanently isolated from male song (Eales, 1987). In this context, it could be argued that the tutor-isolated birds reared with females may have acquired some rudimentary form of an auditory template (although nowhere near as reinforced as in those raised with tutors), resulting in activation of the AFP. Although the role of female interaction is currently unknown, it may be that by some mechanism the female is able to indicate positive reinforcement during the song-learning process (King and West, 1983; Adret, 2004). Certainly in normally-reared birds, those exposed to a tutor and females learn better than those reared with a tutor alone (West and King, 1988; Adret, 2004).

Another interesting feature of tutor-isolated birds is that even after the period of song learning (post-crystallisation), they prefer (Braaten and Reynolds, 1999), and are able to learn (to an extent), species-specific song (Eales, 1987; Slater *et al*., 1988; Livingston *et al*., 2000). Inferring that the song-learning phase in close-ended learners such as zebra finches may remain open after sexual maturity until sufficient, suitable stimuli has been provided. Deafening, muting, perturbation of auditory feedback or isolation from conspecific song, post-crystallisation can result in deterioration of adult song (Nordeen and Nordeen, 1992; Leonardo and Konishi, 1999; Williams *et al*., 2003; Funabiki and Funabiki, 2007; Sober and Brainard, 2009), which in some cases can be corrected following re-exposure to tutor song (Funabiki and Funabiki, 2007). In birds isolated during song learning, such as in this study, a normal sensory template could not have been established (as reflected by the production of abnormal song). This was accompanied by high levels of γ4-subunit mRNA within the song system, which would normally be reduced following the acquisition of song.

5.3.3 Spatial expression of γ4- and γ2-subunit genes in aviary-reared and tutor-isolated birds

Spatial mRNA distribution profiles for both the $GABA_A$ receptor γ2- and γ4-subunits were identical to those identified in Chapter Four (Figs. 5.6, 5.10 and 4.5) and in agreement with

previous findings (Thode *et al*., 2008). Thus, despite the birds being raised in different aviaries, of various ages and collected at different seasons of the year (for the individual experiments), gene expression patterns were consistent, further confirming that the distribution in the song system of both subunit transcripts shown herein was accurate. Spatially, the expression patterns of both the γ2- and γ4-subunit genes were identical in the song system of aviary-reared and tutor-isolated zebra finches. The $GABA_A$ receptor γ2-subunit gene exhibited low, but diffuse expression throughout the song system (and the remainder of the brain). This was anticipated as in mammals, this subunit associates with α1- and β2-subunit polypeptides to form the most abundant (~50%), $GABA_A$ receptor subtype in the mammalian brain (Whiting *et al*., 2003). The relative abundance of the α1β2γ2 subtype is indicative of a generalised function, whereas the restricted yet robust expression of the γ4-subunit gene points to a more specialised role, perhaps in learning and memory processes. Widespread, diffuse expression of the γ2-subunit gene meant that in many regions of the avian brain (including the song-system nuclei), there was overlapping expression with the γ4 subunit; this was most notable in Area X. Despite a small amount of evidence suggesting that two different γ-subunit polypeptides may assemble into a functioning receptor (Khan *et al*., 1994; Quirk *et al*., 1994; Benke *et al*., 1996), the general consensus is that native $GABA_A$ receptors contain a single γ subunit (Olsen and Sieghart, 2008, 2009). However, it is most likely that specific brain regions contain more than one subtype of $GABA_A$ receptor (Laurie *et al*, 1992a, b; Wisden *et al*, 1992; Pirker *et al*., 2000; Pöltl *et al*., 2003; Wafford *et al*., 2004). In mammalian systems, γ2-subunit-containing receptors may be synaptic or extrasynaptic, whereas those lacking a γ2-subunit are extrasynaptic (Olsen and Sieghart, 2008). If avian systems mirror that of mammals, then it can be postulated that γ4-subtype receptors are extrasynaptic and thus would occur on the same neurons as the synaptic γ2-subunit-containing receptors. Furthermore, expression of γ2- and γ4-subunit mRNAs seem unlikely to be linked as γ4-subunit mRNA was down-regulated in response to learning and γ2-subunit mRNA expression was unaffected.

In addition to the four nuclei under scrutiny, expression of the γ4-subunit gene was robust in many other regions of the brain, including the cerebellum, an area responsible for control

of complex behaviour which has recently been implicated in cognitive functions (Spence *et al*., 2009). The hippocampus also displayed prominent levels of γ4-subunit mRNA; this region is involved in processing of spatial memory (Patel *et al*., 1997; Wanatabe and Bischof, 2004). Robust γ4-subunit gene expression in these two areas may indicate a role in other important memory systems for $GABA_A$ receptors containing this subunit.

5.3.4 Down-regulation of $GABA_A$ receptor γ4-subunit transcript in response to song learning

Although the role of $GABA_A$ receptors in learning and memory has been well documented (reviewed by Chapouthier and Venault, 2002; Maubach, 2003; McNally *et al*., 2008), the precise mechanisms of their action are not clarified. The majority of data pharmacologically confirms the involvement of $GABA_A$ receptors in these processes (reviewed by Brioni, 1993). $GABA_A$ antagonists such as bicuculline (Brioni and McGaugh, 1988; Clements and Bourne, 1996) and inverse agonists such as FG7142 and L-655 708 improve memory formation (Chambers *et al*., 2004; McNally *et al*., 2008) and accordingly, agonists such as muscimol promote amnesia and impair performance in memory tasks (Clements and Bourne, 1996; Ramirez *et al*., 2005; Zarrindast *et al*., 2006; Yamamoto *et al*., 2007). Due to the plethora of $GABA_A$ receptor subtypes, their regional-specific distribution and their individual pharmacological profiles (indicative of specialised physiological functions), it is paramount to identify the role of individual subtypes. Data showed a regional-specific decrease in $GABA_A$ receptor γ4-subunit mRNA within the zebra finch song system following acquisition and production of normal song (Figs. 5.4 and 5.12). This was in agreement with previous work which reported a down-regulation of γ4-subunit mRNA in the entopallium and intermediate medial mesopallium of one-day-old chicks (*Gallus gallus domesticus*), 10 h following imprinting training (another established learning and memory paradigm – Horn, 1998; Harvey *et al*., 1998). γ2-subunit mRNA levels in the song system of tutor-isolated birds did not change relative to aviary-reared birds (Fig. 5.8). This concurred with previous data which demonstrated that γ2-subunit gene expression remained unchanged in the brain by imprinting in the one-day-old chick (Harvey *et al*., 1998). In contrast, following water-maze training, γ2-subunit mRNA was

one of four $GABA_A$ receptor subunits down-regulated in the hippocampus of adult rats (along with α4- α5- and β2-subunits) – interestingly, π-subunit mRNA was up-regulated in response to this training (Cavallaro *et al*., 2002). Such conflicts in data may arise as this was in mammalian systems (which do not have the γ4 subunit; Simon *et al*., 2004), within a completely different region of the brain and a different learning paradigm. Although the decreases in $GABA_A$ receptor subunit expression is response to learning were not quantified, the results show an overall down-regulation of $GABA_A$ receptor subunit expression (Cavallaro *et al*., 2002). The work also served to highlight the differential expression of $GABA_A$ receptor subtypes in the brain and the presence of subtype-specific mediated responses to learning and memory processes, as seen herein. However, despite localisation of different $GABA_A$ receptor subtypes to specific regions of the brain and the different physiological properties of these receptors, there is a paucity of data relating individual subtypes to specific learning and memory processes. An exception to this is the α5-subtype of the $GABA_A$ receptors. Receptors comprising the α5 subunit are primarily located in the hippocampus where they constitute ~20% of the $GABA_A$ receptor population in this region (Sur *et al*., 1998, 1999). This regional-specific localisation of a specific receptor subunit gene in an area of the brain associated with spatial learning (Wanatabe and Bischof, 2004) was indicative for a role of α5-subunit receptors in these processes (much like the discrete distribution of γ4-subunit mRNA within the nuclei of the song system). Homozygous α5-subunit knockout mice demonstrated improved performance in hippocampal-dependent spatial learning tasks (Collinson *et al*., 2002) and point mutations within the α5-subunit gene facilitated trace fear conditioning in mice (Crestani *et al*., 2002). This lead to identification of a partial inverse agonist (L-655 708), an imidazobenzodiazepine with a high affinity for α5-subunit-containing receptors, which enhances performance of rats in the Morris water maze (Chambers *et al*., 2004). Again this data confirms a down-regulation of $GABA_A$ receptor-mediated inhibition is associated with learning and memory processes and exemplifies the pharmacological benefits of identifying specific receptors and their function in the brain.

Expression of the γ4-subunit gene in nuclei of the zebra finch song system has been thoroughly documented (Thode *et al*., 2008), but it remained unknown as to whether

$GABA_A$ receptors containing this subunit played a functional role in song learning. This work has indicated a role for γ4-subunit-containing receptors in both the acquisition and production of song. It may be theorised that as the birds were in isolation from a tutor, they did not acquire an auditory template and so gene expression within nuclei controlling auditory template acquisition would be most affected (LMAN and Area X), with these structures not being as active as those in the brains of birds stimulated by a tutor. Moreover, it may be speculated changes in gene expression in nuclei controlling the motor production of song (HVC and RA) may be less prone to tutor-isolation as both isolated and deafened birds proceed to make vocalisations, even if they are abnormal (Immelmann, 1969; Eales, 1987; Searcy and Marler, 1987; Tchernichovski and Nottebohm, 1998; Konishi, 2004; Kojima and Doupe, 2007; Fehér *et al*., 2009). Yet it was the HVC and RA (which lie within the VMP) which exhibited the greatest decreases in γ4-subunit mRNA following normal song learning. This can be explained by the inextricable links between the two pathways. The origin of both pathways is the HVC. Separate classes of neurons project to RA and Area X; the connections to RA are only established at ~PHD 35 in parallel to onset of the sensorimotor phase (Akutagawa and Konishi, 1985; Mooney and Rao, 1994; Scott and Lois, 2007). The HVC→RA pathway (VMP) is involved in the motor production of song (Nottebohm *et al*., 1976; Simpson and Vicario, 1990), where the RA is the output of the song system projecting to hyperglossal motor neurons which innervate the syrinx (nXIIts) and Ram (nucleus retroambigualis) which is the site of vocal and respiratory muscle coordination (Nottebohm *et al*., 1976). The AFP is primarily involved in the acquisition of song (Bottjer *et al*., 1984; Scharff and Nottebohm, 1991), however, more recently, it has been shown to be involved in adult song plasticity, although the exact mechanisms remain unknown (Nordeen and Nordeen 1992; Brainard and Doupe, 2000; Funabiki and Konishi, 2003; Williams *et al*., 2003; Sober and Brainard, 2009). Projections from HVC link it to Area X, which subsequently connects to LMAN via the DLM (refer to Fig. 1.2, Chapter 1). Premotor neurons of RA receive input from LMAN (as well as those from the HVC). Thus, the involvement of HVC and RA in both pathways may account for the exacerbated effects (on γ4-subunit expression) described in these particular nuclei. Furthermore, the lessened effects on γ4-subunit expression within LMAN and Area X (AFP) of tutor-isolated zebra finches may be attributable to the formation of some rudimentary auditory template by

incorporation of some female call notes (Eales, 1987) and the presumed presence of a genetically-encoded intrinsic template (Marler, 1970; Konishi, 2004), resulting in production of abnormal song which still retained some species-specific features.

It is estimated that > 100 genes are regulated (up or down) by singing in the zebra finch brain (Wada *et al*., 2006), giving some insight into the complex underlying molecular mechanisms (Jarvis *et al*., 2002). Immediate-early genes such as *ZENK*, *c-fos* and *c-jun* have all been identified in the song system and their expression is rapidly increased in relevant regions when birds hear species-specific song (Mello *et al*., 1992; Mello and Clayton, 1994, Nastiuk *et al*., 1994; Bolhuis *et al*., 2001). Furthermore, (as detailed in section 5.1) many genes have been be implicated in song-driven gene expression (Jarvis and Nottebohm, 1997; Kimpo and Doupe, 1997; Li *et al*., 2000; Lombardino *et al*., 2005; Tarcisco *et al*., 2005; Poopatanapong *et al*., 2006; Wada *et al*., 2006). It is now proposed that due to genes being differentially expressed within song nuclei in variable combinations and at different basal levels, generalised neural activity cannot be the only mechanism implemented in regulation of gene expression within the song system (Wada *et al*., 2004). In the avian brain, imprinting studies in one-day-old chick have demonstrated regional-specific increases of mRNA encoding *Arc/Arg3.1* (Bock *et al*., 2005) and *ZENK* (Thode *et al*., 2005) in response to learning; in similar experiments to where γ4-subunit mRNA was found to be down-regulated in response to imprinting training (Harvey *et al*., 1998). However, in contrast to data presented here, ZENK mRNA is transcribed in all the telencephalic nuclei of the song system in response to song production, in deafened or isolated zebra finches at the same levels as in normally reared birds (Johnson and Whitney, 2005) confirming its status as a motor-driven gene, unlike γ4-subunit mRNA (Jarvis and Nottebohm, 1997). At a protein level, ZENK mRNA was not faithfully translated in RA. In birds which had been deafened or isolated (despite high levels of mRNA), there was a significant decline in ZENK protein production when compared to normally reared birds. These findings suggested a less common, post-transcriptional regulation of the ZENK gene during motor behaviour in RA (Johnson and Whitney, 2005). Whereas differential expression of the γ4-subunit mRNA in differently reared birds suggests transcriptional level gene regulation. Further levels of complexity are introduced when considering the type of

song, direct song (in the presence of females) results in an induction of *FOS* but an absence of *ZENK* in the AFP and in indirect song *ZENK* is induced (Kimbo and Doupe, 1997; Jarvis *et al*., 1998). Findings such as these can link behaviour with regulation of gene expression. Moreover, in the characterisation of genes that were differentially expressed in HVC and the surrounding shelf region, in order to determine the specialisation of molecular and biochemical pathways in the functioning of the HVC, the ε subunit of the $GABA_A$ receptor was tentatively identified as one of the differential markers (Lovell *et al*., 2008). As lower vertebrates do not posses this subunit and instead have the orthologue γ4 it is likely to have been this that was detected (sequence details were not given). It was postulated that the expression was therefore potentially similar to *ZENK* in that during song production there is high mRNA expression in the HVC (Jarvis and Nottebohm, 1997) and when listening there is significantly higher gene expression in the shelf region relative to HVC (Mello and Clayton, 1994). This may be interpreted as different $GABA_A$ receptor subtypes or perhaps the same subtype (but both including the γ4-subunit) playing region-specific physiological roles within nuclei of the song system. mRNA levels in HVC shelf region were not quantified in this study.

5.3.5 Temporal expression of $GABA_A$ receptor γ4-subunit gene

Another avenue of evidence suggesting a critical physiological role for $GABA_A$ receptors containing the γ4-subunit polypeptide is that the corresponding mRNA has been shown to exhibit a developmentally-regulated pattern of expression. It is well expressed in LMAN and Area X (AFP) in juvenile zebra finches but is absent from RA in birds of PHD 22. However, at ~PHD 35 the γ4-subunit mRNA is highly expressed in the RA and these high levels are continued into adulthood (Thode *et al*., 2008). The rapid increase in mRNA levels in the RA was simultaneous to establishment of the VMP pathway by innervation of the RA by projections from the HVC (Nordeen and Nordeen, 1988; Wang *et al*., 1999). A similar response was observed for the ZENK protein, whereby it was only detected in the RA at the stage of plastic or crystallised song. Interestingly this was not seen at mRNA level (where it was high in the RA in all stages of development), indicating that transcription was driven by singing but translation was conditional upon behavioural development (Whitney *et al*., 2000). As aforementioned, in the case of the γ4 subunit gene,

regulation appears to be at an mRNA level. Coupling this with the profound decrease in γ4-subunit mRNA levels in nuclei of the VMP following acquisition and production of normal song found here, the evidence strongly implies a functional role for these receptors.

5.3.6 Regulation of gene expression

As aforementioned, the *FOXP2* gene was the first gene to be implicated in a human communication disorder (Lai *et al*., 2001; 2003). Like the $GABA_A$ receptor γ4-subunit gene, *FOXP2* is well expressed within the adult zebra finch song system, and in Area X of canaries, expression of *FOXP2* is seasonally regulated, with highest mRNA expression levels when song is plastic (Scharff and Haesler, 2005). A study with adult zebra finches demonstrated that the expression of *FOXP2* was significantly down-regulated in Area X in male birds which produce normal song in comparison to non-singing zebra finches (Teramitsu and White, 2006), mirroring data obtained here for the γ4-subunit gene. Interestingly, comparison of sequence data between humans, songbirds and non-songbirds demonstrated no appreciable differences at a peptide level of *FOXP2* between singing and non-singing birds and in addition no residue similarities between songbird and human *FOXP2* (both of which produce vocalisations; Webb and Zhang, 2005). Thus, it is proposed that the evolutionary differences can be attributed to regulatory elements, rather than coding sequence evolution. Both $GABA_A$ receptors and FOX molecules show regional-specific expression (Laurie *et al*., 1992a, b; Wisden *et al*., 1992; Haesler *et al*., 2004; Thode *et al*., 2008) suggestive of multiple regulatory elements and with high sequence homology between the genes of different species. The evolutionary changes (i.e. producing vocalisations) are therefore potentially due to mechanisms that control gene expression rather than sequence changes in proteins (Carroll, 2005). Transcriptional mechanisms controlling both these sets of genes are not well understood and whilst the $GABA_A$ receptors and *FOXP2* appear to play an important role in song acquisition and production in the zebra finch, their expression is ultimately controlled by regulatory elements and it is these that should be more closely scrutinized (discussed in further detail in section 7.3). It was postulated by Thode *et al.* (2008) that the high levels of γ4-subunit gene expression in the male zebra finch brain may be attributable to steroid hormones (and not learning and

memory processes), due to the strong sexual dimorphism of the song system and its' sensitivity to steroids (Nottebohm and Arnold, 1976; Brenowitz et al., 1997; Thompson *et al*., 2007). Neurosteroids (potent allosteric, positive modulators of $GABA_A$ receptors; Belelli and Lambert, 2005; Hosie *et al*., 2006, 2007, 2009; Li *et al*., 2009), can accumulate in the brain after being locally synthesised or after metabolism of peripheral steroids. They can modulate $GABA_A$ receptors by direct activation (Hosie *et al*., 2009), or via hormone-activated nuclear receptors which function as transcription factors and can interact with specific sites within $GABA_A$ receptor subunit gene promoter regions. Such steroid hormone response elements have been identified in the majority of subunit sequences, which interact with ERs (estrogen receptors), PRs (progesterone receptors), GRs (glucocorticoid receptors) and AR (androgen receptors). Mammalian γ1-, γ2- and ε- (the orthologue of γ4) subunit gene promoters contain AR-consensus motifs (Steiger and Russek, 2004). Due to the presence of testosterone (and its metabolites) in the song system during development (Nottebohm 1989), a surge of circulating testosterone just prior to song crystallisation (Pröve 1983) and fluctuations in circulating testosterone during seasonal plasticity (Brenowitz *et al*., 1997), it may modulate transcription of $GABA_A$ receptor subunit genes. On the other hand, previous research indicated that in deafened birds endogenous circulating testosterone levels remained unaffected (Woolley and Rubel, 2002; Brenowitz *et al*., 2007), thus it is possible that isolated zebra finches had the same levels of neurosteroids as their aviary-reared counterparts and that changes in γ4-subunit mRNA levels were unlikely to be simply a consequence of hormonal regulation and certainly appear to play a role in learning and memory mechanisms.

As previously mentioned, song is a behaviour which is influenced by early auditory and social experience and development of the neural substrate is also subject to these factors. The development of the song system in zebra finches is on-going throughout development with synaptogenesis and loss of neurons all contributing to the establishment of the nuclei of the vocal pathways (Fig. 1.4; Nordeen, 2006). The process of song learning significantly reduces spine density in LMAN. If the sensory period of song learning is extended, then so is the length of time in which the dendritic spines are lost (Wallhäusser-Franke *et al*., 1995). Anatomical modification of synapses are integral to the functional development of the nervous system and it appears that the effects of learning are presented as dendritic

pruning (Bock and Braun, 1999; Nordeen, 2006), perhaps eradicating superfluous synapses as a means of information storage (Scheich *et al*., 1991). This is exemplified by a significant reduction of dendritic spine frequencies in the Dorsocaudal epistriatal pallium (formerly dorsocaudal neostriatum) following filial imprinting in the one-day-old chicken (Bock and Braun, 1999). Such anatomical synaptic changes such as decrease in synaptic connections following learning correlate well with the observed decrease of γ4-subunit mRNAs. However, there is some evidence to the contrary. Deafened birds cannot learn and produce normal song, but neuronal number and morphology within nuclei remains the same (Burek *et al*., 1991). Moreover, in late-isolated birds (after PHD 30), dendritic spine density remained relatively unaffected even when the zebra finches memorised new songs (Heinrich *et al*., 2005). It is possible that it is the neuroendocrine system which is ultimately responsible for synaptic remodelling, but this requires further investigation, for a more comprehensive review see Nordeen (2006).

5.3.7 Lateralisation of function

The data indicated that there was no significant lateralisation of function. There was a marginal increase in γ4-subunit mRNA expression within the nuclei of the right side of the brain (Fig. 5.13), which is consistent with previous findings (Williams *et al*., 1992). This is in contrast to many other songbirds in which it is the nuclei of the left hemisphere which are more important in the production of song (Nottebohm and Nottebohm, 1976, 1977) and in some species there is no evidence of lateralisation at all (Suthers *et al*., 1990). The slight increase in mRNA expression in nuclei of the right hemisphere of the zebra finch brain was also observed for the γ2-subunit; although, gene expression is not necessarily a definitive indication of lateralisation of function.

5.3.9 Concluding remarks

An understanding of the biology of human behaviour starts with the recognition that there are many parallels between humans and many other species and human-associated tendencies are recognisable throughout the animal kingdom (Adkins-Regan, 2002); an excellent example of this is the parallel between human vocalisations and the acquisition and production of song by songbirds (Kuhl, 2004).

The discrete and proliferate distribution of $GABA_A$ receptor γ4-subunit transcript within nuclei of the avian song system is indicative of an important physiological role. Moreover, zebra finches unable to produce normal song demonstrate higher mRNA levels encoding the $GABA_A$ receptor γ4-subunit in nuclei contained in both the AFP and VMP, thereby confirming the theory that down-regulation of GABAergic mechanisms plays a role in learning and memory systems (Clements and Bourne, 1996; Harvey *et al*., 1998; Cavallaro *et al*., 2002; Collinson *et al*., 2002; Zarrindast *et al*., 2006). This effect appears to be exclusive to the γ4 subunit, no such effects were observed with the γ2-subunit transcript and coupled with the widespread, diffuse expression was indicative of a more generalised functional role in the CNS as opposed to the robust, specific expression of the γ4-subunit gene which suggested a more specific functional role. Given that $GABA_A$ receptors that contain this subunit have a relatively unique pharmacology (Forster *et al*., 2001); specific-subtype-selective drugs could be used to ameliorate brain function in learning and memory processes. This information may be extrapolated to human subjects due to the many parallels between human and avian telencephalic systems and the presence of the γ4-subunit orthologue, ε in the CNS of mammals.

6. PRELIMINARY WORK TO CONFIRM THE ROLE OF γ4-SUBUNIT-CONTAINING $GABA_A$ RECEPTORS IN THE SONG SYSTEM

6.1 Introduction

Although real-time RT-PCR and *in situ* hybridisation were useful tools in providing a qualitative and quantitative insight into expression levels and spatial distribution of $GABA_A$ receptor subunit-mRNAs, it was important to consider if these results corresponded at a protein level. mRNA expression studies have proven invaluable in the development of markers e.g. for cancer and other diseases (Wallace *et al*., 2001; Cossette *et al*., 2002; Edenberg *et al*., 2004; Backus *et al*., 2005; Symmans *et al*., 2005; Torrey *et al*., 2005). However, these results only represent a 'pattern' as it is the proteins they encode and their interactions within cells which are the causative agents of a phenotype (Greenbaum *et al*., 2003). It is generally accepted that mRNA is eventually translated into protein, thus a correlation between the two would be expected; however this is not necessarily always the case (Greenbaum *et al*., 2003). In the field of proteomics, mathematical models have been employed with varying levels of success in an attempt to provide a solution to nonlinear cellular dynamics (Hatzimanikatis *et al*., 1999; Yu *et al*., 2007). The primary problem lies in that there are numerous considerations such as mRNA degradation, differing transcription rates and gene sequence lengths to name but a few. It has been demonstrated that in order to create a model it is necessary to have expression information both at mRNA and protein levels. Looking at either level individually to understand gene networks can lead to erroneous conclusions about the genes involved in a particular phenotype (Hatzimanikatis and Lee, 1999; Rhodes and Trimmer, 2006). To this end, investigations into the potential role of γ4-subunit-containing $GABA_A$ receptors required analysis at a protein level.

6.1.1 Immunohistochemical/immunocytochemical studies

The primary problem with $GABA_A$ receptor subunit-specific antibodies is that they are not always as specific as required. High levels of sequence similarity between individual subunits of the same class (as demonstrated in Chapter Three), means that targeting a single epitope is very difficult; this is most notable with the β subunit group (McKernan and Whiting, 1996). Despite this, a plethora of immunohistochemical,

immunocytochemical and immunoprecipitation studies have been undertaken in an effort to determine the possible co-assembly of the $GABA_A$ receptor subunits into the pentameric subtype (for review see Sieghart and Sperk, 2002).

Some studies have employed immunohistochemical techniques to localise the distribution of 13 $GABA_A$ receptor subunits (not π, θ, or ε subunits) within the rat hippocampus (Sperk *et al*., 1997); whole brain (Pirker *et al*., 2000); cerebellum (Pöltl *et al*., 2003), basal ganglia and associated limbic areas (Schwarzer *et al*., 2001). These studies all concluded a regional-specific distribution of corresponding subunit polypeptides and regional co-localisation was also apparent. Other studies have examined protein distribution of only a selection of subunits in the rat brain (Zimprich *et al*., 1991; Fritschy *et al*., 1992; Fritschy and Möhler, 1995; Nusser *et al*., 1996; Scotti and Reuter, 2001). The classical α1β2/3γ2 combination was found within single neurons across many brain areas (Fritschy *et al*., 1992). α6-subunit-containing receptors were localised primarily to the cerebellum (agreeing with previous findings concerning mRNA distribution, Laurie *et al*., 1992), where they were predominantly extrasynaptic (Nusser *et al*., 1996). More recent work has detected $GABA_A$ receptor clusters (β2/3 and γ2 subunits) in rat hippocampal cells before the establishment of functional presynaptic nerve terminals (Scotti and Reuter, 2001). One study purported significant co-localisation of the γ2S and γ2L splice variants (Miralles *et al*., 1994), indicative of a single subtype, when mapped by immunoprecipitation in the rat brain. The mammalian ε subunit was only recently identified (Davies *et al*., 1997; Whiting *et al*., 1997) and consequently has not undergone the scrutiny of the other receptor subunits. However, ε-subunit mRNA (mammalian orthologue of γ4) has been localised to various nuclei of the hypothalamus (e.g. in septum and preoptic areas, and paraventricular and supraoptic nuclei; Moragues *et al*., 2003), locus coeruleus, dorsal raphe, (Moragues *et al*., 2000). *In situ* hybridisation studies suggest the ε subunit may associate in various regions of the rat brain with α3 and θ subunits, its chromosomal partners (Moragues *et al*., 2002). Although, no co-precipitation studies or immunohistochemical studies have validated this co-localisation at a protein level.

Immunohistochemical and immunocytochemical studies have revealed that some $GABA_A$ receptor-subunit proteins are widely distributed throughout the brain (such as

α1, β1, β2, β3 and γ2), whereas other subunits show a more restricted distribution pattern (including α2, α3, α4, α5, α6, γ1, ε, θ and δ): which concurs with data concerning mRNA distribution (refer to Sieghart and Sperk, 2002). Although immunohistochemical techniques allow more precise localisation of protein at a subcellular level within any given brain region, there are some caveats such as false positives generated by non-specific antibody binding to tissues (Rhodes and Trimmer, 2006) or false-negatives which may represent an absence of immunoreactivity (due to methodical reasons) rather than an absence of the protein of interest. To this end, immunohistochemical data should only be interpreted alongside complementary data documenting mRNA expression such as *in situ* hybridisation (Fritschy, 2008).

6.1.2 Co-precipitation studies

Co-localisation does not necessarily represent co-assembly, at best it is indicative. In other efforts to determine which subunits co-localise, many studies have used immunoprecipitation techniques using $GABA_A$ receptor subunit-specific antibodies to purify native receptors containing a particular subunit from solubilised brain membrane extracts which can be subsequently quantified. In this manner subunit associations were found for γ2 with α1, α2, α5 and β2/β3 subunits in rat brain, demonstrating that potentially more than one type of α subunit may be present in a single receptor complex (Benke *et al*., 1991, 1996); this has also been shown for α6-subunit-containing receptors in rat cerebellum (Khan *et al*., 1994; Pöltl *et al*., 2003) and in co-purification of β-subunits (Li and De Blas, 1997; Jechlinger *et al*., 1998). However, conflicts in data exist whereby some studies have shown γ2 to co-precipitate with γ3 (Quirk *et al*., 1994a; Benke *et al*., 1996) and others find no such co-purification (Mossier *et al*., 1994). No evidence has shown potential association between γ1-subunit peptides and γ2/γ3 subunits (Mossier *et al*., 1994; Quirk *et al*., 1994a). Interestingly, δ and γ2-subunits have been co-precipitated, inferring potential co-assembly (Mertens *et al*., 1993; Quirk *et al*., 1994b), which is in conflict with the theory that the δ-subunit polypeptide effectively replaces the γ-subunit (Nusser *et al*., 1996). More recent immunoprecipitation studies have revealed potential major $GABA_A$ receptor subtypes existing in rat and mouse cerebellum, the majority containing α6 subunits (Pöltl *et al*., 2003), which concurs with previous data which localises α6-subunit mRNA primarily

to the cerebellum (Laurie *et al*., 1992a). Data generated from this technique can be variable, when considering that an antibody specific to a particular α subunit is able to co-precipitate with the majority of the other subunits and the same is true of a β- or γ-subunit specific antibodies. This confirms the promiscuity of the subunits and thus the subsequent potential variety of $GABA_A$ receptor subtypes in the mammalian brain (Sieghart and Sperk, 2002). Due to the fact that one particular subunit may be present in several receptor subtypes (Olsen and Sieghart, 2008), immunoprecipitation data is difficult to interpret as the number of subunits co-purifying may represent an unknown number of receptor subtypes, but this type of work can certainly contribute when attempting to define native $GABA_A$ receptor subtype assembly (Olsen and Sieghart, 2008), especially if presented alongside comprehensive mRNA data.

6.1.3 Generation of $GABA_A$ receptor γ4-subunit-specific antibodies

It had been recommended that a useful approach is to obtain convergent data, i.e. a combination of *in situ* hybridisation or real-time RT-PCR studies to examine the mRNA distribution, followed by immunohistochemical techniques of the same product at a protein level (Rhodes and Trimmer, 2006). In this manner, the two independent data sets can be combined and related contextually to each other, leading to less erroneous interpretation of results. This was the technique applied in this study for examination of the mRNA/protein expression of the $GABA_A$ receptor γ4 subunit. Two strategies were employed for generation of antibodies specific to the $GABA_A$ receptor γ4 subunit; production of a peptide antibody and production of a protein antibody. Peptide antibodies are generated directly from the amino acid sequence of a protein. Although a well chosen design enables the anti-peptide antibody to work well providing specificity and control, the primary disadvantage is that these are linear epitopes. Natural proteins are of course the ideal antigens as these are surface structural epitopes, but these are rarely pure and producing the amount of protein required can be difficult and time-consuming.

For the production of the peptide antibody, a short region of the peptide sequence, in the non-conserved region of the cytoplasmic loop between the putative transmembrane domains 3 and 4, which was homologous between the zebra finch and the chicken was selected and the whole process was out-sourced commercially.

For the development of a protein antibody, the pMAL™ protein fusion and purification system (New England Biolabs® Inc.) was employed which enables the expression and purification of a protein from a cloned gene. In brief, a cloned gene (e.g. a region of the $GABA_A$ receptor γ4 subunit) was ligated into the vector (pMAL-c2x), downstream and in the same translational reading frame as the *malE* gene (which encodes maltose binding protein). Transformation of the vector in *E.coli* cells resulted in robust expression of a fusion protein (MBP-γ4) which was harvested and affinity purified with an amylose column and eluted with maltose (for further details consult section 2.8, Chapter Two).

An antibody specific to the $GABA_A$ receptor γ4 subunit, ensures primarily that mRNA is being translated into protein and immunohistochemical and immunocytochemical localisation of that protein within the zebra finch song system. Furthermore, in the future, the antibody could be utilised in co-precipitation studies. It would also be employed to verify if the observed increase in mRNA expression in tutor isolated birds correlated at a protein level (chapter 4); and also to verify 'silencing' of the $GABA_A$ receptor subunit mRNA (and thus protein) in the RNA interference experiments (refer to section 6.1.4).

6.1.4 Reverse-genetic strategies employed for assessing $GABA_A$ receptor function

Eliminating/reducing gene expression (and subsequent protein production) and observing any physiological consequences is a common approach in determining the role of a particular gene. The most extreme examples are deletions or knockout animals, where there is a complete absence of gene expression. $GABA_A$ receptor α1-, α2-, α5-, β2-, β3-, γ2- and δ-subunit genes have all been knocked out in mice. β3- and γ2-subunit knockouts produced lethal phenotypes (a major caveat of knockout studies; Culiat *et al*., 1994; Günther *et al*., 1995). α5- and γ3-subunit knockouts resulted in altered drug responses (as do most $GABA_A$ receptor knockouts), and α5-subunit lacking mice demonstrated improved learning and memory (Culiat *et al*., 1994; Collinson *et al*., 2002). However, the majority of $GABA_A$ receptor knockout experiments induced compensatory up/down regulation of other subunits (another common problem with

knockout techniques). For example δ-subunit knockouts showed increases in γ2-subunit gene expression (Tretter *et al*., 2001); α6-subunit knockouts resulted in decreased δ-subunit gene expression (Jones *et al*., 1997); β2-subunit knockouts correlated with a decrease in expression of all six α-subunit genes and mice not expressing the α1-subunit gene exhibited increases in α2- and α3-subunit gene expression accompanied by a decrease in expression of β2-, β3- and γ2-subunit genes (Sur *et al*., 2001). A less extreme method of reducing gene expression utilises antisense oligonucleotides, which elicit degradation of complementary mRNA. A plethora of studies successfully show antisense-oligonucleotide-mediated reduction in the expression of $GABA_A$ receptors, both *in vitro* and *in vivo* (reviewed by Karle, 2002). Another method is ribozyme technology, but this has only been applied to the α1 subunit of the $GABA_A$ receptor. Although it resulted in a 90% reduction in α1-subunit protein levels (Subramaniam *et al*., 2001), this method still requires some refinement. All loss-of-function methods for assessing $GABA_A$ receptor function are discussed in Burt, 2003.

The relatively new advent of RNA interference (RNAi) enables sequence-specific silencing, without the caveats of complete gene knockout and with advantages over techniques such as antisense-DNA, dominant negative mutants and small molecular inhibitors, all of which exhibit variable efficiency (Scherer and Rossi, 2003; Mocellin and Provenzano, 2004; Cullen, 2005). Knockout animals ensure complete gene silencing, whereas RNAi is a knock-down method, which allows some translation to occur. The primary disadvantage applied to the majority of knockouts is when levels of one gene are completely diminished; other genes can be upregulated in compensation, 'masking' the phenotypic effects of the knockout. This would especially be of concern in a large gene family such as $GABA_A$ receptors. With the 90% mRNA knock-down induced by RNAi, upregulation is less of a problem and it also reduces the risk of lethal phenotypes (Dykxhoorn *et al*., 2003). Furthermore, it is a relatively inexpensive and rapid technique in comparison to using transgenic animals (Zeringue and Constantin-Paton, 2004). RNAi has not been widely employed to study $GABA_A$ receptors, potentially attributable, in part, to the high sequence homology between subunits and also that it is still a relatively new technique. A further caveat is that one subunit may form the basis of a variety of receptor subtypes, so subtype-specific effects are difficult to resolve. In an effort to determine the role of α4-subunit-containing $GABA_A$ receptors

in the mechanisms controlling alcohol intake, a recent study by Rewal *et al*. (2009) demonstrated a ~60-75% silencing in $GABA_A$ receptor α4-subunit protein expression following *in vivo* injection of viral vectors expressing siRNAs into the nucleus accumbens of adult rats, and observed an associated behavioural response. Upon silencing of α4-subunit gene, no compensatory changes in the levels of associated mRNAs (e.g. α1, β2, γ2 and δ subunits) were observed (Rewal *et al*., 2009). *In vivo* and *in vitro* silencing of the $GABA_A$ receptor γ2-subunit gene in rat brain has confirmed the importance of this subunit in synaptic clustering of $GABA_A$ receptors without creating a lethal phenotype (Li *et al*., 2005), unlike some previous attempts (Günther *et al*., 1995). shRNAs transfected with a lipid based reagent into primary hippocampal cultures showed up to 80% gene silencing. Other than these studies, there is no further work utilising RNAi to determine $GABA_A$ receptor function.

6.1.5 RNA interference (RNAi)

This method is based on post transcriptional gene silencing (PTGS) which was originally discovered in petunias and was initially referred to as co-suppression (Napoli *et al*., 1990; Scherer and Rossi, 2003). The phenomenon was later found in many other plants and also in fungi (where it was termed quelling; Romano and Macino, 1992; Cogoni *et al*., 1996). It was Fire and colleagues who made the critical discovery that although the sense and antisense strands of RNA, when injected, were able to silence target mRNAs in the nematode *Caenorhabditis elegans*, it was annealed double-stranded RNA which caused the greatest silencing, twice as much as the single-stranded RNAs on their own (Fire *et al*., 1998). Studies carried out with *Drosphilia melanogaster* (fruit fly) confirmed it was a two-step process (Elbashir *et al*., 2001a, b). Thus, it has been established that RNAi (or PTGS and quelling) is a well conserved system within all eukaryotes. It is currently postulated that RNAi evolved from an inherent immune response within cells to protect against viral attack, transposon proliferation (Scherer and Rossi, 2003; Zamore and Haley, 2005) and is essential for maintaining genomic stability (Ying and Lin, 2004; Wienholds and Plasterk, 2005; Zamore and Haley, 2005).

6.1.5.1 Mechansims of RNAi

RNAi is triggered by the presence of double-stranded (ds)RNA in the cytoplasm of the cell (Fig. 6.1), regardless of the format of the RNA (i.e. as endogenous pre-cursor

hairpin loops (miRNAs) derived from genomic transcripts in the nucleus, long dsRNA (e.g. from a virus) or synthetic short interfering (si)RNAs). The process of RNAi is summarised in Figure 6.1. In all instances, the first step is cleavage of dsRNA molecules by the dsRNA-specific endonuclease Dicer. The 5' end of the dsRNA binds at the PAZ (Piwi/Argonaute/Zwille) domain at the C-terminal of Dicer, a positively charged flat, central domain binds the remainder of the dsRNA molecule and cleavage occurs at the N-terminal where there are two catalytic ribonuclease III (RNase III) domains (Bernstein *et al*., 2001; MacRae *et al*., 2006). Different homologs of Dicer exist in different species and in *C. elegans* and *Drosphilia* species there are two Dicer enzymes, both of which require ATP for functioning (Shrey *et al*., 2009). However, in mammalian cells, there is only one version of Dicer (Zhang *et al*., 2002) and it is localised to the cytoplasm, where RNAi occurs (Billy *et al*., 2001). Dicer and its associated DNA-binding protein TRBP (human immunodeficiency virus transactivating response RNA-binding protein), which comprises three RNA-binding regions and protein activator of protein kinase PKR (PACT) process dsRNA into short (21-23 nt) interfering RNAs (siRNAs) which have a characteristic 2-3 nt overhangs at the 3' ends and a 5' phosphate group (Elbashir *et al*., 2001a, b; Kim and Rossi, 2007). Features necessary for incorporation of the siRNA into RNA-induced silencing complex (RISC), which is the second step in the RNAi pathway (Elbashir *et al*., 2001b; Yang and Mattes, 2008). RISC is a multi-subunit complex (including helicase, exonuclease, endonuclease and homology searching domains), which is able to bind and unwind siRNA duplexes and cleave target mRNA (Sledz and Williams, 2005). The associated Argonaute 2 (AGO2) protein is responsible for cleavage activity (Chendrimada *et al*., 2005). RISC exists in two forms and is converted to the active form upon binding of an appropriate siRNA molecule and cleavage of the passenger (sense) strand by AGO2 (Nykanen *et al*., 2001; Matranga *et al*., 2005; Rand *et al*., 2005). Asymmetric binding of siRNA molecules via their first unpaired nucleotide at the 5' end allows only one strand (antisense) to become incorporated into the RISC complex (guide strand; Schwartz *et al*., 2006. The guide strand is selected by the associated AGO2 protein due its lower thermodynamic stability (Preall and Sontheimer, 2005); ATP is required for siRNA unwinding (Nykanen *et al*., 2001). The guide strand then directs the RISC-siRNA complex towards corresponding mRNA where AGO2 cleaves target mRNA, which is then completely degraded by cellular exonucleases thereby silencing gene expression (Orban and Izaurralde, 2005). The protected guide stand can then potentially enable the

RISC complex to act repeatedly on many copies of the target mRNA amplifying the silencing effect (Shankar *et al*., 2005).

The miRNA pathway involves the processing of endogenously produced miRNA molecules encoded for within the genome (within intronic sequence of protein-coding genes or independent noncoding RNAs (Fig. 6.1b). Longer (several hundred bp), primary miRNA molecules encoded within intronic sequence are transcribed by RNA polymerase II or III in the nucleus (Kim, 2005) and then cleaved into 70nt pre-cursor miRNA molecules by nuclear RNases, Drosha and Pasha (Lee *et al*., 2003; Denli *et al*., 2004). These stem-loop structures are then recognised and actively exported into the cytoplasm by Exportin-5 (Lund *et al*., 2004), where they are processed by a Dicer-like complex in the same way as siRNAs. The remainder of the pathway is similar to that described for siRNA duplexes with the exception that, in animals, mature miRNA is not completely homologous to the target mRNA sequence (as it is in plants; Pillai *et al*., 2007) and as a result, degradation of target mRNA does not occur, but rather translation is suppressed (Olsen and Ambros, 1999). miRNAs in animals generally bind to partially complimentary sites within the 3'-UTR (Pillai, 2005) although, silencing via coding regions or the 5'-UTR is also possible (Kloosterman *et al*., 2004).

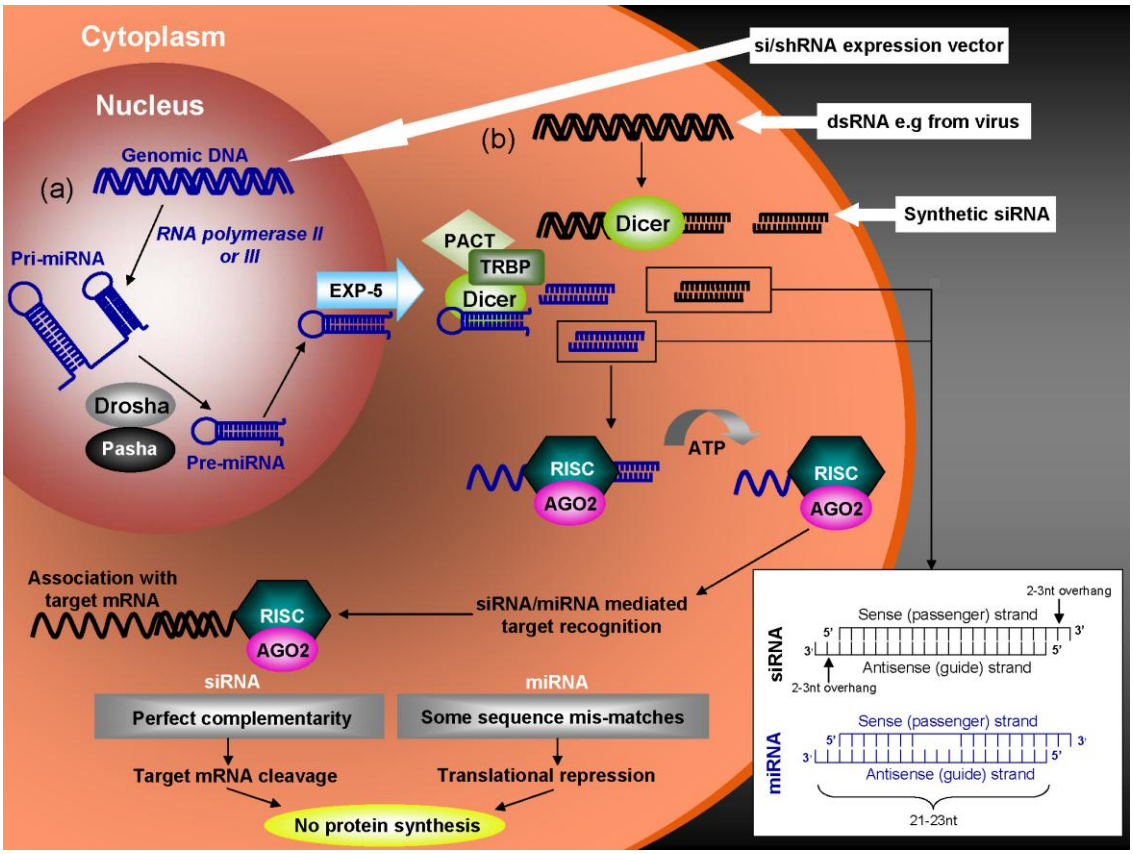


Figure 6.1. Mechanisms of RNA interference (RNAi). **(a)** In non-mammalian systems, dsRNA present in the cytoplasm is detected and cleaved by the Dicer complex into shorter (21-23nt) short-interfering (si)RNA duplexes with characteristic 2nt 3'overhangs, a hydroxyl group and a 5' phosphate group. The less thermodynamically stable antisense strand binds to the RNA-induced silencing complex (RISC) complex where the duplex is unwound and the sense strand degraded by argonaute 2 (AGO2). The guide strand directs the RISC complex to complementary target mRNA to which it binds and degrades. **(b)** Endogenous regulation of gene expression can also occur via micro (mi)RNAs. These are generally encoded for in non-coding regions of the genome and are transcribed by RNA polymerase II or III forming primary miRNA (pri-miRNA) transcripts which are subsequently processed Drosha into pre-cursor miRNA (pre-miRNA) which are exported to the cytoplasm by Exportin-5 (EXP-5). They are cleaved into siRNAs by the Dicer complex in a manner similar to dsRNAs. Both siRNA and miRNA pathways culminate in the binding of RISC to homologous mRNA molecules. Perfect complementarity between the guide strand and target mRNA elicits degradation (generally siRNAs) and an imperfect complement results in translational arrest (generally miRNAs); but both ultimately suppress protein synthesis. Induction of RNA silencing can be achieved by transfection of a cell with a viral or plasmid vector expressing a short hairpin (sh)RNA, which mimics endogenous miRNA molecules (Abbas-Terki *et al*., 2002; Kunath *et al*., 2003) or by direct induction of dsRNA or siRNAs into the cytoplasm (Elbashir *et al*., 2001a, b). siRNAs are particularly useful in mammalian cells as longer dsRNA elicits and innate immune response. **Abbreviations:** PACT, protein activator of protein kinase PKR; TRBP, human immunodeficiency virus transactivating response RNA-binding protein.

6.1.5.2 Functions of RNAi

RNAi functions as an endogenous defence mechanism to protect the cell against viral attack and transposon activity (mobile genetic elements; Hannon, 2002). It also serves to regulate gene expression via miRNAs (Scherer and Rossi, 2003; Ying and Lin, 2004; Wienholds and Pasterk, 2005; Zamore and Haley, 2005). Up to 1/3 of genes in humans are potentially regulated by endogenous, evolutionarily conserved miRNA molecules, of which 1000 have been predicted, although precise mRNA targets and functions have only been described for a few (Pillai *et al*., 2007). miRNA genes are located in intronic sequence and account for ~1-5% of genes in plants, invertebrates and vertebrates (Ying and Lin, 2004). They display regionally- and developmentally-regulated expression which is subject to change in disease states (reviewed by Ambros, 2004; Bartel, 2004; Wienholds and Plasterk, 2005), most notably in cancer whereby their overexpression contributes to tumour development (He *et al*., 2005).

6.1.5.3 Interferon response

In mammals, long dsRNA molecules induce the interferon response (Elbashir *et al*., 2001a, b), characterised by a non-specific termination of transcription and translation (Williams, 1997, 1999). This pathway triggers the degradation of mRNA by inducing oligoadenylate synthase which activates the ribonuclease, RNAse L and also activates RNA-dependent protein kinase (PKR) which phosphorylates the essential translation initiation factor eIF2α leading to a global inhibition of mRNA translation and global RNA degradation (Minks *et al*., 1979; Williams, 1997, 1999). The interferon response is a host defence mechanism which was named after its ability to 'interfere' with viral replication. However, dsRNAs of less than 30nt are able to avoid induction of the interferon response and enter the RNAi pathway (Elbashir *et al*., 2001a, b). More recent research has demonstrated that activation of the interferon response is not wholly dependent on duplex length, but can also be affected by cell type (Reynolds *et al*., 2006) and sequences which are GU rich are also more likely to induce the interferon response (Judge *et al*., 2005). All these criteria must be considered when constructing synthetic si/shRNAs for silencing in mammalian systems.

6.1.5.4 Induction of RNAi in cells

RNAi technology is most frequently employed for the silencing of individual genes via siRNAs in cell culture (Shan, 2009). Chemically (Elbashir *et al*., 2001a) and

enzymatically (Gou *et al*., 2003) synthesised siRNAs induce transient silencing (Tuschl, 2002), with normal gene expression resuming after ~3-5 cell divisions, which is thought to be attributed to dilution of the duplexes rather than their degradation (McManus and Sharp, 2002; Song *et al*., 2003; Genc *et al*., 2004). Silencing via siRNAs is 100-1000x more efficient than with antisense oligonucleotides (Bertrand *et al*., 2002). In efforts to establish greater longevity, plasmid vectors constitutively expressing siRNAs can be transfected into cells which can elicit silencing for up to two months (Brummelkamp *et al*., 2002; Sui *et al*., 2002; Choi *et al*., 2005), although their transfection efficiency is typically lower than for naked siRNAs, most notably in neuronal cultures (McManus and Sharp, 2002). To circumvent this, siRNAs can be electroporated in neuronal cells but cell death then becomes a factor (McManus and Sharp, 2002). Further to this, viral vectors were considered as they don't require transfection and they were shown to be more effective in cells which were resistant to plasmid vectors, such as primary cells (Barton and Medzhitov, 2002; Liu *et al*., 2004). However, these lack a selective pressure and can present a potential biohazard (reviewed by Mocellin and Provenzano, 2004). More recently research has reported that artificial dsRNAs (< 30nt in mammalian cells) and shRNAs which are subsequently processed by Dicer are far more efficient at silencing than Dicer-independent siRNAs (Kim *et al*., 2004; Siolas *et al*., 2004). Thus the use of short hairpin (sh)RNAs (which mimic naturally occurring miRNAs; Cullen, 2005) within expression/viral vectors has become popular for the establishment of stable gene silencing in culture or transgenic mice (Brummelkamp *et al*., 2002; Paddison *et al*., 2002; Yu *et al*., 2002; Li *et al*., 2005). In addition to constitutive expression of sh/siRNA duplexes, plasmids enable *in vitro*/*vivo* tracking by directly linking the si/shRNA expression cassette to a reporter gene (Dai *et al*., 2005; Das *et al*., 2006).

6.1.5.5 Design of RNAi experiments

Criteria for the rational design of siRNA/shRNA molecules are continually being improved, for a full description refer to section 2.9.1, but even adhering to each rule when designing a siRNA, does not ensure successful knockdown or indeed predict the degree of silencing. Therefore it is recommended that several siRNAs are synthesised simultaneously, each targeting a different exonic (intronic regions cannot be used as it is a cytoplasmic process; Dykxhoorn *et al*., 2003) region of the gene. Only two or three base-pair shifts in the positioning of the siRNA can alter silencing efficiency, revealing

that it is likely to be the sequence composition of the siRNA molecule which primarily governs the success of silencing (Holen *et al*., 2002; Reynolds *et al*., 2004). Once a successful siRNA sequence has been selected, dose response curves should be produced to ensure the lowest concentration of siRNAs are introduced to the cell to reduce any off-target effects. Although small amounts should not saturate the endogenous RNAi pathway, they are potentially competing with endogenous miRNA molecules for incorporation into RISC (Castanotto *et al*., 2007; Shan, 2009) and expression of non-target genes can be affected by introduction of exogenous siRNAs (Jackson *et al*., 2003; Scacheri *et al*., 2004). Further considerations concern the half life of the target protein and mRNA (Choi *et al*., 2005) and the G+C content of the siRNA molecule (Reynolds *et al*., 2004).

Cells must be transfected when using siRNAs or plasmid shRNA vectors, with standard transfection reagents (generally liposomal), as they are negatively charged polymers so cannot enter hydrophobic cell membranes (Kim and Rossi, 2007; Castanotto and Rossi, 2009). Alternatively, peptide-conjugated siRNA molecules enable high transfection rates (Kim *et al*., 2006; Davidson *et al*., 2004; Turner *et al*., 2007). As different cell lines can react differently to the various reagents, suitable transfection reagents should be tested on a trial and error basis. Aptamers (synthetic DNA/RNA molecules which bind to specific molecular targets) conjugated to siRNA duplexes also allow efficient cell-specific delivery (Que-Gewirth and Sullenger, 2007). Another option is electroporation (Dai *et al*., 2005; Das *et al*., 2006; Zietelhofer *et al*., 2007) but as previously mentioned, cell death can be a problem when using this technique (McManus and Sharp, 2002).

Knockdown of mRNA can be measured by northern blot analysis or real-time RT-PCR, the latter proving more popular and the most appropriate technique. If mRNA silencing is successful then subsequent protein knock-down should also be analysed, most commonly by western blotting or immunofluoresence (Shan, 2009). Comparison between decreases in mRNA and protein can ascertain that all observable effects are attributable to the artificially induced RNAi mechanisms. Silencing is usually detected (in cell culture) approximately 18-24 h following transfection, but factors such as target protein turnover rate, transfection efficiency, longevity of the siRNA duplex, dilution of

the siRNA and cell type all affect the efficiency of the RNAi reaction (McManus and Sharp, 2002; Genc *et al*., 2004; Mocellin and Provenzano, 2004).

6.1.5.6 Applications of RNAi

RNAi has been exploited in commercial applications, such as in coffee plants, where the enzymes involved in caffeine biosynthesis were silenced to reduced caffeine content in beans by up to 70% (Ogita *et al*., 2003). In the molecular biology field, RNAi is utilised as a powerful tool in reverse genetic strategies in order to determine the function of eukaryotic genes; this can be considerably quicker and cheaper than knock-out animals and reduces the chance of a lethal phenotype (Dykxhoorn *et al*., 2003). Furthermore, consequences of silencing particular proteins within known or unknown pathways can also be explored (Devi, 2006). RNAi may also be employed as an effective therapeutic agent in diseases that involve over-expression of genes, such as cancer, influenza, HIV, SARS and Polio (Cristofaro and Ramratnam, 2006; Devi, 2006; Kumar, 2008). Successful *in vivo* gene silencing with direct injection of siRNAs and shRNAs in many different rodent tissues (such as liver, eye, kidney and lung) has been reported (reviewed by Shankar *et al*., 2005) and in chicken embryos (Dai *et al*., 2005; Das *et al*., 2005). Subsequently there is a profound interest in employing RNAi in the mammalian brain, but much work needs to be completed to ensure efficient, safe and specific *in vivo* delivery (reviewed by Thakker *et al*., 2004; Fountaine *et al*., 2005; Sah, 2006; Kim and Rossi, 2008; Castanotto and Rossi, 2009; Shrey *et al*., 2009). *In vitro* RNAi methods in primary neurons have proven successful using liposomal transfection reagents or electroporation (Krichevsky and Kosik, 2002; Li *et al*., 2005; Tönges *et al*., 2006). Direct stereotactic injections of naked siRNAs, or plasmid/viral vector based si/shRNAs into mammalian brain has shown ~50% decrease in expression of target genes (Makimura *et al*., 2002; Xia *et al*., 2002, 2004; Hommel *et al*., 2003; Van den Haute *et al*., 2003; Li *et al*., 2005; Raoul *et al*., 2005). The relatively short-lived effects induced by siRNAs may be beneficial in treatment of tissues such as liver or neuronal cells of the CNS due to their slow rate of division where they could silence genes for up to two weeks before becoming too diluted (Song *et al*., 2003; Genc *et al*., 2004). Moreover, in instances requiring only a short treatment, such as some viral infections, the short-term effects elicited by siRNAs may be advantageous (Shankar *et al*., 2005). Although, before the introduction of RNAi techniques as gene therapy, safe delivery, off target

effects and functional consequences must be thoroughly investigated (Shrey *et al*., 2009).

6.1.5.7 RNAi in zebra finch brain

RNAi was employed to functionally determine the role of γ4-subunit-containing $GABA_A$ receptors in the zebra finch song system; with the ultimate aim of knocking down γ4-subunit mRNA and protein in specific nuclei of the song system *in vivo* and observing any effects on song acquisition and production. The only previous example of RNAi technology being harnessed for functional genetic analysis in the zebra finch brain, is the silencing of *FOXP2* which encodes forkhead box transcription factor (Haesler *et al*., 2007). Bilateral stereotactic injections of shRNAs within viral vectors were administered directly into Area X of anesthetised juvenile (PHD 23) male zebra finches. Vocalisations were recorded at PHD 65, 80 and 90-93. Suppression of translation was analysed by immunohistochemical staining and mRNA knock-down was quantified with real-time RT-PCR. Zebra finches with reduced *FOXP2* expression exhibited poor imitation of a tutor and their songs contained abnormally variable syllables (Haesler *et al*., 2007). Concurring with previous work in humans which suggested a mutation in *FOXP2* resulted in speech and language impairments (Lai *et al*., 2001, 2003; MacDermot *et al*., 2005). Due to the complexity of neural networks these effects cannot be attributed to a single gene and thus other genetic links should be investigated. As the $GABA_A$ receptor γ4-subunit gene has exhibited regional-, developmental- and learning-associated expression, both in chicken (Harvey *et al*., 1998) and zebra finch (Thode *et al*., 2008; refer to Chapter 5) and coupled with evidence that modulation of $GABA_A$ receptors affects cognitive function (Chapouthier and Venault, 2002, 2004) it was an ideal candidate for such a study. Prior to *in vivo* injections of shRNAs targeting the γ4-subunit mRNA, it was first necessary to verify the silencing efficiency of the constructs by *in vitro* methods (Haesler *et al*., 2007). As zebra finch brains were scarce, and no commercial cell lines were available which endogenously expressed the γ4-subunit, primary culture of chicken neurons was utilised. Due to the high degree of conservation between the γ4-subunit genes of chicken and zebra finch (97% over 271 AA), shRNA constructs were designed that would target both chicken and zebra finch γ4-subunit mRNAs.

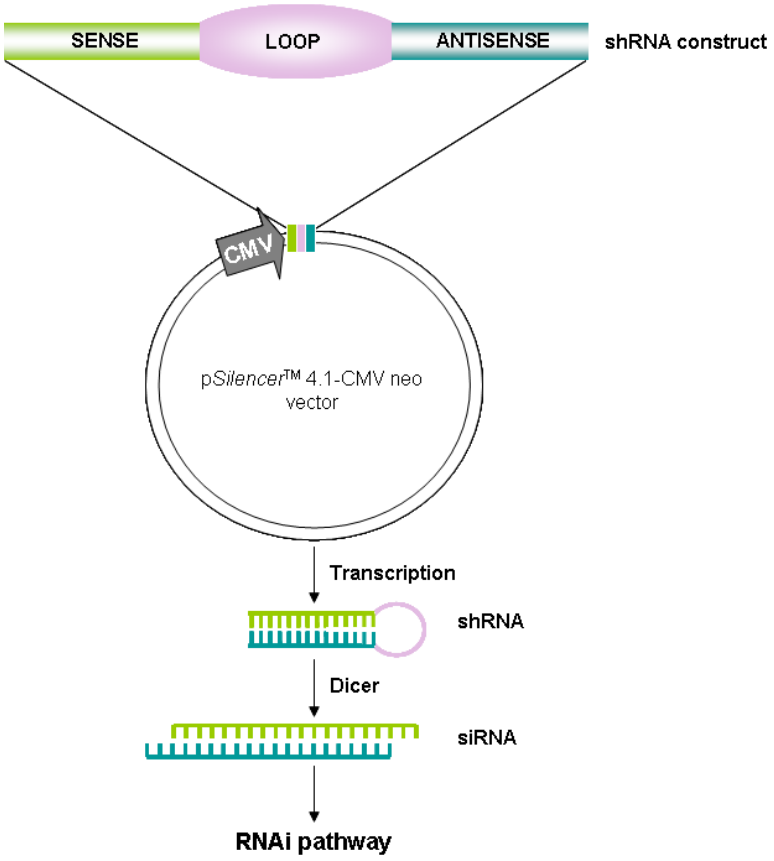


Figure 6.2. Annealed single-stranded shRNA constructs were ligated into the p*Silencer*™ 4.1-CMV neo vector. The plasmid was transfected into cells with a lipid-based carrier where the powerful CMV (human cytomegalovirus) promoter drives constitutive expression of the shRNA *in vitro/vivo*. Resulting shRNA enters the endogenous RNAi pathway and is cleaved into siRNAs by Dicer, resulting in subsequent silencing of the $GABA_A$ receptor γ4-subunit mRNA, therefore reducing corresponding protein expression.

In summary, successful generation of a γ4-specific antibody and shRNA constructs would enable a comprehensive functional analysis/confirmation of the role of the γ4-subunit-containing $GABA_A$ receptors within the zebra finch song system.

6.2 Results

6.2.1 Generation of a γ4-subunit specific antibody

6.2.1.1 Amplification of a partial region of the zebra finch $GABA_A$ receptor γ4-subunit transcript

Following design of the DNA construct, the respective cDNA fragment was amplified by RT-PCR from zebra finch brain (Fig. 6.3) and sent for automated sequencing (Fig. 6.4) to ensure an accurate DNA sequence was going to be expressed alongside maltose-

binding protein (MBP) as a fusion protein in *E. coli* cells by the pMAL™-c2X vector system.

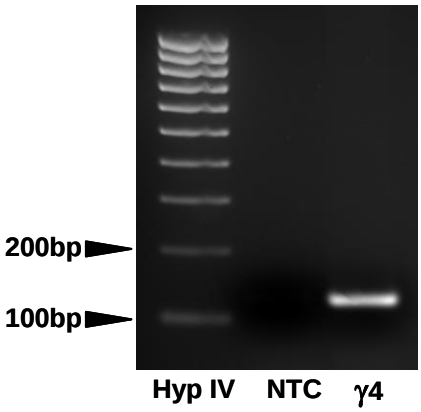


Figure 6.3. A single, 120bp DNA fragment encoding a region of the $GABA_A$ receptor γ4-subunit transcript, amplified from zebra finch brain by RT-PCR. Product was electrophoresed on a 2% (w/v) agarose gel stained with ethidium bromide and visualised under UV light. RT-PCRs were run alongside 7µl Hyper ladder IV (Bioline). NTC, no template control.

(a)

```
Zebra finch (γ4 subunit)      CACCTGCCGAAACCACTGGAGCACAGCCACAGGAGAGCCAGGCTGCCACC
Zebra finch (γ4 construct)    GAATTCCCGAAACCACTGGAGCACAGCCACAGGAGAGCCAGGCTGCCACC
                               *  * ****************************************

Zebra finch (γ4 subunit)      TGCCGGTGCTCAGGTGATGCCAACCTTCACCACCATCAACATCAACCACA
Zebra finch (γ4 construct)    TGCCGGTGCTCAGGTGATGCCAACCTTCACCACCATCAACATCAACCACT
                              **********************************************

Zebra finch (γ4 subunit)      TCATGCACTG
Zebra finch (γ4 construct)    AGATGAATTC
                              *** * *
```

(b)

```
Zebra finch (γ4 subunit)     KKPLEHSHRRARLPPAGAQVMPTFTTININHI
Zebra finch (γ4 construct)   KKPLEHSHRRARLPPAGAQVMPTFTTININH*
                             ******************************
```

Figure 6.4. Alignments of the cloned construct sequence with the corresponding portion of the original zebra finch $GABA_A$ receptor γ4-subunit nucleotide (a) and peptide (b) sequences as a reference (GenBank accession number AM086933; Thode *et al*., 2008), in single letter code. Sequences encode a region of the large variable intracellular loop between transmembrane domains (TM) 3-4. (a). Modifications to the construct nucleotide sequence include a translational stop codon (TAG) and *Eco*R1 restriction sites (GAATTC), highlighted in grey and orange respectively. (b) The asterix at the end of the peptide antibody sequence (highlighted in grey) indicates the presence of the integrated stop codon.

Agarose gel electrophoresis (Fig. 6.3) revealed a single amplicon of the expected size (120 bp) and completely clean negative control reaction. In order to verify the identity of the amplified product it was sent for automated sequencing (Fig. 6.4).

DNA sequencing results confirmed the RT-PCR product to be identical to the designed construct with 100% alignment to the previously documented zebra finch $GABA_A$ receptor γ4-subunit mRNA sequence (GenBank accession number AM086933; Thode *et al*., 2008), with the exception of any of the intentionally modified bases, as shown in Fig. 6.4). From this, the amino acid sequence was translated using online tools (available at; http://www.justbio.com/translator/index.php) demonstrating that no change in peptide sequence had occurred despite modifications to the DNA sequence (except for the translational stop codon).

The molecular weight of the γ4-subunit peptide construct was calculated using online software (www.biopeptide.com/PepCalc/PepMWCalc2.dll/Calculate) to be 3.6 kDa.

6.2.1.2 Pilot experiment

It was necessary to determine the behaviour of the MBP-fusion protein before experiments were undertaken on a larger scale. This section of work was also useful in determining the optimum conditions (e.g. inoculation volume, IPTG concentration, incubation time and sonication duration), for harvesting the greatest concentration of fusion protein from *E. coli* cells.

Data obtained for the pilot experiment demonstrated that the pMAL™-c2X vector was expressing the γ4-subunit shRNA construct and this in turn was being translated into a fusion protein within *E. coli* cells. IPTG induction was optimised and 3 h was determined as the ideal time point for harvesting the cells (as the induced band was much larger at this point than after only one hour induction time, Fig. 6.5a and b; lanes 3 and 4). Time-points were tested for up to 5 h following IPTG induction (data not shown), however, levels after 5h induction were no higher than that of 3 h induction.

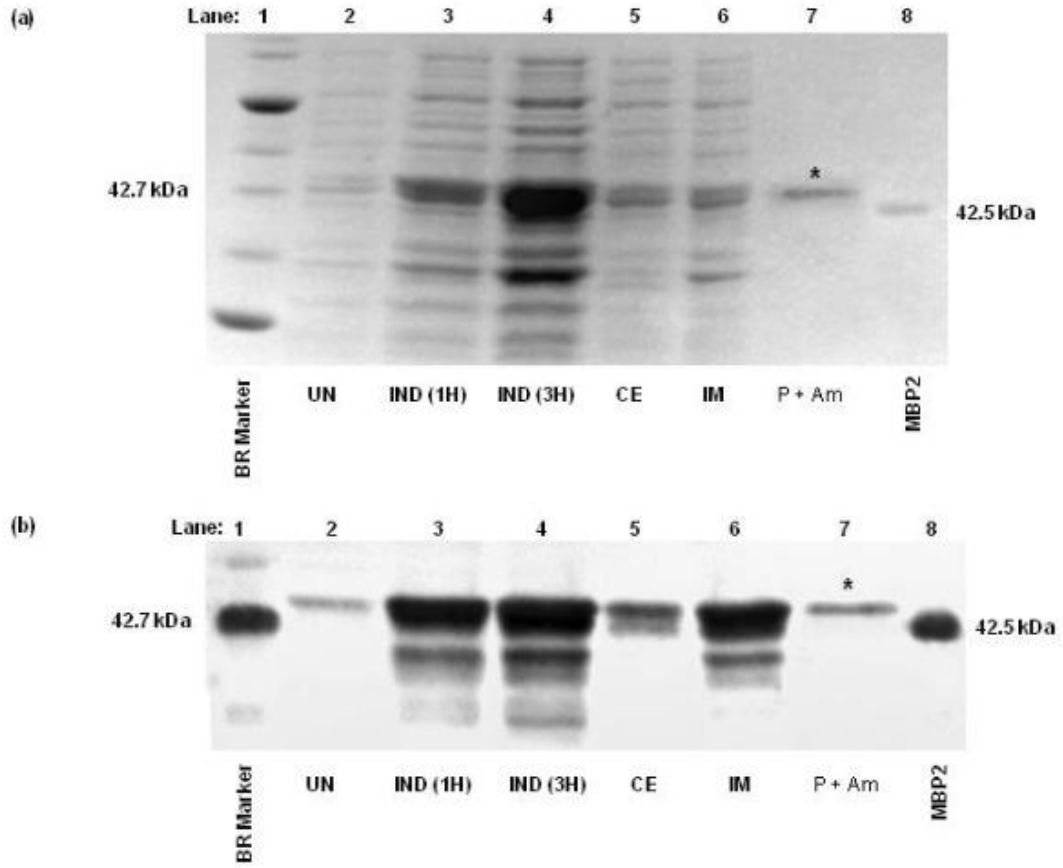


Figure 6.5. Results of pilot experiment with 10% (w/v) SDS-PAGE (a) and western blot analysis (b) to determine activity of the MBP-fusion protein. An induced band was visible at the position corresponding to the molecular weight of the MBP-fusion protein (i.e. ~46 kDa) in each sample (5mg protein loaded per sample). The western blot (b) identifies the target protein (0.5mg protein loaded per sample) and lane 7 indicated that the protein bound successfully to amylose. **Abbreviations: UN**, uninduced cells; **IND (1h)**, cells harvested 1 h following IPTG induction; **IND (3h)**, cells harvested 3h following IPTG induction; **CE**, crude extract; **IM**, insoluble matter; **P + Am**, protein bound to amylose. Samples were run concurrently with 7µl pre-stained broad range protein marker (New England Biolabs® Inc.) and 15µl MBP2 marker (MW 42.5kDa).

In addition the final IPTG concentration used for induction was 0.375 mM, various concentrations were tested but this gave the greatest yield of target protein (data not shown). Crude extract and insoluble matter samples both contained the target protein (Fig. 6.5a and b, lanes 5 and 6 respectively). It was inevitable that not all the protein could be released and retained in the crude extract, so some remained detectable in the insoluble matter. In all samples, except the uninduced and protein bound to amylose, there was some evidence of protein degradation, highlighted by western blot analysis (Fig. 6.5b). The conditions of a protein extraction are not favourable to the integrity of the protein and so some loss was to be anticipated. However, only a protein that is fairly

intact is able to bind to amylose therefore it would be expected that protein purified by an amylose-affinity column would be in tact. The single product yielded in lane 7 (protein bound to amylose; Fig. 6.5a and b) indicated that this was the only protein present which would bind to amylose and the molecular weight of the band was slightly above that of the MBP2 marker (42.5 kDa; lane 8), which was expected as the MBP-fusion protein molecular weight was 46 kDa. western blot analysis confirmed that this was the MBP-fusion protein with a highly specific MBP-targeting primary antibody.

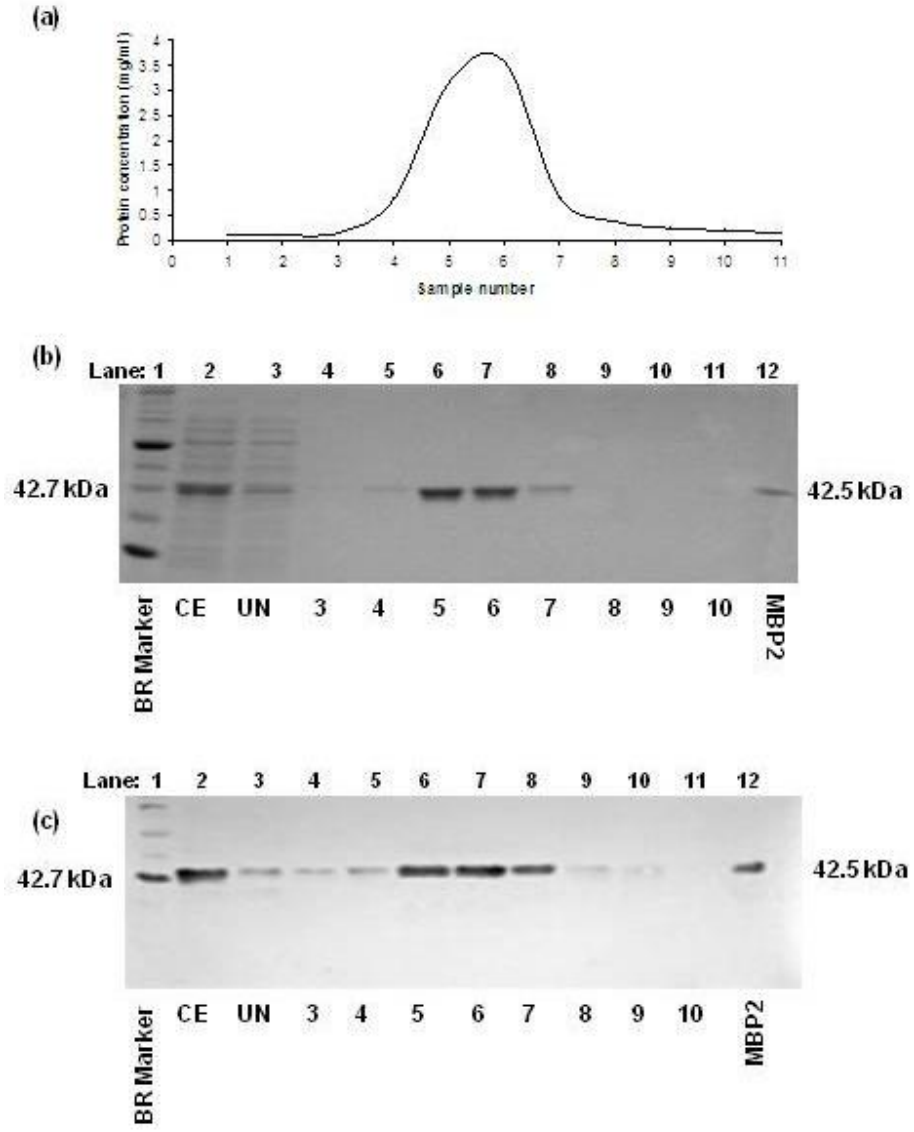
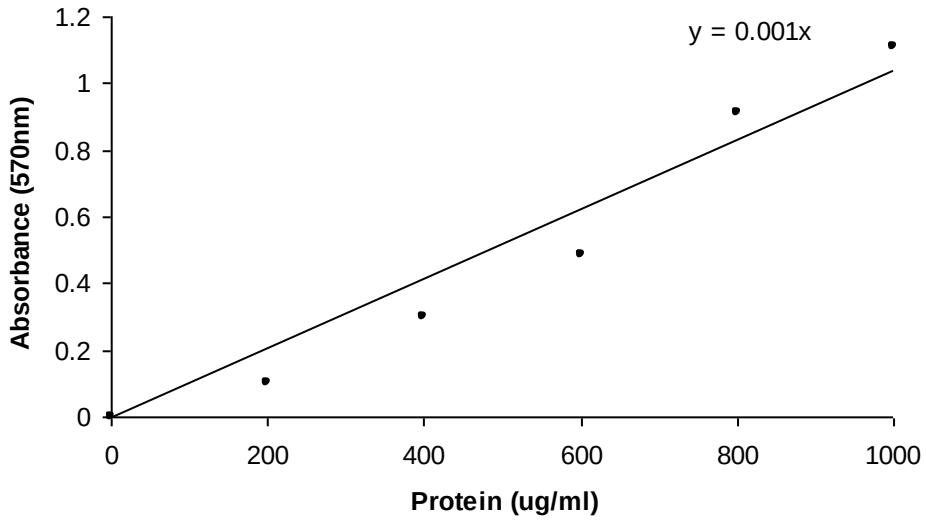


Figure 6.6. Analysis of eluted MBP-γ4 fusion protein following affinity purification with an amylose column. (a) Results of the BCA assay demonstrated that protein was eluted within fractions 4-8. (b) A 10% (w/v) SDS-PAGE, stained with Coomassie brilliant blue confirmed protein elution and showed that only a single protein was eluted from a mixed population, i.e. the crude extract (CE). 10µl sample loaded per lane. (UN) unbound protein, collected once the crude extract was run through the amylose column. (c)

Western blot analysis with an alkaline-phosphatase conjugated secondary antibody verified the identity of the eluted protein; the expected MW was ~46 kDa, 1µl sample loaded per lane. Samples were run concurrently with 7µl pre-stained broad range (BR) protein marker and 15µl MBP2 marker (MW 42.5 kDa).

Results conformed successful isolation of the MBP-γ4-subunit fusion-protein from the crude extract. The BCA assay revealed a peak of protein elution between samples 4-8 (Fig. 6.6a). The SDS-PAGE analysis was necessary to determine the purity of eluted protein samples and the gel revealed only a single product was present (Fig. 6.6b), so the sample was verified as pure. Furthermore, western blot analysis was then performed and this confirmed the identity of the protein due to the high specificity of the primary antibody and the product being of the expected molecular weight (Fig. 6.6c).

(a)



(b)

$y = 0.001x$; $x = y/0.001$
$\therefore$ $x = 0.226\mu g/\mu l$

Figure 6.7. Calibration graph and subsequent calculations following a BCA assay determining the final protein concentration of the MBP-γ4 fusion protein following dialysis. (a) Shown is a calibration curve constructed from the mean of triplicate absorbance readings from bovine serum albumin (BSA) standards in TBS. Dialysed protein was diluted 2x, 4x and 8x in TBS and added to the assay in triplicate. (b) Following a correction for the dilution factor, the concentration of MBP-fusion protein was calculated using the equation taken from the calibration curve.

There was some protein loss during the dialysis process, but the final concentration was found to be 0.23 mg/ml, which was enough protein to enable subsequent antibody production in the future.

6.2.2 RNA interference (RNAi)

In an effort to determine the function of γ4-subunit-containing $GABA_A$ receptors within a learning and memory paradigm; RNAi was employed to silence γ4-subunit mRNA/protein expression, with the ultimate aim of injecting shRNA constructs *in vivo* into specific nuclei of the zebra finch song system and observing effects on song acquisition and production. Before *in vivo* work could commence it was essential to verify the efficiency of the shRNA constructs *in vitro*. As zebra finches were not readily available, primary cerebellar neurons from 1-day-old chicken (*Gallus gallus domesticus*; which is phylogenetically similar to the zebra finch) were cultured. Single stranded 55 nt palindromic nucleotide sequences were synthesised commercially (full sequence details, Fig. 2.2, Chapter 2), annealed together and ligated into the p*Silencer*™ 4.1 - CMV neo vector. Automated sequencing of both strands confirmed presence/absence of annealed inserts in the vector and revealed any inaccuracies in the sequences. shRNA-containing plasmids (γ4-subunit targeting, GAPDH targeting and non-specific targeting) were co-transfected with pRK5-Clomeleon vector (a visualisation vector to verify transfection efficiency) into primary chicken cerebellar neuronal cells and after 72 h cells were harvested. RNA was extracted and levels of γ4-subunit and GAPDH (positive control) mRNA assessed by real-time RT-PCR.

6.2.2.1 Optimisation of real-time RT-PCR primers

It was first necessary to verify suitable house-keeping genes for real-time RT-PCR reactions. Three house-keeping genes were tested; β-actin (GenBank accession no. L08165); 18S Ribosomal RNA (GenBank accession no.AF173612); β-2-Microglobulin (GenBank accession no. Z48922); along with primers to amplify mRNAs being targeted by shRNAs i.e. $GABA_A$ receptor γ4-subunit and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; GenBank accession no. NM_204305), further details of primer pairs in Table 2.6, Chapter Two. A derivative data plot showing Sybr melt analysis indicated all primer pairs gave a single product at similar melting temperatures

and subsequent gel electrophoresis confirmed this and showed all amplicons to be between 140-155bp (data not shown).

6.2.2.2 Primary culture and transfection

Optimisiation of one-day-old chicken cerebellar neuronal culture was relatively labour intensive and the protocol outlined in section 2.9.3 was a result of lengthy optimisation concerning medium used, different plate coatings (such as collagen and poly-D-lysine), tituration technique and trypsinisation times. Cultures of neurons alongside glial cells were produced which could be maintained for up to 12 d. Transfection of plasmid-based shRNAs was carried out in triplicate ~5 days after the culture was established, in the following combinations:

(1) 200ng γ4-subunit-targeting shRNA in p*Silencer*™ 4.1 CMV-neo vector + 200ng pRK5-Clomeleon vector
(2) 200ng GAPDH-targeting shRNA in p*Silencer*™ 4.1 CMV-neo vector + 200ng pRK5-Clomeleon vector
(3) 200ng Non-targeting shRNA in p*Silencer*™ 4.1 CMV-neo vector + 200ng pRK5-Clomeleon vector
(4) 200ng pRK5-Clomeleon vector alone

6.2.2.3 Quantitative real-time RT-PCR

Preliminary real-time RT-PCR data shown in Figure 6.8 revealed that both the $GABA_A$ receptor γ4-subunit and GAPDH shRNAs resulted in sequence-specific silencing of the respective mRNAs. Agarose gel electrophoresis indicated a substantial decrease in γ4-subunit mRNA expression in cells transfected with a γ4-subunit targeting shRNA, 72h following transfection. No such decease in $GABA_A$ receptor γ4-subunit mRNA was observed in cells transfected with GAPDH-targeting or non-targeting shRNAs (Fig. 6.8b and c). Although, some product was still observable (Fig. 6.8a, lanes 2-4), so silencing was not 100% (this was expected). Further confirmation of sequence-specific gene silencing was seen in cells transfected with a shRNA targeting GAPDH (Fig. 6.8b). Corresponding mRNA was noticeably decreased (lanes 5-7) whereas the levels of γ4-subunit mRNA remained stable (lanes 2-4).

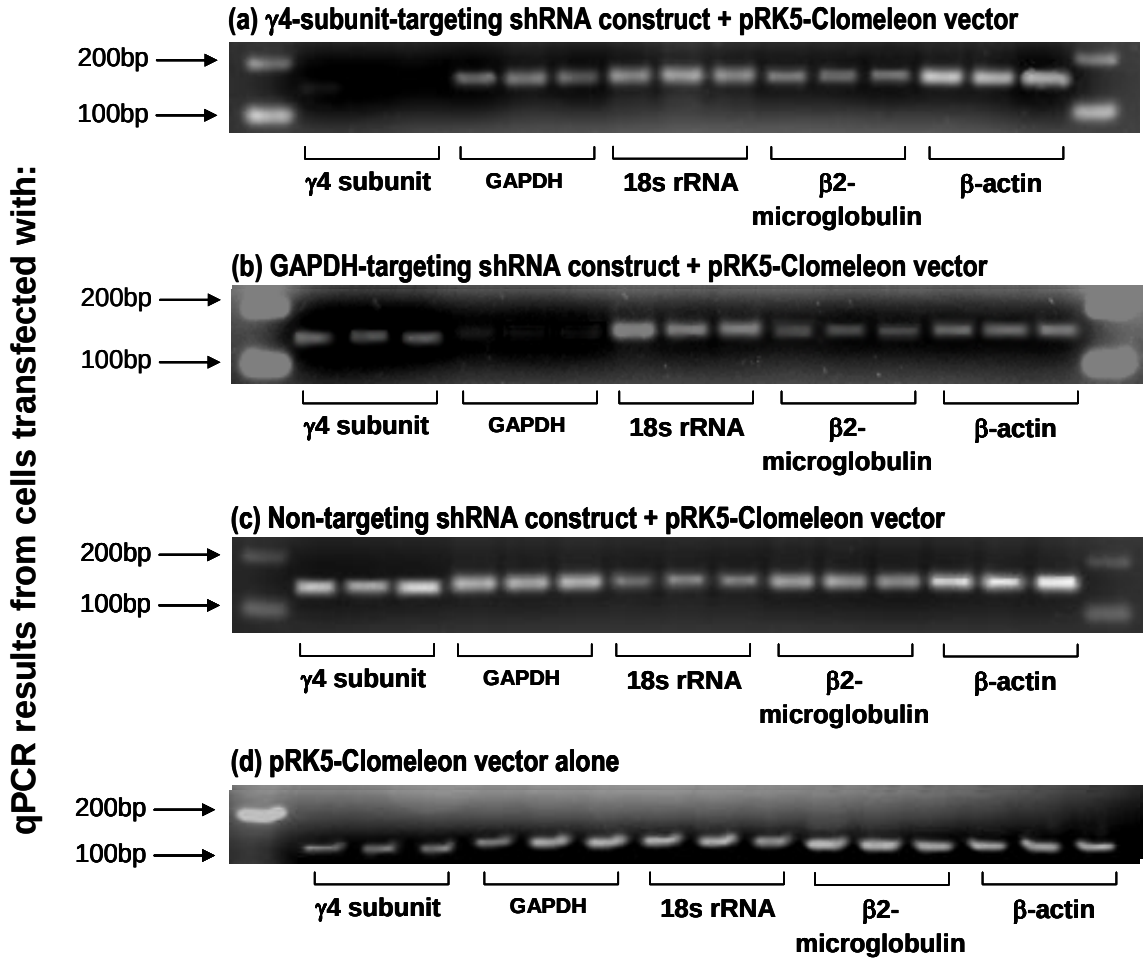


Figure 6.8. Silencing of $GABA_A$ receptor γ4-subunit and GAPDH mRNAs in primary chicken cerebellar neurons. Cells were harvested 72 h following transfection with plasmid-based shRNAs and cellular mRNA levels assessed by real-time RT-PCR. (a) $GABA_A$ receptor γ4-subunit mRNA levels in neurons were considerably reduced after transfection with the corresponding shRNA, whereas levels of GAPDH remained relatively unaffected, along with mRNA expression of the three housekeeping genes (18s rRNA, β2-microglobulin and β-actin). (b) GAPDH mRNA levels were decreased in cells in response to transfection with a sequence-specific shRNA, but levels of other mRNAs remained unaffected. (c) Neither γ4-subunit or GAPDH mRNA levels were noticeably affected by transfection with a non-targeting shRNA or (d) pRK5-Clomeleon vector alone. (n = 2). No template controls (for validating RT-PCR reactions) were negative (data not shown).

Expression of neither of the target mRNAs (γ4-subunit and GAPDH) were greatly affected by the transfection of a non-targeting shRNA, nor with the pRK5-Clomeleon vector alone, confirming that transfection was not affecting mRNA expression. Real-time RT-PCRs also illustrated consistent expression of the housekeeping genes under all different experimental conditions and validated the real-time RT-PCRs themselves. Due

to time restraints and limitations of resources, the *in vitro* RNAi experiment was only completed twice in its entirety and was not fully quantified, but nonetheless it provided a good indication as to the future success of this work.

6.3 Discussion

It is well documented that to understand true gene expression and thus the phenotype of a cell, mRNA and protein data is required; if either of these are overlooked it can lead to erroneous conclusions (Hatzimanikatis *et al*., 1999; Hatzimanikatis and Lee, 1999; Greenbaum *et al*., 2003; Yu *et al*., 2007). It may be assumed that for the most part, mRNA is faithfully translated into protein. However, within the zebra finch song system, some dissociation has been revealed. *ZENK* (also known as *Zif268*, *Egr-1*, *NGFI-A*, *Krox-24* and *tis8*; Whitney *et al*., 2000) is an immediate early gene, and corresponding mRNA expression has been well studied within the zebra finch song system (reviewed by Johnson and Whitney, 2005). Within HVC and RA, ZENK mRNA is well expressed, increasing as song development progresses. In HVC, ZENK protein levels increase in correlation with mRNA levels (indicating faithful translation of mRNA into protein. However, in RA, despite increases in mRNA levels, there are no increases in ZENK protein until the birds begins singing plastic song. This is indicative of post-transcriptional regulatory mechanisms and the authors speculate that this is behaviourally, and not developmentally linked (Whitney *et al*., 2000). Previous work has shown that $GABA_A$ receptor γ4-subunit gene expression is down-regulated in response to learning of song (refer to Chapter Five). Akin to *ZENK*, this perhaps points towards behaviourally-linked regulation of gene expression, although with the γ4-subunit this appears to be transcriptional regulation; but it is necessary to determine if this correlation appears at a protein level. Certainly a decrease in mRNA levels would inevitably lead to a decline in protein levels as the available template for translation is reduced; however, it must be ascertained if the decrease in mRNA levels is due to decreased rate of transcription, increased protein synthesis or increased mRNA degradation.

With regards to determining native $GABA_A$ receptor stoichiometry, evidence for true subunit association must firstly be established by co-localisation of subunit mRNAs and corresponding proteins in tissues/cells. Subcellular distribution should then be investigated (as a single neuron can express several different $GABA_A$ receptor

subtypes), followed by a demonstration of subunit interactions e.g. by co-precipitation studies (Olsen and Sieghart, 2008). In order for all of these criteria to be fulfilled, it was imperative to have a γ4-subunit-selective antibody. However, no such antibody was commercially available, so one had to be synthesised. Generation of a specific antibody would allow analysis as mentioned above, but would also be of use in the RNAi work to enable validation of γ4-subunit protein silencing.

6.3.1 γ4-subunit-specific antibody generation

The large number of subunits forming the $GABA_A$ receptor subunit gene family and the high similarity that exists within classes of subunits (>70%) and even between classes (~30%) makes generation of highly specific antibodies a difficult task. The majority of antibodies directed against the $GABA_A$ receptor subunits are polyclonal. This can be problematic as the composition of individual polyclonal antibodies is variable with each donor animal (Olsen and Sieghart, 2008). However, a paucity of well characterised and highly selective monoclonal antibodies has meant that the majority of data is obtained by use of the polyclonal variety. The strategy undertaken herein was to synthesis a protein antibody (specific to the $GABA_A$ receptor γ4-subunit) by use of a fusion protein system. This enabled the expression and purification of a protein from a cloned gene. Results showed the γ4-subunit-MBP fusion protein had a molecular mass of 46 kDa when analysed by SDS-PAGE which correlated with the predicted molecular mass (MBP = 42.5 kDa + γ4-subunit peptide 3.63 kDa). Subsequent immunoblotting with an anti-MBP antibody confirmed the product as the MBP-fusion protein (Fig. 6.5a and b). Following affinity purification with an amylose column, SDS-PAGE and western blotting revealed a single product was purified of the appropriate molecular weight which was identifiable as the MBP-fusion protein with use of the specific anti-MBP antibody (Fig. 6.6b and c). There were no contaminants or evidence of degradation found within the eluted samples (which can sometimes be a problem with this system; de Pieri *et al*., 2004), which were all pooled (those fractions containing eluted fusion protein) and concentrated. The next stage of the work would be to cleave the fusion protein by hydrolysis of the integrated cleavage site and then the protein would be ready for commercial synthesis into a protein antibody. This is a commonly used technique for the synthesis of protein antibodies and has proven successful in previous studies (Kang *et al*., 2005; de Pieri *et al*., 2004). Several $GABA_A$ receptor subunit-specific

antibodies have been generated using this method including those targeting β3 (Todd *et al*., 1996), γ1 (Mossier *et al*., 1994) and γ3 (Tögel *et al*., 1994).

6.3.2 Silencing of the $GABA_A$ receptor γ4-subunit gene

The wide heterogeneity and relative promiscuity of $GABA_A$ receptor subunits culminate in a plethora of potential subtypes, each with a slightly different electrophysiological, biochemical and pharmacological profile. Coupled with their differential expression patterns throughout the brain, it suggests they have evolved to fulfil specific physiological functions (Wafford *et al*., 2004; Olsen and Sieghart, 2008). As aforementioned, various reverse-genetic strategies have been employed in attempts to identify the roles of particular subunits/subtypes (reviewed by Burt, 2003). Work herein has attempted to elucidate the role of γ4-subunit-containing $GABA_A$ receptors in song acquisition and production in the male zebra finch, which is a well established learning and memory paradigm. The advent of RNAi technology allows for effective, inexpensive and rapid analysis of gene function. The ultimate aim of the RNAi work was to utilise *in vivo* bilateral injections of $GABA_A$ receptor γ4-subunit-targeting shRNAs directly into specific nuclei of the zebra finch song system, silence γ4-subunit mRNA/protein expression, and observe subsequent effects on song acquisition and production. An experiment of this nature has only been carried out once previously whereby *FOXP2* (which encodes the forkhead box transcription factor) was silenced *in vivo* by direct bilateral injections of lentivirus mediated shRNA into Area X of zebra finch. Silencing persisted throughout the entire song learning phase (PHD 23-~90) and reduced mRNA and protein levels resulted in birds which less accurately mimicked their tutor's song and produced song with abnormally variable syllables (Haesler *et al*., 2007). Akin to the γ4-subunit, previous work with the zebra finch had shown *FOXP2* to be of potential importance during song-learning (Haesler *et al*., 2004) and mutations in the human homologue of the gene resulted in language impairments (Lai *et al*., 2001; 2003; MacDermot, 2005), although no such work has been carried out with the mammalian orthologue of γ4, ε. In chicken, a learning-associated down-regulation of γ4-subunit mRNA expression was observed during imprinting in learning-relevant regions of the brain (Harvey *et al*., 1998). There is high sequence conservation between zebra finch and chicken γ4-subunits (97% over 271 AA). Corresponding mRNA is robustly expressed in all nuclei of the zebra finch song system (Thode *et al*., 2008), so it

can be assumed their respective functions are highly conserved. Accordingly, a similar learning-associated down-regulation of γ4-subunit mRNA was evident in the zebra finch (refer to Chapter five), this gene was therefore an ideal candidate for investigation with RNAi. If the role of γ4-subunit-containing $GABA_A$ receptors in song production in zebra finch could be confirmed, data could be extrapolated to humans which have the ε subunit, the orthologue of avian γ4 (Darlison *et al*., 2005).

Although the degree of silencing was not fully quantified, real-time RT-PCRs indicated a decrease in levels of γ4-subunit mRNA in cells transfected with the corresponding shRNA. This decrease in expression was not observed in cells transfected with a GAPDH-targeting shRNA, non-targeting shRNA or with the pRK5-clomeleon vector alone. However, silencing was not absolute; some γ4-subunit cDNA was amplified in the real-time RT-PCR reactions. This is to be expected, as RNAi is not a complete *knockout* technique, but a *knockdown* technique, where 75-90% gene silencing may be considered average. However, this is considered advantageous as it reduces the chances of inducing a lethal phenotype and compensatory upregulation of other genes (Dykxhoorn *et al*., 2003). Another factor impeding the degree of silencing is the transfection efficiency. Primary cell cultures more accurately represent the biology of normal cells but they tend to be far more sensitive to chemical or physical stress and so are notoriously difficult to transfect (Zeitelhofer *et al*., 2007), where often transfection efficiency can be around 20% (McManus and Sharp, 2002). This is important in RNAi experiments as proteins synthesised by untransfected cells will compromise analysis (Zeitelhofer et *al*., 2007). In efforts to circumvent this in other studies, electroporation was introduced. This method uses an electrical field which transiently creates pores in cell membranes allowing DNA and RNA molecules to pass directly into the cell. Although this can increase transfection efficiency up to 95%, it also promotes cell death (~50%; McManus and Sharp, 2002). Despite this, electroporation has been widely employed *in vitro* (Guigetnet and Mayer, 2008; Zeitelhofer *et al*., 2007) and *in vivo* shRNA-mediated silencing via electroporation of chicken embryos has documented silencing efficiencies of up to 90% (Pekarik *et al*., 2003; Dai *et al*., 2005; Das *et al*., 2006). Nevertheless, successful transfection with more traditional lipid-based carriers has also been achieved (Dalby *et al*., 2004; Tönges *et al*., 2006), however these can prove toxic when utilised long-term (Davidson *et al*., 2004). In short term *in vitro*

experiments such as these, where longevity of the culture is not necessary, lipid-based transfection reagents can be used for induction of RNAi (Krichevsky and Kosik, 2002). Therefore, continuation of this work would involve testing different transfection strategies to improve efficiency. It is also important to complete a titration of shRNA constructs to ensure the lowest concentration is used to elicit silencing; too high concentration of shRNAs can result in off target effects and potentially saturate siRNA machinery (Castanotto *et al*., 2007; Shan, 2009). Also of consideration is that introducing artificial si/shRNAs into cells may cause competition with endogenous miRNAs which are involved in regulation of cellular gene expression (Shan, 2009). Therefore, some off-target effects may be attributable to interference with the miRNA pathway, so this needs to be verified in future work. Furthermore, before commencing *in vivo* work, it is essential to verify corresponding protein is being silenced. In this regard, the turnover rate of the protein should be ascertained so that residual proteins are not masking the effects of the shRNAs (Choi *et al*., 2005; Zeitelhofer *et al*., 2007). The half-life of $GABA_A$ receptors in chicken neuronal culture is ~25 h and subunit-mRNA ~9 h (Lyons *et al*., 2000). A computer-predicted miRNA precursor has been identified which encodes the $GABA_A$ receptor ε subunit (the mammalian orthologue of γ4; Dostie *et al*., 2003). miRNA precursors are highly conserved between fairly divergent species (Dostie *et al*., 2003) so it is likely that a miRNA precursor may be present to regulate γ4-subunit gene expression. Taking this into consideration, the levels of γ4-subunit protein must be carefully quantified across all experimental conditions to ensure that any knockdown is mediated by the synthetic shRNA and not endogenous miRNA molecules.

Silencing of a constitutively expressed gene (at the same dosage as shRNA targeting the gene of interest) indicates successful RNAi-mediated knockdown (Shan, 2009). GAPDH mRNA was successfully silenced (with no observable effects on γ4-subunit mRNA levels), indicating that the mRNA knockdown was due to induction of RNAi via shRNAs and not endogenous regulatory mechanisms. Thus it can be assumed that the apparent decrease in γ4-subunit mRNA was also attributable to the shRNAs, although these findings need to be further verified. It was also paramount to include negative controls. In previous work it was suggested that a scrambled control sequence or a sh/siRNA with a single base-pair mismatch was suitable. However, more recent

discoveries involving the miRNA pathway (refer to section 6.1.5.1) reveal that even mis-matching duplexes can cause silencing, or off-target effects (Alvarez *et al*., 2006; Birmingham *et al*., 2006). In neurons, inducing RNAi causes competition with the endogenous miRNA pathway and results in phenotypic effects such as a decrease in length and branching of dendritic spines (Alvarez *et al*., 2006). This is primarily of importance in long-term silencing, but would need to be considered if work is taken to an *in vivo* level in zebra finch. Thus, it is recommended that a non-targeting sequence be employed (Anon, 2003; Shan, 2009). Only seven consecutive complementary bases are necessary to induce protein silencing via translational arrest (Jackson and Linsley, 2004), so a non-targeting construct requires careful selection. The negative control shRNA used here has proven successful in previous RNAi studies in chicken (Rumi *et al*., 2006) and results herein showed no observable effects on levels of GAPDH or γ4-subunit mRNA. Transfection of pRK5-Clomeleon vector alone ensured no transfection-specific problems were arising, as this does not utilise the RNAi machinery.

Although the results here were only preliminary (n = 2), and not fully quantified, they indicate that the synthesised shRNA duplexes may efficiently silence the $GABA_A$ receptor γ4-subunit and GAPDH genes. This paves the way for this work to be taken to an *in vivo* level, which will clearly identify the role that $GABA_A$ receptors comprising this subunit may play in learning and memory. The only example to date of RNAi being used to investigate molecular mechanisms of learning and memory is in nymphal crickets (*Gryllus bimaculatus*) where long-term potentiation (LTP) was disrupted by silencing of the *NOS* (nitric oxide synthase) gene (Takahashi *et al*., 2009). It may also be insightful to carry out this work in chicken in imprinting experiments as previous work has indicated a role for γ4-subunit-containing receptors in this paradigm (Harvey *et al*., 1998). If γ4-subunit mRNA/protein silencing experiments are successful *in vivo* in zebra finch (with the established protocol), it would be possible to replicate the work with other $GABA_A$ receptor subunit genes, which could potentially identify a particular subtype which is involved in learning in and memory in zebra finch. This may be subsequently be applied to humans to aid in the pursuit of understanding learning and memory processes and treating pathologies affecting these mechanisms via subtype-specific rational drug design.

7. DISCUSSION

The heterogeneity of the $GABA_A$ receptor family is both its redeeming feature and greatest encumbrance. $GABA_A$ receptors mediate the majority of inhibitory neurotransmission within the brain and the plethora of available subunits enables temporal- and regional-specific expression of many different subtypes (Laurie *et al*, 1992a, b; Wisden *et al*, 1992; Sperk *et al*., 1997; Kultas-Ilinsky *et al*., 1998; Huntsman *et al*., 1999; Pirker *et al*., 2000; Pöltl *et al*., 2003). Each subtype has an individual pharmacological and electrophysiological profile (due to the combination of subunits which compose each receptor), which potentially fulfil different physiological functions (Wafford *et al*., 2004; Olsen and Sieghart, 2008). However, the extreme promiscuity of the subunits causes problems in elucidating defined native $GABA_A$ receptor subtypes; this is frequently observed in co-precipitation and expression studies (Sieghart and Sperk, 2002). This work aimed to characterise the expression of $GABA_A$ receptor subunits within a novel paradigm, the zebra finch song system, and determine the role $GABA_A$ receptor-mediated neurotransmission may play in learning and memory processes.

7.1 Isolation of partial cDNAs encoding $GABA_A$ and $GABA_C$ receptor subunits

Isolation of partial cDNAs encoding zebra finch $GABA_A$ and $GABA_C$ receptor subunits yielded several key findings. Peptide alignments with corresponding chicken and rat sequences demonstrated high sequence identity (>90% and >80% similarity respectively), confirming their identity and indicative of a strong conservation of function. The work has highlighted several errors in the annotation of predicted zebra finch and chicken $GABA_A$ receptor subunit sequences available online, and confirmed the need to experimentally determine the sequence of cDNAs and not just rely on *in silico* evidence, generated from algorithms (section 3.4). Nonetheless all isolated sequences shared ~95% similarity with predicted zebra finch $GABA_A$ receptor subunit sequences, released following sequencing of the whole genome. Isolation of π-subunit partial cDNA heralded various interesting points. Firstly, this cDNA was isolated from brain, where levels appeared to be fairly robust (Fig. 4.7, Chapter Four). In contradiction to much previous work which localises π-subunit mRNA to the periphery such as lung (Jin *et al*., 2005, 2006, Xiang *et al*., 2007) and reproductive tissues

(Hedblom and Kirkness, 1997; Fujii and Mellon, 2001), and it has shown promise as a molecular marker for tumours (Backus *et al*., 2005; Johnson and Haun, 2005; Symmans *et al*., 2005). However, there is one example of π-subunit gene expression in mammalian brain. A learning-associated decrease in π-subunit gene expression was observed in rat hippocampus following training in the Morris water-maze (Cavallaro *et al*., 2002). Other than this, there is no evidence of π-subunit gene expression in the brain, so the observed expression in zebra finch brain was an unexpected finding. Not only this, but in zebra finch, a novel π-subunit splice variant was identified which contained a 37AA deletion within exon 7 which corresponds to a region of sequence encoding part of TM2 and TM3, and the extracellular loop lying between them (Figs. 3.3 and 3.4). This is the first report of a π-subunit splice variant in any species and mRNA encoding both the long and short forms were present in zebra finch brain. Splice variants add a further layer of complexity to the $GABA_A$ receptor family, but are of importance as they have been implicated in disease states (Huntsman *et al*., 1998; Dredge *et al*., 2001; Volk *et al*., 2002; Zhao *et al*., 2009) and potentially play a role in trafficking and receptor assembly (Mu *et al*., 2002). Further investigation pertaining to spatial expression of π-subunit mRNA in zebra finch brain is warranted. There is much lower sequence identity between zebra finch and human π-subunit sequences (~80% over 275 AA), compared to other $GABA_A$ receptor subunit sequences. Consequently, the functions may be slightly different between mammals and lower vertebrates. In zebra finch, π-subunit-containing receptors may play a more important role in the brain, hence the more prominent expression and the existence of a splice variant.

7.2 Spatial distribution of $GABA_A$ receptor subunit mRNAs in song system

Involvement of $GABA_A$ receptors in learning and memory processes (i.e. the song system) has been previously studied in zebra finch by pharmacological and electrophysiological techniques (Livinston and Mooney, 1997; Bottjer *et al*., 1998; Dutar *et al*., 1998; Luo and Perkel, 1999; Spiro *et al*., 1999; Vicario and Raksin, 2000; Farries *et al*., 2005; Prather *et al*., 2008). However, this is the first study to qualitatively and quantitatively map mRNA expression of an entire gene family within the brain of any species. The zebra finch is a well established learning and memory model and the neural substrate of song learning, the song system, has been extensively characterised

(Nottebohm *et al*., 1976). Prior to this work, only $GABA_A$ receptor γ4-subunit mRNA had been spatially mapped within the song system and findings showed it to be extremely well expressed in nearly all relevant nuclei (Thode *et al*., 2008).

Expression studies of this nature are notoriously difficult to interpret owing to ubiquitous and overlapping mRNA expression patterns. Although they give a good insight into potential co-expression of $GABA_A$ receptor subunit mRNAs, the limited resolution of *in situ* hybridisation cannot confirm co-assembly. As many $GABA_A$ receptor subtypes can be present on a single neuron, immunocytochemistry at a subcellular level may give a clearer idea of which individual subunits are assembling. However, even this technique is not fully conclusive as different receptor species may be adjacent to each other at the membrane. It is also difficult to use *in vivo* electrophysiological methods due to the presence of multiple receptor assemblies on a single neuron or neuronal population. Single-cell RT-PCR may offer a clearer picture regarding cell-specific expression, but the general consensus is that a variety of complementary techniques (including *in situ* hybridisation and real-time RT-PCR) should be applied which can be contextually related in pursuit of native stoichiometry (Olsen and Sieghart, 2008, 2009). This study successfully demonstrates the wide heterogeneity of $GABA_A$ receptor subunit gene expression within the song system, with each subunit demonstrating a unique expression profile throughout the zebra finch brain. Detection of all subunit mRNAs in each nucleus confirms the documented promiscuity of this family and highlights the complexity of subtype assembly. Moreover, the work provided a platform for further and more specific investigations into $GABA_A$ receptors and the song system. The most striking expression profile was that exhibited by the γ4-subunit gene. No other $GABA_A$ receptor subunit gene was so strongly expressed in all nuclei (LMAN, Area X, HVC and RA), inferring a functional role for receptors comprising this subunit in the acquisition and production of song.

Electrophysiological studies have confirmed that the recombinant α3β2γ4 receptor is fully functional (Forster *et al.*, 2001). However, these forced combinations are not necessarily representative of native $GABA_A$ receptor stoichiometry *in vivo*. When considering the spatial distribution of α3-, β2- and γ4-subunit mRNAs within the song system nuclei, α3- and γ4-subunit genes were well expressed in RA and Area X but the

β2-subunit gene was not specifically expressed within any nuclei of the song system (Figs. 4.3 and 4.4, Chapter Four). It may be assumed that the most likely native stoichiometry would be α3β4γ4 as these genes form a cluster on chromosome 4A of the zebra finch genome (Fig. 3.8, Chapter Three). The same cluster is located to chromosome 4 of the chicken genome and the orthologous genes (α3θε) are located in an identical cluster on the X chromosome of the human genome. The order and transcriptional orientation of the genes is conserved between all three species, thus coordinated expression could be anticipated. β4-subunit gene expression appeared more robust in song-system nuclei when compared to the β2-subunit gene, most notably in HVC and Area X, so potential for co-assembly into an α3β4γ4 subtype exists. Interestingly, similar studies in chicken brain have revealed quite distinct distribution patterns of mRNA encoding β4 and γ4 subunits, suggesting they do not assemble into a single receptor complex (Darlison *et al*., 2005). It was purported some time ago that native $GABA_A$ receptors may comprise of their chromosomal partners (Barnard *et al*., 1998), however there are numerous exceptions to this rule, suggesting chromosomal partnering is not a prerequisite for native receptor assembly (Olsen and Sieghart, 2008). In the case of α1-, β2- and γ2-subunit genes (which are clustered on chromosome 5 in humans and 6 in zebra finches), expression in zebra finch brain was modest and diffuse, with no discernible expression in any of the song system nuclei (Figs. 4.1, 4.3 and 4.5, Chapter Four). As this is the most prevalent subtype in the mammalian brain (Whiting 2003a), the function may be conserved in zebra finch brain (especially as the sequences are so well conserved). The diffuse distribution implicates a generalised function (as in mammals), in contrast to the highly discrete and specific expression of γ4-subunit gene which suggests a more specialised function, perhaps exclusive to the song system. The unique expression of the γ4-subunit gene may point towards homomeric assemblies within song-system nuclei, but this is highly unlikely as the mammalian orthologue, ε is unable to form functional homomers (Jones and Henderson, 2007), and generally homomeric assemblies (of any subunits) are retained within the endoplasmic reticulum for degradation (Kittler *et al*., 2002; Jacob *et al*., 2008). Thus it can be postulated that instead, γ4-subunits form the basis of numerous $GABA_A$ receptor subtypes, within the nuclei of the zebra finch song system, perhaps purveying a unique physiological property not conferred by γ1- or γ2-subunit-containing receptors.

7.3 Potential association of $GABA_C$ and $GABA_A$ receptor subunits

No previous work has considered $GABA_C$ receptors in the song system. There is considerable debate pertaining to the classification of $GABA_C$ receptors, with many groups believing they should be grouped as a subdivision of $GABA_A$ receptors (Olsen and Sieghart, 2008, 2009; Collingridge *et al*., 2009), due to ~35% sequence similarity with $GABA_A$ receptor subunits. However, this would assume that they could co-assemble with $GABA_A$ receptor subunits to form functional receptors, which there is some evidence of *in vitro* (Ekema *et al*., 2002; Milligan *et al*., 2004; Pan and Qian, 2005) but not *in vivo*. Data obtained from recombinant expression studies should be acknowledged with some caution as the polypeptides are often overexpressed, which may cause forced assemblies which are not apparent *in vivo* (Olsen and Sieghart, 2008). Moreover, it must be considered that the properties of native receptors may differ from recombinant receptors due to endogenous phosphorylation or interactions with other endogenous cellular or synaptic proteins, especially as the majority of studies are not carried out in neuronal cultures (Wanamaker *et al*., 2003; Everitt *et al*., 2004). Thus, the forced assembly of $GABA_A$ and $GABA_C$ receptor subunits in recombinant studies may lead to spurious assumptions. There are no gene expression studies which have comprehensively mapped all the $GABA_C$ receptor subunits (ρ1-ρ3) in parallel to $GABA_A$ receptor subunits (as this would be the first step in determining if there was co-expression and thus the potential for assembly; Olsen and Sieghart, 2008). Data obtained from this study gave little indication of co-expression of $GABA_A$ and $GABA_C$ receptor subunit genes within the song system of the zebra finch. ρ1-ρ3 subunits were barely detectable by *in situ* hybridisation in any of the song-system nuclei (Fig. 4.8, Chapter 4). However, all three $GABA_C$ receptor-subunit genes (with particular reference to ρ1) were well expressed within cerebellum (Fig. 4.8, Chapter Four) where they may potentially associate with $GABA_A$ receptor subunits. Although, there are many different cell types within the cerebellum, and so it is equally likely that $GABA_C$ receptor subunits may be present in completely separate neuronal populations from those harbouring $GABA_A$ receptors, forming distinct receptor species. Techniques such as subcellular immunocytochemistry (which has a higher resolution than *in situ* hybridisation), co-precipitation studies and single-cell RT-PCR would be appropriate for further investigations into heteromerisation within cerebellum. But within the

parameters of this study there was no appreciable association between $GABA_A$ and $GABA_C$ receptor subunits in any nuclei of the zebra finch song system.

Surface expression of $GABA_A$ receptors (which are constantly being recycled between the synapse and intracellular endocytic locations, either for recycling or degradation; Bedford *et al*., 2001) should be confirmed, via techniques such as receptor-autoradiography. $GABA_A$ receptors only assemble at an efficiency of ~25%, (Gorrie *et al*., 1997), so it would be of paramount importance to determine which assemblies are actually expressed at the membrane, determining the phenotype of the cell. mRNA levels are certainly indicative of a trend, but ideally should be complemented with protein data to avoid erroneous interpretation of results (Greenbaum *et al*., 2003). Nonetheless, data obtained provides interesting insights and implicates $GABA_A$ receptors, most crucially those comprising the γ4-subunit in learning and memory systems in zebra finch.

7.4 Importance of the $GABA_A$ receptor γ4-subunit

The $GABA_A$ receptor γ4-subunit cDNA was first isolated from the chicken (*Gallus gallus domesticus*), displaying greatest identity to rat γ1, γ2 and γ3 subunits (67%, 69% and 70% respectively; Harvey *et al*., 1993). Following this, the mammalian ε subunit was identified which exhibited highest similarity to the γ4-subunit (49%). This degree of similarity is relatively low (when other receptor subunits are compared across species identity is often >90%), however, there is greater evolutionary diversity when considering the ε subunit as rat and human ε subunit sequences only share 68% identity (Sinkkonen *et al*., 2000). Unlike ε, the γ4-subunit shares considerably higher interspecies sequence identity (e.g. 97% over 271 AA between zebra finch and chicken), indicating a strong conservation of function in lower vertebrates. Within the embryonic chicken brain, γ4-subunit mRNA is well distributed. γ4-subunit transcript is detected at embryonic day (E) 13 and levels continue to increase during embryogenesis (Harvey *et al*., 1993); correlating with expression in the brainstem which is not detected until E8, some time after many other $GABA_A$ receptor subunit mRNAs are detected (Enomoto *et al*., 2001). Strong expression of the γ4-subunit gene within one-day-old chicken brain is apparent in areas essential for visual processing and imprinting, including optic tectum, nucleus rotundus and entopallium (Harvey *et al*., 1998).

Subsequent behavioural studies demonstrated a learning-associated down-regulation of γ4-subunit mRNA 10 h following imprinting in learning-relevant regions of the chicken brain including intermediate medial mesopallium (a sensory sorting centre), entopallium (visual projection area) and posterior medial nidopallium. Such decreases were not observed for γ2-subunit mRNA (Harvey *et al*., 1998), indicative of a role for γ4-subunit-containing $GABA_A$ receptors in learning and memory processes. Following this, the γ4-subunit gene was found to be robustly expressed in almost all the nuclei of the zebra finch song system (Thode *et al*., 2008, Fig. 4.5, Chapter Four). Interestingly γ4-subunit mRNA was only detectable in RA in birds at PHD 35 or older, which coincides with innervation of RA by projections from HVC and establishment of the VMP pathway (Akutagawa and Konishi, 1985; Mooney and Rao, 1994; Scott and Lois, 2007), which strongly implicated a role for receptors comprising this subunit in song production (Thode *et al*., 2008). Data obtained in the current study has further confirmed the relative importance of γ4-subunit containing $GABA_A$ receptors in learning and memory. Corresponding mRNA was robustly distributed throughout the song system, where expression levels appeared to be subject to temporal regulation. Robust distribution and coordinated peaks of gene expression at specific developmental time points during song learning, in the relevant nuclei is indicative of a functional role in acquisition and production of song (Fig. 4.10, Chapter Four). A similar situation is observed in mammalian hippocampus (a brain region associated with spatial learning; Wanatabe and Bischof, 2004), where there is robust expression of α5-subunit receptors. Subsequent knockout of this gene resulted in improved performance in hippocampal-dependent spatial learning tasks (Collinson *et al*., 2002). Akin to this, there was a significant learning-associated down-regulation of γ4-subunit mRNA levels in four nuclei of the song system in both the AFP (LMAN and Area X) and VMP (HVC and RA, Fig, 5.12, Chapter Five). This again exemplifies that a down-regulation of neuronal inhibition is associated with improved cognitive function (Harvey *et al*., 1998; Chapouthier and Venault, 2002, 2004; Collinson *et al*., 2002; McNally *et al*., 2008). The effect was specific to the γ4-subunit as no such decreases were observed for γ2-subunit mRNA (Fig. 5.8, Chapter Five), concurring with previous imprinting-training studies in chicken (Harvey *et al*., 1998). A decrease in mRNA levels may reflect a reduction in the rate of transcription, decreased mRNA stability or an increase in translation. Observed decreases in γ4-subunit mRNA levels represent a long-term response to learning, most

likely reflected by an alteration in gene transcription rather than reduced mRNA stability. It is generally accepted that a reduction in $GABA_A$ receptor-mediated neurotransmission is involved in improved learning (Brioni and McGaugh, 1988; Clements and Bourne, 1996; Chapouthier and Venault, 2002, 2004; Collinson *et al*., 2002; Crestani *et al*., 2002; Zarrindast *et al*., 2006), so the subsequent decrease in γ4-subunit mRNA levels is unlikely to be attributed to an increased rate of translation.

7.5 Regulation of $GABA_A$ receptor subunit gene expression

Gene expression can be regulated at many different levels including rate of initiation of transcription, splicing or stability of mRNA products, translation rate, post-translational modifications (including phosphorylation, glycosylation), receptor assembly, trafficking of receptors and protein stability, e.g. degradation or recycling (Steiger and Russek, 2004). Regulation at the level of transcription is important (Lemon and Tjian, 2000) and is controlled by a variety of transcription factors which directly interact with specific DNA sequences adjacent to the gene. Within $GABA_A$ receptor subunit gene promoter sequences, consensus sites for interaction of transcription factors from a variety of families have been identified via computational analysis. Interestingly, the γ-subunit gene class appear to harbour the greatest number of transcriptional elements. Moreover, the majority of transcriptional binding motifs are unique to each subunit class, thus enabling subunit-specific transcription of α-, β- or γ-subunit genes (Steiger and Russek, 2004). Presence of multiple transcriptional elements, which may work alone or in tandem, facilitates the characteristic spatial expression of $GABA_A$ receptor subunit genes and responses in gene transcription to temporal and environmental cues. *Cis*-regulatory elements which bind tissue-specific factors also permit regional-specific regulation of gene expression. Genes which have CNS-restricted expression encompass a characteristic 21 bp motif (neuron-restrictive silencer element; NRSE), which binds neuron-restrictive silencing factor (NRSF, a zinc finger repressor); NRSF is predominantly found in non-neuronal tissues where it can silence expression of genes which have an NRSE (Joyce, 2007). *In silico* analysis has demonstrated the presence of NRSE-like sequences for the majority of $GABA_A$ receptor subunit genes located in the promoter region (α2, α3, α4, β2, γ1 and δ subunits), or downstream of the start site (α1, α5, β1, β3, γ2, δ and ε subunits; Steiger and Russek, 2004). No NRSE domain was identified in the π-subunit gene, thus accounting for its peripheral distribution. More

recently, regulation of gene expression via the micro (mi)RNA pathway is under closer scrutiny. miRNAs are short, non-coding RNAs which can endogenously regulate gene expression via translational arrest (RNAi pathway; Pillai *et al*., 2005, 2007). A computer-predicated miRNA has been identified for the ε-subunit gene, the mammalian orthologue of γ4 (Dostie *et al*., 2003), and although there is some evolutionary sequence divergence between γ4 and ε, there remains much identity and it is likely such a molecule exists for regulation of γ4-subunit gene expression.

Furthermore, endogenous and exogenous molecules are able to influence $GABA_A$ receptor subunit expression. As detailed in section 1.2.7, neurosteroids are able to potentiate the activity of $GABA_A$ receptors, but they can also govern at a transcription level by binding to hormone-activated nuclear receptors. These function as transcription factors and can interact with specific sites within $GABA_A$ receptor subunit gene promoter regions. Such steroid hormone response elements have been identified in the majority of subunit sequences, which interact with ERs (estrogen receptors), PRs (progesterone receptors), GRs (glucocorticoid receptors) and ARs (androgen receptors). No consensus sites for steroid hormone response elements were found in α2- or θ-subunit sequences (Steiger and Russek, 2004), which correlates with data herein, as the α2-subunit gene showed no evidence of developmentally-regulated expression within the song system. γ1-, γ2- and ε-subunit gene promoters contain AR-consensus motifs and the orthologue of ε, γ4, exhibited highly coordinated gene expression at important developmental stages (Fig. 4.10, Chapter Four). Testosterone is an androgen and thus can modulate transcription of $GABA_A$ receptor subunits in mammals. Due to the high sequence conservation, this would also be applicable in lower vertebrates, including the zebra finch. The song system is sexually dimorphic (Arnold, 1997) and the sexual differentiation in brain nuclei structures in the zebra finch are purported to be a result of exposure to steroid hormones (Cooke *et al*., 1998). In fact both male and female zebra finch nuclei are sensitive to testosterone and estrogen. Testosterone is converted to estrogen by aromatase, which is also present in song system nuclei (Gahr and Metzdorf, 1999; Jacobs *et al*., 1999). More recently, *in vitro* studies have demonstrated an essential role for autonomously synthesised estrogen in development of the HVC→RA connection in zebra finch (Holloway and Clayton, 2001), which may be connected with the large increase in γ4-subunit gene expression in RA following establishment of this

connection. Testosterone, (and its metabolites) are implicated in the crystallisation and stereotypy of zebra finch song (Pröve, 1983; Korsia and Bottjer, 1991; Bottjer and Hewer, 1992; Williams *et al*., 2003; Cynx *et al*., 2005). Furthermore, increased levels of corticosterone, a glucocorticoid (released under stressful conditions) can facilitate or detrimentally affect learning and memory (Coburn-Litvak *et al*., 2003; Martin *et al*., 2009; Quirarte *et al*., 2009). Corticosterone administration during development results in adult zebra finches producing shorter and less complex songs than their untreated counterparts (Spencer *et al*, 2003). Mammalian α3-, β1-, δ- and ε-subunit promoters all contain glucocorticoid receptor consensus sites. Increased levels of corticosterone produced in response to stress would a) potentiate $GABA_A$ receptor activity, thereby increasing inhibition and reducing memory formation and b) effect transcription of $GABA_A$ receptor subunit genes. Accordingly, studies in rats have demonstrated that expression and pharmacology of $GABA_A$ receptors in the hippocampus are affected by stress levels of corticosterone. In some regions of the hippocampus, the β2- and γ2-subunit mRNAs are increased in response to high corticosterone levels (Orchinik *et al*., 1995, 2001). As there is a wealth of evidence to suggest the presence of $GABA_A$ receptors within the song system and evidence that upregulation of $GABA_A$ receptors impairs memory; it may be the case that high levels of corticosterone result in deficits in the memorisation and production of tutor song by increasing $GABA_A$ receptor-mediated neurotransmission. This was also a reason for ensuring the zebra finches raised in auditory isolation were in the presence of females, as the stressful situation of isolation for such sociable animals may have influenced the results. Despite this, direct links between $GABA_A$ receptor subunit expression and neurosteroids in the song-system have not been investigated. There is also evidence to the contrary. The rufous-sided towhee (*Pipilo erythropthalmus*) has a large repertoire, but is a close-ended learner and shows increased song nuclei size and increased testosterone on a seasonal basis, but no change in song (Brenowitz *et al*., 2007). Furthermore, there is no alteration in circulating endogenous testosterone levels in birds which have been mechanically or chemically deafened (Woolley and Rubel, 2002, Brenowitz *et al*., 2007), nor is there a change in nuclei size (Burek *et al*., 1991; Brenowitz *et al*., 2007) which highlights a dissociation between production of song and endogenous neurosteroids.

7.6 Concluding remarks

As $GABA_A$ receptors are targets of many clinically important drugs (benzodiazepines, barbiturates, steroids and volatile anaesthetics; Reynolds, 2008), it is important to resolve specific receptor subtypes involved in mediating different physiological functions in an effort to generate novel subtype-specific (and thus function-specific) therapeutic strategies (such as those being developed for α5-subunit containing receptors; Chambers *et al*., 2004). $GABA_A$ receptors have long been implicated in modulation of learning and memory (for review, see Chapouthier and Venault, 2002; Maubach, 2003; McNally *et al*., 2008), and the avian song system is a well established paradigm for studying neuronal mechanisms of cognitive processes and development of sophisticated motor skills. Due to the numerous parallels with human speech production (Doupe and Kuhl, 1999; Brainard and Doupe, 2002; Kuhl, 2004; Bolhius and Gahr, 2006), defining $GABA_A$ subtypes and their functional roles in this system may extrapolate to human behaviour and subtype-specific strategies can be employed to target cognitive defects and give further understanding into diseases which affect motor coordination. Results obtained from this study complement those obtained by Wada et al. (2004). They observed differential expression of a selection of glutamate receptor subunits/subtypes (which elicit excitatory post-synaptic responses), in various vocal nuclei of songbirds, the functions of which correspond to the properties of each individual nucleus. Data acquired for both inhibitory- and excitatory-eliciting receptors is essential as there is complex interplay between glutamatergic and GABAergic systems in song learning (Bolhius and Gahr, 2006).

This study gives an exclusive insight into the potential role of $GABA_A$ receptors in a learning and memory paradigm. Concurring with data obtained in the mammalian brain, $GABA_A$ receptor subunit mRNAs exhibit ubiquitous, yet unique expression profiles thoughout nuclei of the song system, indicative of many potential assemblies. This was the first complete study of the neuronal $GABA_A$ receptor gene family, and the first study to also consider $GABA_C$ receptor subunits, as only when all subunits are considered can meaningful conclusions be drawn. In addition to spatial regulation, $GABA_A$ receptor subunit mRNAs showed developmentally-correlated patterns of expression, which provided further evidence of a functional role. The most interesting subunit was γ4, which has avoided much scrutiny since its discovery due to its absence

in mammalian species, despite being the orthologue of the mammalian ε-subunit gene (Darlison *et al*., 2005). ε-subunit-containing receptors have also not been thoroughly investigated as they show no sensitivity to benzodiazepines (Davies *et al*., 1997; Whiting *et al*., 1997). Yet, the discrete and proliferate distribution of $GABA_A$ receptor γ4-subunit transcript within nuclei of the avian song system is indicative of an important physiological role. Moreover, zebra finches unable to produce normal song demonstrate a significant increase in $GABA_A$ receptor γ4-subunit transcript expression in nuclei contained in both the AFP and VMP, thereby confirming the theory that down-regulation of GABAergic mechanisms plays a role in learning and memory systems. This effect was exclusive to the γ4 subunit, no such effects were observed with the γ2-subunit transcript. Such discrete distribution of γ4-subunit mRNA suggested a specific functional role. *In vitro* RNA interference experiments indicated that the shRNA constructs were efficient in silencing γ4-subunit mRNA. Once subsequent protein reduction is validated, work could be taken to an *in vivo* level and the function of γ4-subunit receptors further verified. RNAi has successfully been employed in the zebra finch to silence *FOXP2* (which encodes the forkhead box transcription factor) *in vivo*, resulting in birds producing abnormal song (Haesler *et al*., 2007). A mutation in the same gene in humans causes speech and language impairments (Lai *et al*., 2001, 2003; MacDermot *et al.*, 2005), demonstrating how data can be extrapolated from zebra finch to human subjects. As mammals have the γ4-subunit orthologue, ε, this work is relevant by contributing to our understanding of $GABA_A$ receptor involvement in complex cognitive processes.

With regards to determining native $GABA_A$ receptor stoichiometry, evidence for true subunit association must firstly be established by co-localisation of subunit mRNAs and corresponding proteins in tissues/cells. This work has, for the first time, characterised the expression of all the $GABA_A$ receptor subunit genes in the song system revealing subunit-specific and time-specific changes in levels of gene expression, which have been correlated with different stages of the song learning process. A novel splice variant of the π-subunit has been identified which was present, along with the full length transcript in zebra finch brain. Furthermore, evidence has been provided for the involvement of γ4-subunit-containing receptors in learning and memory. Preliminary *in vitro* studies have verified that γ4-subunit gene expression can be silenced with specific

shRNA constructs and paves the way for future *in vivo* studies to further confirm the role of γ4-subunit-containing $GABA_A$ receptors. Subcellular distribution should then be investigated (as a single neuron can express several different $GABA_A$ receptor subtypes), followed by a demonstration of subunit interactions e.g. by co-precipitation studies. A γ4-subunit fusion protein has been generated and purified which can be synthesised into an antibody to allow for future analyses at a protein level. Given that $GABA_A$ receptors containing the γ4-subunit have a relatively unique pharmacology (Forster *et al*., 2001); specific subtype-selective drugs could be applied to ameliorate brain function in learning and memory processes. This information may be extrapolated to human subjects due to the many parallels between human and avian telencephalic systems and the presence of the γ4-subunit orthologue, ε in the CNS of mammals.

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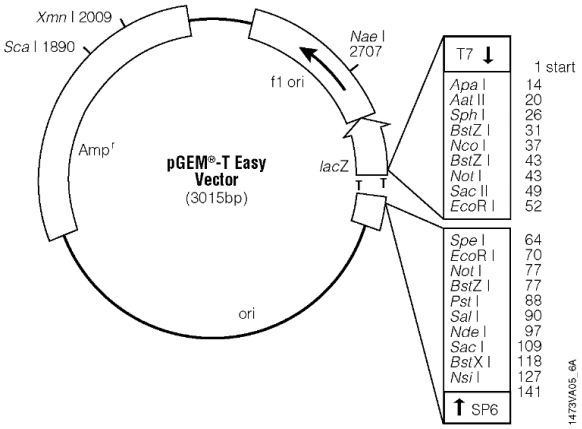
APPENDIX

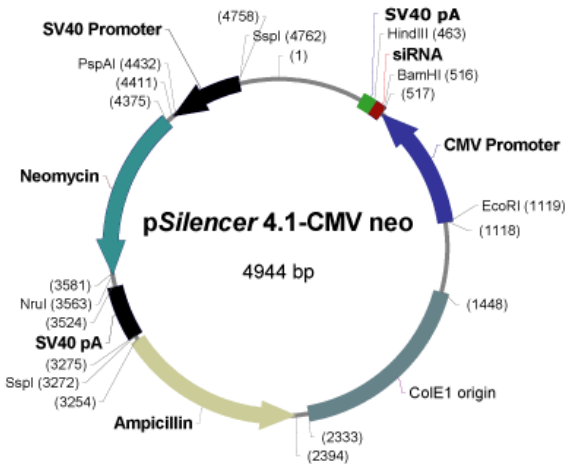


pGEM®-T Easy vector map. The multiple cloning site is flanked by many recognition sites for restriction enzymes. *Eco*RI sites were used to release the cloned sequences. The ampicillin resistance gene enables antibiotic selection and the l*acZ* gene enables blue/white screening for recombinant plasmids.

The following primers were used for sequencing:

SP6: 5´-TATTTAGGTGACACTATAG-3´

T7: 5´-TAATACGACTCACTATAGGG-3´

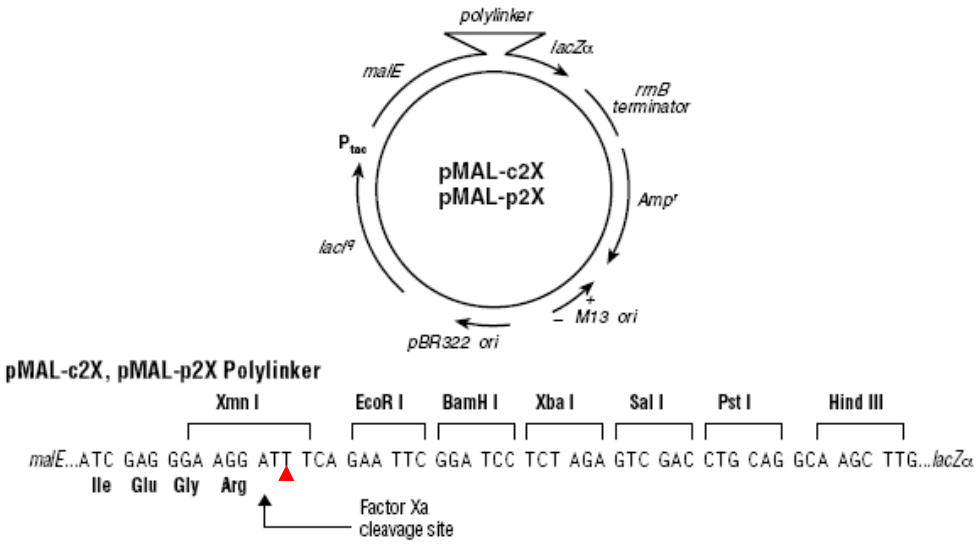


p*Silencer*™ 4.1-CMV neo vector map. Double-stranded oligonucleotides are annealed and ligated into the vector. The powerful human cytomegalovirus (CMV) promoter then drives the expression of the shRNA. The ampicillin and neomycin genes allow for antibiotic selection.

The following primers were used for sequencing:

Forward: 5'-AGGCGATTAAGTTGGGTA-3'

Reverse: 5'-CGGTAGGCGTGTACGGTG-3'



pMAL vector map (~6.5kb). The *malE* gene encodes maltose-binding protein, the arrows denote the direction of transcription. The target gene is inserted in the same translational reading frame as the *malE* gene so the DNA will be expressed by the cell as a single protein. The red arrow indicates the position of insertion of the $GABA_A$ receptor γ4-subunit antibody construct. This site was selected as it required the least mis-matches to be introduced to the construct and positioning at the start of the polylinker ensures minimum vector-derived sequence in the expressed fusion protein. The factor Xa cleavage site is shown, after amylose-affinity purification the target protein will be cleaved from the MBP and purified.

The following primers were used for sequencing:

Forward: 5´-GGTCGTCAGACTGTCGATGAAGCC-3´

Reverse: 5´-CGCCAGGGTTTTCCCAGTCACGAC-3´

GenBank accession numbers (mRNA and peptide sequences):

Rat (α3):	NM_017069 (SEQ)	NP_058765 (PEP)
Chicken (α3):	XM_420268 (SEQ)	XP_420268 (PEP)
Rat (α4):	NM_080587 (SEQ)	NP_542154 (PEP)
Chicken (α4):	XM_420724 (SEQ)	XP_420724 (PEP)
Rat (α6):	NM_021841 (SEQ)	NP_068613 (PEP)
Chicken (α6):	NM_205058 (SEQ)	NP_990389 (PEP)
Rat (γ1):	NM_080586 (SEQ)	NP_542153 (PEP)
Chicken (γ1):	XM_420725 (SEQ)	XP_420725 (PEP)
Rat (δ):	NM_017289 (SEQ)	NP_058985 (PEP)
Chicken (δ):	XM_001234040 (SEQ)	XP_001234041 (PEP)
Chicken (π):	XM_426542 (SEQ)	XP_426524 (PEP)
Chicken (π):	XM_414507 (SEQ)	XP_414507 (PEP)
Zebra finch (π):	XM_002188687 (SEQ)	XP_002188723 (PEP)

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Zebra finch β1  CTGGAGATCGAGAGCTATGGTTACACGGTGGATGACATTGTCTTCTTCTGGCAAGGGAAT
Zebra finch β4  CTGGAGATCGAGAGCTATGGTTACACGGTGGATGACATTGTCTTCTTCTGGCAAGGGAAT
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Zebra finch β1  GACTCTGCTGTCACGGGGATGGAGGTGCTGGAGCTGCCCCAGTTCACCATCATCGAGCAG
Zebra finch β4  GACTCTGCTGTCACGGGGATGGAGGTGCTGGAGCTGCCCCAGTTCACCATCATCGAGCAG
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Zebra finch β1  AGGCTGGTCAGCAGGGAAGTGGTCTTCACCACTGGTTCGTATCTGCGCTTATCCCTGAGT
Zebra finch β4  AGGCTGGTCAGCAGGGAAGTGGTCTTCACCACTGGTTCGTATCTGCGCTTATCCCTGAGT
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Zebra finch β1  TTCCGGATTAAGAGGAACATTGGTTACTTCATCCTGCAGACCTACATGCCATCCATCCTC
Zebra finch β4  TTCCGGATTAAGAGGAACATTGGTTACTTCATCCTGCAGACCTACATGCCATCCATCCTC
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Zebra finch β1  ATCACCATCCTGTCCTGGGTCTCCTTCTGGATCAACTATGATGCTTCTGCAGCACGAGTG
Zebra finch β4  ATCACCATCCTGTCCTGGGTCTCCTTCTGGATCAACTATGATGCTTCTGCAGCACGAGTG
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Zebra finch β1  GCACTGGGGGTCACCACGGTGCTGACCATGACAACCATCAACACCCACCTGCGGGAGACT
Zebra finch β4  GCACTGGGGGTCACCACGGTGCTGACCATGACAACCATCAACACCCACCTGCGGGAGACT
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Zebra finch β1  CTGCCCAAGATCCCCTACGTCAAGGCTATTGATGTTTATCTCATGGGCTGCTTTGTCTTC
Zebra finch β4  CTGCCCAAGATCCCCTACGTCAAGGCTATTGATGTTTATCTCATGGGCTGCTTCGTCTTC
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Zebra finch β1  GTGTTCCTGGCACTCCTGGAATATGCTTTTGTCAACTACATATTCTTCGGGCGAGGGCCG
Zebra finch β4  GTGTTCCTGGCACTCCTGGAATATGCTTTTGTCAACTACATATTCTTCGGGCGAGGGCCG
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Zebra finch β1  CGGCAGCAGAAGAAGCAGA-GCGAGCGCGTCAGCAAGGCCAACAACGAGCGCCACCGTTA
Zebra finch β4  CGGCAGCAGAAGAAGCAGAAGCGAGCGCGTCAGCAAGGCCAACAACGAGCGCCACCGTTA
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Zebra finch β1  CGAGGAGAAGAGGGTGAGAGAGCAGGTTGACCCTTACGGTAACATCCTCCTCAGCACGCT
Zebra finch β4  CGAGGAGAAGAGGGTGAGAGAGCAGGTTGACCCTTACGGTAACATCCTCCTCAGCACGCT
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Zebra finch β1  GGAGATGAACAACGAGCTGCTGGCCACGGACATGATGAGCAGCGTGGGCGACTCTCGAAA
Zebra finch β4  GGAGATGAACAACGAGCTGCTGGCCACGGACATGATGAGCAGCGTGGGCGACTCTCGAAA
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Zebra finch β1  CTCTGTCATGTCCTTCGAAGGCTCAGGAATCCAGTTCCGCAAGCCGCTGGCCTCTCGGGA
Zebra finch β4  CTCTGTCATGTCCTTCGAAGGCTCAGGAATCCAGTTCCGCAAGCCGCTGGCCTCTCGGGA
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Zebra finch β1  TGGCTTTGGCCACCACCCCACCCTGGACCGCCACGTCCCGCTGAGCCACCACGCCGCCGC
Zebra finch β4  TGGCTTTGGCCACCACCCCACCNTGGACCGCCACGTCCCGNTGAGCCACCACGCCGCCGC
                ********************** ***************** ******************

Zebra finch β1  CCGCAACCGCGCCAACTGCCGCCTGCGCCGGCGGTCCTCCAAGCTGAAGCTCAAAATCCC
Zebra finch β4  CCGCAACCGCGCCAACTGCCGCCTGCGCCGGCGGTCCTCCAAGCTGAAGCTCAAAATCCC
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Zebra finch β1  AGACCTGACAGACGTCAGCACCATTGACAAGTGGTCACGGATCATTTTTCCAATCACTTT
Zebra finch β4  AGACCTGACAGACGTCAGCACCATTGACAAGTGGTCACGGATCATTTTTCCAATCACTTT
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Zebra finch β1  TGGATTCTTCAACCTTGTT
Zebra finch β4  TGGATTCTTCATCCTTGTT
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Alignment of the predicted zebra finch $GABA_A$ receptor β1-subunit nucleotide sequence (single-letter code) with the zebra finch β4-subunit sequence cloned within the laboratory. There is 99% sequence identity, confirming the predicted β1-subunit sequence to be that encoding β4. (*) denote identity between the sequences.