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$GABA_A$ Receptors: Gene Expression and Evolution

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Abstract

γ-Aminobutyric acid (GABA) is the principle inhibitory neurotransmitter in the vertebrate central nervous system. It binds to three classes of receptor, namely, the $GABA_A$, $GABA_B$ and $GABA_C$ receptor. The $GABA_A$ receptor exists as a pentamer, formed by the association of different types of subunits (α1-α6, β1-β3, γ1-γ3, δ, ε, π and θ). Two further subunits have been identified in the chicken namely the β4-subunit (replacing the mammalian β1-subunit), and the γ4-subunit (replacing the mammalian γ3-subunit).

The aims of this project were to study the molecular evolution of $GABA_A$ receptor β-subunit genes, investigate changes in gene expression and search for a potential evolutionary cluster by *in silico* analysis. The former was carried out by cloning $GABA_A$ receptor β-subunit complementary DNAs (cDNAs) from various lower vertebrate species, and searching published genome databases for these genes. *In situ* hybridisation experiments determined the distribution of the $GABA_A$ receptor β4-subunit mRNA in the one-day-old chick brain and the zebra finch brain (both provide useful models of learning, namely, imprinting and song learning, respectively).

Unexpectedly four $GABA_A$ receptor β-subunit cDNAs were isolated from the zebra finch. *In silico* analysis of the puffer fish draft genome sequence also revealed the presence of four $GABA_A$ receptor β-subunit-like genes. The genome of sea squirt revealed a cluster of $GABA_A$ receptor α- and β- like and glycine receptor-like subunit genes, which are highly, conserved with vertebrate $GABA_A$ receptor genes both in gene structure and transcriptional orientation. The β4 subunit was present in other species e.g. common wall lizard and African clawed frog hence, not avian specific.

The β4-subunit mRNA was detected in the medial striatum and the mesopallium, in adult and juvenile zebra finch brain, both areas that contain nuclei important for song production. The chicken genome revealed a cluster of $GABA_A$ receptor α3, β4 and γ4 subunit genes orthologous to the cluster of genes on the X chromosome in man. In the one-day-old chick brain, the distribution of this subunit mRNA appeared similar in some brain areas to that of the α3 and γ4-subunit mRNA, suggesting that these genes are co-ordinately regulated forming native receptor subtypes in some brain areas.

Acknowledgements

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Thanks to everyone in the lab for making it a great place to work. Thanks to Anne for her cheerfulness and helping me bake all those troughs! A big thank you to all in the post grad room (both past and present), for some memorable times. Last but not least I would love to express my gratitude to my family, friends and Ankar for their support, love and always being there to keep me going!

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Introduction

1.1 The neurotransmitter GABA

γ-Aminobutyric acid (GABA) is the predominant inhibitory neurotransmitter in the vertebrate central nervous system (CNS), and is also found in invertebrate nervous systems. GABA was first discovered to be an inhibitory neurotransmitter in the mammalian brain in 1950 by Awapara *et al.* Research conducted by Otsuka *et al.* (1966) supported this as they demonstrated that GABA is released upon the stimulation of the inhibitory nerve in the crustacean *Homarus americanus* (lobster). Interestingly, the release of GABA was proportional to the frequency and duration of stimulation. The enzyme glutamic acid decarboxylase (GAD) is responsible for the synthesis of GABA from L-glutamic acid (Erlander and Tobin, 1991; Martin and Rimvall, 1993), of which there are two forms GAD_{65} and GAD_{67} in mammals (Bu *et al.*, 1992). The former synthesises 10% of GABA and is the major isoform in the pancreas, whereas 90% of GABA is synthesised by the latter in the brain (Christgau *et al.*, 1992). GABA is a relatively small molecule as illustrated by the structural formula (Figure 1).

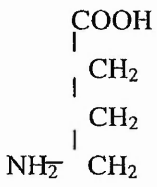

Figure 1: The structural formula of the inhibitory neurotransmitter GABA.

GABA has many important functions, including roles in the processing of sensory information, the co-ordination of motor output, and negative feedback mechanisms. The GABAergic system has been associated with numerous neurological disorders, e.g. epilepsy and schizophrenia. Numerous studies have demonstrated that GABA has an inhibitory effect on seizures (Roussinov *et al.*, 1976; Schechter *et al.*, 1977). Perry *et al.* (1979) found during post-mortems that the level of GABA in the nucleus accumbus and thalamus brain regions was significantly reduced in patients who had suffered from schizophrenia. There are three classes of GABA receptor: $GABA_A$, $GABA_B$ and $GABA_C$ receptors. $GABA_A$ receptors are agonist-gated ion channels, whereas $GABA_B$ receptors are G-protein coupled receptors that couple to Ca^{2+} and K^+ channels. Hence

fast responses are mediated by ionotropic $GABA_A$ receptors and slow responses by G-protein coupled $GABA_B$ receptors (Connors *et al.*, 1998). $GABA_C$ receptors are also agonist-gated ion channel receptors, but they are pharmacologically distinct from $GABA_A$ receptors.

$GABA_A$ receptor dysfunction has also been implied in other conditions including premenstrual syndrome, Alzheimer's disease and sleep disturbances. Adkins *et al.* (2001) found that the endogenous neurosteroid pregnalone had a higher affinity to the $GABA_A$ receptor δ subunits, which resulted in exacerbated premenstrual symptoms e.g. anxiety. Mizukami *et al.* (1998) demonstrated by *in situ* hybridisation that levels of the $GABA_A$ receptor β3-subunit mRNA was reduced in the hippocampus region of the brain of patients who had been diagnosed as suffering from severe Alzheimer's Disease. Radioligand binding studies by Sullivan *et al.* (2004) revealed that the drug Indiplon (a sedative agent with a potential to treat insomnia) showed highest binding to the $GABA_A$ receptor α1 subunit. The $GABA_A$ receptor has long been accepted as the target for numerous clinically important compounds such as the anxiolytic benzodiazepines (BZs), as well as barbiturates, neurosteroids and certain volatile anaesthetics (Sieghart, 1995; Watanabe *et al.*, 2002; Davies, 2003; Lambert *et al.*, 2003). Barbiturates enhance the binding affinity of GABA and at high concentrations mimic GABA by opening the channel in the absence of GABA (Yang and Olsen, 1987). Bicuculline a competitive antagonist of the $GABA_A$ receptor acts by decreasing both the frequency and duration of channel opening (Macdonald *et al.*, 1989). In contrast, benzodiazepines act by increasing the frequency of the $GABA_A$ receptor channel opening (Macdonald and Barker, 1977). Benzodiazepines e.g. diazepam are used for their anti-convulsant, sedative and muscle relaxation properties, however they have side effects e.g. amnesia, hence more knowledge of the $GABA_A$ receptor is needed to increase selectivity (Möhler *et al.*, 2002). As a consequence of its modulation by BZs, the $GABA_A$ receptor has attracted a great deal of activity, particularly from the pharmaceutical industry. Initial efforts to purify this receptor (Sigel *et al.*, 1983; Sigel and Barnard, 1984; Schoch *et al.*, 1985) indicated that it comprised only two polypeptide chains of ~53,000 and ~57,000 Da, which, in retrospect, was difficult to reconcile with early pharmacological studies that had suggested the existence of two receptor subtypes, named BZI and BZII (Sieghart and Karobath, 1980). Another peculiarity was observed upon expression of the first cloned complementary DNAs (cDNAs) for the $GABA_A$ receptor (Schofield *et al.*, 1987). Experiments conducted in *Xenopus laevis* oocytes showed that the bovine α

and β subunits (now referred to as α1 and β1) formed a receptor that responded only weakly to BZs; similar results were reported in the rat and human for receptors encompassing α1 and β1 subunits (Malherbe *et al.,* 1990). Subsequently, the group of Peter Seeburg verified that a third type of polypeptide (a γ subunit) was needed to present the full range of BZ responses on $GABA_A$ receptors (Pritchett *et al.,* 1989). By 1999, 16 different mammalian $GABA_A$ receptor subunits had been recognised using molecular cloning techniques (Sieghart, 1995; Davies *et al.,* 1997; Hedblom and Kirkness, 1997; Hevers and Lüddens, 1998; Bonnert *et al.,* 1999; Watanabe *et al.,* 2002; Darlison *et al.*, 2005).

1.2 The $GABA_A$ Receptor

The $GABA_A$ receptor mediates the majority of fast synaptic inhibition in the mammalian CNS. The binding of two molecules of GABA is believed to result in a structural change (Horenstein *et al.,* 2001; see Figure 2) that leads to the opening of an integral anion-selective channel and the subsequent flow of ions (particularly chloride ions) down a concentration gradient. Usually, this causes a hyperpolarisation of the post-synaptic membrane, however, early in development activation leads to neuronal excitation because, at this ontogenic stage, the concentration of chloride ions is greater within the cell than in the extracellular space (see Ben-Ari, 2002). The excitatory action may be needed for cell maturation and regulating DNA synthesis by Ca^{2+} entry to regulate neocortical neurogenesis (LoTurco *et al.*, 1995; Brickley *et al.*, 1996).

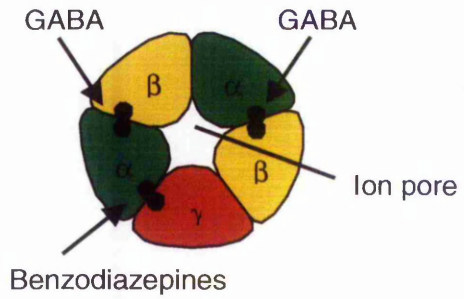

Figure 2: The pentameric structure of the $GABA_A$ receptor with the most common subunit arrangement of two α, two β and one γ subunit. GABA binds between α and β subunits and two molecules of GABA are required for opening of the integral ion channel. Benzodiazepines, used to treat neurological disorders, e.g. anxiety and insomnia (Farkas and Crowe, 2000) bind between α and γ subunits.

Molecular cloning studies in mammals have resulted in the classification of numerous $GABA_A$ receptor subunits, named $\alpha1$–$\alpha6$, $\beta1$–$\beta3$, $\gamma1$–$\gamma3$, δ, ε, π and θ, each of which is encoded by a separate gene (Sieghart, 1995; Davies *et al.*, 1997; Hedblom and Kirkness, 1997; Hevers and Lüddens, 1998; Bonnert *et al.*, 1999; Watanabe *et al.*, 2002; Darlison *et al.*, 2005). There is evidence for at least 30 $GABA_A$ receptor subtypes having different physiological and pharmacological properties (Sieghart and Sperk, 2002; Fritschy and Brunig, 2003; Whiting, 2003). Each receptor subunit polypeptide comprises a signal peptide (~22 base pairs), large amino-terminal extracellular domain (220 to 250 amino acids containing sites for glycosylation), and a dicysteine loop. There are four hydrophobic transmembrane domains (M1–M4; M2 forms the wall of the channel pore) with a large intracellular loop of variable length between M3 and M4, and a short carboxy-terminus that is extracellular (see Figure 3).

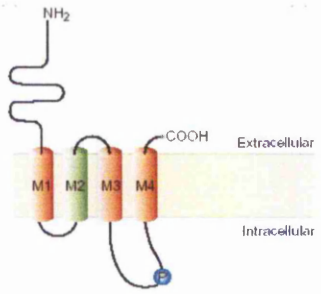

Figure 3: Topology of a $GABA_A$ receptor subunit (from Bormann, 2000). Each subunit is comprised of four transmembrane domains (indicated M1-M4) and a large intracellular loop between transmembrane domains 3 and 4, which contains a consensus site for phosphorylation (P).

These polypeptides have been classified solely on the basis of sequence identity, with subunits being categorised to a particular class (e.g. α, β or γ) if they exhibit 60% to 80% sequence identity to other members; subunits from different classes, i.e. α and β subunits, typically share only 30% to 40% sequence identity (Darlison *et al.*, 2005). Interestingly, the ε and θ subunits (Davies *et al.*, 1997; Wilke *et al.*, 1997; Bonnert *et al.*, 1999; Sinkkonen *et al.*, 2000) display ~50% identity to γ and β subunits, respectively. $GABA_A$ receptors, like nicotinic acetylcholine receptors, glycine receptors, the 5-hydroxytryptamine type 3 ($5\text{-}HT_3$) receptor and a recently-discovered protein, ZAC, which is a zinc-activated channel (Davies *et al.*, 2003), belong to the superfamily of agonist-gated ion channels and are believed to possess a pentameric structure

(Nayeem *et al.,* 1994; Baumann *et al.,* 2001, 2002). Surprisingly, recombinant $GABA_A$ receptors can be formed from many different combinations of polypeptides (for review, see Sieghart, 1995; Moss and Smart, 2001; Whiting, 2003), although it is generally accepted that most receptors possess 2 α subunits, 2 β subunits and 1 γ subunit (Backus *et al.,* 1993; Chang *et al.,* 1996; Baumann *et al.,* 2002; see Figure 2). It is hypothesised that the diversity of receptor subtypes could be important for subcellular localisation, e.g. the α1 subunits are equally distributed at synapses, whereas the α2 subunits are only found at axo-axonal synapses (Moss and Smart, 2001). Regardless of the potential for a vast number of different receptor subtypes, it has been suggested that the majority of all mammalian $GABA_A$ receptors are of the $\alpha1\beta2\gamma2$ type (Whiting, 2003; see Figure 4).

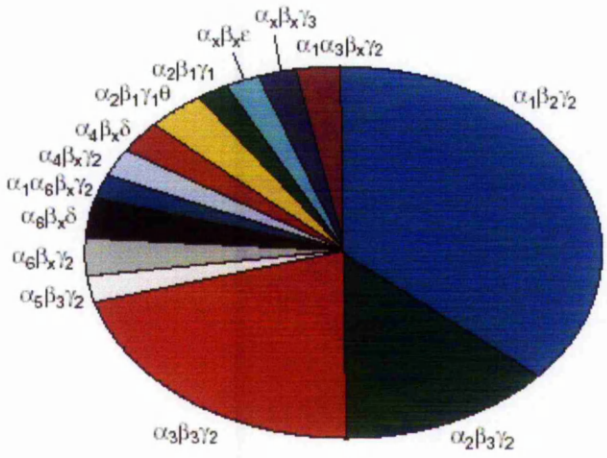

Figure 4: Pie chart showing the relative abundance of $GABA_A$ receptor subtypes in the rat brain. The subscript x indicates that the subunit is not yet known (Whiting, 2003).

Additional heterogeneity of native $GABA_A$ receptors occurs due to alternative splicing of primary gene transcripts (see Whiting *et al.,* 1990; Darlison and Albrecht, 1995; Poulsen *et al.,* 2000, and references cited therein), although the functional significance of many of these variants is uncertain. Interestingly, tissue-specific splicing occurs most frequently in neuronal cells (Stamm *et al.,* 2000; Xu *et al.,* 2002). One of the best studied examples is the alternatively-spliced 24-basepair exon, within the γ2-subunit gene, that encodes an additional 8 amino acids that are located within the long intracellular loop between M3 and M4 (Whiting *et al.,* 1990), and which contains a consensus sequence for phosphorylation by protein kinase C. The two polypeptides produced, γ2S and γ2L, were initially reported to confer differential sensitivity to

ethanol on recombinant $GABA_A$ receptors (Wafford *et al.,* 1991), though not all groups have been able to reproduce these findings (e.g. Sigel *et al.,* 1993). Interestingly, a mouse lacking the 24-basepair exon has been created (Homanics *et al.,* 1999), and this displays no behavioural differences when compared to the wild-type mouse except for having a higher level of anxiety. This "knock-out" mouse only produces the γ2S form of the γ2 subunit, yet it and the wild-type animal exhibit similar sensitivity to ethanol.

1.3 Evolution of the $GABA_A$ Receptor Gene Family in man

Original gene mapping studies, which showed that the α2- (*GABRA2*) and β1- (*GABRB1*) subunit genes were both positioned in chromosome bands 4p12-p13 (Buckle *et al.,* 1989), indicated that $GABA_A$ receptor subunit genes might be clustered within the human genome. It is now evident that 14 of the 16 human $GABA_A$ receptor genes are clustered on four chromosomes, namely, in 4p12-p13, 5q31-q35, 15q11-q13, and Xq28 (see Wilcox *et al.,* 1992; Russek and Farb, 1994; Bailey *et al.,* 1999b; Russek, 1999, Simon *et al.*, 2004 and references cited therein). Two clusters of four genes each encode two α subunits, one β subunit and one γ subunit (*GABRA2*, *GABRA4*, *GABRB1* and *GABRG1* on chromosome 4, and *GABRA1*, *GABRA6*, *GABRB2* and *GABRG2* on chromosome 5; see Figure 5). The two other clusters each contain three genes: that on chromosome 15 comprises one α-subunit gene (*GABRA5*), one β-subunit gene (*GABRB3*) and one γ-subunit gene (*GABRG3*), and that on the X chromosome consists of one α-subunit gene (*GABRA3*), the θ-subunit gene (*GABRQ*) and the ε-subunit gene (*GABRE*; see Figure 5). However, it should be taken into account that the results of *in silico* analysis of the human genome database by Simon *et al.* (2004) reveal a difference in the order of the subunit genes on chromosome 5 and 15 (see Figure 5) from those found by radiation hybrid mapping (Bailey *et al.*, 1999b).

Since, as noted earlier, the θ and ε polypeptides display ~50% identity to β and γ subunits, respectively (which is a significantly higher identity than is seen between different subunit classes), the θ and ε subunits may be considered to be orthologous to the β and γ subunits. Orthology refers to sequence divergence reflecting a speciation event (Huynen and Bork, 1998) i.e. $GABA_A$ receptor human β2 subunit and the chicken β2 subunits. In contrast, parology can be defined as sequence divergence following gene

duplication i.e. genes existing side by side in the same lineage (Fitch, 2000), e.g. the human $GABA_A$ receptor β1 and β2 subunits.

Based on the above observations, it has been proposed that the mammalian $GABA_A$ receptor genes evolved from a common ancestral gene cluster. As a result of the two whole-genome duplication (tetraploidisation) events that are believed to have occurred early in the evolution of chordates, the ancestral cluster is presumed to have possessed an "α-like", a "β-like" and a "γ-like" subunit gene, (Russek and Farb, 1994; Bailey *et al.,* 1999b; Russek, 1999). To accommodate the fact that two clusters contain only three $GABA_A$ receptor genes, while the other two contain four such genes, it is thought that a tandem duplication of an α-subunit gene took place, in one of the clusters, after the first but before the second tetraploidisation event. If this model is accurate, then it follows that the clusters on chromosomes 4 and 5 arose from a common ancestral cluster, and that the clusters on chromosome 15 and the X evolved from a second, common, ancestral segment of DNA. Furthermore, genes occupying the same position within clusters that have arisen from a common ancestor should be more comparable to one another than to the corresponding genes in clusters that arose from different ancestors. This is supported by the fact that, the sequence similarity between the α2 and α1 subunits, which have the same location within the clusters on chromosomes 4 and 5, respectively, is greater than that between any other pair of α subunits (Ortells and Lunt, 1995). Similarly, the α4 and α6 subunits, which also derive from a common ancestor, are more closely related to one another than to any other α polypeptide. Note that the existence of additional, as yet undetected, α-subunit genes on the X chromosome and on chromosome 15 can be ruled out because the corresponding regions of the human genome have been sequenced (see http://www.ncbi.nlm.nih.gov/genome/guide/human/) and analysed (Simon *et al.*, 2004).

Ohno (1970) originally proposed that two rounds of genome duplication, which permitted gene multiplication, took place during chordate evolution. The first event is thought to have occurred after the divergence of cephalochordates and the second was prior to the origin of the jawed vertebrates (the gnathostomes, i.e. fish and mammals; Holland *et al.,* 1994; see Figure 6). These two rounds of gene duplication have been referred to as the "one-to-two-to-four" rule (see Meyer and Schartl, 1999). Evidence in support of this hypothesis has been acquired from extensive research on another multigene family, the *Hox* family of homeobox genes (see Martinez and Amemiya,

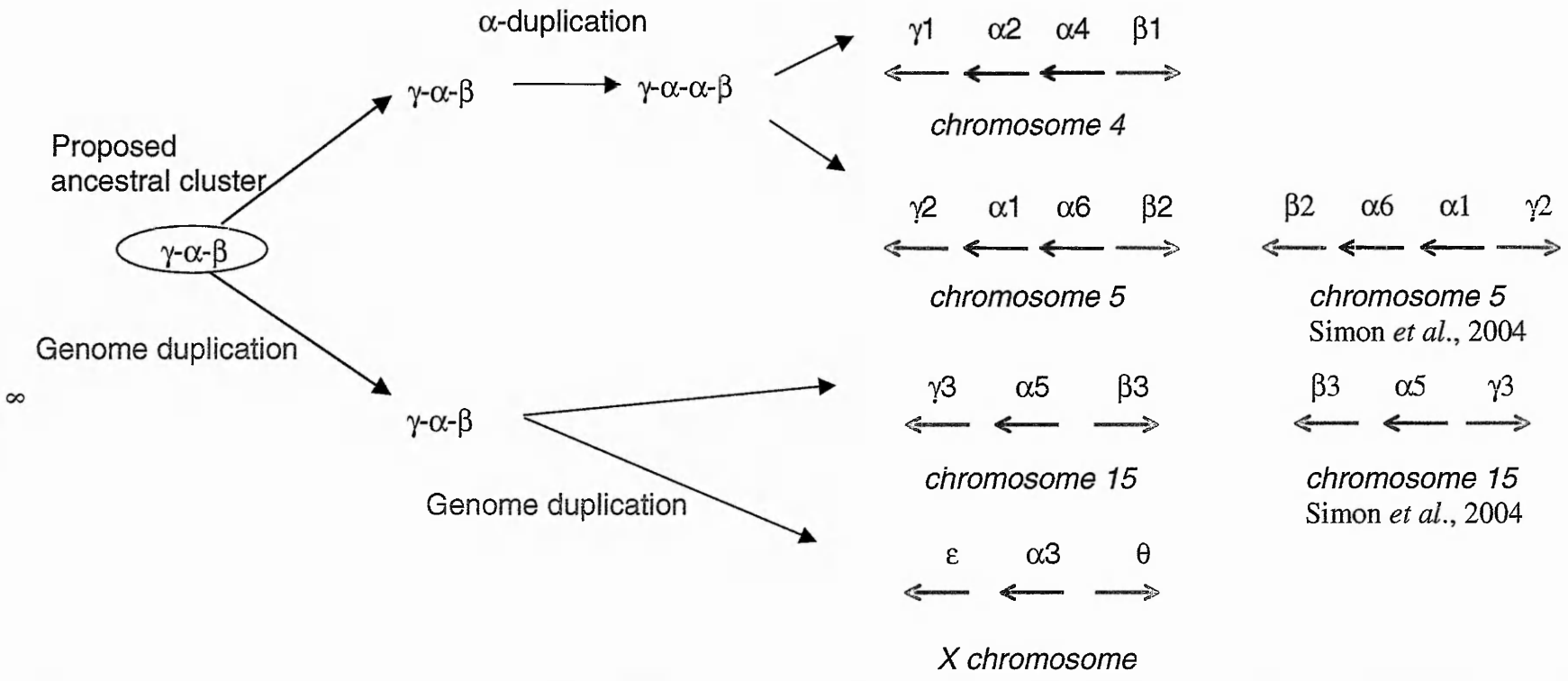

Figure 5: Proposed model for the evolution of $GABA_A$ receptor genes in man. $GABA_A$ receptor gene clusters are located on four chromosomes in humans; 4, 5, 15, and the X and two genome duplications appear to have occurred to give rise to the $GABA_A$ receptor subunits seen today. Since there is an additional α subunit on chromosomes 4 and 5 a further gene duplication is proposed to have occurred. The direction of the coloured arrows denotes the transcriptional orientation.

2002), which play a crucial role in the development of the anterior-posterior axis and body plan in many organisms (Prince, 2002). In mammals, there are 39 *Hox* genes, which occur in four clusters named *HoxA*, *HoxB*, *HoxC* and *HoxD*; in man, they are located on chromosomes 7, 17, 12 and 2, respectively (Hughes *et al.,* 2001). These genes, which encode transcription factors, are transcribed in a specific temporal and spatial sequence that mirrors their chromosomal order (Kmita and Duboule, 2003). Four *Hox* gene clusters can also be found in the puffer fish (*Takifugu rubripes*), a ray-finned fish, which arose after the second tetraploidisation event (Holland *et al.,* 1994; Aparicio *et al.,* 1997), but two clusters are present in the cephalochordate amphioxus (*Branchiostoma floridae*; Pendleton *et al.,* 1993), which arose before the second genome doubling, and only one cluster is seen in the urochordate sea squirt (*Ciona intestinalis*; Spagnuolo *et al.,* 2003).

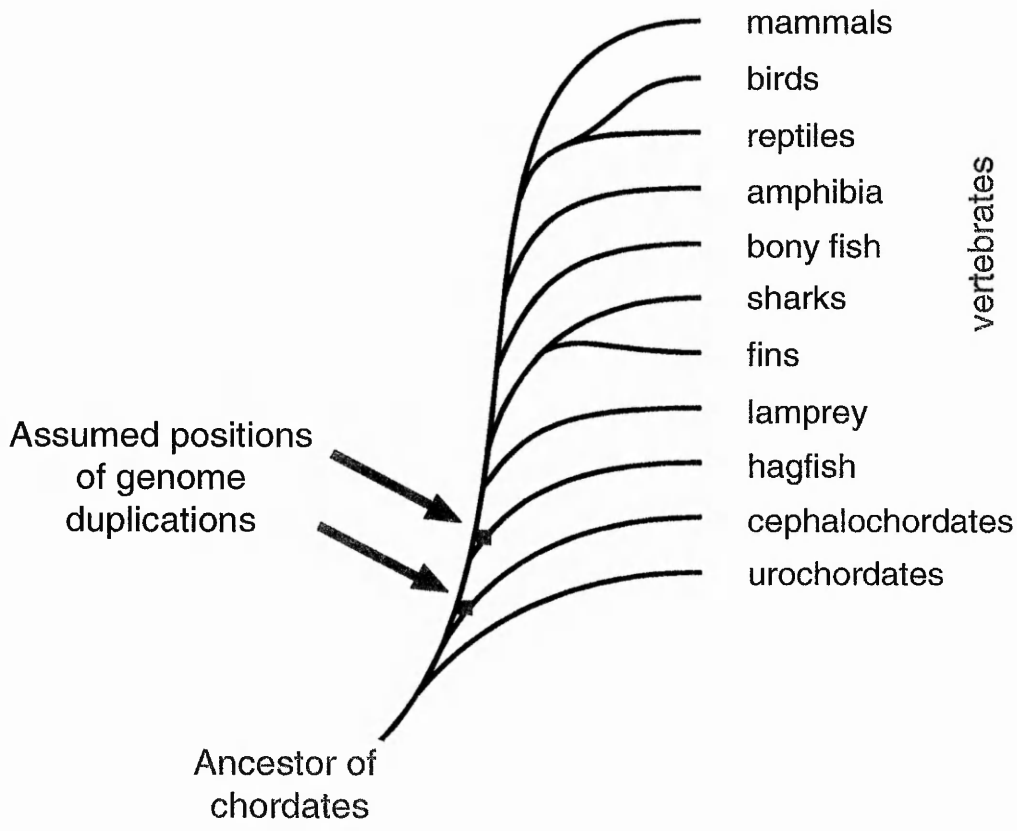

Figure 6: The occurrence of the two genome duplications or tetraploidisation events (indicated by blue arrows) occurring during chordate evolution.

Additional support for the proposal that the majority of $GABA_A$ receptor genes arose as a consequence of two whole-genome duplications comes from the finding that the intron/exon organisation of these genes is highly conserved. Sommer *et al.* (1990) were the first to determine the structure of a $GABA_A$ receptor gene, namely that for the murine δ subunit. Subsequently, Lasham *et al.* (1991) and Kirkness *et al.* (1991) revealed the organisations of two other genes, that for the avian β4 subunit and that for the human β1 subunit, and realised that they all had the same basic arrangement, with the coding region being specified by nine exons (see Figure 7). Other studies (e.g. Glatt *et al.*, 1997; Sinkkonen *et al.*, 2000) have consequently confirmed this gene structure. In addition, the transcriptional orientation of the various genes within the four clusters (Glatt *et al.*, 1994; Russek and Farb, 1994) has been shown to be consistent with their derivation from a single ancestral cluster.

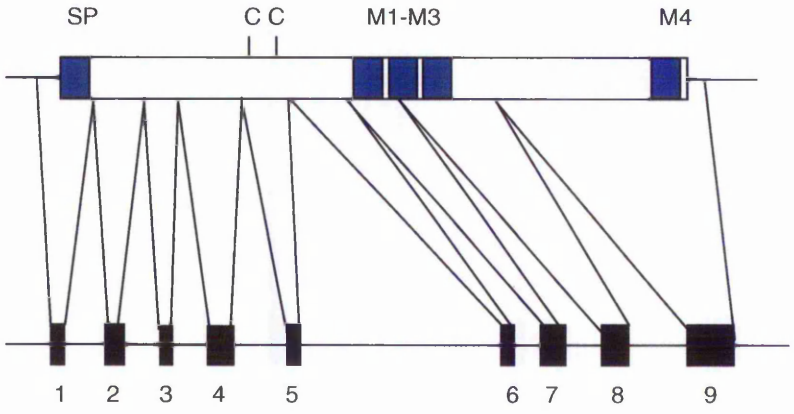

Figure 7: Schematic representation of the genomic organisation of a typical vertebrate $GABA_A$ receptor subunit gene (not to scale: see Sommer *et al.*, 1990; Kirkness *et al.*, 1991; Lasham *et al.*, 1991). Each gene is encoded by nine exons (indicated by black boxes, numbered 1 to 9) and eight introns (indicated by lines). Abbreviations: signal peptide (SP), dicysteine loop (C-C), membrane spanning domains (indicated by blue boxes; M1 to M4).

The two $GABA_A$ receptor genes that do not reside in clusters are *GABRD* and *GABRP*, which encode the δ and π subunits, respectively. The former is present on the short arm of chromosome 1 (Sommer *et al.*, 1990), while the latter is located in 5q34-q35 (Bailey *et al.*, 1999b) but ~3.5 Mb in distance to and separate from the cluster containing *GABRA1*, *GABRA6*, *GABRB2* and *GABRG2*. It is assumed that *GABRD* and *GABRP* arose via duplication of an ancestral $GABA_A$ receptor gene(s) and that they were subsequently translocated to another chromosome or chromosomal region.

1.4 Non-mammalian Vertebrate $GABA_A$ Receptor Subunit Genes?

Two additional $GABA_A$ receptor subunits have been identified in the chicken (*Gallus gallus domesticus*), namely, the β4 and γ4 subunits (Lasham *et al.*, 1991; Harvey *et al.*, 1993). These were originally assumed to be different to mammalian $GABA_A$ receptor subunits because of their low sequence similarity to any of the β or γ subunits (they display ~70% identity or less at amino acid level). Their discovery initiated a search for orthologous genes in man, Simon *et al.* (2004) searched the human genome for a β4-subunit gene however, did not find one and the highest similarity was to the θ subunit. Furthermore, subsequent studies revealed that despite having both a β4 and a γ4 subunit, the chicken appeared to have only 3 β-subunit genes (that encode the β2, β3 and β4 polypeptides) and 3 γ-subunit genes (for the γ1, γ2 and γ4 polypeptides; see Darlison and Albrecht, 1995). This raised questions as to the phylogenetic positions and physiological significance of the β4 and γ4 subunits. Two isoforms of the β4 subunit were identified namely β4S and β4L which, differ by four amino acids (VREQ), occurring in the intracellular loop (Bateson *et al.*, 1991b). The positions of the intron/exon boundaries are conserved with those seen in other $GABA_A$ receptor subunit genes, i.e. α subunits (see Figure 7).

The identification of the $GABA_A$ receptor ε (Davies *et al.*, 1997; Wilke *et al.*, 1997; Whiting *et al.*, 1997) and θ (Bonnert *et al.*, 1999) subunits confused matters yet further, since they displayed highest sequence similarity to the chicken γ4 (49% identity) and β4 (56% identity) subunits, respectively. This degree of identity is significantly higher than that seen for $GABA_A$ receptor polypeptides from different subunit classes (e.g. 30% to 40% identity is observed between α and β subunits). However, it is much lower than is generally found for two vertebrate orthologues; $GABA_A$ receptor subunits from different vertebrate classes typically display >90% identity (e.g. the human and chicken α1 subunits are 98% identical; Bateson *et al.*, 1991a). Although the θ subunit can not functionally replace β subunits in recombinant $GABA_A$ receptors (Bonnert *et al.*, 1999), the ε subunit is able to substitute for γ subunits; however, ε-subunit-containing receptors are BZ-insensitive (Davies *et al.*, 1997; Whiting *et al.*, 1997).

1.5 Potential Consequences of Gene Clustering

It was originally proposed (McKernan and Whiting, 1996) that the clustering of $GABA_A$ receptor genes was important to permit co-ordinate transcription of genes, the products of which would assemble to form specific receptor subtypes. The most significant evidence of this is that the subunits that form the major $GABA_A$ receptor subtype ($\alpha1\beta2\gamma2$; Whiting, 2003) are expressed as a cluster on chromosome 5 (see Figure 5). Both the ε- and θ-subunit genes have been found to be co-expressed in many areas of the brain, including the septum, amygdala, thalamus and various hypothalamic nuclei (Whiting *et al.,* 1997; Bonnert *et al.,* 1999; Moragues *et al.,* 2002). Moreover, the ε- and θ-subunit transcripts co-localise in monoaminergic neurons (Moragues *et al.,* 2002), which have previously been demonstrated, by immunocytochemistry, to contain the α3 subunit (Fritschy *et al.,* 1992). This is consistent with the clustering of the ε-, α3- and θ-subunit genes on the X chromosome (Wilke *et al.,* 1997). Nevertheless, it is now evident that there are numerous $GABA_A$ receptor subtypes in brain (see Whiting, 2003), and co-ordinate transcription of the genes within the four clusters clearly cannot account for all of the known subtypes. Furthermore, many neurons express a multitude of receptor subunit genes; e.g. Brooks-Kayal *et al.* (2001) have identified the expression of at least 10 different subunit genes in dentate granule cells of the hippocampus. Therefore, the regulation of $GABA_A$ receptor gene expression, at distinct developmental stages and in different neuronal and glial cell populations, must be extremely complex.

1.6 $GABA_A$ receptor β subunits

Three $GABA_A$ receptor β-subunit genes are known to be expressed by all vertebrate species (Barnard *et al.*, 1998; Whiting, 2003), namely the β1, β2 and β3 subunits in mammalian species and β2, β3 and β4 in avian species. *In vitro* mutagenesis studies have identified threonine and tyrosine residues in the rat β2 subunit, which are also present in the other β subunits, that are essential in the binding of GABA (Amin and Weiss, 1993). The β subunits also affect the properties of the anaesthetic etomidate and the anticonvulsant loreclezole (Wingrove *et al.*, 1994; Belelli *et al.*, 1997, 2003). Benke *et al.* (1994) have shown that the β2 subunit is the most abundant β subunit, present in 55-60% of rat brain $GABA_A$ receptors; in contrast, the β1 subunit is present in 16-18% and the β3 subunit in 19-25% of receptors. The β2 and β3 subunits have been found to

play a role in embryogenesis (Hevers and Lüddens, 1998). Homanics *et al.* (1997) and DeLorey *et al.* (1998) demonstrated that β3-subunit gene 'knock-out' mice exhibited epileptic seizures and displayed hypersensitive behaviour resembling Angelman Syndrome, which is a tendancy of seizures in children. Tehrani *et al.* (1995) have shown, using immunoprecipitation, that the $GABA_A$ receptor β4 subunit is a major component of $GABA_A$ receptors in the chick cerebral cortex.

1.7 Learning paradigms

$GABA_A$ receptors are known to play a role in learning as they are abundantly expressed in the hippocampus of the rat brain (Wisden *et al.*, 1992; Sperk *et al.*, 1997; Pirker *et al.*, 2000). Gene-knockout studies have linked certain subunits to specific processes of learning; for example, while α5-subunit-containing receptors play a role in learning and memory (Collinson *et al.*, 2002), α2-subunit-containing receptors mediate the anxiolytic effects of compounds such as diazepam (Low *et al.*, 2000). The down-regulation of the $GABA_A$ receptor γ4-subunit transcript seen after imprinting on a visual stimulus (Harvey et al., 1998), strongly suggest that one or more receptors that contain the γ4 subunit are involved in early learning in the chicken and, possibly, other species. However, the physiological function of the β4 subunit is, uncertain therefore this investigation focused on the expression and spatial distribution of the β4-subunit mRNA.

Gene deletion technologies, such as those that can be applied to the mouse, are not yet available in avian species. Hence, it is quite difficult to assess the physiological functions of $GABA_A$ receptors that contain either the β4 or the γ4 subunit. Nonetheless, *in situ* hybridisation studies, in the one-day-old chicken (Harvey *et al.*, 1993), have shown that the γ4-subunit gene is expressed in brain areas that play a significant role in visual processing (e.g. the optic tectum, nucleus rotundus and entopallium) and imprinting (e.g. medial striatum).

This is consistent with earlier pharmacological experiments that have demonstrated that injection of muscimol (a $GABA_A$ receptor agonist), into the intermediate and medial mesopallium (IMM) of the one-day-old chick, causes amnesia in a one-trial passive avoidance test, while injection of bicuculline (a $GABA_A$ receptor antagonist) improves

performance (Clements and Bourne, 1996). Also, injection of a β-carboline (methyl β-carboline-3-carboxylate), that is an inverse agonist at the $GABA_A$ receptor BZ site, has been shown to improve memory performance, in chicks, in an imprinting paradigm that involves a moving decoy (a ball; Venault *et al.*, 1986).

1.7.1 Song learning

Song learning is another learning paradigm and, involves acquisition of the ability to produce song during a critical period. The model of song learning may be applied to speech production in humans. There are two phases of song learning: exposure to song (where birds listen to and memorise adult song from a tutor) and sensory-motor acquisition (where birds have the motor skills necessary to vocalise; Brainard and Doupe, 2002). Konishi (1965) found that if birds were deafened after exposure to song, but before they vocalised then they developed abnormal songs, confirming the need for auditory feedback. In zebra finches, the sensory exposure stage occurs up to 25 days post-hatching; the sensory-motor stage varies from day 25 to day 90, when song structure is crystallised. There are specific brain regions that are involved in these processes; Area X and the lateral magnocellular nucleus of the anterior nidopallium (LMAN; anterior pathway) play a role in the learning and production of song, and the higher vocal centre (HVC) and robust nucleus of the arcopallium (RA) are involved in crystallised song production (refer to Figure 8 for schematic of brain areas). The advantages of using songbirds for learning studies include their relatively rapid development and the existence of specialised brain areas for this naturally learnt behaviour (Brainard and Doupe, 2002). Note that only male zebra finches produce song, and anatomical differences can be seen between the sexes in brain regions that are involved in song production.

During the course of this investigation the genome sequences of several species became available including the chicken, puffer fish and sea squirt. $GABA_A$ receptor subunit genes could then be searched for and specific antisense oligonucleotides for *in situ* hybridisation could be designed from the genome sequences.

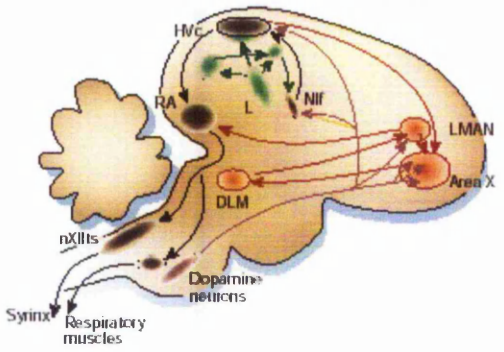

Figure 8: Schematic sagittal section of a songbird brain (from Brainard and Doupe, 2002). Areas indicated include: HVC (higher vocal centre), LMAN (lateral magnocellular nucleus of the anterior neopallium), RA (robust nucleus of the arcopallium), Area X (Area X of the medial striatum), Nif (interfacial nucleus of the neopallium), NXIIts (tracheosyringeal portion of the 12th cranial nerve), and the DLM (dorso-lateral division of the medial thalamus). The motor pathway is indicated in black and is essential for song production; additional brain areas (in red) are possibly also part of the motor pathway; the network on higher auditory brain areas in shown in green.

1.8 *In silico* analysis of genome databases

The trend of sequencing genomes was taken advantage of to analyse the evolution of $GABA_A$ receptor subunit genes.

1.8.1 Chicken (*Gallus gallus domesticus*)

Chickens shared a common ancestor with humans ~300 million years ago (Schmutz and Grimwood, 2004). The chicken genome sequence was released in June 2004 (http://www.ensembl.org/Gallus_gallus/). The chicken possesses a haploid genome that is ~1.2 x 10^9 base pair in size, which is approximately a third of the size of the human genome. The chicken genome encompasses 1 pair of sex chromosomes namely Z and W (ZW in the female and ZZ in males) and 38 autosomes (Schmutz and Grimwood, 2004).

1.8.2 Puffer Fish (*Takifugu rubripes*)

The Japanese puffer fish belongs to the Teleost group, which is the most diverse group of vertebrates with more than 24,000 species (Venkatesh *et al.*, 2000). Aparicio *et al.*

(1995) have proposed that the puffer fish is a suitable organism for a comparison of vertebrate genomes. The puffer fish has a 7.5 times smaller genome size than that of man, thus, intron/exon arrangement is easier to study in this fish as there is less 'junk' DNA (Elgar, 1996). The draft genome sequence of the puffer fish was released in October 2001 (http://bahama.jgi-psf.org/fugu/html/README.html). The puffer fish genome encompasses ~30,000 genes, which is very similar to the predicted number in humans however, the puffer fish genome is 332.5 million bases in size compared to 3.2 billion bases in the human genome (Collins *et al.*, 2003). Hence, it is a more compact genome, with an average of one gene every 6-7kb of sequence (Elgar *et al.*, 1999) arranged on 44 chromosomes. Brenner *et al.* (1993) found small intron sizes in the puffer fish genome and although the common ancestor between fish and humans lived ~400 million years ago, the intron positions (e.g. of the fibroblast growth factor and integrin genes) were conserved with mammals, hence the puffer fish plays a role in understanding the evolution of complex genomes (Elgar, 1996). In addition, the sequencing of the puffer fish genome is extremely useful as 1000 new human genes have been discovered because of it including ZNF 366 and ZNF 267 mapping to human chromosomes 5q13.2 and 9q22.32, respectively (Gilligan *et al.*, 2002).

1.8.3 Sea squirt (*Ciona intestinalis*)

Vertebrates share the phylum Chordata with the cephalochordates (headless and segmented animals) e.g. amphioxus (*Branchiostoma floridae*), and urochordates (headless and unsegmented animals; e.g. ascidians), which represent the most distant living relatives to vertebrates. There is agreement that the three classes (urochordate, cephalochordate and vertebrate) arose from a common ancestor more than 560 million years ago (Bosma *et al.*, 1999). Ascidians are sessile marine animals, which live in shallow water areas attaching themselves to rock by the tunic covering their body. There are two types of ascidians: solitary e.g. *Ciona intestinalis* (commonly known as the sea squirt, see Figure 9) and colonial e.g. *Botryllus schlosseri.* The sea squirt is characterised as a filter feeder, reproducing sexually, and possessing two openings or siphons, namely branchial (inhalant) and atrial (exhalant). The tunic is composed of a layer of cellulose, which acts as a protective layer and is required for adhesion. Although, sea squirts are hermaphrodites the egg has a vitelline coat, which has a key mechanism in self-recognition therefore preventing self-fertilisation (Murabe and Hoshi, 2002).

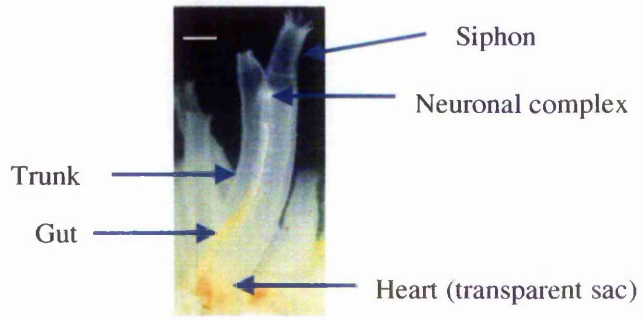

Figure 9: A photograph of an adult sea squirt, with the tunic removed. Scale bar is equal to 2cm.

The nervous system consists of a cerebral ganglion, a neural gland and ciliated duct, collectively known as the neuronal complex, which is located between the two siphons. Osborne *et al.* (1979) first detected GABA in the homogenate from the neuronal complex. Bollner *et al.* (1993) showed by immunocytochemistry that GABA is detected quite early in development i.e. 2 to 4 weeks post-metamorphosis (i.e. tail resorption), and that the majority of the immunoreactivity was seen near the nerve exits. The function of the neuronal complex is not actually known, however it is hypothesised to act as the equivalent of the mammalian pituitary gland, and is possibly involved in metamorphosis in ascidians (Cloney, 1982). Boorman and Shimeld (2002) showed high similarities in the expression of the *Pitx* gene (needed to control left-right asymmetry) in the neuronal complex of the sea squirt with the vertebrate pituitary.

The larval CNS is composed of approximately 330 cells, of which 215 represent the sensory vesicles, 65 constitute the nerve cord of the tail and approximately 40 are present in the visceral ganglion. It is estimated that of these 330 cells only 100 are neuronal (Nicol and Meinertzhagen, 1991). The sensory vesicle (equivalent to the forebrain and midbrain) contains two sense organs, the ocellus which is a light-sensitive single eye (containing photoreceptors), and a gravity-sensing organ the otolith (Hudson and Lemaire, 2001). Although, there is a relatively low number of cells in the sea squirt larval CNS, they are responsible for the swimming behaviour (Satoh, 1994). Meinertzhagen *et al.* (2000) described the immunostaining of the neurons in the larval CNS of the sea squirt, and found that 29 neurons in the visceral ganglion are responsible for generating motor output to control swimming. In 1993, Arshavsky *et al.* found that there are approximately 30 GABAergic neurons in the CNS of the marine mollusc *Clione limacina*. Evidence for the role of GABA in locomotion comes from Arshavski *et al.* (1991) who found that injection of GABA into *C. limacine* induced feeding

behaviour, specifically acceleration of swimming. More recently, GABA has been shown to cause a left-ward 45° inclination in the angle of swimming and, inhibit gravitational effects allowing *C. limacina* to migrate long distances (Deliagina *et al.*, 2000).

There are numerous reasons why sea squirts are model organisms to study. Their development is rapid; the larvae hatch only 18 hours after fertilisation (Corbo *et al.*, 1997a) and the life span ranges from 2 to 6 months depending on water temperature. Sea squirts also have clearly defined embryonic lineages and Corbo *et al.* (1997b) showed that each of the ependymal cells of the neural tube could be traced back to the 110-cell gastrula. Dehal *et al.* (2002) released the draft genome sequence (http://genome.jgi-psf.org/ciona4/ciona4.home.html) of the sea squirt stating that the genome size is approximately 117 million bases in length with 15, 852 genes, which is comparable to that of the fruit fly (*Drosophila melanogaster*; Chiba *et al.*, 2004). As the genome is nearly 20 times smaller than that of humans, with more densely packed genes, it is relatively easy to analyse.

1.9 Aims and Objectives

The aims of my PhD included:

1. Studying the molecular evolution of $GABA_A$ receptor β subunits, by cloning and sequencing of β-subunit cDNAs from various species (e.g. birds, reptiles, amphibians) and carrying out a phylogenetic analysis of the $GABA_A$ receptor β subunits. As the draft genome sequence of the puffer fish was available, *in silico* analysis was conducted to search the database for $GABA_A$ receptor β-subunit-like genes.
2. Determining the expression patterns of the $GABA_A$ receptor β4-subunit gene in different species. For this the spatial distributions and abundances of the β4-subunit mRNA in the one-day-old chicken, 35-day-old and 90-day-old zebra finch were examined by *in situ* hybridisation.
3. To search for the potential cluster of $GABA_A$ receptor subunit genes in the genome of the sea squirt, as it is thought to have arisen before the genome duplications occurred. *In silico* analysis was used for this, and the distribution of

the genes found was characterised by *in situ* hybridisation and immunohistochemistry.

It was hoped that this investigation would yield important insights into the physiological functions of $GABA_A$ receptors that contain the β4 subunit, increase our understanding of the role of these receptors in the modulation of different types of learning, and provide further information on the evolution of the $GABA_A$ receptor gene family.

Materials and Methods

All chemicals were purchased from Sigma-Aldrich or Fisher Scientific, enzymes were bought from Promega and oligonucleotide primers were ordered from Sigma-Genosys unless otherwise stated. Note that all centrifugation steps carried out at 4°C were in a Mikro 22R microcentrifuge (Hettich), whereas all steps carried out at room temperature were in a Mikro 20 microcentrifuge (Hettich), unless stated otherwise.

Species	Latin Name	Source
Chicken	*Gallus gallus domesticus*	R. Mileusnic, Open University
Zebra finch	*Taeniopygia guttata*	H. R. Güttinger, Technische Universität Kaiserslautern, Germany and T. Birkhead, University of Sheffield
African clawed Frog	*Xenopus borialis*	University of Nottingham
Common wall lizard	*Podarcis muralis*	A-F. Lopez, Universidad de León, Spain
Sea squirt	*Ciona intestinalis*	S. M. Shimeld, University of Oxford

Table 1: Summary of the species studied in this investigation and their sources. Note that although *Xenopus borealis* is known as the African clawed frog it is zoologically classified as a toad, but for simplicity will be referred to as a frog in this thesis.

2.1 Isolation of total RNA

The brain was dissected from all of the vertebrate species studied in this investigation. The tunic of an adult sea squirt was peeled back and the following tissues were dissected: gonads, gut, heart, neuronal complex, siphons, stomach and trunk (this includes atria, endostyle and pharynx).

Total RNA was isolated from the dissected tissues (see Chomczynski and Sacchi, 1987). The tissues were homogenised using a glass-teflon homogeniser (Scientific Laboratory Supplies Ltd) and 1ml of RNAbee (ISO-TEX Diagnostics; containing guanidinium thiocyanate to extract total RNA) was added per 50mg of tissue. The homogenised tissue was aliquoted into 2ml Eppendorf tubes and incubated on ice. 0.2ml of

chloroform was added to the tube per ml of RNAbee originally added to the tissue. The tubes were shook vigorously for 15 to 30s and incubated on ice for 5min. The tubes were then centrifuged at 10800rpm (4°C) for 15min. The upper layer (aqueous) was transferred to a 1.5ml Eppendorf tube and an equal volume of isopropanol was added, the tubes were incubated at room temperature for 5min. Centrifugation of the tubes was then performed at 10800rpm (4°C) for 5min. After removal of the supernatant the pellet was washed using the same volume of 75% (v/v) ethanol as the RNAbee added initially. The pellet was dislodged by shaking the tube gently and centrifuged at 8500rpm (4°C) for 6min. This wash step was repeated.

The pellet was air dried for approximately 10min, and the RNA was dissolved using 66µl of RNAse and DNAse-free water per 100mg of tissue. The concentration of the RNA was measured (see 2.2) and aliquoted to give 40µg per Eppendorf tube and stored in 2.5 times the volume of absolute ethanol at –80°C until required. To concentrate the isolated RNA, an ethanol precipitation was carried out on one aliquot. For this a 2.5 times volume of absolute ethanol was added to the tube followed by a tenth of the volume of 3M sodium acetate (pH 5.2). The tube was mixed (by inversion) and incubated at –80°C for a minimum of 20min. Centrifugation of the tube was carried out at 13000rpm (4°C) for 20min. The supernatant was discarded and the pellet was washed with 100µl of 75% (v/v) ethanol and air-dried at room temperature for 1min. Again the supernatant was discarded and the pellet was air-dried for approximately 5min. Finally 55µl of 0.1% (v/v) diethylpyrocarbonate (DEPC)-treated water was added to resuspend the RNA.

2.2 Measuring the concentration of nucleic acids

Samples were diluted (1 in 100) in 0.1% (v/v) DEPC-treated water. Nucleic acid concentration was measured at 260nm using a spectrophotometer (S2100, Biowave) with 0.1% (v/v) DEPC-treated water being used for calibration of the spectrophotometer. The concentration was calculated as follows:

$OD_{260\ nm}$ x dilution factor x50µg/ml for double-stranded DNA
$OD_{260\ nm}$ x dilution factor x20µg/ml for single-stranded DNA
$OD_{260\ nm}$ x dilution factor x40µg/ml for single-stranded RNA
(Birnboim and Doly, 1979; Summerton *et al.*, 1983).

2.3 Reverse transcription of RNA into single-stranded complementary DNA

Total RNA and larval RNA isolated from the sea squirt (kindly donated by Dr Sebastian Shimeld, University of Oxford) were reverse transcribed into complementary DNA (cDNA). To remove any genomic DNA from the total RNA the following were added to a 1.5ml Eppendorf tube and incubated at 37°C for 15min:

total RNA (40µg)	55µl
5x Transcription buffer (200mM Tris-HCl pH 8, 50mM $MgSO_4$, 5mM $CaCl_2$; Promega)	16µl
RNasin (40U/µl; Promega)	1µl
dithiothreitol (DTT; 100mM; Promega)	5µl
RQ1 DNase (1U/µl; Promega)	3µl

To remove any proteins a phenol/chloroform extraction was conducted. An equal volume of phenol/chloroform/isoamyl alcohol (PCI; ratio of 25:24:1; BDH) solution was added to the tube and vigorously shaken for 30s. The tube was then centrifuged at 13000rpm (4°C) for 5min. The upper aqueous phase was transferred to a new 1.5ml Eppendorf tube and an ethanol precipitation was carried out (see 2.1). The pellet was air-dried and resuspended in 54µl of 0.1% (v/v) DEPC-treated water. This was then heated to 65°C for 5min.

To synthesise cDNA the following were added to a 1.5ml Eppendorf tube and incubated at 42°C for 90min:

total RNA (40µg; from previous step)	54µl
RNasin (40U/µl)	2µl
5x Moloney Murine Leukemia Virus (M-MLV) buffer (250mM Tris-HCl (pH 8.3), 375mM KCl, 15mM $MgCl_2$, 50mM DTT; Promega)	20µl
dNTPs (5mM; Sigma)	20µl
random primers (9mer, 500µg/µl; Promega)	2µl
M-MLV Reverse Transcriptase (200U/µl; Promega)	2µl

To precipitate the cDNA, 100µl of 0.1% (v/v) DEPC-treated water, 20µl of 3M sodium acetate (pH 5.2), and 120µl of isopropanol were added to the tube, which was then incubated on ice for 15min. The tube was then centrifuged at 13000rpm (4°C) for 15min. The pellet was washed with 100µl of 80% (v/v) ethanol and after air-drying was resuspended in 200µl of 0.1% (v/v) DEPC-treated water. The wash step was repeated twice, however the third wash was with 100µl of 75% (v/v) ethanol. Finally the pellet was air-dried, resuspended in 25µl of 0.1% (v/v) DEPC-treated water and stored at –20°C.

2.4 Polymerase Chain Reaction (PCR)

All oligonucleotides used in this investigation are described in Table 2. All oligonucleotides were designed to specifically anneal to the relevant cDNA or genomic DNA sequence. The criteria for primer design were ~50:50 G+C: A+T ratio (with a higher G+C: A+T at the 3' end), and a minimum length of 21 base pairs (bp). The degenerate oligonucleotide sequences were based on an alignment of known $GABA_A$ receptor β subunits and had integral restriction sites (for sticky end cloning).

The reaction mixtures were prepared in 0.5ml thin walled tubes (Biozyme Diagnostik) containing:

template ~20ng of genomic DNA or cDNA	1 to 5µl
10x buffer (10mM Tris-HCl (pH 8.0), 100mM NaCl, 0.1mM ethylenediaminetetracetic acid (EDTA), 1mM DTT, 50% glycerol, and 1% Triton X100; Promega)	5µl
$MgCl_2$ (25mM; Promega)	3µl
dNTPs (1.25mM)	8µl
forward primer (200ng/µl)	1µl
reverse primer (200ng/µl)	1µl

adjusted to 50µl using RNAse-free and DNAse-free water (Saiki *et al.*, 1985).

Name	Amplification of:	Sequence 5' to 3'	Nucleotide positions of primers	Reference
βF	Vertebrate $GABA_A$ receptor β subunit cDNAs	ACXACXACXGCXGCXTGXATGATGGA	681bp to 706bp	Vertebrate $GABA_A$ receptor β-subunit sequences
βR	"	TTAGTXCACXTAXTAXAXCCAXTA	1620bp to 1643bp	"
βActinF	Sea squirt (ss) β-actin cDNAs	ATGGACGAGGATGTTGCCGCA	180192bp to 180212bp	Scaffold number 176
βActinR	"	AATCGGTAAGATCTCGTCCGGCC	180877bp to 180899bp	"
ssαF	Sea squirt $GABA_A$ receptor α-like subunit	ACCCATTACCTTAAAGGGCGGA	66264bp to 66285bp	Scaffold number 300
ssαR	"	CAAAGCACAAGACAAGAGCATCG	66658bp to 66680bp	"
ssβF	Sea squirt $GABA_A$ receptor β-like subunit cDNAs	TGGACGAACAGAACTGCACGC	51556bp to 51576bp	Scaffold number 300
ssβR	"	CTGTAAGGTGACGAGGAATCCG	57770bp to 57791bp	"
ssβF2	"	CGGGAGTTGAAAACTTGAAGCTGGC	53169bp to 53193bp	Scaffold number 300
ssβR2	"	CGCCACTCTTGCCACTTTGTC	57655bp to 57675bp	"
ssγF	Sea squirt glycine-like receptor subunit	ATTGTTCCAACCCAGTGGTCAC	11219bp to 11240bp	Scaffold number 827
ssγR	"	CCCCACCTTATAAGACAACCA	11417bp to 11437bp	"
GADF	Sea squirt GAD cDNAs	AACAATCGGCTTGGAGGTTCCG	83bp to 104bp	Accession number AB072597
GADR	"	AGTGTTAGCCGTCGACGTCAC	502bp to 522bp	"

Table 2: A list and the sequence of primers used in the PCRs carried out in this investigation. Degenerate primers were based on an alignment of vertebrate $GABA_A$ receptor β-subunit sequences using the PILEUP programme (Wisconsin Package, Genetic Computer Group). All sea squirt sequences were designed from the genome database (http://genome.jgi-psf.org/ciona4/ciona4.home.html). The glutamic acid decarboxylase sequence was obtained from the NCBI database (http://www.ncbi.nlm.nih.gov/).

Negative controls were also prepared; where the DNA template was replaced with RNAse-free and DNAse-free water. The tubes were then placed in a PCR thermocycler (Techne Genius), and after a 10min incubation at 94°C ('hot start'), 0.3µl of *Taq* DNA polymerase (5U/µl; Promega) was added to the tubes. This was followed by cycles of different temperatures (see Table 3 for the PCR conditions). Note that a nested PCR was conducted to amplify $GABA_A$ receptor β-subunit cDNAs and GAD (glutamic acid decarboxylase) cDNA from the sea squirt. Hence 5µl of the product from the first PCR was used as a template for the second PCR.

Note that the concentration of the primers was determined by measuring the absorbance at 260nm using a spectrophotometer (see 2.2).

2.4.1 Gel electrophoresis

All PCR products were separated in 2% (w/v) agarose (Geneflow) gels (in 1x TAE buffer (50x: 2M Tris-base, 1M glacial acetic acid, 0.25M EDTA; pH 8), with a 1kb DNA ladder as a molecular weight marker (1kb DNA ladder was prepared using: 16.7% (v/v) loading dye (0.09% (w/v) bromophenol blue, 0.09% (w/v) xylene cyanol FF, 60% (v/v) glycerol, 60mM EDTA), 16.7% (v/v) 1kb ladder (Gibco) and 66.7% (v/v) TE buffer (10mM Tris-HCl, 1mM EDTA; pH 8) at 100V for 1hr, which were visualised using a transilluminator (Syngene Inc).

2.4.2 Purification of PCR fragments

DNA fragments of the expected size were excised from gels and placed in pre-weighed 1.5ml Eppendorf tubes. DNA was recovered using a GFX Purification Kit, following the manufacturers's instructions (Amersham Pharmacia Biotech). This kit worked by trapping DNA in a silica matrix of a spin column whilst all impurities were eluted through, after which the DNA was then eluted.

Template	Amplification of:	Denaturation of genomic DNA (1 cycle)	Denaturation	Annealing	Extension	Further extension (1 cycle)	Cycles
Zebra finch cDNA (1µl)	$GABA_A$ receptor β-subunit cDNAs	/	94°C for 1min	55°C for 1.5min	72°C for 2min	/	35
Frog cDNA (2µl)	"	/	"	48°C for 1.5min	"	72°C for 10min	40
Lizard cDNA (2µl)	"	/	"	"	"	/	40
Sea squirt tissue cDNA (5µl)	β-actin cDNA	/	"	68°C for 1.5min	"	/	35
Sea squirt larval cDNA (5µl)	GAD cDNA	/	"	62°C for 1min	"	/	40 (twice)
Sea squirt genomic DNA (2µl)	$GABA_A$ receptor α-like subunit gene	94°C for 10min	"	"	"	/	40
Sea squirt tissue cDNA (1, 2 or 3µl)	$GABA_A$ receptor β-like subunit gene	/	"	64°C for 1.5min	"	72°C for 10min	25 (twice)
"	$GABA_A$ receptor γ-like subunit gene	94°C for 10min	"	55°C for 1min	"	/	40

Table 3: A list of PCR conditions for all reactions carried out in this investigation.

2.5 Cloning

All PCR products were cloned using the pGEM T-Easy vector (Promega) except those from the zebra finch, which were cloned using the pCR 2.1 TOPO Cloning Vector Kit (Invitrogen) following the manufacturer's instructions. *Taq* polymerase adds a single deoxyadenosine (A) to the 3' end of the PCR product and the linearised vector has a single deoxythymidine (T) at the 3' overhang. Both vectors possess ampicillin resistance genes.

Vector	Type of cloning	Promoters
pGEM T-Easy	TA	SP6 and T7 RNA polymerase
pCR 2.1 TOPO	TA	T7 RNA polymerase

Table 4: Summary of the vectors used for cloning PCR products

The pCR 2.1 TOPO Cloning Vector Kit included TOP10 chemically competent *E. coli* cells (genotype: F$^-$ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *deo*R *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G).

2.5.1 Preparation of competent *Escherichia coli* XL1-blue cells

A single *E. coli* XL1-blue colony (genotype: *rec*A1, *end*A1, *gyr*A96, *thi*-1, *hsdR*17 (rk-, mk+), *supE*44, *rel*A1, *lac* [F' *proAB*, *lacI*^{q}ZΔM15::Tn10(tet^r)]) was picked off a LB agar plate (1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl, 1.5% (w/v) agar (Becton Dickinson Company) containing tetracycline (5μg/ml) and incubated in 3ml of LB media (1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl) containing tetracycline (5μg/ml) in an orbital shaker (Gallenkamp) at 200rpm (37°C) overnight. 100ml of LB media tetracycline (5μg/ml) was inoculated with 0.5ml of the overnight culture. A 1ml aliquot of the inoculated media was added to a 1ml plastic cuvette (Sarstedt) to calibrate the spectrophotometer. The remainder was incubated on a shaking platform (200rpm; Luckham R100) 37°C) for 2hr. The absorbance was then measured at 550nm and every 20min thereafter until an optical density of 0.5 was obtained. The inoculated LB media was split into two 50ml tubes (Sarstedt) and centrifuged at 3000rpm at room temperature for 15min. The supernatant was discarded

and the cells were resuspended in 20ml of filter sterilized (0.22nm) ice-cold TFBI solution (30mM KOAc, 50mM $MnCl_2$, 100mM KCl, 10mM $CaCl_2$, 15% (v/v) glycerol; pH 5.8) per tube and briefly vortexed (Vortex Genie 2; Scientific Industries). The resuspended cells were incubated on ice for 30min. The cells were then centrifuged at 1750rpm (4°C) for 20min. The supernatant was discarded and the cells were resuspended in 2ml of filter sterilized ice-cold TFBII solution (10mM Na-MOPS, 75mM $CaCl_2$, 10mM KCl, 15% (v/v) glycerol; pH 6.5) per tube and the cells were gently resuspended. 200µl aliquots of the competent cells were prepared and stored at –80°C until required.

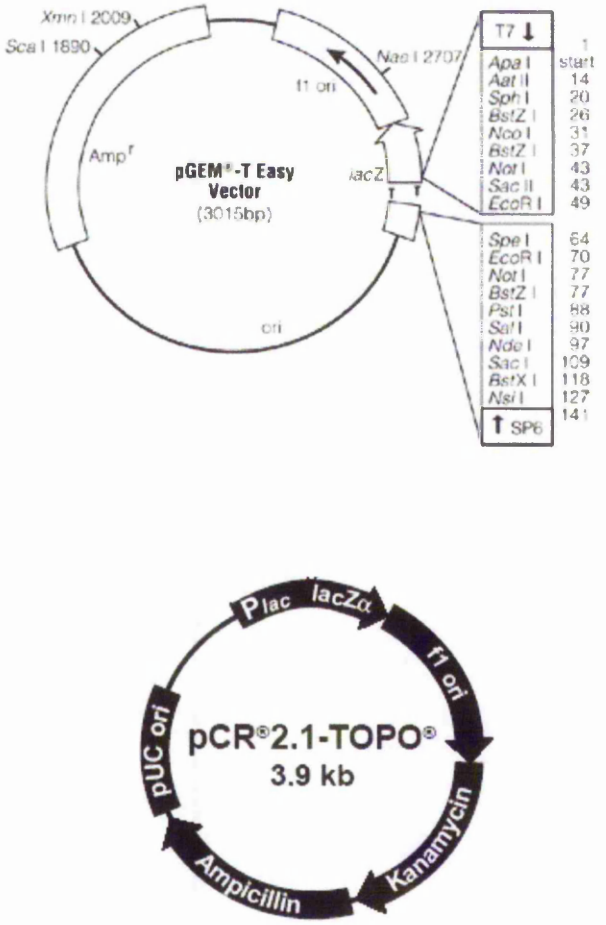

Figure 10: Maps of the pGEM T-Easy and pCR 2.1 TOPO cloning vectors. The position of the *lacZα* gene represents the multiple cloning sites.

2.5.2 Cloning using TOPO TA (2.1) Cloning Vector kit

The TOP10 chemically competent *E. coli* cells were transformed with the ligation mixture in SOC medium (2% (w/v) tryptone, 0.5% (w/v) yeast extract, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$ (Fluka Chemika), 20mM glucose) and grown in an orbital shaker (Gallenkamp) at 200rpm (37°C) for 1h. 40µl of 20mg/ml 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal; Fluka Chemika) was spread onto LB agar plates, and 0.1% (v/v) ampicillin with 50µl of transformed cells onto 6 plates and incubated overnight at 37°C.

The cloned insert disrupts the plasmid's *lacZ* gene (which encodes β-galactosidase; see Figure 10), hence X-gal (a colourless substrate) cannot be metabolized to galactose, and, therefore recombinant colonies are white. Recombinant clones (single white colonies) were picked from the plates using sterile toothpicks and a non-recombinant (blue colony) was picked as a negative control. These were grown overnight in 2ml of liquid LB medium containing 0.1% (v/v) ampicillin in 30ml sterile glass bottles (Scientific Laboratory Supplies Ltd) in an orbital shaker at 200rpm and 37°C.

2.5.3 Cloning using the pGEM T-Easy vector

The following formula was used to determine the amount of PCR product (insert) needed for a successful ligation.

$$\frac{\text{ng of vector} \times \text{kb size of insert}}{\text{kb size of vector}} \times \text{insert:vector molar ratio} = \text{ng of insert}$$

Usually the insert:vector molar ratio was 3:1. Two tubes were set up, one with the insert, and the second without the insert. The lambda Hind III ladder (Promega) was used as a control; if the ligase was efficient it would rejoin the fragments hence becoming circular therefore visualized as a single band on a gel rather than a 'ladder' of DNA fragments. The following reagents were added to 0.5ml Eppendorf tubes.

Test tube:

2x ligation buffer (60mM Tris-HCl (pH 7.8), 20mM $MgCl_2$, 20mM DTT, 2mM ATP, 10% polyethylene glycol; Promega)	5µl

insert (50ng/µl)	1µl
pGEM T-Easy vector (50ng/µl; Promega)	1µl
RNAse and DNAse-free water	2µl
T4 DNA ligase (1U/µl; Roche)	1µl

Control tube:

2x ligation buffer	5µl
Lambda Hind III (500µg/ml; Promega)	1µl
RNAse and DNAse-free water	3µl
T4 DNA ligase	1µl

both tubes were incubated at 4°C overnight.

The ligation product of the lambda Hind III marker was run in a 1% (w/v) agarose gel at 100V for 45min and visualised on a transilluminator.

The ligation product of the inserts to be cloned was then transformed into *E. coli* XL1-blue cells. The circular pGEM-3ZF vector (1µg/µl, with no insert; Promega) was used as a positive control to check the transformation efficiency.

6µl of the ligation product was added to 100µl of competent XL1-blue cells in a 1.5ml Eppendorf tube and 1µl of pGEM 3ZF vector (1ng/µl) was added to 50µl of cells in a second 1.5ml Eppendorf tube. Both tubes were incubated on ice for 20min. The tubes were placed at 42°C for 45s to 'heatshock' the cells and were then incubated on ice for 2min. 200µl of SOC media was added to the 'test' tube and 100µl to the control tube (due to the difference in the volume of XL1-blue cells added). Both tubes were then incubated in an orbital shaker at 200rpm (37°C) for 1.5hr.

100µl of isopropyl-β-D-thiogalactopyranoside (IPTG; 200mg/ml; Promega) and 16µl of X-gal (50mg/ml) was spread onto LB agar plates (containing 0.1% (v/v) ampicillin) and approximately 60µl of the transformed cells were spread onto 5 plates (all of the cells from the control were spread on another plate) and incubated at 37°C overnight. Single white colonies were picked and grown overnight in LB medium (see 2.5.1).

2.5.4 Isolation of plasmid DNA

Mini-preparations of DNA were prepared, according to the manufacturer's instructions of the Plasmid Miniprep Spinkit 250 (Genomed). Midipreps (larger volumes of plasmid DNA) were prepared using the Plasmid 500 kit following the manufacturers (Qiagen) instructions. Both kits comprised a series of solutions to lyse bacterial membranes, neutralize the DNA and remove any impurities including proteins by columns, which retain the plasmid DNA until eluted. An ethanol precipitation step was then conducted to concentrate the purified plasmid DNA (see 2.1).

2.5.5 Restriction endonuclease digestion

To determine whether an insert was present in the isolated plasmids, an *Eco*RI restriction digest was performed. *Eco*RI was chosen because there are recognition sites in the cloning vectors for *Eco*RI that flank the insert (see Figure 10).

Each tube contained:	
plasmid DNA (~200ng)	0.5µl
*Eco*RI (12U/µl; Promega)	0.25µl
buffer H (90mM Tris-HCl (pH 7.4),	1µl
10mM $MgCl_2$, 50mM NaCl; Promega)	
sterile distilled water	8.25µl

the tubes were incubated at 37°C for 1hr.

Samples were electrophoresised in a 1% (w/v) agarose gel (in 1x TAE buffer) along with a 1kb DNA ladder for 45min at 100V.

A double digest was subsequently performed using *Eco*RI and *Sau*3AI. *Sau3*AI has a recognition site of four bases hence it cuts more frequently than *Eco*RI, which has a recognition site of six bases. This yields a more differential banding pattern therefore, made it possible to distinguish between the different $GABA_A$ receptor β-subunit clones. Each tube contained:

plasmid DNA (~200ng)	0.5µl
*Eco*RI (12U/µl)	0.25µl

*Sau*3AI (10U/µl; Promega)	0.25µl
buffer B (6mM Tris-HCl (pH 7.4),	1µl
6mM $MgCl_2$, 50mM NaCl; Promega)	
sterile distilled water	8µl

the tubes were incubated at 37°C for 1hr.

Samples were separated in a 1% (w/v) agarose gel (in 1x TAE buffer) as above.

2.5.6 Sequencing of cDNA and genomic clones

For automated sequencing the samples were prepared in 0.5ml thin walled PCR tubes and contained:

ABI Prism Big Dye (Applied Biosystems)	4µl
M13 forward primer (1.6pmol; 5´-GTAAAACGACGGCCAG-3´)	0.85µl
or	
M13 reverse primer (1.6pmol; 5´-CAGGAAACAGCTATGAC-3´)	
plasmid DNA	450ng

in a total volume of 10µl.

The tubes were placed in a thermocycler and subjected to the following conditions: 25 cycles of 96°C for 30s, 50°C for 15s, and 60°C for 4min.

An ethanol precipitation step was then performed to concentrate the reaction products. For this, 3µl of 3M sodium acetate (pH 4.6), 62.5µl of 95% (v/v) ethanol and 14.5µl of deionised water was added to the tubes. The tubes were pulse centrifuged and incubated at room temperature for 45min. The tubes were then centrifuged at 13000rpm (4°C) for 20min. The supernatant was discarded and 250µl of 70% (v/v) ethanol was added. The tubes were briefly vortexed and centrifuged at 13000rpm (4°C) for a further 5min. The supernatant was again removed and the pellets air-dried. Lark Technologies Incorporated ran the samples, on a Prism 310 automatic DNA sequencer (Applied Biosystems). The sequences obtained were compared to other $GABA_A$ receptor and GAD sequences using the National Centre for Biotechnology Information (NCBI) BLAST programme (http://www.ncbi.nlm.nih.gov/BLAST/). The nucleotide sequences were translated to protein sequences using the translate programme of the Justbio website (http://www.justbio.com/translator/index.php). Protein sequences were aligned

using the software programme ClustalW (http://www.ch.embnet.org/software/ClustalW.html) and percentage identities were calculated manually. Note that the signal peptide of $GABA_A$ receptor subunits was not included when aligning sequences using ClustalW.

2.6 Radioactive *in situ* hybridisation

This technique was used to localise the $GABA_A$ receptor β4-, α3-subunit mRNAs of the chicken and the β4-subunit mRNA of the zebra finch. All solutions were prepared in 0.1% (v/v) DEPC-treated water, unless otherwise stated to avoid contamination with RNAses (Simmons *et al.*, 1989).

2.6.1 Design of antisense oligonucleotides

Using the sequence of the zebra finch $GABA_A$ receptor β4-subunit cDNA, a specific 45-base antisense oligonucleotide was designed. This sequence was complementary to the sequence that encodes part of the intracellular loop (KLKIPDLTDVSTIDK; between M3 and M4):

5'-CTTGTCAATGGTGCTGACGTCTGTCAGGTCTGGGATTTTGAGCTT-3'

A 45-base antisense oligonucleotide was similarly designed to recognise the chicken $GABA_A$ receptor β4-subunit mRNA. The sequence of this was:

5'-GCTCATCATGTCCGTGGCCAGCAGCTCGTTGTTCATATCCAGAGT-3'

This was complementary to nucleotide positions 1037bp to 1081bp (which encodes the peptide sequence TLDMNNELLATDMMS) and recognised both isoforms of the β4 subunit, (Wisden, 1989) of the chicken β4-subunit cDNA (Bateson *et al,* 1991b).

The chicken $GABA_A$ receptor α3 subunit cDNA was found in the chicken expressed sequence tag (EST) database (Boardman *et al.* 2002 http://www.chickest.udel.edu/ and the accession number is XM_420268; see Table 5). Based on this sequence a 45-base antisense oligonucleotide was designed complementary to nucleotide positions 2317bp to 2361bp (encoding TKTEVIYTWTLGKDK):

5'-TTTATCCTTCCCCAGGGTCCAGGTGTAGATTACCTCTGTCTTTGT-3

This sequence was confirmed after the sequencing of the chicken genome (http://www.ensembl.org/Gallus_gallus/).

2.6.2 Cryostat sectioning

Silanised slides were used for the fixing of sections to microscope slides (BDH). For this, pre-cleaned microscope slides were baked in an oven (Sakura) at 200°C for 3-4h. After baking, the slides were transferred to glass racks and dipped in a series of solutions: acetone for 1-2min; acetone/silane (3-aminopropyltriethoxysilane) mix (49:1) for 5min; twice in acetone for 1-2min each; three times in 0.1% (v/v) DEPC-treated water for 1-2min each time. The slides were air dried and then baked at 200°C for 8hr. Frozen brains were mounted onto a tissue disk using Tissue Tek (Sakura). 10µm coronal sections from male zebra finch brains were then cut using a cryostat (CM1900, Leica) at –16°C and stored at –80°C until further use. One-day-old chicken brains, a gift from Dr. R. Mileusnic (Open University, UK) were sectioned at 16µm thick horizontal sections.

2.6.3 Fixation

A series of cryostat sections were selected, placed in glass racks and fixed by dipping in ice-cold 2% (w/v) paraformaldehyde (PFA; pH 7.4) in 1x phosphate buffered saline (PBS; 135mM NaCl, 5.6mM KCl, 20.6mM Na_2HPO_4, 3.5mM KH_2PO_4; pH 7.6) for 10min. The sections were then dipped twice in 1x PBS for 5min each. The sections were dehydrated in an ascending ethanol series followed by air-drying.

2.6.4 Preparation of radiolabelled probe

The oligonucleotide probes were labelled in 0.5ml Eppendorf tubes, which contained:

RNAse and DNAse-free water	16.25µl
5x terminal deoxynucleotidyl transferase (TdT) reaction buffer (500mM cacodylate buffer, 5mM $CoCl_2$, 0.5mM DTT; Promega)	5µl
antisense oligonucleotide (5ng/µl)	1µl

[α-^{35}S] dATP (12.5mCi/ml; PerkinElmer Life Sciences Inc.)	1.25µl
TdT (20U/ml; Promega)	1.5µl

this was incubated at 37°C for 1hr.

2.6.5 Purification of radiolabelled probe

To remove any unincorporated [α-^{35}S] dATP, Sephadex G-25 Coarse (Amersham Pharmacia) columns were prepared. Glass wool was inserted into a 1ml syringe (BD Plastipak) and the syringe was filled with 1ml of swollen Sephadex G-25 Coarse in TE buffer (pH 8). The syringe was placed in a 5ml glass tube (Sarstedt) and centrifuged (Universal 32; Hettich) at 2000rpm for 2min to remove excess buffer. The syringe was then placed in a fresh glass tube and 2µl of 1M DTT was added to the tube. 1µl of the labelling reaction was added to 4ml of scintillation fluid (Ultima Gold; Packard Bioscience) to determine the activity of the [α-^{35}S] dATP. The remaining labelling reaction was added to the Sephadex column, and centrifuged at 2000rpm for 1min. 1µl of the eluate was added to 4ml of scintillation fluid and the remainder was stored at 4°C until required. Samples were measured using a Liquid Scintillation Analyser (Tri-Carb 2250CA; Packard).

2.6.6 Hybridisation

200, 000cpm of the purified labelled probe was used per slide in 200µl of hybridisation buffer. Negative control hybridisations contained in addition, a 200-fold excess of the same unlabelled oligonucleotide. Hybridisation buffer (50% (v/v) formamide, 4x standard saline citrate (SSC; 3M NaCl, 0.3M tri-sodium citrate (pH 7)), 5x Denhardt's solution (1% (w/v) polyvinylpyrrolidone, 1% (w/v) bovine serum albumin (BSA; fraction V), 1% (w/v) ficoll), 25mM sodium phosphate (0.5M Na_2HPO_4 (pH 9) and 0.5M NaH_2PO_4 (pH 4); pH 7), 1mM sodium pyrophosphate (0.6M $Na_4P_2O_7$; pH 10.4), 10mM DTT, 100µg/ml polyadenylic acid, 120µg/ml heparin (Grade I-A; from porcine intestinal mucosa), 200µg/ml denatured DNA from salmon testes, 10% (w/v) dextran sulphate (Amersham Pharmacia Biotech AB) was adjusted to 50ml using 0.1% (v/v) DEPC-treated water). The hybridisation buffer which, contained the labelled probe, was added to each slide (200µl) and a coverslip (parafilm; Pechiney Plastic Packaging) was positioned over the sections, which were then placed in humidified chambers. The

chambers were then incubated in a hybridisation oven (Bibby Stewart Scientific) at 42°C overnight.

2.6.7 Washing

Sections were washed to remove the hybridisation buffer and excess radiolabelled probe as follows. Parafilm was removed from the sections by dipping the slides in 1X SSC for 30s at room temperature. Slides were then washed twice in 1x SSC for 30min each in a 55°C shaking water bath (Clifton) at 200rpm. The slides were then transferred to 1x SSC at room temperature for 30s, followed by 0.1x SSC for 30s. Sections were dehydrated in an ascending ethanol series, and finally air-dried. Note, that all wash buffers were prepared in 0.1% (v/v) DEPC-treated water containing 0.04% (v/v) DTT.

2.6.8 Exposure and analysis

The slides were exposed to X-ray film (Kodak Biomax MR, PerkinElmer Life Sciences Inc) for approximately 1 month. The film was developed using an automatic developing machine (Compact X4, Xograph). After placing the film on a light box (UVP Inc), images were captured using a CCD camera (Euresys Multicam) and analysed using the software package Scion Image. Chicken brain regions were identified using Kuenzel and Masson (1988) and zebra finch brain regions according to Stokes *et al.* (1974). Any changes in the level of $GABA_A$ receptor β4-subunit mRNA in brain regions of juvenile and adult zebra finches was investigated by quantitative analysis. The density (pixels/cm^3) of the mRNA labelling was measured in the mesopallium and medial striatum in the zebra finch brain (both the right and left hemispheres) using the Scion Image package. Statistical significance was determined by analysis of variance (1-way-ANOVA) followed by the Bartlett's test for equal variances and $P<0.05$ was considered as the limit of statistical significance.

2.7 Whole mount non-radioactive *in situ* hybridisation

To localise the expression of the $GABA_A$ receptor subunit-like and GAD mRNAs in the embryos and larvae of the sea squirt, whole-mount *in situ* hybridisation was carried out (see Corbo *et al.*, 1997b). This was non-radioactive and the *in vitro* transcribed RNA probes were labeled with digoxigenin, which binds to an antibody (Anti-Digoxigenin-

AP) that is detected by a blue colour reaction via alkaline phosphatase. All solutions were prepared in 0.1% (v/v) DEPC-treated water, unless otherwise stated.

2.7.1 Fertilisation, dechorionation and fixation

The gonoducts from 3 adult sea squirts (collected from Sparke's Yacht Haven, Hayling Island, UK) were dissected. All of the eggs and sperm collected were mixed together using a Pasteur pipette (to produce fertilised embryos) in a petri dish (3.5cm diameter; Sarstedt) with 5ml of seawater and incubated at room temperature for 10min. Using a sieve (0.3nm; VWR International Ltd), the excess sperm were discarded and the fertilised eggs remained in the sieve. It is important to remove the excess sperm to prevent more than one sperm fertilising an egg, which disrupts the normal development of the embryo. The fertilised eggs in the sieve were transferred using a Pasteur pipette to three petri dishes (3.5cm diameter) each containing 5ml of seawater, and incubated at room temperature. The eggs were incubated at room temperature for 7h, for the development of early tailbud (ETB) stage embryos, 10h for late tailbud (LTB) embryos and 30h for larvae.

The ETB and LTB stage embryos were dechorionated as follows. The chorion (outer layer) surrounding the embryo was removed to enable the probe to reach its target. For this 10ml of dechorionation solution (0.05% (v/v) pronase and 1% (v/v) sodium thioglycolate; pH 10) was added to the embryos. The solution was pipetted up and down gently using a Pasteur pipette. The embryos were observed under a microscope (Nikon SMZ1500) after 2-3m and every 30s thereafter until the chorion was digested. The fertilised dechorionated embryos were then washed twice using 10ml of seawater. Finally the ETB, LTB and larvae were fixed using 4% (w/v) PFA in 15ml tubes (Sarstedt) at 4°C overnight. The fixed embryos and larvae were washed three times with absolute ethanol (5min each time), and resuspended in 10ml of absolute ethanol and stored at –20°C until required.

2.7.2 Linearisation of plasmid DNA

An enzyme was chosen to linearise the cloned fragment depending on the orientation of the insert within the pGEM T-Easy vector. The $GABA_A$ receptor α-subunit like and GAD plasmid DNAs were linearised using *Spe*I (cleaves once on the SP6 promoter side

of the vector; see Figure 10), and the $GABA_A$ receptor β- and γ-subunit like plasmid DNAs were linearised using *Nco*I (cleaves once on the T7 promoter side of the vector).

The following were added to a 0.5ml Eppendorf tube and incubated at 37°C for 1hr:

buffer D (60mM Tris-HCl (pH 7.9), 1.5M NaCl, 60mM $MgCl_2$, 10mM DTT)	1µl
plasmid DNA (~4µg)	2µl
*Nco*I (10U/µl)	0.25µl
RNAse and DNAse free water	6.75µl
or:	
buffer B (60mM Tris-HCl (pH 7.5), 500mM NaCl, 60mM $MgCl_2$, 10mM DTT)	1µl
plasmid DNA (~4µg)	2µl
*Spe*I (10U/µl)	0.25µl
RNAse and DNAse free water	6.75µl

Samples were electrophoresised in a 1% (w/v) agarose gel (in 1x TAE buffer) along with a 1kb DNA ladder and the corresponding non-linearised plasmid DNAs for 45min at 100V.

2.7.3 Riboprobe synthesis

The linearised DNA was *in vitro* transcribed to produce RNA, using either the T7 or SP6 RNA polymerase, depending on which enzyme was used to linearise the plasmid DNA.

The reaction was set up in 0.5ml Eppendorf tubes and components added in the following order:

linearised DNA (2.5µg)	10µl
5x transcription buffer (40mM Tris-HCl (pH 8.0), 8mM $MgCl_2$, 50mM NaCl, 2mM spermidine, 30mM DTT; Stratagene)	4µl
DTT (0.1M)	2µl
10x Digoxigenin (DIG) RNA labelling mix (10mM ATP, 10mM CTP,	2µl

10mM GTP, 6.5mM UTP, 3.5mM DIG-11-UTP, pH 7.5; Roche)

RNasin (40U/μl; Promega)	1μl
RNA polymerase (SP6 or T7 (1U/μl); Stratagene)	1μl

Note that T7 RNA polymerase was used to linearise $GABA_A$ receptor α subunit and GAD plasmids, and SP6 RNA polymerase was used to linearise $GABA_A$ receptor β subunit and glycine receptor subunit plasmids. The tubes were incubated in a water bath (Grant) at 37°C for 2hr.

To remove any unincorporated DIG, "Mini quick spin columns" were utilised according to the manufacturers (Roche) instructions. The principle is the same as that described in 2.6 for the Sephadex G25 coarse columns.

2.7.4 Pre-hybridisation

Pre-hybridisation was required to block any non-specific binding of RNA by saturation with yeast RNA (Ambion). Note that in all of the following steps as much liquid was removed as possible, without disturbing the settled embryos and larvae.

The ETB and LTB embryos and larvae were all treated in separate 1.5ml Eppendorf tubes. After removing excess ethanol, 1ml of xylene:ethanol (1:1 ratio; VWR International Ltd) was added to the embryos and larvae and incubated at room temperature for 5min (the time taken for embryos and larvae to settle). The xylene:ethanol was removed slowly, 1ml of xylene:ethanol (3:1) was added and incubated at room temperature for 1hr. The xylene:ethanol was again removed slowly and the embryos and larvae were washed in 1ml of xylene:ethanol (1:1) at room temperature for 5min. 1ml of absolute ethanol was added to the embryos and larvae and incubated at room temperature for 5min to remove the xylene. The embryos and larvae were then washed in 1ml of methanol at room temperature for 5min. After removing the methanol, three washes were carried out in PBT (PBS with 0.1% (v/v) Tween 20) at room temperature for 5min each. To permeabilise cell membranes the embryos were digested using 1ml of PBT and proteinase K (4μg/ml; Roche) at room temperature for 5min (larvae were digested for 10min, as they are more resilient). Embryos and larvae were then fixed in 4% (w/v) PFA (in PBS) at room temperature for 25min. The embryos and larvae were washed three times in 1ml of PBT at room temperature for 5min each

and then washed twice using 1ml of pre-hybridisation buffer (50% (v/v) formamide, 5x SSC, 100µg/ml yeast RNA, 50µg/ml heparin, 0.1% (v/v) Tween 20) at room temperature for 5min each. Finally 1ml of pre-hybridisation buffer was added to all tubes and incubated at 65°C for a minimum of 2hr. The embryos and larvae could then be stored at -20°C until required.

2.7.5 Hybridisation

The pre-hybridisation buffer was removed and the embryos and larvae were incubated in 1ml of hybridisation buffer and 3µl of the digoxygenin-labelled riboprobe (~20ng/µl) and hybridised at 65°C overnight. The hybridisation solution was removed and the embryos and larvae were washed six times in 1ml of washing solution (50% (v/v) formamide, 5x SSC, 0.1% (v/v) Tween 20) at 65°C for 20min each. After removal of the washing solution, the embryos and larvae were washed three times in 1ml of PBT at room temperature for 5min each.

2.7.6 Blocking of non-specific staining

The washing solution was replaced with 1ml of blocking solution (heat treated sheep serum:PBT (1:4 ratio)) at room temperature for a minimum of 2hr. During this time the antibody (anti-Digoxigenin-AP, Fab fragments from sheep; Roche) was diluted in blocking solution (1:3000 ratio), this was incubated on ice, on a shaking platform (100rpm; Luckham R100) for a minimum of 2hr. Negative controls were treated identically however the anti-Digoxigenin antibody was not added.

2.7.7 Antibody application

The blocking solution was replaced with 1ml of the antibody and incubated at 4°C overnight. The antibody was removed and the embryos and larvae were washed with 1ml of PBT at room temperature for 5min, and this was repeated three more times for 15min each. The embryos and larvae were then washed three times in 1ml of APT (100mM NaCl, 100mM Tris-HCl (pH 9.5), 50mM MgCl, 1% (v/v) Tween 20) at room temperature for 15min each. Finally 0.5ml of staining solution (1ml of APT and 4.5µl of 4-nitroblue tetrazolium chloride (NBT; 100mg/ml; Roche) and 3.5µl of 5-bromo-4-chloro-3-indolyl-phosphate (BCIP, the substrate for alkaline phosphatase; 50mg/ml;

Roche) was added to the Eppendorf tubes. The contents of the tubes were transferred to an 8 well plate (Nunc) using a 1ml pipette and wrapped in aluminium foil to develop the colour reaction. Images were taken at x400 magnification using a DIC resolution microscope and camera (Zeiss Axioskop2, Axiocam).

2.8 Immunohistochemistry

Immunohistochemistry was performed to detect GABA in whole-mount sea squirt tissues.

2.8.1 Fixation and preparation of samples

Embryos and larvae were fixed in 4% (w/v) PFA (in PBS) at 4°C overnight (see 2.7.1). The one-day-old chicken brain sections were fixed using different methods. Either 95% (v/v) ethanol: 5% (v/v) glacial acetic acid, on a shaking platform (100rpm) for 25min. Or 4% (w/v) PFA: 1% (v/v) glutaraldehyde in 0.1M PBS (pH 7.4) for 1hr. The chicken brain sections and sea squirt embryos and larvae were rehydrated using 70% (v/v) ethanol, and washed three times in PBS (pH 7.4) for 10min each. Note that all stages of the sea squirt (i.e. ETB, LTB embryos and larvae) were treated separately in a 24 well plate (Nunc). To block any endogenous peroxidases, the PBS was replaced with 0.3% (v/v) H_2O_2 in PBS for 20min. Samples were then washed three times in 1ml of PBS for 10min each. All samples were then saturated with 3% (w/v) BSA (to prevent non-specific binding) and 0.5% (v/v) Triton X100 (to permeabilise the sections) in PBS at room temperature for 1hr.

2.8.2 Antibody application

0.5ml of primary mouse anti-GABA antibody (Chemicon) prepared in 1% (w/v) BSA in PBS, at different dilutions: 1:100, 1:200, 1:400, 1:500 and 1:1000 was added to the chicken brain sections and to each of the wells containing the embryos and larvae and inoculated at 4°C on a shaking platform (100rpm) for 24 hours. Negative controls were also prepared, where samples were incubated in 1% (w/v) BSA in PBS at 4°C for 24hr (without the primary antibody). Samples were washed three times in PBS for 10min each. The secondary goat anti-mouse antibody (biotin SP (long-spacer) conjugated; Jackson Immunoresearch Labs. Inc.) was prepared at the following dilutions 1:100,

1:200, 1:400, 1:800 and 1:1000 in PBS containing 1% (w/v) BSA. 1ml of the secondary antibody was added to the chicken brain sections and sea squirt embryos and larvae, at room temperature on a shaking platform (100rpm) for 1hr. Note that the experiment was repeated, and the samples were incubated with the secondary antibody at 4°C on a shaking platform (100rpm) overnight.

2.8.3 Detection

After 1hr, 1 drop of avidin solution (Vector Laboratories Inc.) and 1 drop of biotin solution (Vector Laboratories Inc.) was added to 3ml of 0.1M Tris buffer (pH 7.4) in a 15ml tube and incubated at room temperature for 30min. Samples were washed (after incubation in secondary antibody) three times in 1ml of 0.1M Tris buffer (pH 7.4) for 10min each. 0.5ml of the biotin-avidin complex was then added to the samples and incubated at room temperature on a shaking platform (100rpm) for 1hr. The complex was poured off and the samples were washed three times in PBS for 5min each. 3-3'-diaminobenzidine tetrahydrochloride (DAB, a substrate for horseradish peroxidase) was prepared during the washes by adding 1 tablet of DAB (3.5mg) to 5ml of Tris buffer (pH 7.4), which was incubated in the dark for 10min. 1 tablet of urea hydrogen peroxide (1mg) was added to the DAB buffer, and once the tablets were completely dissolved, 1ml was added to the samples in the dark, initially for 15min.

2.8.4 Washing

Washing the samples three times in 1ml of PBS for 5min each stopped the colour reaction. The sea squirt samples were observed under a microscope (Ts100, Nikon) at x200 and x400 magnification, and the images were captured using a digital camera (Ts100, Nikon). The chicken brain sections were embedded in Xam (BDH) and cover slips (Chance Proper Ltd.) were placed over the sections, before viewing under the microscope at x40 magnification.

2.9 *In silico* analysis

The sequencing of the human genome has initiated the sequencing of genomes from a variety of species including the chicken (*Gallus gallus domesticus*), puffer fish

(*Takifugu rubripes*) and sea squirt (*Ciona intestinalis*). This was taken advantage of to search for $GABA_A$ receptor like-subunit genes in the above genomes.

2.9.1 Analysis of genome databases

A draft of the puffer fish genome was originally released in October 2001 and a third version in August 2002 (http://genome.jgi-psf.org/fugu6/fugu6.home.html). This was used to search for $GABA_A$ receptor β-subunit gene sequences. The published rat $GABA_A$ receptor β1-, β2- and β3-subunit (see Table 5 for accession numbers) and the chicken $GABA_A$ receptor β4-subunit sequences were entered into a search engine (http://genome.jgi-psf.org/cgi-bin/browserLoad/4210e8a0120c0bfa46231f5c), which identified the scaffold number and the position of related sequences on the scaffold. The minimum consensus sequences (Smith *et al.*, 1989) for splice junctions were then searched for manually. The $GABA_A$ receptor-like subunit exons were searched for manually using the grouping of amino acids according to their residue types (Feng *et al.*, 1984). The identified puffer fish nucleotide sequences were translated into protein sequences using the Justbio website (http://www.justbio.com). The predicted amino-acid sequences obtained from the puffer fish database were then entered into the NCBI search engine (http://www.ncbi.nlm.nih.gov/BLAST/) and 'tblastn' was used to reconfirm the identity of the sequence.

In December 2002, a draft of the sea squirt genome was released (http://genome.jgi-psf.org/ciona4/ciona4.home.html). $GABA_A$ receptor sequences for the rat α1, β2 and γ2 subunits were entered into the database using the BLAST function (http://genome.jgi-psf.org/cgi-bin/runAlignment?db=ciona4). The sequences were analysed as above (in the puffer fish), and pair-wise alignments were conducted for each sea squirt sequence found with the rat $GABA_A$ receptor α1-, β2- and γ2-subunits polypeptide and the glycine receptor α1-subunit polypeptide using ClustalW. Note that the signal peptides were not included in the alignments. Cloned fragments encoding the $GABA_A$ receptor α, β and γ-like subunits from the sea squirt were also compared to the rat $GABA_A$ receptor α, β and γ subunits and the rat glycine receptor α subunit.

Sequence	Accession number
Rat $GABA_A$ receptor α1 subunit	NP_899155
Rat $GABA_A$ receptor α2 subunit	AAC42030
Rat $GABA_A$ receptor α3 subunit	AAC42031
Rat $GABA_A$ receptor α4 subunit	NP_542154
Rat $GABA_A$ receptor α5 subunit	NP_058991
Rat $GABA_A$ receptor α6 subunit	NP_068613
Rat $GABA_A$ receptor β1 subunit	NP_037088
Rat $GABA_A$ receptor β2 subunit	NP_037089
Rat $GABA_A$ receptor β3 subunit	NP_058761
Rat $GABA_A$ receptor γ1 subunit	CAA40739
Rat $GABA_A$ receptor γ2 subunit	AAC42036
Rat $GABA_A$ receptor γ3 subunit	CAA44930
Rat $GABA_A$ receptor ε subunit	NP_075579
Rat $GABA_A$ receptor θ subunit	NP_113921
Rat glycine receptor α1 subunit	NP_037265
Rat glycine receptor α2 subunit	NP_036700
Rat glycine receptor α3 subunit	NP_536686
Human $GABA_A$ receptor α1 subunit	CAA32874
Human $GABA_A$ receptor α3 subunit	AAB27279
Human $GABA_A$ receptor β1 subunit	AAB30712
Human $GABA_A$ receptor β2 subunit	AAB33983
Human $GABA_A$ receptor β3 subunit	AAA52511
Human $GABA_A$ receptor γ2 subunit	NP_944494
Human $GABA_A$ receptor ε subunit	CAA70904
Human $GABA_A$ receptor θ subunit	BC109211
Human GAD_{65}	Q05329
Human GAD_{67}	Q99259
Chicken $GABA_A$ receptor α1 subunit	1805342A
Chicken $GABA_A$ receptor α3 subunit	XM_420268
Chicken $GABA_A$ receptor β2 subunit	JH0829
Chicken $GABA_A$ receptor β3 subunit	NP_990677
Chicken $GABA_A$ receptor β4 subunit	CAA39970
Chicken $GABA_A$ receptor γ2 subunit	CAA38704
Chicken $GABA_A$ receptor γ4 subunit	CAA51939
Mouse glycine receptor α4 subunit	NM_010297
Sea squirt GAD	AB072597

Table 5: Accession numbers from the NCBI database for the different sequences used in this investigation. GAD is the abbreviation for glutamic acid decarboxylase.

Chicken $GABA_A$ receptor α3-, β4- and γ4-subunit gene structures and transcriptional orientations were analysed using the chicken genome, which was published in May 2004 (http://www.ensembl.org/Gallus_gallus/). Chicken $GABA_A$ receptor α3, β4 and γ4 subunits (refer to Table 5 for accession numbers) were entered into the search engine (http://www.ensembl.org/Multi/blastview?species=Gallus_gallus). The minimum consensus sequences for splice junctions were then searched for manually. In addition, the chicken genome was searched for orthologues of the rat $GABA_A$ receptor θ and ε subunits (see Table 5 for accession numbers).

2.9.2 Phylogenetic analysis

To analyse the phylogenetic relationships of $GABA_A$ receptor subunits, alignments of the relevant subunit sequences were generated using ClustalX 1.8 (Thompson *et al.*, 1997). All of the sequences were compared over a region of either ~300 amino acids or ~900 basepairs, which corresponded to the minimum length of sequence that was deduced from cDNAs cloned in our laboratory (I. K. Pahal, C. Thode and M. G. Darlison, unpublished results). Manual editing was carried out to further optimise the alignments. Phylogenetic trees were calculated using the maximum likelihood method (PROML and DNAML) of the Phylip 3.6 package (Felsenstein, J. (2002) Version 3.6a, distributed by the author, Department of Genetics, University of Washington, USA). To search for the best tree, different rates of evolution for each amino-acid position were taken into consideration. Estimations of these rates were determined using the Tree-Puzzle 5.0 programme (Schmidt *et al.*, 2002), and were based on the Jones-Taylor-Thornton model for amino-acid substitutions (Jones *et al.*, 1992). Note that the rat $GABA_A$ receptor α1 subunit served as the outgroup for both of the trees constructed.

Results

3 Evolution of $GABA_A$ receptor β-subunit genes

3.1 Introduction

It has been hypothesised that the vertebrate $GABA_A$ receptor subunit genes that exist today have evolved by two genome duplications (tetraploidisation events) from a common ancestral cluster of $GABA_A$ receptor genes (Buckle *et al.,* 1989; Wilcox *et al.,* 1992; Russek and Farb, 1994; Bailey *et al.*, 1999b; Russek, 1999). The ancestral cluster is proposed to exist of one α-, one β- and a γ-like subunit gene. The first genome duplication took place giving rise to two β subunits and then a second duplication occurred, which gave four β-like subunits. However, only three β-subunit genes are known to be expressed by all vertebrate species (Darlison and Albrecht, 1995; Barnard *et al.*, 1998; Whiting, 2003). The θ subunit is most similar to the β subunits (see Bonnert *et al.*, 1999; Sinkkonen *et al.*, 2000; Darlison *et al.*, 2005).

The β subunits are essential for the binding of GABA (Amin and Weiss, 1993; Ortells and Lunt, 1995) and surface expression of the receptor (Connor *et al.*, 1998). Recently, the $GABA_A$ receptor β2 subunit has been shown to play a role in anxiety. 'Knock-out' studies by Blednov *et al.* (2003) found that the β-selective drug etomidate did not produce sedation in mice. Homanics *et al.* (1997) and DeLorey *et al.* (1998) showed that β3-subunit gene 'knock-out' mice exhibit epileptic seizures and display hypersensitive behaviour resembling Angelman Syndrome in humans. The β1 subunit is highly abundant (49%) in the rat hippocampus (Li and De Blas, 1997). As the hippocampus is essential for memory formation (Barnes, 1979) this suggests a role for the β1 subunit in learning and memory. The β4 subunit has to date only been found in the chicken, and is only 74% identical to the mammalian β subunits (β1, β2 and β3; Bateson *et al.*, 1991b).

The initial aim of this investigation was to search for $GABA_A$ receptor β-subunit genes in a variety of species, particularly the β4 subunit as little is known of this subunit. $GABA_A$ receptor β-subunit cDNAs were amplified from the brains of different species

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Refer to Table 5 for all accession numbers.

(e.g. birds, reptiles, amphibians) using PCR and degenerate oligonucleotide primers. As a draft genome sequence of the puffer fish (http://bahama.jgi-psf.org/fugu/html/README.html) was available *in silico* analysis was conducted in this species to search for $GABA_A$ receptor β-like subunit genes.

3.2.1 Amplification of $GABA_A$ receptor β-subunit cDNAs from zebra finch

Degenerate primers (βF and βR) were used to amplify $GABA_A$ receptor β-subunit cDNAs from the juvenile zebra finch; the expected fragment size was approximately 1100bp (see 2.4; Table 2).

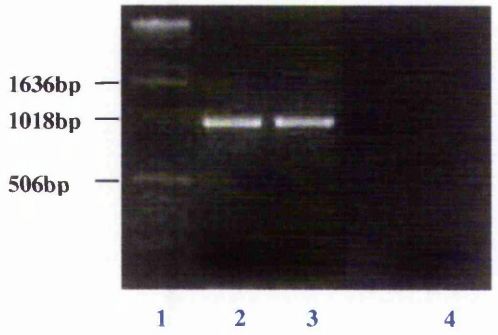


Figure 11: A 2% (w/v) agarose gel illustrating the amplification of $GABA_A$ receptor β-subunit cDNAs from the zebra finch (*Taeniopygia guttata*). Lane 1 contains a 1kb DNA ladder (marker), lanes 2-3 show the product amplified from zebra finch first-strand cDNA, and lane 4 is the negative control (no DNA).

The PCR yielded a fragment of the expected size, namely 1100bp (Figure 11). The no DNA control was indeed negative confirming that there was no contamination. PCR products were purified; cloned using the pCR 2.1 TOPO Cloning Vector Kit, and plasmid DNA from recombinant clones was isolated using the Plasmid Miniprep Spinkit 250. The plasmid DNAs were digested using the restriction enzyme *Eco*RI to determine whether an insert was present in the plasmid (there are sites for *Eco*RI in the vector that flank the insert). A double digestion was then conducted using *Eco*RI (recognition size of six bases) and *Sau*3AI (cuts at a tetranucleotide sequence hence cuts more frequently than *Eco*RI) to produce different banding patterns allowing one to differentiate between the different cDNA clones encoding $GABA_A$ receptor β subunits.

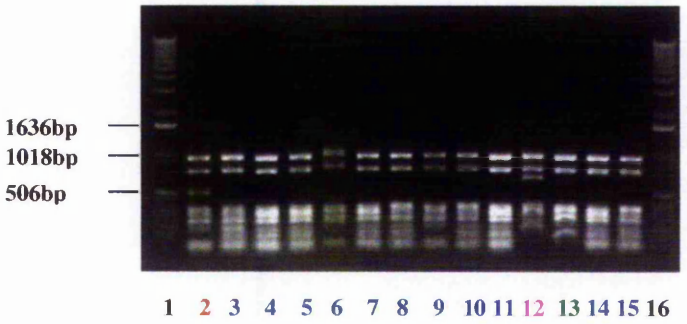


Figure 12: A 2% (w/v) agarose gel showing the restriction digest with *Eco*RI and *Sau*3A1. Lanes 1 and 16 contain the 1kb DNA ladder, and lanes 2-15 illustrate cloned zebra finch (*Taeniopygia guttata*) cDNA fragments. Samples 2, 4, 12, and 13 (indicated in four different colours) demonstrate four different banding patterns (clones).

Figure 12 illustrates that a minimum of four different types of cDNA clones were isolated. After DNA sequence analysis, the isolates were found to encode: the $GABA_A$ receptor β1 subunit (2 clones sequenced; this subunit was previously only found in mammals), the β2 subunit (4 clones), the β3 subunit (2 clones) and the β4 subunit (2 clones). Sequence identities are shown in Table 6 below.

Polypeptide	**Sequence identity (%)**
β1 (rat vs. zebra finch)	82.1
β2 (chicken vs. zebra finch)	99.4
β3 (chicken vs. zebra finch)	97.7
β4 (chicken vs. zebra finch)	98.4

Table 6: Comparison of the zebra finch (*Taeniopygia guttata*) partial protein sequences with those of the rat (*Rattus norvegicus*) $GABA_A$ receptor β1 subunit and the chicken (*Gallus gallus domesticus*) β2, β3 and β4 subunits, using ClustalW (http://www.ch.embnet.org/software/ClustalW.html). The β1 subunit was compared against the rat, as this subunit has not been found, to date, in the chicken.

There is a very high level of sequence identity of the β2, β3 and β4 subunits between the chicken and zebra finch, and relatively high identity between the rat and zebra finch β1 subunits. The amino-acid sequences of the $GABA_A$ receptor β subunits of the zebra finch were aligned to look for regions of similarity and differences (see Figure 13).

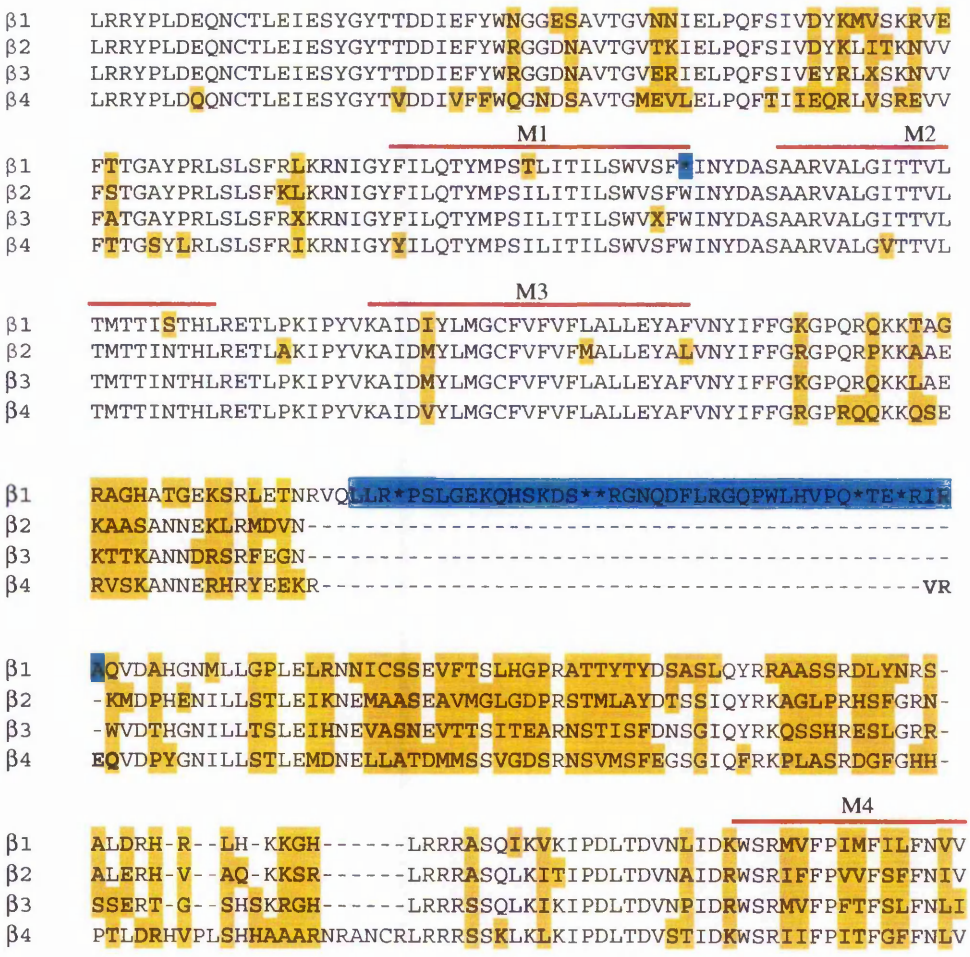


Figure 13: Alignment of zebra finch (*Taeniopygia guttata*) $GABA_A$ receptor β-subunit sequences generated using the computer programme ClustalW (http://www.ch.embnet.org/software/ClustalW.html). Dashes denote gaps, which have been inserted to maximise the alignment. Red bars above the sequences indicate the transmembrane domains, namely M1 to M4. Yellow shading denotes differences between the sequences. The stop codon in M1 and the 'intronic' sequence (see text) of the β1 subunit are indicated by blue shading. Stop codons generated are represented by an asterisk (X).

Sequencing revealed that the $GABA_A$ receptor β1-subunit protein possesses a stop codon in M1 (possibly a PCR error by *Taq* DNA polymerase) and an additional possible 'intronic' sequence with multiple stop codons. The additional sequence is approximately 130bp in length. The alignment (Figure 13) showed that as expected the majority of the differences in sequence occurred in the intracellular loop, i.e. between M3 and M4. Interestingly, the β4 subunit possessed the additional four amino acids (VREQ; indicated in bold) also present in the long isoform of the chicken β4-subunit polypeptide (β4L; Bateson *et al.*, 1991b).

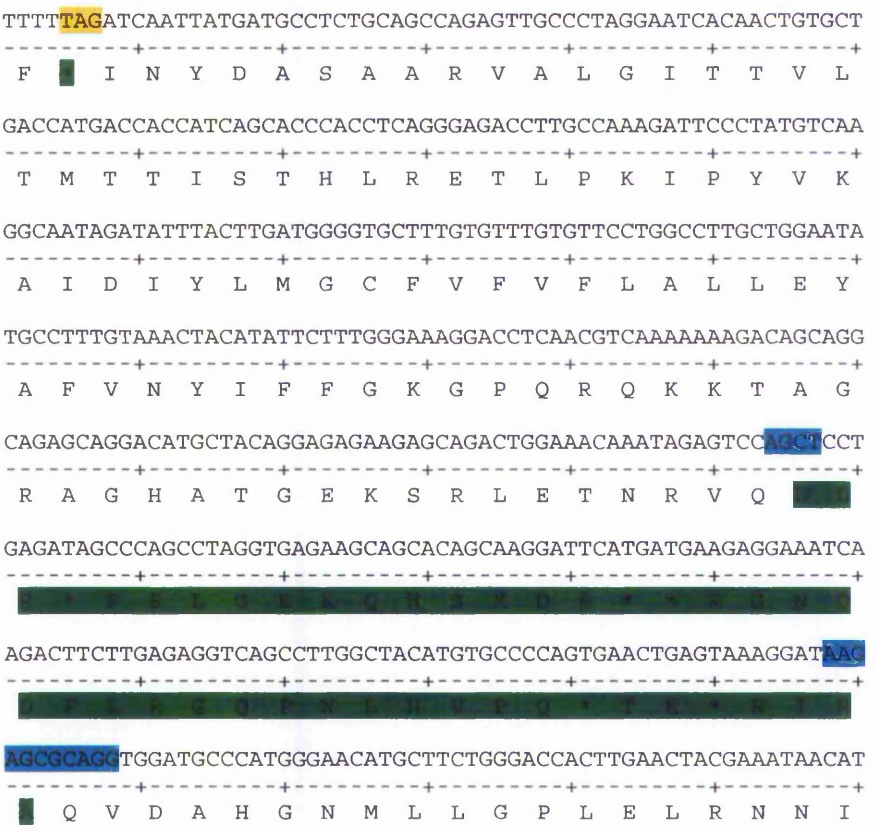


Figure 14: Partial sequence of the zebra finch (*Taeniopygia guttata*) cDNA encoding part of the $GABA_A$ receptor β1 subunit. The nucleotide and deduced amino-acid sequences are shown in single-letter code. The stop codon and possible intronic sequence is shown in green shading. The substituted codon is highlighted in yellow.

The stop codon TAG (in yellow), in the zebra finch $GABA_A$ receptor β1 subunit cDNA that would lead to a premature end of translation, was expected to be the amino acid trytophan (see Figure 14; encoded by TGG). This suggests that there was a G-A substitution in the second position of the codon. The sequences highlighted in blue (Figure 14) correspond to the consensus donor-acceptor splice sequences (Shapiro and Senopathy 1987). The intron 5' donor sequence is AG|**GT**, where the second guanine may have mutated and 3' acceptor site is T/C_6N**CAG**|G (the vertical line denotes the intron/exon boundary, and the conserved nucleotides are in bold). There are also multiple stop codons in the additional piece of sequence, an indication of pseudogenes (expressed non-functional genes). All three of the partial cDNA clones encoding the $GABA_A$ receptor β1 subunit were identical in their nucleotide sequence.

To determine whether a $GABA_A$ receptor β1 subunit is present in other avian species, the zebra finch β1-subunit sequence was entered into the chicken genome database (http://www.ensembl.org/Gallus_gallus/). A sequence was identified on chromosome 13 (2114842 to 2115105bp), however this was only 70% identical (and not more than 97% as expected in a functional gene) in sequence to the zebra finch β1 polypeptide sequence. In addition, the chicken EST database (http://www.chickest.udel.edu/) was also searched, and two exons (5 and 7) were found, (i.e. only a partial sequence which shared 89% sequence identity to the murine $GABA_A$ receptor β1 subunit) providing further evidence that the β1 subunit may be a pseudogene in both the zebra finch and chicken.

3.2.2 Amplification of $GABA_A$ receptor β-subunit cDNAs from the African clawed frog

Degenerate primers (βF and βR) were used to amplify $GABA_A$ receptor β-subunit cDNAs from the African clawed frog; the expected fragment size was ~1100bp.

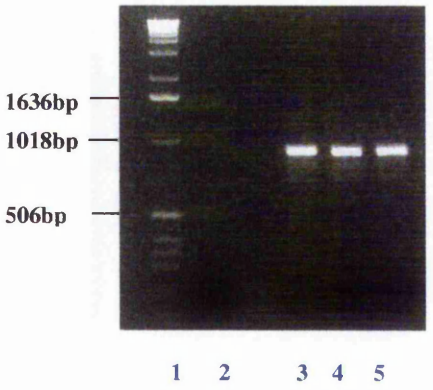


Figure 15: A 2% (w/v) agarose gel demonstrating the amplification of $GABA_A$ receptor β-subunit cDNAs from the African clawed frog (*Xenopus borealis*). Lane 1 contains a 1kb DNA ladder (marker), lane 2 is the negative control (no DNA) and lanes 3-5 show the product amplified from the African clawed frog first-strand cDNA.

The PCR yielded a fragment of the expected size, namely 1100bp (Figure 15), which was assumed to represent β-subunit cDNAs from the frog (lanes 3-5 represent aliquots of the same PCR). The 'no DNA' control was indeed negative confirming that there was no contamination. Sequencing revealed $GABA_A$ receptor β2- (4 clones), β3- (2 clones) and β4- (2 clones) subunit cDNAs.

There is a very high level of sequence identity of the β2, β3 and β4 subunits between the rat and frog and the chicken and frog, and once more the highest is the β2-subunit polypeptide (see Table 7).

Polypeptide	**Sequence identity (%)**
β2 (rat vs. frog)	90.1
β3 (rat vs. frog)	78.9
β4 (chicken vs. frog)	87.1

Table 7: Sequence similarities of the partial amino-acid sequences of the $GABA_A$ receptor β-subunit sequences from the rat (*Rattus norvegicus*), chicken (*Gallus gallus domesticus*) and African clawed frog (*Xenopus borealis*).

3.2.3 Amplification of $GABA_A$ receptor β-subunit cDNAs from the common wall lizard

Degenerate primers (βF and βR) were used to amplify $GABA_A$ receptor β-subunit cDNAs from the common wall lizard (*Podarcis muralis*); the expected fragment size was approximately 1100bp.

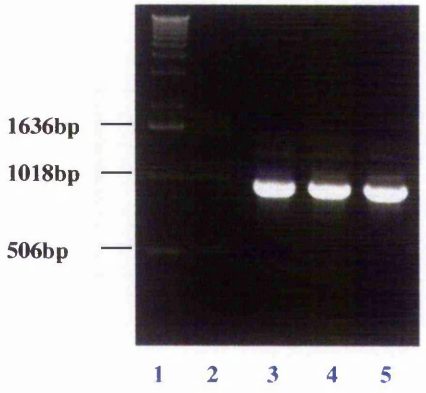


Figure 16: A 2% (w/v) agarose gel demonstrating the amplification of $GABA_A$ receptor β-subunit cDNAs from the common wall lizard (*Podarcis muralis*). Lane 1 contains a 1kb DNA ladder (marker), lane 2 the negative control (no DNA) and lanes 3-5 illustrate the product amplified from common wall lizard first-strand cDNA.

The PCR yielded a fragment of the expected size, namely 1100bp (Figure 16), which was assumed to represent β-subunit cDNAs from the lizard. The 'no DNA' control was

indeed negative confirming that there was no contamination. The samples were sequenced and analysed as above in 3.2.2. From the results $GABA_A$ receptor β3- (2 clones) and β4- (5 clones) subunit cDNAs were found. However, no β2-subunit cDNAs were found despite several cloning attempts.

Polypeptide	**Sequence identity (%)**
β3 (rat vs. lizard)	85.4
β4 (chicken vs. lizard)	84.1

Table 8: Sequence similarities of the partial amino-acid sequences of the $GABA_A$ receptor β-subunit sequences from the rat (*Rattus norvegicus*), chicken (*Gallus gallus domesticus*) and common wall lizard (*Podarcis muralis*).

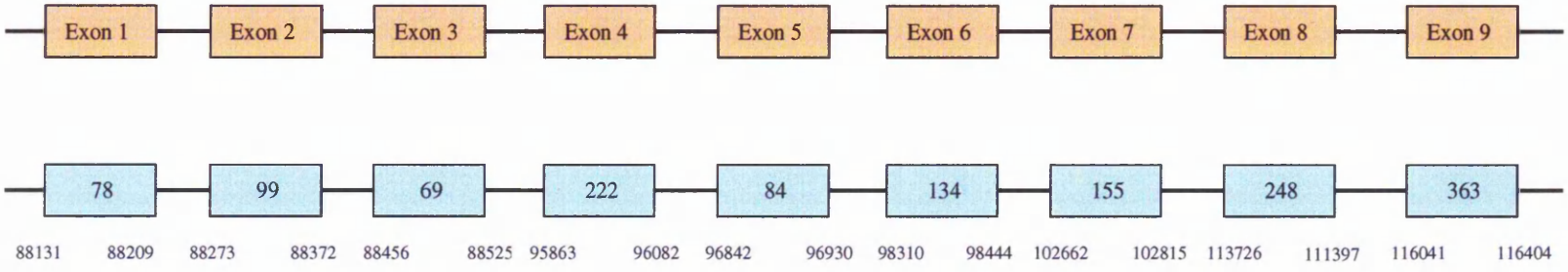
There was a high level of sequence identity of the β3 and β4 subunits between the rat and lizard and the chicken and lizard shown in Table 8.

3.2.4 *In silico* analysis of the puffer fish genome to search for $GABA_A$ receptor β-like subunit genes

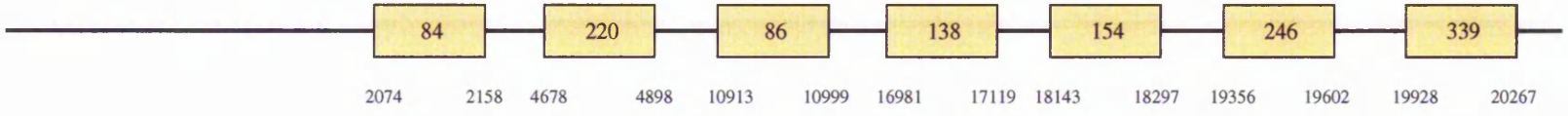
$GABA_A$ receptor β-subunit sequences were searched for in the puffer fish (*Takifugu rubripes*) genome version 3.0 (http://genome.jgi-psf.org/fugu6/fugu6.home.html). The locations of the $GABA_A$ receptor β-subunit genes were: β1 on scaffold 798; β2 on scaffold 531; β3 subunit on scaffold 8; and β4 on scaffold 3072 (see Figure 17 for nucleotide positions and length of the exons). These were named β1- to β4-like due to the highest sequence similarity detected compared with previously reported $GABA_A$ receptor β-subunit genes in the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST/). Unexpectedly four β-like subunits were found, however unlike in the zebra finch, none of the coding sequences possessed stop codons or the additional sequence. All four sequences also contained the domains (YGTT and TGSY) needed to bind GABA (Amin and Weiss, 1993), indicating that they are likely to be functional genes. The sequences deduced from the puffer fish genome were then aligned using ClustalW to look for similarities and differences.

From Figure 17 it can be seen that the $GABA_A$ receptor subunit gene structure is highly conserved, the largest exon is exon 9 (~0.3kb) and shortest is exon 3 (~0.07kb). Exons 1

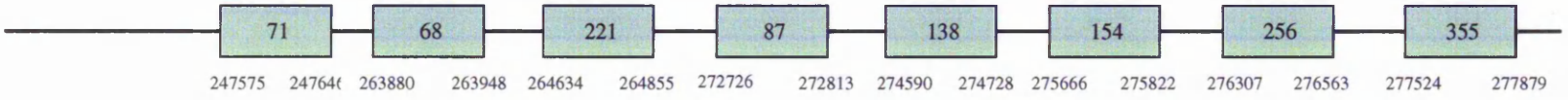
and 2 encoding the $GABA_A$ receptor β2-like subunit polypeptide and exons 1 to 3 encoding the $GABA_A$ receptor β4-like subunit polypeptide could not be found. In addition, the sequence similarity is not generally as high at the N' amino terminus, hence it was difficult to search for 5' exons manually. Nevertheless the relative lengths and positions of exons are highly conserved to other known vertebrate $GABA_A$ receptor gene structures (Sommer *et al.*, 1990; Kirkness *et al.*, 1991; Lasham *et al.*, 1991; see Figure 7). The exons were searched for manually using the minimum consensus sequences (Smith *et al.*, 1989) for splice junctions. Figures 18 to 21 demonstrate the conserved sequences denoting the intron/exon splice sites in the $GABA_A$ receptor β-subunit genes in the puffer fish. In contrast, there was no conservation of intron size, (ranging from 0.1kb to 16.2kb), this is in agreement with Lasham *et al.* (1991) who found that the intron sizes between the chicken $GABA_A$ receptor β4 subunit and murine δ-subunit gene was not consistent. In addition, the most diverged sequence, is at the 3'end of exon 5 in both the puffer fish β1 and β4-like subunit genes, this is consistent with the findings of Smith *et al.* (1989) and Lasham *et al.* (1991). The largest intron in the β1-like subunit gene was 10.9kb between exon 7 and 8, and the shortest was 0.1kb located between exon 2 and 3 (refer to Figure 18). Within the puffer fish $GABA_A$ receptor subunit genes, the largest intron is 6kb between exon 4 and 5 in the β2-like subunit gene; 16.2kb between exon 2 and 3 in the β3-like subunit gene; and only 1.3kb between exon 7 and 8 in the β4-like subunit gene. The shortest intron length in the β2-like subunit gene was 0.3kb between exon 8 and 9; in the β3-like subunit it was 0.5kb between exon 7 and 8; and 0.4kb between exon 6 and 7 in the β4-like subunit gene. The intron between exons 8 and 9 occurs within the region encoding the intracellular loop, which varies between subunits hence it is not surprising that this intron size does vary from 0.3kb to 2.1kb (refer to Figures 18 to 21).



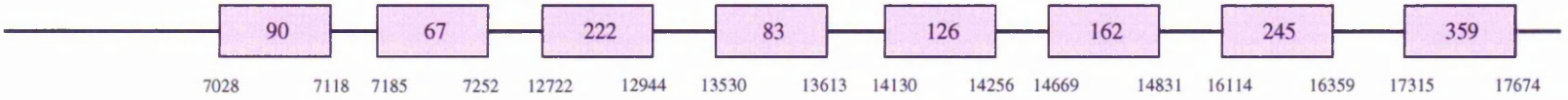
$GABA_A$ receptor β1-like subunit gene structure (Scaffold 798)



$GABA_A$ receptor β2-like subunit gene structure (Scaffold 531)



$GABA_A$ receptor β3-like subunit gene structure (Scaffold 8)



$GABA_A$ receptor β4-like subunit gene structure (Scaffold 3072)

Figure 17: Schematic of $GABA_A$ receptor β-subunit like sequences found in the puffer fish (*Takifugu rubripes*) genome. The length of the exons are indicated by numbers in the boxes and numbers under the boxes represent nucleotide positions in a particular scaffold in the puffer fish genome database (http://genome.jgi-psf.org/fugu6/fugu6.home.html version 3). Exons 1 and 2 on scaffold 531and exons 1 on scaffolds 8 and 3072 could not be found. Note that the schematic is not to scale.

Exon 1.....AAAAG**gt**gagtgctttt.....intron (0.1kb).....gtctattcgc**ag**CGTCA.....

Exon 2.....TGGAG**gt**acaagcgcgg... intron (0.1kb).....acccccctccc**ag**GTCCC.....

Exon 3.....GGTGA**gt**gaattaaaca.....intron (7.4kb).....tgtgtctccc**ag**GACTA.....

Exon 4.....CTCAG**gt**gggctctgat.....intron (1.8kb).....ccctcttttc**ag**GATAA.....

Exon 5.....TGTGA**gt**agaaggttct..... intron (1.3kb).....cctgtgcagatgGCTAC.....

Exon 6.....CACAG**gt**aaacgcggcg.....intron (4.2kb).....tctcgtcttc**ag**GTGCC.....

Exon 7.....ACTCG**gt**aagaagcagg.....intron (10.9kb)...tctttcctga**ag**GCATC.....

Exon 8.....CAAGG**gt**gagttgggat..... intron (2.1kb).....ttattcaggc**ag**AGCCG.....

Exon 9.....ACTAC**gt**ctgatggaacc.....

Figure 18: Sequences across the intron/exon boundaries of the puffer fish $GABA_A$ receptor β1-subunit like gene. Nucleotides in upper-case letters represent exonic sequences whilst those intronic sequences are shown in lower-case. The conserved minimum consensus sequences for splice junctions (Smith *et al.*, 1989) gt (5' donor site) and ag (3' acceptor site) are indicated by blue lettering. The approximate sizes of introns are shown in brackets.

Exon 3.....TTTGAtcccaaaaaccc.....intron (2.5kb).....tgcactttcc**ag**GACTA.....

Exon 4.....CTGAG**gt**gagacccaaa.....intron (6kb).....atatgtttcc**ag**GATAA.....

Exon 5.....GAGCT**gt**gagtaatccc..... intron (6kb).....gcctcttttc**ag**ACGGC.....

Exon 6.....CACAG**gt**aatgacagaa.....intron (1kb).....tcctgtctgc**ag**GCTCT.....

Exon 7.....TTTAG**gt**gaggcagcta.....intron (1kb).....ttcctactcc**ag**GTATC.....

Exon 8.....ACAAG**gt**gcgctggaga.....intron (0.3kb).....ttcctcgggc**ag**ATGGA.....

Exon 9.....ATTAT**gt**caaataataa.....

Figure 19: Sequences across the intron/exon boundaries of the puffer fish $GABA_A$ receptor β2-subunit like gene. Nucleotides in upper-case letters represent exonic sequences whilst those intronic sequences are shown in lower-case. The conserved minimum consensus sequences for splice junctions (Smith *et al.*, 1989) gt (5' donor site) and ag (3' acceptor site) are indicated by blue lettering. The approximate size of introns are shown in brackets.

Exon 2.....TGGAG**gt**acgtggagct.....intron (16.2kb).....ctctccccccc**ag**GTCCT.....

Exon 3.....ACATG**gt**acgttccaat..... intron (0.7kb).....ctttctcctc**ag**GACTA.....

Exon 4.....CTGAG**gt**acaaacacgg.....intron (7.8kb).....gtctgtccac**ag**AATAA.....

Exon 5.....TGTAA**gt**gatgataact.....intron (1.8kb).....gctttgacac**ag**ACGGA.....

Exon 6.....TACAG**gt**gagaccgtcg.....intron (0.9kb).....ttgtccttgc**ag**GTTCC.....

Exon 7... CCTGG**gt**aagaaccaga.....intron (0.5kb).....gtctgtgctc**ag**GAGTG.....

Exon 8.....AACAG**gt**ttgcaccccc.....intron (1kb).....ctccccacgc**ag**GTGGA.....

Exon 9.....TTGAT**gt**gcaccccacc.....

Figure 20: Sequences across the intron/exon boundaries of the puffer fish $GABA_A$ receptor β3-subunit like gene. Nucleotides in upper-case letters represent exonic sequences whilst those intronic sequences are shown in lower-case. The conserved minimum consensus sequences for splice junctions (Smith *et al.*, 1989) gt (5' donor site) and ag (3' acceptor site) are indicated by blue lettering. The approximate sizes of introns are shown in brackets.

Exon 2.....TGGGG**gt**acgtcgactg.....intron (0.1kb).....gtgtatttcc**ag**GTCGA.....

Exon 3.....ATATG**gt**aagatgtttc.....intron (5.5kb).....ttctgtccac**ag**GACTA.....

Exon 4.....CTCAG**gt**aaactgttct.....intron (0.6kb).....tttccgtttc**ag**AATCA.....

Exon 5.....GAGTT**gt**aagttcgagg.....intron (0.5kb).....tcgtccacagatGGATA.....

Exon 6.....CACAG**gt**gaggtgctcg.....intron (0.4kb).....tgtcccctgc**ag**GTGCC.....

Exon 7.....ATTAG**gt**atgatgtgac.....intron (1.3kb).....tttacctcac**ag**GCATC.....

Exon 8.....CTAAG**gt**aggaatcctc.....intron (1kb).....tctcccgccc**ag**GCCGA.....

Exon 9.....CTAAG**gt**gccgacgtcc.....

Figure 21: Sequences across the intron/exon boundaries of the puffer fish $GABA_A$ receptor β4-subunit like gene. Nucleotides in upper-case letters represent exonic sequences whilst those intronic sequences are shown in lower-case. The conserved minimum consensus sequences for splice junctions (Smith *et al.*, 1989) gt (5' donor site) and ag (3' acceptor site) are indicated by blue lettering. The approximate sizes of introns are shown in brackets.

The alignment (Figure 22) showed that as expected the majority of the differences in sequence occurred in the intracellular loop, i.e. between M3 and M4. Interestingly, the $GABA_A$ receptor β4 subunit possesses an additional four amino acids (LREQ) within the intracellular loop domain, similar to the chicken β4L subunit sequence: VREQ (Bateson *et al.*, 1991b). The identities with the corresponding rat and chicken sequence were then calculated by aligning pairs of sequences using ClustalW.

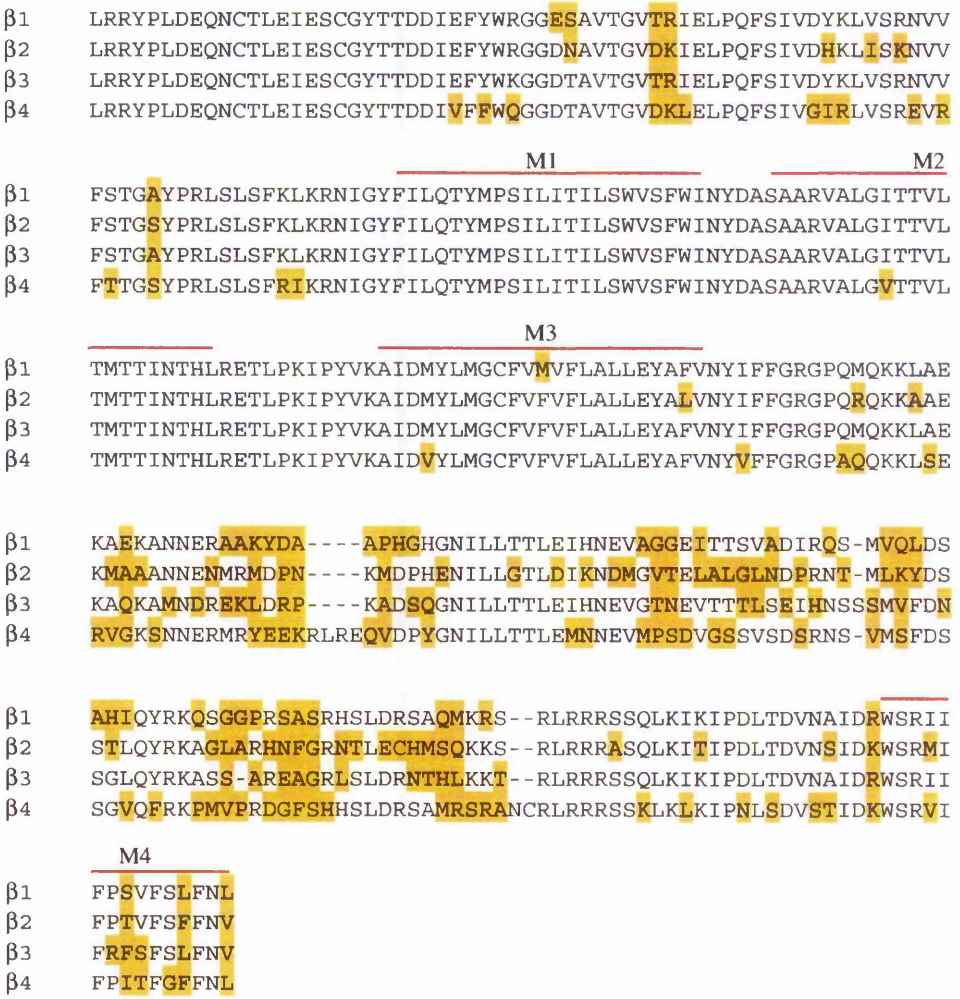


Figure 22: Alignment of the deduced amino-acid sequences of the $GABA_A$ receptor β-like subunit sequences obtained from *in silico* analysis of the puffer fish (*Takifugu rubripes*) genome using the computer programme ClustalW (http://www.ch.embnet.org/software/ClustalW.html). Gaps have been introduced into the sequences to maximise the alignment and are denoted by dashes. The proposed membrane-spanning domains (M1-M4) are indicated by solid red lines above the sequences, and differences between subunits are highlighted in yellow.

There was a relatively high level of sequence identity between the puffer fish and rat and chicken β-subunit polypeptide sequences (see Table 9). The puffer fish β2-like polypeptide shared the highest sequence identity. To ensure that the four puffer fish sequences were actually different to one another, a pair-wise alignment was carried out using ClustalW (see Table 10). The sequences were unique, and the alignments showed that the sequences were approximately 70% identical to each other.

Polypeptide	Sequence identity (%)
β1 (rat vs. puffer fish)	70.6
β2 (rat vs. puffer fish)	87.1
β3 (rat vs. puffer fish)	79.2
β4 (chicken vs. puffer fish)	68.9

Table 9: Sequence similarities between the amino-acid sequences of the $GABA_A$ receptor β subunit from the rat (*Rattus norvegicus*) and chicken (*Gallus gallus domesticus*) and the puffer fish (*Takifugu rubripes*).

	β2	β3	β4
β1	69.8	74.1	72.7
β2	/	76.8	69.3
β3	/	/	72.2

Table 10: Sequence similarities of the deduced amino-acid sequences of the $GABA_A$ receptor β-like subunit sequences from the puffer fish *(Takifugu rubripes)*.

3.2.5 Similarities between the $GABA_A$ receptor β subunits of different species

The four $GABA_A$ receptor β-subunit (β1, β2, β3 and β4) sequences from different species were aligned using ClustalW to compare similarities and differences between species.

The deduced amino-acid sequences of part of the $GABA_A$ receptor β1 subunit from the rat, human, zebra finch and puffer fish are illustrated in Figure 23. There is conservation of sequence in all (particularly transmembrane domains) but the intracellular loop region of the polypeptide. Although this alignment includes sequences from three classes of vertebrates (mammals, birds and fish) it is evident that the four polypeptides share a high degree of identity despite their evolutionary distance.

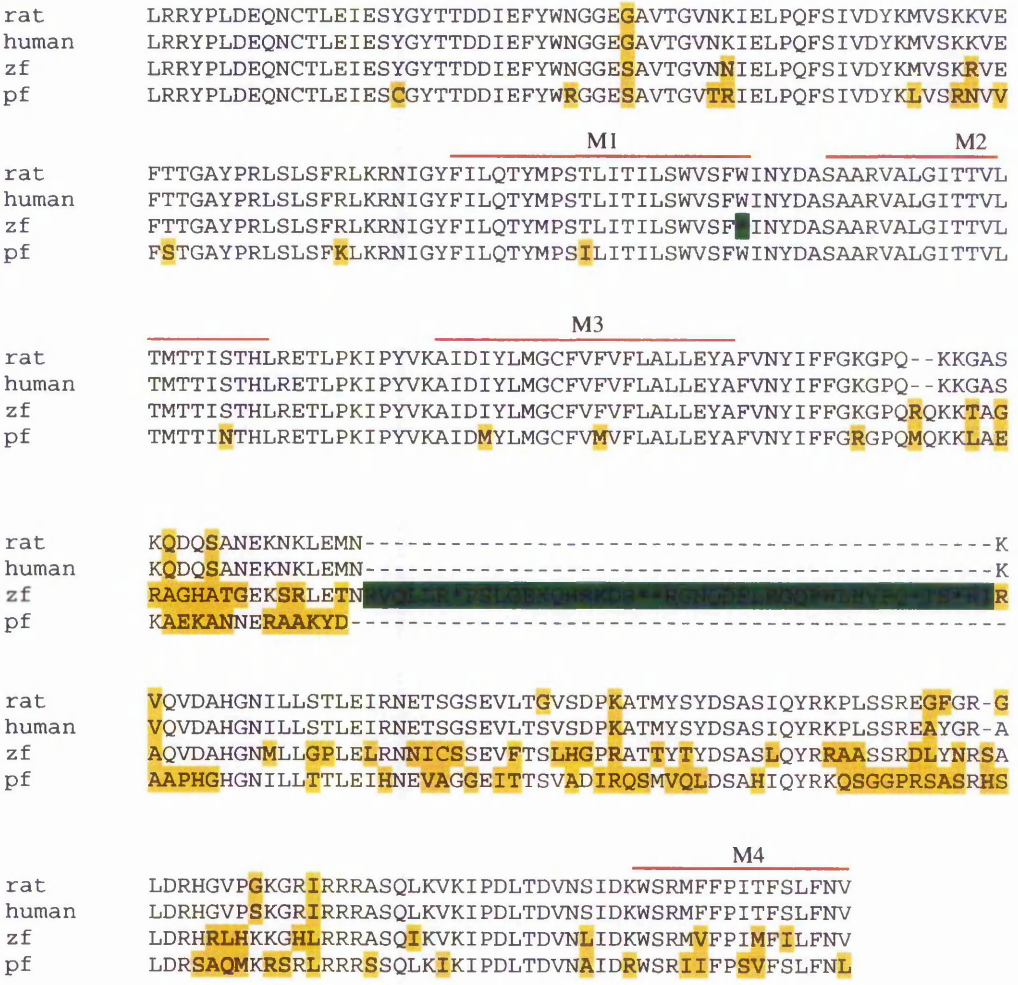


Figure 23: Alignment of the deduced amino-acid sequences of the $GABA_A$ receptor β1 subunit from the rat (*Rattus norvegicus*), human (*Homo sapiens*), zebra finch (zf; *Taeniopygia guttata*) and that obtained from *in silico* analysis of the puffer fish (pf; *Takifugu rubripes*) genome, using the computer programme ClustalW (http://www.ch.embnet.org/software/ClustalW.html). Gaps introduced into the sequences to maximise the alignment are denoted by dashes. The proposed membrane-spanning domains (M1-M4) are indicated by solid red lines above the sequences and differences in sequence between species are highlighted in yellow. The stop codon in M1 of the zebra finch and the intronic sequence (see text) are in green shading.

Since both human and rat are mammalian species, these sequences were expected to be most similar. The puffer fish $GABA_A$ receptor β1 subunit was less similar to mammalian sequences than the zebra finch β1 subunit (refer to Table 11).

	Human	Zebra finch	Puffer fish
Rat	98.3	82.1	74.2
Human	/	83.1	74.9
Zebra finch	/	/	70.9

Table 11: Sequence similarities (as a percentage) of the amino-acid sequences of the $GABA_A$ receptor β1 subunit from the rat (*Rattus norvegicus*), human (*Homo sapiens*), zebra finch (*Taeniopygia guttata*) and that obtained from *in silico* analysis of the puffer fish (*Takifugu rubripes*) genome (http://bahama.jgi-psf.org/fugu/html/README.html).

There are noticeably very few differences in the $GABA_A$ receptor β2 subunit between species of different classes i.e. mammals, birds, fish and amphibians, even within the intracellular loop, a region that is usually highly variable in sequence (see Figure 24). Most of the differences are in the puffer fish sequence. Unlike in the chicken where two isoforms of the β2 subunit exist (Harvey *et al.*, 1994a), namely β2L (possessing an additional 17 amino acids, DRRIIGTYHCPEMYSTK) and β2S (lacking this sequence), only the short form was found in all of the species studied.

There is high sequence identity between subunits from the six different species (see Table 12). Once more as expected, the rat sequence is most similar to the human (both mammalian species), and the zebra finch sequence to that of the chicken (both avian species). The avian polypeptide sequences are closer to mammals than to the frog and; the puffer fish sequence is the most distant.

As seen in the alignments in Figures 24 and 25, the transmembrane domains exhibit a high degree of conservation. However, compared to the β2-subunit sequences, there appears to be more variation in the β3-subunit sequences (26% compared to 54% respectively) in the intracellular loop region.

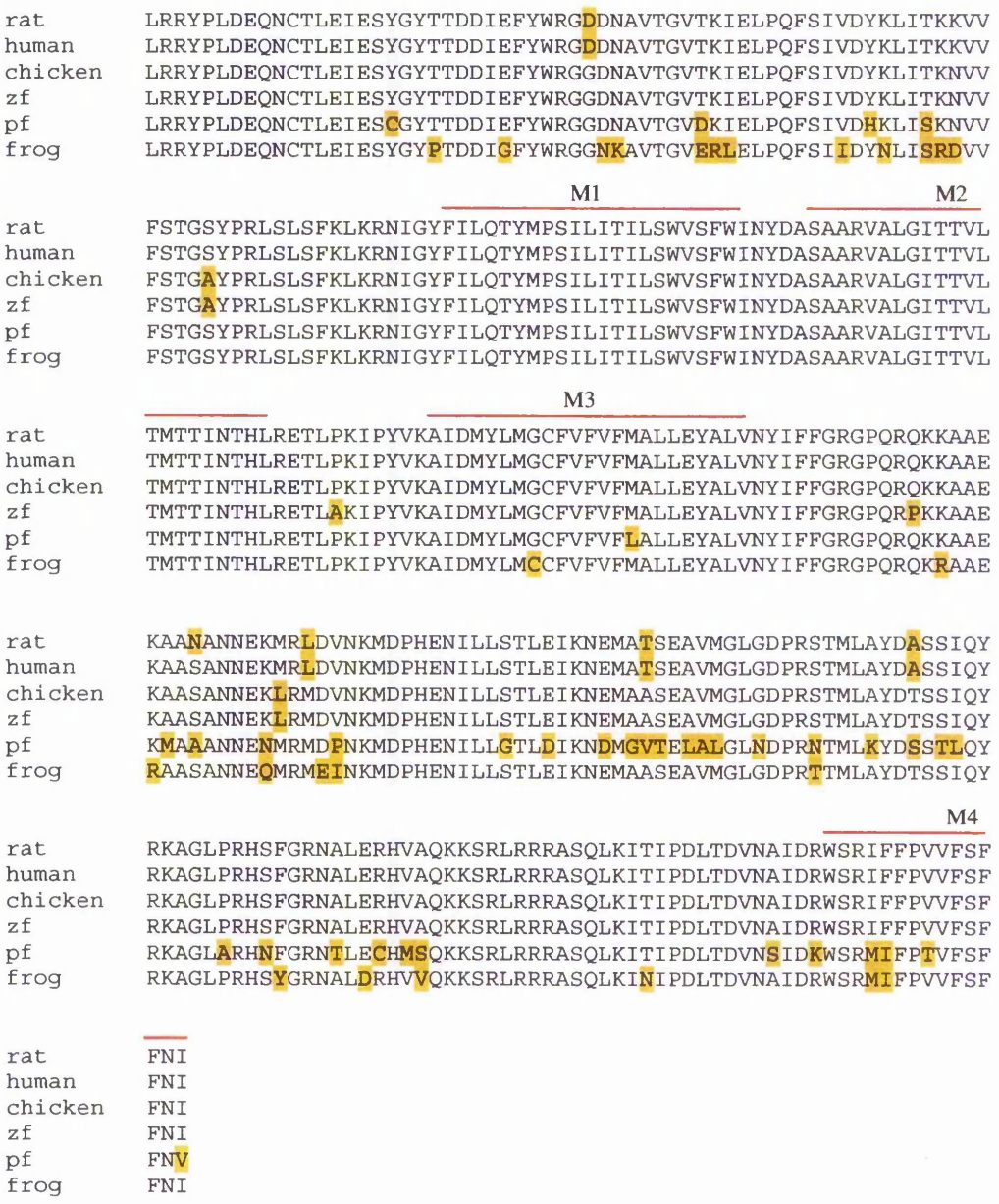


Figure 24: Alignment of the amino-acid (single-letter code) sequences of the $GABA_A$ receptor β2 subunit from the rat (*Rattus norvegicus*), human (*Homo sapiens*), chicken (*Gallus gallus domesticus*), zebra finch (zf; *Taeniopygia guttata*), African clawed frog (*Xenopus borealis*) and that obtained from *in silico* analysis of the puffer fish (pf; *Takifugu rubripes*) genome, using the computer programme ClustalW (http://www.ch.embnet.org/software/ClustalW.html). The proposed membrane-spanning domains (M1-M4) are indicated by solid red lines above the sequences and differences between species are highlighted in yellow.

	Human	Chicken	Zebra finch	Puffer fish	Frog
Rat	99.7	97.7	97	87.5	90.1
Human	/	98	97.4	87.5	91.1
Chicken	/	/	99.4	87.5	91.1
Zebra finch	/	/	/	86.8	90.8
Puffer fish	/	/	/	/	83.8

Table 12: Sequence similarities (as a percentage) of the deduced amino-acid sequences of the $GABA_A$ receptor β2 subunit from the rat (*Rattus norvegicus*), human (*Homo sapiens*), chicken (*Gallus gallus domesticus*), zebra finch (*Taeniopygia guttata*), African clawed frog (*Xenopus borealis*) and that obtained from *in silico* analysis of the puffer fish (*Takifugu rubripes*) genome (http://bahama.igi-psf.org/fugu/html/README.html).

	Human	Chicken	Zebra finch	Puffer fish	Frog	Lizard
Rat	96.3	87	86	80.9	78.9	85.4
Human	/	88.7	88	82.6	80.1	87.4
Chicken	/	/	97.7	83.1	79.3	92.1
Zebra finch	/	/	/	82.7	78.3	91.5
Puffer fish	/	/	/	/	76.2	81.5
Frog	/	/	/	/	/	78.9

Table 13: Sequence similarities (as a percentage) of the amino-acid sequences of the $GABA_A$ receptor β3 subunit from the rat (*Rattus norvegicus*), human (*Homo sapiens*), chicken (*Gallus gallus domesticus*), zebra finch (*Taeniopygia guttata*), African clawed frog (*Xenopus borealis*), common wall lizard (*Podarcis muralis*) and that obtained from *in silico* analysis of the puffer fish (*Takifugu rubripes*) genome (http://bahama.igi-psf.org/fugu/html/README.html).

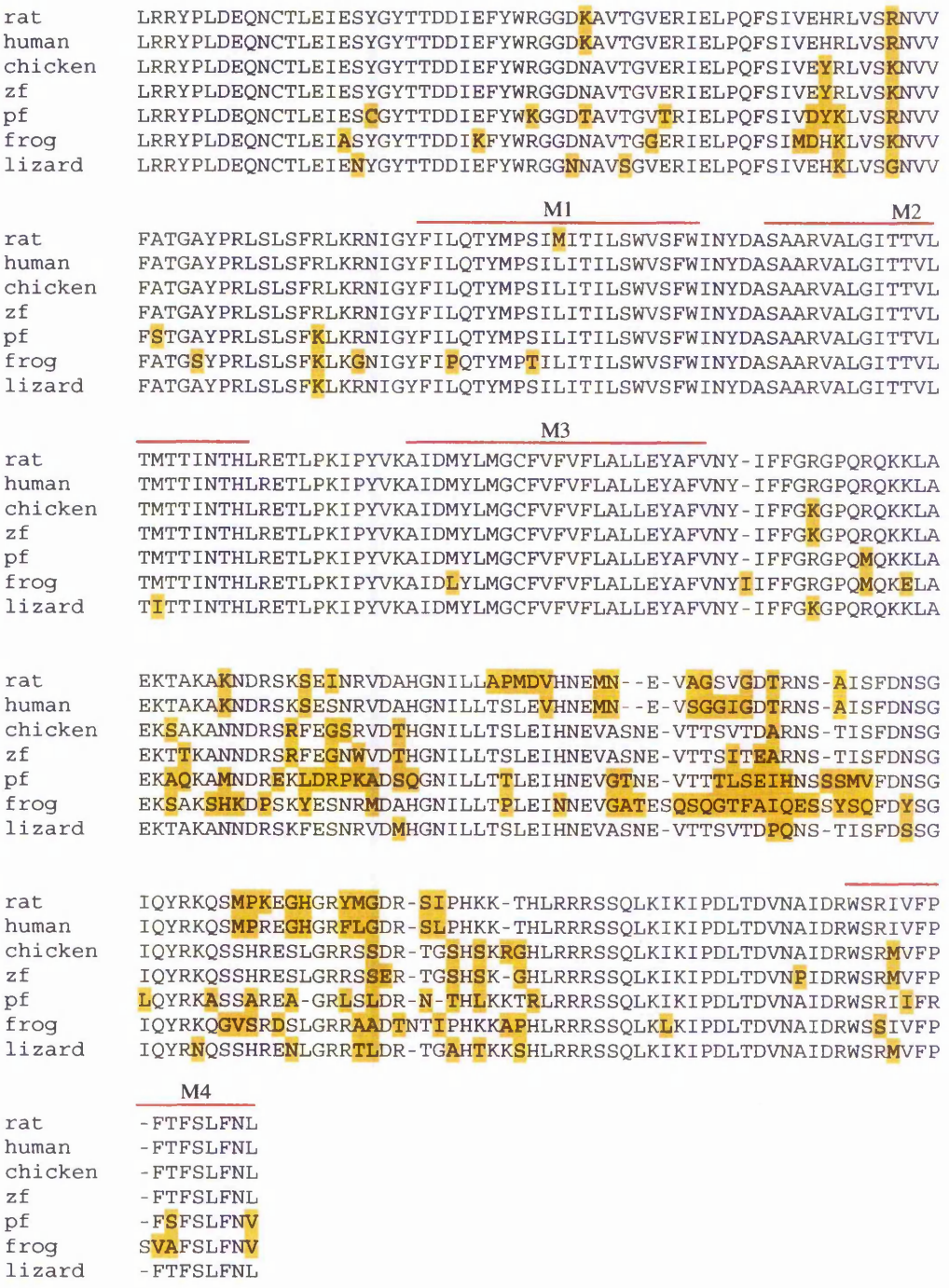


Figure 25: Alignment of the partial amino-acid (single-letter code) sequences of the $GABA_A$ receptor β3 subunit from the rat (*Rattus norvegicus*), human (*Homo sapiens*), chicken (*Gallus gallus domesticus*), zebra finch (zf; *Taeniopygia guttata*), African clawed frog (*Xenopus borealis*), common wall lizard (*Podarcis muralis*) and the puffer fish (pf; *Takifugu rubripes*), using the computer programme ClustalW (http://www.ch.embnet.org/software/ClustalW.html). Gaps introduced into the sequences to maximise the alignment are denoted by dashes. The proposed membrane-spanning domains (M1-M4) are indicated by solid red lines above the sequence, and differences between species are highlighted in yellow.

The sequence identity between pairs of β3 subunits from the seven different species studied, from five classes of vertebrate, was compared (see Figure 25). Once again $GABA_A$ receptor β3-subunit sequence from the human is most similar to the rat (96.3%) and that from the zebra finch shares highest identity to that from the chicken (97.7). The lizard shares a very high sequence identity with the chicken (92.1%; see Table 13).

Interestingly, the long form of the β4 subunit (containing 4 extra amino acids (V/L)REQ) occurs in all species studied in this investigation except the lizard. The length of the $GABA_A$ receptor β4-subunit sequence is also shorter (in the region encoding the intracellular loop; see Figure 26). To date a short form of the β4 subunit (as seen in the chicken with lacking the amino acids VREQ; Bateson *et al.*, 1991b) has not been found in any other species studied.

The identity of pairs of β4 subunits from five different species was compared (see Table 14). Once more the $GABA_A$ receptor β4 subunit is highly conserved in sequence, with the zebra finch and chicken sequences being most similar.

	Zebra finch	**Puffer fish**	**Frog**	**Lizard**
Chicken	98.4	85.3	87.1	84.1
Zebra finch	/	85.4	86.9	83.7
Puffer fish	/	/	83.2	77.7
Frog	/	/	/	78.1

Table 14: Sequence similarities (as a percentage) of the amino-acid sequences of the $GABA_A$ receptor β4 subunit from the chicken (*Gallus gallus domesticus*), zebra finch (*Taeniopygia guttata*), African clawed frog (*Xenopus borealis*), common wall lizard (*Podarcis muralis*) and that obtained from *in silico* analysis of the puffer fish (*Takifugu rubripes*) genome (http://bahama.jgi-psf.org/fugu/html/README.html).

```
chicken   LRRYPLDQQNCTLEIESYGYTVDDIVFFWQGND-SAVTGMEVLELPQFTIIEQRLVSREV
zf        LRRYPLDQQNCTLEIESYGYTVDDIVFFWQGND-SAVTGMEVLELPQFTIIEQRLVSREV
pf        LRRYPLDEQNCTLEIESCGYTTDDIVFFWQGGD-TAVTGVDKLELPQFSIVGIRLVSREV
frog      LRRYPLDQQNCTLEIESYGYTIDDIKFFWQGGEEAAVTGVSALELPQFTIIETRLVSKNV
lizard    LRRYPLDQQNCTLEIESYGYTVDDIVFFWQGNS-SAVTGMEVLELPQFTIIEQKLVTREV

                                        M1                    M2
chicken   VFTTGSYLRLSLSFRIKRNIGYFILQTYMPSILITILSWVSFWINYDASAARVALGVTTV
zf        VFTTGSYLRLSLSFRIKRNIGYYILQTYMPSILITILSWVSFWINYDASAARVALGVTTV
pf        RFTTGSYPRLSLSFRIKRNIGYFILQTYMPSILITILSWVSFWINYDASAARVALGVTTV
frog      VFTTGSYPRLSLSFRIKRNIGYFILQTYMPSVLITILSWVSFWINYDASAARVALGVTTV
lizard    VFTTGSYLRLSLSFRIKRNIGYFILQTYMPSILITILSWVSFWINYDASAARVALGVTTV

                                  M3
chicken   LTMTTINTHLRETLPKIPYVKAIDVYLMGCFVFVFLALLEYAFVNYIFFGRGPRQQKKQS
zf        LTMTTINTHLRETLPKIPYVKAIDVYLMGCFVFVFLALLEYAFVNYIFFGRGPRQQKKQS
pf        LTMTTINTHLRETLPKIPYVKAIDVYLMGCFVFVFLALLEYAFVNYVFFGRGPAQQKKLS
frog      LTMTTINTHLRETLPKIPYVKAIDVYLMGCFVFVFLALLEYAFVNYVFFGRGPQQQKKLN
lizard    LTMTTINTHLRETLPKIPYVKAIDVYLMGCFVFVFLALLEYALVNYIFFGRGPRQQKR--

chicken   ERISKANNERHRYEEKRVREQVDPYGNILLSTLDMNNELLATDMMSSVGDSRNS-VMSFE
zf        ERVSKANNERHRYEEKRVREQVDPYGNILLSTLEMDNELLATDMMSSVGDSRNS-VMSFE
pf        ERVGKSNNERMRYEEKRLREQVDPYGNILLTTLEMNNEVMPSDVGSSVSDSRNS-VMSFD
frog      ERTTKANNERHRYEERRVREQVDPYGNILLSTLDMSSELMSAEMGSSVGDSRNS-VLSYD
lizard    ---------RARHLEP--RTQPDPYGNLLLAGISDSNCPLFSTSTS--PDLRPCGLRGFE

chicken   GSGI--RKPLASR-DGFGHHPTLDRHVPLTHHAAARNRANCRLRRRSSKLKLKIPDLTDV
zf        GSGIQFRKPLASR-DGFGHHPTLDRHVPLSHHAAARNRANCRLRRRSSKLKLKIPDLTDV
pf        SSGVQFRKPMVPR-DGFSHHS-LDR-------SAMRSRANCRLRRRSSKLKLKIPNLSDV
frog      TSGIQFRKQLTSRGDGFGHTP-LERHVPM---TASRGRANCRLRRRSSKLKLKIPDLTDV
lizard    SSGTA----LYSR-----KAP-----------ACRATRANCRLRRRSSKLKLKIPDLADV

                M4
chicken   STIDKWSRIIFPITFGFFNL
zf        STIDKWSRIIFPITFGFFNL
pf        STIDKWSRVIFPITFGFFNL
frog      STIDKWSRIIFPITFGFFNL
lizard    STIDKWSRVLFPITFGFFNL
```

Figure 26: Alignment of the partial amino-acid (single-letter code) sequences of the $GABA_A$ receptor β4 subunit from the chicken (*Gallus gallus domesticus*), zebra finch (zf; *Taeniopygia guttata*), African clawed frog (*Xenopus borealis*), common wall lizard (*Podarcis muralis*) and that obtained from *in silico* analysis of the puffer fish (pf; *Takifugu rubripes*) genome, using the computer programme ClustalW (http://www.ch.embnet.org/software/ClustalW.html). Gaps introduced into the sequences to maximise the alignment are denoted by dashes. The four additional amino acids of the long isoform of the β4 subunit are in bold. The proposed membrane-spanning domains (M1-M4) are indicated by solid red lines above the sequences and differences between species are highlighted in yellow.

3.3 Discussion

One of the main aims of this project was to determine the sequences of $GABA_A$ receptor β subunits in phylogenetically distant species (e.g. birds, reptiles, fish), particularly the β4 subunit as very little is known about this subunit. The β subunits are essential for $GABA_A$ receptor function, as they are needed for the binding of GABA and surface expression of the receptor (Connor *et al.*, 1998). Also, a functional β subunit has been found in invertebrates e.g. *Lymnaea stagnalis* (Harvey *et al.*, 1991), demonstrating the importance of the β subunit over evolution. In addition, a β-like subunit gene has been seen in Drosophila melanogaster known as LCCH3 (Henderson *et al.*, 1993). Initially a PCR was conducted using zebra finch brain first-strand cDNA and degenerate β-subunit primers (see section 2.4; Table 3). From this a single band of approximately 1100bp was detected (see Figure 11). From a double restriction endonuclease digest, four different types of cDNA clones could be identified (see Figure 12), which encode the $GABA_A$ receptor β1, β2, β3 and β4 subunits (see Table 6).

The presence of four different β subunits was unexpected because, to date, only three β subunits have been detected in any one species (Darlison and Albrecht, 1995; Barnard *et al.*, 1998; Whiting, 2003). However, four β-like subunit sequences were also found in the puffer fish. A fourth β subunit may have arisen by an internal duplication event in birds (and fish), or loss of a β-subunit gene in mammals. As puffer fish are polyploid it is possible that another duplication took place, which separated the β1-like and β4-like subunits. However, then eight β-like subunit sequences would be expected if another genome doubling had occurred.

When the sequences of the $GABA_A$ receptor β subunits found by *in silico* analysis of the puffer fish database (http://bahama.jgi-psf.org/fugu/html/README.html), were compared with sequences within the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST/) highest similarity to $GABA_A$ receptor β1, β2, β3 and β4 subunits was observed. It was determined that there were four different β subunits in the puffer fish, displaying approximately 70% identity (see Table 10) as expected between subunits within a given class (Darlison *et al.*, 2005). Based on sequence comparisons these were then named β1-, β2-, β3- and β4-like (see Table 9). Interestingly, analysis of the intron/exon boundaries (see Figure 17) revealed

conservation of these positions with those of previously studied $GABA_A$ receptor subunits (see Harvey *et al.*, 1994a).

Prior to this investigation little was known of the $GABA_A$ receptor β4 subunit, it had previously only been found in the chicken (Lasham *et al.*, 1991). Mammals possess the β1, β2 and β3 subunits, whereas the β2, β3 and β4 subunits are present in the chicken. In addition, the θ subunit, which shares the highest sequence similarity to the β subunits (Bonnert *et al.*, 1999), was seen in mammals but not found in the chicken genome database (http://www.ensembl.org/Gallus_gallus/). The β1 subunit was not expected in the zebra finch, as it had not previously been detected in the chicken. The zebra finch β1 subunit was not as high in sequence identity to the rat β1 subunit as the other polypeptides encoding the $GABA_A$ receptor β subunits in the zebra finch (see Table 6). This is possibly because the sequence contains stop codons, and an inserted sequence (see Figure 13). The initial stop codon found in the zebra finch coding sequence, may have arisen by a PCR error produced by the enzyme *Taq* DNA polymerase. The expected amino acid was tryptophan (see Figure 14; encoded by TGG) but the cloned sequence was TAG i.e. there was a G-A substitution. The presence of multiple stop codons and an additional piece of sequence (in the intracellular loop) indicates that it probably represents a mispliced intron, i.e. that the cDNA derives from an expressed pseudogene, genes that are prone to increased mutation rates because of less selective pressure (Zhang and Gerstein, 2004). Pseudogenes are usually expressed non-functional genes however they are not rare and have been identified in both mammals and the chicken to generate immunoglobulin heavy and light chains during B-cell development (Mighell *et al.*, 2000). To date, no $GABA_A$ receptor pseudogene has been identified nevertheless, they may occur. Interestingly, processed pseudogenes have been found in the diazepam-binding inhibitor (Kolmer *et al.*, 1993), which is an antagonistic modulator of the $GABA_A$ receptor in brain membranes (Mandrup *et al.*, 1992).

The β1-like subunit sequence found in the chicken genome database (http://www.ensembl.org/Gallus_gallus/) was only 70% identical to the zebra finch β1 polypeptide sequence. This is very low since the $GABA_A$ receptor β-subunit polypeptide sequences from the zebra finch are more than 95% identical in sequence to those of the chicken (see Table 6). In addition, only a partial sequence of a β1-like subunit (exons 5 and 7) could be found in the chicken EST database (http://www.chickest.udel.edu/) providing evidence that the zebra finch β1-subunit gene

is likely to be a pseudogene, as only fragments of the gene could be found in the chicken EST database (exons 5 and 7; http://www.chickest.udel.edu/). If time had permitted a southern blot could have been conducted (using genomic DNA rather than zebra finch cDNA) and further codon analysis to confirm whether the $GABA_A$ receptor β1 subunit from the zebra finch is indeed a pseudogene.

An interesting observation is that the misplicing occurs in the intracellular loop in the eighth exon, i.e. in the same position for the insertion of the 17 additional amino acids in the β2 subunit (Harvey *et al.*, 1994a) and the 8 amino acids in the γ2L subunit (Glencorse *et al.*, 1992). As all of the isolated zebra finch β1-subunit cDNA clones were identical in sequence, it is plausible that they derive from the same mRNA. Further attempts to isolate additional β1-subunit cDNAs from the zebra finch were unsuccessful. This may be due to the β1-subunit mRNA being a very rare transcript in the zebra finch brain.

Sequence comparisons were made between the various $GABA_A$ receptor β subunits (see Table 6). The sequence identity of the all of the β subunits is highly similar even between different vertebrate classes especially considering that the evolutionary distance between avian and mammalian species is approximately 300 million years (Schmutz and Grimwood, 2004). The highest identity was seen between the zebra finch and chicken β4 subunits (99.4%), as they are both avian species this was to be expected. Conservation of sequence is likely to be due to evolutionary pressure, and this may be related to functional importance.

The $GABA_A$ receptor β2-subunit sequences illustrated in Figure 24 were highly conserved (even in the intracellular loop region). Since conservation of sequence is indicative of function, this was expected because the β2 subunit forms the most common $GABA_A$ receptor subtype α1β2γ2 (Whiting, 2003). Harvey *et al.* (1994a) and McKinley *et al.* (1995) have demonstrated that two forms of the β2 subunit are found in the chicken and human respectively, denoted β2L (which contains an additional 17 amino acids) and β2S (lacking this sequence). However, only cDNAs for the β2S subunit have been found in all of the species studied in this investigation. This is not unusual because Harvey *et al.* (1994a) were also unable to detect the long form of the β2 subunit in the rat and cow. In addition, Harvey *et al.* (1994a) found that although there was a similar abundance between the two β2-subunit transcripts, the β2S subunit

was slightly more dominant. It was surprising that the β2 subunit was not found in the lizard, as it is usually the most abundant subunit (Benke *et al.*, 1994). It is possible that because the primers were degenerate, (the reverse primer was designed at the C-terminus of the subunit; see 2.4 Table 2) even one mismatch could have meant that the primers did not anneal hence, failing to amplify all of the β-subunit cDNAs.

As seen in Table 14, the $GABA_A$ receptor β4 subunit is relatively well conserved in the intracellular loop domain (see Figure 26). Until this work was begun, the β4 subunit was thought to be unique to the chicken (Darlison and Albrecht, 1995). However, the cloning studies described in this study, and the *in silico* analysis of the puffer fish genome database, has revealed a β4-subunit gene in other species. Bateson *et al.* (1991b) found two isoforms of the β4 subunit in the chicken (β4S and β4L). In all of the species studied only the long form of the β4 subunit (with an insertion of four amino acids, (L/V)REQ) was detected. Nevertheless, these findings indicate, therefore, that the $GABA_A$ receptor β4 subunit may have a significant physiological role in avian (and other) species.

In conclusion studying the evolution of the $GABA_A$ receptor subunit genes can give an insight in to their importance, as conservation of sequence would imply a functional significance. Since the sequences of the $GABA_A$ receptor β4 subunit polypeptides are highly conserved between species, it indicates that the β4 subunit may have an important biological function that is not required in mammals.

4 The co-expression of the $GABA_A$ receptor β4-subunit gene and other subunit genes

4.1 Introduction

Initially, the $GABA_A$ receptor β4 and γ4 subunits were only found in the chicken. The pattern of expression of the latter was studied by Harvey *et al.* (1998) and was seen in areas of the chicken brain (*Gallus gallus domesticus*) involved in learning and memory, including mesopallium. C. Thode and M. G. Darlison (unpublished results) have found that the γ4-subunit gene is expressed in the zebra finch brain and, furthermore that there are differences in the level of the γ4-subunit mRNA between juvenile and adult zebra finches (*Taeniopygia guttata*; i.e. before and after song acquisition). This occurred in certain brain nuclei namely the robust nucleus of the arcopallium (RA), which is involved in crystallised song production. Vicario and Raksin (2000) found that the injection of bicuculline (GABA antagonist) caused degradation of song structure, suggesting that GABA regulates excitability in the RA. Therefore, the possible function of the β4 subunit was initially addressed in the chicken and zebra finch using *in situ* hybridisation to determine the distribution of the β4-subunit mRNA. Any changes occurring in levels of β4-subunit mRNA in male zebra finches was also investigated. Nottebohm and Arnold (1976) determined that the brains of zebra finches were sexually dimorphic. The RA and HVC were shown to be five times larger in volume in the male than female zebra finch brain (both regions involved in song production). The zebra finch is an ideal species to study as the song system provides a model for understanding speech production in humans.

Previously it has been found that $GABA_A$ receptor genes have a conserved transcriptional orientation and this was proposed to be involved in the co-ordinate regulation of genes (McKernan and Whiting, 1996). Gene mapping studies in mammals (Buckle *et al.*, 1989; Wilcox *et al.*, 1992; Russek and Farb, 1994; Bailey *et al.*, 1999b; Russek, 1999) found that the α3-, θ- and ε-subunit genes are clustered together on the X chromosome (Xq28). The mammalian θ subunit is hypothesised to be orthologous to the β4 subunit and the mammalian ε subunit orthologous to the γ4 polypeptide. To determine whether the α3-, β4- and γ4-subunit genes do cluster together in birds, the gene structures and transcriptional orientations were analysed using the recently

Refer to Table 5 for all accession numbers.

published chicken genome database (http://www.ensembl.org/Gallus_gallus/). In addition, *in situ* hybridisation was performed on one-day-old chicken brain sections using antisense oligonucleotides complementary to the $GABA_A$ receptor β4- and α3-subunit mRNAs (the distribution of the γ4-subunit mRNA has already been published; Harvey *et al.*, 1993, 1998).

4.2.1 The expression of the $GABA_A$ receptor β4-subunit gene in the adult and juvenile zebra finch brain

In situ hybridisation (see section 2.6) was performed to investigate the spatial distribution of the β4-subunit mRNA in the male adult and juvenile zebra finch (*Taeniopygia guttata*) brain. Since only adults produce song, the level of the β4-subunit mRNA was expected to be higher in certain brain regions e.g. RA (required for vocalisation) and more abundant in brain areas involved in acquision of sound in juveniles e.g. Area X.

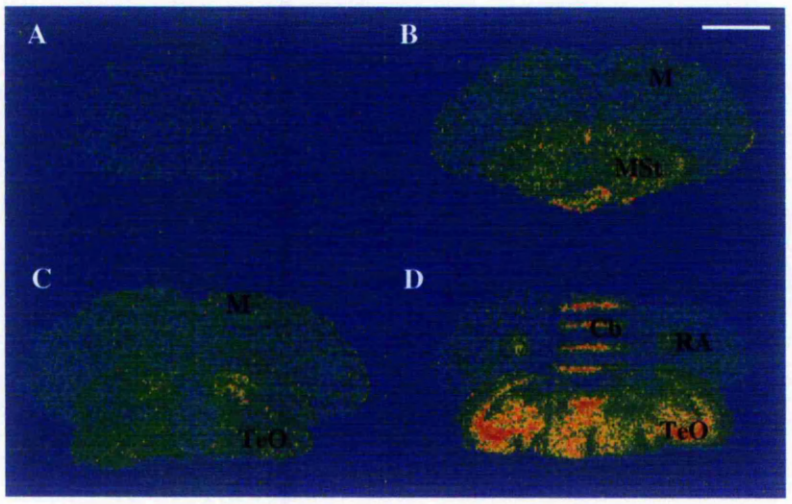


Figure 27: Distribution of the $GABA_A$ receptor β4-subunit mRNA in 10μm coronal sections of the adult zebra finch brain (*Taeniopygia guttata*; B-D); A is a negative control, determined by *in situ* hybridisation. Abbreviations: Cb, cerebellum; M, mesopallium; MSt, medial striatum; RA, robust nucleus of the arcopallium; TeO, optic tectum. Figures 27A to 27D correspond to plates A14.2, A13.6, A11.8 and A4.6 respectively (see see Kuenzel and Masson, 1988). Scale bar: 1.6 mm. Red colour denotes the highest labelling, whereas blue signifies no or low labelling.

Expression of the β4-subunit gene was observed in several major regions including: the medial striatum which contains Area X (important for song learning); the mesopallium

(important in song perception; Bolhuis *et al.*, 2000); the hyperpallium apicale which has a similar function to that of the mammalian hippocampus, i.e. it plays an important role in learning (Oades, 1976); the lateral striatum which is responsible for integrating sensory signals to produce specific behaviours (Palmiter, 2001); and the optic tectum which integrates visual and acoustic information (see Figure 27). Note that negative control *in situ* hybridisations (containing a 200 fold excess of the labelled probe) did not yield any specific autoradiographic signal.

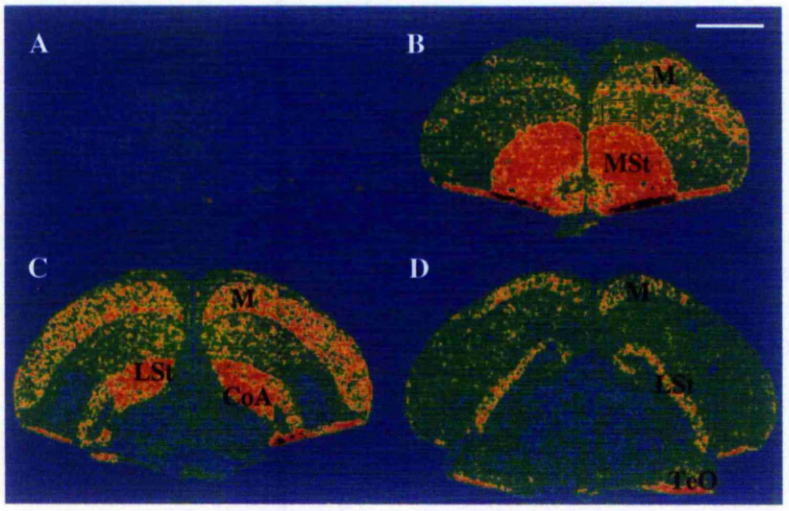


Figure 28: Distribution of the $GABA_A$ receptor β4-subunit mRNA in 10μm coronal sections of the juvenile zebra finch brain (*Taeniopygia guttata*; B-D) A is a negative control, determined by *in situ* hybridisation. Abbreviations: CoA, commisura anterior; LSt, lateral striatum; M, mesopallium; MSt, medial striatum; TeO, optic tectum. Figures 28A to 28D correspond to plates A14.2, A13.0, A10.0 and A8.2 respectively (see see Kuenzel and Masson, 1988). Scale bar: 1.6 mm. Red colour denotes the highest labelling, whereas blue signifies no or low labelling.

The β4-subunit gene was also expressed in the juvenile male zebra finch brain (see Figure 28) in similar regions to the adult. However, it was also detected in the commisura anterior, which is involved with the recognition of instinctive behaviour i.e. reflexes. These *in situ* hybridisation experiments suggested differences in the level of the β4-subunit mRNA between male adult (more than 90-day-old) and juvenile (35-day-old) zebra finches. For instance there appeared to be a higher amount of the β4-subunit mRNA in the medial striatum and mesopallium of juvenile birds compared to adult birds. However, levels of the β4-subunit mRNA appeared higher in the cerebellum of adults compared to juveniles. *In situ* hybridisation and image analysis was conducted on

a series of six pairs of male birds (juveniles and adults) to determine whether differences were statistically significant. The two brain regions analysed were mesopallium and medial striatum, because there was highest abundance of the $GABA_A$ receptor β4-subunit mRNA in these areas.

Initially, it appeared as though there were differences in the level of the $GABA_A$ receptor β4-subunit mRNA between adult and juvenile and left and right hemispheres of the male zebra finch brain. However, the error bars (see Figures 29 and 30) demonstrate that this was not the case. The P values obtained from the 1-way ANOVA test were 0.39 and 0.85 for the mesopallium and medial striatum, respectively, i.e. higher than 0.05, which meant that the differences were not statistically significant. The P values obtained from the Bartlett's test for equal variances were 0.56 and 0.1 again not statistically significant.

Since the pattern of expression of the $GABA_A$ receptor β4-subunit mRNA appeared similar in parts of the zebra finch brain to that of the γ4-subunit mRNA (C. Thode and M. G. Darlison, unpublished results), parallel *in situ* hybridisation experiments were performed to determine whether there was co-expression of the two genes.

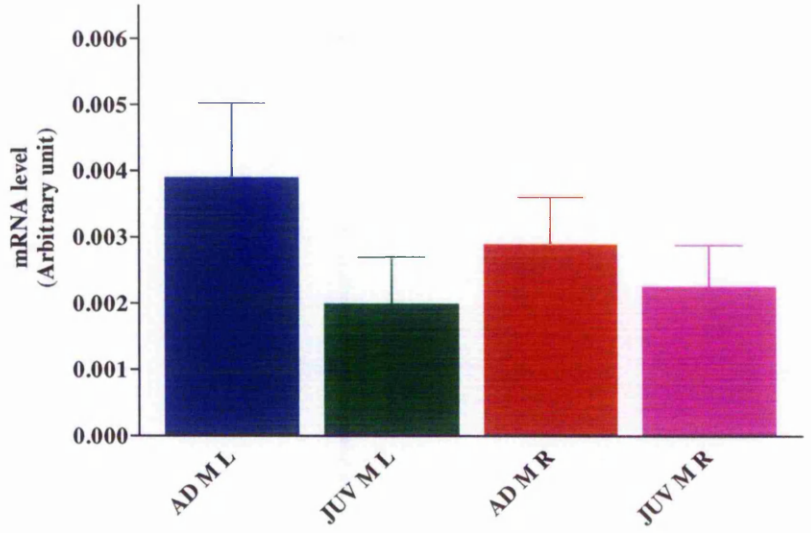


Figure 29: Comparison of the abundance of the $GABA_A$ receptor β4-subunit mRNA between adult (AD) and juvenile (JUV) zebra finches (*Taeniopygia guttata*) in the left (L) and right (R) hemispheres of the mesopallium (M). The graph was generated by the computer programme Prism (GraphPAD, CA, USA).

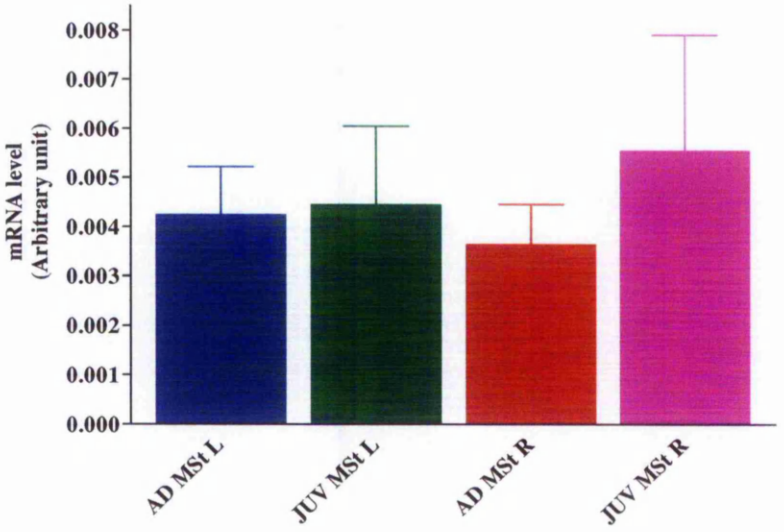


Figure 30: Comparison of the abundance of the $GABA_A$ receptor β4-subunit mRNA between adult (AD) and juvenile (JUV) zebra finches (*Taeniopygia guttata*) in the left (L) and right (R) hemispheres of the medial striatum (MSt). The graph was generated by the computer programme Prism (GraphPAD, CA, USA).

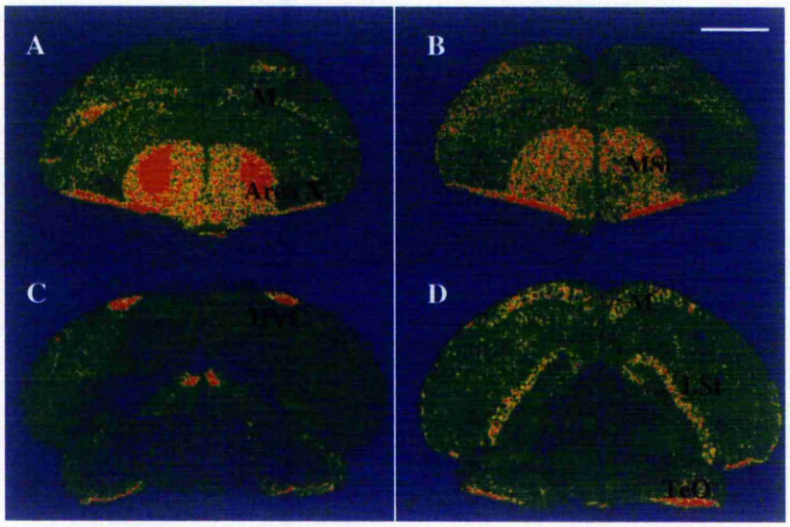


Figure 31: Distribution of the $GABA_A$ receptor γ4- (A and C) and β4- (B and D) subunit mRNAs in 10μm coronal sections of the adult zebra finch brain (*Taeniopygia guttata*), as determined by *in situ* hybridisation. Abbreviations: HVC, higher vocal centre; LSt, lateral striatum; M, mesopallium; MSt, medial striatum; TeO, optic tectum. Figures 31A to 31D correspond to plates A13.6, A13.6, A8.8 and A8.2 respectively (see see Kuenzel and Masson, 1988). Scale bar: 1.6 mm. Red colour denotes the strongest labelling, whereas blue signifies weak or no labelling.

Although, the overall expression pattern of the $GABA_A$ receptor β4- and γ4-subunit mRNAs is not the same, there is a clear overlap in some areas of the zebra finch brain. In particular, the mesopallium, medial striatum, and optic tectum (see Figure 31). However, there is prominent expression of the γ4-subunit gene but not the β4-subunit gene, in two brain regions, Area X and the higher vocal centre. The former is involved in song learning and maintenance of song and, the latter plays a role in song production. In contrast, the β4-subunit mRNA is detected at high levels in the lateral striatum, needed for processing of sensory information, indicating a role in learning.

4.2.2 *In silico* analysis of the chicken genome database and expression of $GABA_A$ receptor subunit genes in the one-day-old chicken brain

Since a gene cluster found in the mammalian genome encodes an α3, θ and ε subunit, if the latter are orthologous to the β4 and γ4 subunits respectively, then there should be an ortholous cluster in the avian genome. Therefore *in silico* analysis was conducted on the chicken genome database (http://www.ensembl.org/Gallus_gallus/), to determine the chromosomal locations and organisations of the $GABA_A$ receptor α3-, β4- and γ4-subunit genes.

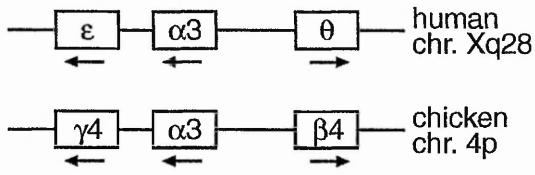


Figure 32: Comparison (not to scale) of the cluster of the $GABA_A$ receptor α3-, θ- and ε-subunit genes on the X chromosome, in man (*Homo Sapiens*), with the corresponding cluster of orthologous genes in the chicken namely those encoding the α3, β4 and γ4 subunits (*Gallus gallus domesticus*). Arrows indicate the transcriptional orientations of the genes.

In silico analysis of the chicken genome revealed that the β4- and γ4-subunit genes flank the α3-subunit gene and there is conservation of transcriptional orientation (see Figure 32). The $GABA_A$ receptor α3-, β4- and γ4-subunit genes are located on chromosome 4p, contigs 11.256, 11.268 and 11.254 respectively. An alignment was then conducted to demonstrate their exon boundaries (see Figure 33).

The organisations of the chicken $GABA_A$ receptor γ4- and α3-subunit genes were found to be similar to that of the published β4-subunit gene (Lasham *et al.*, 1991). Figures 34, 35 and 36 demonstrate the conserved sequences denoting the intron/exon splice sites in the $GABA_A$ receptor subunit genes in the chicken. After aligning the sequences, the variable exon boundaries were between exons 1 and 2, 8 and 9 (see Figure 33). The sizes of the eight introns, which split the exons of the $GABA_A$ receptor genes, were not conserved, neither in the chicken, or the orthologous genes in the human (data not shown). In the chicken, the largest intron in the β4-subunit gene is >22kb occurring between exons 3 and 4 and the smallest is ~0.5kb long, separating exons 8 and 9. The largest intron in the α3-subunit gene is >24kb between exons 2 and 3, and the shortest is >1kb separating exons 6 and 7. Finally, in the γ4-subunit gene the largest intron is >19kb between exons 3 and 4 and the shortest which separates exons 4 and 5 is ~0.6kb in size. The cluster of $GABA_A$ receptor genes found in the chicken was compared for sequence similarities to those previously published.

It is evident that the $GABA_A$ receptor α3-subunit polypeptide is significantly more similar between the chicken and man than the ε- or γ4- and θ- or β4-subunit polypeptides. The corresponding human and rat sequences have also been compared against each other. Even between orthologous subunits of mammalian and avian species there is an extremely high level of sequence identity i.e. more than 97%. Interestingly, the α3 subunit is 87% similar at the polypeptide level between chicken and rat or human (see Table 15). This is possibly due to the decreased selective pressure on that part of the chromosome. Interestingly, Eichler *et al.* (1997) found a high rate of mutations in the gene encoding adreno leukodystrophy located on the X chromosome (specifically the Xq28 locus). Unexpectedly, the rat and human ε and θ subunits share a relatively low sequence identity when compared to the α3-subunit polypeptide (see Table 16). This is highly unusual since orthologous subunits are usually more than 90% identical (Bateson *et al.*, 1991a; see Table 17) particularly when both are from the same family of species, namely mammalian.

Figure 33: Alignment of the deduced amino-acid sequences of the chicken $GABA_A$ receptor α3, β4 and γ4 subunits using the computer programme ClustalW. Gaps introduced into the sequences to maximise the alignment are denoted by dashes. Amino-acids are numbered from the mature amino-terminus; negative numbers indicate to the signal peptide. Red lines above the sequence indicate the proposed membrane-spanning domains (M1 to M4). The change between black lettering on a white background and black lettering on a yellow background marks the nine exons.

Exon 1.....GTAAG**gt**ggcaagaggc.....intron (3kb).....caaacatacc**ag**GCTTT.....

Exon 2.....TGGAG**gt**cagttggttg.....intron (24.3kb).....ttttttttcc**ag**ACAGT.....

Exon 3.....ACATG**gt**aagtgccact.....intron (2.5kb).....ttctccacaa**ag**GAATA.....

Exon 4.....ATGAG**gt**gagttcgtgg.....intron (8.3kb).....tctgtcttgt**ag**GCTAA.....

Exon 5.....TGGGAagctgtaagtac.....intron (4.1kb).....tttcctccacc**ag**ATGCC.....

Exon 6.....CACAG**gt**acttgtggga.....intron (1.1kb).....ttgtgtccct**ag**GGGAG.....

Exon 7.....TTTTG**gt**gagtctgtga..... intron (2.2.kb).....gtgtttccat**ag**GTGTC.....

Exon 8.....TGAAG**gt**gagccccgtg.....intron (7.7.kb).....caccttccct**ag**AAAAA.....

Exon 9.....AACAAtaaaacctatca.....

Figure 34: Sequences across the intron/exon boundaries of the chicken $GABA_A$ receptor α3-subunit gene. Nucleotides in upper-case letters represent exonic sequences whilst those intronic sequences are shown in lower-case. The conserved minimum consensus sequences for splice junctions (Smith *et al.*, 1989) gt (5' donor site) and ag (3' acceptor site) are indicated by blue lettering. The approximate sizes of introns are shown in brackets.

Exon 1.....CAAAG**gt**aaggattgca.....intron (4kb).....tttttatttc**ag**CCCAT.....

Exon 2.....TGGAG**gt**atggtgatgg.....intron (20kb).....tttgtgtttc**ag**GTAAC.....

Exon 3.....ACATG**gt**gagtttggga.....intron (3.4kb).....tttctctcct**ag**GACTA.....

Exon 4.....CTGAG**gt**aggtgtgagg... intron (31.5kb)....catcttctttag**GATCA**.....

Exon 5.....TGAGAgctgtgcgtatg.....intron (4.5kb).....cctttgcagatgGTTAC....

Exon 6.....CACCG**gt**gagtctctgc.....intron (1.7kb).....tctcattgac**ag**GTTCA....

Exon 7.....GGTAG**gt**acctttccat..... intron (2.8kb).....ctctccttac**ag**GGGTC....

Exon 8.....AGCAG**gt**ttgtcccctt.....intron (0.5kb)... ..cccttgttct**ag**GTTGA....

Exon 9.....TAAATtgatgcctgcag.....

Figure 35: Sequences across the intron/exon boundaries of the chicken $GABA_A$ receptor β4-subunit gene. Nucleotides in upper-case letters represent exonic sequences whilst those intronic sequences are shown in lower-case. The conserved minimum consensus sequences for splice junctions (Smith *et al.*, 1989) gt (5' donor site) and ag (3' acceptor site) are indicated by blue lettering. The approximate sizes of introns are shown in brackets.

Exon 1.....GCCAG**gt**aagcaccccg.....intron (4.9kb).....tctctgctcc**ag**GTGTG.....

Exon 2.....TGGCA**gt**aagtgtccct.....intron (0.2kb).....attttctttac**ag**TCAAG.....

Exon 3.....AAATG**gt**gagtggtacc.....intron (19.4kb).....tctctttcac**ag**GAGTA.....

Exon 4.....CTCAG**gt**acgtgcagaa.....intron (0.6kb).....tttctctctc**ag**GCTGA.....

Exon 5.....TAGTT**gt**gagtatccat.....intron (1.3kb).....ccctttcccc**ag**ATGGC.....

Exon 6.....AGCAG**gt**gaggcaaagg.....intron (1.5kb).....ctttttttct**ag**GGGAG.....

Exon 7.....CCTCG**gt**aagagaaaag.....intron (2kb).....ttttcctctt**ag**GTATC.....

Exon 8.....GACTG**gt**aggtggaagc.....intron (1kb).....tcctcattac**ag**CCACC.....

Exon 9.....ATCTCtagcgattcttc.....

Figure 36: Sequences across the intron/exon boundaries of the chicken $GABA_A$ receptor γ4-subunit gene. Nucleotides in upper-case letters represent exonic sequences whilst those intronic sequences are shown in lower-case. The conserved minimum consensus sequences for splice junctions (Smith *et al.*, 1989) gt (5' donor site) and ag (3' acceptor site) are indicated by blue lettering. The approximate sizes of introns are shown in brackets.

Human	**Chicken**		
	α3	β4	γ4
α3	87.3	/	/
θ	/	49.4	/
ε	/	/	52.8

Table 15: Comparison of the sequence identities (%) of mature $GABA_A$ receptor subunits between the chicken (*Gallus gallus domesticus*) and human (*Homo sapiens*).

Human	**Rat**		
	α3	θ	ε
α3	98.1	/	/
θ	/	78.3	/
ε	/	/	71.9

Table 16: Comparison of the sequence identities (%) of the mature $GABA_A$ receptor subunits on the X chromosome, namely α3, θ and ε between human (*Homo sapiens*) and rat (*Rattus norvegicus*).

Human	Chicken		
	α1	β2	γ2
α1	98.1	/	/
β2	/	98.4	/
γ2	/	/	97.5

Table 17: Comparison of the sequence identities (%) of the most abundant $GABA_A$ receptor subunits (α1, β2 and γ2) between human (*Homo sapiens*) and the chicken (*Gallus gallus domesticus*).

Since the $GABA_A$ receptor α3-, β4- and γ4 subunit genes were found to form a cluster on chromosome 4p in the chicken genome, *in situ* hybridisation was conducted to determine whether the subunits are co-expressed to potentially form a native receptor subtype in the chicken brain. Whiting *et al.* (2003) discussed the abundance of $GABA_A$ receptor subtypes in the rat brain and illustrated that the most abundant subunits (α1, β2 and γ2; Chang *et al.*, 1996) were found to form a subtype in a third of all $GABA_A$ receptor subtypes in the rat brain (see Figure 4). In addition, the $GABA_A$ receptor ε and θ subunits clustered together on the X chromosome in humans (see Figure 5) were shown by immunohistochemistry to co-express throughout the monkey brain (Moragues *et al.*, 2002). Although it was proposed that the $GABA_A$ receptor α3-, β4- and γ4 subunit genes would co-express this was not the case in all chicken brain regions. Partial co-localisation of the α3- and β4-subunit mRNAs can be seen in certain brain regions e.g. in the mesopallium, hyperpallium intercalatum, lateral striatum, optic tectum and cerebellum but not in the nucleus dorsomedialis anterior thalami and nucleus septalis lateralis (refer to Figure 37). A comparison of the expression patterns of the $GABA_A$ receptor α3-, β4- and γ4-subunit genes is shown in Table 18. The hyperpallium apicale, hyperpallium intercalatum, and mesopallium are areas of the brain where the α3-, β4- and γ4-subunit genes appear to be highly co-expressed. Major differences in expression of the β4- and γ4- and α3- γ4 subunit genes are in the following areas: entopallium, nucleus dorsomedialis anterior thalami, nucleus reticularis superior pars dorsalis, nucleus rotundus, nucleus septalis lateralis, and lateral striatum. In addition, other areas where there are differences in the expression of the $GABA_A$ receptor subunit genes are in the deep cerebellar nuclei, globus pallidus and nucleus dorsomedialis anterior thalami.

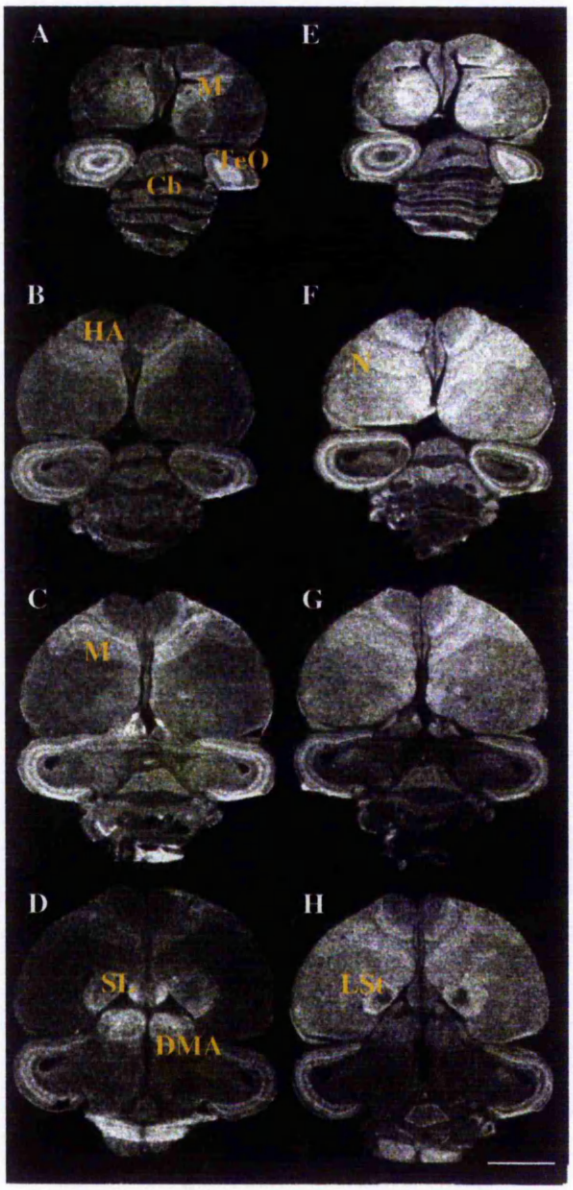


Figure 37: Autoradiographs from 16μm horizontal sections of the one-day-old chicken (*Gallus gallus domesticus*) brain, illustrating the partial co-localisation of the $GABA_A$ receptor α3- (A-D) and β4-subunit (E-H) mRNAs. A and E represent plate D6.8; B and F correspond to D6.4; C and G correspond to D5.8; and D and H correspond to D5.2. (see Kuenzel and Masson, 1988). Abbreviations: Cb, cerebellum; DMA, nucleus dorsomedialis anterior thalami; HA, hyperpallium apicale; HI, hyperpallium intercalatum; LSt, lateral striatum; M, mesopallium; N, nidopallium; SL, nucleus septalis lateralis; TeO, optic tectum. Scale bar = 2.7mm

Region	α3	β4	γ4
Telencephalon			
HA	+	+	+
HD	+	+	-
HI	+++	++++	++++
M	+++	+++	++++
N	+	+	+
NI	-	+	+
E	-	-	++++
L	-	+	-
LSt	+++	+++	+
GP	++	-	-
SL	+++	++	-
DMA	+++	+	-
RSd	-	-	++++
Rt	-	-	++++
Mesencephalon and brainstem			
TeO	++++	++++	++
Cb	++	++	+
DCN	-	-	+++

Table 18: Regional distribution of the $GABA_A$ receptor α3- and β4-subunit transcripts in the one-day-old chicken (*Gallus gallus domesticus*) brain. The distribution of the γ4-subunit transcript was studied by Harvey *et al.* (1993). Hybridisation signals from the *in situ* hybridisations were arbitrarily scored as very intense, ++++; intense, +++; moderate, ++; weak, +; not detectable, -. Abbreviations: Cb, cerebellum; DCN, deep cerebellar nuclei; DMA, nucleus dorsomedialis anterior thalami; E, entopallium; GP, globus pallidus; HA, hyperpallium apicale; HD, hyperpallium densocellulare; HI, hyperpallium intercalatum; L, field L; LSt, lateral striatum; M, mesopallium; N, nidopallium; NI, nidopallium intermedium; SL, nucleus septalis lateralis; RSd, nucleus reticularis superior, pars dorsalis; Rt, nucleus rotundus; TeO, optic tectum.

4.3 Discussion

The $GABA_A$ receptor β4 and γ4 subunits of the chicken were identified more than a decade ago, and their functional significance and phylogenetic relationship to other $GABA_A$ receptor polypeptides has been proposed (Bonnert *et al.*, 1999; Sinkkonen *et al.*, 2000; Darlison *et al.*, 2005). Harvey *et al.* (1998) found a down-regulation of the γ4-subunit mRNA after imprinting (a form of recognition memory) in the chicken. In addition, C. Thode and M. G. Darlison (unpublished results) have found a difference in the level of the γ4-subunit mRNA in the adult and juvenile male zebra finch brain, in regions involved in song production (including RA and HVC). Comparatively little is known of the role of the β4 subunit and this has been studied here in the zebra finch and chicken.

Songbirds were studied because song production is a naturally learnt behaviour, which could possibly provide an insight to speech production in humans (White, 2001). A 45-base antisense oligonucleotide was designed complementary to a sequence that encodes part of the zebra finch $GABA_A$ receptor β4-subunit (see 2.6.1). This was successfully used to detect the corresponding mRNA in the zebra finch brain. The β4-subunit mRNA was detected in numerous regions of the male brain, including the medial striatum (MSt; which contains Area X), mesopallium (M), optic tectum (TeO), and cerebellum (Cb). Area X is involved in song acquisition and maintenance and more specifically has been proposed to store information regarding the tutor's song (Bolhuis *et al.*, 2000). Sorabji *et al.* (1990) demonstrated that lesions in Area X of the juvenile zebra finch disrupted song development. Grisham and Arnold (1994) found high levels of GABA-like immunoreactivity in certain brain nuclei including Area X, the higher vocal centre (HVC) and the robust nucleus of the arcopallium (RA). The anterior pathway in birds i.e. the lateral magnocellular nucleus of the anterior nidopallium (LMAN)-Area X pathway is also important for the acquisition and maintenance of song. By day 35 the motor pathway is closed i.e. the neurons from the HVC have extended to the RA, hence the zebra finch is able to produce song.

Both adult (more than 90-day-old) and juvenile (35-day-old) male zebra finch brains were subjected to *in situ* hybridisation (Figures 27 and 28), and differences (although not statistically significant) were found between the two ages. Juvenile birds are still acquiring sensorimotor skills required to produce song, whereas the adults are already

able to sing hence, there was a higher abundance of the β4-subunit mRNA in the juvenile MSt (which contains Area X). Only male birds were used due to sexual dimorphism i.e. anatomical differences in the brain therefore, the male zebra finch solely produces song. Initially, it was thought that there was a higher level of the $GABA_A$ receptor β4-subunit mRNA in the MSt of juvenile birds compared to adults; this is interesting because Area X, which is involved in the learning of song, is present within the MSt. Image analysis software was utilised namely, Scion which is a densitometric analysis programme, distinguishing between different subunit expression patterns. However, unlike the γ4-subunit mRNA (C. Thode and M. G. Darlison, unpublished results), densitometric analysis found no statistically significant difference between adult and juvenile zebra finches in both the left and right hemispheres of the M or MSt (see Figures 29 and 30).

It was originally suggested (McKernan and Whiting, 1996) that the clustering of $GABA_A$ receptor genes was important to permit the co-ordinate transcription of genes, the products of which would assemble to form specific receptor subtypes (see Darlison *et al.*, 2005). The main $GABA_A$ receptor subtypes are α1,β2,γ2; α2,β3,γ2; and α3,β3,γ2 (Whiting, 2003). This study has aimed to demonstrate the clustering of the $GABA_A$ receptor subunits in the chicken. It appears that this is a complex process as some subunits may cluster with other subunits to form only a minority of receptor subtypes.

The discovery of the mammalian $GABA_A$ receptor ε and θ subunits led to the suggestion that these were orthologues of the γ4 and β4 subunits. Based on this assumption, it was proposed that the genes for the γ4 and β4 subunits clustered together with the α3-subunit gene, like those of the mammalian ε and θ subunits, may be co-ordinately transcribed to form a $GABA_A$ receptor subtype (see Darlison *et al.*, 2005). *In silico* analysis of the recently released chicken genome, revealed that the $GABA_A$ receptor α3-, β4- and γ4-subunit genes cluster together, on chicken chromosome 4 (contigs 11.256, 11.268 and 11.254). This is in agreement with the findings of Sinkkonen *et al.* (2000) that despite the relatively low sequence similarity, the ε and θ subunits are orthologous to the chicken γ4 and β4 subunits. As seen previously for other $GABA_A$ receptor genes (Sommer *et al.*, 1990; Lasham *et al.*, 1991) the exon boundaries of these genes (Figure 33) are with two exceptions conserved. The difference in the boundary positions of exon 1 is not surprising since this mainly encodes the signal peptide, which is not highly conserved in sequence. Simon *et al.* (2004) have recently

confirmed these findings having stated that, "the exon positions were identical in θ and β4 (disregarding the first exon)". The differences in lengths of exon 8 and exon 9, respectively, could be explained by the fact that they encode part of the intracellular loop, which is variable in size. Lasham *et al.* (1991) demonstrated that 5 out of 8 exon boundaries between the chicken β4 and murine δ subunits (Sommer *et al.,* 1990) were identical, whereas those for exons 1, 6 and 8 were similar but not identical. In addition, the intron/exon boundary positions are also conserved in the bovine $GABA_A$ receptor α3-subunit gene (Levitan *et al.,* 1988). Hence genomic organisation must have been established before the divergence of the different types of $GABA_A$ receptor subunits. Interestingly, there does not appear to be a correlation between the sizes of the introns in the chicken α3-, β4- and γ4-subunit genes. This was also the case between the $GABA_A$ receptor chicken β4- and murine δ-subunit genes (Lasham *et al.*, 1991).

The cluster of the $GABA_A$ receptor α3-, γ4- and β4-subunit genes is on an autosome (chromosome 4) in the chicken, in contrast to mammals where it on the X chromosome. This is surprising, because there is generally less selective pressure on the X chromosome (Russek, 1999) hence, the $GABA_A$ receptor β4 and γ4 subunits diverged from the θ and ε subunits (see Darlison *et al.*, 2005). This is supported by the finding that there is only 87% sequence identity at the amino-acid level between the chicken α3 subunit and the mouse, human or bovine α3 subunit (see Table 15). The relatively low sequence identity cannot only be due to species difference but also their chromosomal location as, the chicken α1-subunit polypeptide shares 98% sequence identity with the human and rat α1-subunit polypeptides (see Table 17). The fact that the $GABA_A$ receptor β4 and γ4 subunits are highly conserved between the chicken and zebra finch imply that they may have evolved to fulfil a function in birds.

It is assumed that if the $GABA_A$ receptor α3-, β4- and γ4-subunit genes are co-ordinately transcribed they should be co-expressed in the same brain regions. Although the expression pattern of the chicken $GABA_A$ receptor γ4-subunit gene (Harvey *et al.*, 1993) is not completely identical to those of the α3-and β4-subunit mRNAs (see Figure 37) there was some overlap. The hyperpallium apicale (HA), hyperpallium intercalatum (HI) and M are areas of the brain where the α3-, β4- and γ4-subunit genes appear to be highly co-expressed (see Table 18). In addition, all three subunit mRNAs (α3, β4 and γ4) are present in the cerebellum (Cb). The Cb is involved in the control and maintenance of muscle tone. There is also expression of the $GABA_A$

receptor α3-, β4- and γ4-subunit genes in the optic tectum (TeO). The TeO is involved in vision (Glencorse *et al.*, 1993) and Cohen (1967) found that the tectum-entopallium-mesopallium pathway was responsible for visual discrimination. In contrast, to the α3- and β4-subunit mRNAs, the γ4-subunit mRNA is highly expressed in the entopallium (E), IMM, medio-rostral nidopallium/mesopallium (MNM) and nucleus rotundus (Rt) (Harvey *et al.*, 1993, 1998). The medial striatum (MSt) is an important region that has been linked with learning and memory in the chicken (Freeman and Young, 2000; Rose, 2000). Izawa *et al.* (2003) also found that lesions to the MSt caused chicks to choose the wrong colour of bead i.e. impaired discrimination in the one-trial passive-avoidance test and stated that the avian MSt may represent the mammalian nucleus accumbens. Interestingly, previous research by Serrano *et al.* (1995) showed that lesions to the left MSt only, caused amnesia in such avoidance tasks in chicks. Harvey *et al.* (1998), have shown that the level of the γ4-subunit mRNA is downregulated after learning (auditory imprinting) in the entopallium (E), medio-rostral nidopallium/mesopallium (MNM) and Field L of the one-day-old chicken. Since, the β4-subunit gene is expressed in similar brain regions to the γ4-subunit gene (e.g. MSt, M, TeO; see Figure 31), this suggests that the β4-subunit gene may also play a role in learning and memory in the chicken.

As the $GABA_A$ receptor α3-, β4- and γ4-subunit mRNA patterns of distribution are not identical; this suggests that they may form a $GABA_A$ receptor subtype in some but not all avian brain areas. For example they may form subtypes in the mesopallium and hyperpallium intercalatum but not in the ectopallium (refer to Table 18). Interestingly, the $GABA_A$ receptor α3-, β4- and γ4-subunit genes also shared a relatively low sequence identity even between the orthologous rat and human sequences. In contrast, the most abundant subunits α1, β2 and γ2, which form the most common receptor subtype in mammals (Wisden *et al.*, 1992; Laurie *et al.*, 1992), share very high sequence identities between species (~98%; see Table 17).

Moragues *et al.* (2002) stated that there must be a subtype containing the ε and α3 subunits in the brain. This study disagrees with this because: firstly in the chicken there is little co-expression of the α3- and γ4-subunit genes; and secondly there should be a β subunit present as the θ subunit has never been found to functionally replace a β subunit (Bonnert *et al.*, 1999). Interestingly, the $GABA_C$ receptor ρ1 subunit has recently been found to co-assemble *in vivo* with the α1 and γ2 subunits in rat neuronal cells (Milligan

et al., 2004) and can functionally replace the β subunit. Forster *et al.* (2001) found that the $GABA_A$ receptor γ4 subunit co-expressed with the α3 subunit to form a functional recombinant receptor. The possibility of the $GABA_A$ receptor β4-subunit mRNA co-localising with other α-subunit mRNAs was investigated in our laboratory by C. Dixon (2004). *In situ* hybridisation experiments demonstrated that the distribution patterns of the β4-, α2- and α4-subunit mRNAs are not identical. The expression of the α1-, α5- and α6-subunit genes is already known (Bateson *et al.*, 1991a; Bahn *et al.*, 1996; Aller *et al.*, 2000), and does not appear identical to the distribution of the β4-subunit mRNA in the chicken. Although, the β4-subunit mRNA was found in similar areas to the α2-subunit mRNA i.e. HA, M, nidopallium (N), lateral striatum (LSt), E, Cb and TeO, Dixon (2004) concluded that since the patterns of expression were not highly similar the two encoded subunits may co-localise to form a minority receptor subtype. In contrast, the α4-subunit mRNA was only detected in the Cb and TeO (where the majority of subunit mRNAs are localised), therefore it is likely that the β4 subunit co-localises with the α4 subunit to form a receptor subtype only in these regions. Previous work also showed that the expression of the β4-subunit gene was not similar to those of the γ1- or γ2-subunit genes (Glencorse *et al.*, 1991; Glencorse *et al.*, 1993).

It is evident that the mammalian ε and θ subunits are the most highly diverged $GABA_A$ receptor polypeptides (see Table 16). Russek (1999) has suggested, based on a phylogenetic analysis, that there is an increased mutation rate in that part of the genome where the ε-subunit gene is located (Xq28 in man) which, could explain the low sequence similarity. In addition, the relatively low level of sequence identity and uncertainty of function, seen for the ε and θ subunits between species i.e. rat and human (Table 16), indicates that there is less selective pressure on the two corresponding genes, which has resulted in the accumulation of mutations.

E. J. Barnes raised an antibody against the chicken $GABA_A$ receptor β4 subunit, however he found that it was not specific enough (personal comment). As the β subunits are highly similar in sequence it is difficult to raise selective antibodies. Hence, the non-specificity and poor tissue penetration can be a limitation of antibody staining, however it permits the spatiotemporal distribution of proteins to be studied. Although *in situ* hybridisation is a powerful method of localising cellular distribution (both DNA and RNA), there are longer exposure times and the technique is highly susceptible to contamination by DNAses. Unfortunately the BD17 antibody (specific to β2 and β3

subunits; Ewert *et al.*, 1990) cannot be used because the epitope required, 'QSVNDP' (Ewert *et al.*, 1991) is not present on the N terminus of the β4 subunit. The $GABA_A$ receptor β4 subunit was found to be functional by Liu *et al.* (1998) as a result of electrode voltage-clamp studies in *Xenopus* oocytes. In addition, the β4 subunit was seen to form homomers *in vivo*, a property distinct from the vertebrate β1, β2 and β3 subunits.

In conclusion, it is important to investigate the distribution patterns of the various $GABA_A$ receptor subunits as the subunit composition is likely to play a role in determining cell function. The expression of the $GABA_A$ receptor β4-subunit mRNA was ubiquitous in the avian brain, which is similar to the β subunits expressed in mammalian brain (Pirker *et al.*, 2000) hence, further indicating the importance of the β4 subunit. Although birds diverged from mammals over 310 million years ago (Schmutz and Grimwood, 2004), it is astounding to see a high level of sequence identity of the β subunits particularly the β4 subunit indicating a strong selective pressure for the sequences and their gene structures. It could be proposed that since the $GABA_A$ receptor β4 and γ4 subunit genes evolved at a greater rate than other $GABA_A$ receptor subunit genes they may have evolved to fulfil another function such as learning.

5. The characterisation of a $GABA_A$ receptor in the sea squirt

5.1 Introduction

The sea squirt (*Ciona intestinalis*) is a solitary ascidian that belongs to the phylum Chordata and represents one of the most distant living relatives of vertebrates. The sea squirt was studied in this investigation firstly because it is thought to have arisen before the two-genome duplications that occurred (providing the opportunity to study subtypes arisen from evolution) and secondly because it has a small genome size (20 times smaller than humans), making it easier to analyse. The sea squirt is also an ideal model organism and has been studied extensively by developmental biologists, as the larval body plan is so similar to that of vertebrates (Katz, 1983; Hudson *et al.*, 2003). The nervous system consists of a cerebral ganglion, a neural gland and a ciliated duct, collectively known as the neuronal complex. The neuronal complex is thought to be homologous to the vertebrate CNS (Katsuyama *et al.*, 1995; Wada *et al.*, 1996; Wada *et al.*, 1998; Jiang and Smith, 2002).

In silico analysis was conducted on the sea squirt genome (http://genome.jgi-psf.org/ciona4/ciona4.home.html), which was released in 2002 by Dehal *et al.* It was hypothesised that if the sea squirt lineage arose before the two tetraploidisation events, it would possess a single cluster of $GABA_A$ receptor genes. Hence $GABA_A$ receptor subunit genes were searched for in the database, and the structures (intron/exon boundaries) of the genes found in the sea squirt were analysed, and compared to those in vertebrates (Sommer *et al.*, 1990; Kirkness *et al.*, 1991; Lasham *et al.*, 1991).

Based on sequences obtained from the sea squirt database, primers were designed to amplify the $GABA_A$ receptor subunit genes, β-actin (a housekeeping gene), and glutamic acid decarboxylase (GAD, the enzyme converting glutamic acid to GABA). To detect where $GABA_A$ receptor subunit genes are expressed, cloned DNA fragments were *in vitro* transcribed to produce riboprobes for whole mount *in situ* hybridisation on sea squirt embryos and larvae. To map the distribution of GABA-like immunoreactivity, immunohistochemistry was conducted using a monoclonal anti-GABA antibody on sea squirt embryos and larvae; chicken brain sections were used as a positive control.

Refer to Table 5 for all accession numbers.

5.2.1 *In silico* analysis of the sea squirt genome database

The sea squirt (*Ciona intestinalis*) genome database was searched for $GABA_A$ receptor-like genes. Three $GABA_A$ receptor-like genes were found; an α-like, a β-like and a γ-like.

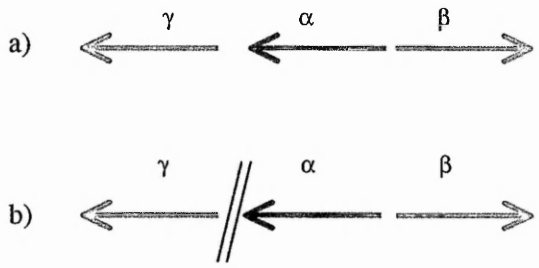


Figure 38a: Predicted ancestral gene cluster of $GABA_A$ receptor genes; b: Arrangement of $GABA_A$ receptor-like genes found in the sea squirt; the α-like and β-like subunit genes are on the same scaffold, whereas the γ-like subunit gene is on a separate scaffold (indicated by the //).

However, only two of these the α- and β-like subunit genes were clustered together (Figure 38a; on scaffold 300, nucleotides 58541bp to 66406bp and 46618bp to 57725bp respectively). The third (γ-like subunit gene) was found on scaffold 827 (nucleotides 6514bp to 11438bp). In addition, the transcriptional orientation of the $GABA_A$ receptor like-subunit genes was conserved with that of other vertebrate orthologues (Glatt *et al.*, 1994; Russek and Farb, 1994). All of the sequences were then entered in to the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST/) to determine which subunits they displayed highest sequence similarity to. The α-like subunit sequence was found to be 47% identical to the rat $GABA_A$ receptor α6 subunit. The β-like subunit sequence was 49% identical to the chicken $GABA_A$ receptor β3 subunit. However, the γ-like subunit was most similar to the mouse glycine receptor α2 subunit (56% sequence identity at the peptide level). To confirm which subunit classes the sequences belonged to, pair-wise alignments were conducted between the translated polypeptide sequences of the three genes found in the sea squirt genome database and the rat (*Rattus norvatus*) $GABA_A$ receptor α1, β2 and γ2 subunits and the glycine receptor α1 subunit.

Sea squirt	$GABA_A$ receptor α1 subunit (%)	$GABA_A$ receptor β2 subunit (%)	$GABA_A$ receptor γ2 subunit (%)	glycine receptor α1 subunit (%)
α	47.2	36	42	36
β	38.8	59.1	37.9	37.8
γ/glycine	35.4	37	35.4	58

Table 19: Translated polypeptide sequence identities of the three $GABA_A$ receptor-like genes found in the sea squirt (*Ciona intestinalis*) genome database (http://genome.jgi-psf.org/ciona4/ciona4.home.html) with $GABA_A$ and glycine receptor subunits from the rat (*Rattus norvatus*). The values shown in red indicate the highest sequence identity.

The $GABA_A$ receptor α-like subunit polypeptide sequence found in the sea squirt genome database was indeed most similar to the rat α1 subunit (see Table 19). The $GABA_A$ receptor β-like subunit was highly similar to the rat $GABA_A$ receptor β2 subunit. Once more, the $GABA_A$ receptor γ-like subunit was significantly more similar to the rat glycine receptor α1 subunit than the rat $GABA_A$ receptor γ2 subunit (hence was named glycine receptor-like subunit). It is possible that this subunit diverged during evolution to form both the glycine receptor α-subunit and $GABA_A$ receptor γ-like subunit gene. The gene structures were analysed in detail by looking for intron/exon boundaries and relative sizes. A schematic was then drawn comparing these gene structures to published vertebrate gene structures (see Harvey *et al.*, 1994a).

There is a significant level of conservation of gene organisation between vertebrates and the primitive chordate *Ciona intestinalis* (see Figure 39). The genes from the sea squirt are extremely compact (only approximately 7kb in length) in comparison to the average vertebrate $GABA_A$ receptor gene, which spans approximately 50 to 100kb (Darlison, 1994). Nevertheless, the size of the exons and positions of the intron/exon boundaries are very similar. Interestingly, some of the exons are split by an intronic sequence (for example exons 4, 7, 8 and 9; refer to Figure 39). Figures 41 to 43 demonstrate the conserved sequences denoting the intron/exon splice sites in the $GABA_A$ and glycine receptor-like subunit genes in the sea squirt. The size of the introns vary throughout the $GABA_A$/glycine receptor subunit-like genes (see Figures 41, 42 and 43) and this is consistent with the findings of Lasham *et al.* (1991), who stated that there was no correlation of intron size between $GABA_A$ receptor subunit genes.

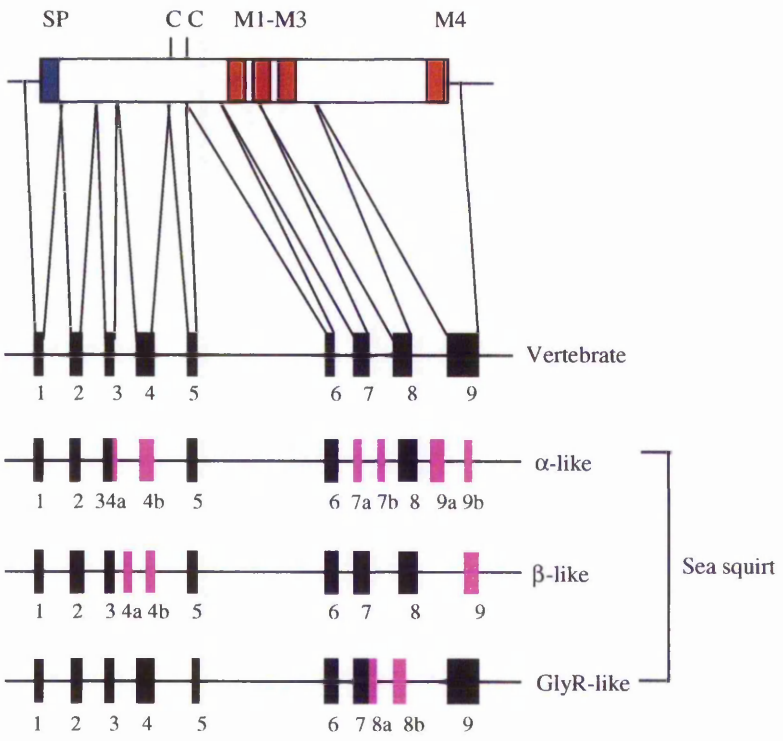
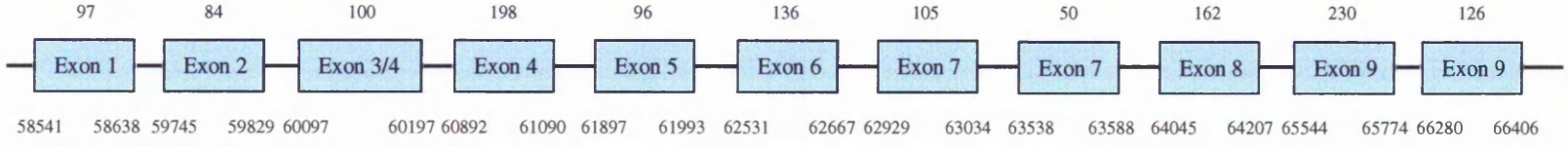
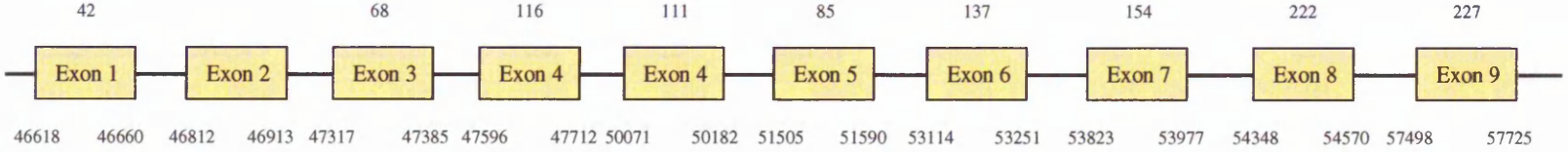


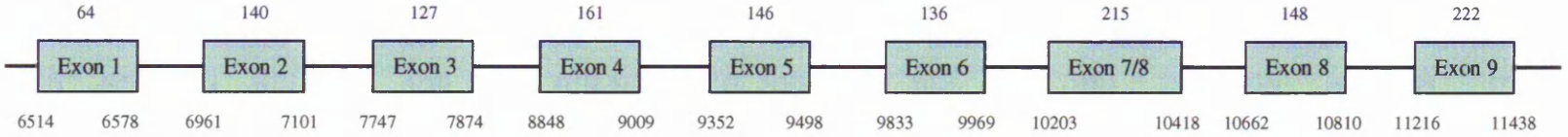
Figure 39: Comparison of the genomic organisations (not to scale) of $GABA_A$/glycine receptor-like subunit genes from the sea squirt (*Ciona intestinalis*), with that of a typical vertebrate $GABA_A$ receptor gene. SP denotes the signal peptide and the four transmembrane domains are labelled M1 to M4. Pink boxes represent split exons.



$GABA_A$ receptor α-like subunit gene structure (Scaffold 300)



$GABA_A$ receptor β-like subunit gene structure (Scaffold 300)



Glycine receptor-like subunit gene structure (Scaffold 837)

Figure 40: Schematic of $GABA_A$/glycine receptor subunit-like gene sequences found in the sea squirt (*Ciona intestinalis*). The lengths of the exons are indicated by numbers above the exons (boxes) and numbers under the boxes represent nucleotide positions on a particular scaffold in the sea squirt genome database (http://genome.jgi-psf.org/ciona4/ciona4.home.html version 1). Note that the schematic is not to scale.

Exon 1.....TTAACtagcgaaaaggg.....intron (600bp).....atcctgcaac**ag**AAAAG.....

Exon 2.....GGAAG**gt**tcgtttgaat.....intron (300bp).....aatgttttac**ag**AAGGA.....

Exon 3.....CAACG**gt**gggtttaccg.....intron (650bp).....tgttcttaac**ag**ATGGA.....

Exon 4.....ATGAG**gt**tgtttgggat.....intron (800bp).....acctttttac**ag**GTTAA.....

Exon 5.....CGAAG**gt**aacttaatgt.....intron (500bp).....ttaatacttc**ag**GATCA.....

Exon 6.....ATCTG**gt**aaatataatg.....intron (250bp).....tttgttttgc**ag**GCGAG.....

Exon 7.....CCCAG**gt**ataacgtgtg.....intron (500bp).....ttttcattct**ag**ATTTC.....

Exon 7.....CTCTG**gt**aagactgagt.....intron (450bp).....attacatttc**ag**GTATC.....

Exon 8.....GTCAG**gt**aagctgtttt.....intron (1300bp)...ttttacaccc**ag**AGAAC.....

Exon 9.....ATTTG**gt**agtaaacaaa.....intron (500bp).....attaccttaa**ag**GGCGG.....

Exon 9.....AGGCActtgaatcgtaa.....

Figure 41: Sequences across the intron/exon boundaries of the sea squirt $GABA_A$ receptor α-subunit like gene. Nucleotides in upper-case letters represent exonic sequences whilst those intronic sequences are shown in lower-case. The conserved minimum consensus sequences for splice junctions (Smith *et al.*, 1989) gt (5' donor site) and ag (3' acceptor site) are indicated by blue lettering. The approximate sizes of introns are shown in brackets.

Exon 1.....CAAAG**gt**aagttgaata.....intron (150bp).....cttgtgtttt**ag**AATTT.....

Exon 2.....TGGCG**gt**aatgatgtgt.....intron (250bp).....ttttaatttc**ag**CCGAT.....

Exon 3.....ACATG**gt**aagcttcaat.....intron (150bp).....tatcttttct**ag**GATTA.....

Exon 4.....TATGG**gt**gagtgtaact.....intron (2400bp)....atgacgtcac**ag**GTACC....

Exon 4.....CTCAG**gt**gcgatgaatg....intron (1300bp)....tgttttaaac**ag**AGTGA.....

Exon 5.....GAGCT**gt**aagttaaaga....intron (1500bp)....aaccattcac**ag**ACGGT.....

Exon 6.....TACAG**gt**cagtatcaac...intron (600bp).....ttttcaacac**ag**GATCT.....

Exon 7.....GTTGG**gt**gaggaaaata....intron (400bp).....tctgtacatc**ag**GTATT.....

Exon 8.....TCGAG**gt**gagccgagcg...intron (2300bp)....tacttattac**ag**GGCCG .....

Exon 9.....TTTGA**gt**tgccatcaca.....

Figure 42: Sequences across the intron/exon boundaries of the sea squirt $GABA_A$ receptor β-subunit like gene. Nucleotides in upper-case letters represent exonic sequences whilst those intronic sequences are shown in lower-case. The conserved minimum consensus sequences for splice junctions (Smith *et al.*, 1989) gt (5' donor site) and ag (3' acceptor site) are indicated by blue lettering. The approximate sizes of introns are shown in brackets.

Exon 1.....CATTG**gt**gtgcgtcgtt.....intron (400bp)....ttcgccaaac**ag**GGTGA....

Exon 2.....TCAAG**gt**gacttttaca.....intron (600bp)....ttgttacaac**ag**TTTTG.....

Exon 3.....GGGTG**gt**atgcaaatat.....intron (900bp)....accttacaac**ag**GACTA.....

Exon 4.....ATGAG**gt**tggcgttgta.....intron (360bp)....tgttccaccc**ag**GTTAC.....

Exon 5.....GAGTT**gt**aagttgttga.....intron (250bp).....cctactacac**ag**TTGGC.....

Exon 6.....CACAG**gt**gggttgtgtg.....intron (250bp).....tcacgaacac**ag**GTTCA....

Exon 7.....CTAAG**gt**tggtacctca.....intron (220bp)....attacatcac**ag**GTGTC.....

Exon 8.....AAAAG**gt**gggggttgta....intron (420bp)....gatacttaac**ag**CAATT.....

Exon 9.....GTGGG**gt**ttttataactt.....

Figure 43: Sequences across the intron/exon boundaries of the sea squirt glycine receptor-like subunit gene. Nucleotides in upper-case letters represent exonic sequences whilst those intronic sequences are shown in lower-case. The conserved minimum consensus sequences for splice junctions (Smith *et al.*, 1989) gt (5' donor site) and ag (3' acceptor site) are indicated by blue lettering. The approximate sizes of introns are shown in brackets.

5.2.2 Expression of the β-actin, $GABA_A$/glycine receptor-like subunit and glutamic acid decarboxylase (GAD) genes in the sea squirt

Following the identification of $GABA_A$/glycine receptor-like subunit genes in the sea squirt (*Ciona intestinalis*) genome, the next step was to determine whether these genes were expressed and if so, in which tissues. RNA was isolated from a range of tissues from an adult sea squirt; these were reverse transcribed to cDNA. Glutamic acid decarboxylase (GAD) is the enzyme that converts glutamic acid to the neurotransmitter GABA, and since GABA binds to $GABA_A$ receptors, GAD must be present for $GABA_A$ receptors to be activated. Therefore, PCR was conducted to amplify products of the $GABA_A$ receptor subunit cDNAs and the GAD cDNAs. cDNA from the housekeeping gene (β-actin) was amplified from all tissues by PCR (expected fragment size of approximately 500bp) to act as a positive control.

The cDNA to be used in the subsequent PCR to amplify $GABA_A$/glycine receptor and GAD cDNAs were found to be of good integrity (see Figure 44). The tissues with the most β-actin cDNA were gut, neuronal complex, stomach, trunk and that reverse transcribed from whole larvae. There was less β-actin cDNA in the heart and siphons. All of these tissues were screened for the expression of the $GABA_A$/glycine receptor-like subunit cDNA using PCR (see 2.4). Primers were designed based on exonic

sequences (over at least one intron) to amplify $GABA_A$ receptor α-, β- and glyR-like subunit cDNA fragments (refer to Table 2). Initially one set of primers was designed; however as some of the PCRs were unsuccessful further sets of primers (i.e. ssβF2 and ssβR2) were designed to conduct nested PCRs (see 2.4 and Table 3).

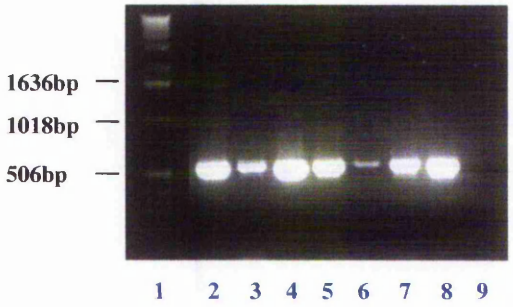


Figure 44: A 2% (w/v) agarose gel illustrating the amplification of the β-actin cDNA from different tissues of the sea squirt (*Ciona intestinalis*). Lane 1, contain a 1kb DNA ladder (marker), lane 2 is the product amplified from first-strand cDNA from the gut, 3 is heart, 4 is larvae, 5 is neuronal complex, 6 is siphons, 7 is stomach, 8 is trunk and lane 9 is the negative control (no DNA).

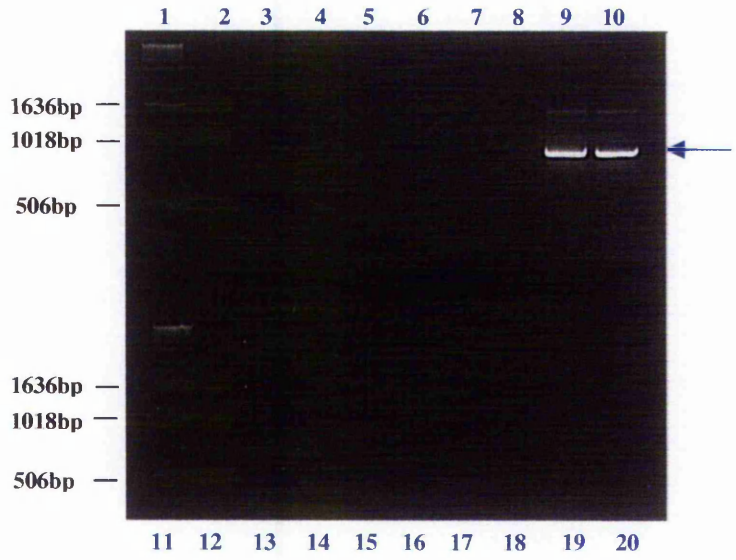


Figure 45: A 2% (wv) agarose gel demonstrating the amplification of the $GABA_A$ receptor β-subunit cDNA from sea squirt (*Ciona intestinalis*). Lane 1 and 11 contain the 1kb DNA ladder, lanes 2 and 12 are negative controls, lanes 3 and 4 show the product amplified from first-strand cDNA from the gonad, 5 and 6 are gut, 7 and 8 are heart, 9 and 10 are larvae, 13 and 14 are neuronal complex, 15 and 16 are siphons, 17 and 18 are stomach and 19 and 20 are trunk.

The $GABA_A$ receptor β-subunit gene was not expressed in any of the adult tissues, a band of the expected size (approximately 800bp) was only found with larval cDNA (see Figure 45). As there was not enough RNA from the neuronal complex of one organism, RNA was extracted from 6 animals and this was pooled together to synthesise cDNA. The fainter band of approximately 1400bp (lane 9 and 10; see Figure 45) could represent unspliced RNA. This product (of 800bp) was cloned, sequenced and compared against the $GABA_A$ receptor β-like subunit sequence deduced from the sea squirt genome.

Interestingly, the sequences were not 100% identical (refer to Figure 46) this could possibly be due to PCR errors or that the cDNA was isolated from sea squirt of a UK population whereas, the genome sequences were isolated from a Japanese population of sea squirt. To further determine which $GABA_A$ receptor subunit polypeptide was most similar to that found in the sea squirt, the β1-, β2- and β3-subunit amino-acid sequences from the rat and the β4-subunit sequence from the chicken were aligned against the β-like sequence obtained from the sea squirt database.

```
Clone     FKVTNSRLLIKNITFATGSYPRVSLSFLLKRNLGFFILQTYLPCSLITILSWVSFWINHE
Genome    FKVTNSRLLIKNITFATGSYPRVSLSFLLKRNIGFFILQTYLPCSLITILSWVSFWINHE

Clone     ATAARVALGITTVLTVTTISTNVRQSLPKIPDVKALDVYLICCFVFVFLALLEYAMVNCT
Genome    ATAARVALGITTVLTVTTISTNVRQSLPKIPDIKALDVYLICCFVFVFLALLEYAMVNCT

Clone     YYGNMARQTKAKLRRKLTEALEAEESAKSANFAAQFSGGSKETIRYCDEESTSPGSTHQI
Genome    YYGNMARQTKAKLRRKLTEALEAEESAKSANFAAQFSGGSKETIRYCDEESTSPGSIHQI

Clone     AAGWSDNEENAIRHISTCPHANHARPLPTRRRNVGFNVATQGRARSMHNAANGKRRGQPT
Genome    AAGWSDNEENAIRHISTCPHANHARPLPTRRRNVGFNVATQGRARGMHNAANGKRRGQPS

Clone     RNSVGRKRKGYASRAKKSLSALKVPKISDVSIIDKVARV
Genome    RNSVGKKRKGYASRAKKSLSALKVPKISDVSIIDKVARV
```

Figure 46: Alignment of the deduced amino-acid (single-letter code) sequences of the $GABA_A$ receptor β-like subunit cloned from the sea squirt and that deduced from the sea squirt genome database. There was a 97.5% sequence identity between the cloned sequence and that deduced from the genome database (scaffold 300, nucleotide positions 53197bp to 57676bp). Yellow shading denotes the differences between sequences.

The position of the sequences relative to one another is determined by sequence identity i.e. sequences that are most similar are placed next to each other. Hence, the alignment generated (Figure 47) confirmed that the β-like subunit sequence from the sea squirt was most similar to the β4-subunit sequence from the chicken. This is interesting,

because the β4-subunit gene is not found in 'higher' vertebrates e.g. mammals hence it could be speculated that it is the 'oldest' of the $GABA_A$ receptor β-subunit genes, since the sea squirt was predicted to possess the ancestral cluster this would be logical. As expected the majority of differences can be seen in the regions encoding the intracellular loop (between M3 and M4).

After amplification of the $GABA_A$ receptor β-like subunit cDNA from the sea squirt the following step was to amplify the sea squirt α-like subunit cDNA. However, after attempting to amplify a $GABA_A$ receptor α-like subunit cDNA from a wide range of tissues from the sea squirt using two sets of primers a third set was designed (see 2.4). Since the third set of primers were also not successful in amplifying an α-like cDNA from the sea squirt even after performing a nested PCR, genomic DNA from larvae was used as a template (rather than cDNA).

Fortunately, a correctly size fragment of approximately 400bp was amplified from genomic DNA (refer to Figure 48). This was cloned and the sequence obtained was compared against the 3' end of the $GABA_A$ receptor α-subunit sequences from the rat and the corresponding region of the predicted $GABA_A$ receptor α-like subunit from the sea squirt by pair-wise alignments.

The partial $GABA_A$ receptor α-like subunit sequence cloned from the sea squirt was compared against the sequence from the genome database. The $GABA_A$ receptor α-like subunit cloned from the sea squirt was indeed most similar to the sequence predicted from the sea squirt genome database, however the sequence identity was significantly lower than for the comparison of the β-like subunit polypeptides (i.e. 87.6% compared to 97.5%, respectively). The $GABA_A$ receptor α-subunit sequences from the rat were then aligned against the α-like sequence obtained from the sea squirt database. The alignment generated confirmed that the α-like subunit sequence from the sea squirt was most similar to the α6-subunit sequence from the rat (see Figure 50). As expected the region of highest sequence similarity are the transmembrane regions.

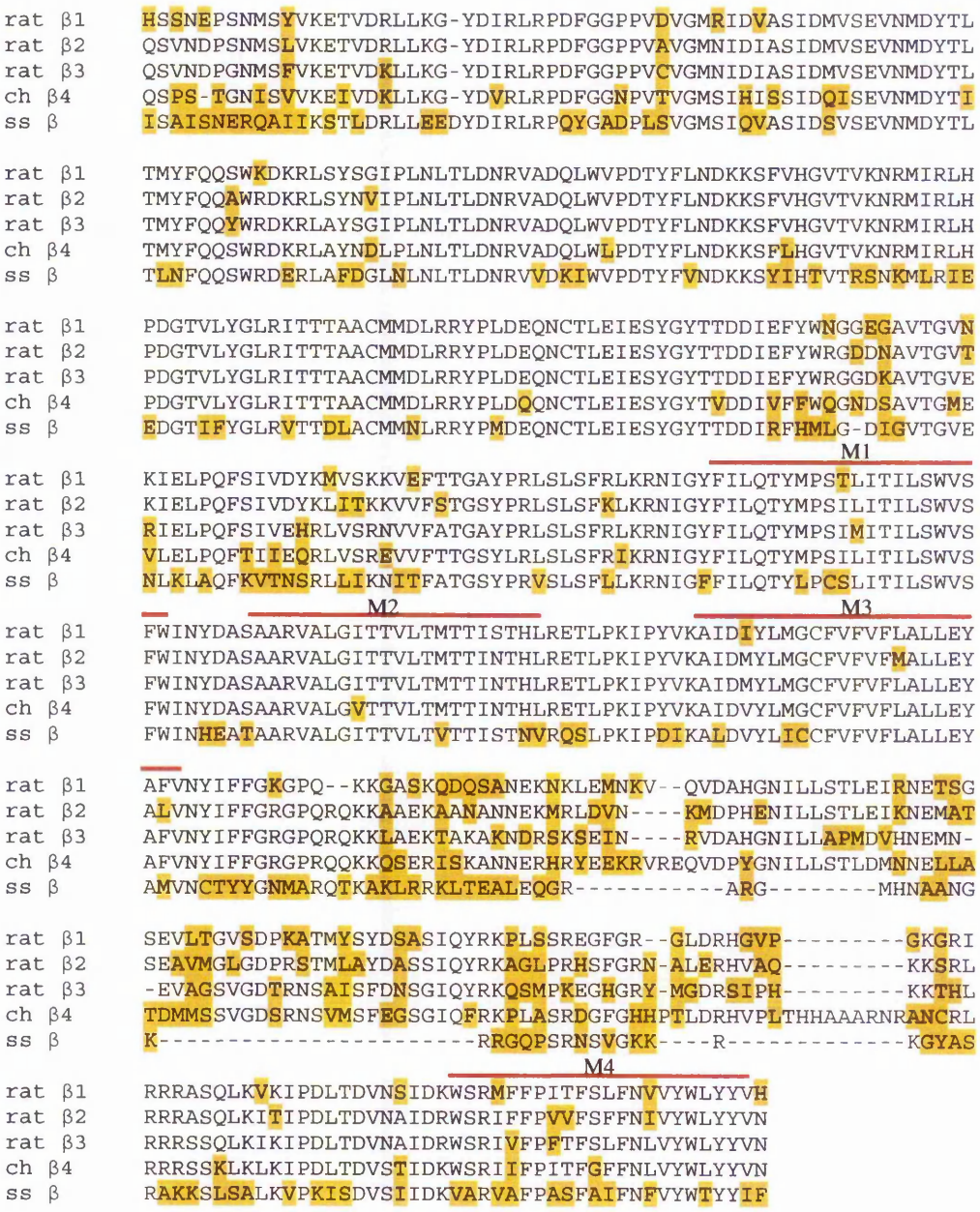


Figure 47: Alignment of the partial amino-acid (single-letter code) sequences of the $GABA_A$ receptor β-subunit sequences from the rat (*Rattus norvegicus*), chicken (ch; *Gallus gallus domesticus*) and that obtained by *in silico* analysis of the sea squirt (ss; *Ciona intestinalis*) genome, generated using the computer programme ClustalW (http://www.ch.embnet.org/software/ClustalW.html). Dashes denote gaps, which have been inserted to maximise the alignment. Red bars above the sequences indicate the transmembrane domains (M1 to M4). Yellow shading denotes differences between the sequences.

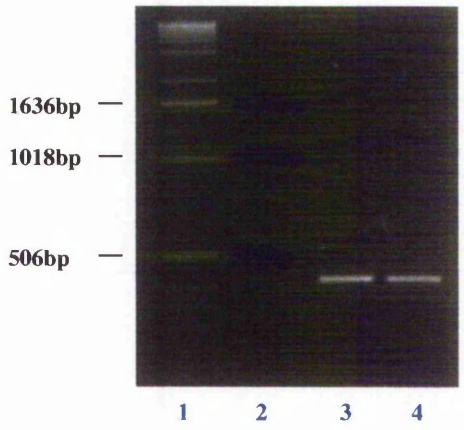


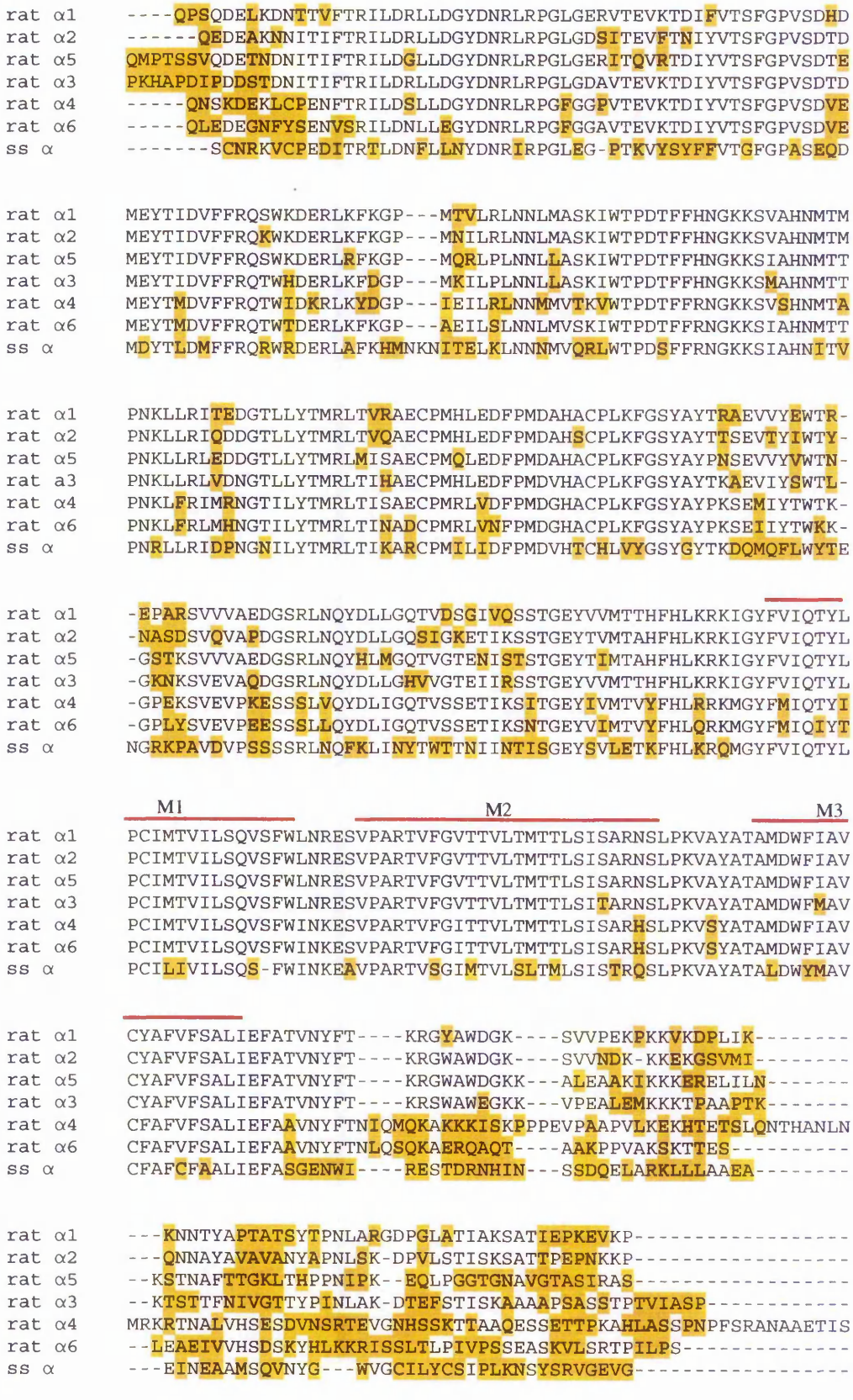
Figure 48: A 2% (w/v) agarose gel illustrating the amplification of a $GABA_A$ receptor α-subunit gene fragment from the sea squirt (*Ciona intestinalis*) and lane 1 contain a 1kb DNA ladder (marker), lane 2 is the negative control (no DNA) and 3 to 4 represent the product amplified from genomic DNA.

```
clone    PITLKGGVSRIDRVSRVAFPLAFFFFNGIYWFVYLSHQTHEVAYRLGTIVTCPHIQTFKI
genome   PITLKGGVSRIDRVSRVAFPLAFFFFNGIYWFVYLSHQTHEVAYRLGTIVTCQHIKTFKM

clone    NYVNCVNIKCVYRCARVIVLSKL-TGILSDKKYECMF
genome   NHVNCVNIKCVYKCAKVNILSKLRTGTVSDTKYVCMF
```

Figure 49: Alignment of the partial amino-acid sequences of the $GABA_A$ receptor α-like subunit cloned from the sea squirt and that obtained from the sea squirt genome database. There was an 87.6% sequence identity between the cloned sequence and that deduced from the genome database (scaffold 300 nucleotide positions 66264 to 66681bp). Yellow shading denotes the differences between sequences.

A fragment of the glyR-like subunit gene was even more difficult to clone, even though a wide range of tissues and two sets of primers were tested (see 2.4). A third set of primers was designed but a PCR using genomic DNA as a template also did not produce a band, hence a fourth set was designed. The fourth set of primers did not yield a band either therefore all four forward primers were tested with the first reverse primer (which was based on a conserved sequence (TM4; see Table 2). From Figure 51 the expected sizes of fragments amplified by PCR could be calculated. The PCR using a combination of the primer glyR with glyF yielded a band of approximately 2000bp; with glyF2 1180bp; with glyF3 220bp and with glyF4 255bp. Hence four PCR were carried out using the different forward primers and the single reverse primer on sea squirt genomic DNA.


rat α1 ----QPSQDELKDNTTVFTRILDRLLDGYDNRLRPGLGERVTEVKTDIFVTSFGPVSDHD
rat α2 ------QEDEAKNNITIFTRILDRLLDGYDNRLRPGLGDSITEVFTNIYVTSFGPVSDTD
rat α5 QMPTSSVQDETNDNITIFTRILDGLLDGYDNRLRPGLGERITQVRTDIYVTSFGPVSDTE
rat α3 PKHAPDIPDDSTDNITIFTRILDRLLDGYDNRLRPGLGDAVTEVKTDIYVTSFGPVSDTD
rat α4 -----QNSKDEKLCPENFTRILDSLLDGYDNRLRPGFGGPVTEVKTDIYVTSFGPVSDVE
rat α6 -----QLEDEGNFYSENVSRILDNLLEGYDNRLRPGFGGAVTEVKTDIYVTSFGPVSDVE
ss α -------SCNRKVCPEDITRTLDNFLLNYDNRIRPGLEG-PTKVYSYFFVTGFGPASEQD
rat α1 MEYTIDVFFRQSWKDERLKFKGP---MTVLRLNNLMASKIWTPDTFFHNGKKSVAHNMTM
rat α2 MEYTIDVFFRQKWKDERLKFKGP---MNILRLNNLMASKIWTPDTFFHNGKKSVAHNMTM
rat α5 MEYTIDVFFRQSWKDERLRFKGP---MQRLPLNNLLASKIWTPDTFFHNGKKSIAHNMTT
rat α3 MEYTIDVFFRQTWHDERLKFDGP---MKILPLNNLLASKIWTPDTFFHNGKKSMAHNMTT
rat α4 MEYTMDVFFRQTWIDKRLKYDGP---IEILRLNNMMVTKVWTPDTFFRNGKKSVSHNMTA
rat α6 MEYTMDVFFRQTWTDERLKFKGP---AEILSLNNLMVSKIWTPDTFFRNGKKSIAHNMTT
ss α MDYTLDMFFRQRWRDERLAFKHMNKNITELKLNNNMVQRLWTPDSFFRNGKKSIAHNITV
rat α1 PNKLLRITEDGTLLYTMRLTVRAECPMHLEDFPMDAHACPLKFGSYAYTRAEVVYEWTR-
rat α2 PNKLLRIQDDGTLLYTMRLTVQAECPMHLEDFPMDAHSCPLKFGSYAYTTSEVTYIWTY-
rat α5 PNKLLRLEDDGTLLYTMRLMISAECPMQLEDFPMDAHACPLKFGSYAYPNSEVVYVWTN-
rat a3 PNKLLRLVDNGTLLYTMRLTIHAECPMHLEDFPMDVHACPLKFGSYAYTKAEVIYSWTL-
rat α4 PNKLFRIMRNGTILYTMRLTISAECPMRLVDFPMDGHACPLKFGSYAYPKSEMIYTWTK-
rat α6 PNKLFRLMHNGTILYTMRLTINADCPMRLVNFPMDGHACPLKFGSYAYPKSEIIYTWKK-
ss α PNRLLRIDPNGNILYTMRLTIKARCPMILIDFPMDVHTCHLVYGSYGYTKDQMQFLWYTE
rat α1 -EPARSVVVAEDGSRLNQYDLLGQTVDSGIVQSSTGEYVVMTTHFHLKRKIGYFVIQTYL
rat α2 -NASDSVQVAPDGSRLNQYDLLGQSIGKETIKSSTGEYTVMTAHFHLKRKIGYFVIQTYL
rat α5 -GSTKSVVVAEDGSRLNQYHLMGQTVGTENISTSTGEYTIMTAHFHLKRKIGYFVIQTYL
rat α3 -GKNKSVEVAQDGSRLNQYDLLGHVVGTEIIRSSTGEYVVMTTHFHLKRKIGYFVIQTYL
rat α4 -GPEKSVEVPKESSSLVQYDLIGQTVSSETIKSITGEYIVMTVYFHLRRKMGYFMIQTYI
rat α6 -GPLYSVEVPEESSSLLQYDLIGQTVSSETIKSNTGEYVIMTVYFHLQRKMGYFMIQIYT
ss α NGRKPAVDVPSSSSRLNQFKLINYTWTTNIINTISGEYSVLETKFHLKRQMGYFVIQTYL
M1
M2
M3
rat α1 PCIMTVILSQVSFWLNRESVPARTVFGVTTVLTMTTLSISARNSLPKVAYATAMDWFIAV
rat α2 PCIMTVILSQVSFWLNRESVPARTVFGVTTVLTMTTLSISARNSLPKVAYATAMDWFIAV
rat α5 PCIMTVILSQVSFWLNRESVPARTVFGVTTVLTMTTLSISARNSLPKVAYATAMDWFIAV
rat α3 PCIMTVILSQVSFWLNRESVPARTVFGVTTVLTMTTLSITARNSLPKVAYATAMDWFMAV
rat α4 PCIMTVILSQVSFWINKESVPARTVFGITTVLTMTTLSISARHSLPKVSYATAMDWFIAV
rat α6 PCIMTVILSQVSFWINKESVPARTVFGITTVLTMTTLSISARHSLPKVSYATAMDWFIAV
ss α PCILIVILSQS-FWINKEAVPARTVSGIMTVLSLTMLSISTRQSLPKVAYATALDWYMAV
rat α1 CYAFVFSALIEFATVNYFT----KRGYAWDGK----SVVPEKPKKVKDPLIK--------
rat α2 CYAFVFSALIEFATVNYFT----KRGWAWDGK----SVVNDK-KKEKGSVMI--------
rat α5 CYAFVFSALIEFATVNYFT----KRGWAWDGKK---ALEAAKIKKKERELILN-------
rat α3 CYAFVFSALIEFATVNYFT----KRSWAWEGKK---VPEALEMKKKTPAAPTK-------
rat α4 CFAFVFSALIEFAAVNYFTNIQMQKAKKKISKPPPEVPAAPVLKEKHTETSLQNTHANLN
rat α6 CFAFVFSALIEFAAVNYFTNLQSQKAERQAQT----AAKPPVAKSKTTES----------
ss α CFAFCFAALIEFASGENWI----RESTDRNHIN---SSDQELARKLLLAAEA--------
rat α1 ---KNNTYAPTATSYTPNLARGDPGLATIAKSATIEPKEVKP----------------
rat α2 ---QNNAYAVAVANYAPNLSK-DPVLSTISKSATTPEPNKKP----------------
rat α5 --KSTNAFTTGKLTHPPNIPK--EQLPGGTGNAVGTASIRAS----------------
rat α3 --KTSTTFNIVGTTYPINLAK-DTEFSTISKAAAAPSASSTPTVIASP----------
rat α4 MRKRTNALVHSESDVNSRTEVGNHSSKTTAAQESSETTPKAHLASSPNPFSRANAAETIS
rat α6 --LEAEIVVHSDSKYHLKKRISSLTLPIVPSSEASKVLSRTPILPS------------
ss α ---EINEAAMSQVNYG---WVGCILYCSIPLKNSYSRVGEVG----------------

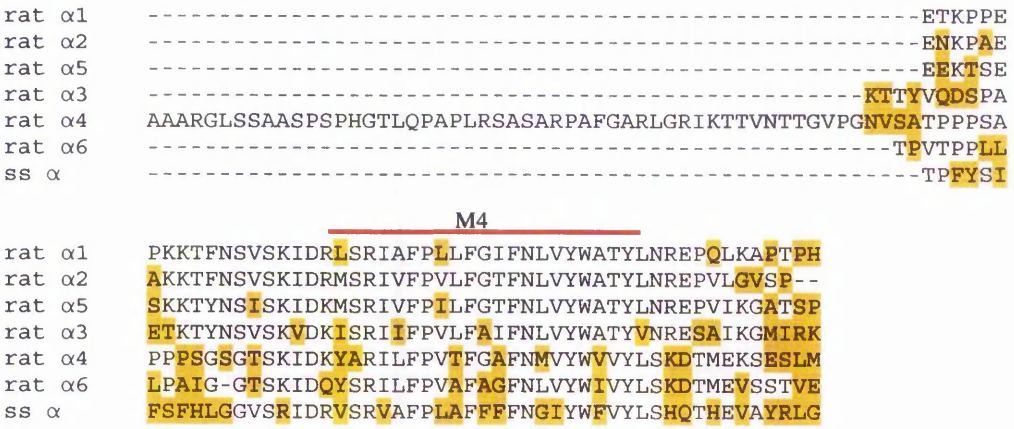


Figure 50: Alignment of the amino-acid (single-letter code) sequences of the $GABA_A$ receptor α subunit from the rat (*Rattus norvegicus*) and *in silico* analysis of the sea squirt (ss; *Ciona intestinalis*) genome, generated using the computer programme ClustalW (http://www.ch.embnet.org/software/ClustalW.html). Dashes denote gaps that have been inserted to maximise the alignment. Red bars above the sequence indicate the transmembrane domains (M1 to M4). Yellow shading denotes differences between the sequences.

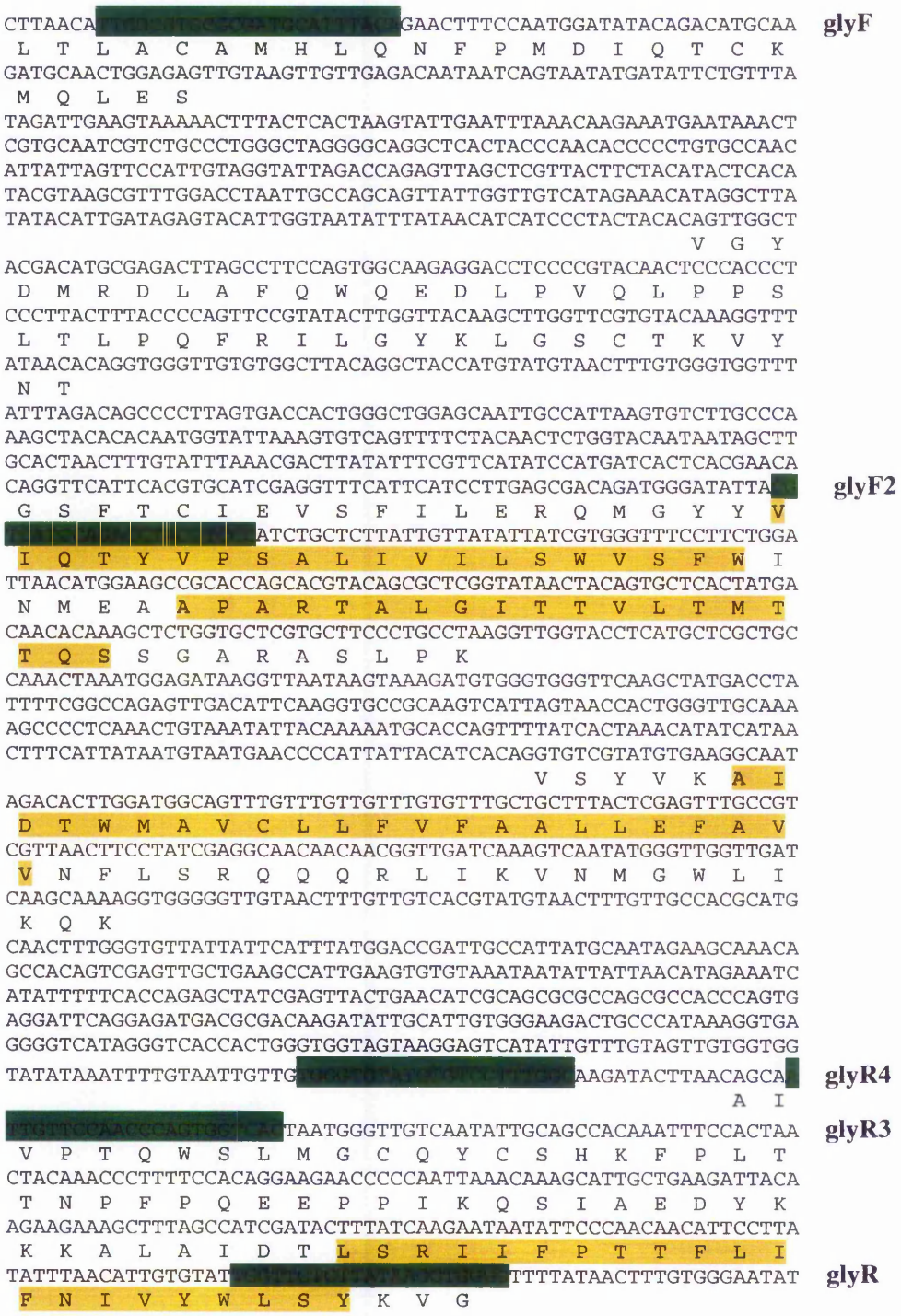


Figure 51: Nucleotide and amino-acid sequences of part of the glyR-like subunit from the genome database of the sea squirt (*Ciona intestinalis*) generated using the Translate programme of the Justbio website (http://www.justbio.com/translator/index.php). The transmembrane domains are highlighted in yellow and the primer sequences are green, with the name of the primer on the right hand side.

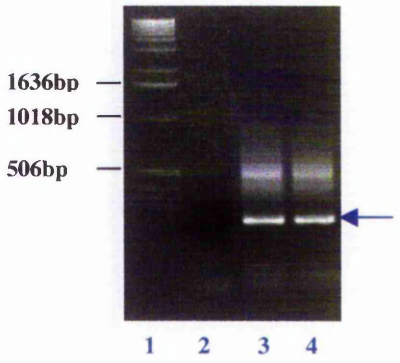


Figure 52: A 2% (w/v) agarose gel demonstrating the amplification of a fragment of the glyR-like subunit gene from the sea squirt (*Ciona intestinalis*). Lane 1 contains a 1kb DNA ladder (marker), lane 2 contains the negative control (no DNA) and lanes 3 and 4 harbour the product amplified from the glyR-like subunit genomic DNA.

The combination of the reverse primer (glyR), and the forward primer, namely glyF3, yielded a band of the correct size i.e. approximately 220bp (indicated by a blue arrow, see Figure 52). The cloned glyR-like subunit sequence from the sea squirt was then compared against the sequence deduced from the database (shown in Figure 53).

```
Clone     IVPTQWSLMDCPNCQPQRKFIPHKVPLTTPPSSQEDPPIKQSIAEDYKRKALAIDTLSRI
Genome    IVPTQWSLMGCQYCS-------HKFPLTTNPFPQEEPPIKQSIAEDYKKKALAIDTLSRI

Clone     IFPTTFLIFNIVYWLSYK
Genome    IFPTTFLIFNIVYWLSYK
```

Figure 53: Alignment of the amino-acid (single-letter code) sequences of the glyR-like subunit cloned from the sea squirt and that obtained from the sea squirt genome database (scaffold 827 nucleotide positions 11216 to 11438bp). There was 85.9% sequence identity between the cloned sequence and that deduced from the genome database. Yellow shading denotes the differences between sequences.

The glyR-like subunit sequence was 85.9% identical to the glyR-like subunit sequence deduced from the sea squirt genome database (shown in Figure 53). In addition, the alignments demonstrated that the glyR-like subunit from the sea squirt was most similar to the $GABA_A$ receptor γ4 subunit from the chicken (see Figure 54). Interestingly, the γ4-subunit gene is found in the same cluster as the β4-subunit gene in the chicken (see 4.2.2; Figure 32) and the sea squirt β-like subunit polypeptide was most like the vertebrate β4-subunit polypeptide from the chicken. Once more the areas of least difference were the transmembrane domains.

Two molecules of the neurotransmitter GABA are required to initiate the conformational change in the $GABA_A$ receptor ion channel (Horenstein *et al.*, 2001). Since GAD is the enzyme that is responsible for the synthesis of GABA, identification of the expression of GAD in the sea squirt would indicate where and whether GABA is being produced. Hence, a GAD cDNA fragment was amplified by PCR using larval cDNA and primers based on the gene sequence, which should have yielded a band of approximately 460bp. Although two isoforms of GAD exist in vertebrates GAD_{65} and GAD_{67} (Bu *et al.*, 1992), only one gene was found in the sea squirt genome database. However, two bands can be seen in Figure 56, which may represent alternative splice forms. The cloned cDNA that encodes GAD was compared against the sequence deduced from the sea squirt genome. The cloned GAD sequence was 96.6% identical to the sea squirt sequence obtained from the NCBI database (see Figure 57), however this was lower than expected since the sequences are from the same species, one would therefore expect a similarity of 100%. It should be taken into account that the GAD sequence obtained by PCR in this study was generated after 80 cycles, hence this lower similarity could be due to PCR errors. Interestingly, the cloned GAD sequence from the sea squirt was significantly more similar to the human GAD_{67} isoform than GAD_{65} as seen by the alignment above (see Figure 58).

In mammals both $GABA_A$ and glycine receptor-like subunit genes are expressed as heteromers (Moss and Smart, 2001). Therefore, *in silico* analysis was conducted to determine whether there were other $GABA_A$ receptor and glycine receptor-like subunit genes. Preliminary analysis revealed this to be the case. An additional four GABA receptor like-subunit genes were found in the sea squirt genome database (refer to Table 20). Further analysis confirmed that they were most similar to the $GABA_C$ receptor ρ subunits and the $GABA_A$ receptor π subunit. Interestingly, three partial sequences for $GABA_C$ receptor-like ρ-subunit genes were found, which is the same as that in man namely, ρ1, ρ2 and ρ3. The genes encoding the human ρ1 and ρ2 subunits (*GABRR1* and *GABRR2*) are clustered together on chromosome 6q14-q21 (Cutting *et al.*, 1992), while *GABRR3* is located on chromosome 3q11-q13 (Bailey *et al.*, 1999a). However, the $GABA_C$ receptor-like ρ-subunit genes were found on three different scaffolds in the sea squirt (see Figure 59). As stated earlier only partial sequences were found, exons 1, 3, 4 and 6 were missing (except scaffold 113 where only exons 1 and 3 were absent).

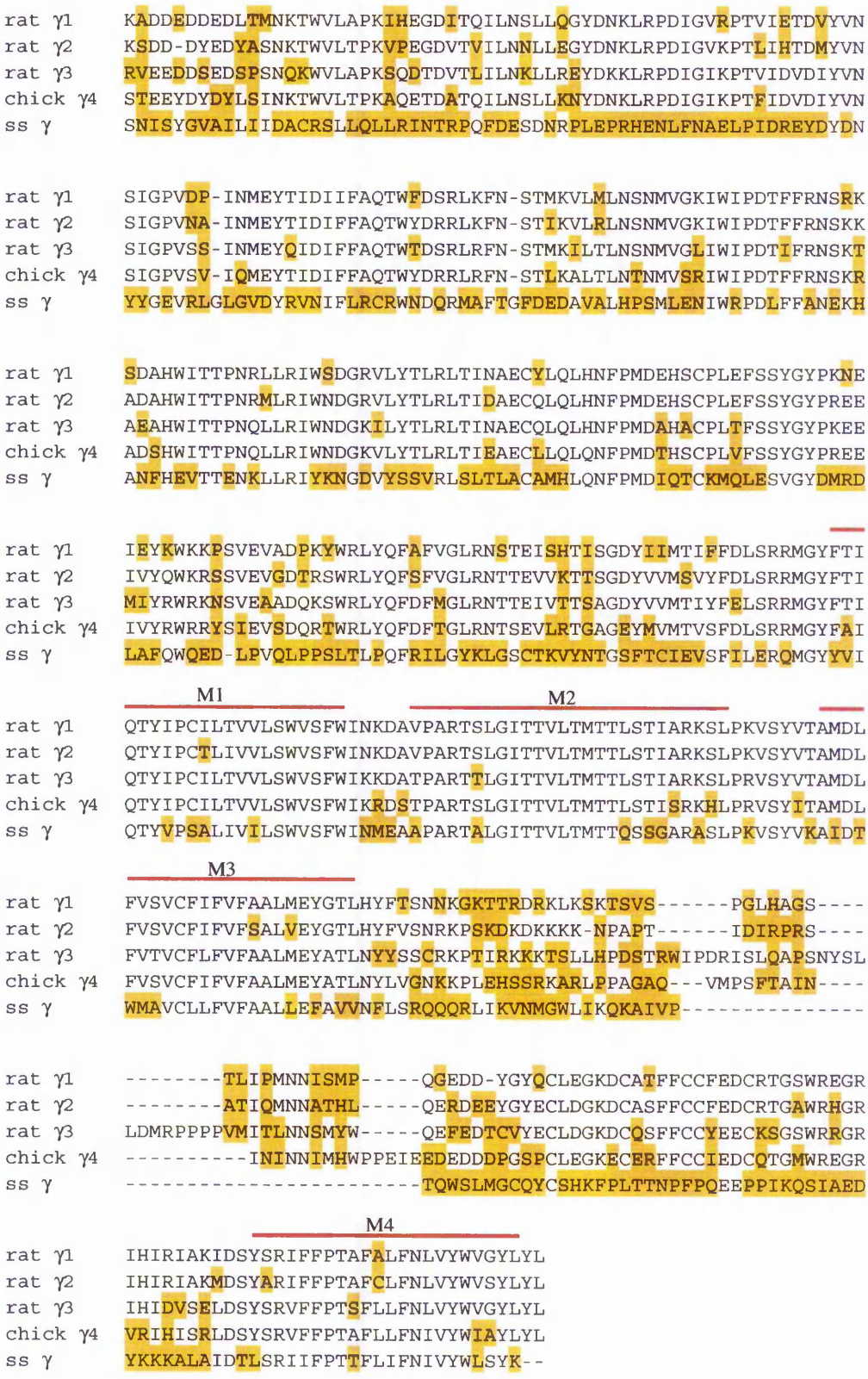

rat γ1 KADDEDDEDLTMNKTWVLAPKIHEGDITQILNSLLQGYDNKLRPDIGVRPTVIETDVYVN
rat γ2 KSDD-DYEDYASNKTWVLTPKVPEGDVTVILNNLLEGYDNKLRPDIGVKPTLIHTDMYVN
rat γ3 RVEEDDSEDSPSNQKWVLAPKSQDTDVTLILNKLLREYDKKLRPDIGIKPTVIDVDIYVN
chick γ4 STEEYDYDYLSINKTWVLTPKAQETDATQILNSLLKNYDNKLRPDIGIKPTFIDVDIYVN
ss γ SNISYGVAILIIDACRSLLQLLRINTRPQFDESDNRPLEPRHENLFNAELPIDREYDYDN
rat γ1 SIGPVDP-INMEYTIDIIFAQTWFDSRLKFN-STMKVLMLNSNMVGKIWIPDTFFRNSRK
rat γ2 SIGPVNA-INMEYTIDIFFAQTWYDRRLKFN-STIKVLRLNSNMVGKIWIPDTFFRNSKK
rat γ3 SIGPVSS-INMEYQIDIFFAQTWTDSRLRFN-STMKILTLNSNMVGLIWIPDTIFRNSKT
chick γ4 SIGPVSV-IQMEYTIDIFFAQTWYDRRLRFN-STLKALTLNTNMVSRIWIPDTFFRNSKR
ss γ YYGEVRLGLGVDYRVNIFLRCRWNDQRMAFTGFDEDAVALHPSMLENIWRPDLFFANEKH
rat γ1 SDAHWITTPNRLLRIWSDGRVLYTLRLTINAECYLQLHNFPMDEHSCPLEFSSYGYPKNE
rat γ2 ADAHWITTPNRMLRIWNDGRVLYTLRLTIDAECQLQLHNFPMDEHSCPLEFSSYGYPREE
rat γ3 AEAHWITTPNQLLRIWNDGKILYTLRLTINAECQLQLHNFPMDAHACPLTFSSYGYPKEE
chick γ4 ADSHWITTPNQLLRIWNDGKVLYTLRLTIEAECLLQLQNFPMDTHSCPLVFSSYGYPREE
ss γ ANFHEVTTENKLLRIYKNGDVYSSVRLSLTLACAMHLQNFPMDIQTCKMQLESVGYDMRD
rat γ1 IEYKWKKPSVEVADPKYWRLYQFAFVGLRNSTEISHTISGDYIIMTIFFDLSRRMGYFTI
rat γ2 IVYQWKRSSVEVGDTRSWRLYQFSFVGLRNTTEVVKTTSGDYVVMSVYFDLSRRMGYFTI
rat γ3 MIYRWRKNSVEAADQKSWRLYQFDFMGLRNTTEIVTTSAGDYVVMTIYFELSRRMGYFTI
chick γ4 IVYRWRRYSIEVSDQRTWRLYQFDFTGLRNTSEVLRTGAGEYMVMTVSFDLSRRMGYFAI
ss γ LAFQWQED-LPVQLPPSLTLPQFRILGYKLGSCTKVYNTGSFTCIEVSFILERQMGYYVI
M1
M2
rat γ1 QTYIPCILTVVLSWVSFWINKDAVPARTSLGITTVLTMTTLSTIARKSLPKVSYVTAMDL
rat γ2 QTYIPCTLIVVLSWVSFWINKDAVPARTSLGITTVLTMTTLSTIARKSLPKVSYVTAMDL
rat γ3 QTYIPCILTVVLSWVSFWIKKDATPARTTLGITTVLTMTTLSTIARKSLPRVSYVTAMDL
chick γ4 QTYIPCILTVVLSWVSFWIKRDSTPARTSLGITTVLTMTTLSTISRKHLPRVSYITAMDL
ss γ QTYVPSALIVILSWVSFWINMEAAPARTALGITTVLTMTTQSSGARASLPKVSYVKAIDT
M3
rat γ1 FVSVCFIFVFAALMEYGTLHYFTSNNKGKTTRDRKLKSKTSVS------PGLHAGS----
rat γ2 FVSVCFIFVFSALVEYGTLHYFVSNRKPSKDKDKKKK-NPAPT------IDIRPRS----
rat γ3 FVTVCFLFVFAALMEYATLNYYSSCRKPTIRKKKTSLLHPDSTRWIPDRISLQAPSNYSL
chick γ4 FVSVCFIFVFAALMEYATLNYLVGNKKPLEHSSRKARLPPAGAQ---VMPSFTAIN----
ss γ WMAVCLLFVFAALLEFAVVNFLSRQQQRLIKVNMGWLIKQKAIVP----------
rat γ1 --------TLIPMNNISMP-----QGEDD-YGYQCLEGKDCATFFCCFEDCRTGSWREGR
rat γ2 --------ATIQMNNATHL-----QERDEEYGYECLDGKDCASFFCCFEDCRTGAWRHGR
rat γ3 LDMRPPPPVMITLNNSMYW-----QEFEDTCVYECLDGKDCQSFFCCYEECKSGSWRRGR
chick γ4 ----------ININNIMHWPPEIEEDEDDDPGSPCLEGKECERFFCCIEDCQTGMWREGR
ss γ ----------TQWSLMGCQYCSHKFPLTTNPFPQEEPPIKQSIAED
M4
rat γ1 IHIRIAKIDSYSRIFFPTAFALFNLVYWVGYLYL
rat γ2 IHIRIAKMDSYARIFFPTAFCLFNLVYWVSYLYL
rat γ3 IHIDVSELDSYSRVFFPTSFLLFNLVYWVGYLYL
chick γ4 VRIHISRLDSYSRVFFPTAFLLFNIVYWIAYLYL
ss γ YKKKALAIDTLSRIIFPTTFLIFNIVYWLSYK--

Figure 54: Alignment of the partial amino-acid (single-letter code) sequences of the $GABA_A$ receptor γ-subunit sequences from the rat (*Rattus norvegicus*), chicken (ch; *Gallus gallus domesticus*) and that deduced from *in silico* analysis of the sea squirt (ss; *Ciona intestinalis*) genome, generated using the computer programme ClustalW (http://www.ch.embnet.org/software/ClustalW.html). Dashes denote gaps that have been inserted to maximise the alignment. Red bars above the sequence indicate the transmembrane domains (M1 to M4). Yellow shading denotes differences between the sequences.

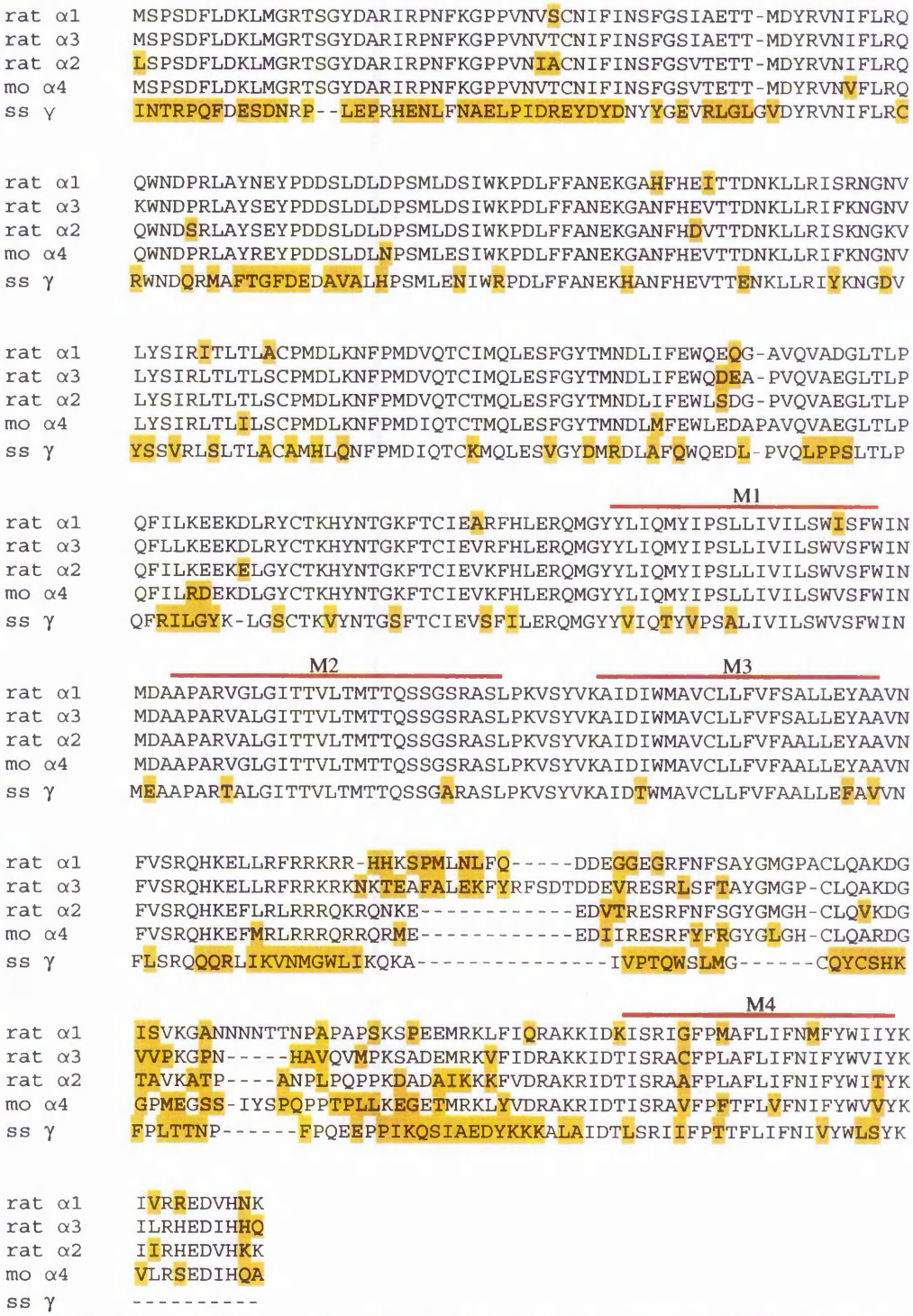


Figure 55: Alignment of the partial amino-acid (single-letter code) sequences of the glyR α-subunit sequences from the rat (*Rattus norvegicus*), mouse (mo; *Mus musculus*) and that deduced by *in silico* analysis of the sea squirt (ss; *Ciona intestinalis*) genome, generated using the computer programme ClustalW (http://www.ch.embnet.org/software/ClustalW.html). Dashes denote gaps that have been inserted to maximise the alignment. Red bars above the sequence indicate the transmembrane domains (M1 to M4). Yellow shading denotes differences between the sequences.

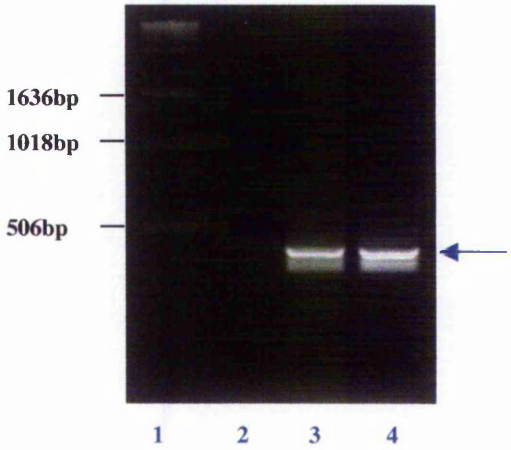


Figure 56: A 2% (w/v) agarose gel illustrating the amplification of glutamic acid decarboxylase (GAD) cDNA from sea squirt (*Ciona intestinalis*) larvae. Lane 1 contains a 1kb DNA ladder (marker), lane 2 is the negative control (no DNA) and lanes 3 and 4 contain the product amplified from cDNA. The arrow indicates the band excised out of the gel with a size of approximately 460bp.

```
Clone     QSAWRFRSSSVGLIKVEPNNEDYSMNNGDFENSSSTDLLPFGKSEEKTQLFLKEIFEILL
Genome    QSAWRFRSSSVGLIKVEPNNDDYSMNNGDFENSSSADLLPFGKSDEKTQLFLKEIFEILL

Clone     KYISKSFDRKSKILDFHHPHQLLEGIEGFSLNINGEAESLEQILVDCRDTLKYGVKTGHP
Genome    KYISKSFDRKCKILDFHHPHQLLEGIEGFSLNINGEAESLEQILVDCRDTLKYGVKTGHP

Clone     RFFNQLSSGLDVVSLAADWVTSTANT
Genome    RFFNQLSSGLDIVSLAADWVTSTANT
```

Figure 57: Alignment of the amino-acid (single-letter code) sequences of glutamic acid decarboxylase cloned from the sea squirt and that deduced from the sea squirt genome database. There is 96.6% sequence identity between the cloned sequence and that deduced from the genome database (scaffold 761 nucleotide positions 3925bp to 4587bp). Yellow shading denotes the differences between species.

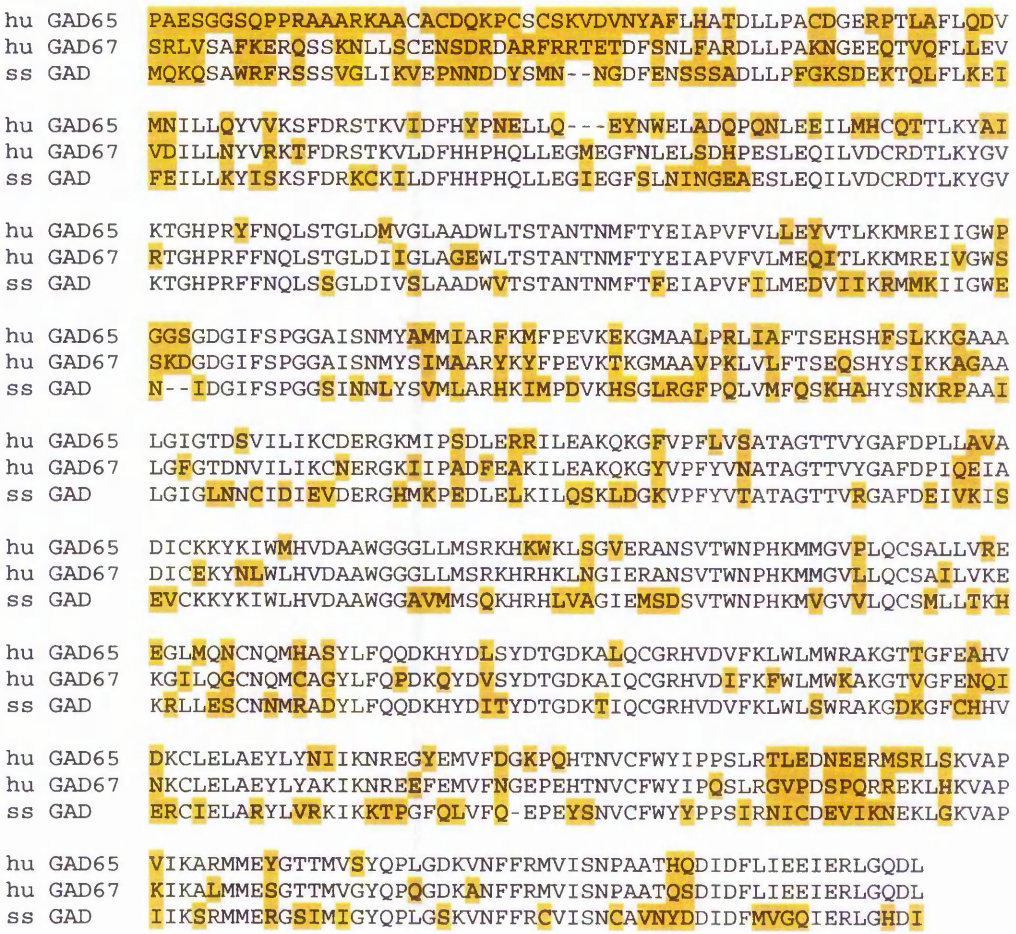


Figure 58: Alignment of the partial deduced amino-acid (single-letter code) sequences of the two isoforms of glutamic acid decarboxylase (GAD) found in humans (hu; *Homo sapiens* GAD_{65} and GAD_{67}) and the sequence found in the sea squirt (ss; *Ciona intestinalis*). The alignment was generated using the computer programme ClustalW (http://www.ch.embnet.org/software/ClustalW.html). Dashes denote gaps that have been inserted to maximise the alignment. Yellow shading denotes differences between the sequences.

Figure 59: Alignment of the deduced partial amino-acid sequences (single-letter code) of other GABA receptor-like genes found in the sea squirt (*Ciona intestinalis*). The alignment was generated using the computer programme ClustalW (http://www.ch.embnet.org/software/ClustalW.html). Dashes denote gaps that have been inserted to maximise the alignment. Yellow shading denotes differences between the sequences. Red lines above the sequences denote the transmembrane domains (M1 to M4). The sequences derive from scaffold numbers s113, s14, s781 and s45 (http://genome.jgi-psf.org/ciona4/ciona4.home.html).

Scaffold	Sequence identity (%)
45	33% to the human $GABA_A$ receptor π subunit
113	28% to the human $GABA_C$ receptor ρ1 subunit
781	34% to the human $GABA_C$ receptor ρ2 subunit
14	31% to the human $GABA_C$ receptor ρ3 subunit

Table 20: Maximum similarity of the predicted polypeptide sequence of other GABA receptor-like genes found in the sea squirt (*Ciona intestinalis*) genome database (http://genome.jgi-psf.org/ciona4/ciona4.home.html) to mammalian GABA receptor subunits. The sequences derive from scaffold numbers 14, 45, 113 and 781.

5.2.3 The distribution of the $GABA_A$ receptor β-subunit mRNA using whole mount *in situ* hybridisation on embryos and larvae of the sea squirt

To determine where the $GABA_A$ receptor β-like subunit gene was expressed in the sea squirt (*Ciona intestinalis*) whole-mount (non-radioactive) *in situ* hybridisation was conducted using a digoxigenin labelled riboprobe. Since PCR detected the $GABA_A$ receptor β-like subunit gene in the larvae (see Figure 45) *in situ* hybridisation was carried out on different stages of embryos and larvae (7, 10 and 30 hours after *in vitro* fertilisation).

$GABA_A$ receptor β-like subunit mRNA cannot be detected in the neural tube at the early tailbud stage (Figure 60A). By the mid tailbud stage there is a prominent patch of expression in the posterior brain this is in the equivalent position of the midbrain-hindbrain boundary of vertebrates (Wada *et al.*, 1998). This is posterior to the anterior tip of the notochord (see arrow in Figure 60B), which is interesting because the notochord is required for tail movement (Katz 1983; Di Gregorio and Levine 1998). The patch is bilateral, and the $GABA_A$ receptor β-like subunit mRNA is found only in 2 to 4 cells on each side. A second spot of expression is seen in the anterior brain in the mid-late tailbud stage (Figure 60C), which is the phase when the sea squirt begins to move. In the early larva (Figure 60D) there is prominent expression just posterior to the ocellus (light receptor) and the otolith (gravity sensor) in the sensory vesicle, and another spot is detected adjacent to the anterior notochord. The sensory vesicle is composed of approximately 17 photoreceptors (making up the ocellus), a single otolith

cell and 17-21 cells thought to be hydrostatic pressure receptors (Nicol and Meinertzhagen, 1991). Initially after hatching, sea squirt larvae swim upwards followed by a period when they swim or sink downwards, possibly because the larvae need to attach to rock to begin metamorphosis. However, larvae are not photoresponsive until 4 hours after hatching (Kajiwara and Yoshida, 1985; Nakagawa *et al.*, 1999). Tsuda *et al.* (2003a) showed that larvae start swimming in response to the cessation of light and stop swimming with its onset. Tsuda *et al.* (2003b) also confirmed that the ocellus was responsible for the photosensitivity of sea squirt larvae by ablating the otolith and ocellus; removal of the latter resulted in larvae continuing to swim even after illumination. Tsuda *et al.* (2003b) further confirmed that the otolith was solely responsible for the gravity response in sea squirt larvae, because ablation of the otolith caused a reduction in upward swimming and increased the frequency of random swimming movements. At late larval stages (Figure 60E and 60F) the site of expression behind the ocellus/otolith has largely disappeared. The remainder of the blue staining (seen in Figure 60) is non-specific staining in the tunic. Omission of the primary antibody revealed no specific staining.

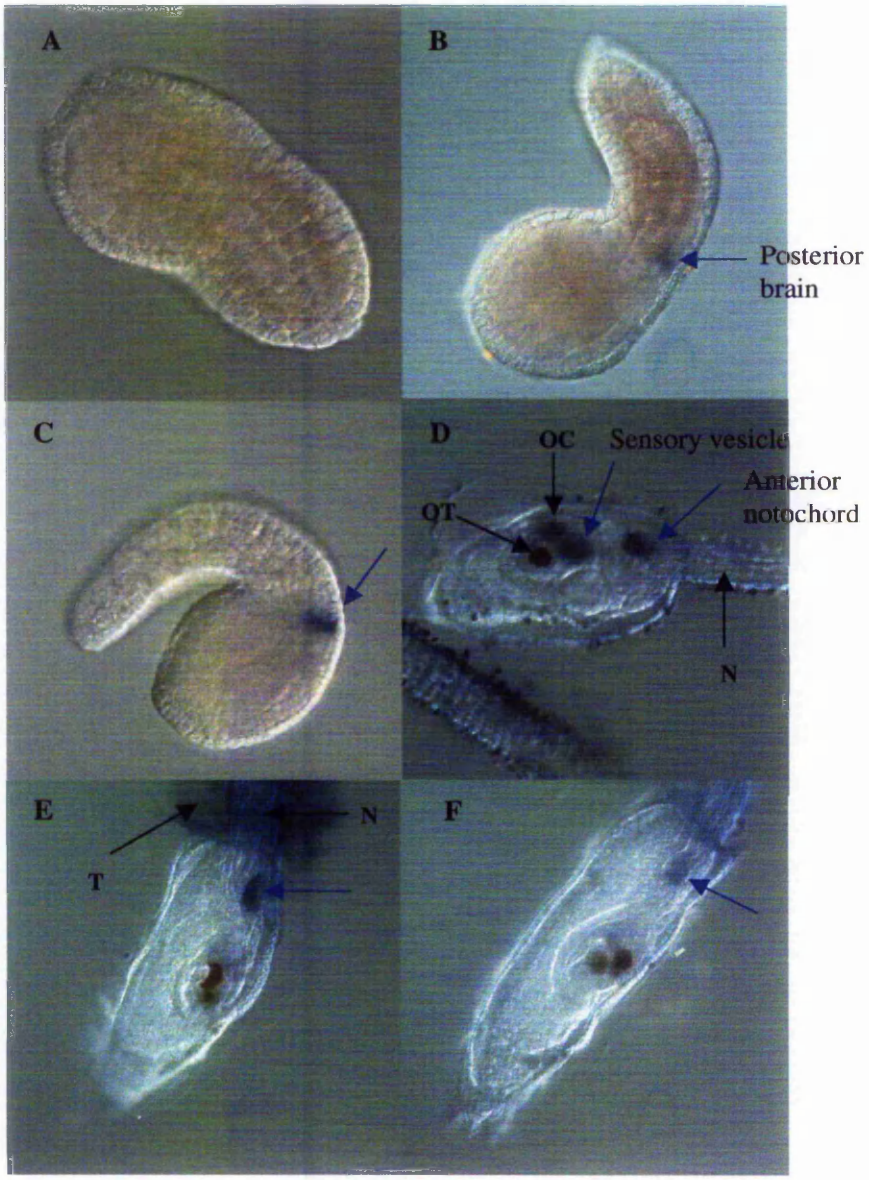


Figure 60: Whole-mount *in situ* hybridisation in sea squirt (*Ciona intestinalis*) using a $GABA_A$ receptor β-like subunit RNA probe. A, early tailbud; B, mid tailbud stage; and C, mid-late tailbud stage. Staining can be seen in the posterior brain (B and C), in a region that is bilateral and which contains 2 to 4 cells on each side (blue arrows). D to F larval stages, expression is seen in the sensory vesicle (D), then adjacent to the notochord (E), former area of expression was absent and the latter was less abundant (F). Larvae were stained for 4 hours and embryos for 24 hours. The images were taken at X 400 magnification. Although all stages were treated at the same time, the differences in background are due to the staining reactions being carried out in different wells of the tissue culture plate. Abbreviations: notochord (N), ocellus (OC), otolith (OT) and tunic (T).

5.2.4 Immunohistochemistry in the sea squirt using an anti-GABA antibody

To map the distribution of GABA-like immunoreactivity, a monoclonal anti-GABA antibody was tested on embryonal and larval stages of the sea squirt (*Ciona intestinalis*). Chicken brain sections were used as a positive control for the antibody, and the negative control used involved application of the secondary antibody only. Unfortunately staining was only seen in cells of the sensory vesicle, the otolith and ocellus (see Figure 60). It was confirmed that this was not due to expression of the $GABA_A$ receptor as negative control experiments (without the primary antibody) showed staining in the same cells (shown in Figure 61).

These experiments were repeated several times using different fixation conditions (see 2.6.1) and different dilutions of the primary antibody (refer to 2.6.3), however no specific labelling was ever observed. In addition since no staining was detected after 15 minutes incubation in DAB buffer (even in the chicken brain sections i.e. positive controls) the samples were incubated in DAB buffer in the dark for a further 2hrs.

The primary mouse anti-GABA antibody had previously been shown to be effective on monkey and rat brain (Miettinen *et al.*, 1997; Jongen-Relo *et al.*, 1999). The presence of GABA in the chicken brain has been demonstrated extensively (Granda and Crossland, 1989; Matute and Streit, 1986; Watson *et al.*, 1991). Therefore, immunocytochemistry was also carried out on one-day-old chicken brain horizontal sections (16μm) as a positive control. Unfortunately no specific staining was seen in these sections. Note that all of the brain section was treated with the antibody however only the cerebellum and optic tectum were photographed because both of these areas are easily identified and abundantly express $GABA_A$ receptor subunits. Negative control experiments were also carried out using chicken brain sections with the secondary goat anti-mouse (biotin SP conjugated) antibody only (see Figure 61C and F), however there was no difference in staining between the positive and negative controls.

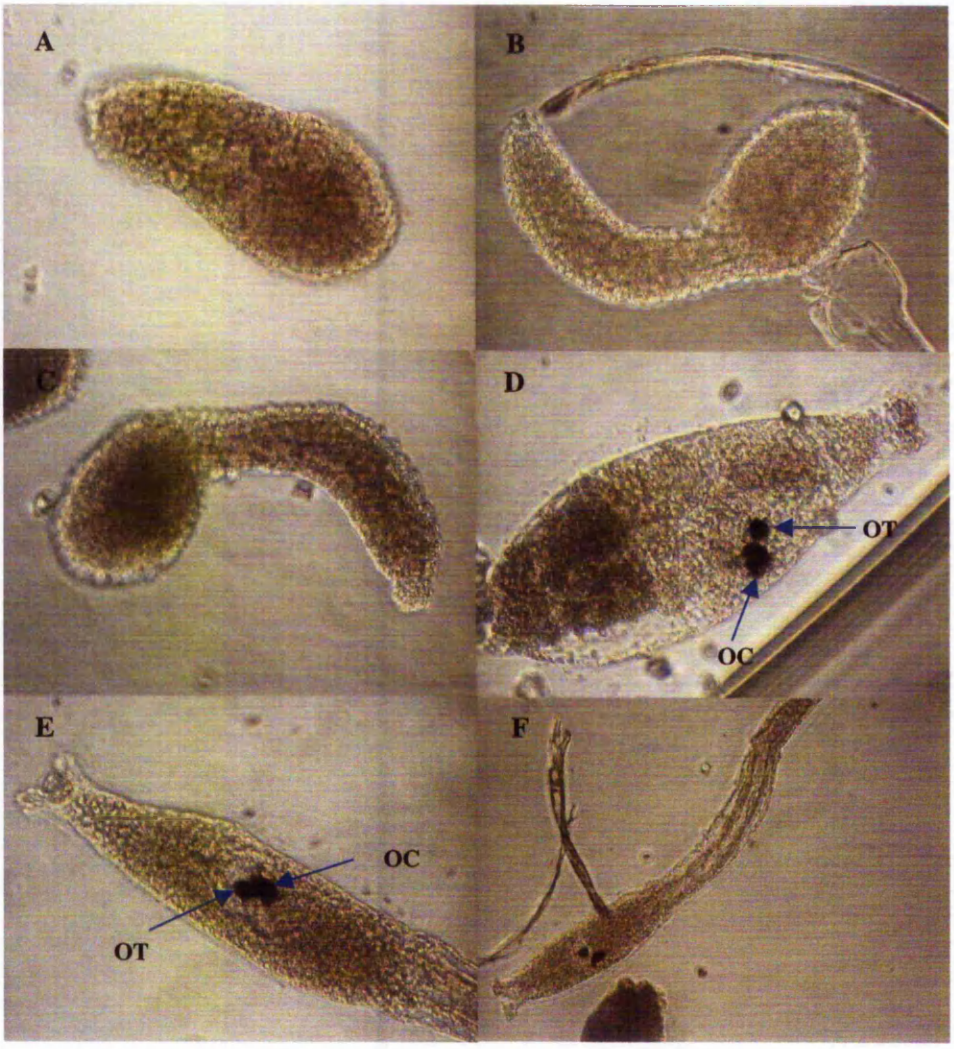


Figure 61: Immunohistochemistry in the sea squirt (*Ciona intestinalis*) using a monoclonal anti-GABA antibody. A, early tailbud; B, mid tailbud stage; C, mid-late tailbud stage; D, early larvae; E and F are later larval stages. The two black spots represent the ocellus (OC; light-sensitive) and otolith (OT; gravity-sensitive organs). A, B, C and E are at X 200 magnification, D is at X 40 and F is at X 100 magnification. Although all stages were treated at the same time, the differences in background are due to the staining reactions being carried out in different wells of the tissue culture plate.

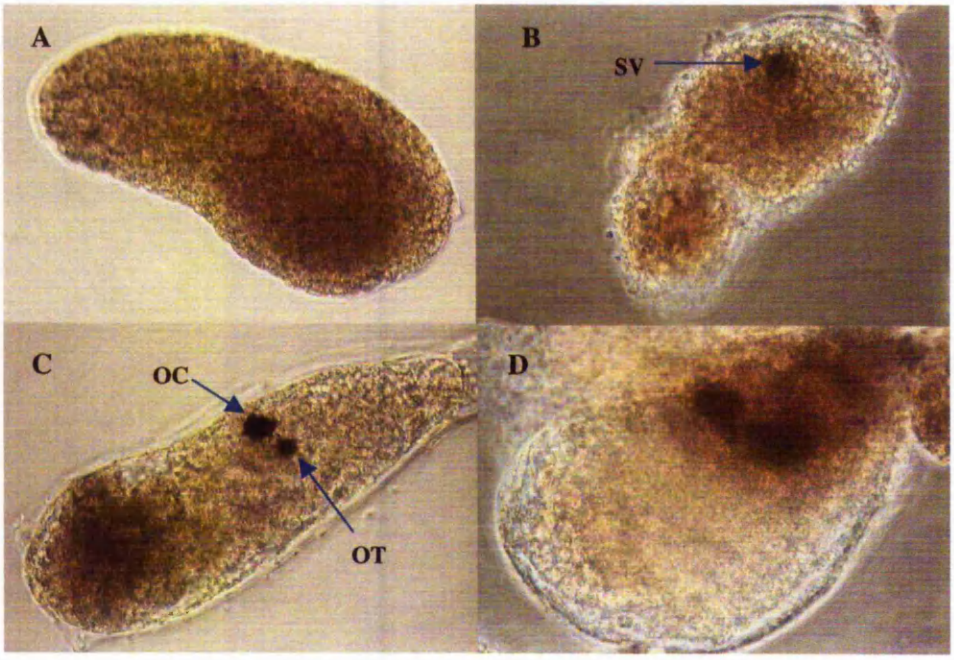


Figure 62: Immunohistochemistry in the sea squirt (*Ciona intestinalis*) using a secondary goat anti-mouse (biotin SP conjugated) antibody only. A, early tailbud; B, late tailbud; C and D are late larval stages. The two black spots form the sensory vesicle (SV) and represent the ocellus (OC; light-sensitive) and the otolith (OT; gravity-sensitive). A to C are at X 200 magnification, D is at X 40 magnification. Although all stages were treated at the same time, the differences in background are due to the staining reactions being carried out in different wells of the tissue culture plate.

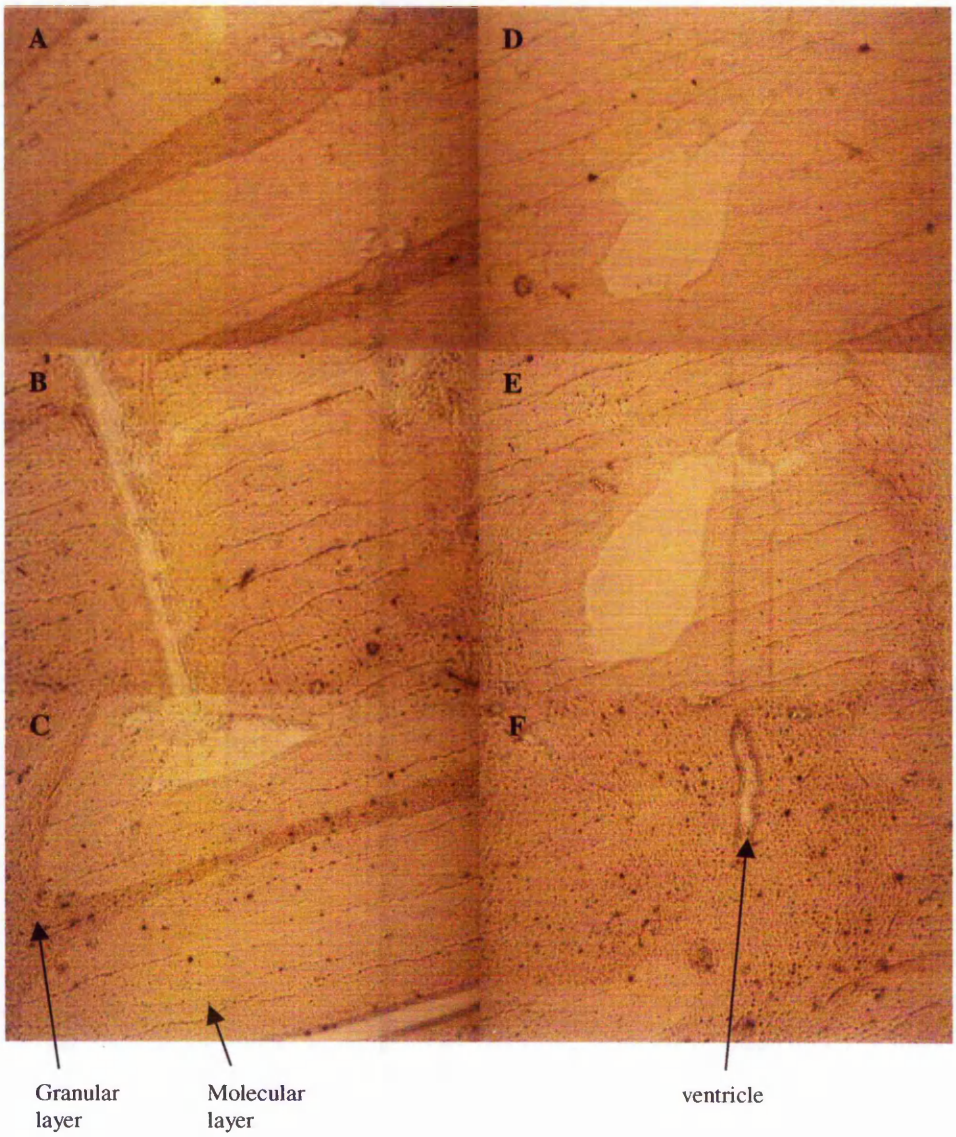


Figure 63: Immunohistochemistry on chicken (*Gallus gallus domesticus*) brain sections (16μm) using a monoclonal anti-GABA antibody. A to C illustrate the cerebellum and D to F the optic tectum. A 1:100 dilution of antibody was applied to A and D, 1:500 to B and E, and only the secondary antibody was applied to C and F i.e. negative controls. All images were observed at X 40 magnification.

5.3 Discussion

One of the aims of this study was to search for the potential cluster of $GABA_A$ receptor subunit genes that correspond to the ancestral cluster. Since the sea squirt is thought to have arisen before the two genome duplications took place (see 1.3; Figure 6), it was hypothesised that it would contain a single cluster of $GABA_A$ receptor (α-, β- and γ-like) subunit genes. A pair of $GABA_A$ receptor α- and β-subunit-like genes and a glycine receptor-like subunit gene was found by *in silico* analysis (Figure 38b). The gene structures were then analysed by searching for the minimum consensus sequences (Smith *et al.*, 1989) for splice junctions. The gene organisation was found to be highly conserved with those of vertebrate $GABA_A$ receptor subunit genes "during evolution" (see Sommer *et al.*, 1990; Kirkness *et al.*, 1991; Lasham *et al.*, 1991; Figure 39). Harvey *et al.* (1991) also found a high conservation of the intron/exon boundaries between the invertebrate *Lymnaea stagnalis* β-subunit gene and vertebrate genes, and concluded that the organisation of $GABA_A$ receptor subunit genes was determined before the divergence of molluscs and chordates. Although, there is thought to be 560 million years between the evolution of the sea squirt and vertebrates (Bosma *et al.*, 1999) the results here indicated a conserved gene structure. Unexpectedly, some of the exons in the $GABA_A$ and glycine receptor-like subunit genes are split by an intronic sequence (for example exons 4, 7, 8 and 9; see Figure 40). This could possibly be due to an intron insertion in urochordates i.e. *Ciona intestinalis* or the intron may have been deleted during evolution in vertebrates. Interestingly, the $GABA_A$ receptor β-like subunit from the sea squirt is most similar to the chicken β4 subunit (refer to Table 19). This supports the idea that the β4 and γ4 subunits are orthologues of the ancestral genes and diverged to the θ and ε subunits seen in man (see 1.3; Figure 5).

Interestingly, invertebrates do not appear to possess a homologue of the $GABA_A$ receptor α or γ subunit (Bamber *et al.*, 1999), and as the β subunit can form homo-oligomeric receptors (Harvey *et al.*, 1991), this may be similar in the sea squirt. This could also provide an explanation for why only the $GABA_A$ receptor β-like subunit mRNA was detected by non-radioactive *in situ* hybridisation (see Figure 60). Four α and one β subunit of the glycine receptor have been cloned to date (Lynch, 2004). GlyR β subunits however, cannot produce homomeric channels but do form heteromers with α subunits (Nevin *et al.*, 2003). Nevertheless, there are glyR β subunits in the brain

where α subunits are not expressed e.g. Purkinje cell layer of the cerebellum and the septum (Malosio *et al.*, 1991) therefore, it is possible that the glyR β subunits may assemble with $GABA_A$ receptor subunits. The sea squirt genome database was searched for a glyR β-subunit sequence however, this was not found. Therefore, providing further evidence that the $GABA_A$ and glycine receptor may indeed share a common β subunit (Harvey *et al.*, 1994b) in the sea squirt. In addition, the gene structures are highly conserved between the glycine and $GABA_A$ receptor subunit genes (Matzenbach *et al.*, 1994) as they may have evolved from a common evolutionary ancestor. It is possible that after gene duplication, one of the resultant genes, (initially redundant), could have either evolved to gain a new function or be lost from the genome as a consequence of acquiring multiple mutations (see Meyer and Schartl, 1999). Interestingly, three partial sequences for $GABA_C$ receptor-like ρ-subunit genes were also found in the sea squirt, the same number that is found in man. The $GABA_C$ receptor ρ subunits are 30-38% identical in sequence to mammalian $GABA_A$ receptor subunit polypeptides (Bormann, 2000). Although $GABA_A$ and $GABA_C$ receptors are similar in structure both possess four transmembrane domains, an extracellular amino-terminus, and an intracellular carboxy terminus, they are pharmacologically distinct. The $GABA_C$ receptor is insensitive to bicuculline (a $GABA_A$ receptor antagonist) and benzodiazepines do not act at this receptor (Mehta and Ticku, 1999; Bormann, 2000). Since $GABA_C$ receptor subunits are primarily found in the mammalian retina (Enz *et al.*, 1995; Wegelius *et al.*, 1998) it would be interesting to see whether the ρ-subunit-like genes that have been identified are expressed in the sea squirt ocellus (light sensory organ). Whilst, the π-subunit gene (*GABRP*) is located on chromosome 5q34-q35 in man (Bailey *et al.*, 1999b) it does not cluster with the other $GABA_A$ receptor subunit genes on this chromosome (it lies approximately 3.5 Mb from the cluster containing *GABRA1*, *GABRA6*, *GABRB2* and *GABRG2*). It has been proposed that *GABRP* arose via duplication of an ancestral $GABA_A$ receptor gene(s), which was then translocated to another chromosomal region (Darlison *et al.*, 2005). It is possible that a similar situation occurred with the $GABA_A$, $GABA_C$ and glyR subunit genes, where a common ancestor existed, and after gene duplication the products of the latter two receptors acquired functions distinct from those of $GABA_A$ receptors (Darlison *et al.*, 2005).

Although the integrity of the sea squirt tissue cDNAs was checked by amplification of the housekeeping gene β-actin as a positive control (refer to Figure 44), expression of the $GABA_A$ receptor subunit genes was only found in larvae (see Figure 60). Since only

the larvae swim (Satoh, 1994) and the adult sea squirt is immotile this may indicate a role for GABA in locomotion. In addition, expression of the $GABA_A$ receptor β-subunit gene was only found in 2 to 4 cells of the mid-tailbud embryos demonstrating the low abundance of the subunit mRNA. The $GABA_A$ receptor α-like subunit and glyR-like subunit mRNAs may also not have been detected because they were shorter probes (approximately 400bp and 220bp respectively, compared to approximately 800bp for the $GABA_A$ receptor β-like subunit).

All of the cloned sequences were compared at the amino-acid level against those predicted from the genome database. The α-like subunit was 87.6% identical; the β-like was 97.5% identical; the glyR-like subunit was 85.9% identical. GAD was only 96.6% identical to the published GAD gene sequence (see Figures 49, 46, 53 and 57, respectively) however 100% identity was expected since they are from the same species. Variation could have arisen by PCR errors particularly since the $GABA_A$ receptor β-like subunit and GAD fragments were amplified by nested PCRs. However, the lower sequence identities are likely to be due to a high rate of mutation in the sea squirt because the clones generated in this study were from a UK population, whereas the genome sequencing project was carried out using the Pacific population of sea squirts. Riemer *et al.* (2000) also found a large variation in the cytoplasmic intermediate filament protein sequences and suggested that this may also have been due to different populations of sea squirt. Also, the sea squirt is known to have a very high allelic polymorphism, where approximately 1.2% of the nucleotides differ between chromosome pairs within an individual (Dehal *et al.*, 2002). This is 15-fold higher than that in humans, and is probably due to a large population size (Boffelli *et al.*, 2004). In addition, Boffelli *et al.* (2004) found that two pairs of sea squirts were only 85% identical at the genomic DNA level. The highest rates of mutation found in this investigation occurred in non-coding regions therefore, any primer designed outside the conserved region might not anneal to the DNA template e.g. ssglyF4, thus explaining the difficulties that arose when amplifying the α- and glyR-like subunits (Figures 48 and 52 respectively). The possibility of sub-species of sea squirt occurring can also not be ruled out as the UK and Pacific populations do interbreed (S. Shimeld, personal communication).

At the early tailbud stage (Figure 60A) there are no neurons yet which could indicate why the $GABA_A$ receptor β-like subunit mRNA could not be detected. It is possible that

the $GABA_A$ receptor subunits may be present in cells other than neurons e.g. they are seen in the muscle cells of the invertebrate *Caenorhabditis elegans* (Bamber *et al.*, 1999). Initial detection of the $GABA_A$ receptor β-like subunit mRNA was at the mid-tailbud stage of the sea squirt in the posterior brain (Figure 60B). The staining persisted into larvae, where these cells came to lie in the posterior part of the sensory vesicle, behind the ocellus (light receptor) and the otolith (gravity/balance sensor). It is possible that the expressing cells are involved in the relaying of information to the otolith and ocellus, as this area makes up the sensory vesicle (see Figure 64 for a schematical representation of the body plan; Hudson and Lemaire, 2001). The cluster of cells (Figure 60D) appears to be the 'ganglion cells' adjacent to the anterior notochord which are known to be bilateral but asymmetric. As this is in the motor relay centre, these cells could be involved in co-ordinating the axial musculature for locomotion.

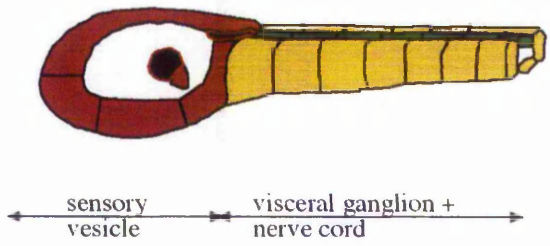


Figure 64: Schematic representation of the sea squirt embryo body plan. The red shading indicates the cell layer surrounding the sensory vesicle, the yellow shading represents the endodermal layer and the green is the notochord.

Interestingly, not all invertebrates express GABA receptors solely in neuronal cells. The *unc-49* gene (which encodes a GABA receptor polypeptide) is found in the body wall muscles and tail of *Caenorhabditis elegans* (Bamber *et al.*, 1999). The evidence found in the marine mollusk *Clione limacine*, a predator that feeds on smaller molluscs e.g. *Limacina helicina*, supports the role of GABA in movement. The injection of GABA into the hemocoel of *C. limacina* caused its mouth to open and tentacles to protract, mimicking the effect of contact with the prey (Arshavsky *et al.*, 1993). Since the motor neurones are also a target of GABA, this suggests once more that GABA may be important for regulating behaviours such as feeding and locomotion.

McKernan and Whiting (1996) suggested that the clustering of $GABA_A$ receptor genes was important to permit their co-ordinate transcription, the products of which would

assemble to form specific receptor subtypes (see Darlison *et al.*, 2005). Originally it was predicted that the sea squirt α-like and β-like subunit genes would be co-regulated (as they are on the same scaffold) however, this may not be the case in the most basal chordates (Adoutte *et al.*, 2000). Although, the $GABA_A$ receptor β-like subunit mRNA was detected by whole-mount *in situ* hybridisation, the α-like, glyR-like subunit and GAD mRNAs could not be detected. It should be noted that a very limited distribution was detected for the $GABA_A$ receptor β-subunit mRNA, it is possible that the α-, glyR-like subunit and GAD genes are expressed but less abundantly hence, the technique may not have been sensitive enough. Interestingly, Bamber *et al.* (1999) found that the UNC-49 subunit is most similar to the product of the *rdl* gene in the fruit fly (*Drosophila melanogaster*), which resembles $GABA_A$ receptor subunits (Ffrench-Constant *et al.*, 1991). In addition, after searching the genome of *C. elegans*, Bamber *et al.* (1999) discovered a β-like subunit (named ZC482.1) but no subunits homologous to the vertebrate $GABA_A$ receptor α and γ subunits.

It is possible that $GABA_A$, glycine receptor subunit or GAD genes are not expressed on the surface of sea squirt embryos or larvae. Whole-mount *in situ* hybridisation was employed to detect the $GABA_A$ receptor subunit and GAD mRNAs however, only surface distribution is seen with this technique. Expression of these cDNAs was detected by PCR; therefore, it may be necessary to section the sea squirt tissue to visualise any binding of the RNA probes. Another interesting finding was that the $GABA_A$ receptor β-like subunit polypeptide possessed the domains (YGTT and TGSY; Figure 47), which are required for the binding of GABA (Amin and Weiss, 1993).

GAD is the enzyme required for the synthesis of GABA (Erlander and Tobin, 1991; Martin and Rimvall, 1993), which is the endogenous ligand that binds to $GABA_A$ receptors. It is therefore surprising that there was no expression of GAD mRNA particularly because the $GABA_A$ receptor β-like subunit mRNA was clearly detected. Since during the PCR reaction a band for GAD was only obtained after 80 cycles (see Figure 56), it is possible that GAD is not highly abundant in the sea squirt. The two human GAD isoforms, GAD_{65} and GAD_{67} share a high sequence identity (81%), suggesting that they arose from a common ancestral gene (Bu *et al.*, 1992). Previous work by Bosma *et al.* (1999) also found only one isoform of GAD in the sea squirt. In addition, only one form of GAD exists in poikilothermic vertebrates e.g. trout and frog (Legay *et al.*, 1986). However, Bosma *et al.* (1999) stated that GAD from the sea squirt

was approximately 80% identical to both human forms of GAD, whereas in this investigation the GAD clones were clearly more similar to human GAD_{67} than GAD_{65} (see Figure 58).

The spatial distribution of the $GABA_A$ receptor β-like subunit mRNA is very similar to that of the *Pax2* mRNA, which is also expressed at the mid-tailbud stage in the posterior brain (Wada *et al.*, 1998; Imai *et al.*, 2002; Mazet *et al.*, 2003). *Pax* genes encode transcription factors known to be involved in the early development of the nervous system. Torres *et al.* (1996) found by 'knock-out' studies in mice that the *Pax2* gene is needed for development of the eye specifically, closure of the neural tube and optic fissure. This is interesting because the area of localisation of the $GABA_A$ receptor β-like subunit mRNA in sea squirt is in the eyespot (see Figure 60). Previous work carried out by Maricich and Herrup (1999) found by *in situ* hybridisation that *Pax2* identifies a subset of GABAergic interneurons in the murine cerebellar cortex.

GABA has also previously been detected in the adult neuronal complex (specifically the ganglion) of the sea squirt by immunostaining (Bollner *et al.*, 1993; Osborne *et al.*, 1979). However, $GABA_A$ receptor subunit cDNAs or genes only appeared to be expressed in the larvae (detected by PCR). $GABA_A$ receptor subunit gene expression may not have been seen by PCR in the adult because the adult is sessile and GABA may not be needed for locomotion. Bollner *et al.* (1993) found GABA in the regenerated neuronal complex of the sea squirt by immunocytochemistry. It is known that following ablation, the neuronal complex of the sea squirt is able to completely regenerate (Bollner *et al.*, 1995) hence GABA may be important for regeneration.

To map the distribution of GABA-like immunoreactivity, immunohistochemistry was performed using a monoclonal anti-GABA antibody. This has previously been shown in the zebra finch (Grisham and Arnold, 1994; Sakaguchi, 1996). Sakaguchi (1996) used a 1:100 dilution of the primary antibody whereas Grisham and Arnold (1994) used a 1:500 dilution therefore several concentrations were applied to chicken sections. Bollner *et al.* (1993) used a 1:2000 dilution and this worked on adult sea squirt, suggesting that the concentration of the antibody should not have been the problem. The optic tectum and cerebellum were the brain regions used for controls as they have 'well-established GABAergic neurones' (Grisham and Arnold, 1994). The brown staining seen was due to the staining reagent diaminobenzidine, and not the anti-GABA antibody, because a

similar pattern was seen in the negative controls where no primary antibody was applied. $GABA_A$ receptor subunit and GAD gene expression was observed by PCR in the larvae. The BD17 antibody, which detects the $GABA_A$ receptor β2 and β3 subunits could not be used, as the sea squirt β-like polypeptide does not possess the epitope required, QSVNEP (Ewert *et al.*, 1992).

In conclusion, the hypothesis that the $GABA_A$ receptor α-like and β-like subunit would exist in the sea squirt was proven. Furthermore, although the sea squirt arose before the tetraploidisation events occurred to give rise to the $GABA_A$ receptor subunits seen in vertebrates today, the sequence and gene organisations are relatively highly conserved. The selective pressure for preservation of these sequences indicates that they may be important for the functioning of the $GABA_A$ receptor.

General Discussion

The study of $GABA_A$ receptors is essential as they play a role in various neurological conditions e.g. epilepsy, anxiety, Alzheimer's disease and schizophrenia (Frølund *et al.*, 2002). This investigation has looked at the evolution and expression of $GABA_A$ receptor β-subunit genes (specifically the β4-subunit gene) in numerous species. Previously, the $GABA_A$ receptor β4-subunit gene was only known to be present in the chicken (Lasham *et al.*, 1991). However, the findings from this study have revealed this gene in a variety of species including zebra finch, frog and lizard (refer to Figures 11, 15 and 16 respectively). The β4-subunit gene was also detected in the puffer fish by *in silico* analysis of the genome database. Detailed analysis of the $GABA_A$ receptor gene structures has revealed that the intron/exon boundaries are highly conserved, even between organisms that are thought to have arisen before and after the two-genome duplications took place i.e. *Ciona intestinalis* (commonly known as the sea squirt) and mammals respectively.

Phylogenetic analysis has been conducted to produce dendrograms, which illustrate the evolutionary relationships of the $GABA_A$ receptor β-subunit genes from a variety of species based on nucleotide and amino-acid sequences (see Figures 65 and 66 respectively). Generally the β-subunit genes (β1 to β4) from the different species, group together because they share highest sequence similarities. In addition, species that arise from the same vertebrate class i.e. aves or mammals are closer together as they are most similar in sequence (indicated by the length of horizontal lines, for example rat and human, and zebra finch and chicken $GABA_A$ receptor β2-subunit sequences display equal lengths). Interestingly, phylogenetic analysis illustrated that the θ-subunit genes clustered with the β4 subunits (see Figures 65 and 66) with the exception of the β1 subunit from the zebra finch. Oncemore demonstrating that the β4 subunit is orthologous to the mammalian θ subunit as shown previously in this investigation (see Figure 32). Since, the β4 subunit was originally hypothesised to replace the β1 subunit in mammals (Darlison and Albrecht, 1995) this may explain the phylogenetic position of the zebra finch β1-subunit genes (refer to Figure 65). However, caution should be exercised since the same result was not seen on the dendrogram generated based on amino-acid sequence. In addition, full-length polypeptide sequences were not used for the phylogenetic analysis because only partial sequences of the $GABA_A$ receptor β-

subunit sequences were cloned (from the frog, lizard, sea squirt and zebra finch) this may have influenced the results. Interestingly, the $GABA_A$ receptor β1 subunit deduced from the puffer fish genome database was more similar in sequence to the β3 subunit from the puffer fish than other β1 subunits (refer to Figure 66). This was not expected because Table 10 shows that all of the β subunits deduced from the puffer fish genome database were approximately 70% identical to each other. It could be speculated that a local duplication occurred in the puffer fish to give rise to a fourth β subunit, after which the β1 and β3 subunits diverged.

It was originally proposed that the β4-subunit gene found in the chicken, replaced the mammalian β1-subunit gene, and that similarly the chicken γ4 subunit substituted for the mammalian γ3 subunit (Darlison and Albrecht, 1995); thus, each species was suggested to possess only three β- and three γ-subunit genes. Unexpectedly, a $GABA_A$ receptor β1-subunit cDNA was found in the zebra finch. Thus, the chicken EST (Boardman *et al.*, 2002) and genome database (http://www.chickest.udel.edu/) was searched for a potential $GABA_A$ receptor "β1-like" subunit. However, only a partial sequence was detected. Since this only possesses sequences corresponding to exons 6 and 8 (for numbering of exons, see Lasham *et al.*, 1991), this may indicate that, in the chicken, the β1-subunit gene has evolved into a pseudogene, as has been observed for other vertebrate genes that became non-functional after a duplication event (Mighell *et al.*, 2000). This suggests that an initial "β-like" subunit gene has been lost from the chicken genome during its recent evolution. Since, preliminary analysis of the chicken genome did not reveal either a "γ3-like" or an "ε-like" subunit gene, a "γ-like" subunit gene may also have been lost from this species or a further duplication may have occurred in birds (Darlison *et al.*, 2005). In addition, since no ε- or θ-subunit cDNA, gene or expressed sequence tag (EST) has been found in the chicken it is proposed that the mammalian ε and θ subunits are most likely to be orthologous to the chicken γ4 and β4 subunits, respectively (Darlison *et al.*, 2005). This investigation has confirmed this, as the cluster of $GABA_A$ receptor α3-, β4- and γ4-subunit genes in the chicken is orthologous to the cluster of genes (α3, ε and θ) found in the human genome on the X chromosome (see Figure 5). Hence, the mammalian ε-subunit gene may have evolved from an ancestral "γ-like" subunit gene and the θ-subunit gene from a "β-like" subunit gene and the ε subunit replaced the γ subunit in $GABA_A$ receptors (Darlison *et al.*, 2005).

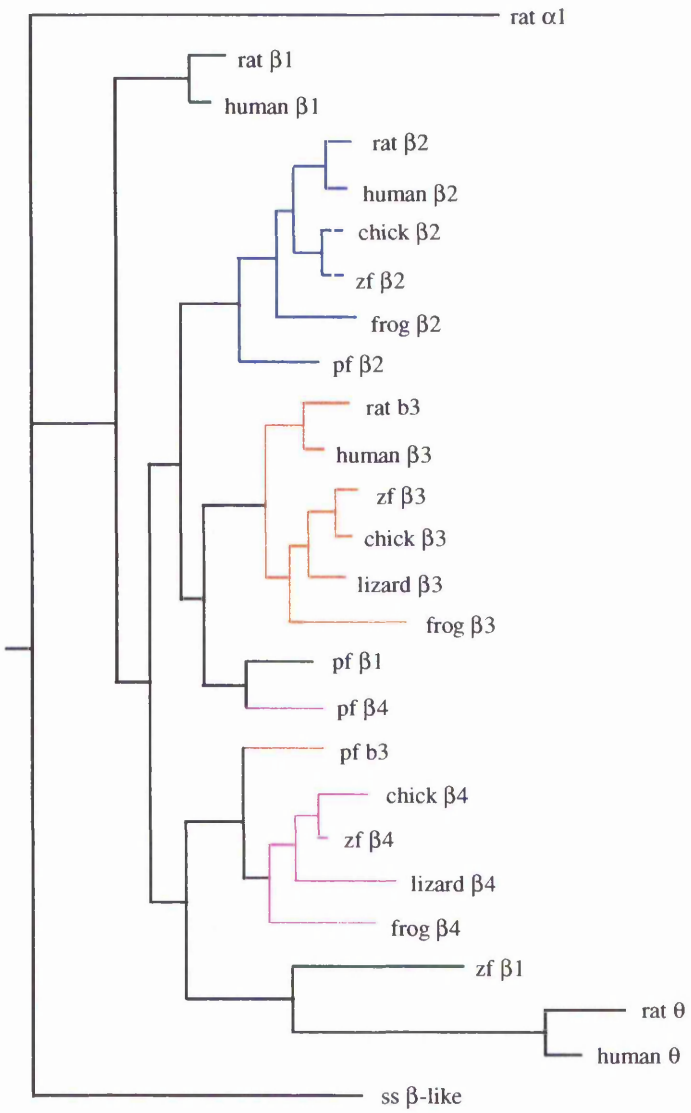


Figure 65: Phylogenetic relationships of $GABA_A$ receptor β subunits. For this analysis, nucleotide alignments of the various subunit sequences were generated using ClustalX 1.8 (Thompson *et al.*, 1997). All of the sequences were compared over the same region of ~900 base pairs; these sequences vary in length because they encompass the intracellular loop region between M3 and M4. Manual editing was employed to further optimise the alignments. Phylogenetic trees were calculated using the maximum likelihood method (DNAML) of the Phylip 3.6 package (Felsenstein, J. (2002) Phylip (Phylogenetic Interference Package) Version 3.6a, distributed by the author, Department of Genetics, University of Washington, USA). Differences in the lengths of the horizontal lines represent differences in the nucleotide sequences of the subunits. Note that the rat α1 subunit served as the outgroup for tree construction.

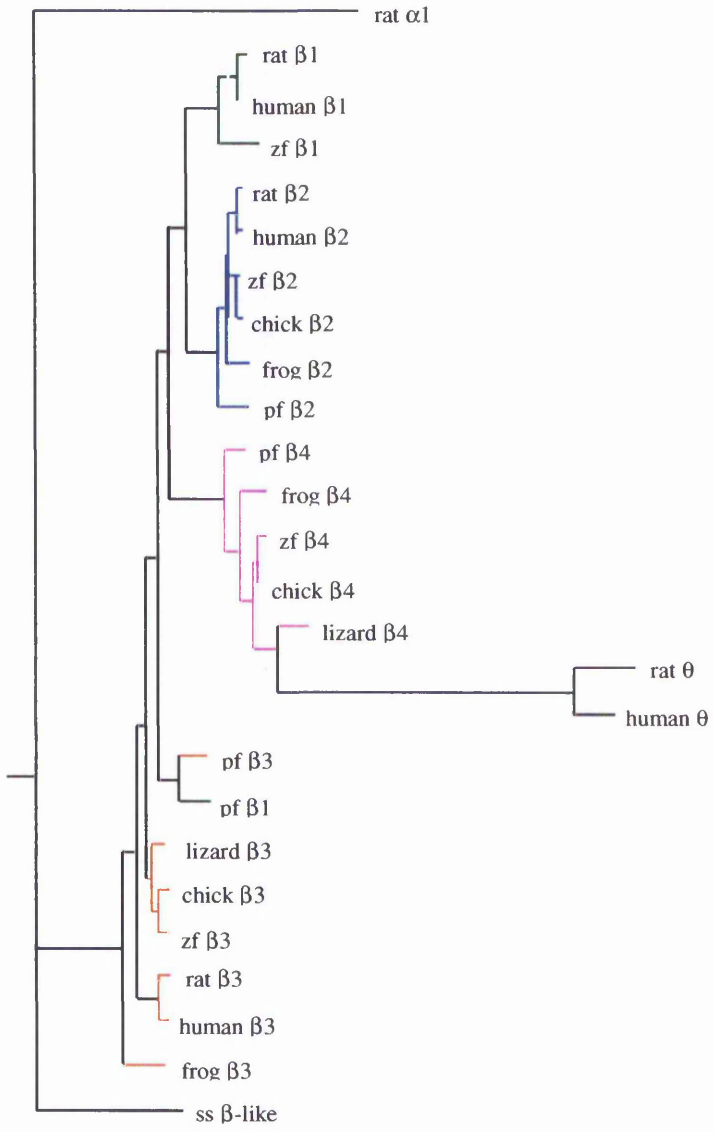


Figure 66: Phylogenetic relationships of $GABA_A$ receptor β-subunit polypeptides. For this analysis, amino-acid alignments of the various subunit sequences were generated using ClustalX 1.8. All of the sequences were compared over the same region of ~300 amino acids, which corresponds to the minimum length of sequence that was deduced from the cDNAs that were cloned. Phylogenetic trees were calculated using the maximum likelihood method (PROML) of the Phylip 3.6 package (Felsenstein, J. (2002) Phylip (Phylogenetic Interference Package) Version 3.6a, distributed by the author, Department of Genetics, University of Washington, USA). Estimations of the rates of evolution were determined using the Tree-Puzzle 5.0 programme (Schmidt *et al.,* 2002), and were based on the Jones-Taylor-Thornton model for amino-acid substitutions (Jones *et al.,* 1992). Differences in the lengths of the horizontal lines represent differences in the amino-acid sequences of the subunits. Note that the rat α1 subunit served as the outgroup for tree construction.

The mammalian ε and θ subunits have been found to be the most highly diverged $GABA_A$ receptor polypeptides (see Table 16). Russek (1999) has suggested based on phylogenetic analysis, that there is an increased mutation rate on the X chromosome where the ε-subunit gene is located (Xq28 in man). The lack of conservation of the θ-subunit gene may be due to the fact that it is clustered together with the ε-subunit gene. However, the α3-subunit sequence (flanked by the ε- and θ-subunit genes) is relatively highly conserved between species (for example, 98.1% identity between human and rat and 87.3% sequence identity between the chicken and human subunit polypeptides; refer to Tables 16 and 15, respectively). Nevertheless, the lower level of sequence similarity, seen for the ε and θ subunits between the human and rat subunit polypeptides (71.9% and 78.3% respectively; see Table 16), indicates that there is less selective pressure on the two corresponding genes, which has resulted in the accumulation of mutations (Darlison *et al.*, 2005).

Since very little was known about the $GABA_A$ receptor β4 subunit at the onset of this work, the distribution of the β4-subunit mRNA was investigated in the zebra finch (comparing both adults and juveniles), and the one-day-old chicken brain. The $GABA_A$ receptor β4-subunit gene was abundantly expressed in both birds, with the highest level of expression being seen in the mesopallium region of the chicken and the medial striatum, of the zebra finch brain (see Figures 37 and 28, respectively). The latter region contains Area X, which is important for song acquisition (Brainard and Doupe, 2002). Since the mesopallium is needed for visual and spatial learning, levels of the β4-subunit mRNA could be compared. Harvey *et al.* (1998) and Thode *et al.* (2005) used the chicken to study up or downregulation of mRNAs ($GABA_A$ receptor γ4 subunit and the immediate early gene *ZENK*, respectively) after imprinting (a form of recognition memory). Similarly, *in situ* hybridisation could be carried out on the brains of trained vs. untrained one-day-old chickens, to assess whether there is any change in the level of the β4-subunit mRNA. Since the $GABA_A$ receptor γ4-subunit mRNA has been localised in the chicken retina (Harvey *et al.*, 1994b), *in situ* hybridisation experiments could be conducted to investigate whether the α3 and β4 subunit mRNAs are also present in the retina, i.e. playing a role in the detection of visual signals. The expression profile of the $GABA_A$ receptor β4-subunit mRNA was compared to that of the γ4- and α3-subunit mRNAs, as the mammalian α3, ε (γ4-like) and θ (β4-like) subunit genes cluster on chromosome X, and this cluster is orthologous to that on chromosome 4 in the chicken

(see Figure 32). Since the α1, β2 and γ2 subunits (forming the majority of $GABA_A$ receptor subtypes in the brain; Whiting 2003) are clustered together on chromomsome 5, it was hypothesised that the corresponding genes of the α3, β4 and γ4 subunits would co-express to form a native receptor subtype in birds. However, this was not found as there was only partial overlap of the $GABA_A$ receptor α3-, β4- and γ4-subunit mRNAs suggesting that the α3β4γ4 subtype is only present in some brain regions (e.g. mesopallium and hyperpallium intercalatum; shown in Table 18).

It was proposed by Holland *et al.* (1994) that the ancestral cluster of $GABA_A$ receptor subunit genes would exist in the sea squirt as it arose before the two genome duplications occurred (see Figure 6). A pair of $GABA_A$ receptor α- and β-subunit genes was found on scaffold 300 in the sea squirt genome database. A glycine receptor-like subunit gene was also characterised from the sea squirt genome database but on a different scaffold (827) as seen in Figure 38. Since a $GABA_A$ receptor γ-subunit gene was not found, it is possible that the genes found in the sea squirt do not represent the ancestral cluster, as the genome of the sea squirt would also have continued evolving. In addition, the glyR-like subunit may have diverged before the sea squirt arose in evolution. Nevertheless, the transcriptional orientation and intron/exon boundaries of the two $GABA_A$ receptor genes were relatively conserved with the orthologous cluster found in man (see Figure 39). Therefore, the structure of the $GABA_A$ receptor may have been determined before divergence occurred. Interestingly, fragments of a $GABA_\Lambda$ receptor π-subunit-like gene and $GABA_C$ receptor ρ-subunit-like genes were also detected by *in silico* analysis of the sea squirt genome. Since full sequences could not be found it supports the finding of GABA receptor pseudogenes in other species (for example in the zebra finch). However, it should be taken into consideration that the sea squirt genome database is not complete and the data presented in this thesis are accurate only at present.

Whole-mount *in situ* hybridisation was conducted to determine the distribution of the $GABA_A$ and glyR-like subunit transcripts. The $GABA_A$ receptor β-like subunit mRNA was localised to approximately two-four cells, located in the posterior brain of the sea squirt embryo, more specifically in the sensory vesicle and notochord, which are required for locomotion in the juvenile sea squirt. This was an interesting finding, as neither the $GABA_A$ receptor nor the glyR-like subunit genes had been detected in any of the adult sea squirt tissues tested by PCR, yet all of the subunit cDNAs were amplified

in larval tissue from the sea squirt. As described earlier, the adult is immotile whereas the juvenile is not therefore indicating that GABA may play a role in locomotion. Indeed, GABA has previously been reported to be required for movement in *Cliona limacine*, a marine mollusc. To examine in greater detail where $GABA_A$ receptor subunit genes are expressed, sea squirt embryos could be injected with the promotor region (i.e. the intergenic region between the $GABA_A$ receptor α- and β-like subunit genes, where the promoter is located) fused with the *LacZ* gene to visualise where the genes are expressed (see Corbo *et al.*, 1997a). This is important because the promoter-coding region is responsible for adjusting the level and spatial expression of genes (Darlison *et al.*, 2005). Greer *et al.* (2000) successfully demonstrated that the murine *Hoxa3* and *Hoxd3* (homeobox genes) could functionally substitute for each other. Hence, it was the sequence of the promoter that was necessary for gene regulation. To ascertain whether the $GABA_A$ receptor subunits form a functional receptor, electrophysiology could be conducted on *Xenopus laevis* oocytes (see Gisselmann *et al.*, 2004). *In vitro* transcribed $GABA_A$ receptor α and β subunits would be injected into the cytoplasm of *X. laevis* oocytes; the response to agonists and antagonists could then be measured by voltage-clamp electrophysiology. Since the $GABA_A$ receptor β-subunit mRNA was detected in the sensory vesicle, behavioural experiments on larvae are presently being conducted by S. Shimeld in Oxford (due to time constraints, this experiment was not carried out during the period of this study). The aim is to observe swimming patterns using light/gravity senses (detected by the ocellus and otolith, respectively), both before and after the addition of muscimol (a $GABA_A$ receptor agonist) and bicuculline (an antagonist) to study any differences in locomotion.

Since, a glyR-like subunit sequence was found in the sea squirt genome, the GRD polypeptide from *Drosophila melanogaster* (GABA/glycine receptor of *Drosophila*, which is highly similar to the α subunit; Harvey *et al.*, 1994c) was aligned with the $GABA_A$ and glycine receptor subunit-like amino-acid sequences from the sea squirt genome database. However, only approximately 40% sequence identity was seen between GRD and $GABA_A$ receptor α- and β-like and glyR-like subunit genes (data not shown), indicating that the genes found in the sea squirt are not highly similar to GRD. Interestingly, Gisselmann *et al.* (2004) found that the GRD and LCCH3 (a homologue of the $GABA_A$ receptor β subunit; Henderson *et al.*, 1993) isolated from *D. melanogaster* formed a functional cation channel when co-expressed in *X. laevis* oocytes. Therefore, it is possible that the $GABA_A$ receptor α- and β-like subunits form a channel and the $GABA_A$ receptor β subunit and glyR-like subunit form a different

receptor in the sea squirt. Thus, it may be too simple to conclude that in the sea squirt two α, two β and one γ like subunit are required for $GABA_A$ receptor functionality (Backus *et al.,* 1993; Chang *et al.,* 1996; Baumann *et al.,* 2002). Further evidence of subunits from different receptor types forming functional complexes, comes from the research conducted by Milligan *et al.* (2004), who demonstrated that the ρ1 subunit (a component of the $GABA_C$ receptor), associates with the γ2 subunit (a $GABA_A$ receptor subunit).

In summary, this thesis reports the isolation of $GABA_A$ receptor β-subunit cDNAs, in particular the β4 subunit cDNA, from a variety of species (including, zebra finch, African clawed frog and common wall lizard) therefore is not avian specific. There is an orthologous gene cluster to that on the X chromosome in mammals ($GABA_A$ receptor α3-, ε- and θ-subunit genes) on chromosome 4 in the chicken ($GABA_A$ receptor α3-, γ4- and β4-subunit genes), and the organisation of the $GABA_A$ receptor gene structure has been found to be remarkably conserved over evolution. This has been demonstrated from a primitive chordate to complex mammals, thereby reinforcing the importance of the $GABA_A$ receptor. This is necessary to provide a more comprehensive knowledge of the evolution of the principal inhibitory neurotransmitter namely, GABA.

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