Expression of the $GABA_A$ receptor γ4-subunit gene in discrete nuclei within the zebra finch song system

Christian Thode[1*], Hans Rudolf Güttinger[2] and Mark G. Darlison[1]

[1]Neuroscience and Signal Transduction Laboratory, School of Science and Technology, College of Science, Nottingham Trent University, Clifton Lane, Nottingham NG11 8NS, United Kingdom; and [2]Abteilung für Allgemeine Zoologie, Fachbereich Biologie, Technische Universität Kaiserslautern, Erwin-Schrödinger-Straβe 13, 67663 Kaiserslautern, Germany.

*Correspondence and proofs to: Dr Christian Thode
Neuroscience and Signal Transduction Laboratory
School of Science and Technology, College of Science
Nottingham Trent University, Clifton Lane
Nottingham NG11 8NS, England

Telephone: +44 115 848 3916

FAX: +44 115 848 3384

E-mail: christian.thode@ntu.ac.uk

Running title: $GABA_A$ receptors within the song system

Abbreviations

A, arcopallium

Cb, cerebellum

cDNA, complementary DNA

DLM, medial nucleus of the dorsolateral thalamus

GABA, γ-aminobutyric acid

HA, apical part of the hyperpallium

Hb, habenula

HD, densocellular part of the hyperpallium

Hp, hippocampus

HVC, used as the proper name

L, Field L

LMAN, lateral magnocellular nucleus of the anterior nidopallium

LSt, lateral striatum

M, mesopallium

M1, first membrane-spanning domain

M3, third membrane-spanning domain

M4, fourth membrane-spanning domain

MMAN, medial magnocellular nucleus of the anterior nidopallium

MSt, medial striatum

N, nidopallium

NC, caudal nidopallium

NIf, nucleus interface of the nidopallium

nXIIts, tracheosyringeal subdivision of the hypoglossal nucleus

PBS, phosphate-buffered saline

PCR, polymerase chain reaction

PHD, post-hatch day

RA, robust nucleus of the arcopallium

SNAg, *S*ong system *N*uclear *Ag* (antigen)

TeO, optic tectum

Uva, uvaeform nucleus

X, Area X

zRalDH, zebra finch retinaldehyde-specific aldehyde dehydrogenase

Abstract

The acquisition, production and maintenance of song by oscine birds is a form of audition-dependent learning that, in many ways, resembles the process by which humans learn to speak. In songbirds, the generation of structured song is determined by the activity of two interconnected neuronal pathways (the anterior forebrain pathway and the vocal motor pathway), each of which contains a number of discrete nuclei that together form the song system. It is becoming increasingly evident that inhibitory GABAergic mechanisms are indispensable in counterbalancing the excitatory actions of glutamate and, thus, likely shape the neuronal firing patterns of neurons within this network. Furthermore, there is compelling evidence for the involvement of $GABA_A$ receptors, although the molecular composition of these has, to date, remained elusive. Here we describe the isolation of a complementary DNA for the zebra finch $GABA_A$ receptor γ4 subunit, and map the expression pattern of the corresponding gene within the zebra finch (*Taeniopygia guttata*) brain. Our findings show, remarkably, that the γ4-subunit transcript is highly enriched in the major nuclei of the song system, including the lateral magnocellular nucleus of the anterior nidopallium (LMAN), the medial magnocellular nucleus of the anterior nidopallium (MMAN), Area X, the robust nucleus of the arcopallium (RA) and the HVC, as well as Field L, which innervates the area surrounding HVC.

In summary, we have demonstrated the presence of the mRNA for the γ4 subunit of the $GABA_A$ receptor, the major inhibitory receptor in brain, in most of the nuclei of the two neural circuits that mediate song production in the zebra finch. This not only marks the beginning of the characterisation of the $GABA_A$ receptor subtype(s) that mediate(s) the actions of GABA in the song system but it also provides a robust molecular marker with which to distinguish song system-specific brain structures.

Keywords: Behavioural learning; development; inhibitory neurotransmitter receptor; ligand-gated ion channel; robust nucleus of the arcopallium (RA); song system-specific marker

The generation of structured song by adult songbirds is, like human speech, a motor behaviour that is learnt during an early sensitive period (reviewed in Doupe and Kuhl, 1999; Mooney, 1999). The neuronal network that mediates song acquisition and production comprises a well-characterised set of anatomically-discrete brain nuclei, which form two functionally-distinct but interconnected circuits, called the song system (reviewed in Brainard and Doupe, 2002; Zeigler and Marler, 2004). The vocal motor pathway descends from the HVC (used as the proper name; see Reiner et al., 2004) to the robust nucleus of the arcopallium (RA), and from there to the tracheosyringeal subdivision of the hypoglossal nucleus (nXIIts), which innervates the syrinx (Nottebohm et al., 1976). RA also projects to the dorsal medial nucleus and then to respiratory centres in the medulla, which innervate the respiratory muscles (see Zeigler and Marler, 2004). The anterior forebrain pathway comprises the lateral magnocellular nucleus of the anterior nidopallium (LMAN), Area X, and the medial nucleus of the dorsolateral thalamus (DLM; Bottjer et al., 1989). This circuit is part of a feedback loop, which may be involved in evaluating auditory information; it receives input from HVC (part of the vocal motor pathway) and projects to the RA via the LMAN. Two other important structures, that provide sensory input to the song system, are Field L, an integral component of the avian central auditory system, and the nucleus interface of the nidopallium (NIf); the former innervates the region around the HVC, while the latter provides a major auditory input to HVC itself (Kelley and Nottebohm, 1979; Cardin et al., 2005). Finally, it has been shown that the motor pathway is required for song production throughout life (Nottebohm et al., 1976), while the anterior forebrain pathway is necessary for song acquisition in juvenile birds and for song maintenance in adults (Bottjer et al., 1984; Brainard and Doupe, 2000).

It has been known for many years that the inhibitory neurotransmitter, γ-aminobutyric acid (GABA), is widely distributed within the song system (Sakaguchi et al., 1987; Grisham and Arnold, 1994). And, recently, the distribution of GABAergic cells within the zebra finch telencephalon has been mapped, using a glutamic acid decarboxylase riboprobe (Pinaud et al., 2004) and an anti-GABA antibody (Pinaud and Mello, 2007). Consistent with this, the presence of functional GABA type A ($GABA_A$) receptors, within the song system, has been

demonstrated electrophysiologically by a number of groups (Livingston and Mooney, 1997; Bottjer et al., 1998; Luo and Perkel, 1999; Vicario and Raksin, 2000; Pinaud et al., 2004; Farries et al., 2005; Mooney and Prather, 2005), although a detailed pharmacological characterisation is lacking. For example, picrotoxin and bicuculline block receptors on neurons within the LMAN (Livingston and Mooney, 1997; Bottjer et al., 1998), the DLM (Luo and Perkel, 1999), the HVC (Mooney and Prather, 2005), the RA (Vicario and Raksin, 2000) and Area X (Farries et al., 2005). From this body of work, it appears that $GABA_A$ receptors, which are a family of post-synaptic proteins, play an important role in the learning and production of song in the zebra finch.

In mammals, 16 different $GABA_A$ receptor subunits have been identified (named, $\alpha1$–$\alpha6$, $\beta1$–$\beta3$, $\gamma1$–$\gamma3$, δ, ε, π and θ; for review, see Darlison et al., 2005; Sieghart, 2006), which assemble to form pentameric ion-channel subtypes. Birds possess two other subunits, named $\beta4$ and $\gamma4$, that appear to be orthologous to the mammalian θ and ε subunits, respectively (Darlison et al., 2005). Interestingly, the $\gamma4$ polypeptide has been implicated in imprinting, an early learning paradigm (see Horn, 1998), in the one-day-old chicken (*Gallus gallus domesticus*), by the demonstration of a large decrease in the corresponding mRNA, in appropriate brain regions, after training on a visual stimulus (Harvey et al., 1998). Here we provide evidence that the $\gamma4$-subunit gene is expressed in most of the major nuclei of the song system of the zebra finch, *Taeniopygia guttata*. Our data suggest that this subunit is a component of one or more $GABA_A$ receptor subtypes that regulate song acquisition and production in this songbird. Furthermore, the expression pattern of this $GABA_A$ receptor gene reveals it to be an excellent molecular marker for the song system.

Experimental Procedures

Animals

Zebra finches (*Taeniopygia guttata*) were obtained from breeding colonies at the Abteilung für Allgemeine Zoologie, Technische Universität Kaiserslautern, Germany, and the Max Planck Institute for Ornithology, Seewiesen, Germany. Birds of different ages (n = 22; males = 18, females = 4) were sacrificed, and their brains were removed and frozen over liquid nitrogen.

RNA isolation and complementary DNA (cDNA) synthesis

Total RNA was isolated using RNAzol™ B (WAK-Chemie Medical, Bad Soden, Germany), and treated with RNase-free DNase I (Promega, Mannheim, Germany) to remove any contaminating genomic DNA. It was then transcribed into first-strand cDNA using random nonamers (Stratagene, Amsterdam Zuidoost, The Netherlands) and Moloney murine leukemia virus reverse transcriptase (Promega).

Cloning of a partial cDNA

An ~870-bp fragment, encoding part of the $GABA_A$ receptor γ4 subunit, was amplified from zebra finch brain first-strand cDNA using degenerate oligonucleotide primers, that were designed using the sequences of different vertebrate $GABA_A$ receptor γ subunits, and *Taq* DNA polymerase (Promega). The primer sequences were: 5'-GTGTCTAGAAT (A/T/C)TGGAT(A/T/C)CC(A/C/G/T)GA(T/C)AC-3', which is based on the amino-acid sequence: V(G/S)(K/L/R)IWIPDT (single-letter code) that is present in the amino-terminal extracellular domain, and 5'-CTGGAATTCTCG(A/T)(A/T)(A/G)CA(A/G)CA(A/G)AA (A/G)AA-3', which is based on the amino-acid sequence: FFCC(F/Y/I)E(D/E)C(R/K/Q) that is located within the large intracellular loop between the third (M3) and fourth (M4) membrane-spanning domains. Note that restriction endonuclease recognition sites for *Xba*I and *Eco*RI (underlined) were incorporated into the forward and reverse primer sequences, respectively. The reaction conditions were 35 cycles of 94°C for 1 minute (denaturation), 50°C for 1.5 minutes (annealing), followed by 72°C for 1.5 minutes (extension). Products of

the expected size were cloned directly into pCR®2.1-TOPO® (Invitrogen, Leek, The Netherlands), and sequenced to confirm their identity.

In situ *hybridisation*

In situ hybridisation was performed using 45-base transcript-specific oligonucleotide probes. To ensure specificity of the hybridisation signal, three different oligonucleotides were used that are complementary to different parts of the nucleotide sequence that encodes the large intracellular loop (located between M3 and M4) of the zebra finch $GABA_A$ receptor γ4 subunit. This region is highly variable in length and sequence between different receptor polypeptides (for details, see Darlison et al., 2005). The sequences used were: probe 1, 5'-CGGGGAGCCGGGCTCGTCATCCTCATCCTCCTCTATCTCCGGGGG-3', which is complementary to the sequence that encodes the amino-acid sequence PPEIEEDEDDEPGSP (single-letter code); probe 2, 5'-CTGTGGCTGTGCTCCAGTGGTTTCTTGTTTCCCACCA GGTAGTTG-3', which is complementary to the sequence that encodes NYLVGNKKPLEH SHR; and probe 3, 5'-TGCATGATGTGGTTGATGTTGATGGTGGTGAAGGTTGGCAT CACC-3', which is complementary to the sequence that encodes VMPTFTTININHIMH. These three oligonucleotides yielded identical hybridisation patterns. However, all of the data shown here derive from the use of one oligonucleotide (probe 2).

Oligonucleotide probes were labelled, with [α^{35}S]dATP (1250 Ci/mmol; NEN/PerkinElmer, Boston, USA), to a specific activity of between 1.3 and 2.6 x 10^9 cpm/μg. 10 μm coronal sections of zebra finch brains were prepared using a cryostat (Jung CM 3000, Leica, Bensheim, Germany) and thaw-mounted onto slides coated with 3-aminopropyl-triethoxysilane (Sigma, Deisenhofen, Germany). Sections were then fixed in 2% (w/v) paraformaldehyde in phosphate-buffered saline (PBS; 130 mM sodium chloride, 7 mM disodium hydrogen orthophosphate, 3 mM sodium dihydrogen orthophosphate) for 10 minutes at 4°C, washed twice in PBS for 5 minutes each, and dehydrated in an ascending ethanol series. The *in situ* hybridisation and wash conditions were as described previously (Wisden et al., 1991; Harvey et al., 1998). For each bird, sections throughout the entire brain

were hybridised to ensure that all of the song system nuclei (both large and small) were included in our analysis.

To directly compare the level of the γ4-subunit transcript in brains from birds at different developmental stages, and from different genders, fixed sections from small groups of brains were hybridised and washed together, and then exposed to the same sheet of Kodak BioMax™ MR X-ray film (Integra Biosciences, Fernwald, Germany) at room temperature for two to eight weeks. Relative signal intensities in brain regions of interest were arbitrarily scored, as previously (see, for example, Harvey and Darlison, 1997; Bock et al., 2005; Thode et al., 2005), as either high (++++), moderate (+++), low (++), very low (+) or no signal above that of surrounding areas (-). Note that hybridisation signals were entirely reproducible for birds of a given age and sex. Negative control hybridisations were also performed. These contained, in addition, a 200-fold excess of the same unlabelled oligonucleotide, which competes with the radiolabelled probe and should abolish the hybridisation signal. Such control hybridisations did not yield any significant autoradiographic signal (data not shown). Rather than digitally quantify the *in situ* hybridisation signals for the various song system nuclei, we have recently carried out a much more sophisticated analysis of $GABA_A$ receptor gene expression. For this, we (in collaboration with the group of Prof. Dr. Manfred Gahr, Seewiesen, Germany) have used a combination of laser capture microdissection of song system nuclei followed by quantitative reverse transcription-PCR (manuscript in preparation); the data obtained for the γ4-subunit gene are consistent with those reported here (Table 1).

Brain regions were systematically identified using several approaches. We used both the stereotaxic atlas of the canary (Stokes et al., 1974), and the zebra finch brain atlas, produced by B. E. Nixdorf-Bergweiler and H.-J. Bischof, which can be found on the National Center for Biotechnology Information web-site (see http://www.ncbi.nlm.nih.gov/books/bookres.fcgi/ atlas/atlas.pdf). In addition, for confirmation of the location of structures such as Field L, MMAN, NIf and Uva, autoradiographs from relevant section were compared with published work (for example, Bottjer et al., 1989; Fortune and Margoliash, 1992). Finally, we have analysed our *in situ* hybridisation data in the light of the recent publication by Poirier et al. (2008), who

constructed a three-dimensional magnetic resonance imaging stereotaxic atlas of the zebra finch brain. Note that brain regions have been named using the nomenclature for the avian telencephalon developed by the Avian Brain Nomenclature Forum (see Reiner et al., 2004).

Results

Sequence of the zebra finch $GABA_A$ receptor γ4 subunit

Amplification of zebra finch brain first-strand cDNA, using primers that were designed to recognise vertebrate $GABA_A$ receptor γ-subunit sequences, led to the isolation of a fragment that encodes a polypeptide of 271 amino acids that exhibits 97% identity to the chicken $GABA_A$ receptor γ4 subunit (Figure 1). This partial cDNA specifies part of the amino-terminal extracellular domain, the first three membrane-spanning domains, and part of the large intracellular loop between M3 and M4, which is highly variable in sequence and length between different subunits. Remarkably, there are only 8 amino-acid differences between the zebra finch and chicken polypeptides (4 of which are conservative substitutions), and all of these occur between M3 and M4. From this, we conclude that we have isolated a cDNA that encodes a large part of the zebra finch $GABA_A$ receptor γ4 subunit.

Figure 1 near here

Mapping of $GABA_A$ receptor γ4-subunit gene expression in the major song system nuclei

Using three transcript-specific oligonucleotide probes, designed to recognise the zebra finch $GABA_A$ receptor γ4-subunit cDNA sequence, the distribution of the corresponding mRNA was initially examined, in the brains of post-hatch day (PHD) 25 male zebra finches (i.e. juvenile birds), by *in situ* hybridisation. After a two-week exposure to X-ray film, the strongest signals were observed in three of the major nuclei of the song system, namely, the LMAN, Area X in the medial striatum, and the HVC. Strong signals were also detected in several forebrain areas, such as Field L (Table 1).

Table 1 near here

In view of the intriguing preliminary data obtained, and the fact that hybridisation signals appeared stronger in the anterior forebrain than in the posterior part, the pattern of expression of the $GABA_A$ receptor γ4-subunit gene was investigated in male zebra finches at

different, well-defined, developmental stages. For this, we chose time-points before birds are able to produce song (PHD22 and PHD25), at the onset of singing (PHD35 to 38), when birds are capable of song (PHD60/61), and after the song has crystallised (adult birds; PHD>100; Mooney, 1999). For most brain regions, the distribution of the γ4-subunit transcript was qualitatively similar at all ages (Table 1), with high levels of expression again being seen in LMAN (Figure 2), Area X (Figure 2), the HVC (Figure 3) and Field L (Figure 4). Furthermore, where *in situ* hybridisation was carried out simultaneously, with brain sections from birds of different ages, the results appeared quantitatively similar, although the signals in the LMAN and the HVC appeared a little weaker at PHD22 and PHD25 compared to later stages (Table 1). The one striking exception was the RA, which is part of the vocal motor pathway. Expression of the $GABA_A$ receptor γ4-subunit gene was observed, in this nucleus, only in PHD35 and older birds and the level of the corresponding mRNA increased during development (compare Figures 3C and D; see also Table 1); cells within the RA of birds younger than PHD35 did not contain this transcript at a level above that of the surrounding areas.

Figures 2 to 4 near here

Expression of the $GABA_A$ receptor γ4-subunit gene in other brain regions

Detailed anatomical analysis of the data from male zebra finches showed the presence of the $GABA_A$ receptor γ4-subunit mRNA, in a number of other brain areas, at all developmental time-points. One of these was the medial magnocellular nucleus of the anterior nidopallium (MMAN; Figure 2A and Table 1), which innervates the HVC (Nottebohm et al., 1982; Bottjer et al., 1989), and which is also part of the song system. Note that MMAN lies adjacent to, but is not connected with, the LMAN. In addition to the HVC, the MMAN projects to the paraHVC region (Foster et al., 1997), a medial portion of the HVC, which also expresses the γ4-subunit gene (see, for example, the right and left hemispheres, respectively, in Figures 3B and 5E). Other regions that were labelled included the densocellular part of the hyperpallium (HD; Figure 2), the mesopallium (M; Figure 2), the

medial and lateral striatum (MSt and LSt; Figures 2 and 3), and the habenula (Hb; Figures 3 and 4). Expression of the γ4-subunit gene was also found in the ventral part of the entopallium (data not shown), the optic tectum (TeO; Figures 3 and 4), the caudal nidopallium (NC; Figure 3), and the medial nucleus of the dorsolateral thalamus (DLM; data not shown). It should be noted that the latter nucleus is part of the anterior forebrain pathway. Analysis of autoradiographs, from sections covering the entire diencephalon and telencephalon, did not reveal signals above those of the surrounding areas in either the uvaeform nucleus (Uva; Williams and Vicario, 1993) or the nucleus interface of the nidopallium (NIf; Table 1); both of these structures send efferents to the HVC (Nottebohm et al., 1982; Bottjer et al., 1989; Cardin et al., 2005).

Expression of the $GABA_A$ receptor γ4-subunit gene in the female zebra finch brain

We have also used *in situ* hybridisation to investigate the expression of the $GABA_A$ receptor γ4-subunit gene in the brains of female zebra finches. These birds generate vocalisations but can not produce the stereotypic song that is characteristic of males; this behavioural difference can be correlated with gender-specific differences in the size of song system nuclei (Nottebohm and Arnold, 1976; Nixdorf-Bergweiler, 1996; Zeigler and Marler, 2004). The γ4-subunit mRNA distribution in adult females is very similar to that of adult males (Figure 5) with the notable exception of the song system nuclei. Expression is observed in females in LMAN (Figure 5B), albeit at a lower level than in males (compare with Figure 5A), but there is no autoradiographic signal above that of surrounding areas in the MMAN (Figure 5B), the HVC (Figure 5F) and the RA (Figure 5H). Also, there is no enhanced labelling in the region corresponding to Area X, which is observed in male zebra finches (compare Figures 5C and D). This is consistent with the known absence, in females, of a structure comparable to Area X in males (Nottebohm and Arnold, 1976). Note that no qualitative differences were observed in the expression pattern of the γ4-subunit gene in juvenile (PHD35) vs. adult (PHD>100) female brains (data not shown).

Figures 5 near here

Discussion

We have described here the isolation of a cDNA clone that encodes a large portion of the zebra finch $GABA_A$ receptor γ4 subunit, and its use in mapping the expression pattern of the corresponding gene in the male zebra finch brain at four different ontogenetic stages, namely, before birds are able to produce song (PHD22 and PHD25), at the onset of singing (PHD35 to 38), when birds are capable of song (PHD60/61) and after the song has crystallised (adult; PHD>100; Mooney, 1999). Our data are important because they: i.) begin the molecular characterisation of the $GABA_A$ receptor(s) that participate(s) in audition-dependent learning; ii.) yield insight into the development of the GABAergic system in a central song nucleus, the RA; and iii.) provide a molecular marker for most of the nuclei that comprise the two neuronal circuits that are required for vocalisation in songbirds. We have also described the expression of the γ4-subunit gene in female zebra finches, and the data obtained are qualitatively similar to those found for males, with the expected exception of the song system nuclei (see Nottebohm and Arnold, 1976; Nixdorf-Bergweiler, 1996, and Figure 5).

$GABA_A$ receptors containing the γ4 subunit possibly play a role in song learning

A significant body of literature has strongly implicated $GABA_A$ receptors in the regulation of excitation within the zebra finch song system (Livingston and Mooney, 1997; Bottjer et al., 1998; Luo and Perkel, 1999; Cardin and Schmidt, 2004; Pinaud et al., 2004; Farries et al., 2005; Mooney and Prather, 2005). These receptors are the major inhibitory neurotransmitter receptors in brain, and they exist in a variety of forms called subtypes, that are assembled from seven different types of subunit (α, β, γ, δ, ε, π and θ; Darlison et al., 2005; Sieghart, 2006). Each of them possesses an integral chloride-selective channel that is activated upon the binding of GABA. Although α and β subunits can form functional agonist-activated channels (Schofield et al., 1987), most *in vivo* $GABA_A$ receptors possess two α subunits, two β subunits and a γ subunit (see Sieghart, 2006). The absence of a γ subunit, in $GABA_A$ receptors *in vivo*, has been shown to result in a number of changes, including a significant reduction in the whole-cell GABA-induced current and insensitivity to

the benzodiazepine class of compounds (Günther et al., 1995).

Our observation that the γ4-subunit gene is expressed in the major nuclei of the song system, in male zebra finches, strongly suggests that one or more $GABA_A$ receptors containing this polypeptide regulate song acquisition and production. An alternative, but not mutually-exclusive, interpretation of our findings is that the high levels of expression of this gene are a consequence of the unique properties of the song system, for example, its sensitivity to steroid hormones and its sexual dimorphic nature. In this context, it is well-established that several $GABA_A$ receptor genes are regulated by steroids (Orchinik et al. 1995). Indeed, putative steroid hormone response elements have been identified in the promoter regions of the majority of $GABA_A$ receptor subunit genes (see Steiger and Russek, 2004); however, it is not yet known if this is the case for the γ4-subunit gene. The sexual dimorphic nature of the song system is also mirrored by our *in situ* hybridisation data (Figure 5). Nevertheless, it has long been known that pharmacological modulation of $GABA_A$ receptors has a profound effect on learning and memory processes (Chapouthier and Venault, 2002).

The involvement of $GABA_A$ receptors in the physiological processes underlying song learning and production is supported by data, from lightly-sedated birds, showing that injection of the $GABA_A$ receptor agonist, muscimol, into NIf eliminated spontaneous activity and auditory responses in the projection area HVC; conversely, injection of the antagonist, bicuculline, into the same structure, increased auditory responsiveness in HVC (Cardin and Schmidt, 2004). However, although several groups have recorded $GABA_A$ receptor responses from neurons within the LMAN (Livingston and Mooney, 1997; Bottjer et al., 1998), the DLM (Luo and Perkel, 1999), the HVC (Mooney and Prather, 2005), the RA (Vicario and Raksin, 2000) and Area X (Farries et al., 2005), the drugs used in these studies (picrotoxin and bicuculline) do not discriminate between receptor subtypes. Thus, the molecular identity of these receptors in the song system is unknown. Here we provide convincing evidence that the γ4 subunit is a component of one or more $GABA_A$ receptor subtypes that is/are present in most of the major song system nuclei (i.e. the LMAN, Area X, the HVC and the RA). These data are in line with our previous studies that have shown that γ4-subunit gene expression is

modulated, in appropriate brain areas such as the intermediate medial mesopallium (formerly, the intermediate and medial part of the hyperstriatum ventrale; see Reiner et al., 2004), in response to visual imprinting in the one-day-old chick (Harvey et al., 1998), which is a form of juvenile learning (Horn, 1998). Although $GABA_A$ receptors have been functionally identified, in the zebra finch, in the NIf (Cardin and Schmidt, 2004), significant levels of the γ4-subunit transcript could not be detected in this nucleus. We therefore conclude that these receptors likely contain another γ subunit or the δ or ε subunit.

That the γ4 subunit is a *bona fide* $GABA_A$ receptor polypeptide has been demonstrated by the functional expression of the chicken sequence, with an α and a β subunit, in *Xenopus laevis* oocytes (Forster et al., 2001). These experiments have revealed that the γ4 subunit exhibits most of the properties expected of a $GABA_A$ receptor γ subunit, including the ability to confer sensitivity to benzodiazepines; it differs from the mammalian γ1, γ2 and γ3 subunits in that recombinant αβγ4-subunit receptors have a high sensitivity to zinc (Forster et al., 2001). Together with the strong sequence conservation between the zebra finch and chicken γ4 subunits (97% identity over 271 amino acids), our data point to a conserved function for $GABA_A$ receptors containing this polypeptide in learning and memory processes in birds. Recently, we, in collaboration with others, have begun to quantify the expression levels of $GABA_A$ receptor subunit genes, in the zebra finch brain, using laser capture microdissection of individual song system nuclei in combination with quantitative (real-time) PCR, at a variety of developmental stages. These experiments should help elucidate the nature of the other $GABA_A$ receptor polypeptides that assemble with the γ4 subunit *in vivo*, and reveal the extent of the involvement of this ligand-gated ion channel in the learnt behaviour of song production.

Development of the GABAergic system within the RA

The expression pattern of the $GABA_A$ receptor γ4-subunit gene, within the male zebra finch brain, suggests that the encoded polypeptide (and the receptor subtype(s) of which it is a part) has functional significance in communication between, and within, the two interconnected circuits that make up the song system. This is supported by the fact that, in

juvenile birds as young as PHD22, the γ4-subunit mRNA is already present in two of the main nuclei of the anterior forebrain pathway (the LMAN and Area X), at the time when this pathway is anatomically connected and essential for song acquisition (Mooney and Rao, 1994). The γ4-subunit transcript is also detectable, by *in situ* hybridisation, in the RA but only in PHD35 and older birds, despite the fact that this nucleus is histochemically recognisable much earlier (i.e. PHD12; Konishi and Akutagawa, 1985). It has been reported that, at ~PHD15, the HVC terminals migrate and reach the dorsal border of the RA, where they remain until the onset of singing (Konishi and Akutagawa, 1985), and at ~PHD35, which marks the beginning of the sensorimotor phase (Mooney, 1999), the terminals begin to innervate this nucleus. However, this observation has been challenged by Foster and Bottjer (1998), who have provided evidence that the HVC to RA connection is established much earlier (namely, at PHD20 to PHD23). Nevertheless, the onset of expression of the γ4-subunit gene in the RA coincides with the time-point when the vocal motor pathway becomes functional. Whether the transcription of this gene in this nucleus is dependent on innervation from the HVC requires further investigation. However, it must be independent of innervation by LMAN projection neurons because the terminals of this nucleus are functionally connected to the RA as early as PHD15 (Mooney, 1992; Mooney and Rao, 1994). An interesting observation in connection with our findings is that the zebra finch retinaldehyde-specific aldehyde dehydrogenase gene, zRalDH, has also been found to be developmentally regulated in the RA (Denisenko-Nehrbass et al., 2000). The expression of this gene peaks in the RA at PHD38, around the time that the $GABA_A$ receptor γ4-subunit transcript starts to accumulate in this nucleus.

It is currently not technically easy to localise the γ4-subunit mRNA to a particular cell type using either emulsion autoradiography or non-radioactive *in situ* hybridisation and, in the absence of a suitable antibody, it is not possible to determine the subcellular location of receptors containing the γ4 subunit. However, there is clear evidence for GABAergic interneurons within the RA (Spiro et al., 1999; Vicario and Raksin, 2000). It is, perhaps, worth noting that these inhibitory neurons are believed to be essential for the generation of temporally-precise patterns of neural activity (Spiro et al., 1999; Vicario and Raksin, 2000),

which are necessary for the production of song.

The $GABA_A$ receptor γ4-subunit mRNA is a molecular marker for the song system

As mentioned above, we have described here strong hybridisation signals, corresponding to the presence of the $GABA_A$ receptor γ4-subunit transcript, in most of the major nuclei of the song system. Furthermore, the strength of the signals clearly distinguishes these structures from the surrounding brain areas. Moreover, the sizes of some of the nuclei undergo alterations during brain development (Nixdorf-Bergweiler, 1996), and this appears to be paralleled by the extent of spatial labelling seen here (compare, for example, Area X in Figures 2C and D, and the HVC in Figures 3A and B). Clearly, this observation merits further investigation. Thus, the γ4-subunit mRNA appears to be a specific marker for the song system that could, perhaps, be used, among other things, to delineate the boundaries of the component nuclei (Gahr, 1997). This is clearly a fortuitous finding, since early attempts to identify molecules that are unique to, and account for the sexual dimorphism, of this neuronal network in another songbird (the canary) were not particularly successful (Clayton and Huecas, 1990). Akutagawa and Konishi (2001) generated a monoclonal antibody that recognises a yet unknown antigen (named SNAg for *S*ong system *N*uclear *Ag*) that is found within the nuclear compartment. While this antibody is able to label the HVC, the RA, the LMAN, the MMAN and the NIf of male zebra finches and other estrildine birds, it does not yield a signal in other song system nuclei, such as Area X and the DLM, nor does it label structures within the canary brain. SNAg is interesting because its synthesis is developmentally regulated, and it can be induced by the hormone oestrogen in female zebra finches (Akutagawa and Konishi, 2001). However, based on its subcellular location and expression pattern, it can not be the $GABA_A$ receptor γ4 subunit.

A number of other authors have identified genes, the expression of which is enriched in song system nuclei. For example, using *in situ* hybridisation, Metzdorf et al. (1999) investigated the expression of genes encoding the enzyme aromatase, and receptors for oestrogen and androgen, in songbirds (namely, the canary, *Serinus canaria*, and the ring dove, *Streptopelia risoria*). Of these, only the androgen receptor gene is transcribed in a number of

song system nuclei (i.e. the HVC, the LMAN, the MMAN, the NIf, and the RA; expression was not detected in Area X). The mRNAs for the oestrogen receptor and aromatase were only found in the HVC and the MMAN, respectively. However, the transcript for the androgen receptor gene has been detected, in the zebra finch, in Area X (Kim et al., 2004). Denisenko-Nehrbass et al. (2000) have reported the isolation of a cDNA for zRalDH; this is expressed in the HVC, the LMAN and the RA but not Area X. A plethora of cDNAs for glutamate receptor subunits have also been cloned and the corresponding gene expression patterns elucidated (Wada et al., 2004), and many of these are transcribed within the song system. In particular, the *N*-methyl-D-aspartate receptor NR2A-subunit mRNA can be detected, albeit not in high amounts, within the HVC, the LMAN, the RA and Area X. In addition, the mRNA for the middle-weight neurofilament protein is enriched in the HVC, the LMAN, the MMAN and the RA; it is also present in area X but at a very low level (Velho et al., 2007). Finally, radioligand binding studies, using the antagonist [^{3}H]RX821002, have demonstrated that the α_2-adrenergic receptor protein is present in the HVC, the LMAN, the RA and Area X (Riters and Ball, 2002; the MMAN was not reported on). In contrast, the $GABA_A$ receptor γ4-subunit gene is highly expressed in the HVC, the LMAN, the MMAN and the RA, as well as Area X. The latter is, therefore, clearly, a useful marker for studies on the song system. Finally, its use is not limited to the zebra finch since we (C. Thode and M. G. Darlison, unpublished data) have recently isolated an orthologous cDNA from the canary.

In conclusion, $GABA_A$ receptors are known to modulate learning and memory in a number of animal models, including man (see, for example, Chapouthier and Venault, 2002; Maubach, 2003). And, as has been noted (Doupe and Kuhl, 1999; Mooney, 1999), the acquisition of complex vocalisations in songbirds resembles the process by which humans learn to speak. Although the data presented here only demonstrate the specific and enhanced expression of the $GABA_A$ receptor γ4-subunit gene within most of the major song system nuclei, they do provide a very strong correlation with vocal behaviour. Currently, we are trying to modulate the expression of $GABA_A$ receptor genes *in vivo*, which should enable us to demonstrate a functional role for γ4-subunit-containing receptors in the learning, production and/or maintenance of song.

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Legends to Figures

Figure 1. Alignment of part of the zebra finch $GABA_A$ receptor γ4 subunit with the corresponding chicken sequence. The amino-acid sequences (shown in single-letter code) were aligned with the help of the computer programme CLUSTAL_X (Thompson et al., 1997); dots denote gaps that have been introduced to maximise the alignment. Three membrane-spanning domains (M1 to M3) and the conserved cysteine loop, in the amino-terminal extracellular domain, are indicated by solid lines and a dotted line above the sequences, respectively. Positions at which the two sequences differ are high-lighted by white lettering on a black background. The sequence of the cDNA, from which the zebra finch $GABA_A$ receptor γ4-subunit amino-acid sequence has been deduced, has been given the EMBL accession number AM086993. The chicken $GABA_A$ receptor γ4-subunit sequence has been taken from EMBL accession number X73533 (see Harvey et al., 1993).

Figure 2. Expression of the $GABA_A$ receptor γ4-subunit gene in nuclei of the anterior forebrain pathway in juvenile (PHD35; A and C) and adult (PHD>100; B and D) male zebra finches. Shown are inverse images, from autoradiographs, of sections that were hybridised with a transcript-specific oligonucleotide probe. Numbers on the schematics refer to the corresponding sections of the stereotaxic atlas of Stokes et al. (1974). Abbreviations: HA, apical part of the hyperpallium; HD, densocellular part of the hyperpallium; LMAN, lateral magnocellular nucleus of the anterior nidopallium; M, mesopallium; MMAN, medial magnocellular nucleus of the anterior nidopallium; MSt, medial striatum; N, nidopallium; X, Area X. Scale bar: 2 mm.

Figure 3. Expression of the $GABA_A$ receptor γ4-subunit gene in nuclei of the vocal motor pathway in juvenile (PHD35; A and C) and adult (PHD>100; B and D) male zebra finches. Shown are inverse images, from autoradiographs, of sections that were hybridised with a transcript-specific oligonucleotide probe. Numbers on the schematics refer to the corresponding sections of the stereotaxic atlas of Stokes et al. (1974). Abbreviations: A, arcopallium; Cb, cerebellum; Hb, habenula; Hp, hippocampus; LSt, lateral striatum; N,

nidopallium; NC, caudal nidopallium; RA, robust nucleus of the arcopallium; TeO, optic tectum. Note that HVC is used as the proper name. Scale bar: 2 mm.

Figure 4. Expression of the $GABA_A$ receptor γ4-subunit gene in Field L of juvenile (PHD35; A) and adult (PHD>100; B) male zebra finches. Shown are inverse images, from autoradiographs, of sections that were hybridised with a transcript-specific oligonucleotide probe. The number on the schematic refers to the corresponding section of the stereotaxic atlas of Stokes et al. (1974). Abbreviations: Hb, habenula; Hp, hippocampus; L, Field L; LSt, lateral striatum; N, nidopallium; TeO, optic tectum. Scale bar: 2 mm.

Figure 5. Comparison of the expression of the $GABA_A$ receptor γ4-subunit gene in adult (PHD>100) male (A, C, E and G) and female (B, D, F and H) zebra finches. Shown are inverse images, from autoradiographs, of sections at equivalent levels of the zebra finch neuroaxis. Male and female sections were hybridised (with a transcript-specific probe) and washed together, and then exposed to the same sheet of X-ray film. Abbreviations: LMAN, lateral magnocellular nucleus of the anterior nidopallium; M, mesopallium; MMAN, medial magnocellular nucleus of the anterior nidopallium; RA, robust nucleus of the arcopallium; X, Area X. Note that HVC is used as the proper name. Scale bar: 2 mm.

Table 1. Distribution of the $GABA_A$ receptor γ4-subunit mRNA in the song system of the male zebra finch at different developmental stages.

Brain region		**Developmental stage**			
		PHD22 and 25 (n = 3)	PHD35 to 38 (n = 6)	PHD60 and 61 (n = 2)	PHD>100 (n = 7)
Anterior forebrain pathway	Area X	+++	+++	+++	+++
	LMAN	++	+++	+++	+++
	MMAN	++	+++	+++	+++
Vocal motor pathway	HVC	++	+++	++++	++++
	RA	–	+	+++	++++
Auditory areas	Field L	+++	+++	+++	+++
	NIf	–	–	–	–

Legend to Table 1

Listed are the major nuclei of the two neuronal pathways of the zebra finch song system and two areas, Field L and NIf that provide auditory input. Hybridisation signals in these nuclei, obtained with specific oligonucleotide probes that recognise the $GABA_A$ receptor γ4-subunit mRNA (see text), were arbitrarily scored essentially as previously (see, for example, Harvey and Darlison, 1997; Bock et al., 2005; Thode et al., 2005), as either high (++++), moderate (+++), low (++), very low (+) or no signal above that of surrounding areas (-). Data derive from Figures 2 to 4, and data not shown. Brain areas were identified as described in Experimental Procedures.

Figure 1

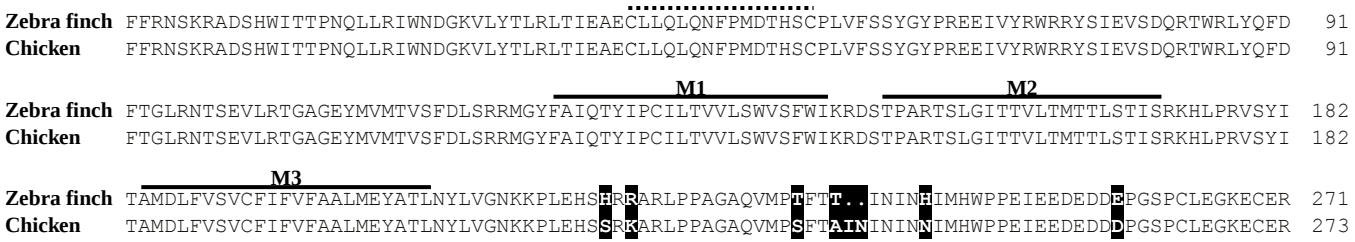
M1
M2
M3
Zebra finch FFRNSKRADSHWITTPNQLLRIWNDGKVLYTLRLTIEAECLLQLQNFPMDTHSCPLVFSSYGYPREEIVYRWRRYSIEVSDQRTWRLYQFD 91
Chicken FFRNSKRADSHWITTPNQLLRIWNDGKVLYTLRLTIEAECLLQLQNFPMDTHSCPLVFSSYGYPREEIVYRWRRYSIEVSDQRTWRLYQFD 91
Zebra finch FTGLRNTSEVLRTGAGEYMVMTVSFDLSRRMGYFAIQTYIPCILTVVLSWVSFWIKRDSTPARTSLGITTVLTMTTLSTISRKHLPRVSYI 182
Chicken FTGLRNTSEVLRTGAGEYMVMTVSFDLSRRMGYFAIQTYIPCILTVVLSWVSFWIKRDSTPARTSLGITTVLTMTTLSTISRKHLPRVSYI 182
Zebra finch TAMDLFVSVCFIFVFAALMEYATLNYLVGNKKPLEHSHRRARLPPAGAQVMPTFTT..INININHIMHWPPEIEEDEDDEPGSPCLEGKECER 271
Chicken TAMDLFVSVCFIFVFAALMEYATLNYLVGNKKPLEHSSRKARLPPAGAQVMPSFTAINININNIMHWPPEIEEDEDDDPGSPCLEGKECER 273

Figure 2

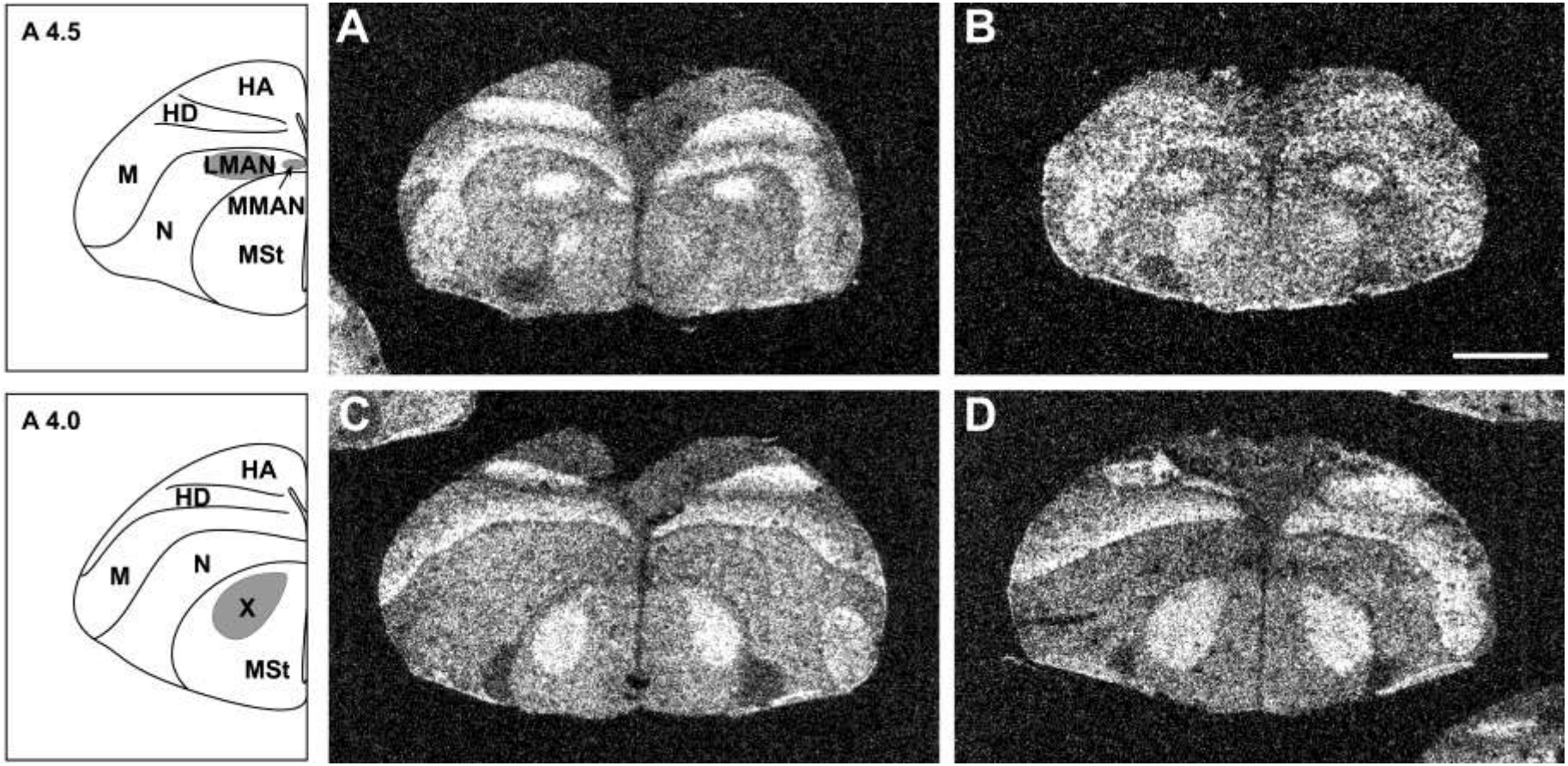

Figure 3

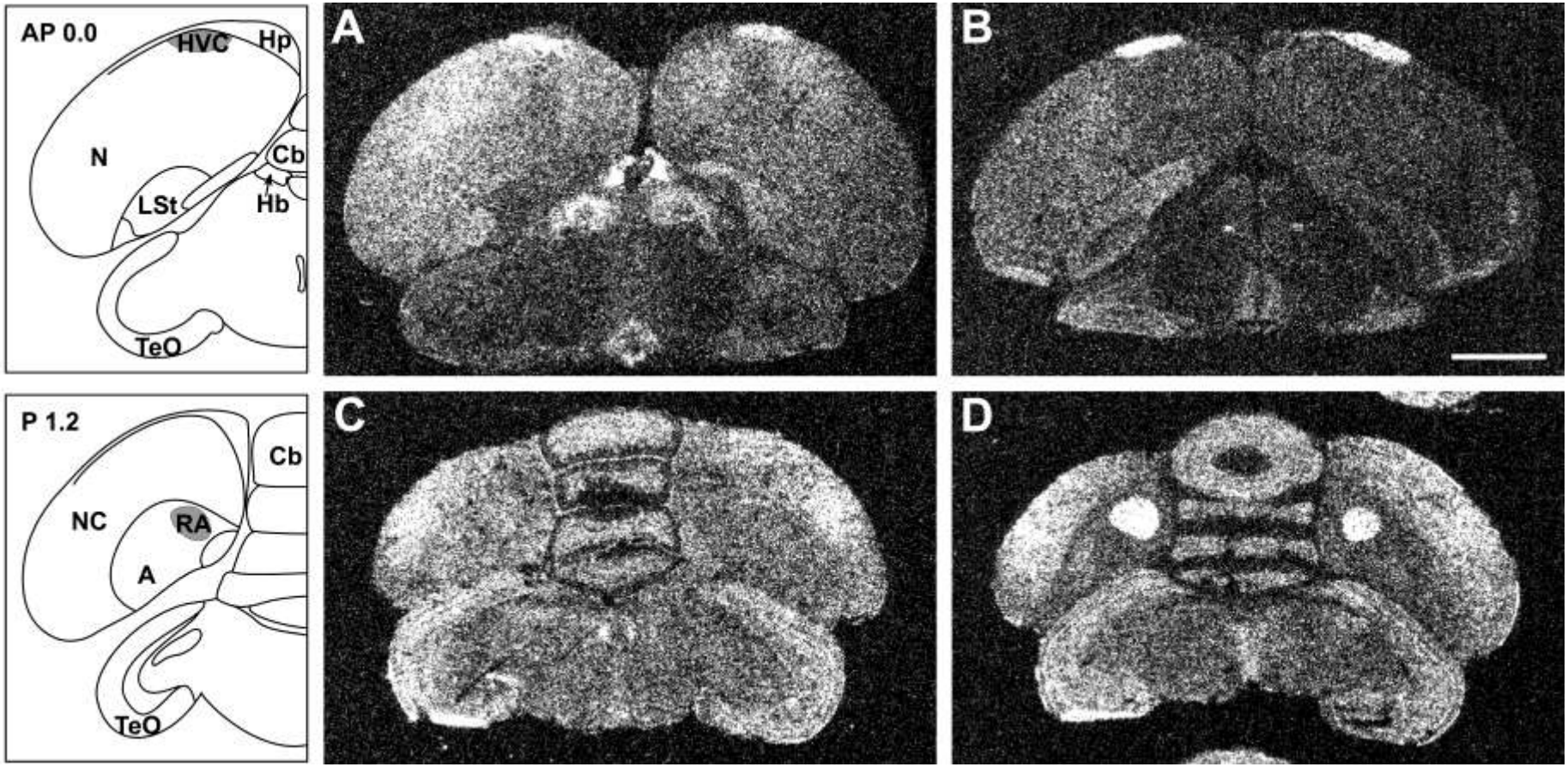

Figure 4

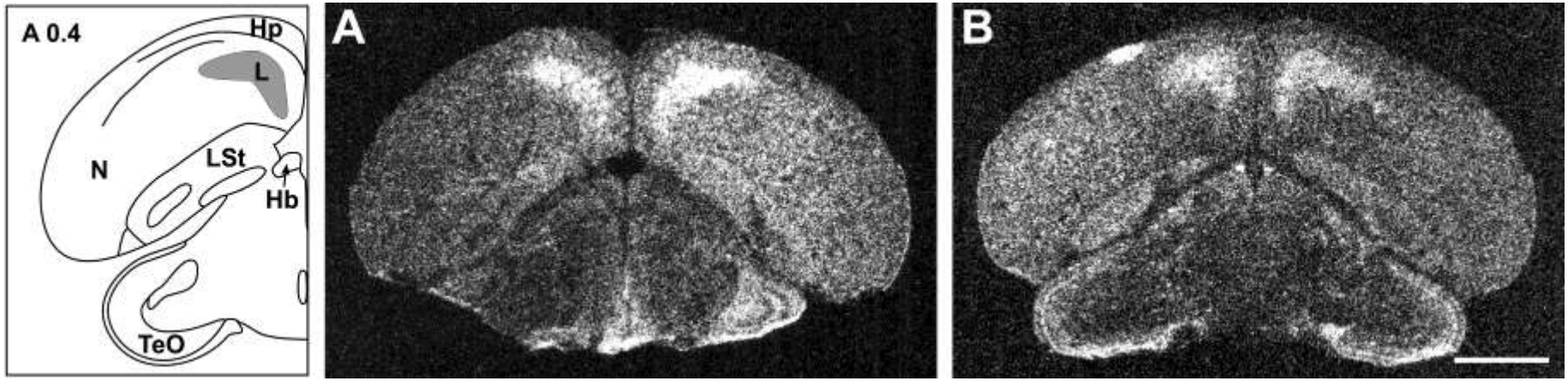

Figure 5