Language-related *Cntnap2* Gene is Differentially Expressed in Sexually Dimorphic Song Nuclei Essential for Vocal Learning in Songbirds

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ABSTRACT

Multiple studies, involving distinct clinical populations, implicate contactin associated protein-like 2 (*CNTNAP2*) in aspects of language development and performance. While *CNTNAP2* is broadly distributed in developing rodent brain, it shows a striking gradient of frontal cortical enrichment in developing human brain, consistent with a role in patterning circuits that subserve higher cognition and language. To test the hypothesis that *CNTNAP2* may be important for learned vocal communication in additional species, we employed in situ hybridization to characterize transcript distribution in the zebra finch, an experimentally tractable songbird for which the neural substrate of this behavior is well established. Consistent with an important role in learned vocalization, *Cntnap2* was enriched or diminished in key song control nuclei relative to adjacent brain tissue. Importantly, this punctuated expression was observed in males, but not females, in accord with the sexual dimorphism of neural circuitry and vocal learning in this species. Ongoing functional work will provide important insights into the relationship between *Cntnap2* and vocal communication in songbirds and thereby clarify mechanisms at play in disorders of human cognition and language. J. Comp. Neurol. 518:1995–2018, 2010.

INDEXING TERMS: autism; avian; birdsong; CASPR2; contactin; neurexin; zebra finch

Multiple independent lines of evidence have recently converged to identify contactin associated protein-like 2 (*CNTNAP2*) as an important modulator of diverse clinical phenotypes involving impaired language performance. Early linkage scans in families with autistic children, a disorder in which language impairment is a defining feature, highlighted the distal portion of chromosome 7q (Alarcón et al., 2002; Badner and Gershon, 2002; Trikalinos et al., 2006). Subsequent fine mapping in these and other families led to the identification of associations between common variation in *CNTNAP2* and both autism diagnosis (Arking et al., 2008) and language onset in autistic probands (Alarcón et al., 2008). Remarkably, work by Vernes et al. (2008) provided additional support for the relationship between common variation in *CNTNAP2* and language performance outside of autism. Genetic variants overlapping with those studied previously (Alarcón et al., 2008) were found to be associated with nonword repetition in individuals with specific language impairment. Importantly, in the same study chromatin immunoprecipitation followed by DNA sequencing identified *CNTNAP2* as a direct target of the transcription factor Forkhead box 2 (FOXP2). Mutations in this latter molecule result in a severe speech and language disorder known as developmental verbal dyspraxia (Lai et al., 2001). Demonstration of an interaction between these two molecules bolsters the role of each in language development and begins to define a molecular network which may subserve vocal communication. Further support for involvement of

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CNTNAP2 in language comes from the identification of rare variants in children with either autism or cortical dysplasia focal epilepsy (CDFE). The latter is a disorder characterized by abnormal brain development, seizures, and behavioral abnormalities that include language regression. In autism, rare nonsynonymous coding variants (Bakkaloglu et al., 2008) or structural rearrangements (Alarcón et al., 2008; Bucan et al., 2009; Poot et al., 2009) in *CNTNAP2* have been observed in cases and family members (Abrahams and Geschwind, 2010). Importantly, within CDFE, study of patients and their families from the Old Order Amish suggests that homozygosity for a truncating mutation in *CNTNAP2* may be sufficient to cause disease in a Mendelian fashion (Strauss et al., 2006), a result that has since been replicated in an independently ascertained case (Jackman et al., 2009).

CNTNAP2 was discovered almost a decade ago and determined, on the basis of sequence homology, to be a member of the neurexin superfamily (Poliak et al., 1999). At the same time, relatively little is known about its function, particularly in the central nervous system. Work in the peripheral nervous system has shown that the Cntnap2 protein is localized at the juxtaparanodal (JPN) region of myelinated axons where it interacts with transient axonal protein 1 (TAG-1), an interaction that mediates axon-glia contact and is required to promote the local clustering of voltage-gated potassium channels at the JPN (Poliak et al., 2001, 2003; Traka et al., 2003).

Of particular importance given the seizures described in the Old Order Amish patients homozygous for truncating mutations (Strauss et al., 2006) is the finding that Cntnap2 localizes to the axon initial segment, a key functional domain involved in generation of action potentials (Inda et al., 2006). Consistent with the idea that CNTNAP2 plays multiple developmental roles is the observation of defects in neuronal migration in cortical material resected from CDFE patients (Strauss et al., 2006). Characterization of transcript distribution in human fetal brain prior to the onset of axonal myelination (Abrahams et al., 2007) provides further support for this notion. In mid-gestation human brain tissue, the *CNTNAP2* transcript is highly enriched in frontal cortex, striatum, and dorsal thalamus, suggesting that the gene product is functionally specialized in these regions. Although a variety of structures have been implicated in different aspects of human cognition (Lieberman, 2002), the fact that *CNTNAP2* marks cortico-striato-thalamic circuits thought to be central to aspects of executive function and language is intriguing, particularly when considered together with the genetic evidence outlined above. Interestingly, these results from human stand in sharp contrast to the broad transcript distribution observed in the developing brains of rodents (Abrahams et al., 2007). Yet detailed knowledge about the manner by which apparent language-related circuits act to subserve vocal communication is lacking, necessitating work in other vocal learners in which more is known about the neural substrate of this behavior.

Toward functional work in songbirds, we undertook a comprehensive analysis of neural expression of *Cntnap2* in the zebra finch (*Taeniopygia guttata*), an important animal model for study of the neural and social bases of vocal learning (Doupe and Kuhl, 1999; Jarvis, 2004). Like humans, but unlike nonhuman primates or rodents, songbirds have the ability to modify vocal communication signals in a process that involves implicit learning and sensorimotor integration. Like humans, zebra finches learn their vocalizations (i.e., song) during critical developmental stages and learning is dependent on social interactions (Nelson, 1997; Doupe and Kuhl, 1999; Kuhl, 2003) and auditory experience (Price, 1979; Marler, 1991; Iyengar and Bottjer, 2002b). In this species, song learning and its underlying neural circuitry are sexually dimorphic (Nottebohm and Arnold, 1976). During development, groups of neurons dedicated to song learning and production, referred to as song control nuclei, emerge as subregions of the cortical-like pallium, striatum, and thalamus and become synaptically connected in males (Fig. 1) but not in females, who do not learn to produce song (Nottebohm and Arnold, 1976). These include pallial subregions known as HVC (abbreviation used as proper name) and the robust nucleus of the arcopallium (RA), which comprise the posterior vocal pathway controlling song production (Nottebohm et al., 1976) via innervation of motor neurons for the syrinx and respiratory muscles used in singing (Wild, 1997).

A second pathway in the anterior forebrain pathway (AFP) is required for song modification during development (Bottjer et al., 1984; Sohrabji et al., 1990; Scharff and Nottebohm, 1991). In the AFP, a subset of HVC neurons project to area X of the striatum. Area X projection neurons innervate the medial part of the dorsolateral thalamic nucleus (DLM). DLM innervates the lateral magnocellular nucleus of the anterior nidopallium (LMAN) whose neurons project back to area X and also rejoin the vocal motor pathway by innervating RA (Bottjer et al., 1989; Vates and Nottebohm, 1995; Vates et al., 1997). This pallio-striato-thalamic AFP loop is analogous to the human cortico-striato-thalamic circuits used for planning and executing complex sequential movements and involved in implicit learning, including speech and language (Bottjer and Johnson, 1997; Doupe and Kuhl, 1999; Jarvis, 2004). An additional thalamo-pallial pathway (not depicted in Fig. 1) connects the medial magnocellular nucleus of the anterior nidopallium (MMAN), HVC, and the posterior part of the dorsomedial thalamic nucleus (DMP) (Foster et al., 1997; Vates et al., 1997). This pathway is hypothesized to function as a reafferent loop for relaying information

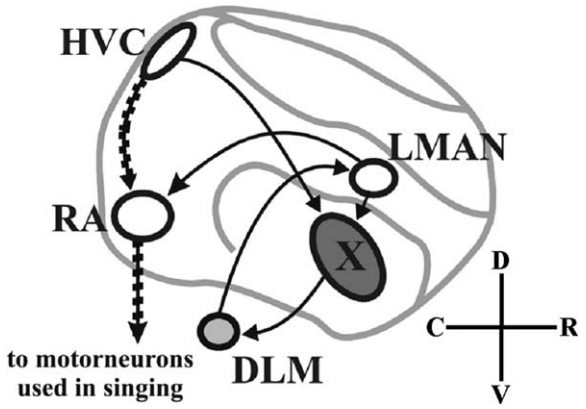

Figure 1. Schematic of connections within the male song circuit in pseudo-sagittal section of zebra finch brain. Cortical-like pallial structures appear in White, striatal in dark gray, and thalamic in light gray. Auditory input (not shown) enters the song circuit at HVC. In the posterior vocal pathway (stippled arrows), a subset of HVC neurons project to RA which projects to brainstem motor neurons that control the syrinx, or song organ, and respiratory muscles used in singing. A separate group of HVC neurons projects to the AFP (plain arrows) which connects HVC, area X, DLM, and LMAN and rejoins the posterior vocal pathway via the connection of LMAN to RA. C (caudal), D (dorsal), R (rostral), V (ventral). See Table 1 for abbreviations of brain regions. Adapted from Teramitsu et al. (2004).

between the motor pathway and components of the AFP (Vates et al., 1997). Song nuclei HVC and RA are larger in the adult male zebra finch than in the female, whereas striatal area X is absent in females (Nottebohm and Arnold, 1976). (For a complete list of abbreviations of brain structures see Table 1).

We hypothesized that *CNTNAP2* might be important for the formation and function of neural circuits necessary for vocal learning in the songbird, similar to its predicted role in patterning language-related areas in the human. While the subset of neurons specialized for language within developing human cortico-striato-thalamo-cortical circuitry is not precisely localized, zebra finch neural circuitry for learned vocalizations is (see above). As a first test, we conducted a comprehensive analysis of *Cntnap2* expression in zebra finch brain in the adult and at key stages during posthatch development (see Materials and Methods). In support of a role for *Cntnap2* in the establishment and function of song circuitry, we observed sexually dimorphic transcript distribution which mapped both temporally and spatially to the development and functional specialization of song control nuclei.

MATERIALS AND METHODS

Animals and tissue preparation

All animal use and experimental procedures were in accordance with the National Institutes of Health (NIH) guidelines for experiments involving vertebrate animals and approved by the UCLA Chancellor's Institutional Animal Care and Use Committee. Zebra finches ($n = 18$ males and 15 females) used in this study were obtained from our breeding colony. Following decapitation, brain tissue was rapidly extracted and flash-frozen on aluminum weigh boats floated on liquid nitrogen, then stored at -80°C until use. For posthatch day 1 and 6-day timepoints, brains were not dissected but left encased in the skull to preserve tissue integrity during sectioning. Coronal or sagittal sections were cut at 20 μm thickness on a cryostat (Leica Microsystems, Wetzlar, Germany) and thaw-mounted onto microscope slides (Superfrost Plus; Fisher Scientific, Pittsburgh, PA) to produce several replicate sets of adjacent or near-adjacent sections. One set was used for Nissl staining to allow for identification of neuroanatomical structures. At 25 days the emergence of sexually dimorphic plumage enables differentiation of male from female zebra finches. To determine the sex of younger birds (1–15 days) we performed polymerase chain reaction (PCR) using genomic DNA as template to amplify regions corresponding to sex-specific gene isoforms present on Z (male) or Z and W (female) chromosomes, as described in Soderstrom et al. (2007).

Reverse transcription PCR and cDNA cloning

With the cDNA for human *CNTNAP2* (AF319045.1) as a query, *blastn* was used to search the zebra finch Trace-WGS database (http://www.ncbi.nlm.nih.gov/genome, taxid = 59729) for sequence fragments potentially corresponding to the zebra finch homolog. To obtain a complete open reading frame (ORF), primers against and between sequences recovered in this fashion were then designed for use in PCR. Total RNA was isolated from whole brain using RNeasy Mini Kit (Qiagen, Valencia, CA), and was random primed and reverse transcribed with Superscript III to generate cDNA according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Sequencing of PCR products resulted in the recovery of a partial ORF. Missing upstream material was obtained by 5′ RACE (GeneRacer, Invitrogen) using the manufacturer-provided 5′ primer (5′-CGACTGGAGCACGAGGACACTGA-3′) in combination with gene-specific primer (oBSA829, 5′-AGGCTTCCAGTTTCTCCCTGTATC-3′). Missing 3′ material was obtained by PCR with nested forward primers (oBSA834 and oBSA835, 5′-CTTCAGCTTCAGCACCAC-CAAGTCC-3′ and 5′-CCGGATTTCATGGCTGTCCTCGT-3′, respectively) in conjunction with a degenerate reverse primer (oBSA836, 5′-rATrArCCAyTCCTTyTTGCT-3′), obtained through alignment of cDNAs corresponding to *CNTNAP2* homologs from human, mouse, chicken, and fugu.

TABLE 1.
List of Abbreviations for Brain Structures in This Study

A	arcopallium	LSt	lateral striatum
Cb	cerebellum	M	mesopallium
DLM	medial part of the dorsolateral thalamic nucleus	MLd	dorsal part of the lateral mesencephalic nucleus
DMP	posterior part of the dorsomedial thalamic nucleus	MMAN	medial magnocellular nucleus of the anterior nidopallium
DTZ	dorsal thalamic zone	MSt	medial striatum
GP	globus pallidus	N	nidopallium
HA	apical part of the hyperpallium	NC	caudal nidopallium
Hb	habenula	RA	robust nucleus of the arcopallium
HD	densocellular part of the hyperpallium	SpL	lateral spiriform nucleus
HVC	nucleus HVC of the nidopallium	SpM	medial spiriform nucleus
ICo	intercollicular nucleus	St	striatum
IM	magnocellular isthmus nucleus	TeO	optic tectum
IPC	parvocellular isthmus nucleus	X	nucleus area X of the striatum
LMAN	lateral magnocellular nucleus of the anterior nidopallium		

Probe design and validation

Public databases for human, chicken, and zebra finch genomes were employed as above to identify conserved regions within the *CNTNAP2* transcript from which to generate in situ hybridization probes. Two nonoverlapping regions within the 3′ untranslated region (UTR) were identified as both conserved across species and unique to *CNTNAP2* (Fig. 2A). Standard PCR was then employed to amplify material from cDNA, followed by cloning of amplified products into pCR4-TOPO vector (Invitrogen) and insert verification by sequencing. Probe 1 corresponds to a 618-basepair (bp) product amplified by primers 1F (5′-CTGGGATGGGATACAAGCTG-3′) and 1R (5′-CCTGCCAG GATTGACAAGAG-3′) from chicken cDNA. Probe 2 corresponds to a 601-bp product amplified by primers 2F (5′-TGTATCTTTAACTCGTACTGGAGCA-3′) and 2R (5′-CCCCCTCCTCTTTATTTATCA-3′) from zebra finch cDNA and its entire sequence is included within the GenBank expressed sequence tag EE054214.1. Validation of probe specificity confirmed that nonoverlapping *Cntnap2* 3′ UTR riboprobes yielded identical hybridization patterns in adult zebra finch brain sections (Fig. 2B). In contrast, sense probes failed to produce any significant signal on the brain sections (Fig. 2C). Unless otherwise noted, all in situ hybridization analyses performed in this study used Probe 2 (herein designated "Probe").

In situ hybridization

In situ hybridization analyses were performed largely as previously described in Perlman et al. (2003) with the following modifications: brain sections were hybridized with 8×10^6 cpm in 125 μL per slide of ^{33}P-UTP-labeled (PerkinElmer, Waltham, MA) riboprobe and the last post-hybridization wash was in 0.25× SSC. For darkfield autoradiography, the protocol described in Teramitsu et al. (2004) was followed, with the exception that slides were incubated at 4°C for 3 weeks and, after developing, sections were fixed in CitriSolv and coverslipped with Permount mounting media (Fisher Scientific). To examine *Cntnap2* expression during development and in the adult, hybridization signals were evaluated at several key timepoints: 1 day (n = 1 each sex), 6 days (n = 2 males, 1 female), 15 days (n = 2 each sex), 25 days (n = 2 each sex), 35 days (n = 2 each sex), 40 days (n = 1 each sex), 50 days (n = 2 males, 1 female), 75 days (n = 2 males, 1 female), and adult (>120 days; n = 4 each sex). These timepoints were selected to mark key events during post-hatch brain development with an emphasis on the song-learning process, comprising the stages prior to or at the onset of sensory acquisition (15 and 25 days, respectively) and during sensorimotor practice (35, 40, 50, and 75 days), leading to the production of crystallized song (>120 days). Hybridization patterns described in the Results were consistently observed at each timepoint and within each sex, unless otherwise noted.

Image processing

Photomicrographs of Nissl-stained or film autoradiography sections were acquired with an AxioCam HRm digital camera mounted on a Discovery.V12 stereomicroscope (Carl Zeiss, Oberkochen, Germany). Emulsion radiography images were digitized from snapshots taken with the same camera mounted on an AxioImager.V1 microscope (Carl Zeiss) capable of darkfield epi-illumination (Darklite; Nikon, Melville, NY). All digitized images were processed in Adobe Photoshop 7.0 (Adobe, San Jose, CA) to remove background and adjust grayscale levels to improve brightness and contrast. Adjustments were made to the entire image and not to selective subregions. Prior to adjustment of levels, darkfield images were black-white inverted to be consistent with the interpretation of signals from film. Thus, dark areas reflect high transcript levels

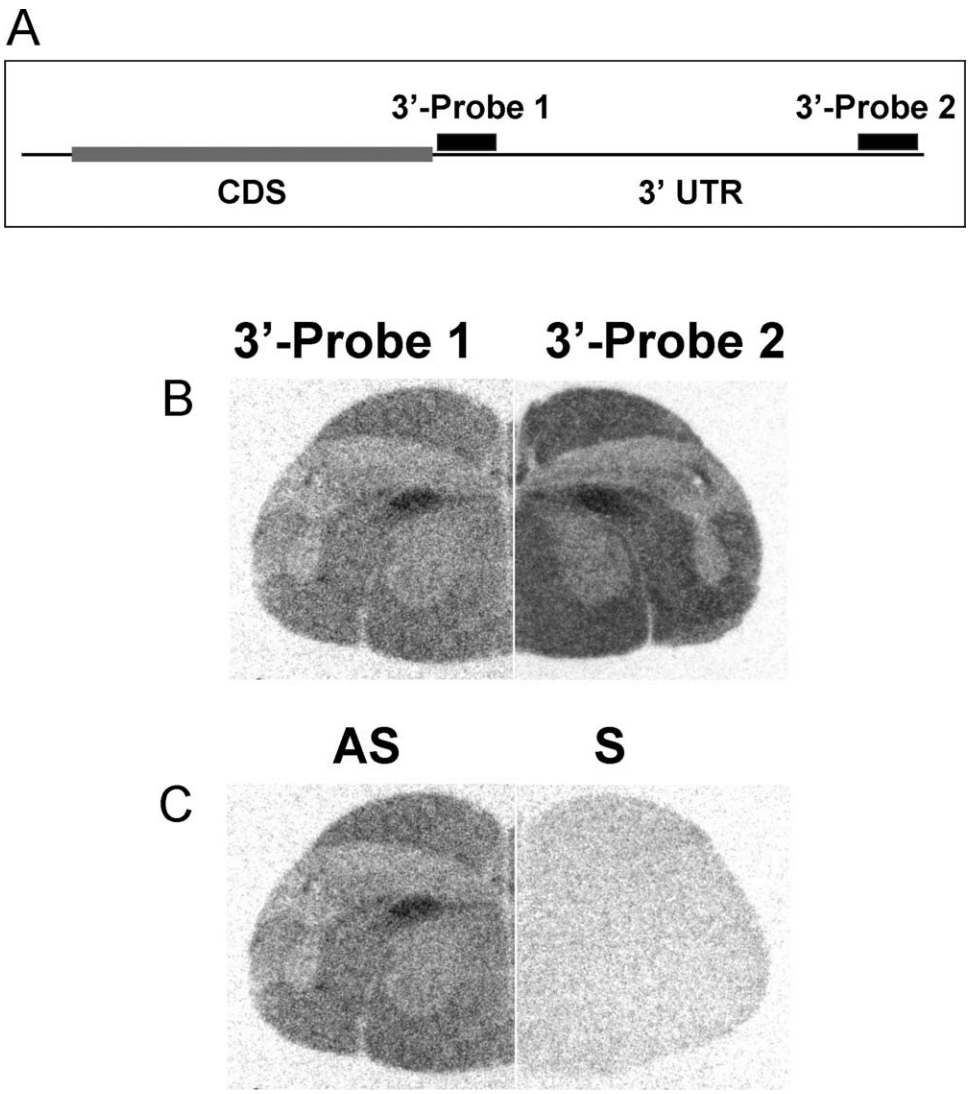

Figure 2. Schematic of zebra finch *Cntnap2*-specific riboprobes and their validation by in situ hybridization. Riboprobes were designed to hybridize to nonoverlapping portions of the 3′ UTR of *Cntnap2*, downstream of the coding sequence (CDS) (**A**). Both antisense (AS) riboprobes yielded identical hybridization patterns as exemplified in the hemicoronal section shown here (**B**). Hybridization of sense (S) probes gave results indistinguishable from background (**C**).

and light areas reflect low transcript levels in images from both emulsion-dipped slides and film autoradiograms. Schematic drawings of neuroanatomical structures revealed by Nissl staining were traced using the freehand tool in CorelDRAW 12 (Corel, Ottawa, Canada). Identification of neural structures was guided by the use of several published atlases of songbird brain, including that available for canary (*Serinus canaria*; Stokes et al., 1974) and zebra finch (http://www.ncbi.nlm.nih.gov/bookshelf/picrender.fcgi?book = atlas&part = A24&blobname = &blobtype = pdf, courtesy of Dr. Barbara Nixdorf-Bergweiler), and employed the revised avian brain nomenclature described in Reiner et al. (2004).

RESULTS

Cloning of zebra finch *Cntnap2*

Prior to characterization of *Cntnap2* transcript distribution in zebra finch, we sought to determine whether a homologous transcript was present in this species and, if so, the extent to which the ORF was conserved in human and songbird. We used *blastn* to identify sequence fragments in the zebra finch Traces-WGS database (http://www.ncbi.nlm.nih.gov/genome, taxid = 59729) with high similarity to the human mRNA (AF319045.1). This search resulted in identification of multiple sequence reads, including TGAB-apl06a12.g1, TGAB-ajb23e04.b1, TGAB-apl06a12.b1, and TGAB-aec18g03.b1. Comparison of the

TABLE 2.

Comparison of *CNTNAP2* Gene, cDNA, and Protein Structure in Human and Zebra Finch

	Ensembl human locus (Mar. 2006 Assembly)	*Ensembl zebra finch locus (Jul. 2009 Assembly)*	*GenBank acession No. GU290551*
Locus Start (bps)	chr7: 145,444,386	chr2:30,635,452	chr2:31,216,806
Locus End (bps)	chr7:147,749,019	chr2:30,184,705	chr2:30,180,291
Locus Size (bps)	2,304,634	450,748	1,036,385
Exon number	24	16	24
Upstream gene	*TPK1*	*Tpk1*	*Tpk1*
Downstream gene	*CUL1*	*Cul1*	*Cul1*
	Refseq human cDNA (NM_014141.4)	*Ensembl zebra finch cDNA (ENSTGUT00000001874)*[1]	*GenBank acession No. GU290551*
bps 5' UTR	516	0	537
bps Coding	3993	2647	3987
bps 3' UTR	5381	0	4411
bps mRNA	9890	2647	8935
	Refseq human protein (NP_054860)	*Ensembl zebra finch protein (ENSTGUP00000001856)*[1]	*GenBank acession No. GU290551*
Number of aminoacids	1331	882	1329
Weight (Daltons)	148116	97407	147661
Number of Motifs	11	7	11
Identical to Human	NA	735/885 (83%)	1126/1334 (84%)
Positive vs. Human	NA	799/885 (90%)	1213/1334 (90%)
Gaps vs. Human	NA	6/885 (<1%)	9/1334 (<1%)

[1]Both cDNA and inferred protein were the output of a modified version of the standard EnsEMBL genebuild pipeline, relying on alignment of EnsEMBL chicken, other avian and mammalian proteins, as well as evidence from zebra finch. For the taeGut3.2.4 assembly the predicted gene set contains 18,447 genes, of which 17,475 are protein coding (Potter et al., 2004).

TABLE 3.

Identity and Conservation of CNTNAP2 Protein Domains in Human and Zebra Finch

Motif	Amino Acid Position	Number of Zebra Finch-Human Differences	Domain Length	Percent Identity
Signal Peptide	1-25	11	25	56
FA58C	35-181	13	147	91
LamG2	215-344	13	130	90
LamG2	400-528	23	129	82
EGF3	553-590	4	38	89
LamG2	826-994	27	169	84
EGF3	963-1001	7	39	82
LamG2	1057-1189	45	133	66
TM	1258-1277	0	20	100
4.1B	1278-1296	0	19	100
PDZ Binding	1328-1331	0	4	100

human protein against six frame translations of these sequence fragments with *tblastn* indicated a conserved ORF across each, unlikely to occur as a result of chance alone (Eval between 3e-17 and 1e-28). PCR with multiple primer sets against cDNA confirmed the presence of zebra finch *Cntnap2* transcript in adult brain. In support of an expressed transcript, each gave rise to signal in experimental but not negative control conditions (not shown).

PCR on this same cDNA was then employed to obtain an internal sequence, estimated to correspond to ≈70% of the expected ORF. Missing 5′ and 3′ ends were obtained via RACE-PCR with a degenerate reverse primer as described in Materials and Methods, and assembled with internal sequence to generate a cDNA 8,935 bp in length (GenBank Access. No. GU290551). Alignment of this cDNA to the July 2009 assembly of zebra finch genome defined a 1-Mb locus spanning 24 exons (Table 2). Both exon number (n = 24) and identity of gene neighbors (*TPK1* and *CUL1*) were conserved between finch and human. Available genomic sequence indicated that songbird locus is less than 50% the size of the human equivalent (March 2006 Assembly), although the interpretation is confounded by the presence of unsized gaps in the zebra finch assembly. Comparison of the predicted protein in each species highlighted more similarities than differences. In each, a protein of 148 kDa with identical domain structure was observed (Table 3). Relative to the overall average amino acid identity of 84%, percent

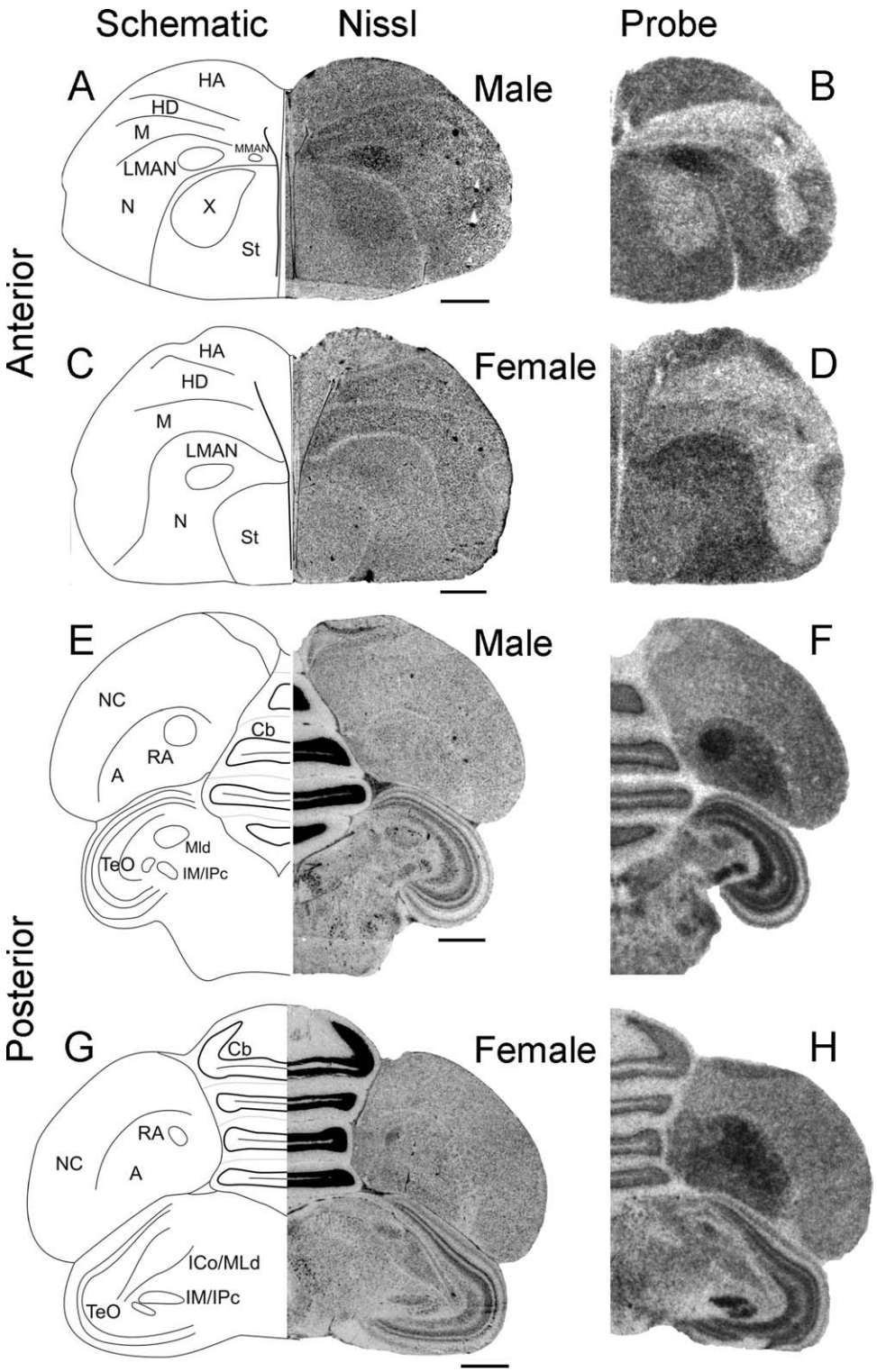

Figure 3. *Cntnap2* transcript distribution marks song nuclei in the adult male zebra finch. In anterior forebrain, enriched *Cntnap2* signals were detected in both LMAN and MMAN of hemicoronal (**B**) sections of male brain. A similar enrichment could not be detected in hemicoronal sections of female brain (**D**). Low *Cntnap2* levels in male area X (B) stood in contrast to high transcript levels in surrounding St and uniform levels in the corresponding area of female St (D). In posterior brain, strong signals were detected in male (**F**) but not female (**H**) RA. Outside the song circuitry, robust *Cntnap2* levels were detected in HA, N and St (B,D) in anterior brain, as well as in the NC and the subpallial areas of TeO, Cb and IM/IPc in posterior brain (F,H) of both sexes. For abbreviations, see Table 1. Scale bars = 1 mm.

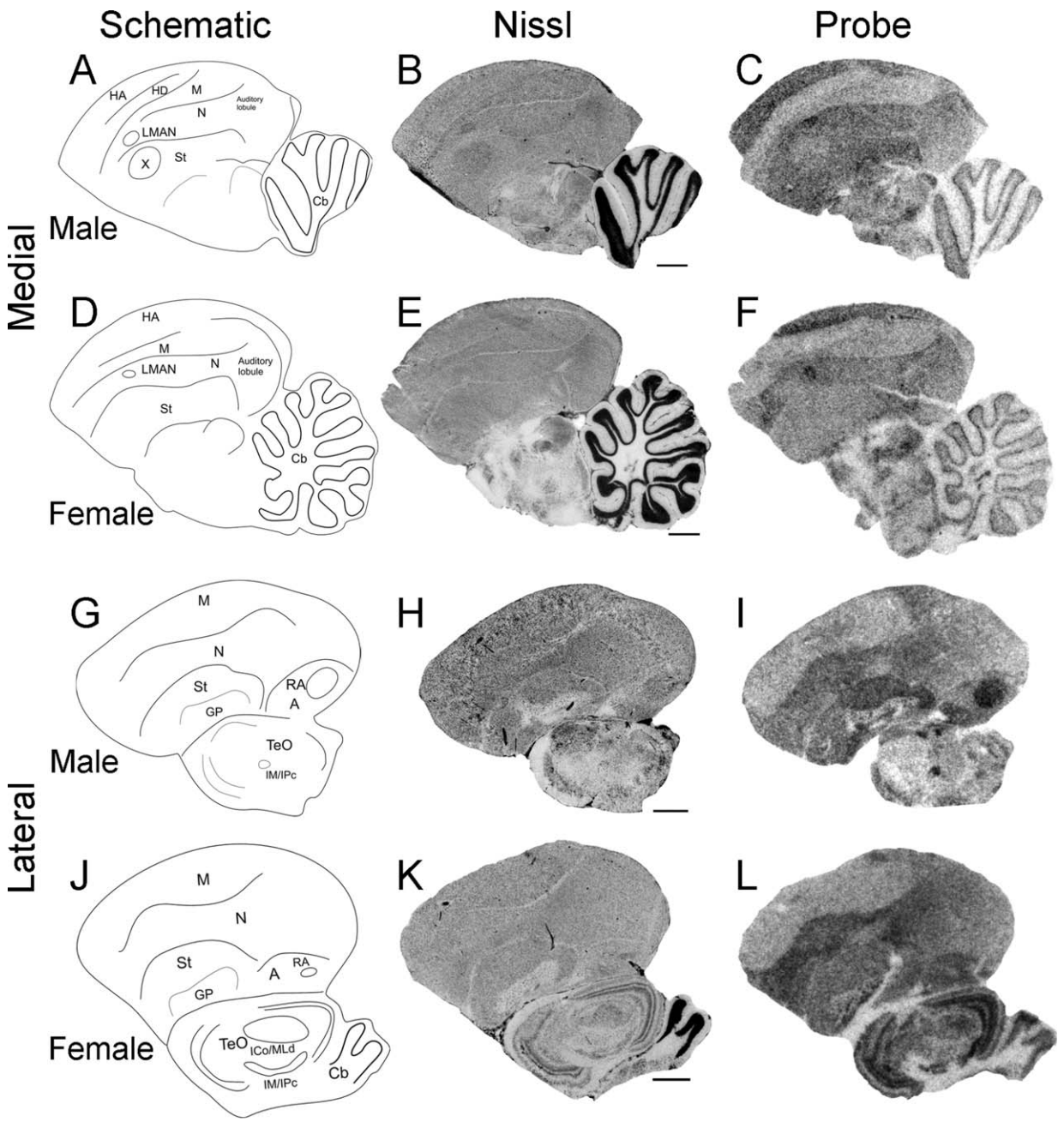

Figure 4. *Cntnap2* expression in parasagittal sections of adult male and female brain. *Cntnap2* transcript is enriched in male song nuclei LMAN (**C**) and RA (**I**) and reduced in area X (C) compared to adjacent tissue. While elevated *Cntnap2* hybridization signal could also be discerned in adult female LMAN (**F**), there was no corresponding enrichment in RA (**L**) and transcript levels appeared uniform in female striatum which lacks area X (F). Outside song circuitry transcript abundance was high in HA, N, St, TeO, and Cb of both sexes. For abbreviations see Table 1. Scale bars = 1 mm.

identity across the N-terminal signal peptide and fourth and final LamG2 domain were low (55% and 66%, respectively). Further support for relatively reduced functional constraint around this fourth LamG2 domain comes from the presence of insertion/deletion differences on either side of this region; all nine alignment gaps observed following protein alignment flanked this motif. In contrast, 100% identity across multiple C-terminal domains (transmembrane, 4.1B binding, and PDZ binding) suggests evolutionary constraint across these intracellular portions of the protein. Strong conservation at the C-terminus is consistent with previous work demonstrating the requirement of this region in the clustering of potassium channels (Poliak and Peles, 2003) and also the fact that the premature stop codon observed in cases of CDFE eliminates this portion of the protein (Strauss et al., 2006).

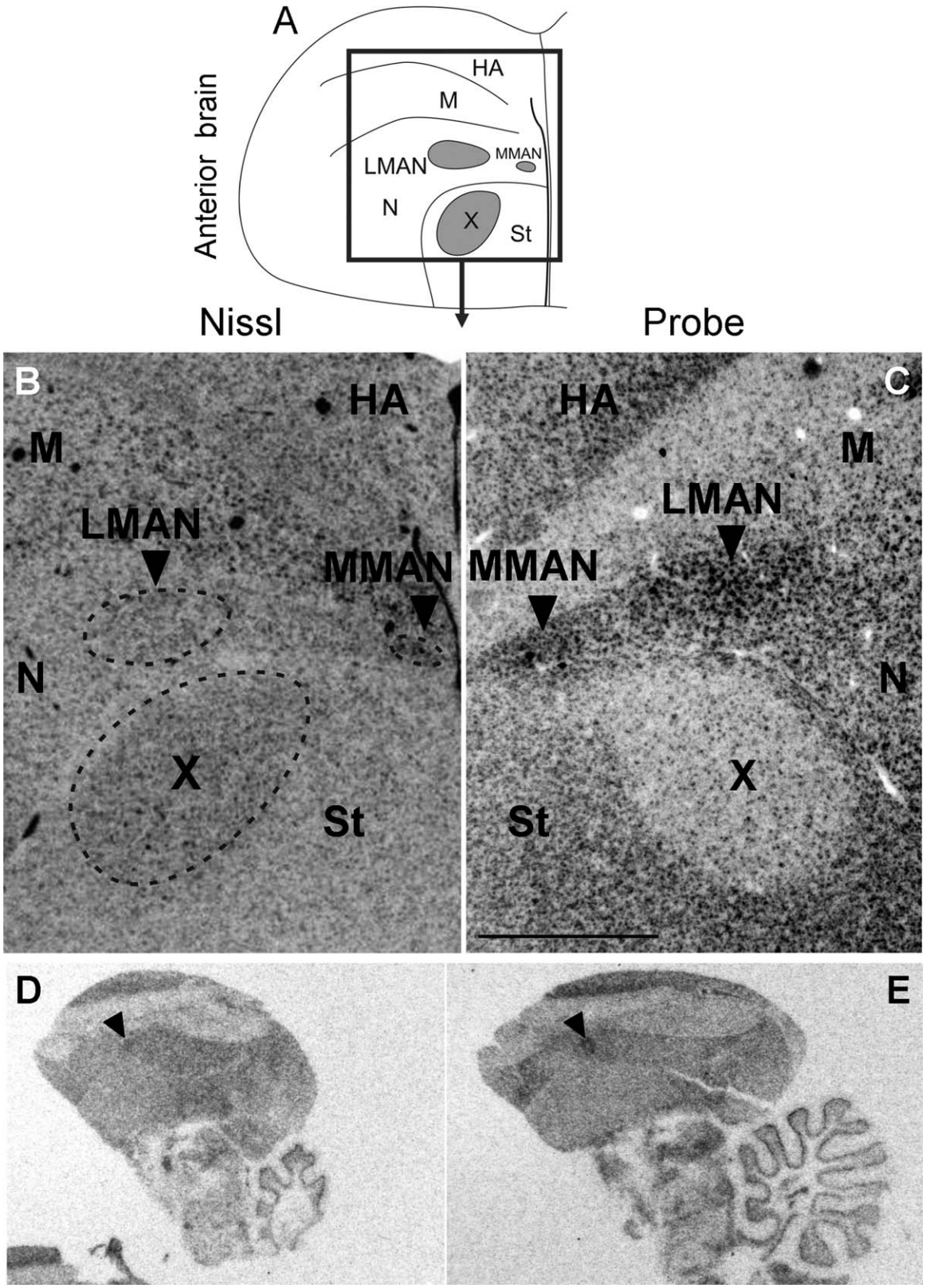

Figure 5. Inverted darkfield images of emulsion autoradiography of coronal sections of anterior adult male brain confirm strong signals in LMAN and MMAN and low signals in area X compared to adjacent tissue (**C**). In situ hybridization of parasagittal sections of adult female brain indicate that there is considerable variability in *Cntnap2* levels in LMAN, as identified by arrowheads in **D** and **E**. Unprocessed images in D and E, representing sections from different individuals, were taken under similar illumination conditions in order to emphasize the interindividual variability in hybridization signals in female LMAN. For abbreviations see Table 1. Scale bar = 1 mm in C (applies to B,C).

TABLE 4.

Summary of Relative Levels[1] of *Cntnap2* Expression in Selected Song-related Regions and Adjacent Tissue of Male and Female Zebra Finch Brain

Age (days posthatch)	15d		25d		35d		50d		75d		Adult	
Sex	M	F	M	F	M	F	M	F	M	F	M	F
Telencephalon												
HA	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
HD	+	+	+	+	+	+	+	+	+	+	+	+
M	+	+	+	+	+	+	+	+	+	+	+	+
LMAN	+++	++	+++	++	+++	+++	++++	+++	++++	+++	++++	+++
N	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
X	++	-	++	-	++	-	++	-	++	-	++	-
St	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
NC	++	++	++	++	++	++	++	++	++	++	++	++
A	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
RA	++++	++++	++++	++++	++++	++++	++++	+++	++++	+++	++++	++
HVC	++	++	++	++	++	++	++	++	++	++	++	++
Diencephalon												
DTZ											++	++
SpM											++++	++++
SpL											++++	++++
Midbrain												
TeO (layer 13)	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
ICo/MLd	++	++	++	++	++	++	++	++	++	++	++	++
IM/IPc	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
Hindbrain												
Cb	++	++	++	++	++	++	++	++	++	++	++	++

[1]Transcript levels were semiquantitatively estimated by densitometric measurements of in situ film hybridization signals in single sections of individual birds using AxioVision software. Levels of relative expression were then classified according to a subjective scale as ++++ (very high), +++ (high), ++ (moderate), + (low) or – (not detectable or structures not present). For abbreviations of brain structures, see Table 1.

Sexually dimorphic *Cntnap2* expression in the adult

To begin to test the hypothesis that *Cntnap2* is important for the formation and functional specialization of zebra finch song circuitry, we compared the pattern of *Cntnap2* expression between adult (>120 days) male and female zebra finch brains. Using hemicoronal (Fig. 3) and parasagittal (Fig. 4) sections, we uncovered differential *Cntnap2* transcript distribution within several subregions dedicated to song control, consistent with the known sexual dimorphism of the zebra finch song circuit in these areas (Nottebohm and Arnold, 1976). Within male brains, *Cntnap2* expression was enriched bilaterally in both the lateral and medial magnocellular nuclei of the anterior nidopallium (LMAN, MMAN, respectively; Figs. 3B, 4C), relative to the surrounding nidopallium (N), and in the robust nucleus of the arcopallium (RA; Figs. 3F, 4I), compared to the surrounding arcopallium (A). In male area X, low levels of *Cntnap2* were in sharp contrast to transcript abundance in the surrounding striatum (St; Figs. 3B, 4C). Emulsion autoradiography of an additional male brain confirmed this expression pattern, revealing strong transcript enrichment in both male LMAN and MMAN and diminished levels in area X (Fig. 5C).

In contrast, *Cntnap2* levels in adult female LMAN appeared to vary. While we could not detect enhanced expression in female LMAN in any coronal sections hybridized to probe (Fig. 3D), in parasagittal sections of two additional female brains *Cntnap2* levels in LMAN appeared increased above those in surrounding N (Fig. 4F), more so in sections from one of the female brains compared to another (Fig. 5D vs. E). In adult female RA, *Cntnap2* hybridization signal was similar to or diminished compared to surrounding A (Figs. 3H, 4L). Moreover, *Cntnap2* transcript was uniformly distributed in female St, which lacks area X (Figs. 3D, 4F). (For a summary of relative *Cntnap2* expression levels in song nuclei and adjacent brain regions in young and adult birds, see Table 4.)

Developmental expression of Cntnap2

Compelled by the finding of site-specific sexual dimorphism of *Cntnap2* expression in the adult songbird brain, we proceeded to characterize gene expression throughout posthatch brain development, at key stages in the ontogeny of song nuclei and vocal learning. Thus, to best capture whether changes in *Cntnap2* transcript distribution may be linked to neuroanatomical changes and functional specialization associated with the formation of song circuitry, we conducted in situ hybridization analyses at posthatch days 1, 6, 15, 25, 35, 50, and 75. (For a summary of changes in the relative expression levels of *Cntnap2* during male and female posthatch development, see Table 4.) Notably, both during the formation and maturation of song-learning

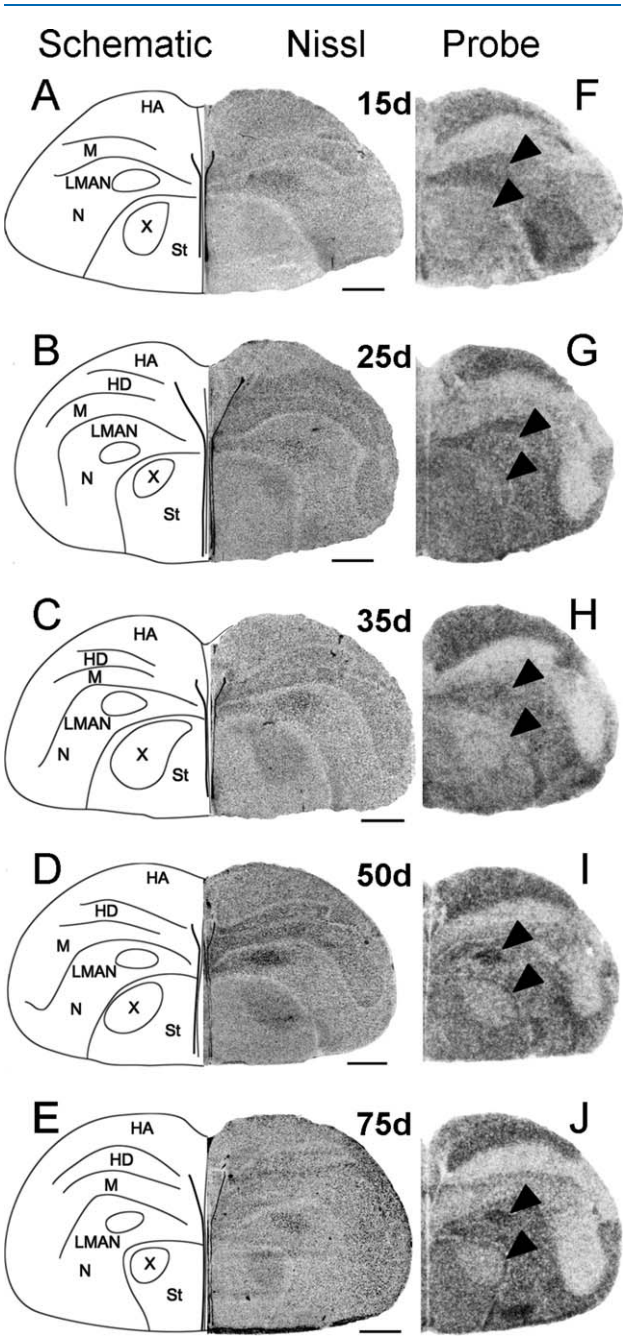

Figure 6. Developmental pattern of *Cntnap2* expression in hemicoronal sections of male anterior forebrain. Prior to 50 days, *Cntnap2* signals in LMAN (**F–H**) were similar to those in the surrounding N. From 50–75 days, *Cntnap2* signals in LMAN were higher than those in the robustly labeled region of N. Transcript levels in area X of developing males were lower than in the surrounding St. This contrast was particularly evident at 50 (**I**) and 75 days (**J**). Robust expression was also detected in HA, N, and St throughout development. Arrowheads point to male LMAN and area X as identified in **A–E**. For abbreviations, see Table 1. Scale bars = 1 mm.

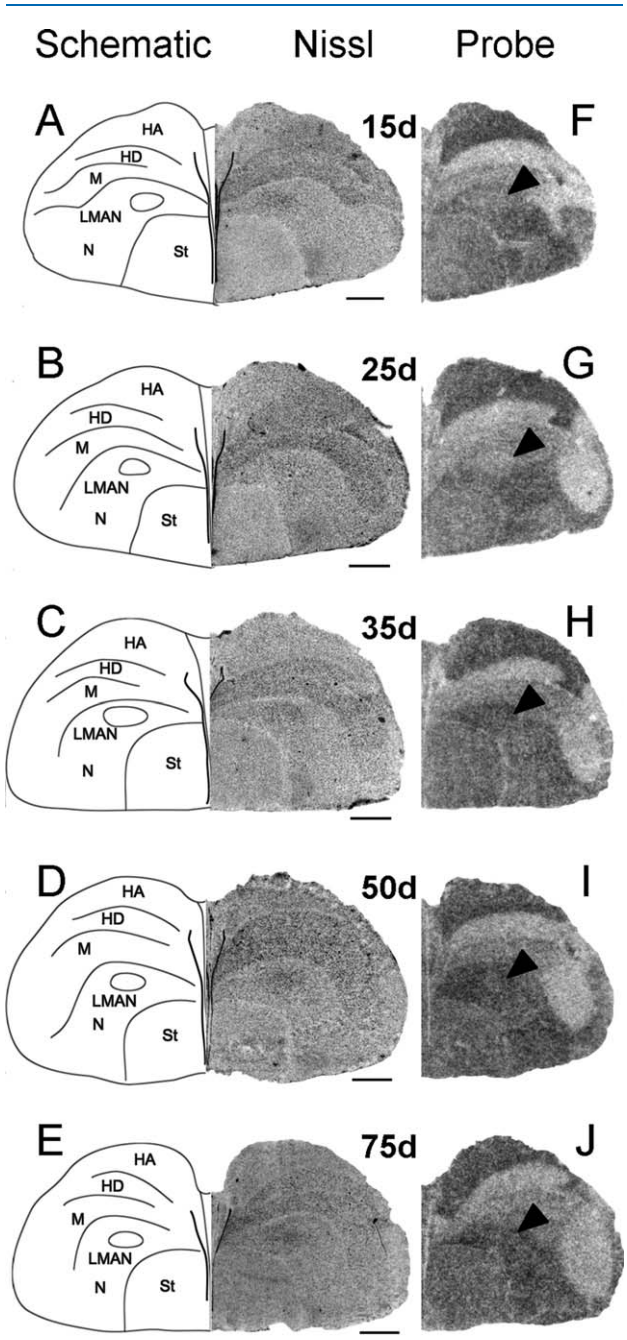

Figure 7. Developmental pattern of *Cntnap2* expression in hemicoronal sections of female anterior forebrain. From 15 to 75 days, *Cntnap2* signals in female LMAN (**F–J**) were comparable or slightly decreased to the abundant transcript levels present in the surrounding N. Female St was robustly labeled by the *Cntnap2* probe (F–J) and no attenuation of transcript hybridization signal was detected in the striatal region corresponding to male area X (which females lack). Arrowheads point to female LMAN as identified in A-E. For abbreviations, see Table 1. Scale bars = 1 mm.

circuitry, changes in *Cntnap2* transcript levels appear to track the structural and functional changes occurring within the song nuclei themselves, albeit with some distinctions, noted below.

Lateral magnocellular nucleus of the anterior nidopallium

Between 15 and 35 days, *Cntnap2* hybridization signals in both male (Fig. 6F–H) and female (Fig. 7F–H) LMAN

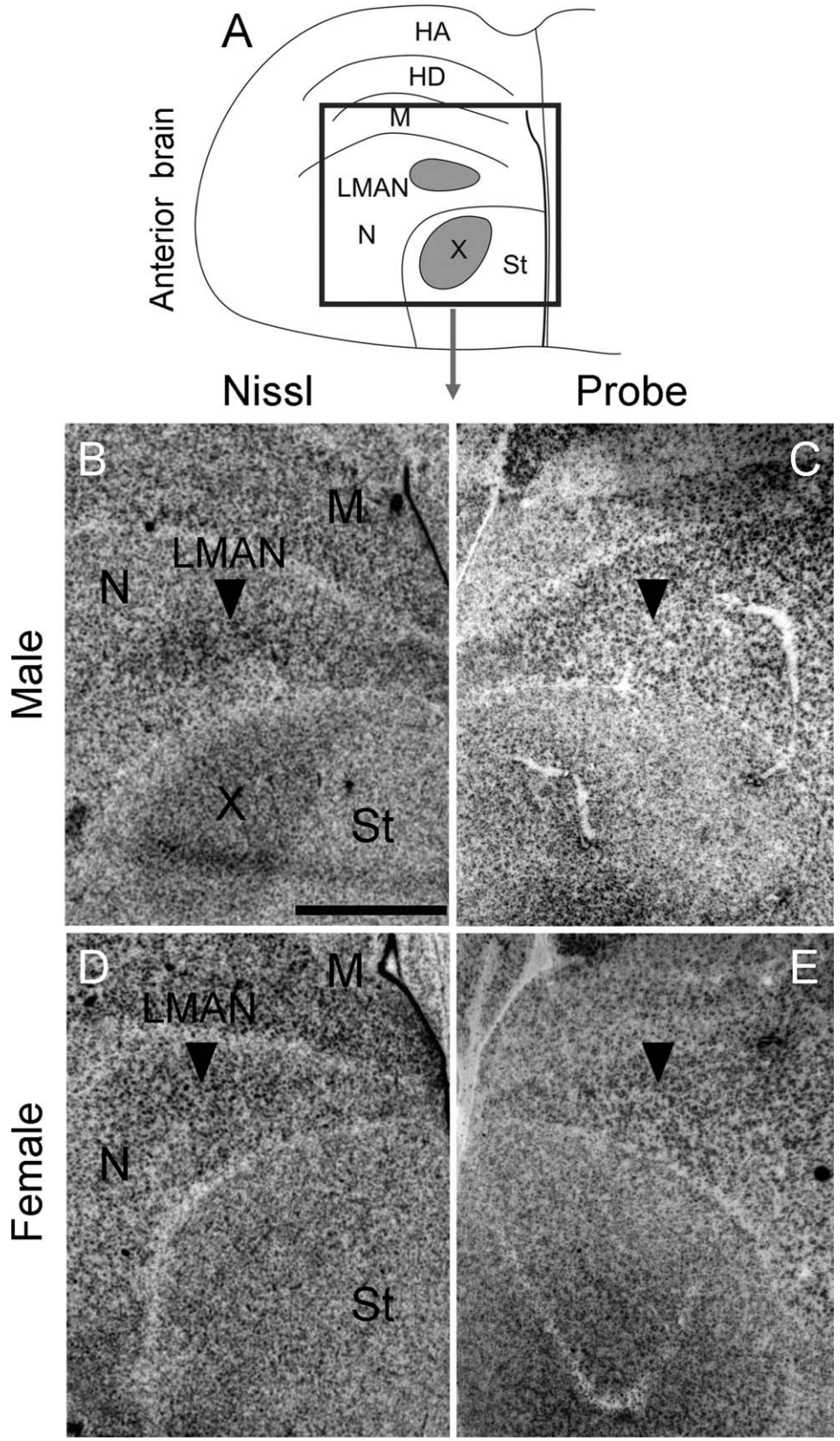

Figure 8. Inverted darkfield images of emulsion autoradiography sections confirm brightfield findings of *Cntnap2* hybridization in LMAN and area X. At 35 days *Cntnap2* emulsion grain signals in both male (**C**) and female (**E**) LMAN appeared slightly diminished or similar compared to transcript levels in the surrounding N. Attenuated labeling in male area X (C), a song nucleus that females lack (**D**), stood in contrast to transcript abundance in both male (C) and female (E) St. Arrowheads point to the location of male (C) and female (E) LMAN, as identified on adjacent Nissl-stained sections (B,D). For abbreviations, see Table 1. Scale bar = 1 mm.

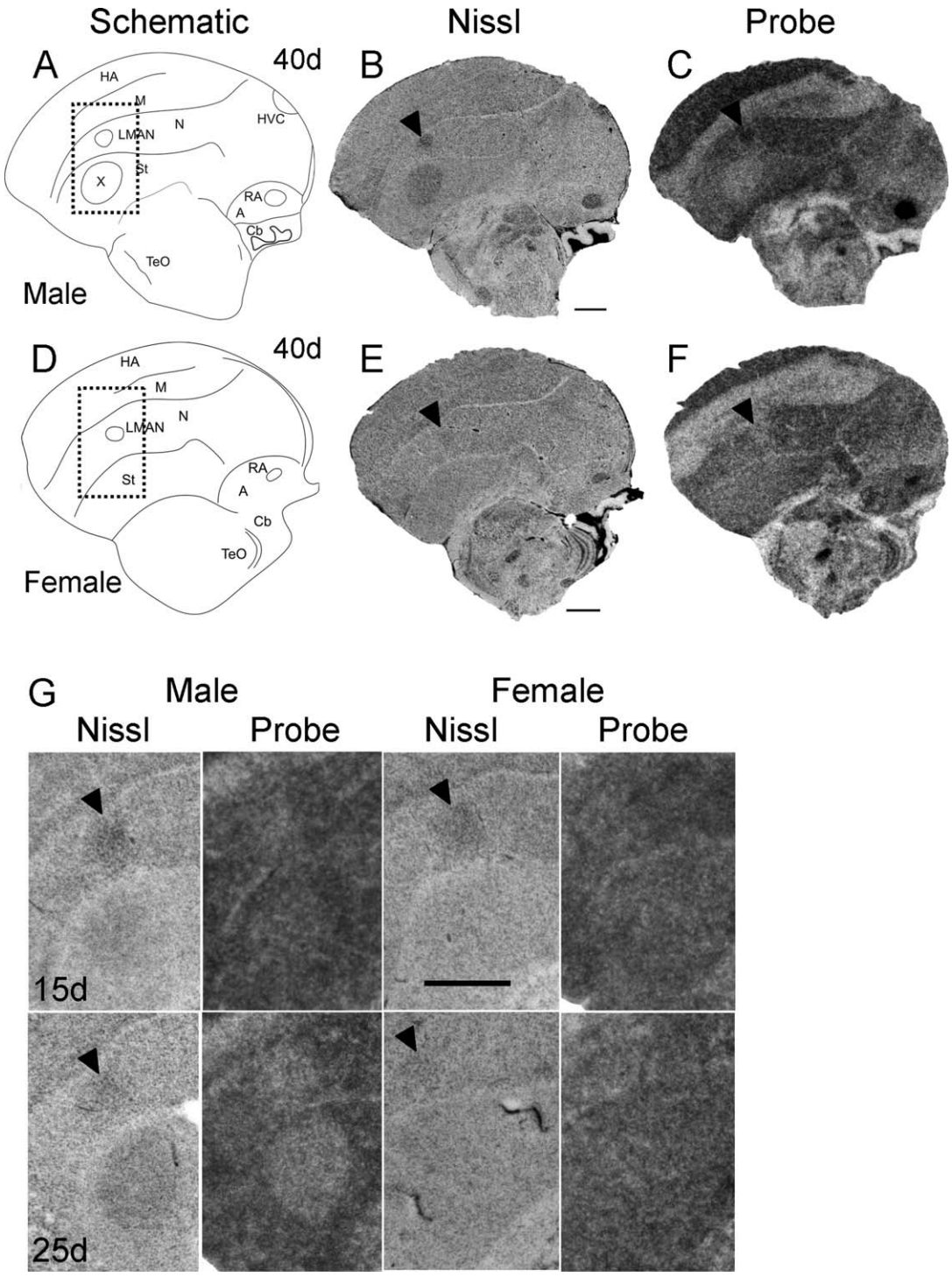

Figure 9. Onset of sexual dimorphism in *Cntnap2* expression in zebra finch song circuitry. At ≈40 days *Cntnap2* probe hybridization signals in male LMAN and RA (**C**) were enriched above those in the surrounding pallial regions, which were also robustly labeled. In male area X (**B**), low *Cntnap2* hybridization signal was in contrast to transcript abundance in surrounding St (C). Of note, although transcript was present in this region, *Cntnap2* levels were not enriched in male HVC (B) compared to surrounding pallial area (C). *Cntnap2* levels did not appear enriched in LMAN (**F**) of ≈40 days female and were uniformly distributed in the St which lacks area X (F). Probe signal was enriched in the smaller female RA (F) compared to surrounding A. At 15 and 25 days no sex differences in *Cntnap2* expression were detected in LMAN (**G**). In male song nucleus area X there is an indication of downregulation of *Cntnap2* transcript at 15 days which becomes more apparent at 25 days (G). Rectangles outlined with a dotted line in schematic drawings **A** and **D** indicate the region of higher magnification Nissl and autoradiography parasagittal images of developing male and female brain depicted in G. Arrowheads in B,C,E,F,G point to male and female LMAN. For abbreviations, see Table 1. Scale bars = 1 mm.

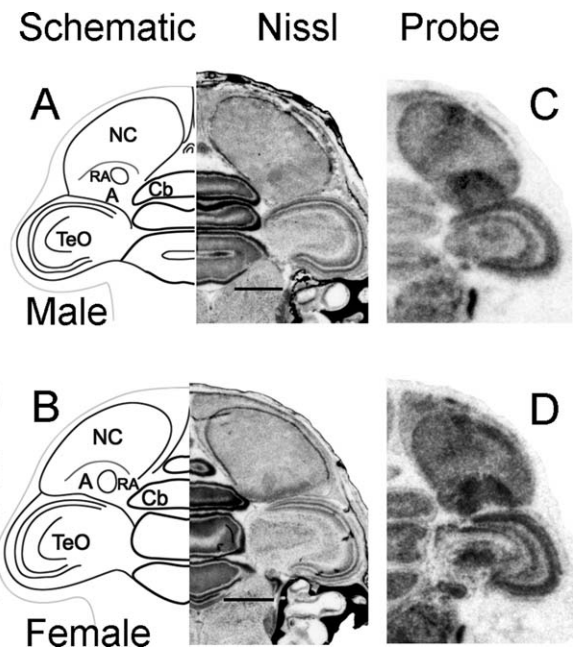

Figure 10. *Cntnap2* transcript is detected early in the development of song nucleus RA in zebra finch brain. At 6 days, *Cntnap2* hybridization signals in RA were enriched above those in A in hemicoronal sections of both male (**C**) and female (**D**) posterior brain, as identified on adjacent Nissl-stained sections (**A,B**). For abbreviations, see Table 1. Scale bars = 1 mm.

were indistinguishable from or appeared slightly lower (Fig. 9G) than those in the surrounding N, which was robustly labeled in both sexes. However, at 50 and 75 days Cntnap2 transcript levels in LMAN were enriched in males, with hybridization signals becoming even stronger than the surrounding N (Fig. 6I,J). In contrast, *Cntnap2* signals in female LMAN were virtually indistinguishable from surrounding N, indicating an absence of comparable transcript enrichment at the same timepoints (Fig. 7I,J). To increase the resolution of hybridization signal and potentially detect more subtle changes in *Cntnap2* transcript levels in male and female LMAN at 35 days, we performed emulsion autoradiography analyses. As with the film autoradiograms, no differences in emulsion grain labeling were detected when comparing hemicoronal sections that included male (Fig. 8C) and female (Fig. 8E) LMAN at this age.

To better define the age at which *Cntnap2* signals become enriched in males, we performed additional in situ hybridization analyses of parasagittal sections of ≈40-day male brain. By this timepoint, *Cntnap2* labeling of male LMAN had increased above the levels of surrounding N (Fig. 9C). This enrichment was not observed in parasagittal sections containing LMAN of a comparably aged female (Fig. 9F), providing evidence for an even earlier onset of sexual dimorphism of *Cntnap2* expression in LMAN than we had initially detected (i.e., 40 vs. 50 days).

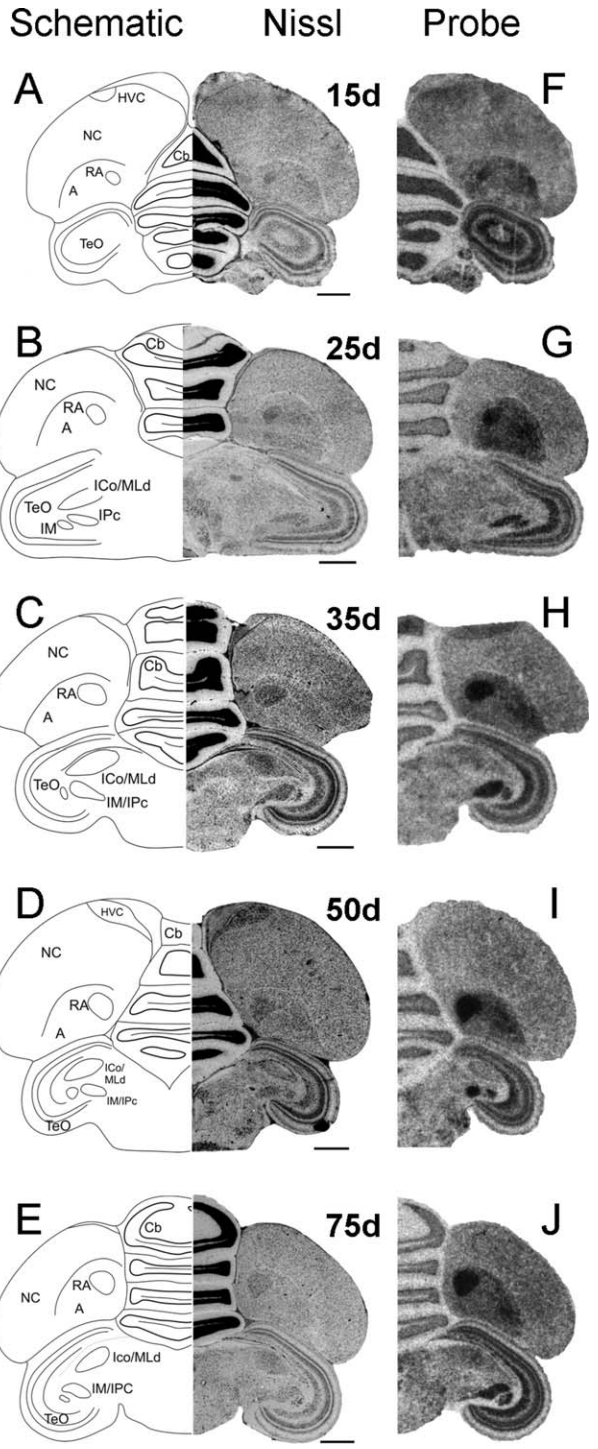

Figure 11. Developmental pattern of *Cntnap2* expression in male posterior brain. Throughout development, *Cntnap2* transcript distribution revealed a consistently robust hybridization signal in hemicoronal sections of male RA (**F–J**), as identified based on adjacent Nissl-stained sections (**A–E**). Strong *Cntnap2* signals were also present in Cb, TeO, and tectal nuclei IM/IPc (F-J). For abbreviations, see Table 1. Scale bars = 1 mm.

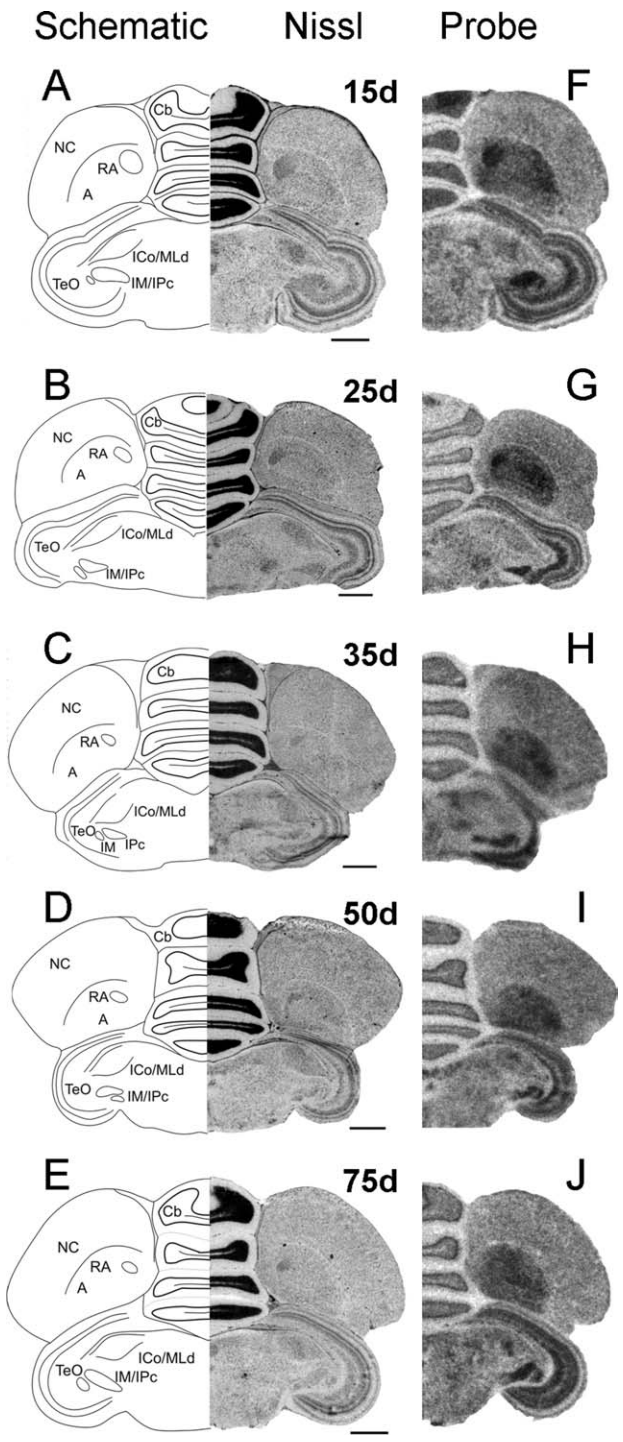

Figure 12. Developmental pattern of *Cntnap2* expression in female posterior brain. *Cntnap2* probe strongly labeled female RA from 15 days until ≈35 days (**F–H**). Thereafter, *Cntnap2* signals were diminished (**I,J**), rendering female RA indistinguishable from surrounding A, as identified based on adjacent Nissl-stained sections (**A–E**). Robust signals were also detected in the Cb, TeO, and tectal nuclei IM/IPc throughout development (F–J). For abbreviations, see Table 1. Scale bars = 1 mm.

This finding contrasted with the lack of sex differences in LMAN at earlier timepoints (15 and 25 days), seen in analyses of parasagittal sections of developing male and female brain (Fig. 9G).

Area X of striatum

From 15 to 75 days, *Cntnap2* levels in male area X appeared attenuated compared to the robust expression in the surrounding St (Figs. 6F–J, 9G). The reduced *Cntnap2* hybridization signals within area X were apparent as early as 15 and 25 days (Fig. 9G) and were especially evident after 35 days, rendering the contour of this song nucleus clearly discernible from the rest of St (Fig. 6I,J). In contrast, from 15–75 days, females (who lack area X) exhibited uniform and robust *Cntnap2* transcript labeling throughout St (Fig. 7F–J). As expected from film autoradiograms, emulsion grain signals reconfirmed the presence of low transcript levels in male area X, compared to surrounding St (Fig. 8C), and uniform transcript distribution within female St (Fig. 8E).

Robust nucleus of the arcopallium

Nissl-staining of coronal sections of male (Fig. 10A) and female (Fig. 10B) posterior brain allowed us to identify song nucleus RA as early as 6 days. At this age, *Cntnap2* signal in RA was elevated in both sexes compared to surrounding A (Fig. 10C,D), representing the earliest sign of *Cntnap2* enrichment detected within any song nucleus. At 15 and 25 days, *Cntnap2* hybridization signals were strong in both male (Fig. 11F,G) and female (Fig. 12F,G) RA. After 35 days, however, *Cntnap2* labeling of RA began to differ markedly between the two sexes. Between 35 and 75 days, strong enrichment of *Cntnap2* transcript persisted in male RA (Fig. 11H–J), in contrast to attenuation of *Cntnap2* levels in female RA (Fig. 12H–J), resulting in hybridization signals that were indistinguishable from those in the surrounding A. Emulsion autoradiographic signals confirmed these findings. At 15 and 35 days, and in the adult, strong hybridization signals were present in the enlarging male RA (Fig. 13B–D), in striking contrast to diminishing signals exhibited by the regressing female RA at the same timepoints (Fig. 13E–G).

Dorsal thalamic zone

In the diencephalon, *Cntnap2* transcript levels appeared relatively increased in the dorsal thalamic zone (DTZ), which contains the medial part of the dorsolateral thalamic nucleus (DLM), a key component of the song circuit. While specific enrichment could not be attributed to DLM itself (Fig. 14J), elevated signals were detected within DTZ, both in the developing brain, at 1 (Fig. 14F) and 6 days (Fig. 14H), and in the adult (Fig. 14L).

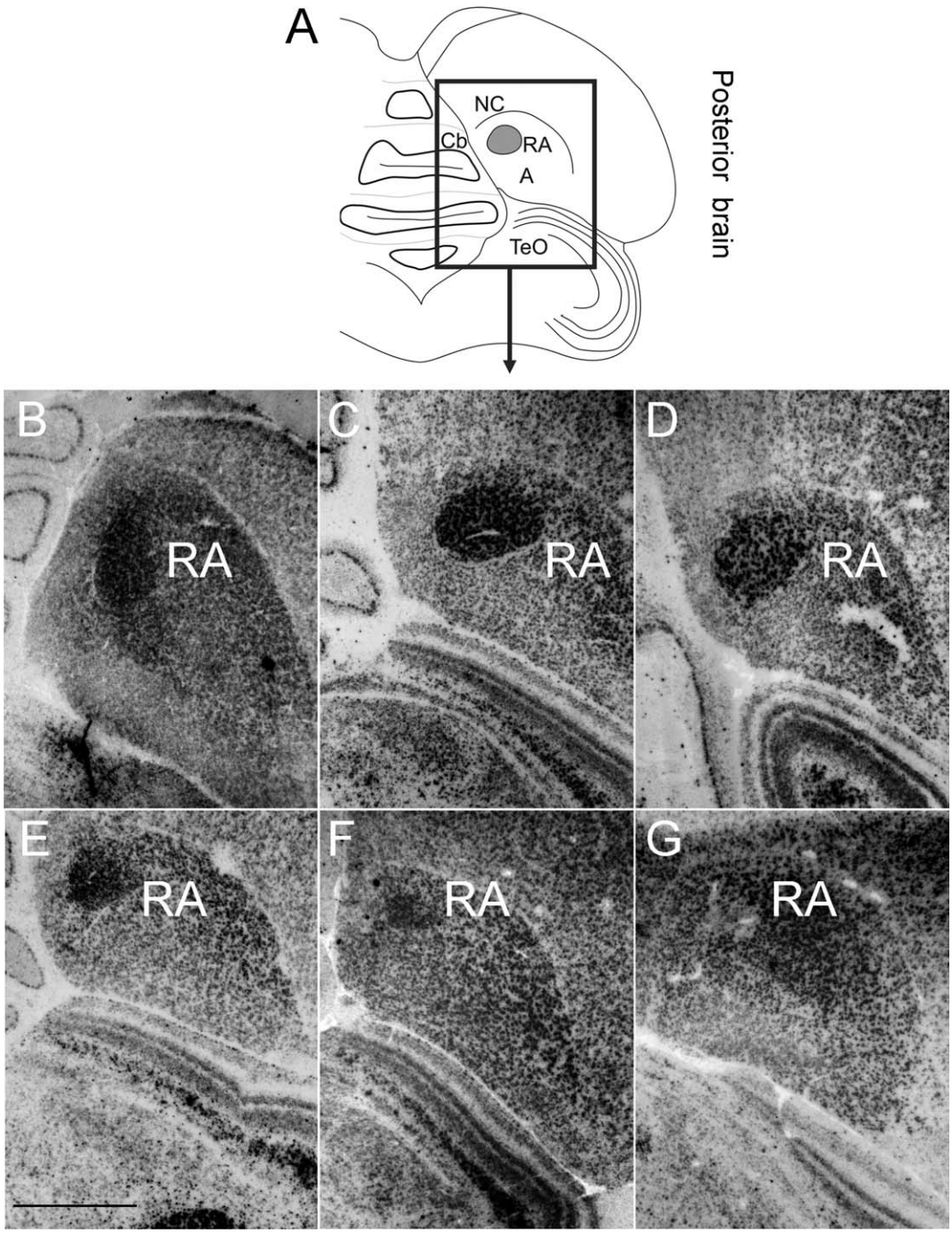

Figure 13. Inverted darkfield images of emulsion radiography signals confirm brightfield findings. In male RA, robust *Cntnap2* hybridization signal was detected at 15 (**B**) and 35 days (**C**) and in the adult brain (**D**). In contrast, in the female RA, hybridization signal diminished between 15 (**E**) and 35 days (**F**), with further attenuation in the adult brain (G). For abbreviations, see Table 1. Scale bar = 1 mm.

Song nucleus HVC

While *Cntnap2* signals were present in HVC, interestingly, there was no enrichment within this region in males, and transcript levels were similar to those of the surrounding N throughout development (Figs. 9C, 11F,I). This represents a notable exception to the punctuated expression of *Cntnap2* in the other male song nuclei. No enrichment was detected in the female HVC in either the developing or adult brain.

Cntnap2 expression outside the song system

Outside the song system, both during development and in the adult, *Cntnap2* transcript distribution in both

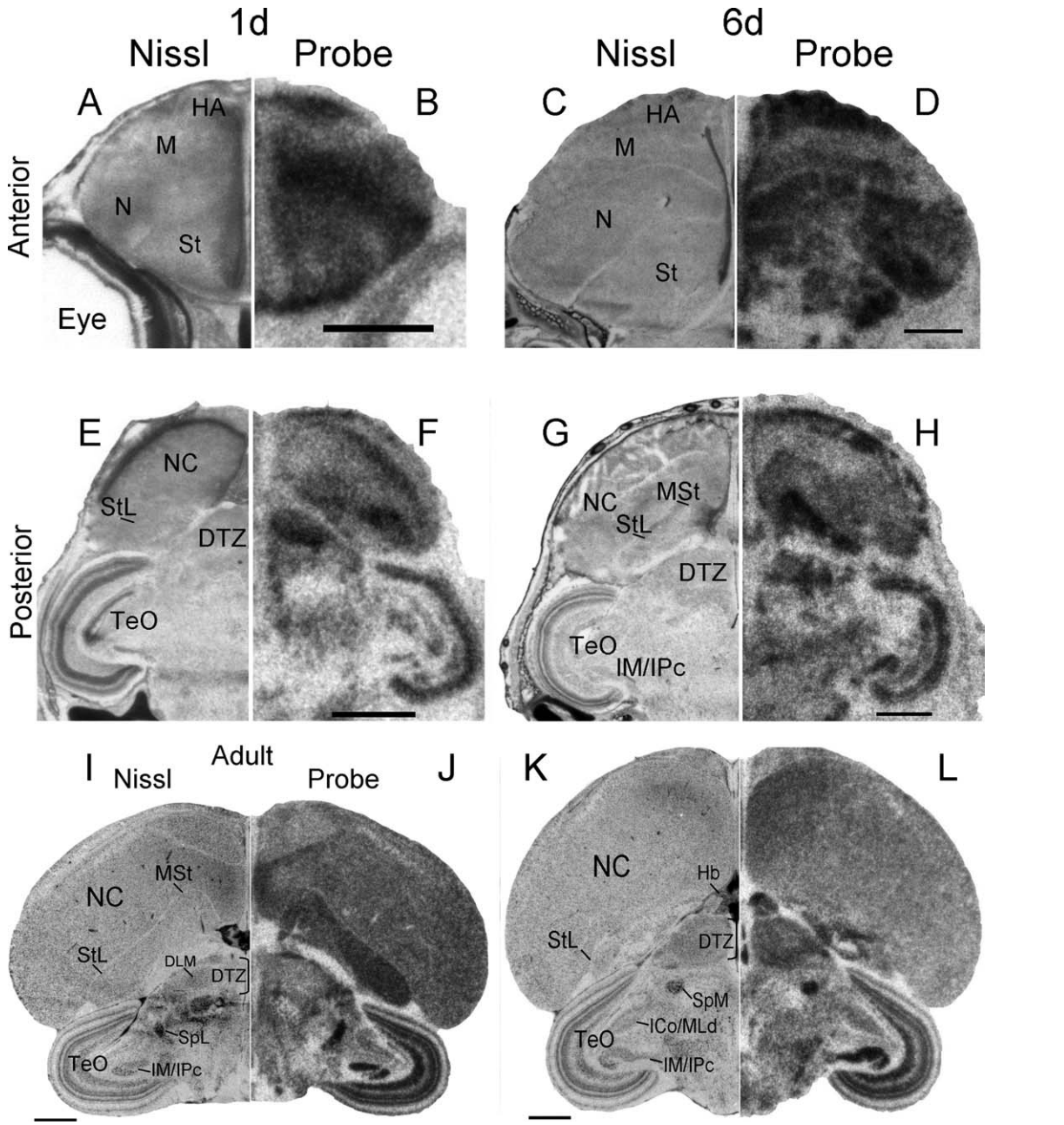

Figure 14. *Cntnap2* expression outside the song system in developing and adult male zebra finch brain. High levels of *Cntnap2* were detected early in development, in both anterior (**B**) and posterior (**F**) hatchling (1 day) brain, highlighting robust expression in HA, N, NC, St (including MSt and StL), DTZ, and TeO. A similar *Cntnap2* expression pattern was conserved in anterior (**D**) and posterior (**H**) male brain at 6 days. The same structures were labeled by *Cntnap2* probe in female brain (not shown). In the adult, *Cntnap2* hybridization signals were enriched in NC, MSt, StL, SpL (**J**), Hb, DTZ, and SpM (**L**), TeO and IM/IPc (J,L). Inverted darkfield images of emulsion autoradiograhy of posterior male brain revealed robust labeling of TeO layers 6, 8–10, and 13 and tectal nuclei IM and IPc (**O**), as well as in the Purkinje cell layer of Cb (**R**). Arrowheads in P and R point to the Purkinje cell layer. More attenuated emulsion grain signals were detected in the midbrain nuclei ICo/MLd (O). For abbreviations, see Table 1. Scale bars = 1 mm.

sexes was widespread, showing regional differences, but lacking focal enrichment or attenuation like that observed for the song system. In anterior brain, robust *Cntnap2* hybridization signals were detected in the apical part of the hyperpallium (HA), N, and St (including medial and lateral St), in both developing (Figs. 6F–J, 7F–J, 9C,F, 14B,D) and adult (Figs. 3B,D, 4C,F) birds. In posterior brain, *Cntnap2* transcript was robustly expressed in caudal N and A, in both developing (Figs. 10C,D, 11F–J, 12F–J, 14F,H) and adult (Figs. 3F,H, 4I,L, 14J,L) birds. Transcript

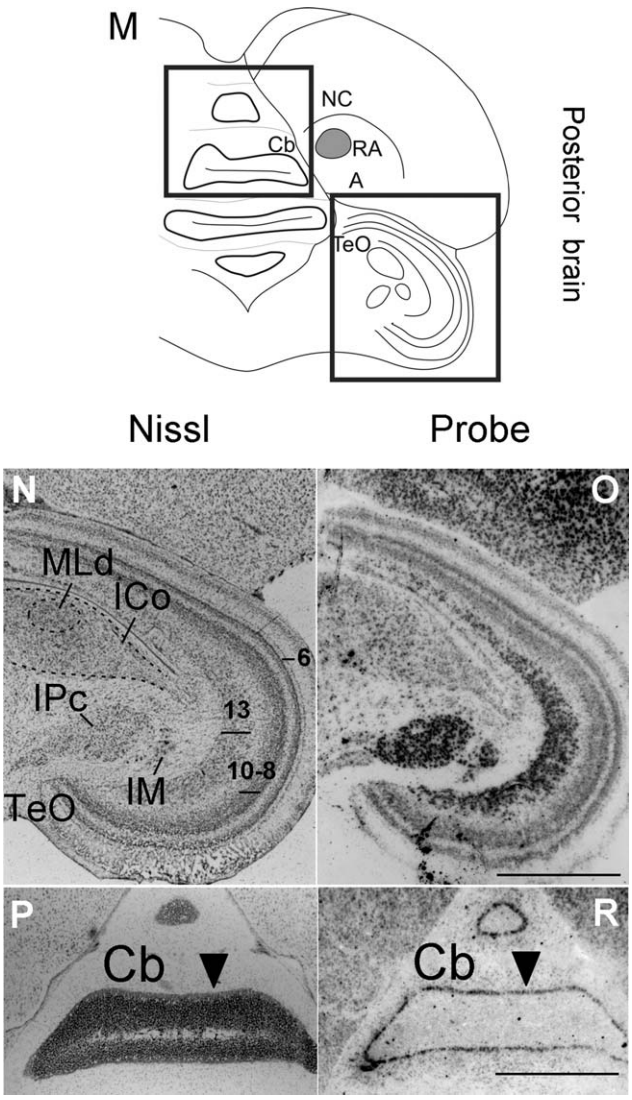

Figure 14. (Continued).

enrichment was also detected in habenula (Fig. 14L), cerebellum (Figs. 3F,H, 10C,D, 11F–J, 12F–J)—with strong signals in the Purkinje cell layer (Fig. 14R)—and optic tectum (Figs. 3F,H, 10C,D, 11F–J, 12F–J), with highest expression in layers 6, 8–10, and 13 (Fig. 14O).

Cntnap2 transcript enrichment was also detected in several midbrain structures dedicated to processing visual and auditory information, such as diencephalic lateral (SpL; Fig. 14J) and medial (SpM; Fig. 14L) spiriform nuclei, tectal nuclei isthmus magnocellular/parvocellular (IM/IPc; Figs. 3F,H, 11G–J, 12F–J, 14J,L,O) and, at lower levels, in the dorsal lateral mesencephalic nucleus within the nucleus intercollicularis (ICo/MLd; Figs. 3F,H, 11G–J, 12F–J). Posterior brain areas implicated in processing auditory input and feedback during song learning and production that comprise the auditory lobule did not appear to show altered *Cntnap2* signal compared to surrounding pallial areas (Fig. 4C,F).

DISCUSSION

Here we report an extensive analysis of *Cntnap2* transcript distribution in the adult zebra finch brain and during the development of the song control system. Consistent with genetic data from human providing support for a role in language performance and disorders in which language is compromised (Abrahams et al., 2007; Alarcón et al., 2008; Arking et al., 2008; Bakkaloglu et al., 2008; Vernes et al., 2008), *Cntnap2* shows punctuated expression within songbird brain subregions specialized for song learning and production.

Sexual dimorphism of *Cntnap2* expression in the adult

In the adult zebra finch, *Cntnap2* is differentially expressed in three of the male song control nuclei, consistent with the sexual dimorphism of these regions and of vocal learning itself (Nottebohm and Arnold, 1976; Gurney, 1982; Wade and Arnold, 2004). *Cntnap2* transcript distribution results in a sharp delineation of song control circuitry, highlighting both a song nucleus-specific enrichment (male lateral magnocellular nucleus of the anterior nidopallium, LMAN, and male robust nucleus of the arcopallium, RA) and attenuation (male area X) of transcript levels within the telencephalic components of the song control system. The functional connectivity and contribution to song learning and production of these nuclei has been studied extensively. While LMAN and area X are part of the AFP, a circuit required for song modification, RA is a key component of the posterior vocal pathway controlling song production (Nottebohm and Arnold, 1976). In young male zebra finches, lesions of male LMAN result in premature stereotypy of song, indicating a critical role for LMAN in song learning during development (Bottjer et al., 1984; Scharff and Nottebohm, 1991), while lesions of area X lead to highly variable and unstable song (Sohrabji et al., 1990; Scharff and Nottebohm, 1991). In contrast, ablation of area X in adults seems to have little effect on song production (Sohrabji et al., 1990; Scharff and Nottebohm, 1991), while lesions of LMAN prevent adult song plasticity (Williams and Mehta, 1999; Brainard and Doupe, 2000; Kao et al., 2005). Lesions of the posterior vocal pathway at either HVC or RA, at any time in life, cause severe disruption or even complete cessation of song (Nottebohm et al., 1976; Simpson and Vicario, 1990).

Enhanced *Cntnap2* expression is also detected in the male MMAN, a small nucleus medially adjacent to LMAN (Fig. 3B) but characterized by different connectivity via efferent projections to HVC (Nottebohm et al., 1982;

Bottjer, 1989; Vates et al., 1997) and afferent input from the posterior part of the DMP (Foster et al., 1997; Vates et al., 1997). Similar to LMAN, efferent projections to area X have also been described for MMAN (Vates and Nottebohm, 1995). Connections to pallial, striatal, and thalamic subregions implicated in song control suggest that MMAN plays a role in vocal behavior, as supported by lesion studies (Foster and Bottjer, 2001), but the exact function of this subregion has yet to be determined. In the adult female, the variability in *Cntnap2* levels observed in LMAN is puzzling (Fig. 3D vs. 5D,E), given that during development, transcript levels in female LMAN appeared consistent across the timepoints sampled here (see below). The possibility that elevated transcript levels in certain individuals may reflect an adult function for the female LMAN, e.g., in song perception related to mate choice (Hamilton et al., 1997), remains to be tested.

In the posterior pathway for song production, a notable exception to the delineation of song circuitry by *Cntnap2* was provided by premotor nucleus HVC, which showed no reliably detected differences in transcript levels compared to surrounding pallial areas. Homogenous expression levels across HVC and adjacent pallial regions is intriguing, given the striking modulation of hybridization signals detected for its targets, song nuclei RA and area X, and the marked sexual dimorphism of this song control region in adults (Nottebohm et al., 1976).

The restricted pattern of *Cntnap2* expression within mature song control nuclei occurred against a backdrop of conserved transcript distribution in the rest of male and female brain, within the regions of the apical part of the HA, N, caudal nidopallium (NC), A, St, cerebellum (Cb), and optic tectum (TeO). In both sexes, *Cntnap2* transcript enrichment was also detected within several structures involved in sensory processing, such as the intercollicular nucleus (ICo), which includes the dorsal part of the lateral mesencephalic nucleus (MLd), together known as ICo/MLd, the diencephalic lateral (SpL) and medial (SpM) spiriform nuclei, and the tectal nuclei isthmus magnocellular and isthmus parvocellular (IM/IPc). MLd, a homolog of the mammalian inferior colliculus (Karten, 1967), is a main relay site for sensory information carried by avian auditory ascending and descending pathways (Karten, 1967; Wild et al., 1993). In addition, in songbirds the shell of MLd receives dense projections from the RA cup (Mello et al., 1998), suggesting a role for this nucleus in sensorimotor integration of auditory inputs and motor commands originating in song control regions. While SpL has an established role in processing visual information via connections to TeO (Toledo et al., 2002), the function of SpM, which in songbirds is known to receive afferent inputs from dorsal A (Bottjer et al., 2000) and to send projections to Cb (Karten and Finger, 1976), remains to be established. Tectal IM and IPc represent important components of the avian visual system and have reciprocal connections with TeO layers (Wang et al., 2004). *Cntnap2* expression in these nuclei specialized for processing auditory and visual information, as well as the relay information to and from motor command centers, seem to implicate this gene in the functional specialization of structures important for feedforward and/or feedback of sensorimotor information.

Developmental expression of *Cntnap2* in song control regions

Consistent with largely monomorphic developmental changes in LMAN between 15 and 35 days (Bottjer et al., 1985; Nixdorf-Bergweiler, 1996), levels of *Cntnap2* transcript in male and female LMAN were similar to or appeared slightly lower than those in the surrounding region of N. While LMAN borders cannot be reliably distinguished on Nissl-stained sections before 10 days (Bottjer et al., 1985; Nixdorf-Bergweiler, 1996), between 15 and 25 days male and female LMAN appear to be comparably sized (Nixdorf-Bergweiler and Von Bohlen und Halbach, 2005) and undergo accelerated growth until they reach peak volume, at ≈20 and ≈30 days, respectively (Bottjer et al., 1985; Nixdorf-Bergweiler, 1996). This time interval marks the onset of the sensory acquisition phase of song learning and precedes the stage when young male zebra finches begin to produce their first learned vocalizations (Immelmann, 1969). Starting at 30–35 days, males enter the vocal practice or sensorimotor stage of song learning (Immelmann, 1969; Price, 1979), during which LMAN has been shown to take on a significant role (reviewed in Brainard and Doupe, 2002), and exhibit premotor activity responsible for driving the production of plastic juvenile song (Aronov et al., 2008).

After reaching peak volume, both male and female LMAN begin to regress. Male LMAN shrinks to half of its original volume by ≈50 days and female LMAN regression follows with a delay of ≈10 days (Bottjer et al., 1985; Nixdorf-Bergweiler, 1996). In the male, developmental changes after ≈30 days have been attributed to processes of cell death (Bottjer et al., 1985; Bottjer and Sengelaub, 1989; Korsia and Bottjer, 1989) and subsequent enlargement of remaining LMAN neurons (Nixdorf-Bergweiler, 1998), as well as refinement of afferent (from DLM; Iyengar et al., 1999; Iyengar and Bottjer, 2002a) and efferent (to RA; Herrmann and Arnold, 1991; Iyengar et al., 1999) connections. The final stage of regression of male LMAN also coincides with the time when lesions of this forebrain nucleus begin to lose their effectiveness in disrupting vocal learning (Bottjer et al., 1984). Given that cell loss in male LMAN appears complete by 37 days (Korsia and Bottjer, 1989), the increase in *Cntnap2* levels

in this song nucleus at ≈40 days onward is likely explained by increased gene expression in surviving LMAN neurons and appears to track the developmental switch in the function of this song nucleus. In contrast, during the development of female LMAN, *Cntnap2* transcript levels did not become enriched relative to surrounding N. While this is consistent with the behavioral difference in song learning between the two sexes, as noted above, there was some variability in the levels of gene expression in the adult female LMAN.

In our study, area X was first identified on Nissl-stained coronal sections of male brain at 15 days and showed reduced expression of *Cntnap2* at all posthatch developmental timepoints investigated (15–75 days). Despite the enlargement of this song nucleus from 15 days until the adult size is attained at ≈40–50 days (Bottjer et al., 1985; Nixdorf-Bergweiler, 1996), no corresponding increases in transcript levels were detected. Attenuated *Cntnap2* levels in area X throughout development contrasted sharply with transcript abundance in the surrounding region of St. The lower signal in area X (Fig. 8C) is intriguing since the cell packing density there, as evidenced by denser Nissl stain (Nottebohm et al., 1976), is higher than in the outlying striatum. Assuming equal expression levels per cell, one would expect increased signal in area X. Reduced expression in area X relative to surrounding regions (which contain similar cell types; Farries and Perkel 2000, 2002) suggests that low transcript levels may be important for the development of this striatal nucleus. While the sexual dimorphism in area X may be expected, given the absence of this song nucleus in female zebra finches, the contrast in *Cntnap2* transcript between area X and adjacent tissue is striking.

As early as 6 days, *Cntnap2* signals were elevated in RA compared to surrounding A, and were even more robust by 15 days in both sexes. Consistent with a lack of significant sex differences in volume and comparable growth rates of this song nucleus in both sexes in the first 3 weeks posthatch (Bottjer et al., 1985; Nixdorf-Bergweiler, 1996), strong *Cntnap2* signals were detected in both male and female RA at 25 days. By ≈20 days, female RA is expected to have reached its peak volume (Bottjer et al., 1985; Nixdorf-Bergweiler, 1996). Following 20 days, male RA continues to enlarge and reaches its maximum volume by ≈50 days (Bottjer et al., 1985; Nixdorf-Bergweiler, 1996) while female RA undergoes a dramatic regression (Konishi and Akutagawa, 1985; Bottjer et al., 1985; Nixdorf-Bergweiler, 1996), culminating in shrinkage to about half of its maximum volume around the same time (Bottjer et al., 1985; Nixdorf-Bergweiler, 1996). Importantly, at ≈35 days, nerve fibers originating from HVC synapse onto RA neurons in male, but not female, zebra finches, resulting in the functional activation of the brain pathway specialized for song production (Konishi and Akutagawa, 1985; Mooney and Rao, 1994; Holloway and Clayton, 2001).

Reflecting these developmental differences, *Cntnap2* labeling of RA began to differ markedly between the two sexes at ~35 days. In males, *Cntnap2* levels were enriched in RA, compared to surrounding A at all timepoints investigated, matching the developmental enlargement (Bottjer et al., 1985; Konishi and Akutagawa, 1985; Nixdorf-Bergweiler, 1996) and predicted increase in synaptic connectivity (Konishi and Akutagawa, 1985; Mooney and Rao, 1994) of the nucleus. In contrast, *Cntnap2* signals in female RA became diminished by 35 days and appeared virtually indistinguishable from those of the surrounding A at 50 and 75 days, likely reflecting the substantial developmental regression and associated processes of cell death (Konishi and Akutagawa, 1985) occurring during this time interval. Thus, the divergent developmental trajectories of male and female RA is accompanied by changes in *Cntnap2* levels (enrichment in male and attenuation in female) that persist into adulthood.

Role of *Cntnap2* in the development and function of song circuitry

In the zebra finch songbird species the sexual dimorphism of song circuitry is tightly linked to behavioral sex differences (males sing a courtship song, females do not), making birdsong an advantageous model to understand both the neuroendocrine and genetic bases for the emergence of sexually dimorphic brain structures and vocal learning (Nottebohm and Arnold, 1976; Gurney and Konishi, 1980; Gurney, 1982; Wade and Arnold, 2004). In the case of steroid-related genes, the androgen receptor (AR) is highly enriched in male song nuclei, both in the developing and adult brain (Kim et al., 2004). Enrichment of the AR transcript (and androgen binding; Arnold et al., 1976) within the male song system and its absence in corresponding areas in the female brain provided compelling evidence for the role of hormones in the sexual differentiation of the steroid-sensitive song circuitry.

To date, most gene expression analyses in songbird brain have focused on revealing the cellular and molecular bases of the specialized ability of song-related circuitry to subserve the capacity for learned vocalizations (see for review White, 2009). Thus, the dynamic expression in male song nuclei has been reported for "motor-driven" genes whose levels change during singing, first exemplified for the immediate early gene *zenk* (*zif*-268, *egr*-1, *NGFI*-A, and *krox*-24; Jarvis and Nottebohm, 1997) and now shown for several other genes (Poopatanapong et al., 2006; Wada et al., 2006).

The investigation of language-related genes in song control circuitry represents a powerful and complementary

approach to using the specialized anatomy of the song control system to identify molecules important for vocal learning. For example, study of the transcription factor FOXP2, known to be disrupted in a severe speech and language disorder (Lai et al., 2001), as well as its downstream target *CNTNAP2*, in the zebra finch song circuitry (Haesler et al., 2007; this study), provide a special opportunity for dissecting the genetic bases of learned vocalization, including song and speech. We previously showed that zebra finch *FoxP2* has a parallel expression pattern to human *FOXP2* and overlapping distribution with *FoxP1* (Teramitsu et al., 2004), encoding a related FoxP subfamily member with which FoxP2 can interact to bind DNA promoter regions (Shu et al., 2001; Carlsson and Mahlapuu, 2002; Li et al., 2004). The finding that *FoxP1* is expressed in a sexually dimorphic pattern within zebra finch song circuitry (Teramitsu et al., 2004) lent support to the idea that, like *FoxP2*, *FoxP1* may also play a role in vocal learning and could contribute to altered development and function in human language-related disorders (Teramitsu et al., 2004). This prediction has recently received support in the discovery of a de novo chromosomal deletion of only the *FOXP1* gene resulting in haploinsufficiency that was associated with severe language impairment in a young patient diagnosed with Chiari I malformation and other congenital physical abnormalities (Abdul-Rahman et al., 2008).

Cntnap2 and language-related neuropathology

The mechanisms whereby disruption of the *Cntnap2* gene results in neural dysfunction remain largely unknown. Both rare and common genetic variation in *Cntnap2* have been implicated in a number of neural diseases, as diverse as autism spectrum disorder (Alarcón et al., 2008; Arking et al., 2008; Bakkaloglu et al., 2008), specific language impairment (Vernes et al., 2008), Gilles de la Tourette syndrome (Verkerk et al., 2003), schizophrenia (Friedman et al., 2007), attention-deficit hyperactivity disorder (Elia et al., 2009), cortical dysplasia-focal epilepsy syndrome (Strauss et al., 2006; Jackman et al., 2009) and intellectual disability (Zweier et al., 2009; Poot et al., 2009). *Cntnap2* has an identified role as a scaffold protein at the juxtaparanodal regions of myelinated axons (Poliak and Peles, 2003). However, the focal enrichment of *CNTNAP2* in language-related structures and circuits in human occurs prior to pre-myelination mid-gestation stage (Abrahams et al., 2007). This raises the possibility that other aspects of human brain development that precede or are unrelated to myelination could be modulated by this molecule. In songbirds, the only available data on this topic suggest that myelin is absent in axonal processes of HVC and LMAN neurons at 20 days and that myelination starts shortly after 20 days in LMAN (Nixdorf-Bergweiler and Von Bohlen und Halbach, 2004). Given the strong *Cntnap2* expression before 20 days in song nuclei and the rest of the brain, it appears that, as suggested in the human, there might also be additional developmental roles for *Cntnap2* in songbirds.

Our discovery of punctuated expression for *Cntnap2* within song control regions reinforces the importance of the songbird model in exploring the biological roles of genes disrupted in language and communication disorders. Understanding the genetic bases of vocal learning may ultimately reveal both shared and unique neuromolecular networks that have emerged in the evolution of learned vocal behavior.

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