Published in final edited form as:

Dev Neurobiol. 2009 February 1; 69(0): 124–140. doi:10.1002/dneu.20686.

Conservation and expression of IQ-domain-containing calpacitin gene products (Neuromodulin/GAP-43, Neurogranin/RC3) in the adult and developing oscine song control system

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Abstract

Songbirds are appreciated for the insights they provide into regulated neural plasticity. Here we describe the comparative analysis and brain expression of two gene sequences encoding probable regulators of synaptic plasticity in songbirds: Neuromodulin (GAP-43) and Neurogranin (RC3). Both are members of the calpacitin family and share a distinctive conserved core domain that mediates interactions between calcium, calmodulin and protein kinase C signaling pathways. Comparative sequence analysis is consistent with known phylogenetic relationships, with songbirds most closely related to chicken and progressively more distant from mammals and fish. The C-terminus of Neurogranin is different in birds and mammals, and antibodies to the protein reveal high expression in adult zebra finches in cerebellar Purkinje cells, which has not been observed in other species. RNAs for both proteins are generally abundant in the telencephalon yet markedly reduced in certain nuclei of the song control system in adult canaries and zebra finches: Neuromodulin RNA is very low in RA and HVC (relative to the surrounding pallial areas), whereas Neurogranin RNA is conspicuously low in Area X (relative to surrounding striatum). In both cases, this selective down-regulation develops in the zebra finch during the juvenile song learning period, 25–45 days after hatching. These results suggest molecular parallels to the robust stability of the adult avian song control circuit.

Keywords

GAP-43; Neuromodulin; critical period; canary; zebra finch; song control system; RA; Area X; HAT-14; songbird; Neurogranin; RC3; nrgn; neural plasticity; IQ domain; calpacitin

INTRODUCTION

Songbirds are widely appreciated as models for the study of regulated neural plasticity (for a large collection of reviews, see (Zeigler and Marler, 2004). Song production is under the control of a neural circuit in the forebrain comprising two major pathways connecting large

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discrete brain nuclei that are unique to songbirds and apparently dedicated to song control. In the zebra finch (the dominant species in laboratory studies), this pathway is completed only during adolescence in males, just before the young bird begins to produce his own vocalizations. He then learns to sing through a process of trial and error, approximately copying the song of an adult tutor. Once learned, his song does not change for the rest of his life. Canaries provide a contrasting example, in which song production and structure vary with the seasons. There is great interest in identifying molecular control points for these various processes.

Here we assess the molecular conservation and expression in the song control system of a pair of well-established regulatory neuronal proteins that integrate multiple pathways associated with neuronal growth and synaptic plasticity in other vertebrates. One protein is known as Neuromodulin (NEUM), also called GAP-43, B-50 and F1 (Gispen et al., 1986; Benowitz and Routtenberg, 1987; Karns et al., 1987; Wakim et al., 1987). In numerous vertebrate species, NEUM has been shown to be a “growth-associated” presynaptic protein (Skene, 1989). The other protein is known as Neurogranin (NRGN), also called RC3 (Watson et al., 1990; Baudier et al., 1991), and has been shown to be critical for normal calcium-dependent synaptic plasticity (Fedorov et al., 1995; Krucker et al., 2002; Zhabotinsky et al., 2006). In contrast to NEUM, NRGN is concentrated in postsynaptic terminals and cell bodies.

Despite their differing subcellular distributions, both NEUM and NRGN share a similar structure and are distinguished by the presence of a distinctive, highly conserved “IQ” domain which is a signature motif for interactions with Calmodulin and other Ca^{++} -binding proteins (Cimler et al., 1985; Deloulme et al., 1991; Apel and Storm, 1992; Cheney and Mooseker, 1992). Additionally, both proteins are substrates for phosphorylation by Protein Kinase C (PKC) (Apel et al., 1990; Baudier et al., 1991), and calcium and PKC signaling pathways both converge and compete at the level of the IQ domain (Chakravarthy et al., 1999). Both proteins appear to function as regulated buffers or capacitors for local synaptic calcium fluxes, hence the name “Calpacitins” has been proposed for the protein family (Gerendasy, 1999).

We begin with a formal characterization of the NEUM and NRGN protein coding sequences in songbirds (canaries and zebra finches), and continue with a survey of their distributions in the major song control nuclei and surrounding telencephalon. In the context of the songbird model, our analysis bears on several fundamental questions. Are these regulatory proteins conserved in songbirds? Are they expressed in the nuclei of the song control circuit in the adult? Are they coordinately regulated to support the timed development of the song control system? Are they suppressed in adulthood, when the song control system is highly stable? Conversely, might they be elevated during juvenile song learning when the song control pathway is actively developing?

Results

Cloning and Comparative Sequence Analyses

Canary sequences for both NEUM and NRGN sequences were identified in screens of a cDNA library prepared from forebrain (George and Clayton, 1992). We specifically searched for NEUM by cross-hybridization using probes prepared from the rat ortholog (Karns et al., 1987; Jin et al., 1994). The NRGN sequence emerged from a differential screen for genes regulated in song control nucleus HVC and was originally called HAT-14 and also referred to as canarigranin (George, 1993; Siepka et al., 1994). Zebra finch orthologs of these sequences emerged from a more recent high-throughput brain mRNA sequencing project (Replogle et al., 2008). Chicken sequences have also emerged from various EST and genome sequencing projects. Sequences of these and other orthologs of interest (Table 1) are shown in Figure 1 (NEUM) and Figure 2 (NRGN).

Comparing across vertebrates, the NEUM peptide sequence is modestly conserved overall, but contains a domain near the N-terminus with a core of 20 residues that are perfectly conserved in birds, fish and mammals and includes the defining IQ motif (Fig. 1A). Around residue 120 is a small sequence chunk that has been deleted in mouse and rats relative to other mammals, but it is present in all three birds. Though there is considerable species variability in the C-terminus of the protein, it is almost identical among the three birds. A global representation of sequence relatedness is shown using the phylogram output of CLUSTALW, which places the two songbirds closest together with the chicken nearby and the other amniotes more distant, and the zebra fish more distant still (Fig. 1B). The distance separating chicken and songbirds is similar to the distance separating rodents and humans, consistent with other phylogenetic evidence (Clayton, in press).

NRGN is a much shorter peptide (Fig. 2A), with a central region of high conservation that includes the IQ domain and a variable C terminus. The original canary sequence, deposited as “canarigranin” in Genbank in 1996, was somewhat perplexing as the C terminus varied so much from the other neurogranin sequences available at the time. In particular, it lacks the high concentration of prolines and glycines found in mammals and it was truncated by 5 residues relative to human and rodent, making a short protein even shorter. The alignment here with chicken and zebra finch (Tagu) supports these points of divergence, showing they hold true for all three birds. Moreover, the C-terminus is even more dramatically shorter in the zebra fish. CLUSTALW analysis of NRGN (Fig. 2B) supports the same approximate phylogenetic relationships as for NEUM.

The IQ domains from avian NEUM and NRGN are aligned in Figure 2C, noting the conserved residues identified in other species as responsible for (1) binding to calmodulin, (2) phosphorylation by PKC, and (3) binding to phosphoinositide 3-kinase lipid products in NRGN (Lu and Chen, 1997).

NEUM RNA expression in the adult canary brain

To survey the pattern of NEUM expression in adult male canary brain, ³⁵S-labeled riboprobes were synthesized from the canary NEUM clone and used to probe tissue sections by in situ hybridization (Clayton et al., 1988). A film autoradiogram (Fig. 3A) demonstrates

the general distribution of the NEUM mRNA in a parasagittal section of a canary sacrificed in spring, when song control nuclei are large and singing behavior is robust. Hybridization signal is intense throughout the telencephalic pallial subdivisions, including the arcopallium, nidopallium and mesopallium, areas considered homologous to mammalian pallial areas that include the cerebral cortex. A general enrichment of NEUM expression in cortical regions of the telencephalon has also been observed in other species (Benowitz et al., 1988). Signal is also high in the granule cell layer of the cerebellum, and especially intense in the midbrain's nucleus pretectalis.

Within the caudal telencephalon of canaries, two nuclei stand out visibly as islands of much lower NEUM mRNA abundance (Fig. 3A, see also Table 2). By histological examination of the counterstained tissue sections, we confirmed that these two islands correspond to the song nuclei HVC and RA, in the nidopallium and arcopallium respectively. A third telencephalic song nucleus, the magnocellular nucleus of the anterior nidopallium (IMAN), shows intense labeling equivalent to the highest signals observed elsewhere in the telencephalon (Fig. 3). The fourth major song nucleus in the telencephalon, Area X, is present but not visible in the autoradiograms shown, as hybridization signal for NEUM is equivalent within Area X and in the surrounding medial striatum. Histological examination confirmed that Area X is present in these sections. A preliminary examination of canaries sacrificed in October (when singing behavior is at its lowest) did not reveal any evidence of a seasonal difference in NEUM expression (data not shown).

Hybridized sections were also processed for emulsion autoradiography and microscopy, and examined for the labeling pattern of cells in and immediately adjacent to nucleus RA (Fig. 4). The level of background labeling is very low in these experiments as indicated by hybridizations using sense-strand control probes in adjacent sections (Fig. 4A). Weak but significant cellular labeling is observed within the interior of RA (Fig. 4B), whereas intense accumulations of silver grains occur over cell bodies in the arcopallium that surrounds RA (Fig. 4C). Thus, even though the signal is much lower inside RA, there is detectable expression of NEUM in RA cells. In general, labeled cell bodies are large with a pale-staining nucleus, features consistent with a neuronal identity.

Developmental regulation of NEUM in the zebra finch brain

The zebra finch provides a contrast to the canary as it is a “critical period” learner and the stages of juvenile song circuit development are defined (Immelmann, 1969; Konishi and Akutagawa, 1985; Holloway and Clayton, 2001). To assess expression in the zebra finch brain, we first established that the canary probe would cross-hybridize effectively. Probes made from the canary cDNA hybridized specifically to a band of appropriate size (migrating at ~1500 nucleotides) in forebrain RNA extracts from zebra finches. In juvenile zebra finch brain, the NEUM RNA is most abundant during the week after hatching, and drops to a lower but still significant level when the birds are about two weeks old (Fig. 5A). This developmental difference was not observed on parallel blots probed for other RNAs including synelfin (George et al., 1995) and NRG1 (Fig. 5B). A similar post-natal decrease in NEUM mRNA abundance occurs in mammalian brain (Jacobson et al., 1986).

To learn whether NEUM gene expression changes in song nuclei during the period of song learning and circuit development in juvenile zebra finches, in situ hybridizations were performed using brain sections taken from males at various ages, beginning at posthatch day 15 (P15). The ages chosen correspond to the beginning of the song model acquisition phase (P15, P25), the ingrowth of fibers from HVC into RA and the start of song production (P35), the middle of the vocal learning period (P45), late in the vocal learning period (P60), shortly after song crystallization (P110), and adults (>P120). At all ages examined, the pattern of NEUM mRNA distribution was very similar in most parts of the brain. A representative comparison of P15 and P110 sections is shown in Fig. 6 (see also Table 2). The anatomical distribution of NEUM mRNA is almost identical at the two ages (Fig. 6A and 6B) with one major exception: NEUM is abundant within nucleus RA at P15, but not at P110 or older. The high signal for NEUM within RA at P15 is clearly due to higher cellular labeling, as determined by examination of the sections using digoxigenin-labeled (Fig. 6C and 6D) or radioactively-labeled probes and emulsion autoradiography (not shown). Large cell bodies with intense NEUM mRNA staining are abundant throughout the nucleus in the younger birds and virtually absent at P110. This observation suggests that NEUM expression in RA may be mostly related to the development of projection neurons, but we cannot exclude its expression in the smaller interneurons as well.

A timecourse of the change in NEUM expression in RA was established using computerized densitometry to quantify autoradiographic film images from birds at different ages. The RNA is equally abundant at both P15 and P25 but then begins to decline significantly by P35, and reaches an apparent minimum in RA by P45 which extends into adulthood (Fig. 7). We also quantified signal over HVC, IMAN and Area X in these same autoradiograms but did not observe any significant developmental changes in these nuclei, nor did we observe changes at these ages in expression of mRNAs for Synelfin, NRGN, HAT-2 (n-chimaerin), or Myelin Basic Protein (not shown).

Thus NEUM mRNA specifically declines in nucleus RA at approximately 35 days of age, in male zebra finches. This closely corresponds in time to the formation of synaptic connections between HVC axons and RA neurons, which occurs only in males. To determine whether the developmental change in NEUM mRNA is also a male-specific event, we performed in situ hybridizations with digoxigenin-labeled probes on sections from female zebra finches at P25 and P45, flanking the time of transition in males. The same pattern of developmental regulation of NEUM mRNA was observed in these female brains as in the male brains: strongly labeled cells are abundant within RA at P25, and virtually absent at P45 (Fig. 8).

NRGN RNA expression in canary and zebra finch brain

The canary NRGN (HAT-14) cDNA probe detects a major band of ~1000 bases on Northern blots of both canary and zebra finch forebrain RNA (Fig. 5B); this transcript was not detected in canary lung, kidney, testis or heart (not shown). By in situ hybridization to sections of adult male canary brain, signal is generally high throughout the telencephalon, particularly in pallial regions as compared to the striatum, and in the Purkinje cell layer of the cerebellum (Fig. 9A). In contrast, expression in the song nucleus Area X is distinctly

lower than surround. The in situ pattern in adult zebra finch is grossly similar to that in adult canary, with general telencephalic (pallial) enrichment, strong expression in cerebellar Purkinje cells, and relative depletion in the song control nucleus Area X (Fig. 9C). However, at younger ages (i.e. 15 and 25 days post-hatch), NRGN mRNA expression in Area X is indistinguishable from that in the surrounding tissue (Fig. 9D). Also, signal is evident in the granule cell layer of the cerebellum in addition to the thin Purkinje cell layer.

NRGN mRNA in Area X was measured by in radioactive situ hybridization over a range of developmental ages in the male zebra finch, to determine when this decrease in expression occurs (Fig. 10). We observed a sharp decline between 25 and 35 days post-hatch, with no further significant change occurring after 35 days of age. When hybridized sections were exposed to autoradiographic emulsion to allow analysis of Neurogranin mRNA at a cellular level, signal was typically detected in the cytoplasm of cells with a neuronal morphology (large cells with large, diffuse, pale-staining nuclei and prominent nucleoli); intense cytoplasmic labeling of cerebellar Purkinje neurons was also confirmed at this level of analysis (not shown).

Subcellular localization of NRGN protein

We raised an antibody (H14C) to the C-terminus of the predicted canary protein and used it to probe protein extracts of zebra finch forebrain (Fig. 11). It recognized a dominant cytosolic band of approximately 18 kDa and showed no significant cross-reactivity with any other molecular species in the forebrain (Fig. 11). Signal from the antibody was abolished completely by preincubation with the immunizing peptide.

We exploited the high level of expression in Purkinje cells to examine the subcellular localization of Nrgn protein. We probed sections of zebra finch brain with our H14C antibody and with an antibody to the synaptic vesicle protein Synaptotagmin, and visualized the two patterns using confocal microscopy (Fig. 12). Nrgn signal completely fills the cell bodies and dendritic arbors of the Purkinje cells, consistent with a post-synaptic localization (Fig. 12A). This is in sharp contrast to synaptotagmin, which has a known pre-synaptic localization and yields a punctate staining pattern in the molecular and granule cell layers (Fig. 12B).

Discussion

NEUM and NRGN have been subjects of intense study in rodents and other model systems for their potential roles in brain development and plasticity (Gispen et al., 1991; Pasinelli et al., 1995). Here we defined the putative protein sequences for NEUM and NRGN in two species of songbird, and we probed the distribution of their RNAs in the major song control nuclei of adult and developing songbirds. Our findings bear on a range of issues, including molecular conservation and divergence within the calpacitins, molecular correlates of song system development, and the broader biological functions of these proteins.

We find rigorous conservation of the core IQ domain in both of these proteins in songbirds compared to other vertebrates, a result anticipated from previous comparative analyses of this protein family (Gerendasy, 1999). In both proteins the 20-residue IQ domain is flanked

by an additional stretch of high conservation that extends toward the N-termini. Although conserved within each protein, these N-terminal domains diverge between NEUM and NRGN and may mediate core functions unique to each protein including localization to different subcellular compartments. In NEUM the conserved N-terminal region and IQ domain represent only a quarter of the total protein and the remainder is much more loosely conserved. The main structural constraint on the rest of the protein may simply be the ability to assume a rod-like shape as proposed (LaBate and Skene, 1989). In our initial determination of the canary NRGN sequence, we were perplexed by the sharp divergence of the short C-terminus from the highly conserved C-terminus observed within mammals (Siepka et al., 1994). Here we show that both canaries and zebra finches share nearly identical C-termini in NRGN and notable similarity is present also in the chicken C-terminus. This suggests that avian and mammalian NRGN may have evolved distinct properties based on their distinct C-termini.

We find that birds and mammals also have strikingly different expression patterns for NRGN in the cerebellum. NRGN is notably absent in cerebellar Purkinje cells in adult rodents and non-human primates (Represa et al., 1990; Watson et al., 1990; Singec et al., 2003; Higo et al., 2004) but we find it to be abundantly present in Purkinje cells of songbirds (Figs 9, 12). This might reflect broader differences in cerebellar physiology between mammals and birds; consistent with this, the steroid-synthetic enzyme P450scc is detected in somata and dendrites of quail, but not mammalian, Purkinje neurons (Usui et al., 1995). Interestingly, one recent study did report expression of NRGN in some Purkinje cells in the developing mouse, but expression was found only in the embryo and neonate and limited to subsets of neurons organized as parasagittal stripes (Larouche et al., 2006).

An important motivation for these studies was the anticipation that NEUM and NRGN might be especially active in the dramatic neural plasticity evident in the adult song control nuclei of songbirds. Canaries, zebra finches and other songbirds show continued development of new neurons within the song control system throughout life (Goldman and Nottebohm, 1983; Alvarez-Buylla and Nottebohm, 1988), and the song control nuclei respond with overt synaptic and neuroanatomical changes to many stimuli including photoperiod (Nottebohm, 1981; Hurley et al., 2008), gonadal steroids (Arnold, 1980; DeVoogd and Nottebohm, 1981; Thompson et al., 2007; Balthazart et al., 2008), social conditions (Lipkind et al., 2002; Boseret et al., 2006; Leitner and Catchpole, 2007), and singing activity (Adkins-Regan, 2005; Sartor and Ball, 2005). In the context of this biology, an active role for NEUM in particular seemed likely, as it was one of the first identified molecular markers of axonal growth and regeneration (Pate Skene and Willard, 1981; Skene and Willard, 1981). Both NEUM and NRGN are regulated by factors that modulate synaptic strength (Krueger and Nairn, 2007), and they continue to be widely used as indicators for sites and periods of plasticity in the mammalian cortex (e.g., Ponomarev et al., 2006; Higo et al., 2007).

However, here we found that the RNAs for both NRGN and NEUM are actually reduced in particular song nuclei of adult canaries and zebra finches. In both species, NRGN was reduced in the striatal song nucleus Area X, whereas NEUM was reduced in the arcopallial nucleus RA. Additionally, in the canary but not the zebra finch, NEUM was reduced in

reflect the robust rearrangement of terminals from IMAN (Herrmann and Arnold, 1991) given the evident presence of NEUM RNA in that nucleus as well (Figs. 3, 6).

In the case of NRGN, we found that an adult-like pattern of expression emerges between P25 and P35, with a local decline in expression specifically in Area X. Prior to this age in the zebra finch, NGRN (a postsynaptic protein) is high in Area X, and NEUM (a presynaptic protein) is high in HVC, which provides the major afferent input to Area X neurons. Both proteins integrate both calcium signaling and PKC signaling pathways, and co-occurrence on each side of the same synapse early in song development could support a concerted program of regulation based on these signaling systems in the HVC-Area X (“anterior forebrain loop”) pathway early in song development; a similar speculation has been entertained for the visual pathway in the lateral geniculate nucleus of the macaque monkey (Higo et al., 2004).

NEUM and NRGN thus provide two more examples of the complexity of gene expression patterns in the developing song control system. Although the system is appreciated for its functional unity and altricial development, clearly this does not involve a common shared profile of gene expression across all the nuclei of the system. Since a review over ten years ago (Clayton, 1997) the situation has only grown more complex, as the following examples illustrate. Various calcium binding proteins (parvalbumin, calbindin, calretinin) are present in complex cellular distributions within individual song nuclei (Wild et al., 2001; Wild et al., 2005). Among RNAs enriched in specific song nuclei during development, the RNA for the medium-weight subunit for Neurofilament is enriched in HVC, RA and IMAN but not Area X (somewhat like NRGN) and reaches a peak in IMAN and RA at day 35 and in HVC at day 50 (Velho et al., 2007). Doublecortin RNA is enriched in HVC and RA in nestlings but not adults whereas the opposite developmental pattern is seen in Area X, with enrichment in adults but not nestlings (Kim et al., 2006). Two ribosomal protein RNAs, RPL17 and RPL37, are selectively enriched in juvenile RA and Area X and but not in adults (Tang and Wade, 2006). FOXP2 RNA is enriched in Area X specifically at days 35 and 50 (Haesler et al., 2004). ZRAlDH RNA is enriched in early juvenile RA and HVC, but then it declines selectively in RA and is essentially absent there in the adult (Denisenko-Nehrbass et al., 2000; Kim and Arnold, 2005). Part of this complexity may be explained by the fact that some of the RNAs discussed here represent markers of different cell populations (e.g., ZRAlDH is a marker of HVC’s X-projecting neurons, calcium-binding proteins are primarily expressed in HVC’s interneurons, and doublecortin is a likely marker of newly-formed neuronal cells). Future studies may reveal that specific programs of gene regulation are tightly coordinated at the level of the distinct cellular phenotypes that make up the song system.

Methods

Animals

All procedures involving animals were conducted with the oversight and approval of the appropriate Institutional Animal Care and Use Committee. Zebra finches were bred and raised in a free-flight group aviary maintained under a 14:10 hour light/dark cycle at the Beckman Institute, Urbana, IL; some of the adults were obtained from Canary Bird Farm

(Englishtown, NJ). Canaries, a gift from Fernando Nottebohm, were maintained at the Rockefeller University Field Research Center on the natural photoperiod of New York state; canaries used in these studies were sacrificed in March-May (breeding season) or October (non-breeding season), 1986–1988.

Cloning and sequence analysis

cDNA clones for canary NEUM and NRGN were originally obtained from screens of a cDNA library constructed from RNA extracted from the HVC-associated telencephalon (HAT) of adult male canaries (George and Clayton, 1992). NEUM clones were identified by reduced stringency hybridizations using a rat GAP-43 cDNA sequence (Karns et al., 1987) as probe; two positive clones were subcloned in pGEM3z (Promega) and sequenced on both strands using Cycle Sequencing (BRL) and internal synthetic oligonucleotide primers. NRGN clones were obtained from a screening procedure using differential hybridization methods (Clayton et al., 1988) for clones of RNAs more abundant in the HAT region compared to hindbrain/midbrain (George and Clayton, 1992; George et al., 1995); the original cDNA clone (“HAT-14”) and two other cross-reactive clones were sequenced on both strands using standard techniques. Orthologous zebra finch sequences were identified by BLAST analysis of the ESTIMA:Songbird database, <http://www.uiuc.edu/goto/songbird> (Replogle et al., 2008). Comparative sequence analyses were performed using CLUSTALW (Chenna et al., 2003) through the EBI server (<http://www.ebi.ac.uk/Tools/>).

Developmental northern blot analyses

PolyA⁺ RNA was prepared from individual zebra finch forebrains collected from birds of known ages from the aviary at the Beckman Institute, University of Illinois, using the FastTrack mRNA isolation kit (Invitrogen). RNA aliquots (1 µg) were electrophoresed, transferred to Immobilon-N membranes and prehybridized in a solution containing 263 mM NaHPO₄, 10mM NaCl, 1mM EDTA, 20 µg/ml polyA. cDNA inserts were ³²P-labeled using the random priming method (Feinberg and Vogelstein, 1984). Probe was added to the hybridization at 10⁶ cpm/ml, incubated overnight at 65°C, and washed repeatedly with 1X SSC, 1% SDS at 65° C before exposure to X-ray film (Kodak XAR).

In situ hybridization

10 µm frozen brain sections were hybridized to ³⁵S-labeled riboprobes followed by X-ray film or emulsion autoradiography, as described elsewhere in detail (Clayton et al., 1988; Clayton and Alvarez-Buylla, 1989). For hybridization of zebra finch sections with probes derived from canary cDNAs, the final wash was in 0.1x SSPE at 65° C, and for hybridization of canary sections the final wash was 11 mM Na⁺ (Clayton et al., 1988); these conditions gave a single, clean band on riboprobed Northern blots (not shown). Parallel hybridizations were performed with sense-strand controls for evaluation of non-specific background levels, which were negligible in these experiments. All hybridizations were replicated on several separate occasions. Some sections were dipped in Kodak NTB2 autoradiographic emulsion followed by cresyl violet counterstaining as described elsewhere in detail (Clayton et al., 1988). Digoxigenin-UTP-labeled probes were synthesized and detected following the protocols of the supplier (Boehringer-Mannheim), and were hybridized under the same conditions as for ³⁵S probes.

Identification of song nuclei on X-ray films was confirmed by cresyl violet staining and microscopic inspection of sections after autoradiography. Film images were digitized on a high-resolution flat-bed scanner, and analyzed using NIH Image software (v. 1.54) by tracing the boundaries of specific brain regions and computing the mean optical density within, by reference to a calibrated Kodak step tablet digitized under identical conditions. Film background was subtracted from all measurements prior to further mathematical analysis. To obtain an accurate quantitative estimate of the relative amount of specific mRNA in different sections (despite the possibility of variations in hybridization efficiency), the autoradiographic film density for each song nucleus on a given section was expressed as a ratio relative to the density measured in the same section for the surrounding arcopallium (for RA), caudal nidopallium (for HVC) and medial striatum (for Area X). This use of ratios assumes there is no developmental change in signal in the surrounding brain regions, an assumption supported by visual comparison of X-ray film images at all ages studied and consistent with the constant levels of NEUM and NRGN mRNA measured in the forebrain as a whole in birds older than 2 weeks (Fig. 5 and other unpublished data). For each nucleus in each bird, such measurements were made in at least two sections and the mean of these ratios was determined. The means from three birds were then used to calculate group means and standard errors for each nucleus at each age (n=3 birds per age). Histogram analysis of the collected data indicated normal distributions in the samples compared by ANOVA, and plots of means vs. standard deviations did not reveal any systematic trend that would disqualify analysis by standard parametric tests. Single-factor ANOVA tests for main effect of age for each probe were performed using Microsoft Excel.

NRGN Immunocytochemistry

The peptide and CANSTRGGDLRNGD (at the canary NRGN C-terminus) was synthesized and conjugated to keyhole limpet hemocyanin (KLH), and purified by gel exclusion chromatography (Imject Activated Immunogen Conjugation Kit, Pierce). The University of Illinois Hybridoma Facility immunized mice and generated polyclonal ascites. Brain tissue was fixed by intracardial perfusion with 3% neutral-buffered paraformaldehyde with 0.75M lysine and 0.1M sodium metaperiodate, and embedded in polyethylene glycol as described (Clayton and Alvarez-Buylla, 1989). 20 μ m sections were probed sequentially with primary antibodies (mouse polyclonals at 1:100; and rabbit polyclonal antibody against p65 synaptotagmin 1:100, a gift from L. Elferink and R. Scheller), followed by fluorescent secondary antibody (goat anti-mouse coupled to lissamine-rhodamine, goat anti-rabbit coupled to Cy5, 1:100; Jackson Immunochemicals). To assess nonspecific immunoreactivity, antibodies were preincubated at 4°C overnight with either the immunizing peptide or a nonabsorbing control peptide (20 μ g/ml). Digitized fluorescence data were collected on a Biorad MRC 600 laser scanning confocal microscope.

Acknowledgments

The work described here developed over a 20-year period at three institutions, the Rockefeller University, the University of Illinois, and the OHSU. A number of students and laboratory personnel made various contributions to it in the early days. In particular we acknowledge with appreciation the contributions of Blair Simpson, Clay Holloway, Hui Jin, Maria Huecas, and Kent Nastiuk. We thank Mark Fishman for helpful advice and for providing the rat GAP-43 (neuromodulin) cDNA clone; Richard Scheller and Lisa Elferink for providing anti-synaptotagmin antisera; the Cell Science Center Hybridoma Facility at the University of Illinois for production of monoclonal

antibodies; and Fernando Nottebohm for providing canaries and administrative support in the early phases of this project. This work was supported by NIH grants NS25742, NS051820 and DC02853.

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Canary	-----TKQVEKNEDGDQKIEQDGIKPEDKAHKAATKIQASFRGHITRKKLKGEKKGDA	53
Tagu	MLCCMRRTKQVEKNEDGDQKIEQDGIKPEDKAHKAATKIQASFRGHITRKKLKGEKKGDA	60
Chicken	-----KVEKNEDGDQKIEQDGIKPEDKAHKAATKIQASFRGHITRKKLKGEKKADA	51
Mouse	-----KVEKNED-EDQKIEQDGVKPEDKAHKAATKIQASFRGHITRKKLKGEKKGDA	50
Rat	-----QVEKNED-EDQKIEQDGVKPEDKAHKAATKIQASFRGHITRKKLKDEKKGDA	50
Human	MLCCMRRTKQVEKNED-DDQKIEQDGIKPEDKAHKAATKIQASFRGHITRKKLKGEKKDDV	59
Elephant	-----VEKNED-EDQKIEQDGIKPEDKAHKAATKIQASFRGHITRKKLKGEKKGDA	49
Dog	MLCCMRRTKQVEKNED-EDQKIEQDGIKPEDKAHKAATKIQASFRGHITRKKLKGEKKGDA	59
Opossum	MLCCMRRTKQVEKNEDDQKIEQDGIKPEDKAHKAATKIQASFRGHITRKKLKGEKKGDA	60
ZebraFish	-VCSASIVEQVEKNEEDQEIQDGIKPEENAHKAATKIQASFRGHITRKKMKDGEKEE	59
****: **::***::***:*****::***:..* :		
Canary	PASETDAADKK--EEGPAGGAAENKESEAPAATEAAAAADSAQQEEGSKD--SSAPAEKKGD	111
Tagu	PASETDAADKK--EEGPAGGAAENKESEAPAATEAAAAADSAQQEEGSKD--SSAPAEKKGD	118
Chicken	PASESEADKK--DEGPAGGAAENKESEASAATEASAADSAQLDEGSKD--SSVPAEKKGN	109
Mouse	PAAEAEAKEK--DDAPVADGVEKKEGDSATTDAAPATSPKAEPSKA--GDAPSEKKGE	107
Rat	PAAEAEAKEK--DDAPVADGVEKKEGDSATTDAAPATSPKAEPSKA--GDAPSEKKGE	107
Human	QAEEAEANKK--DEAPVADGVEKK--GEGTTAAEAPATGSKPDEPGKA--GETPSEKKGE	115
Elephant	QAEEVEVNEKK--EEVPVADGIEKKEGDGSATTDAAPATGPKPEETGKA--GEAPSEKKGE	107
Dog	QAEEAEAKEK--DEAPVADGLEKKEGEGATPSEAAPASGPKPEEPGKA--GETPSEKKGE	116
Opossum	RAAEADANEKKDEAAATADAVEKKESEAPAATEAAPVDGPKAEASKA--GESTSEKK--EA	118
ZebraFish	NGEVPEEQEE--EKPTDASTETQEDPKPEQPNSPANEAAPTAAADPAPSDTPAKEEVEKE	116
. : .: . . . * . : . . . : . . . . . : *		
Canary	GAADTGSEQPAPQAATPAASSEKTA AAAAPERESTPKASTDNSPSLKADEAQDKEEPKQ	171
Tagu	GAADTGSEQPAPQAATPAASSEKTA AAAAPERESATKASTDNSPSLKADEAQDKEEPKQ	178
Chicken	GAADTGSEQPAPQAATPAASSEK--PAAAATESATKASTDNSPSLKADEKTRSLNKP	167
Mouse	G--DAA-----PSEK--AGSAETESAATKASTDNSPSSKAEDGPAKEEPKQ	149
Rat	G--DAA-----PSEK--AGSAETESAATKASTDNSPSSKAEDGPAKEEPKQ	149
Human	G--DAATEQAAPQAP--ASSEK--AGSAETESATKASTDNSPSSKAEDAPAKEEPKQ	167
Elephant	GAPDAATEQVAPQAP--GPSEK--AGSAETESATKASTDNSPSSKAEDAPAKEEPKQ	161
Dog	GAPDAATEQVAPQAP--GPSEK--AGSAETESATKASTDNSPSSKAEDAPAKEEPKQ	170
Opossum	GAPAAASEQPAPKAT--APSEK--PVSPTESATKASTDNSPSSKAEDAPAKEEPKQ	172
ZebraFish	P-----QVEVEK-----PKEEPPSSAAADEKPEEENQAQKEEKEEPK	154
. ** . * . : . * : : . : . *		
Canary	ADVPAADTTATTT--PAAEDATAKATAQPMETVESSQTEEKTDAVEETKPTESAQQEEM	229
Tagu	ADVPAADTTATTT--PAAEDATAKATAQPMETVESSQTEEKTDAVEETKPTESAQQEEV	236
Chicken	TCL--LLTPLTTT--PAAEDATAKATAQPMETVESSQTEEKTDAVEETKPTESAQQEEV	223
Mouse	ADVPAAVTDAAAT--TPAAEDAAAT--KAAQPPPTETAESSQAEEKDAVDEAKPKESARQDEG	207
Rat	ADVPAAVTDAAAT--TPAAEDAAAT--KAAQPPPTETAESSQAEEKDAVDEAKPKESARQDEG	206
Human	ADVPAAVTAAAAAT--TPAAEDAAAKATAQPPPTETGESSQAEEENIEAVDETKPKESARQDEG	226
Elephant	ADVPAAVTAAAAAT--TPAAEDAAAKATAQPPPTETAESSHAEEKT-----	204
Dog	ADVT-----TPAAEDAAAKATAQPPPTDAESSQAEEKIEAVDETKPKESARQDEG	220
Opossum	ADAT-----APPPTETAESSQAEEKTEAVEETKPMESAQQEEE	210
ZebraFish	ADVP-----EASQETHQID--KPDAAEESKPAEGAVSDAG	188
: : : * : : :		
Canary	KEEESKADQENA	241
Tagu	KEEESKADQENA	248
Chicken	KEEESKADQENA	235
Mouse	KED--PEADQEHA	218
Rat	KED--PEADQEHA	217
Human	KEEPEADQEHA	238
Elephant	-----	
Dog	KGEEREADQEHA	232
Opossum	KGDESKADQEHA	222
ZebraFish	TEESV-----	193

Fig 1. Neuromodulin (NEUM) sequence comparisons

A: sequences (Table 1) aligned using CLUSTALW. Symbols beneath indicate conservation:

* = identical or conserved residues; : = conserved substitution; . = semiconserved substitution.

B: phylogram of sequence relationships (from CLUSTALW)

Fig 2. Neurogranin (NRGN) sequence comparison and the core IQ domain

A: sequences (Table 1) aligned using CLUSTALW. Symbols beneath indicate conservation:

* = identical or conserved residues; : = conserved substitution; . = semiconserved substitution.

B: phylogram of sequence relationships (from CLUSTALW)

C: alignment of IQ domains from avian NRG (beginning residue 34) and NEUM (beginning residue 28). 1: calmodulin-binding domain; 2: PKC phosphorylation site; 3: binding site for phosphoinositide 3-kinase lipid products.

Fig. 3. Distribution of NEUM mRNA in canary brain

Shown is a representative parasagittal section (10 μ m) of an adult male canary brain sacrificed in spring, through the level of song nucleus HVC. A: X-ray film autoradiogram after hybridization with an ^{35} S-labeled riboprobe. B: An overlay to panel A indicating specific anatomy referred to in the text; rostral is to the right and dorsal is up. A, arcopallium; M, mesopallium, HVC, nucleus HVC; IMAN, lateral magnocellular nucleus of the anterior nidopallium; MSt, medial striatum; N, nidopallium; RA, robust nucleus of the arcopallium; X, Area X (as visualized by Nissl stain). Size bar: 2 mm

Fig. 4. Cellular analysis of NEUM mRNA expression in nucleus RA of canary
Sections hybridized with ^{35}S -labeled riboprobes were exposed to autoradiographic emulsion, counterstained with cresyl violet, and viewed under high-power light microscopy. A: sense strand control in nucleus RA, silver grains are sparse and evenly distributed, B: antisense signal in nucleus RA, note some accumulation of silver grains over cells in RA, C: antisense signal in adjacent archistriatum, note dense signal over individual cells. Size bar: 20 μm

Fig. 5. Developmental expression of NEUM, NRGN and control RNAs in the zebra finch forebrain (northern blot analysis)

³²P-labeled cDNAs for each probe were hybridized to blots in which each lane represents 1 µg polyA+ RNA isolated from the forebrain of a single zebra finch of the indicated age (in days), except for the 1&2 day lane, in which a 1-day and a 2-day sample were pooled; samples from two different 7-day-old birds are included on this blot. A, NEUM; B, NRGN; C, HAT-2 (n-chimaerin, George et al., 1992); D, HAT-5 (MAP-Kinase-Kinase, (George, 1993)); E, Synelfin (SNCA, (George et al., 1995)).

Fig. 6. NEUM mRNA expression in juvenile and adult male zebra finch

A: X-ray film autoradiogram representing 15-day old male zebra finch. B: adult male zebra finch (compare to A); nucleus RA appears as a “hole” in the adult image, but is indistinguishable from the surround in the juvenile bird. C: 15 day-old male zebra finch brain section after hybridization with a digoxigenin-labeled NEUM probe and viewed at higher magnification; the boundaries of RA are indicated by arrows. D: adult male zebra finch (compare to C); abundant cellular signal fills nucleus RA in the 15 day old animal but few labeled cells are evident in RA in the adult. Size bar: A and B, 3 mm; C and D, 0.3 mm. In all panels, rostral is to the right and dorsal is up.

Fig. 7. Developmental timecourse of NEUM mRNA expression in nucleus RA of male zebra finch Signal was measured densitometrically and is expressed as a ratio versus surrounding arcopallium (see Methods for further description and justification). Means and SEMs ($n=3$ birds) are plotted for each age; SEM bars are too small to distinguish at 15, 35, and 45 days.

Fig. 8. NEUM mRNA expression in nucleus RA of juvenile female zebra finches

A. Brain section of a 25 day-old female after hybridization with a digoxigenin-labeled NEUM probe as in Figs. 6C–D, and viewed at high magnification; the boundaries of RA are indicated by arrows. B. 45 day-old female (as in panel A). Labeled cells are numerous within RA at P25 but absent at P45. Size bar: 0.15 mm; rostral is to the right and dorsal is up.

Figure 9. Distribution of NRGN mRNA in parasagittal sections of canary and zebra finch brain
 A: X-ray film autoradiogram representing a male canary sacrificed in Spring, when song control nuclei are large and singing behavior is robust. B: An overlay to panel A indicating relevant anatomy. **HVC**, nucleus HVC; **RA**, robust nucleus of the arcopallium; **Area X**, a song nucleus in medial striatum, or **MSt**; cerebellar **Purkinje** cell monolayer; **Gran**, cerebellar granule cell layer. C: Adult male zebra finch; note low signal in Area X. D: 25 day old male zebra finch; Area X is indistinguishable from surrounding striatum. Size bar: 2 mm

Figure 10. Developmental timecourse of NRGN mRNA expression in Area X and RA of male zebra finches

Signal was measured densitometrically (Methods) and is expressed as a ratio of Area X (squares, lower trace) relative to surrounding striatum, or of RA (circles, upper trace) relative to surrounding arcopallium. Means and SEMs ($n=3$ birds) are plotted for each age; SEM bars are too small to distinguish at 15 and 25 days.

Figure 11.

Immunoblot analysis of Nrgn protein in zebra finch forebrain extracts. Crude cytosolic and synaptosomal protein extracts of zebra finch forebrain were probed with the H14C antibody, raised against a synthetic peptide representing the C-terminus of canary NRGN. The antibody detects a dominant band of ~18 kDa in cytosol (-peptide) which is greatly diminished by incubation with the immunizing peptide (+peptide).

Figure 12. Immunocytochemical detection of Nrgn protein in zebra finch cerebellum

A: H14C immunoreactivity in zebra finch cerebellum, detected using confocal microscopy (1 μ m optical section thickness). Signal fills the monolayer of Purkinje cell bodies and their extensively branching dendritic arbors within the molecular layer (upper left). B: Synaptotagmin immunoreactivity in the same field has a complementary distribution, with punctate synaptic labeling in the molecular (upper left) and granule cell (lower right) layers, respectively, and minimal staining of Purkinje cell bodies or processes. Size bar: 20 μ m.

Table 1**Sequence Identifiers**

Canary sequences were derived as described in Methods and deposited with the Genbank identifiers shown. Zebra finch (Tagu) sequences were derived from assembled contigs in the ESTIMA:Songbird collection (www.uiuc.edu/goto/songbird). Other sequences represent 1:1 orthologs as annotated in ENSEMBL (www.ensembl.org).

	NEUROGRANIN	NEUROMODULIN
Canary	gb U56726.1	gb U75453.1
Zebra finch (Tagu)	SB.sd.62.C1.Contig11429	SB.193.C1.Contig255
Chicken	ENSGALP00000029243	ENSGALP00000024294
Dog	ENSCAFP00000016434	ENSCAFP00000015971
Elephant	ENSLAFP00000007533	ENSLAFP00000001956
Human	ENSP00000284292	ENSP00000305010
Mouse	ENSMUSP00000070113	ENSMUSP00000058154
Opossum	ENSMODP00000004610	ENSMODP00000022675
Rat	ENSRNOP00000014404	ENSRNOP00000002091
ZebraFish	ENSDARP00000057909	ENSDARP00000002791

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Table 2
Relative in situ hybridization signal for NEUM and NRGN in four song control nuclei

Summary of qualitative assessments of signal intensities from in situ hybridization autoradiograms, comparing each nucleus to immediately surrounding brain tissue. Arrows up (↑) and down (↓) indicate greater and lesser signal (respectively) within the nucleus compared to surround for the probe indicated. (Double arrow indicates larger magnitude of difference). Tilde (~) indicates no evident difference in expression within the nucleus versus surround.

	HVC		RA		Area X		IMAN	
	NEUM	NRGN	NEUM	NRGN	NEUM	NRGN	NEUM	NRGN
zebra finch, day 15	~	~	~	~	~	~	~	~
Adult zebra finch	~	↑	↓↓	~	~	↓↓	~	~
Adult canary	↓	↑	↓↓	↑	~	↓↓	↑	↑