Seasonal Regulation of NMDA Receptor NR2B mRNA in the Adult Canary Song System

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ABSTRACT: **Developmental changes in the composition and function of *N*-methyl-D-aspartate receptors (NMDARs) are believed to regulate neural plasticity. For example, in songbirds, vocal learning entails NMDAR activation, and the sensitive period for such learning in zebra finches (ZFs) parallels developmental changes in NMDAR density and phenotype within several song-related brain regions. In contrast to ZFs, canaries exhibit vocal plasticity recurrently throughout adulthood, prompted by seasonal changes in day length and testosterone (T) levels. We used *in situ* hybridization to determine if such changes in photoperiod affect NMDAR subunit expression in adult canaries. Birds were sacrificed while on short days (SD) when T levels were low, or on long days (LD) when T levels were high. Transcript levels for the constitutive NMDAR subunit (NR1) and two modulatory subunits (NR2A, NR2B) were measured in four song control nuclei: lMAN, Area X, HVc, and RA. NR1 and NR2A mRNA levels were comparable in SD and LD groups in all four song regions studied. However, NR2B mRNA levels within lMAN and RA were significantly higher in SD than in LD birds. Photoperiod did not affect NR2B transcript levels in Area X, HVc, or a nonsong region just lateral to lMAN. Our data support the hypothesis that changes in NMDAR subunit expression may contribute to the neural and behavioral reorganization that accompanies seasonal song remodeling in adulthood.** © 2003 Wiley Periodicals, Inc. J Neurobiol 54: 593–603, 2003

Keywords: **NMDAR; NR1; NR2A; NR2B; photoperiod; testosterone; birdsong; plasticity; canary**

INTRODUCTION

N-methyl-D-aspartate receptors (NMDARs) mediate several forms of experience-dependent synaptic modification (Davis et al., 1992; Bliss and Collingridge, 1993; Sakimura et al., 1995), and developmental changes in NMDAR composition are thought to impact the capacity for synaptic plasticity. NMDARs consist of a constitutive NR1 subunit and modulatory NR2 (NR2A-D) subunits (Moriyoshi et al., 1991; Monyer et al., 1992, 1994; Laurie et al., 1997). A developmental decline in the NR2B:NR2A ratio shortens NMDAR currents, which may reduce the probability of synaptic strengthening (Kirkwood et al., 1996; Flint et al., 1997; Quinlan et al., 1999a, b; Hoffmann et al., 2000; Philpot et al., 2001). Moreover, because NMDAR modulatory subunits directly interact with intracellular signaling molecules (Bayer et al., 2001; Lisman and Zhabotinsky, 2001), changes in subunit composition may affect the biochem-

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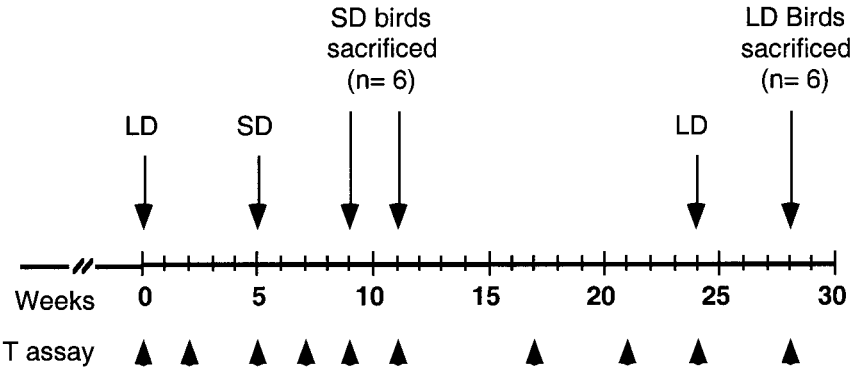

Figure 1 Experimental timelines showing photoperiod manipulations, sample points for plasma testosterone (T) assay, and time of sacrifice.

ical cascades regulating synaptic function (Malenka and Nicoll, 1999).

Birdsong learning requires activation of NMDARs within the forebrain song region lMAN (Basham et al., 1996), and several studies suggest that developmental changes in NMDAR expression within this and perhaps other song regions may influence the timing and/or capacity for vocal learning (see Methods for explanation on avian brain nomenclature). In zebra finches (ZFs) (*Taenopygia guttata*), a developmentally restricted period of song learning overlaps with a decline in NR1 and NR2B subunits and an increase in NR2A transcripts within several song-related nuclei (Aamodt et al., 1992; Basham et al., 1996; Singh et al., 2000; Scott et al., 2001). Importantly, manipulations that can alter the timing of vocal learning also modify the developmental regulation of NMDAR modulatory subunits in lMAN. Early isolation from conspecific song can extend the period of vocal learning and delay the down-regulation of NR2B mRNA in this region (Singh et al., 2000). Conversely, early testosterone (T) treatment can accelerate the achievement of vocal stability and accelerates the developmental changes in NR2B and NR2A expression in lMAN (Singh et al., 2000; Heinrich et al., 2002).

Although vocal plasticity in many songbirds is limited to a sensitive period, some species retain the capacity to change their songs in adulthood. For example, songs produced by both wild and domesticated adult canaries (*Serinus canarius*) are stable during the spring breeding season, but are modified mainly during the fall and winter (Nottebohm et al., 1986, 1987; Leitner et al., 2001). These behavioral changes appear to be driven primarily by photoperiod–induced changes in T secretion (Smith et al., 1995, 1997; Gulledge and Deviche, 1997; Brenowitz et al., 1998; Ball, 2000; Tramontin et al., 2000). Vocal stability in the early spring is associated with high plasma T levels stimulated by increasing day length. After the breeding period, birds become photorefractory and T levels decline. Importantly, vocal plasticity peaks about 1 month after the nadir in T levels (Nottebohm et al., 1987). Here, we determined if photoperiod-induced changes in T in adult canaries are associated with altered NMDAR mRNA expression. In two song nuclei, the lMAN and RA, NR2B transcript levels were significantly higher in birds sacrificed on short days (SD; low T levels) than in birds sacrificed on long days (LD; high T levels).

METHODS

Housing and Photoperiod Manipulations

Thirteen adult male Roller canaries (*S. canarius*) were housed on a short day (8 h light:16 h dark) photoperiod in individual cages with food and water provided ad libitum. To synchronize their reproductive physiology, the photoperiod was gradually shifted to a long day (16 h light:8 h dark) over the course of a week and maintained there for an additional 4 weeks before gradually shifting back to SD (see Fig. 1). Blood samples were taken from each bird approximately every 2 weeks to monitor plasma T levels, and each bird was individually inspected for feather molt every 2–14 days [circulating T levels are at their lowest at midmolt, about when the seventh primary wing-feather has dropped (J.C. Wingfield, personal communication)]. Birds in the SD cohort were sacrificed by decapitation when T levels reached undetectable levels (<0.2 ng/mL), which varied from 19 to 47 days after the SD photoshift. Brains were removed, rinsed in cold phosphate-buffered saline, coated in M-1 Embedding Matrix (Lipshaw), and then frozen on dry ice and stored at -75°C. The entire procedure took less than 7 min for each bird. Testes were also harvested and weighed. After the last SD birds were sacrificed, the remaining birds were maintained on SD until they had completed

their molt, in order to ensure the maximum response to subsequent photostimulation. Blood samples were collected every 3–4 weeks during this period to confirm that plasma T levels remained low. The birds were then shifted back to LD and maintained there for 4 weeks. Blood samples were then collected, and birds immediately sacrificed and tissue harvested as described above.

Blood Sample Collection and Plasma T Assay

Blood samples (≈400 μL) were collected from the alar vein into heparinized microcentrifuge tubes. Each blood sample was immediately centrifuged and the plasma was removed and stored at −20°C until assay. The Coat-a-Count Total Testosterone kit (Diagnostic Products) was used to measure plasma levels of T. This assay has been validated for measurement of plasma T levels in birds (Tramontin et al., 2001). The minimum detectable plasma T concentration was 0.2 ng/mL. Interassay variability was 3.63 ± 0.93%.

Tissue Preparation and *In Situ* Hybridization

Six brains from each of the SD and LD groups were prepared for analysis. Coronal sections (16 μm) were cut on a cryostat and sections were serially mounted on Vectabond-coated slides, air dried, and then stored in airtight boxes at −75°C. Anterior forebrain and posterior sections were collected on two separate groups of slides. Anterior sections included the song-related nuclei lMAN and Area X; posterior sections included the song-related nuclei HVc and RA [due to ongoing changes in avian brain nomenclature (see http://jarvis.neuro.duke.edu/nomen/2002NomenclatureTable.html), acronyms that are consistent with the current literature are used here as the proper name].

The following three synthetic oligonucleotides were used to detect the NR2B, NR2A, and NR1 mRNA in canary brain. The NR2B (GGGCTTGCCAGAGCAGACACCCATGAAA-CAGTGGCGGAATTGCCA, 45 bases) and NR2A (GGGT-TATCATCCGAGTGAACAGCGTACAGG GACTTGCTC-CTCTTGCTGTCCTC, 53 bases) oligonucleotides were based on partial sequences of their respective mRNA isolated from ZF forebrain total RNA (Basham et al., 1999; Singh et al., 2000; Heinrich et al., 2002). The NR1 oligonucleotide (GT-GCTCAGCACCGCGCCGATGTTGACGAT CTTGGG-GTCGCAGCCG, 45 bases) was complimentary to bases 522–566 of duck NR1 cDNA sequence (Kurosawa et al., 1994).

All three HPLC purified oligonucleotides were obtained from Genosys Biotechnologies. Probes were prepared by labeling the 3′ end of oligonucleotides with [^{35}S]-dATP (New England Nuclear, Boston, MA) using a terminal deoxy-nucleotidyl transferase kit (Boehringer Mannheim, Indianapolis, IN) and purified as described previously (Singh et al., 2000; Heinrich et al., 2002). Probes were stored at −20°C and used within 2–5 days of preparation.

Hybridization was carried out as described previously (Singh et al., 2000; Heinrich et al., 2002). Briefly, sections were fixed, acetylated, dehydrated, and then hybridized [probe concentrations = 1×10^7 CPM/mL (NR2B and NR1); 0.5×10^7 CPM/mL (NR2A)] in a humidified chamber for 16–18 h at 42°C. Sections were washed (final stringency = 0.5X SSC at 60°C for NR2B, 0.5X SSC at 55°C for NR2A, and 0.1X SSC at 60° C for NR1), dehydrated, defatted, and then dipped in Kodak NTB2 emulsion. After developing and fixing (Kodak D-19 developer, Kodak fixer), sections were lightly stained with thionin, dehydrated, and coverslipped. The specificity of the *in situ* hybridization in canary brain was confirmed by substituting ^{35}S-labeled sense for antisense probe. The specific activity of these sense probes was similar to that of antisense probes, but sense probes failed to produce any hybridization signal above background.

Somal Grain Density Analysis

lMAN, Area X, HVc, and RA were identified in Nissl stained sections and analyzed using a computer-assisted image analysis system (Image; NIH). All analyses were done blind to treatment group. For each bird, at least eight fields from each region were analyzed. These measurements were taken from the approximate center of the nucleus, sampled from three to four sections, and from both hemispheres. Hybridization levels were measured at 40X. The area of all the cells in a field was measured, and then the area occupied by silver grains over those cells was measured. Somal grain density was calculated as the area occupied by silver grains divided by the total somal area, and this value then was corrected by subtracting background grain density. Background grain density was determined by averaging eight measurements (area occupied by silver grains/total area) taken from nontissue portions of the slides adjacent to the sections chosen for analysis (Singh et al., 2000; Heinrich et al., 2002). Where significant group differences were observed within song regions, a nonsong region ventrolateral to lMAN and lateral to the center of Area X was also measured. Measurements from SD and LD birds were compared by independent t tests (two-tailed).

RESULTS

Effect of Photoperiod on Testes Weight and Plasma Hormone Level

Both testes weight and plasma T levels differed significantly between SD and LD birds. Five of the six SD birds had testes visibly smaller than those of LD birds, and average paired testes weight of SD birds was threefold less than LD birds (0.071 ± 0.038 vs. 0.264 ± 0.038 g; $t = 3.61$; $p < .01$). Plasma T levels were below the detection limit (<0.2 ng/mL) in all SD birds, but were well above this limit in all LD birds (average = 3.62 ± 0.98 ng/mL). These photoperiod-induced changes in testes weight and T levels

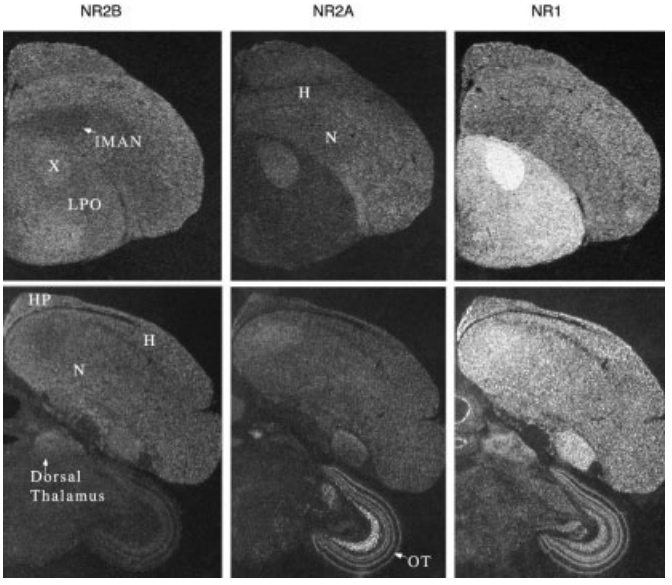

Figure 2 Dark field images of NR2B (left), NR2A (middle), and NR1 (right) *in situ* hybridization signals in coronal sections of an adult canary sacrificed on a long-day photoperiod. The upper panels show sections through the anterior forebrain [including the song regions Area X (X) and lMAN], and the lower panels show sections through posterior regions. HP: hippocampus, OT: optic tectum. Other brain regions are referred to by abbreviations only due to ongoing revisions in avian brain nomenclature (see http://jarvis.neuro.duke.edu/nomen/2002NomenclatureTable.html).

were similar to those that occur in other songbirds across nonbreeding and breeding seasons (Smith et al., 1995; Brenowitz et al., 1998; Tramontin et al., 2000).

NR2B mRNA Expression

The expression of NR2B mRNA in canaries was qualitatively similar to the NR2B hybridization pattern reported in adult male ZFs (Singh et al., 2000). In both SD and LD birds, NR2B expression was moderate in the telencephalon, LPO, and dorsal thalamus, and relatively low or absent in the optic tectum, cerebellum, ventral thalamus, hypothalamus, and brain stem (Fig. 2, left panels). In two birds (one SD and one LD), Area X was distinct under dark-field illumination because the hybridization level within this song region was slightly higher than in the surrounding LPO (see Fig. 2, upper left). In the remaining birds, expression of NR2B was homogeneous throughout the LPO. Visual inspection also revealed that expression of NR2B transcripts in lMAN was somewhat lower than in surrounding regions, while expression in HVc and RA was not notably distinct from their surroundings.

Photoperiod significantly affected NR2B mRNA expression within two song regions, lMAN and RA (Fig. 3). In lMAN, overall NR2B transcript levels were 10.5% higher in SD than in LD birds ($t = 2.86$, $df = 10$, $p = .025$). In RA, overall NR2B transcript levels were 11.8% higher in SD than in LD birds ($t = 2.46$, $df = 10$, $p = .035$). In contrast, the NR2B

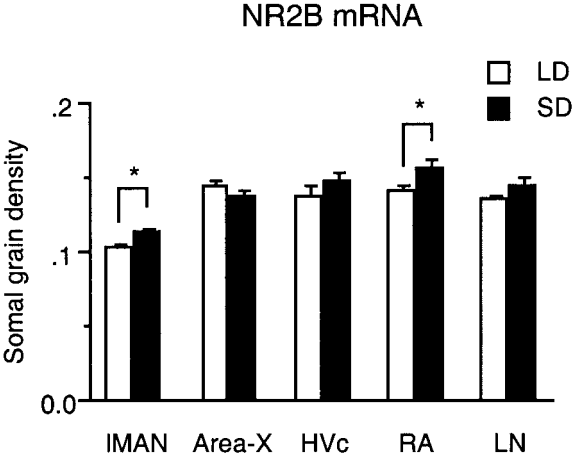

Figure 3 NR2B mRNA hybridization signals over cell soma in four song nuclei and one nonsong region (LN: lateral to lMAN) of adult canaries on long-day (LD) and short-day (SD) photoperiods. In lMAN and RA, NR2B transcript levels were significantly greater in birds on a SD as compared to LD photoperiod. No significant group differences were observed in any other region. Data shown are mean ± S.E.M. $*p < .05$.

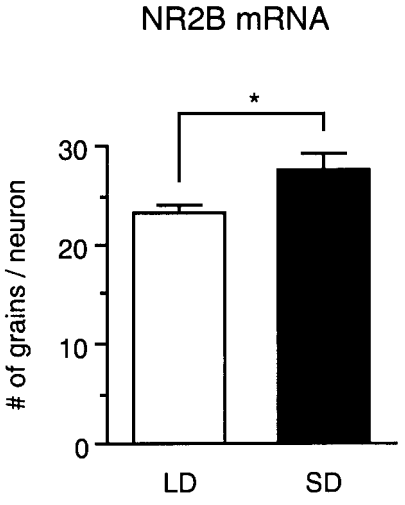

Figure 4 Expression of NR2B mRNA in single nonoverlapping lMAN neurons (24–32 neurons/animal measured at 100X). The NR2B hybridization signals were about 20% greater in birds on a SD photoperiod than in those on a LD photoperiod. Data shown are mean ± S.E.M. *$p < .05$.

hybridization signal in both Area X and HVc was similar between SD and LD birds (Area X: $t = -1.24$, $df = 10$, $p = .28$; HVc: $t = 1.31$, $df = 10$, $p = .26$). Finally, in a nonsong region lateral to lMAN, NR2B transcript levels were 7.3% higher in the SD as compared to the LD group, but this difference was not statistically significant ($t = -1.91$, $df = 10$, $p = .09$).

Photoperiod can profoundly influence the structure of song regions, and therefore the group differences in somal grain density reported above could reflect differences in cell clustering and overlap rather than differences in either the proportion of cells expressing NR2B mRNA, or the average expression per neuron. To evaluate this possibility, we analyzed NR2B hybridization levels over individual nonoverlapping neurons that could be identified by their darkly staining cytoplasm, clear nucleus, and prominent nucleolus. This analysis was performed in lMAN, but could not be done in RA because the relatively small RA neurons could not be distinguished reliably from glia in the lightly stained sections required for analysis of the autoradiographic signal. Also, in a nonsong region lateral to lMAN where the somal grain analysis suggested a small group difference in NR2B expression, the relatively small size of neurons and extensive cell clustering precluded an analysis of individual nonoverlapping neurons. In the lMAN of each animal, all nonoverlapping neurons found in both hemispheres were analyzed at 100X magnification, yielding sample sizes that ranged from 24–32 neurons. Grain area over individual neurons was calculated after subtracting background as described previously (Singh et al., 2000). In this analysis, the cumulative average of NR2B transcript levels stabilized (within 5% of final mean value) by a sample size of 16 neurons, suggesting that our minimum sample of 24 neurons provided a reliable measure of average NR2B expression. The average area occupied by a single silver grain was estimated, and this value was used to calculate the number of silver grains per neuron. This analysis confirmed that photoperiod significantly affects NR2B expression within individual lMAN neurons (Fig. 4). NR2B hybridization levels over lMAN neurons were 20.5% greater in SD birds than in LD birds ($t = 2.60$, $df = 10$, $p = .04$). Although there was no group difference in the average size of lMAN neurons (SD vs. LD = 185 ± 12 μm^2 vs. 183 ± 8 μm^2; $t = 0.14$, $df = 10$, $p = .89$), within each group there was a significant positive correlation between average neuron size and average number grains/neuron (SD: $r^2 = 0.91$, $p = .002$; LD: $r^2 = 0.88$, $p = .006$). When hybridization levels were expressed as a density measurement (grain area/somal area), NR2B mRNA levels again were about 20% higher in SD as compared to LD birds ($t = 7.76$, $df = 10$, $p < .0001$).

To examine photoperiod related changes in the distribution of NR2B labeling intensity and to explore whether group differences were evident among all cell size classes, we sorted neurons according to labeling intensity (bin width 0.02) or cell size. The distribution of labeling intensity (Fig. 5) reflected a unimodal distribution in both treatment groups, with an upward shift in the distribution for SD as compared to LD birds. To explore whether photoperiod altered NR2B expression only in specific cell size classes, we pooled

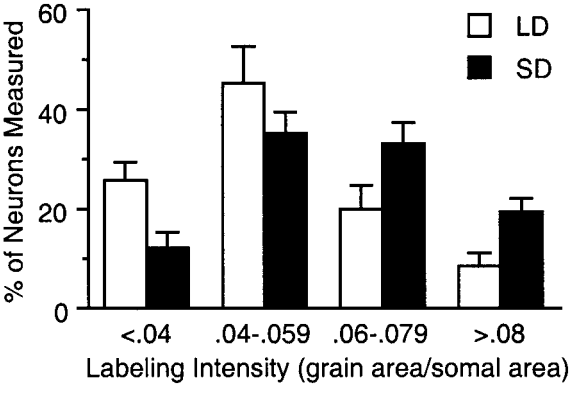

Figure 5 Frequency distributions of the intensity of NR2B hybridization over individual lMAN neurons. Within each treatment group, measurements from individual animals were sorted according to their labeling intensity (bin width 0.02). The entire distribution of labeling intensity is shifted upwards in the SD group as compared to the LD group. Data shown are group means ± S.E.M.

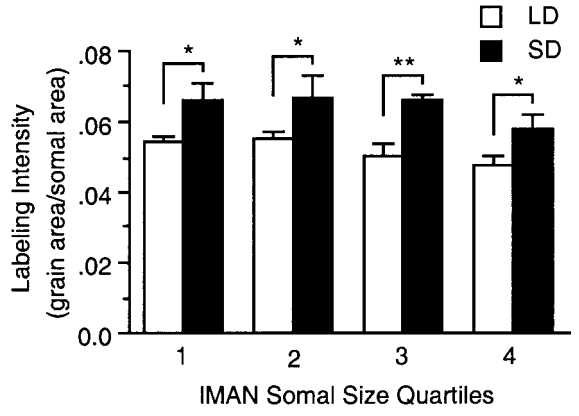

Figure 6 SD photoperiod increased NR2B mRNA expression within virtually all size classes of lMAN neurons. Somal size quartiles were based on all neurons sampled (there was no effect of photoperiod on neuron size) and are as follows: 1st quartile: ≤148.7 μm^2; 2nd quartile: 148.8–179.8 μm^2; 3rd quartile: 179.9–209.9 μm^2; 4th quartile: ≥210 μm^2. Data shown are mean ± S.E.M. *$p < .05$, **$p < .01$.

all neurons within both treatment groups (as noted above, cell size was not affected by photoperiod) to determine boundaries for cell size quartiles. These quartile boundaries then were used to determine the labeling intensity of neurons in each quartile for each animal, and then averages were calculated for each treatment group. As shown in Figure 6, group differences in NR2B gene expression were evident among lMAN neurons in each of the neuronal size quartiles. A two-way ANOVA revealed a significant effect of photoperiod ($F = 25.1$, $p < .0001$), but no effect of quartile, and no interaction.

NR2A mRNA Expression

The NR2A probe generated a highly specific hybridization pattern in canary brain similar to that found in adult ZFs. In marked contrast to the NR2B expression pattern, the NR2A hybridization signal in both SD and LD birds was highest in the optic tectum and granule cell layer of the cerebellum (where NR2B was low or absent), moderate in most of the telencephalon, and low in most of the LPO, thalamus, and brainstem (Fig. 2, middle panels). In all birds, Area X was clearly evident because the hybridization signal was moderate in this region while being virtually absent in the rest of the LPO (Fig. 2, top middle). In contrast, the NR2A hybridization signal over lMAN, HVc, and RA was not visibly distinct from the surrounding regions.

Quantitative analysis revealed no effect of photoperiod on NR2A mRNA levels within any of the four song nuclei analyzed (Fig. 7). The NR2A hybridization signal was similar in SD and LD birds in lMAN ($t = 0.067$, $df = 10$, $p = .949$), area X ($t = 0.308$, $df = 10$, $p = .787$), HVc ($t = -0.9545$, $df = 10$, $p = .362$), and RA ($t = 0.94$, $df = 10$, $p = .362$).

NR1 mRNA Expression

The regional expression of NR1 mRNA in both SD and LD canaries was similar to that found in adult ZFs, and differed markedly from the expression patterns for the NR2B and NR2A probes. As shown in Figure 2 (right panels), NR1 expression was high throughout most of the telencephalon, dorsal thalamus, optic tectum, and granular cell layer of the cerebellum, and was moderate in the ventral thalamus and brainstem. The highest NR1 transcript levels were found in the LPO, particularly within Area X (see Fig. 2, upper right). While the NR1 hybridization signal clearly distinguished Area X from its surroundings, this was not the case for HVc, RA, or lMAN.

Quantitative analysis revealed no effect of photoperiod on NR1 mRNA levels within any of the four song nuclei analyzed (Fig. 8). In the lMAN where NR2B expression was higher in SD as compared to LD birds, NR1 transcript levels were slightly lower (5.7%) in the SD as compared to LD group. However, this difference was not statistically significant ($t = -1.99$, $df = 10$, $p = .097$), and could not be probed further by the single neuron analysis because the large NR1 hybridization signal obscured the intracellular

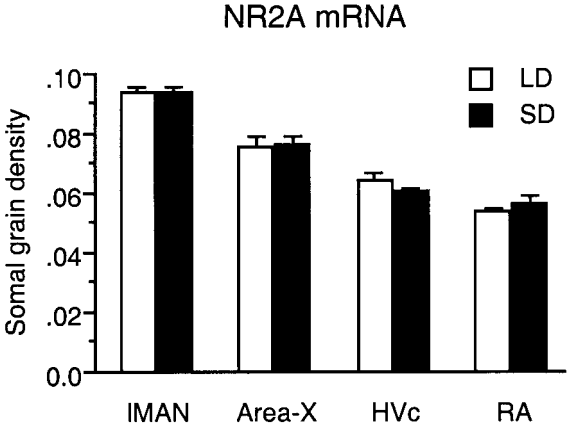

Figure 7 NR2A mRNA hybridization signals over cell soma in four song nuclei of adult canaries on LD and SD photoperiods. Photoperiod did not affect NR2A transcript levels in any of these brain regions. Data shown are mean ± S.E.M.

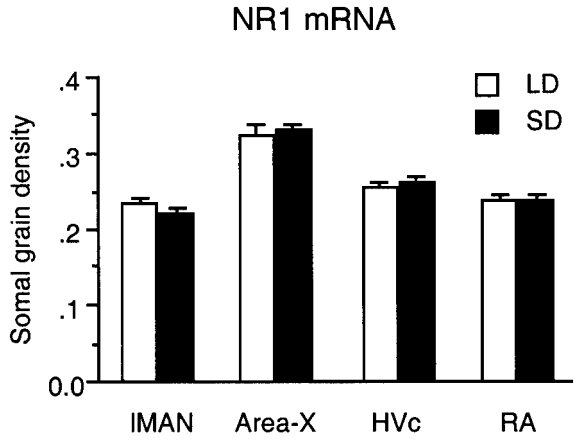

Figure 8 NR1 mRNA hybridization signals over cell soma in four song nuclei of adult canaries on LD and SD photoperiods. Photoperiod did not affect NR1 transcript levels in any of these brain regions. Data shown are mean ± S.E.M.

morphology needed to distinguish neurons from glia. In the other song regions measured, the NR1 hybridization signal was similar in SD and LD birds (Area X: $t = 0.44$, $df = 10$, $p = .68$; HVc: $t = 0.497$, $df = 10$, $p = .66$; RA: $t = 0.18$, $df = 10$, $p = .87$).

DISCUSSION

Previous studies of NMDAR expression in the developing nervous system have revealed that levels of specific NMDAR modulatory subunits correlate with the propensity for neural and behavioral plasticity. The current studies extend this relationship by demonstrating photoperiod-induced changes in NMDAR mRNA expression in song-related brain regions of adult canaries, a species exhibiting recurrent, seasonal periods of neural and behavioral change related to song. In birds maintained on SDs, NR2B mRNA levels are relatively high in both the lMAN and RA. The lMAN is important for vocal plasticity in both young and adult birds (Bottjer et al., 1984; Williams and Mehta, 1999; Brainard and Doupe, 2000), and while RA is necessary for song production (Nottebohm et al., 1976; Simpson and Vicario, 1990), it likely also plays a role in vocal plasticity. Under SD photoperiod conditions, T levels are extremely low, and song is infrequent and variable in structure (Nottebohm et al., 1987). LD photoperiods, which increase T levels dramatically and are accompanied by the refinement and stabilization of a new song pattern, reduce NR2B transcript levels in these same song nuclei. A similar (although larger) decline in NR2B expression occurs in young ZFs as they enter the period of song development. However, unlike the developmental changes evident in ZFs, photoperiod-induced changes in adult canaries do not appear to include message levels for the constitutive NR1 or modulatory NR2A subunits in any of the four song nuclei studied. While a variety of studies implicate changes in the ratio of NR2B:NR2A subunits in fostering experience-driven neural and behavioral organization during development (Hestrin, 1992; Livingston and Mooney, 1997; Quinlan et al., 1999a; Roberts and Ramoa, 1999; White et al., 1999; Singh et al., 2000; Philpot et al., 2001), our results support emerging evidence that such changes in NMDAR expression may also contribute to the *reorganization* of brain and behavior in adulthood (Rumpel et al., 2000; Mittmann and Eysel, 2001).

Although the present study does not elucidate causal relationships, it seems likely that photoperiod regulates NR2B expression in lMAN and RA through its modulation of gonadal androgen secretion. One possibility is that T influences NMDAR transcription directly, as both the lMAN and RA are rich in androgen-accumulating cells (Arnold et al., 1976; Brenowitz and Arnold, 1992). In young ZFs, early exposure to T accelerates the developmental decrease in NR2B mRNA levels within the lMAN (Singh et al., 2000), although similar effects are not evident in RA (unpublished observations). However, it is not known if hormone responsive elements are associated with genes encoding NMDAR subunits. Alternatively, photoperiod-induced changes in T levels may influence NR2B expression indirectly, for example by modifying other regulators of NMDAR gene expression, or by altering the frequency of song behavior, which in turn may affect NR2B expression. In the current study, LD-induced decreases in NR2B expression were measured 4 weeks after T levels began to increase, allowing ample time for an increase in song production. More frequent song behavior would increase neural activity in the lMAN and RA (Margoliash, 1997; Hessler and Doupe, 1999), and neural activity is known to regulate NMDAR subunit expression in other systems (Catalano et al., 1997; Hoffmann et al., 2000). An increase in song behavior also would augment levels of trophic factor expression in HVc neurons that project to RA (Li et al., 2000) and that could interact with lMAN efferents that terminate in RA. To better discriminate between these various alternatives, it will be important to test directly whether T does in fact mediate the observed effects of photoperiod. Additionally, it will be informative to track more accurately the time-course of photoperiod or T-induced changes in NR2B expression particu-

larly as they may relate to changes in the frequency of song behavior.

The lack of photoperiod effects on NR2A hybridization levels was somewhat surprising, because photoperiod drastically altered T levels, and exogenous T increases NR2A transcript levels in the lMAN of young ZFs (Heinrich et al., 2002). However, it is not known whether manipulations of T levels in adult ZFs would affect NR2A expression levels. Perhaps in both species, there is a critical developmental period for such hormone action. Alternatively, NR2A gene expression may not be subject to hormonal regulation in canaries. The developmental regulation of NR2A expression has not been examined in this species.

In discussing the functional implications of the effects of photoperiod on NR2B mRNA expression, we must first consider whether they are likely to accurately predict changes in the composition of NMDARs, and whether such changes are likely to have functional consequences. Although regulation of mRNA abundance does not always translate into proportional changes in protein level, developmental and hormonal regulation of NMDAR subunit mRNA levels within the song system, are, for the most part, paralleled by appropriate changes in receptor pharmacology and physiology. In young ZFs, decreases in NR1 and NR2B transcripts within the lMAN roughly parallel decreases in overall NMDAR density and NR2B-specific binding, respectively (Aamodt et al., 1995; Basham et al., 1999; Singh et al., 2000). Moreover, NMDAR current duration in lMAN and RA decreases (Livingston and Mooney, 1997; Stark and Perkel, 1999; White et al., 1999) in parallel with increases in NR2A and decreases in NR2B mRNA (Singh et al., 2000; Scott et al., 2001; Heinrich et al., 2002). Thus, while it will be important to verify photoperiod-induced changes in NMDAR function as well as to measure NR2B protein directly, we are reasonably confident that the observed changes in NR2B mRNA in canary lMAN and RA are indicative of changes in receptor composition.

There are at least two distinct ways in which changes in the NR2B:NR2A subunit ratio could impact the seasonal reorganization and refinement of circuits controlling song behavior. High NR2B:NR2A ratios are associated with relatively long duration NMDAR currents, and NMDAR currents shorten as this ratio declines. Longer currents could favor synapse formation and strengthening by extending the temporal window for coincidence detection, whereas the transition to shorter currents (lower NR2B:NR2A) might favor NMDAR-mediated synaptic depression and elimination. However, because NMDAR current duration is much more sensitive to changes in NR2A than NR2B subunit expression (Flint et al., 1997; Lu et al., 2001), it is unlikely that the modest seasonal changes in NR2B reported here produce significant changes in NMDAR decay times. A second way changes in NR2B expression might affect the probability of synaptic strengthening is through this subunit's interactions with key intracellular signaling molecules. For example, NR2B associates with CaMKII and can lock it in an active conformation (Bayer et al., 2001) that promotes hyperphosphorylation of CaMKII, a critical step for the induction of LTP (Lisman and McIntyre, 2001). Recent studies in developing somatosensory cortex suggest that reduced plasticity may relate to a decline in NR2B subunit expression (Barth and Malenka, 2001) that is independent of altered NMDAR current duration (Lu et al., 2001).

In the present context, elevated NR2B expression during the nonbreeding season may lower the threshold for synaptic strengthening. This may help maintain relatively inactive circuitry, and perhaps promote synaptogenesis during an early, rapid stage of LD-induced neural regrowth (Tramontin and Brenowitz, 2000). This suggestion is in accord with reported increases in both the NR2B:NR2A mRNA ratio and LTP induction that accompany lesion-induced plasticity and reorganization in adult mammalian visual cortex (Rumpel et al., 2000; Mittmann and Eysel, 2001). As day length increases and NR2B levels decrease, the threshold for LTP is likely to be raised, thereby increasing synaptic competition and promoting an experience-driven synaptic refinement of this new and exuberant circuitry. This model is consistent with recent studies suggesting that developmental decreases in the NR2B:NR2A ratio are more closely associated with the beginning, rather than the end, of periods for experience-dependent plasticity (Roberts and Ramoa, 1999; Cao et al., 2000a, b; Singh et al., 2000). While this scenario seems inconsistent with the absence of photoperiod-induced changes in NMDAR subunit expression within the HVc, a region exhibiting substantial photoperiod-induced neural growth and reorganization (Nottebohm, 1981; but see Leitner et al., 2001), measures of NMDAR mRNA in this nucleus need to be interpreted cautiously. Unlike lMAN and RA, the HVc recruits new neurons throughout adulthood, and the rate of incorporation of these new cells varies seasonally (Alvarez-Buylla et al., 1990; Kirn et al., 1994; Tramontin and Brenowitz, 1999). Hence, LD-induced decreases in NR2B expression within extant HVc neurons could be masked by an influx of newly generated cells exhibiting immature patterns of gene expression (i.e., elevated NR2B).

While changes in NMDAR subunit expression now have been associated with periods of vocal plasticity in both young ZFs and adult canaries, their relationship to distinct aspects of song behavior is unclear. In canaries maintained under natural photoperiod conditions, troughs in plasma T levels are associated with song instability (Nottebohm et al., 1987), perhaps reflecting the elevated NR2B:NR2A ratio within the lMAN and RA. As T levels rise gradually, new syllable types are produced and a transition back to a lower NR2B:2A ratio might facilitate song refinement and restabilization. In this context, a more precise correlation between changes in NR2B levels, photoperiod-associated changes in T titers, syllable addition, and song refinement would be informative. However, it would be important that such a study be done under natural photoperiod transitions, because the relatively rapid shifts in photoperiod employed in the current study may not accurately reproduce the normal time course of changes in NR2B levels. It is important to note also that rising T levels stimulate an increase in the amount of singing behavior. Thus, the transition to a lower NR2B:NR2A ratio also could be an adjustment to the increase in motor-driven activity, rather than a preparatory change fostering vocal plasticity. By studying species in which seasonal changes in T levels and vocal production are not accompanied by song restructuring (e.g., white-crowned sparrows), it may be possible to discriminate between these alternatives. Finally, even if changes in the NR2B:NR2A ratio are functionally related to vocal plasticity, it remains unresolved whether they promote song memorization, sensorimotor learning (vocal practice), or both. In ZFs, the overlap between these two stages of song learning has impaired our ability to relate changes in NMDAR gene expression specifically to song memorization or sensorimotor learning. Likewise, it is not known to what extent the seasonal restructuring of song in adult canaries entails memorization of new song elements, as opposed to the retrieval and imitation of elements acquired during the first year of life (Leitner et al., 2001). Here again, comparative work that exploits the tremendous species diversity in the timing of these different stages of song learning should prove a powerful tool for clarifying the relationships between changes in NMDAR subunits, neural reorganization, song memorization, and sensorimotor learning.

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