IDENTIFICATION
OF CATECHOLAMINERGIC CELL GROUPS
IN THE BRAINSTEM OF THE CANARY, ZEBRA FINCH,
WHITE-THROATED SPARROW AND BUDGERIGAR
BY TYROSINE HYDROXYLASE IMMUNOCYTOCHEMISTRY

JACQUES BALTHAZART (¹) AND GREGORY F. BALL (²)

(¹) Laboratory of Biochemistry, University of Liège, 17 place Delcour, B-4020 Liège, Belgium
(²) Department of Psychology, Behavioral Neuroendocrinology Group, The Johns Hopkins
University, Baltimore, MD 21218,USA

Summary. In Passeriformes and Psittaciformes, the learning and production of complex learned vocalizations is controlled by a network of telencephalic, diencephalic and mesencephalic nuclei, the so-called song-control system. Specialized telencephalic song control nuclei such as the high vocal center, nucleus robustus archistriatalis and area X in the basal ganglia receive dense and discrete catecholaminergic inputs. Catecholaminergic fibers also innervate the telencephalon of other birds and other vertebrate species but there appear to be unique specializations of the catecholaminergic inputs in songbirds. In this paper the distribution of tyrosine hydroxylase-immunoreactive cell groups in the brainstem is described in 4 avian species that are known to be vocal learners. The goal of this work is to identify whether novel cell groups are present in the brainstem that may give rise to the specialized catecholaminergic projections in the telencephalon of these vocal learners. These brainstem cell groups are known to be the source of most telencephalic catecholaminergic input in birds and other vertebrates. Three songbird species, the zebra finch, the canary and the white-throated sparrow and one psittaciform, the budgerigar were analyzed. Immunocytochemical analysis identified in the brainstem of the 4 species the same groups of tyrosine hydroxylase-immunoreactive cells that were previously described in the brain of other non-song birds. These were located in the area ventralis of Tsai (A10), around the substantia nigra (A9) and its caudal extension (retroruberal field, A8), and in the nucleus ceruleus and subceruleus (A6). No additional cell group could be detected in these species by comparison with the other species of non oscines studied previously. This suggests that both in song birds and in budgerigars specialized innervations by catecholaminergic neurones of the telencephalic song control nuclei are not associated with the evolution of novel catecholaminergic cell groups in the brainstem as compared to those that are present in species that do not display these specializations.

Key words: Song system, song control nuclei, song birds, oscines, chemical neuroanatomy, dopamine, noradrenaline.

INTRODUCTION

The catecholamines dopamine (DA), norepinephrine (NE) and epinephrine (E) are known to play a crucial role in the control of various brain functions related to reproduction such as the synthesis and release of gonadotrophin releasing hormone (GnRH) and the activation of male and female sexual behavior (CROWLEY & ZEMLAN, 1981; CROWLEY et al., 1989; MEYERSON et al., 1979; MEYERSON et al., 1985; BITRAN & HULL, 1987; BARCLAY & CHENG, 1992). The activity of catecholaminergic neurons is itself modulated by steroids so that changes in DA and NE turnover rate are often hypothesized to be a part of the cascade of biochemical events that are triggered by steroids in the brain and are responsible for the physiological and behavioral effects of these steroids (MEISEL & SACHS, 1994; PFAFF et al., 1994). Alternative explanations have however been suggested (BALTHAZART & BALL, 1992).

In birds, specific vocalizations are associated with reproduction and activated by steroids (NOTTEBOHM 1975). Three avian orders, the Passeriformes (songbirds), Psittaciformes (parrots, budgerigars) and Trochiliformes (hummingbirds) have independently evolved the ability to learn and produce complex vocalizations. These vocalizations are learned, either during an early age or throughout the entire life of the bird. In two of these orders (Passeriformes, Psittaciformes), it has been demonstrated that the learning and production of these vocalizations is controlled by a complex network of telencephalic, diencephalic and mesencephalic nuclei, the so-called song-control system (NOTTEBOHM, 1980; KONISHI, 1985; STRIEDTER, 1994; BRAUTH et al., 1994; BALL, 1994).

In songbirds, several lines of evidence indicate that the specialized telencephalic song control nuclei such as HVC (High vocal center, formerly Hyperstriatum ventral pars caudale), RA (nucleus robustus archistriatalis), the lateral part of the magnocellular nucleus of the anterior neostriatum (lMAN), and area X of the parolfactory lobe receive dense catecholaminergic inputs. For example, fibers immunoreactive for tyrosine hydroxylase have been described in HVC, RA, lMAN and area X of zebra finches (BOTTJER, 1993; SOHA et al., 1995), and high densities of noradrenergic receptors of the α_2 and $\beta1/\beta2$ subtypes (HVC, RA and area X) (BALL et al., 1994; BALL, 1994) or of dopaminergic D1 receptors (Area X) (CASTO & BALL, 1994a) have been described in several song birds species. High concentrations of NE and DA have also been measured in the song control nuclei of zebra finches and the baseline levels and/or turnover rates of these amines appears to be modulated by steroids and during development (BARCLAY & HARDING, 1988, 1990; HARDING et al., 1995). NE also appears to play a role in the control of vocal behavior of zebra finches (BARCLAY et al., 1992).

Less information is available for Psittaciformes, another avian order of vocal learners, but it is already clear at present that species such as the budgerigar possess telencephalic specializations that are analogous (and probably not homologous) to the HVC and RA of song birds (STRIEDTER, 1994; BRAUTH et al., 1994; BALL 1994) and at least one of these nuclei, the magnocellular nucleus of the parolfactory lobe (LPOm) also receives a dense catecholaminergic input as indicated by the presence of high densities of receptors (BALL, 1994).

NE or DA-containing fibres also innervate the telencephalon of other birds and other species of vertebrates but the highly specific, dense catecholaminergic inputs received by HVC and RA appear to be unique to songbirds. This therefore raises the question of the origin of these projections. One obvious hypothesis is that these dense neuroanatomically discrete inputs originate from the same catecholaminergic brain areas that have been described in other vertebrate species including birds (REINER et al., 1994). Alternatively, specific cell groups may have evolved in vocal learners in order to support this specialized innervation of the telencephalon. In the brain, the catecholamines, NE and E are synthesized solely in the brainstem of all vertebrate species including birds (REINER et al., 1994). DA is primarily synthesized in the brainstem, however, cells synthesizing DA have also been identified in the diencephalon (REINER et al., 1994). The available data do not suggest that novel cell groups are present in the brainstem of songbirds and psittaciforms. One study by Bottjer described the distribution of tyrosine hydroxylase (TH) immunoreactive cells in the zebra finch brain (BOTTJER, 1993). This study did not appear to identify catecholaminergic cells groups that would be specific to song birds but the generality of this finding should be tested in other members of the oscine family. In addition, because similar telencephalic specializations have independently evolved in psittaciforms and because some of these nuclei also receive dense catecholaminergic inputs, it is also appropriate to ask whether the brainstem catecholaminergic nuclei in these species also follow the common vertebrate pattern. Previous work utilizing histofluoresence methods to describe the distribution of catecholamines in a psittaciform species, the budgerigar (*Melopsittacus undulatus*) does not suggest that novel catecholaminergic cell groups are present in this species (TAKATSUKI et al., 1981; SHIOSAKA et al., 1981; TOHYAMA et al., 1974). A single study utilized immunocytochemical methods for the localization of TH and other enzymatic markers of monoamines but this study focussed on monoaminergic cells that either contain or do not contain immunoreactive L-amino acid decarboxylase and did not provide an overview of cells containing catecholamines in the budgerigar brain (SAKAI et al., 1992).

It is difficult to make accurate inter-specific comparisons based on studies utilizing different methods and carried out in different laboratories. Therefore, in the present study questions concerning the presence of catecholaminergic cell groups are addressed consistently in 3 species of songbirds and one psittaciform, the budgerigar. These studies focus on the brainstem, the site of noradrenergic and adrenergic synthesis and of the greater part of dopaminergic synthesis (REINER et al., 1994).

MATERIAL AND METHODS

Subjects and fixation

The immunocytochemical experiments described here were carried out on three species of song birds, namely canaries (*Serinus canaria* Linné, 1758; n=6), zebra finches (*Taeniopygia guttata* Vieillot, 1817; n=8), white-throated sparrow (*Zonotrichia albicollis* Gmelin, 1789; n=1) and on another species of vocal learning bird that does not belong to the passerine order, the budgerigar (*Melopsittacus undulatus* Shaw, 1805; n=2). Subjects

were obtained from a breeding colony established at the University of Liège (Zebra fin-ches) or were bought from local breeders in Liège (canaries, budgerigars). The white-thro-ated sparrow was caught in the wild in North Carolina, kindly provided by Dr. Stephen Nowicki, Duke University. In the laboratory, all birds were maintained under a photope-riod simulating long days (16 hours of light, 8 hours of dark per day) with food and water available at libitum. All subjects were male and sexually mature as evidenced by the pre-sence of fully developed testes.

Birds were deeply anesthetized with Hypnodil (Janssen Pharmaceutica, Beerse, Belgium, 50 mg/kg body weight) and intravenously injected with 50-100 µl of heparin solution (Sigma H-7005, 20 mg/ml or 3340 units/ml). They were then perfused through the heart with a saline solution (9 g/l; 0.15 M NaCl) until the return blood in the atrium was clear, followed by 100-300 ml of fixative (paraformaldehyde 4% and 0.1% glutar-aldehyde in 0.15 M phosphate buffer, pH 7.2). Brains were immediately dissected out of the skull, post-fixed one hour in the fixative solution without glutaraldehyde and placed overnight in a 20% sucrose solution in 0.1 M phosphate buffer-saline, pH 7.2. Brains were then frozen on powdered dry ice and stored in a freezer at -75° C until used. This same procedure was used for all species except for the white-throated sparrow which was anes-thetized with chloral hydrate and perfused with a fixative containing 4% paraformaldehy-de without any glutaraldehyde.

Brains were cut with a cryostat in the coronal plane at 30 µm thickness starting at the rostral end. The plane of section was adjusted to match as closely as possible the atlas of the canary brain (STOKES et al., 1974). Sections used in this study were collected through the mesencephalon from the level of the occulomotor nerves to the level of the nucleus vestibularis. Every fifth section at least (one section every 150 µm or more) was stained by immunocytochemistry for tyrosine hydroxylase.

Tyrosine Hydroxylase Immunocytochemistry

The distribution of tyrosine hydroxylase (TH) immunoreactive (ir) cells was visuali-zed in free floating sections by a standard indirect immunocytochemical procedure using peroxidase as reporter enzyme and diaminobenzidine as the chromogen. This method has been fully described and validated previously (BAILHACHE & BALTHAZART, 1993). Briefly, after two rinses in phosphate buffer 0.01 M-saline (PBS), sections were treated for 15 min with 0.6% H_2O_2 in methanol to block endogenous peroxidase, rinsed in PBS and placed overnight at 4° C in the primary TH antiserum (mouse anti-TH, Incstar cat.Nbr.22941, dilution 1/1000 in PBS containing 0.1% Triton X-100). This antibody was raised against TH purified from rat PC12 cells and recognizes an epitope in the mid-portion of the TH molecule that has been well conserved through evolution so that cross-reactivity is obser-ved over a wide range of species. This antibody does not cross-react with dopamine β-hydroxylase, phenylethanolamine-N-methyltransferase, phenylalanine hydroxylase or tryptophan hydroxylase (Incstar specification sheets). We have also shown that this anti-body exclusively recognizes in the quail brain cell groups that are known to be catechola-minergic and that omission of the primary antibody eliminates all immunocytochemical

staining (BAILHACHE & BALTHAZART, 1993). Its specificity for the TH molecule is therefore firmly established.

Sections were then rinsed in PBS and incubated with a goat anti-mouse peroxidase-conjugated antibody (peroxidase-conjugated affinity-isolated goat immunoglobulins to mouse immunoglobulins, DAKO P-447) at a dilution of 1/200 for one hour. The peroxidase was finally revealed by placing the sections for 6 min in a solution of diaminobenzidine (DAB; 20 mg in 50 ml PBS containing 0.1% Triton X-100 and 20 µl of H_2O_2 at 30%). Sections were then mounted on microscope slides and coverslipped.

RESULTS

Dense groups of TH-ir perikarya were observed throughout the rostral to caudal extent of the brainstem in the three song birds species that were considered (see Fig. 1 for the zebra finch and white-throated sparrow, Fig. 2 for canary). They are described below as they appear in a rostral to caudal order.

First a numerous group of TH-ir cells bodies is present in the area ventralis of Tsai (AVT), just lateral to the roots of the third nerve, which indicate the transition from the hypothalamus to the mesencephalon (Fig. 1A-B). This cell group was visible in several consecutive sections taken along the rostral to caudal extent of the mesencephalon. It is systematically accompanied by a smaller number of TH-ir cells that are located medially to the third nerves just dorsal to the nucleus interpeduncularis.

At its most caudal level, the AVT group of TH-ir perikarya progressively expands in a dorso-lateral direction (Fig. 2A-B) and invades a very wide area at the level of the substantia nigra (SN). The AVT group of TH-ir cells then disappears as the new group reaches its maximal extent (Figs 1C-D, 2C). The SN contains by far the largest number of TH-ir cells in the brains of the 3 species considered here and the density of these cells is so high that they can barely be discriminated in 30 µm-thick sections (Figs 1E-F, 2B). At even more caudal levels, the SN group of TH-ir cells progressively thins out leaving only a small group of positive cells in a more dorsal position that presumably corresponds to the mammalian A8 group (retroruberal).

This cell group disappears at the mesencephalic-metencephalic junction and a compact but smaller and less dense group of TH positive cells appears in a dorso-lateral position at the ventro-lateral corner of the aqueduct (Figs 1H, 2D) at the level of the locus ceruleus. At its rostral end, this group of TH-ir cells is less dense and does not form such a clearly recognizable cell group. These TH-ir cells located just lateral to the fasciculus longitudinalis medialis are, however, usually considered as the rostral end of the locus ceruleus (Fig. 1G).

At similar levels in the rostral to caudal axis, a scattered population of TH-ir positive perikarya is also present in a more ventral and lateral position. These TH-ir cells are distributed over a wide area and do not appear to be specifically associated with any specific cell group as identified in classical histology stains although they clearly overlap with the dorsal and ventral parts of the nucleus subceruleus.
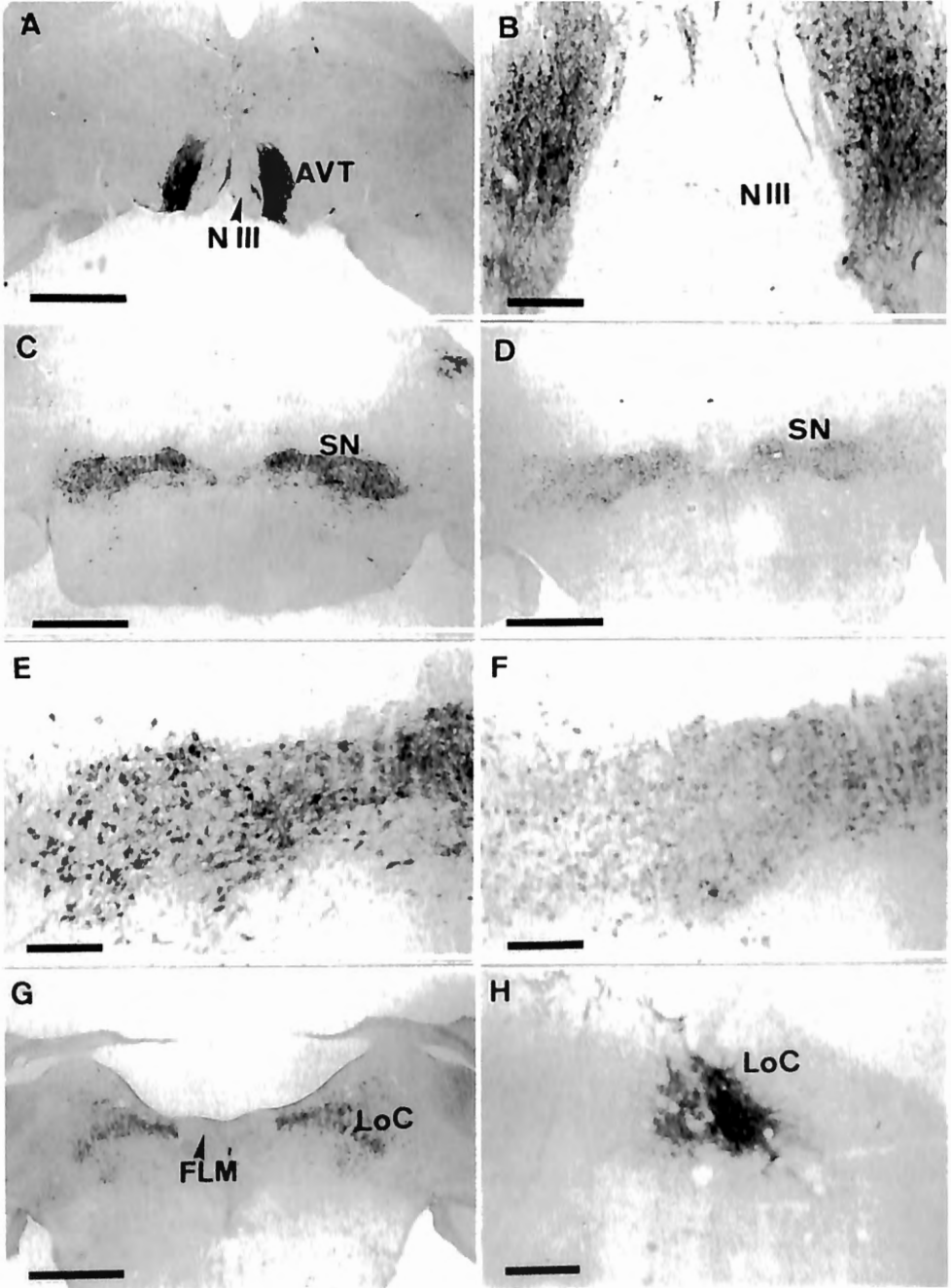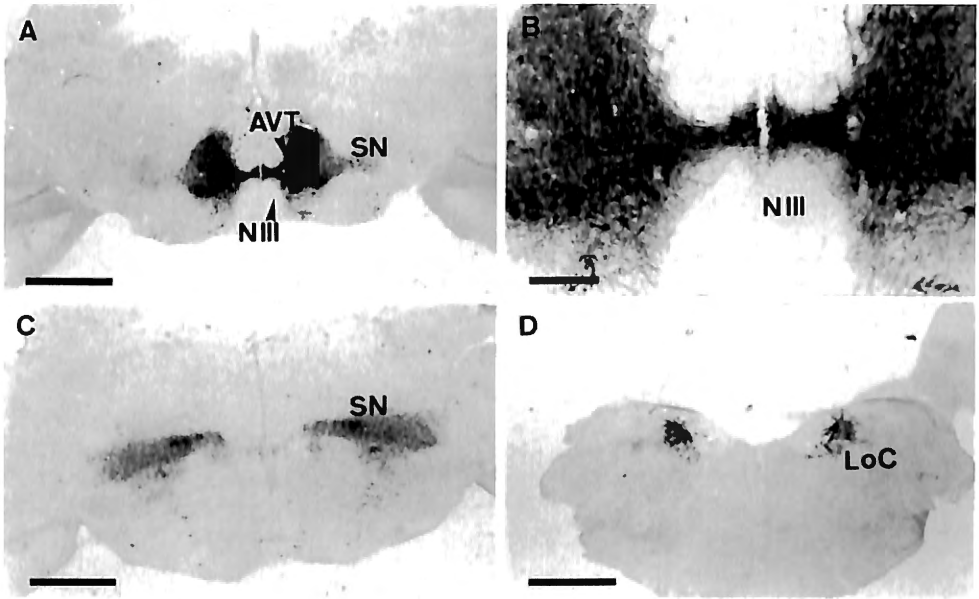
Fig. 2. – Photomicrographs illustrating the distribution of TH-ir cells in the midbrain of the canary. A: Group of TH-ir cells located at the caudal end of the area ventralis of Tsai where it expands in a dorsolateral direction to merge with the substantia nigra. B: Higher magnification of the same area. C: Group of TH-ir cells in the substantia nigra. D: TH-ir cells located at the level of the locus ceruleus. Magnification bar = 1 mm in A, C, D and 200 μm in B. AVT= area ventralis of Tsai, LoC= locus ceruleus, NIII= nervus oculomotorius (third nerve), SN= substantia nigra.

The caudal end of the pons is then essentially devoid of any TH-positive perikarya but small additional populations of immunoreactive cells are located in the medulla oblongata during a fairly long rostral to caudal extension of its more rostral part. Two subgroups of positive cells are recognizable at this level. One is located in dorsomedial position at the level of the nucleus tractus solitarii while the other is more ventral and is centered around the complex of the nucleus reticularis.

A similar distribution of TH-ir cells was observed in the midbrain of budgerigars (Fig. 3). The most rostral group of positive cells occurs at the level of the occulomotor nerves and is clearly identified as the AVT (Fig. 3A-B). It is followed at more caudal levels by the very abundant population of TH-ir cells corresponding to the SN (Fig. 3C-D).

Fig. 1. – Photomicrographs illustrating the distribution of TH-ir cells in the midbrain of the Zebra finch (left; A, C, E, G) and of the white-throated sparrow (right; B, D, F, H). The top panels illustrate the TH-ir cells located in the area ventralis of Tsai (A, B) and in the substantia nigra at low (C, D) and high (E, F) magnification. The two bottom panels illustrate the TH-ir cells located in the locus ceruleus at its rostral end at the level of the fasciculus longitudinalis medialis (G) or more caudally when TH-ir cells form a dense cluster that outlines the locus ceruleus (H). Magnification bar = 1 mm in A, C, D, G and 200 μm in B, E, F, H. AVT= area ventralis of Tsai, FLM= fasciculus longitudinalis medialis, LoC= locus ceruleus, NIII= nervus oculomotorius (third nerve), SN= substantia nigra.

Scattered TH-ir cells then appear at a slightly more caudal level in dorsal position, just lateral to the fasciculus longitudinalis medialis. At more caudal levels, the dorsal part of this cluster becomes very dense and clearly identifies the locus ceruleus located at the ventrolateral edges of the aqueduct as can be observed in sections stained by classical histology techniques (Fig. 3E-F).
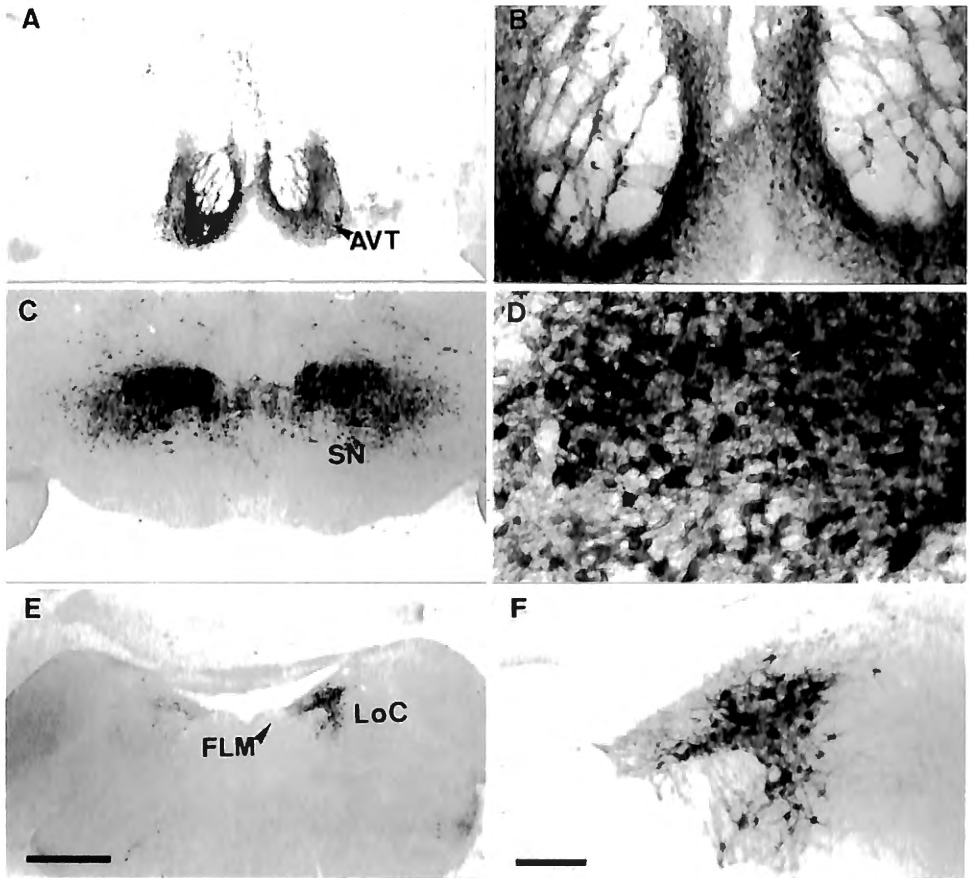

Fig. 3. – Photomicrographs illustrating the distribution of TH-ir cells in the midbrain of the budgerigar. The same areas are shown at low and high magnification in the left and right columns respectively. Panels are arranged in a rostral to caudal order from the top to the bottom. A-B: TH-ir cells at the level of the area ventralis of Tsai. C-D: TH-ir cells at the maximal extension of the substantia nigra. E-F: dense group TH-ir cells outlining the locus ceruleus. A few immunoreactive cells are also visible in a ventrolateral position. These cells belong to the subceruleus cell group. The magnification bar in the left column (panels A, C, and E)=1 mm; magnification bar in the right column (panels in B, D, and F)= 200 μm. AVT= area ventralis of Tsai, FLM= fasciculus longitudinalis medialis, LoC= locus ceruleus, NIII= nervus oculomotorius (third nerve), SN= substantia nigra.

DISCUSSION

Anatomical findings and homologies

The neurochemical studies described in the introduction demonstrate that the telen-cephalic nuclei of the song system receive a dense catecholaminergic innervation that is not visible in the corresponding brain areas of other birds (non-song birds). To our know-ledge, the origin of these projections has not been investigated in detail. The presence of a connection between midbrain dopaminergic cells groups and the newly formed neurons of the dorsal telencephalon (in and around HVC) in the canary brain has, however, been reported in abstract form (BURD et al.,1986).

As a first step in trying to establish the origin of these catecholaminergic pathways innervating the song control nuclei, we analyzed here the TH-ir cells groups in the mesen-cephalon and the metencephalon of 3 species of songbirds and one non-oscine vocal lear-ner the budgerigar. We have identified in the brainstem of all 4 species the same groups of TH-ir cells that were previously described in the brain of the quail (BAILHACHE & BALTHAZART, 1993) and in other non-oscine species (REINER et al.,1994). These cell groups had also been previously described in the brain of zebra finches (BOTTJER, 1993) and to some extent in budgerigars (TAKATSUKI et al.,1981; SHIOSAKA et al.,1981; TOHYAMA et al.,1974).

A leading group of TH-ir cells was located at the edges of the third nerve in the area ventralis of Tsai (AVT), the avian homologue of the ventral tegmental area of mammals and it can therefore be considered as the homologue of the A10 dopaminergic cell group (REINER et al.,1994) as defined in the nomenclature of Dahlström and Fuxe (DAHLSTRÖM & FUXE,1964; BJÖRKLUND & LINDVALL, 1984). It extended in the dorso-lateral direction into the substantia nigra (SN), which can be considered as the avian homologue of the A9 dopaminergic cell group (DALHSTRÖM & FUXE, 1964; BJÖRKLUND & LINDVALL, 1984; REINER et al.,1994) and more caudally into the retroruberal field (A8). These cell groups have also been described in other avian species (BAILHACHE & BALTHAZART 1993; MOONS et al., 1994; REINER et al., 1994).

The TH-ir cell group located at the mesencephalic-metencephalic junction at the vent-ro-lateral corner of the aqueduct is also found in other species in which it also reacts with antibodies directed against dopamine β-hydroxylase (quail: BAILHACHE & BALTHAZART, 1993; European starling, Sturnus vulgaris: BALL G.F. and BERNARD D.J., pers. comm.). This indicates the noradrenergic nature of this cell group that can be considered as the homologue of the locus ceruleus (noradrenergic cell group number A6: DAHLSTRÖM & FUXE 1964; MOORE & CARD, 1984) even if some avian brain atlases do not locate this nucleus in this exact position (KUENZEL & MASSON, 1988) or do not mention it at all (BAYLÉ et al.,1974). The more scattered TH-ir populations that overlap partly with the nucleus subceruleus are also noradrenergic in other avian species (BAILHACHE & BALTHAZART, 1993; BALL G.F. and BERNARD D.J., pers. comm.) and may be the homolo-gue of the mammalian A7 group but this conclusion can only be considered as tentative at present (REINER et al.,1994).

No additional cell group could be detected in these species of vocal learners in com-parison with the other species of non-oscines that have already been studied. This sugge-

sts that both in oscines and in psittaciforms the specialized innervation of the telencephalic song control nuclei have developed from catecholaminergic cell groups that are already present in species that do not display these specializations. This suggestion can be made more strongly for noradrenergic and adrenergic projections, the two catecholamines that appear to be synthesized solely in the brainstem, than for dopaminergic projections (REINER *et al.*, 1994). Although, DA is primarily synthesized in the brainstem, cells synthesizing dopamine have also been identified in the diencephalon (REINER *et al.*, 1994; MOONS *et al.*, 1994). Therefore, the neuroanatomical and neurochemical specializations of the telencephalic song control nuclei seem to involve variations of projections from existing catecholaminergic cell groups rather than the evolution of novel cell populations. This analysis does not preclude the possibility that a more detailed quantitative study within these midbrain cell groups would reveal inter-specific differences indicative of the neural specialization associated with vocal learning. This study, however, clearly shows that the neurochemically specialized song control nuclei that are characterized by the unique presence of steroid receptors and of dense populations of catecholamine receptors (BALL, 1990; 1994) are not associated with the presence of new catecholaminergic cell groups in the brainstem.

Functional interpretations

The functional significance of the noradrenergic and dopaminergic inputs to the song control nuclei remains unclear at present. The steroidal activation of many reproductive behaviors is known to involve the modulation of noradrenergic transmission (NOCK & FEDER, 1981; CROWLEY *et al.*, 1989; ETGEN *et al.*, 1992). In particular the density of α_2 -adrenergic receptors has been shown to be modulated by steroids in brain areas important for the activation of reproductive behavior in mammals and in nuclei involved in the control of vocalizations in non-songbird species such as the Japanese quail (JOHNSON *et al.*, 1988; BALL & BALTHAZART, 1990). Many of the song control nuclei are characterized by high levels of norepinephrine and these are known to be modulated by steroids (BARCLAY & HARDING 1990). It is therefore possible that the activation of song by steroids is also mediated by local changes in noradrenergic (and dopaminergic) transmission. This notion is supported by one recent experiment showing that treatment of male zebra finches with the noradrenergic neurotoxin DSP4 significantly decreases male courtship song and the behavioral deficit is correlated with the depletion of norepinephrine in some song control nuclei such as RA (BARCLAY *et al.*, 1992). The behavioral impairment appears to result from an attentional rather than a motor deficit: latency to intitiate singing during behavioral tests increases in drug-treated animals but once behavior is initiated it is identical to that produced by untreated controls. This correlates well with previous research that relates norepinephrine to attention and memory processes (MCGAUGH, 1985; SARA, 1985).

On the other hand, recent data indicate that the catecholaminergic innervation of the telencephalic song control nuclei, identified by TH immunocytochemistry (SOHA *et al.*, 1995) or by direct assay of the NE and DA content in micropunched nuclei (HARDING *et al.*, 1995) or by quantitative autoradiography of specific receptor subtypes (CASTO & BALL, 1994b), progressively develops during the first two to three months post-hatch in male zebra finches, that is during the period when song is learned. This raises the possi-

bility that the noradrenergic and/or dopaminergic neurotransmission could play a role in the development of the song system or in song learning.

These studies suggest potential roles for catecholamines in the development and activation of song but these now need to be established experimentally. In particular, it is important to evaluate the morphological and behavioral consequences of electrolytic or neurochemical lesions aimed at the noradrenergic or dopaminergic cell groups that innervate the song control nuclei. The present study clearly suggests that, in songbirds and psittaciforms as in other avian species, these should be located in the midbrain but the specific connections should now be investigated by retrograde tract tracing. Such studies would provide a sound basis for the lesion investigations and would also determine whether there is any variation in the pattern of catecholaminergic innervation between the two types of vocal learners. Tracing studies would also be essential to ascertain definitely whether the focal innervation of the song control nuclei derives from the same catecholaminergic cell groups that innervate, in a less dense and non specific manner, the general brain areas where these specialized nuclei have developed.

ACKNOWLEDGMENTS

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