

Muscarinic Cholinergic Receptors in the Songbird and Quail Brain: A Quantitative Autoradiographic Study

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ABSTRACT

In order to clarify the neuroanatomical basis for postulated muscarinic cholinergic control of a wide array of physiological processes in birds, the distribution of muscarinic cholinergic receptors in the brain of three avian species was investigated by quantitative autoradiography. The species consisted of two passerines (songbirds), the European starling (*Sturnus vulgaris*) and the song sparrow (*Melospiza melodia*), and one galliform, the Japanese quail (*Coturnix coturnix japonica*). [³H] N-methyl scopolamine (NMS), a muscarinic cholinergic antagonist was used as the ligand to label the receptors. Initial experiments demonstrated that the binding of this ligand in the three species is saturable in the nanomolar range and has a high affinity ($K_d = \pm 0.6$ nM). Displacement experiments revealed that three muscarinic ligands competed in an order of potency characteristic of the mammalian muscarinic receptor (i.e., atropine > oxotremorine > carbachol) for NMS binding in the avian brain.

In all three species, portions of the basal ganglia, such as the parolfactory lobe and the paleostriatum augmentatum, exhibited the highest density of binding. On the other hand, the paleostriatum primitivum, the avian homologue of the mammalian globus pallidus, contained very few binding sites. Other telencephalic sites, such as the ventral and dorsal hyperstriatum, also revealed relatively high receptor density. However, the neostriatum and especially the ectostriatum showed much lower levels. In the hypothalamus, in all three species, specific binding could be observed in the ventromedial nucleus and adjacent areas. The paraventricular nucleus also showed moderate levels of binding density, especially in the two songbird taxa. At a more rostral level, the preoptic area showed low levels of binding. In the quail, the sexually dimorphic nucleus of the preoptic area was clearly outlined in the autoradiograms by the low level of binding sites compared to the surrounding areas. In the two passerine species, nuclei of the song system were identified by either high or low levels of NMS binding. High binding defined area X and the mesencephalic nucleus, intercollicularis (ICo). In contrast, the robust nucleus of the archistriatum and the magnocellular nucleus of the anterior neostriatum showed low levels of binding in comparison with the surrounding tissue. None of these nuclei were visible in the quail autoradiograms except for ICo, which appeared as in the passerines as a heavily labelled area surrounding the lightly labelled nucleus mesencephalicus lateralis pars dorsalis. In all three species, the hippocampal complex was devoid of NMS binding except for two lateral dark bands that were present along the entire rostral to caudal extent of the hippocampus. These results suggest general similarities between the distribution of muscarinic cholinergic binding sites in birds and mammals. Among the three species, the qualitative pattern of receptor density is similar, with the exception of the telencephalic nuclei controlling vocal behavior in songbirds, which have no apparent homologues in the quail.

Accepted February 19, 1990.

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Key words: acetylcholine, neurotransmitter, N-methyl scopolamine, European starling (*Sturnus vulgaris*), song sparrow (*Melospiza melodia*), Japanese quail (*Coturnix coturnix japonica*)

Traditionally, advances in functional neuroanatomy have been greatly facilitated by comparative studies (e.g., Bullock, '84). However, with the exception of brain steroid receptors (e.g., Morrell and Pfaff, '78), comparative studies of neurochemical receptors are scarce. In this communication, we report on the localization of muscarinic cholinergic receptors in three species of birds. The taxa selected are two forms of wild songbird, the European starling (*Sturnus vulgaris*) and the song sparrow (*Melospiza melodia*) and one domesticated galliform, the Japanese quail (*Coturnix coturnix japonica*).

All three species exhibit sex differences in the volume of nuclei that mediate various dimorphic sexual behaviors (Panzica, '88). Passerine species, such as the starling and the song sparrow, possess a sexually dimorphic complex of interconnected nuclei that have been implicated in the acquisition and control of song, an important male reproductive behavior (Arnold, '80; Nottebohm, '80; Konishi, '85; Ball et al., '88). These two particular passerine species were chosen because of the extensive work that has been conducted on vocal behavior (Adret-Hausberger and Güttinger, '84; Marler and Peters, '87; Nowicki and Ball, '89) and the environmental control of reproductive physiology (Wingfield, '84; Ball and Wingfield, '87). The Japanese quail has also recently been developed as a model avian species in which to investigate brain sexual dimorphisms in relation to the control of reproductive behavior (Panzica, '88). For example, it has been shown to exhibit a sexual dimorphism in the preoptic medial nucleus (POM; Viglietti-Panzica et al., '86), which contains enzymes that metabolize steroid hormones (Schumacher and Balthazart, '87) and the steroid receptors that are necessary for the activation and organization of certain sexually dimorphic reproductive behaviors, such as copulation (Watson and Adkins-Regan, '88; Balthazart et al., '89). In neither the two passerine species nor the quail has the possibility of neurotransmitter

receptor dimorphisms been explored, although such sex differences are well established in certain mammalian systems (e.g., Fischette et al., '83; see McEwen et al., '88 for review).

There are several aspects of the behavioral biology of these species that make an investigation of neurotransmitter receptors in the central nervous system potentially illuminating. In all of these species, reproduction is controlled by photoperiod, and this response to environmental stimulation is not only mediated by changes in the peripheral endocrine system but also involves neurochemical changes in the hypothalamus or extrahypothalamic areas, or both (Follett, '84; Nicholls et al., '88). Acetylcholine is the first neurotransmitter that has been specifically linked to the process by which animals measure daylength regulating reproduction (Zatz and Herkenham, '81; Earnest and Turek, '83; Turek et al., '84; Keefe and Turek, '85). However, the anatomical sites of this cholinergic effect are at present unknown.

Muscarinic cholinergic receptors have been implicated in reproductive processes, making them an appropriate choice for investigation (Dohanich et al., '85a; McEwen et al., '88). For example, cholinergic stimulation facilitates the occurrence of female sexual behavior in certain rodent species (Clemens et al., '81). Cholinergic systems also interact with steroid dependent processes in that female rats implanted with a subthreshold dose of estradiol (E_2) are much more sensitive to the effects of cholinergic stimulation (Clemens et al., '83), and cholinergic receptors appear to be modulated by steroid hormones (Rainbow et al., '84; Olsen et al., '88).

In songbirds, muscarinic receptors have already been investigated at the neuromuscular junction of a peripheral structure essential for singing, the syrinx. This structure is hormone sensitive (Lieberburg and Nottebohm, '79), and androgens are known to modify cholinergic transmission at

Abbreviations

A	archistriatum	MAN	nucleus magnocellularis of the anterior neostriatum
AA	nucleus archistriatalis	mICo	nucleus intercollicularis, medial part
AM	nucleus anterior medialis hypothalami	MLd	nucleus mesencephalicus lateralis, pars dorsalis
APH	area parahippocampalis	N	neostriatum
Cb	cerebellum	NC	neostriatum caudale
CoA	commissura anterior	N III	nervus oculomotorius
E	ectostriatum	PA	paleostriatum augmentatum
EM	nucleus ectomamillaris	PD	nucleus preopticus dorsolateralis
FPL	fasciculus prosencephali lateralis	POM	nucleus preopticus medialis (Berk and Butler '81)
GCT	substantia grisea centralis	PP	paleostriatum primitivum
GLv	nucleus geniculatus lateralis, pars ventralis	PVN	nucleus paraventricularis magnocellularis
HA	hyperstriatum accessorium	RA	nucleus robustus archistriatalis
HA _m	hyperstriatum accessorium medialis (see text)	Rt	nucleus rotundus
HA _l	hyperstriatum accessorium lateralis (see text)	S	septum
Hp	hippocampus	SL	nucleus septalis lateralis
HV	hyperstriatum ventrale	SM	nucleus septalis medialis
HVc	hyperstriatum ventrale, pars caudalis	TeO	tectum opticum
HYp	posterior hypothalamus	TeOl	tectum opticum lateral band (see text)
IN	nucleus infundibuli hypothalami	TeOm	tectum opticum medial band (see text)
lICo	nucleus intercollicularis, lateral part	TrSM	tractus septomesencephalicus
ICo	nucleus intercollicularis	Tu	nucleus tuberis
LAD	lamina archistriatalis dorsalis	V	ventricle
LMD	lamina medullaris dorsalis	VMN	nucleus ventromedialis hypothalami
LPO	lobus parolfactorius	X	area X

the neuromuscular junction (Luine et al., '80; Bleisch et al., '84). Acetylcholinesterase staining and in vivo autoradiographic studies in zebra finches (*Poephila guttata*) have also suggested a role for muscarinic cholinergic activity in the central control of song in passerines (Ryan and Arnold, '81). In addition, nicotinic cholinergic receptors have recently been localized in several of the song control areas of the zebra finch (Watson et al., '88). Passerine male song is a learned behavior in contrast to male vocalizations in nonpasserine avian taxa such as the order Galliformes of which quail are a member. The anatomical basis of vocal behavior is poorly understood in these birds. It is possible that by examining the distribution of a receptor system that labels the oscine song system in a nonvocal learner that insight can be gained into candidate brain areas underlying vocal behavior in these species.

Quantitative in vitro autoradiography is an excellent anatomical tool that can provide insights regarding functional neurochemistry. With the exception of a recent study of the pigeon brain by Dietl et al. ('88), this method has not been extensively applied to the study of muscarinic receptors in the avian brain. In this communication, we describe the results of our efforts to map the distribution of muscarinic cholinergic receptors in the telencephalon, diencephalon, and mesencephalon of European starlings, song sparrows, and Japanese quail. Although we report most instances in which we detect the receptors, we concentrate on areas known to be involved in reproduction and on those that are sexually dimorphic.

MATERIALS AND METHODS

Species and tissue preparation

European starlings ($n = 9$ males and $n = 5$ females) and song sparrows ($n = 5$ males and $n = 7$ females) were collected from the grounds and surrounding areas of the Rockefeller University Field Research Center, Dutchess Co., Millbrook, New York. Japanese quail ($n = 8$ males and $n = 8$ females) were obtained from a commercial supplier, Boneffe, Belgium. Animals were killed by decapitation and their brains rapidly removed and frozen on powdered dry ice. Brains were mounted on a specimen plate and coronal sections (10 or 20 μm) were taken at selected intervals in the rostrocaudal direction on a cryostat. Sampling was limited to the telencephalon, diencephalon, and mesencephalon. Sections were thaw mounted on gelatin-coated slides and freeze-dried at between 0 and -5°C . They were then stored at -40°C for 1 to 2 weeks prior to labeling procedures.

Receptor autoradiography

The method employed is similar to that previously used for studies of rats (Wamsley et al., '80) and guinea pigs (Dohanich et al., '85b). A brief description of the actual procedure is provided below. The slides were removed from the freezer and fan dried for 30 minutes. They were then placed in slide mailers containing the incubation medium. This medium consisted of 8 ml of 50 mM potassium phosphate buffer (pH 7.4) containing [^3H] N-methyl scopolamine (NMS, 85 ci/mmol; New England Nuclear, Boston, MA), a potent muscarinic cholinergic antagonist. Incubation concentration was between 1 and 1.5 nM depending on the experiment. This corresponds to approximately 2 times the K_d (see below). Nonspecific binding was estimated by incubating slides representing all levels of the brain sampled in a solution containing [^3H] NMS and the cold

competitor, atropine, a muscarinic antagonist (5 μM concentration). Sections were incubated for 1 hour at room temperature. The incubation medium was then poured off and the sections were washed in ice cold buffer for 10 minutes.

In competition and saturation experiments, sections were prepared from minced and molded brain tissue (i.e., brain "mash"). The mold used to prepare the mash was a cylinder with a diameter of 15 mm. In order to determine saturability of [^3H] NMS binding in the avian brain, sections of brain mash (10 μm) were incubated with various concentration of the ^3H ligand in the range of 0.2 to 2.5 nM. Nonspecific binding was estimated at every other concentration by incubating the sections in the presence of 5 μM atropine. In these experiments, the sections were wiped off the slides into scintillation vials with glass fiber filters (#30; Schleicher and Schuell, Keene NH). Scintillation fluid (5 ml) was added to vials and after equilibration they were counted in a Beckman LS 100 scintillation counter. The bound counts per minute (cpm) and total free cpm were transformed into femtomoles (fmol) NMS.

In autoradiographic experiments, slides were dipped in ice cold bi-distilled water in order to remove buffer salts and then fan dried for 45 minutes. Slides were mounted on cardboard cassettes and placed under 9 kg weight in contact with LKB Ultrafilm (LKB, Gaithersburg, MD) for 7 days. Ultrafilm was developed with Kodak D-19 developer for 5 minutes, dipped in stop bath (40 ml glacial acetic acid/1 liter bi-distilled H_2O), and fixed in Kodak Rapid Fixer for 2 minutes. The autoradiograms were analyzed with the aid of Drexel's Unix Based Microcomputer iMage Analysis System (DUMAS). Plastic standards (Amersham, Arlington Heights, IL) containing varied concentrations of tritium were co-exposed on the film and used to convert optical density units to fmol of [^3H] NMS specifically bound per mg of protein. Mean protein content was determined by the method of Bradford ('76) in a number of brain areas and was determined to be approximately 8–10% of tissue wet weight (see also Schumacher and Balthazart, '87 for further details). In all subsequent calculations, specific activities per equivalent wet weight provided by Amersham for the plastic standards were transformed into specific activities per mg protein using a ratio of protein versus fresh weight of 10%. Specific binding is defined as binding in the absence of atropine minus binding in the presence of atropine. In all autoradiograms the density of nonspecific binding was equal to film background and no visible image of the brain section was detectable on the film.

The identification of structures was made by reference to the stained sections from which the autoradiograms had been generated. Sections were either stained with methylene blue or for Nissl substance using either cresyl violet (Sigma, St. Louis, MO, # C-1893) or thionin (Sigma, # T-3387). In some cases, the pattern of muscarinic binding outlined more clearly the borders of a given area than the Nissl stained material and the pattern of binding was therefore used to assist with identifying the structure. For the starling and song sparrow sections, identification was assisted by reference to the Stokes et al. ('74) canary (*Serinus canaria*) atlas; for the quail sections the atlas of Baylé et al. ('74), based on Japanese quail material, was employed. Reference was also made to the works of Huber and Crosby ('29), Karten and Hodos ('66), Oksche and Farner ('74), Nottebohm et al. ('82), Kuenzel and van Tienhoven ('82), and Kuenzel and Masson ('88) for clarification of nomenclature and structure identification.

Concentrations of binding sites in males and females were compared by Student's *t* test for each brain area that was quantified. As no significant difference was obtained by this method, no further analyses were performed employing statistically more appropriate tests for multiple comparisons as it would have been impossible to obtain statistical significance using these methods after having failed to obtain significance with the *t* test. No attempt was made to provide statistical comparisons of the levels of binding in different brain regions within a same sex as these have no clear biological interpretation.

RESULTS

Validations

As illustrated in Fig. 1, [3 H] NMS binding was saturable at nM concentrations in the three species. Scatchard analysis revealed linear relationships between bound/free versus bound with correlation coefficients ≥ 0.94 in the three species. The apparent dissociation constant (K_d) was similar in the three species (approximately 0.6 nM). Maximum binding (B_{max}) per slide was in the femtomolar range. A slice of brain mash (15 mm diameter $\times$ 10 μ m section thickness) contains about 0.18 mg protein (based on a 10% protein

content). The maximum binding determined by this method is in the range of 200–500 fmol/mg protein.

As shown in Fig. 2, to confirm the specificity of NMS binding, competition experiments were conducted in two of the species under study with three muscarinic ligands: an antagonist, atropine sulfate, and two agonists, oxotremorine sesquifumate and carbamylcholine chloride (carbachol). In the third species, the song sparrow, because of a limited number of animals available the three ligands were only tested at two concentrations for each of the three ligands. As illustrated in Fig. 2, these three compounds competed for the binding of NMS with an order of potency characteristic of mammalian muscarinic receptors (atropine > oxotremorine > carbachol). A similar result was obtained with the more limited number of points used in the song sparrows. The equilibrium inhibition constants (K_i) for the competitors were calculated using the formula $K_i = IC_{50}/(1 + F/K_d)$, where F equals the free concentration of 3 H ligand, and K_d is the dissociation constant shown in Fig. 1. Values obtained in both species are in the range of those reported in mammalian studies (e.g., Dohanich et al., '85b). The Hill coefficients (H) were calculated from the logit/log plot of the bound ligand versus inhibitor concentration. In this plot the coefficient is equal to the slope divided by -2.3 as described in Bennett and Yamamura ('85). The values obtained were in each case close to one with the possible exception of carbachol in starlings.

Distribution of [3 H] NMS binding sites in the brain of the Japanese quail

autoradiograms illustrating [3 H]NMS binding density at four levels of the quail brain are shown in Fig. 3. The

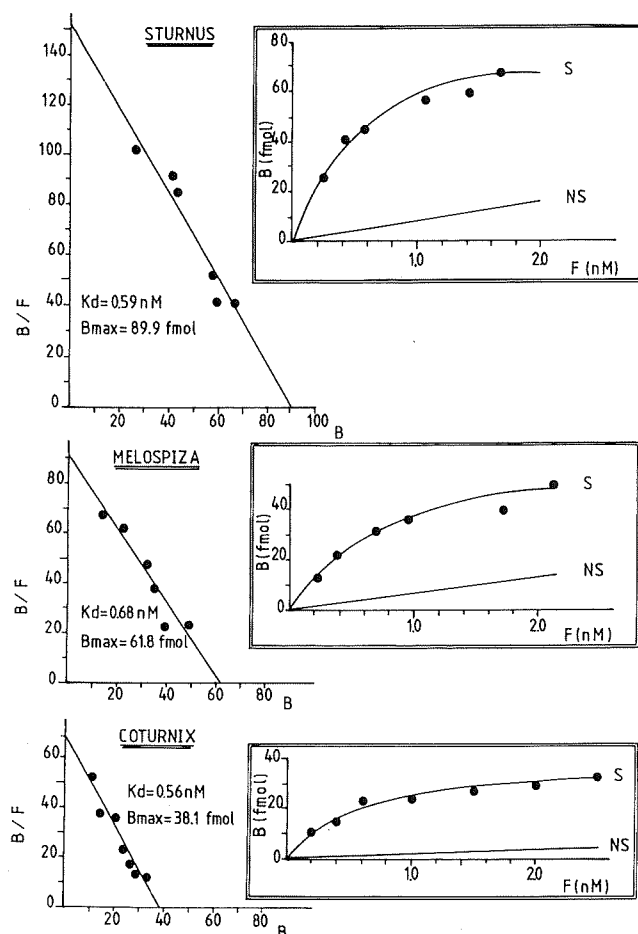


Fig. 1. Saturation analysis of [3 H] NMS as determined by beta counting with procedures described in the text. Each panel contains a saturation curve and a Scatchard plot for one species. S, specific binding; NS, nonspecific binding; B, bound ligand; F, free ligand.

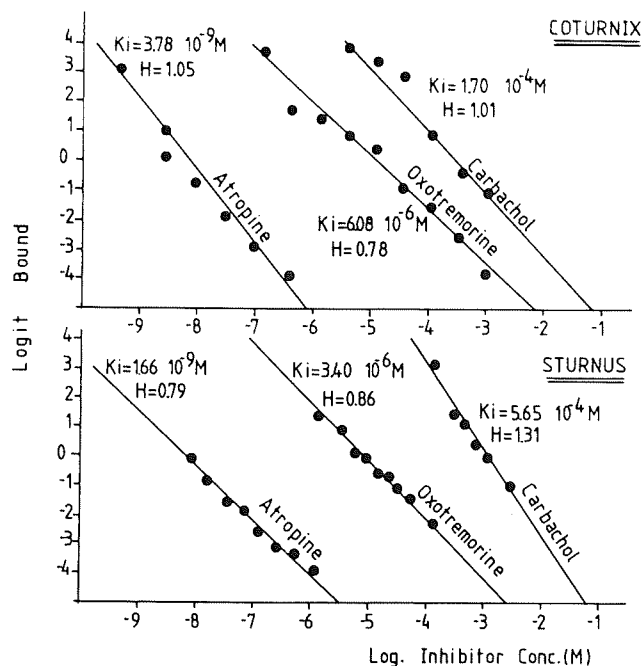


Fig. 2. Characterization of the specificity of [3 H] NMS to muscarinic cholinergic receptor sites. Sections were incubated with [3 H] NMS and various concentrations of competing muscarinic compounds. Data were plotted following logit/logit transformation for determination of each IC_{50} for atropine, oxotremorine, and carbachol. The K_i for each competitor was derived from the IC_{50} . The slope of each plot was converted to a Hill coefficient (H).

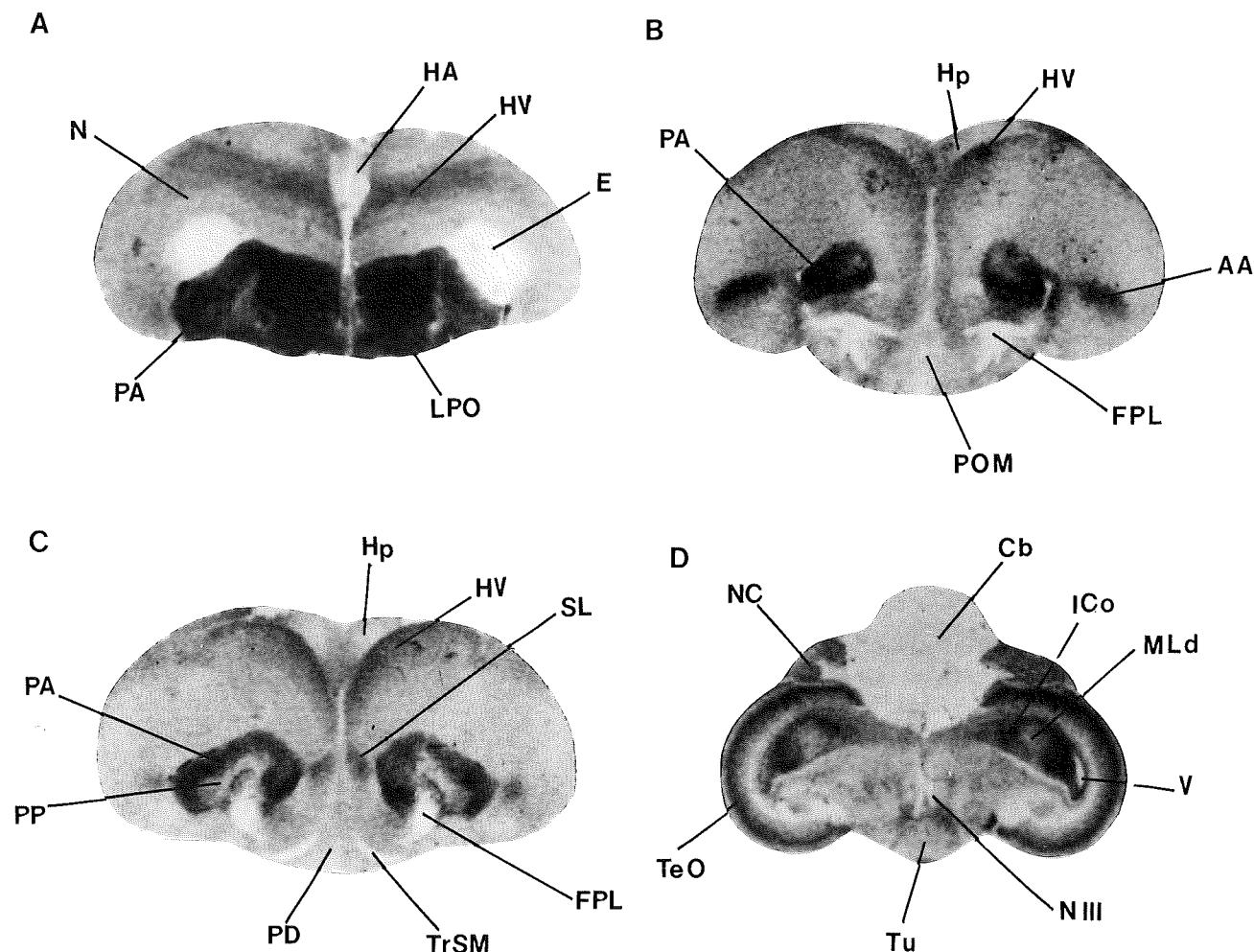


Fig. 3. Autoradiograms cut in the transverse plane illustrating the pattern of [^3H] NMS binding in the quail brain.

density of receptor binding in selected parts of the telencephalon of male and female quail is shown in Fig. 4. Receptor density for various nuclei in the diencephalon and mesencephalon is provided in Fig. 5. All areas where specific binding is higher than approximately 500 fmol/mg protein are illustrated in the two figures containing histograms. However, three areas at or below this level are also shown, the ectostriatum, the cerebellum, and nucleus rotundus. In no case was a significant difference detected between the sexes in any brain area that was quantified. The highest density of binding was apparent in structures that make up the basal ganglia such as the lobus parolfactorius and the paleostriatum augmentatum (Figs. 3A,B, 4). However, binding in the paleostriatum primitivum, another structure in this complex, was relatively low (Fig. 4). Other structures with particularly high binding include the area archistriatalis (Figs. 3B, 4) and the lateral geniculate nucleus (Fig. 5).

Within the hypothalamus binding was high in a discrete area that appeared to correspond primarily with the nucleus ventromedialis hypothalami (VMN). However, we were unable to ascertain if the pattern of binding corresponded precisely with the boundaries of this nucleus. In both the anterior hypothalamus (rostral to the anterior commissure but caudal to the nucleus preopticus anterior-

is[POA]) and the posterior hypothalamus (caudal to the anterior commissure but rostral to the cerebellum), no particular nuclear definition was apparent from a consideration of the binding patterns alone, though detectable levels were apparent (labeled nucleus anterior medialis hypothalami [AM] because the binding included this nucleus and adjacent structures, and more caudally in a region labeled posterior hypothalamus [HYp] respectively in Fig. 5).

In the telencephalon, binding was high in both the dorsal and ventral hyperstriatum, and levels between these two structures were essentially indistinguishable (Fig. 3A,B; entire structure is labeled as hyperstriatum ventrale [HV] only). Values for the ventral hyperstriatum are presented in Fig. 4. In the neostriatum and the caudal neostriatum apparent density was much lower, and this did not seem to vary through the rostral to caudal extent of this region.

In several areas the pattern of binding greatly enhanced distinctions among adjacent structures. This was especially apparent in the nucleus intercollicularis (ICo)—mesencephalic lateralis pars dorsalis (MLd) complex and in the preoptic area. In the former case the ICo, which is characterized by heavy density especially in the medial section, surrounds the MLd, which contains relatively low levels of binding (see Figs. 3D, 5). The preoptic area in quail contains a nucleus, the POM, that is sexually dimorphic in volume.

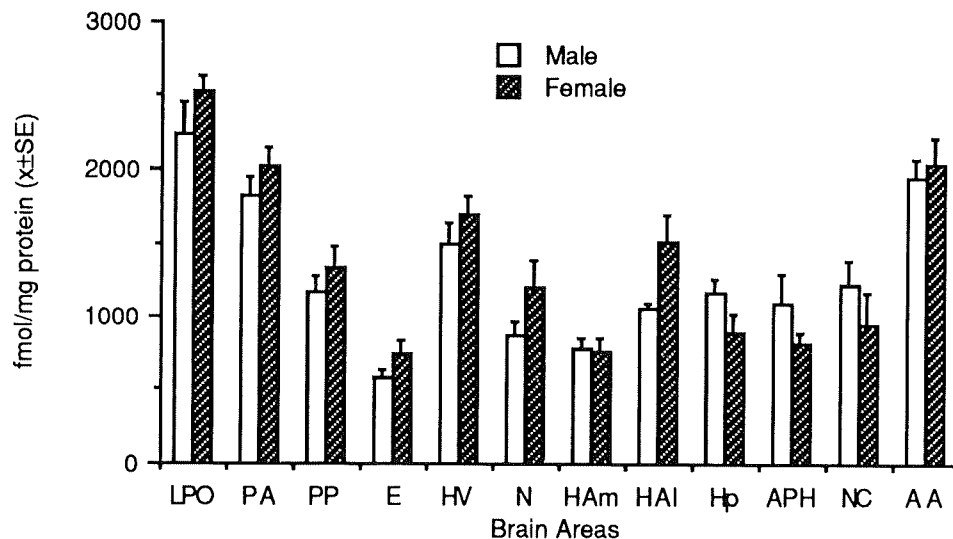


Fig. 4. Histograms showing the amount of [^3H] NMS binding in various telencephalic areas of male and female quail.

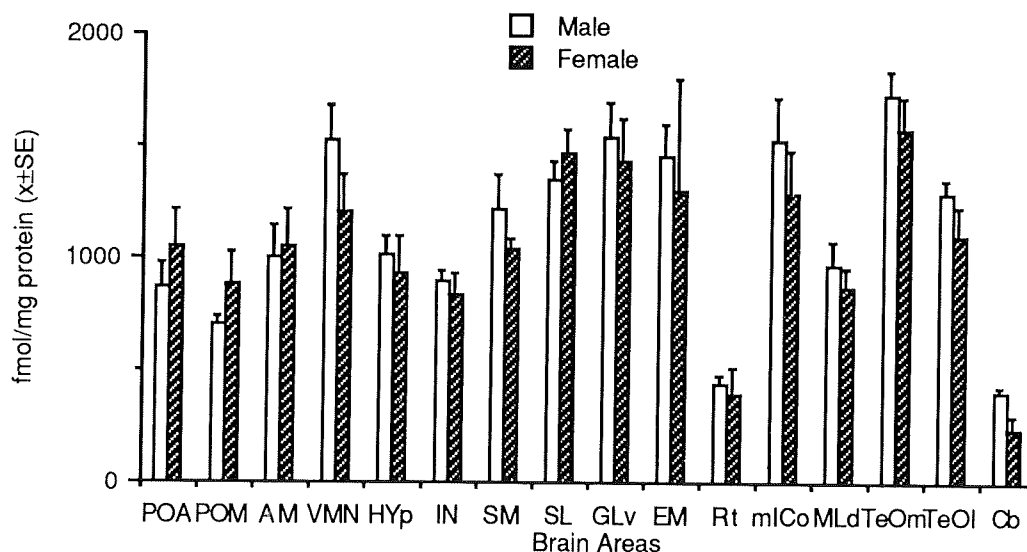


Fig. 5. Histograms illustrating the amount of [^3H] NMS binding in various diencephalic and mesencephalic nuclei as well as the cerebellum of male and female quail.

This sexually dimorphic region was well defined by the low level of binding density it contains relative to the surrounding areas (Fig. 3B).

Distribution of [^3H] NMS binding in the brains of male and female European starlings and song sparrows

The qualitative pattern of binding in the two passerine species was very similar. Because the data for the song sparrow were so similar to that of the starling and fewer sections from the sparrow brains were devoted to this study, we have chosen to present only data for the starling. Figs. 6–8 illustrate NMS binding in the starling brain by the presentation of autoradiograms all from sections cut in the frontal plane. Figs. 9–11 provide a quantitative evaluation of the starling data. In Fig. 8, two frontal autoradio-

grams are presented that compare a starling and a song sparrow from very similar levels in the brain.

In Fig. 6 two autoradiograms showing the pattern of NMS binding from two rostral brain levels in the European starling are provided. Fig. 6A demonstrates the heavy receptor density apparent throughout the lobus parolfactorius (LPO). This panel taken from a male shows the highest binding in a subregion of the LPO named area X. This area was also distinguishable in autoradiograms taken from female brains though the area of the binding appears much smaller, as is apparent in the Nissl stained material. Both A and B in Fig. 6 show the clear distinctions among the different striatal layers in the telencephalon that are accentuated by the pattern of NMS binding. However, as was the case in the quail, the difference between the ventral and dorsal hyperstriatum was not apparent in the autoradiograms.

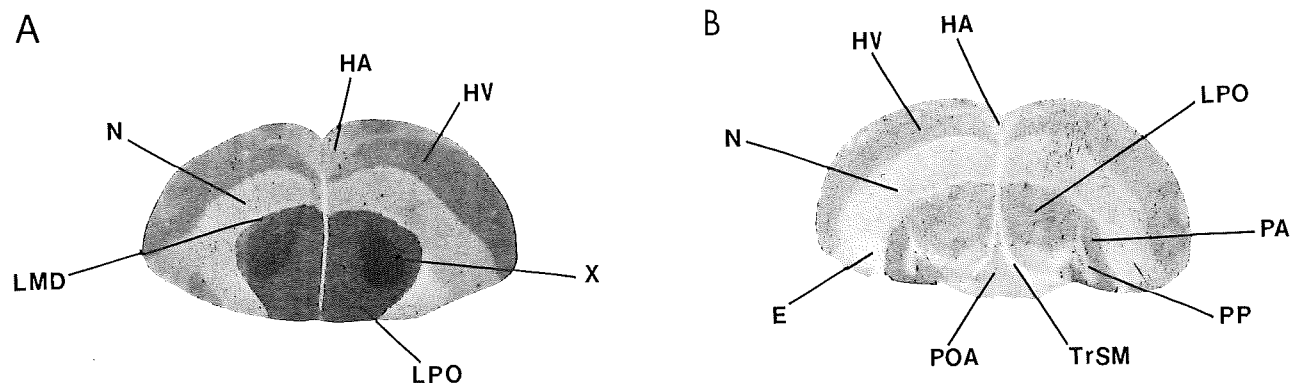


Fig. 6. Two coronal autoradiograms illustrating [^3H]NMS from the forebrain of a male starling.

In other regions, subdivisions were observed based on the pattern of binding that could not be discerned from an examination of Nissl-stained material. An example of this is in the medial and lateral portions of the hyperstriatum accessorium, with the medial area being characterized by relatively low levels of binding and a dark band being apparent in the lateral portion (Fig. 6A,B). These subdivisions as defined by the differences in the binding are labeled in Fig. 4 (for the quail) and Fig. 9 (for the starling) as hyperstriatum accessorium medialis (HAM), which refers to the low density medial portion, and hyperstriatum accessorium lateralis (HAL), referring to the higher density lateral portion. The subdivision in binding continued from the HA through the hippocampal-parahippocampal complex (Fig. 7A–C). The preoptic area was characterized by very low levels of binding (Fig. 6B).

More caudally, another part of the basal ganglia, the paleostriatum augmentatum, is characterized by very high levels of receptor density (Fig. 7A–C). It surrounds the paleostriatum primitivum, which shows negligible levels of specific binding. In the hypothalamus specific binding is apparent in both the nucleus paraventricularis magnocellularis (PVN) and the VMN (Fig. 7A,B). Several of the internal layers of the optic tectum are characterized by relatively high binding (Fig. 7A–C). We were unable to identify precisely which layers of the tectum were labelled. The relatively heavy binding medial layers are identified as tectum opticum medial band (TeOm) and the lower density lateral layers are labelled tectum opticum lateral band (TeOl) in Figs. 5 (for the quail) and 10 (for the starling). Other nuclei that are specifically labeled with binding in a high range include the nucleus geniculatus lateralis, pars ventralis (GLv) (Fig. 7A) and the ectomamillary body (EM) (Fig. 7C).

Fig. 8 presents the pattern of binding at a caudal level in the starling (Fig. 8A) and the song sparrow (Fig. 8B). The overall similarity between the two species is apparent. Two nuclei, characterized by their low levels of binding relative to the surrounding structures, are illustrated in this figure. The robust nucleus of the archistriatum (RA) is quite low in comparison to the surrounding archistriatum and the MLd is relatively low in comparison to the ICo, which surrounds it. The archistriatum itself is characterized by high binding that runs just ventral and medial to the lamina archistriatalis dorsalis, a pattern that is more apparent in Fig. 8A than in B.

Levels of binding in male and female starlings for four nuclei that are a part of the system of nuclei that control

song are presented in Fig. 11. Binding is by far the highest in area X in both sexes. Binding in this area is among the highest reported for any brain area. Hyperstriatum ventrale, pars caudalis (HVc) shows a much lower level of binding. Two of the nuclei, RA and nucleus magnocellularis of the anterior neostriatum (MAN) are among the lowest levels recorded for any brain area. These levels are comparable to those of the ectostriatum. No sex difference in the density of receptors was discernable in these vocal control regions nor in any of the other areas presented in Figs. 9 and 10.

DISCUSSION

Characterization of the avian muscarinic receptor

With the use of the muscarinic receptor antagonist, NMS, we were able to label specifically diverse brain structures in the three species of birds that were studied. The binding observed was saturable in the nanomolar range. The apparent dissociation constant assessed by saturation analysis with beta counting of slices of brain mash was similar in the three species ($K_d = \pm 0.6 \text{ nM}$). This value is in good agreement with the dissociation constant that has been reported in rats and guinea pigs for the same ligands (0.25 nM to 1.4 nM; Wamsley et al., '80; Dohanich et al., '85b; Frey et al., '85). The maximum level of binding observed by scintillation counting in these brain mash slices was in the range of 200–500 fmol/mg protein. In that the brain mash essentially represents a mean value for labeled and unlabeled areas of the brain, this figure agrees well with levels that were evaluated by quantitative autoradiography in specific brain areas. This correspondence independently confirms the validity of the values assigned to brain areas by the quantitative procedures.

The maximum number of binding sites obtained by scintillation counting of the brain mash slices appears higher in the sparrow and the starling than in the quail (see Fig. 1). However, when one compares individual brain regions among the three species, comparable values are observed (compare common areas in Figs. 4 and 5 with those in Figs. 9 and 10). Therefore, rather than being a true species difference, the small variations in the maximum number of binding sites among the three different brain homogenate preparations result in all probability from differential contribution of areas containing a high and low number of binding sites. The absolute levels of binding

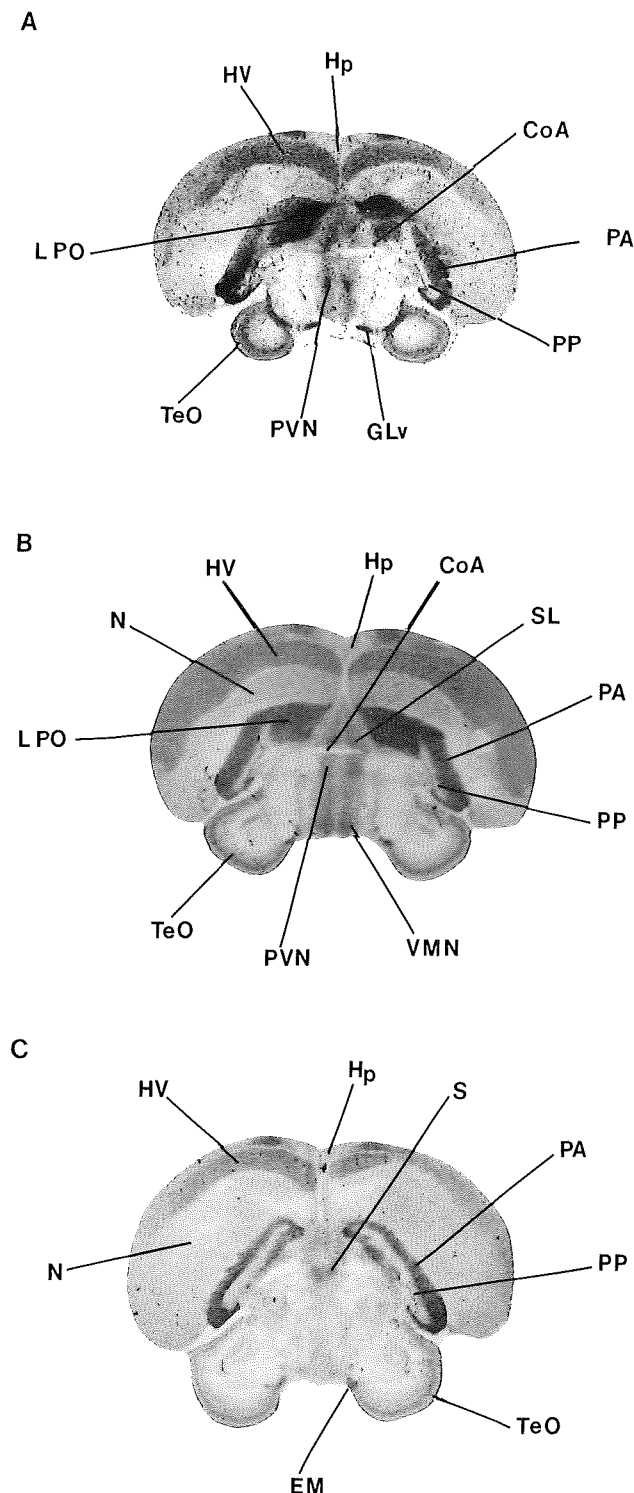


Fig. 7. Three coronal autoradiograms illustrating [^3H] NMS binding in hypothalamic regions of a male starling.

measured here are in the range of levels of binding detected in the rat and guinea pig brain (Dohanich et al., '85b; Frey et al., '85). There has been one previous study examining brain muscarinic receptors by quantitative autoradiography in an avian species, the pigeon (*Columba livia*; Dietl et al., '88). The levels reported in the present investigation are

in general two to three times higher than those reported by Dietl et al. ('88). Whether this represents a true species difference or is a consequence of procedural differences can not be evaluated at present.

Competition experiments confirmed the specificity of NMS binding to the muscarinic receptors in the three species studied in this paper. The muscarinic agonists and antagonists competed for the NMS sites in the predicted manner. The antagonist atropine was more powerful than the agonist oxotremorine, which was itself more potent than the agonist carbachol. The same conclusion could be reached for the three species studied, and the K_i determined in quail and starling were quite similar to those measured previously in the guinea pig brain (Dohanich et al., '85b).

Recent studies in mammals, using more specific ligands, have identified two muscarinic receptors subtypes called M1 and M2. These subtypes are characterized by their preferential affinity for the antagonist pirenzepine (M1) on one hand and for the agonists oxotremorine or carbachol on the other hand (M2). By in vitro autoradiography, these two receptor subtypes have been localized in different areas of the rat brain (Spencer et al., '86). NMS and other antagonists such as quinuclidinyl benzilate (QNB) seem unable to distinguish between these two sites (Watson et al., '83). It is thus very likely that in the present study we labelled both receptor subtypes. No attempt has been made in previous studies on the avian brain to label these two subtypes specifically. However, in the recent pigeon study by Dietl et al. ('88), NMS binding was displaced by carbachol or pirenzepine, and a close positive correlation was found between the binding of NMS remaining after displacement by these two compounds. This is in contrast to rats, in which a negative correlation was found using the same procedure. This finding suggests that in birds, unlike in rats, the putative M1 and M2 receptor subtypes are either not present or show a very similar neuroanatomical distribution.

The fact that the Hill coefficient measured in the present study of agonists and antagonists is close to unity (1 ± 0.2) is consistent with the possibility that there is only one binding site for NMS present in birds. This contrasts with the previous study of the guinea pig brain in which competition with muscarinic agonists yielded Hill coefficients significantly smaller than one. It is thus possible that the heterogeneity of muscarinic receptors that has been described in mammals is not present in birds, as has been argued by Dietl et al. ('88). Strong evidence in favor of this view is, however, lacking at present and additional experiments should be carried out to test this idea more directly. As the functional significance of these two receptor subtypes in mammals is unclear at present, the possible reason or significance for the class difference between birds and mammals cannot be usefully addressed.

Pattern of binding in the avian basal ganglia

A large number of studies have now established that in mammals the highest levels of both muscarinic receptor density and other markers of cholinergic activity such as acetylcholinesterase staining are present in various parts of the basal ganglia (see Rotter, '84 for review). In particular the caudate-putamen complex shows levels of binding that are among the highest in the brain (Kuhar and Yamamura, '75, '76). The results of the present study suggest a close correspondence between the density of muscarinic receptors in various parts of the mammalian basal ganglia and the density of receptors in their homologous structures in

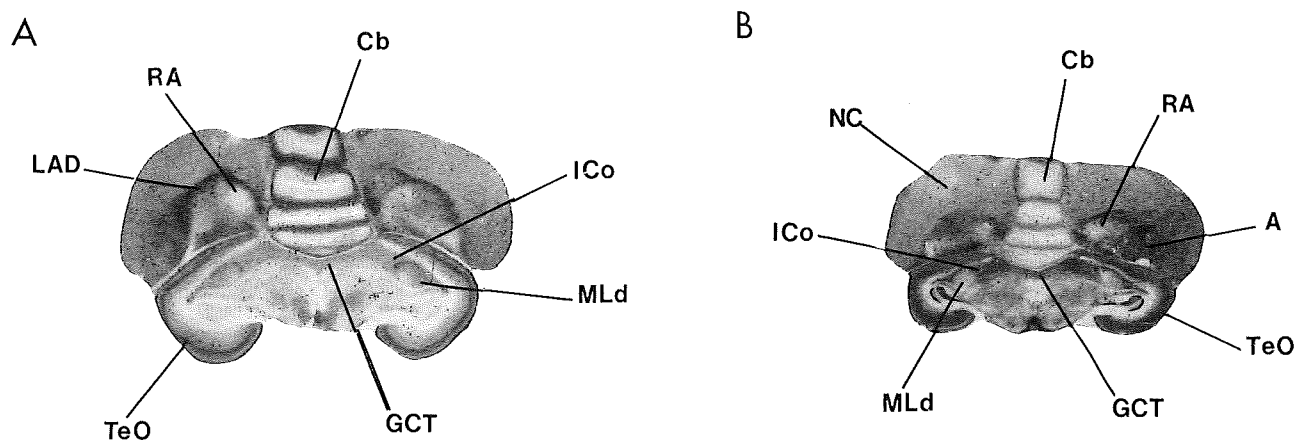


Fig. 8. Two autoradiograms illustrating [^3H] NMS binding in a male starling (A) and a male song sparrow (B).

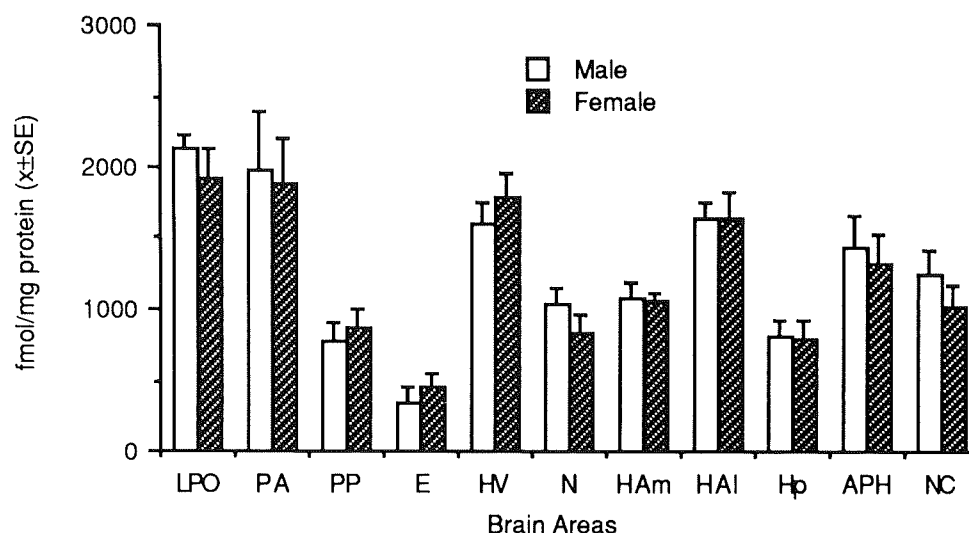


Fig. 9. Histograms showing the amount of [^3H] NMS binding in various telencephalic areas of male and female European starlings.

the avian brain. For example, the avian homologue of the caudate nucleus is the parolfactory lobe, and the homologue of the putamen is the paleostriatum augmentatum (Karten and Dubbeldam, '73; Reiner et al., '84; see especially Parent, '86). Both of these regions exhibit very high amounts of NMS receptor density in all three species studied. Lower levels in all three species are present in the paleostriatum primitivum (PP), an area that corresponds with the low binding globus pallidus in mammals.

Patterns of binding in the preoptic area and hypothalamus

The preoptic area in both the quail and the two songbird species displayed one of the lowest levels of NMS binding. In particular, within the quail preoptic area, the sexually dimorphic medial preoptic nucleus, POM (Viglietti-Panzica et al., '86; Panzica et al., '87), was outlined by this absence of binding. A similar finding concerning the sexually dimorphic area of the gerbil has been reported by Commins and Yahr ('84). The sexually dimorphic area of the gerbil preoptic area is specifically labelled for acetylcholinesterase. However, the most dimorphic part of this area,

called pars compacta, is totally devoid of acetylcholinesterase.

Distinct patterns of medium to heavily labeled nuclei were observed in the VMN and the PVN of the songbirds. In the quail the VMN is distinctly labeled but the PVN could not be clearly discerned from the surrounding hypothalamic area. Cholinergic agents either injected systemically or directly infused into the hypothalamus have been shown to modulate female receptivity in rats. There is heavy cholinergic binding in the VMN of rodents and in more anterior parts of the hypothalamus corresponding to areas containing E_2 receptors. Furthermore, muscarinic receptors in these areas in female rats are modulated by the removal and replacement of steroids. It has been suggested that this modulation of muscarinic receptors by E_2 is somehow involved in the expression of female sexual receptivity, but it has still not been experimentally established that these steroid induced changes of cholinergic receptors by E_2 are directly linked to the activation of behavior. In one bird species, it has been shown that the VMN is implicated in the activation of female sexual behavior by steroids (Gibson and Cheng, '79). Immunocytochemical studies of

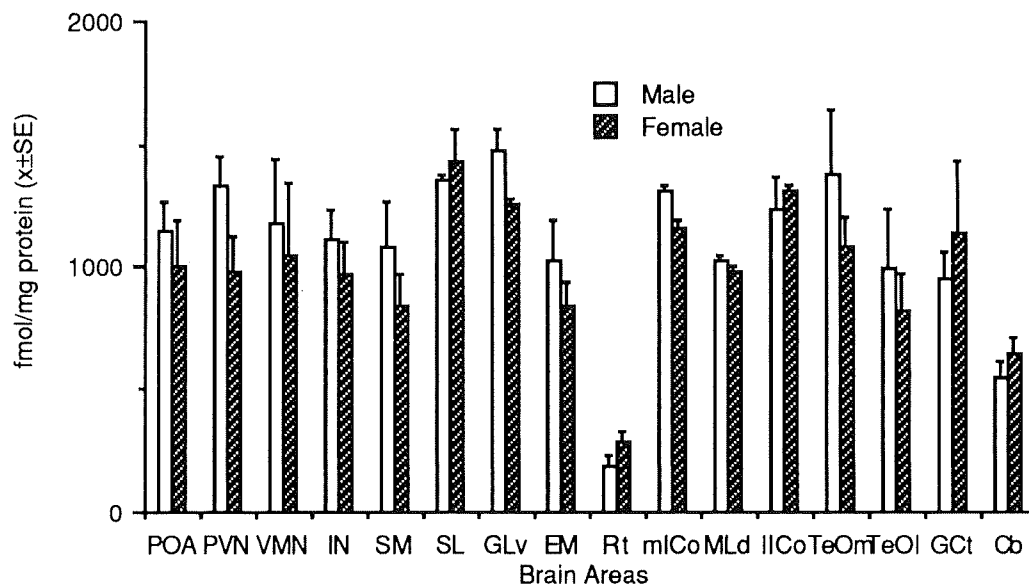


Fig. 10. Histograms illustrating the amount of [^3H] NMS binding in various diencephalic and mesencephalic nuclei as well as the cerebellum of male and female European starlings.

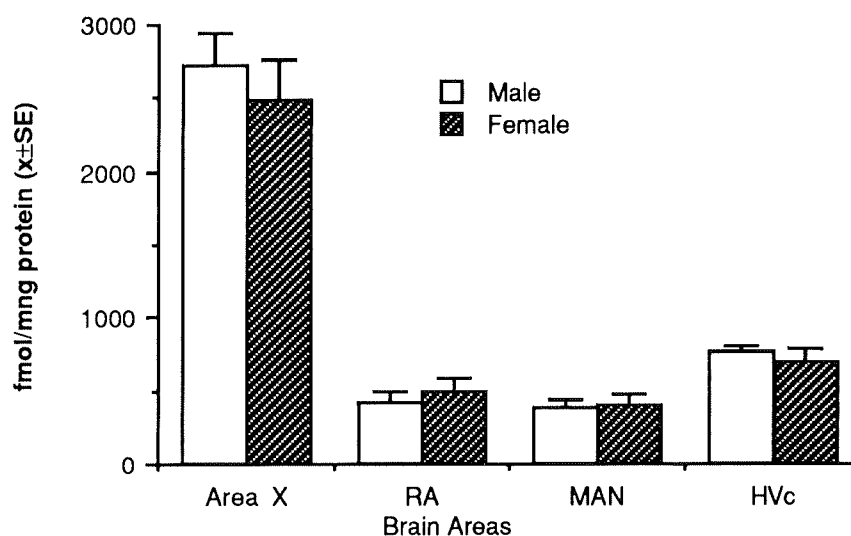


Fig. 11. Histograms illustrating the density of [^3H] NMS binding in four nuclei that are part of the song control system in male and female European starlings.

estrogen receptors in the quail brain (Balthazart et al., '89) and in the brains of two songbird species (Gahr et al., '87) have also demonstrated the presence of estrogen receptor immunoreactivity in this nucleus. The finding that this area contains muscarinic receptors and estrogen receptors suggests that steroid modulation of cholinergic transmission may also play a role in the control of this behavior in birds as it does in mammals. Direct experimental verification of this hypothesis needs to be carried out.

Patterns of binding in vocal control areas

Utilizing acetylcholinesterase staining and *in vivo* muscarinic receptor autoradiography, Ryan and Arnold ('81) first suggested that several of the nuclei implicated in the regulation of passerine song have efferent projections that

utilize acetylcholine and also may receive cholinergic projections. Based on the autoradiographic data, the evidence for a muscarinic cholinergic afferent was best for area X of the parolfactory lobe, HVC, and the mesencephalic nucleus ICo. RA and MAN both showed levels of binding at or below those found in animals that were also treated with the cold competitor. In the present study, high levels of NMS binding were found in area X and moderate levels in HVC. However, similar to the findings of Ryan and Arnold ('81), levels in MAN and RA were very low, especially when they are contrasted to levels in the surrounding neostriatum and archistriatum, respectively. Thus in these two nuclei there does not seem to be a major muscarinic cholinergic projection. Watson et al. ('88) have found evidence for nicotinic receptors in the MAN of the zebra finch, suggesting that these receptors may mediate any cholinergic afferents in

this nucleus. However, they also found very little evidence for nicotinic binding in RA.

The previous two studies by Ryan and Arnold ('81) and Watson et al. ('88) on cholinergic input to the sexually dimorphic nuclei involved in song did not test directly for the possibility of sex differences in receptor density. In this study we looked for such sex differences in the starling but failed to detect any. Given that there is often significant heterogeneity in binding within a given nucleus it is quite possible that a more systematic study of the sexually dimorphic song control nuclei would reveal discrete sex differences in receptor density. The pattern of muscarinic binding did seem to reflect sex differences in volume that are also apparent in Nissl stained material. However, we did not collect serial sections throughout these nuclei that would have allowed us to reconstruct their volume. Therefore we cannot discuss such differences quantitatively.

Quail possess one of the nuclei that are considered a part of the passerine song system and that is the mesencephalic ICo. This nucleus contains cholinergic receptors as it does in the starling and the song sparrow. The exact pattern of cholinergic binding in this nucleus has been described in more detail elsewhere (Ball et al., '89). An examination of the patterns of NMS binding in the telencephalic areas in the quail brain such as the neostriatum and the archistriatum that contain song control areas in the passerine brain do not reveal any differences in binding that might suggest a homologous nucleus. No such nuclei are apparent by an examination of these areas using more traditional Nissl methods either.

Binding in the hippocampus

There has been much recent interest in the avian hippocampal complex because of its implication in the control of tasks involving spatial memory such as homing behavior in pigeons (Bingman et al., '84) and the storing of food by a variety of passerine birds (Sherry and Vaccarino, '89). Certain neurochemical similarities have been described in the hippocampus of birds and mammals. For example, this structure accumulates [³H] corticosterone in both taxa (Rhees et al., '72; McEwen et al., '86). Immunohistochemical studies using different antibodies that recognize five neuropeptides and three neurotransmitter-related enzymes have identified distinct subareas within the avian hippocampus indicative of structural homologies between the avian and the mammalian hippocampus (Krebs et al., '87). In the hippocampal complex in mammals, Kuhar and Yamamura ('75, '76) demonstrated that muscarinic binding can be detected in a laminar pattern in both the hippocampal pyramidal cell and the dentate granule cell in the dentate stratum. In all three species studied in the present investigation, the hippocampal complex was generally devoid of NMS binding except for two lateral dark bands that were present along the entire rostral to caudal extent of the hyperstriatum accessorium (HA) and the hippocampus (see Figs. 3B, 6A, 7A-C). These dark lateral bands appear to demarcate the lateral boundary of the hippocampus that has been described by Krebs et al. ('89) and Sherry et al. ('89) in a variety of passerine species. These authors identified the boundary by a change in cell density. This boundary also appears to correspond to a marked change in cholinergic innervation. There was no apparent distinction between the more rostral HA and the more caudal hippocampus (Hp). At present it does not seem likely that the pattern of NMS binding in the avian hippocampus will be useful in establishing homologies. However, in general the patterns of binding described are

similar for the three avian species investigated and agree well with patterns of binding described for homologous structures in the mammalian brain.

ACKNOWLEDGMENTS

We thank Ms. Jane Uman and Ms. Suzanne Lang for technical assistance. Dr. Allan E. Johnson provided useful advice and encouragement. We thank Professor Peter Marler and Professor Ernest Schoffeniels for their support. This research was supported by a grant from the Whitehall foundation, a Revson Foundation Fellowship, and a BRSG (SO7 RR07065) to G.F.B.; an NIMH grant (MH41256) to B.Mc.E., and grants from the Belgian FNRS, the Medical School of the University of Liège, and the NIH (HD22064) to J.B.

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