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Distribution of Substance P Reveals a Novel Subdivision in the Hippocampus of Parasitic South American Cowbirds

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ABSTRACT

Parasitic cowbirds monitor potential hosts' nests and return to lay when appropriate, a task that is likely to involve spatial recall. Seasonal and sexual behavioral variations in the cowbirds correlate with anatomical changes in the hippocampal formation. During the breeding season, parasites have larger hippocampal formations than nonparasites. In parasitic species in which females alone perform nest bookkeeping, females have larger hippocampal formations than males. We investigated the distribution of the neuropeptide substance P (SP) in three sympatric cowbirds: two obligate parasites (shiny cowbird and screaming cowbird) and one nonparasite (bay-winged cowbird). Distribution of SP was similar to that in other songbirds, except for a previously undescribed field of dense SP-rich terminals within the hippocampus that we call the *hippocampal SP terminal field* (SPh). We found robust species differences in the volume of this new area, measured relative to the remainder of the telencephalon. SPh was largest in the generalist parasite (shiny cowbird) and smallest in the nonparasitic species (bay-winged cowbird). In the specialist parasite (screaming cowbird), SPh was smaller than in the generalist parasite but larger than in the nonparasitic species. SPh overlaps with two subdivisions described in the pigeon that have been related to the mammalian dentate gyrus and subiculum. The area containing SPh receives a major input from the lateral mammillary nucleus, which is probably the avian equivalent of the mammalian supramammillary nucleus (SUM), the main source of extrinsic SP input to mammalian hippocampus. SPh may be the termination of a pathway homologous to the SP-rich projection from SUM to the hippocampus in mammals. J. Comp. Neurol. 496:610–626, 2006.

Indexing terms: spatial memory; avian brain; brood parasitism; lateral mammillary nucleus

Birds naturally exhibit a variety of complex behaviors that have made them invaluable in the study of the relationship between brain structure and behavior. Previous neuroanatomical investigations have identified differences in neural organization related to a number of avian behaviors, including singing (for review see Nottebohm, 1980; Brenowitz, 1997; Nordeen and Nordeen, 1997), food hoarding (for review see Sherry et al., 1989; Krebs, 1990; Sherry, 1997), homing (for review see Bingman et al., 1990; Casini et al., 1997; Gagliardo et al., 1999), and the reproductive habit we investigate here, brood parasitism (Sherry et al., 1993; Reboreda et al., 1996; Clayton et al., 1997).

Avian brood parasitism is a form of breeding in which a species lays its eggs in the nests of another species, the host, which incubates and rears the young. The success of the parasite depends to a large extent on the female laying her own eggs within the laying period of the host. The parasitic cowbirds of the American continents (*Molothrus*

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spp.) often achieve a high degree of synchronization with host laying (see, e.g., Wiley, 1988; Sealy, 1992; Fraga, 1998; Mermoz and Reboreda, 1999; Davies, 2000). To achieve synchronization, parasitic cowbirds monitor host nest-building activity in the home range and, having selected nests at a suitable stage of building/incubation, return to lay (Dufty, 1982; Rothstein et al., 1984). It is very likely that cowbirds rely on memory for the location of nests visited during monitoring to return to parasitize them at a later date, often many days after nest selection. In the brains of parasitic cowbirds, this putative reliance on spatial memory appears to have resulted in an increase in the relative size of the hippocampal formation, a neural structure that plays a role in many avian behaviors relying on spatial memory: general spatial memory (Hampton and Shettleworth, 1996; Papadimitriou and Wynne, 1999; Colombo et al., 2001), homing (Rehkamper et al., 1988; Strasser and Bingman, 1999), and food storing (for review see Clayton, 1998). In the cowbirds, parasitic species have larger hippocampal formations during the breeding season relative to the telencephalon than nonparasitic species (Reboreda et al., 1996; Clayton et al., 1997). Furthermore, in parasitic species in which only the females perform host nest "bookkeeping," females have larger hippocampal formations than males (Sherry et al., 1993; Reboreda et al., 1996).

The avian hippocampal formation is located in the dorsomedial region of the telencephalon, as shown in Figure 1. Although considered the anatomical homologue of mammalian hippocampus based on ontogeny (Redies and Puelles, 2001), connectivity (Benowitz and Karten, 1976; Casini et al., 1986; Hough et al., 2002), and neurochemistry (Erichsen et al., 1991; Krebs et al., 1991), the avian hippocampal formation has a markedly different morphology. Mammalian hippocampus has a well-defined trilaminar cellular arrangement within which major hippocampal regional subdivisions, such as dentate gyrus and Ammon's horn, are clearly demarcated. In contrast, the avian hippocampal complex, consisting of densely packed heterogenous populations of neurons, is not as easily differentiated from adjacent telencephalic structures. Similarly, the internal subdivisions of the avian hippocampus are not readily recognizable in the absence of neurochemical markers. Traditionally, the avian hippocampal complex has been divided into two major areas based on projection patterns: a medioventral region that displays a loose trilaminar V structure (sometimes called the *hippocampus proper*; Hp) and a dorsolateral parahippocampal region (APH). Many cytoarchitectonic (Montagnese et al., 1996; Tombol et al., 2000), connectivity (Benowitz and Karten, 1976; Casini et al., 1986; Szekely and Krebs, 1996; Szekely, 1999; Hough et al., 2002; Kahn et al., 2003; Atoji and Wild, 2004), and immunohistochemical (Erichsen et al., 1991; Krebs et al., 1991; Montagnese et al., 1993) studies have attempted to elaborate on this basic subdivision scheme for the avian Hp, but agreement on the number or type of hippocampal divisions has not been reached (for review see Szekely, 1999). Because of this, many questions remain regarding the similarities and differences between the anatomical organizations of avian and mammalian hippocampal formations.

Previous descriptions of the cowbird Hp have consisted only of gross measures of hippocampal volume. In this study, we investigated the neurochemistry of cowbird Hp in more detail by examining the distribution of the neuropeptide substance P (SP). The occurrence of SP in the parasitic Hp is especially interesting in that SP, a member of the tachykinin family of neuropeptides (for review see Otsuka and Yoshioka, 1993), exerts potent memory-promoting effects in addition to having a neurotrophic role (for review see Huston and Hasenohrl, 1995). When applied either peripherally or centrally, SP improves performance in learning tasks in both rodents (Wetzel and Mat-

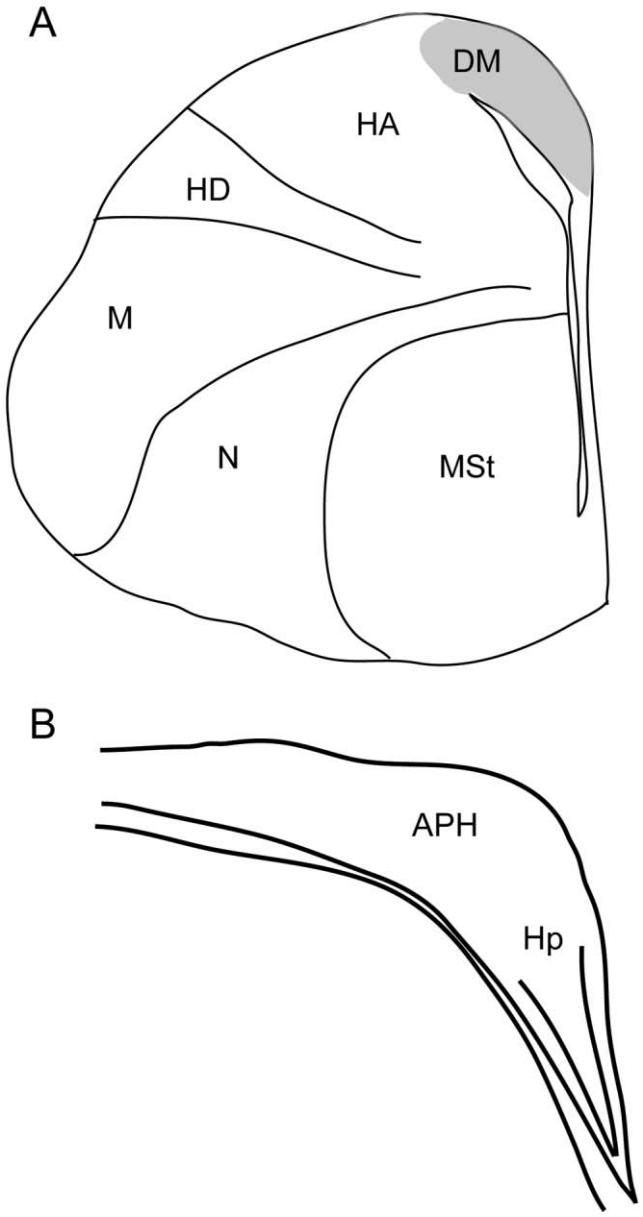

Fig. 1. Location and basic subdivisions of the avian hippocampus. **A:** Schematic representation of a coronal section through the cowbird telencephalon (approximate rostral-caudal level A 5.0). The dorsomedial telencephalon is highlighted in gray. DM, dorsomedial telencephalon; HA, hyperpallium apicale; HD, hyperpallium densocellulare; M, mesopallium; MSt, medial striatum; N, nidopallium. **B:** Basic regional subdivisions of the avian hippocampus (Hp). APH, area parahippocampalis. Nomenclature and subdivisions based on Reiner et al. (2004).

thies, 1982; Schlesinger et al., 1986; Tomaz and Huston, 1986; Tomaz and Nogueira, 1997) and fish (Mattioli et al., 1997; Santangelo et al., 2001). Moreover, chronic SP administration reverses age-related memory deficits in rats (Hasenohrl et al., 1994). Naturally occurring, endogenous levels of SP could also play a role in modulating spatial memory in nature. One SP-rich field located adjacent to the Hp (the SP medial field; SPm) is significantly larger relative to the telencephalon in the food-storing black-capped chickadee than in three nonstoring bird species (Gould et al., 2001). The involvement of SP in many memory processes, including tasks reliant on spatial memory, raises the intriguing possibility that there might be special features of SP distribution in and around the parasitic cowbird Hp related to the performance of parasitic behavior.

We examined the distribution of SP in the brains of three cowbirds native to South America that share the same habitat but differ in reproductive strategies. The shiny cowbird (*Molothrus bonariensis*), a generalist brood parasite, targets over 200 different host species and exhibits morphological and behavioral sexual dimorphism (i.e., only the female performs host nest targeting). The screaming cowbird (*Molothrus rufoaxillaris*) is a specialist brood parasite, with both males and females involved in searching for nests. The bay-winged cowbird [*Molothrus (Agelaioides) badius*][1] is a nonparasitic cowbird. We compared the distribution of SP in and around the Hp in the two parasitic species with that seen in the bay-winged cowbird and other nonparasitic avian species investigated previously.

MATERIALS AND METHODS

Animals

Twenty-eight cowbirds (shiny cowbirds: seven males and seven females; screaming cowbirds: four males and four females; bay-winged cowbirds: three males and three females) were mist-netted in and around the city of Buenos Aires, Argentina, in February 2003. Birds were captured under permit No. 30/2003 from Dirección de Fauna de la Provincia de Buenos Aires. Procedures were carried out according to the guidelines for the care and use of animals established by the University of Buenos Aires and Oxford University.

For a period of 3–4 weeks between the time of capture and death, birds were housed in outdoor aviaries ($3 \times 2 \times 2.5$m) in groups of 14–15 birds. Shiny cowbirds were housed in a monospecific group, whereas screaming and bay-winged cowbirds were housed together.

Visual inspection and post-mortem examination of the gonads confirmed that the animals used in this study were not juveniles of the 2002–2003 breeding season. All birds used in our investigation were therefore at least 1 year old and sexually mature.

Light microscopy

Preparation of brains. Twenty-four birds (ten shiny, eight screaming, and six bay-winged) were terminally anesthetized with an overdose of sodium pentobarbitol (50 mg/kg). All animals were sacrificed within a period of 4 consecutive days to minimize differences in survival time among individuals.

Brains were carefully removed and postfixed by immersion for 48 hours in a low glutaraldehyde fixative [0.01 M phosphate-buffered saline, pH 7.4 (PBS), 4% paraformaldehyde, 0.05% glutaraldehyde, 0.2% picric acid]. After fixation, brains were transferred into PBS-azide (0.1% sodium azide in 0.01 M PBS; Sigma, St. Louis, MO) and stored at 4°C for a period of 2–3 weeks. A comparison of tissue integrity and immunohistochemical staining in brains postfixed by immersion (all 24 brains used in volumetric analyses) and brains fixed by perfusion (the additional four brains prepared for electron microscopy; see below) revealed a similar preservation of tissue integrity and no qualitative differences in SP-like immunoreactivity with either method.

The sectioning of brains and subsequent immunohistochemical procedures were conducted blind to species and sex. To counterbalance our processing regime, brains were assigned to four groups prior to coding and sectioning, ensuring as far as possible an equal distribution of species and sex among groups. Brains were cryoprotected in sucrose-PBS (30% sucrose in 0.01 M PBS) and cut frozen with a sledge microtome. Coronal sections (50 μm) were collected in PBS-azide and stored at 4°C for 1–2 weeks.

Immunohistochemistry. Immunohistochemical processing was carried out over a period of 2 weeks. Two counterbalanced groups were processed simultaneously each week. For each brain, every sixth section was immunolabelled. Sections were washed in 0.01 M PBS (3×10 minutes). Endogenous peroxidases were quenched in a hydrogen peroxide solution (0.3% H_2O_2 in dH_2O for 10 minutes). After several washes in PBS, nonspecific binding was blocked through incubation in normal rabbit serum (NRS) diluted 1:5 in PBS-T (0.01 M PBS with 0.3% Triton) for 1 hour. Sections were transferred directly from blocking solution into the primary antiserum solution, diluted 1:50 in PBS-T.

The primary antiserum, donated by Prof. Claudio Cuello (McGill University), was a rat monoclonal anti-SP (NC1/34) produced by a hybrid myeloma culture. The immunogen employed was SP conjugated to bovine serum albumin (Cuello et al., 1979). Previous radioimmunoassay studies demonstrated that NC1/34 binds the QFFGL C-terminus pentamer of the SP peptide (Cuello et al., 1979). The C-terminus pentamer of SP is highly conserved across vertebrate species (for review see Severini et al., 2002). Indeed, a CLUSTAL multiple sequence alignment for the protachykinin precursor protein (Chenna et al., 2003; alignment not shown) demonstrates that chickens, humans, and mice share the QFFGL signature, suggesting that the epitope recognized by NC1/34 predates the divergence of mammals and birds from a common ancestor. It is thus highly likely that NC1/34 reacts specifically with SP in the cowbirds, as it does in other birds as well as rodents and other mammals.

[1]Early New World blackbird phylogenies placed the bay-winged cowbird within the *Molothrus* genus, alongside the five parasitic cowbird species. However, recent molecular studies suggest that this historical *Molothrus* genus is not actually monophyletic (Lanyon, 1992; Freeman and Zink Robert, 1995). In particular, mitochondrial DNA sequence comparisons recommended the transfer of the bay-winged cowbird from *Molothrus* into a different New World blackbird genus, *Oreopsar* (Johnson and Lanyon, 1999). As a result of icterid nomenclature issues, the genus *Oreopsar* has since been renamed *Agelaioides* (Lowther, 2001).

Sections were incubated for 48 hours at 4°C in primary antiserum, with gentle shaking. After several washes in PBS (3 × 10 minutes), sections were incubated overnight at 4°C in a biotinylated secondary antibody solution (Vector biotinylated anti-rat secondary antibody, diluted 1:250 in PBS-T). Sections were washed in PBS (3 × 10 minutes) before application of an avidin-biotin-peroxidase complex for 1 hour (Vector Elite ABC reagent, prepared according to the manufacturer's instructions). After several washes, sections were preincubated with 3,3′-diaminobenzidine (DAB) for 12 minutes (Vector DAB peroxidase substrate kit). Peroxidase reaction was then initiated by adding H_2O_2 (according to the manufacturer's instructions). The stain was allowed to develop for 3 minutes. Sections were rinsed in dH_2O (3 × 10 minutes) and transferred into a subbing solution (1% glycerin-albumin in distilled H_2O). Sections were mounted onto gelatin-coated slides, dried overnight, and coverslipped with DPX mountant (BDH).

The protocol was also carried out omitting the primary antiserum to rule out nonspecific secondary labelling. In the absence of primary antiserum, no labelling was detected.

Analysis. The distribution of SP-like immunoreactivity throughout the entire forebrain and mesencephalon was studied at the light microscopic level with special attention to labelled neuropil in the dorsomedial telencephalon. High-powered photographs were acquired by using a Leica DMRA2 microscope, with a Leica DC 500 digital camera and QWin v3.1 software. A 1:6 series of sections for each brain was also digitized with a Polaroid Sprintscan slide scanner with Pathscan enabler (resolution 2,700 dpi). Resulting TIFF files were imported into Adobe Photoshop CS, where output levels, brightness, and contrast were adjusted to include information-containing pixels and to reflect the true appearance of the tissue as far as possible. Schematic illustrations based on digital photographs were drawn with a Wacom Graphire 2 digitizing tablet and drawing software (Adobe Illustrator CS).

For volume comparisons, digital images were analysed with the image processing program ImageJ (developed by Wayne Rasband, National Institute of Mental Health, Bethesda, MD). For quantitative analyses, the boundaries of the telencephalon, hippocampal formation, and SP-rich fields in the dorsomedial telencephalon were drawn with a Wacom Graphire 2 digitizing tablet. The area of each region of interest was recorded for every brain section in which it appeared. The volume of each structure (V_c) was calculated from cross-sectional areas using the unbiased Cavalieri formula (Cavalieri, 1635; Howard and Reed, 1998):

$$V_c = I(\Sigma A)$$

where A is cross-sectional area and I is the distance between sections (300 μm). Data were collected blind with regard to species and sex.

For interspecies comparisons, scatterplots were first used to assess the relationship between the volume of the Hp or SP-rich areas and the telencephalon. Volumes relative to the telencephalon (volume of the measured area divided by the volume of the remainder of the telencephalon) were used in comparisons to control for the effect of telencephalon size. Statistical analyses were performed using the SPSS statistical package. Data were assessed for normality and for homoscedasticity before analysis via the general linear model. Two-way ANOVAs were performed with species and sex as independent factors. Post hoc comparisons (with Scheffé's correction for multiple comparisons) were used to assess the direction of species differences. To investigate intraspecies sex differences, we compared residuals of a linear regression (constrained through the origin) of the volume of the area of interest against telencephalon volume (two-tailed independent-samples *t*-test). The overall probability threshold for significance was set at 0.05.

Electron microscopy

Preparation of brains. Four shiny cowbirds (two male and two female) were anesthetized with an overdose of sodium pentobarbitol (50 mg/kg) and transcardially perfused with a high glutaraldehyde solution (0.01 M PBS, 3.4% paraformaldehyde, 1.25% glutaraldehyde, and 0.2% picric acid). Brains were removed and postfixed in the same solution for 24 hours before transfer to PBS-azide for storage. Brains were sectioned at 70 μm using a vibratome.

Preembedding immunohistochemistry. Sections were cryoprotected in PBS-sucrose and subjected to three freeze-thaw cycles on liquid nitrogen to permeabilize cell membranes. Thereafter, SP immunohistochemistry was carried out as described above for light microscopy, save for the omission of Triton in all dilution buffers and doubling of incubation times for antibody solutions. The same control procedure as for the previous section was carried out without primary antibody to confirm the absence of nonspecific secondary labelling.

Preparation for electron microscopy. On completion of immunostaining, sections were treated with osmium tetroxide [2% in 0.1 M phosphate buffer (PB)] for 40 minutes, washed (2 × 10 minutes PB; 2 × 10 minutes dH_2O), then treated with uranyl acetate (1% in dH_2O) for 30 minutes. After several washes, sections were dehydrated in an ascending series of alcohols and two changes of propylene oxide before transfer to resin (Araldite epoxy resin, TAAB) for impregnation overnight. Sections were flat embedded on microscope slides. After polymerization (48 hours at 60°C), the extent of SP immunolabelling was inspected with a light microscope. For selected sections, SPh was excised and reembedded in blocks of resin for sectioning on an ultramicrotome (Reichart-Jung Ultracut E). Sections (90 nm) were collected on copper grids (3.05 mm Athene grids, TAAB). After lead citrate treatment, sections were examined with a Philips CM10 electron microscope.

RESULTS

Light microscopic investigation of SP distribution in the cowbird brain

In total, 24 brains, ten shiny cowbird (five males, five females), eight screaming cowbird (four males and four females), and six bay-winged cowbird (three males, three females), were processed immunohistochemically for light microscopy. Because similar areas of SP immunoreactivity were observed in the brain and Hp of all three cowbirds, the figures depicting SP immunoreactivity in the shiny cowbird may be considered representative of SP immunoreactivity in all three cowbird species. In the fol-

lowing sections, we have adopted the revisions for avian brain nomenclature recently recommended by the Avian Brain Nomenclature Forum (Reiner et al., 2004).

SP-immunoreactive elements in dorsomedial telencephalon.

Hyperpallium apicale (HA; previously hyperstriatum accessorium or dorsal visual wulst). The distribution of SP immunoreactivity in the HA of the shiny cowbird is shown in Figure 2A. Rostrally, two areas of densely staining neuropil were evident, one lying in a ventrolateral position in HA along the boundary with the hyperpallium densocellulare (HD; previously hyperstriatum dorsale) and a second area lying in a dorsomedial position, at the boundary of the Hp. These areas are similar to the lateral SP (SPl) and medial SP (SPm) fields previously described in four species of songbird (Gould et al., 2001). Intense SP-positive (SP^+) neuropil in HA has also been reported for the pigeon (Erichsen et al., 1991). The rostral-caudal distribution of SPl and SPm fields in the shiny cowbird (Fig. 3) is consistent with previous findings in other songbirds. Progressing caudally, the SPl and SPm fields appeared to move laterally and dorsally. The SPl field eventually disappeared altogether, whereas the SPm field remained throughout the entire rostral-caudal extent of Hp.

Hp. Within Hp (defined in this study as the area lying dorsal to the lateral ventricle and medial to SPm), no SP-immunoreactive perikarya were observed. This observation is again in line with findings in some songbirds but differs markedly from the situation in the pigeon and junco, in which numerous SP-immunoreactive perikarya are detected in the Hp (Erichsen et al., 1991; Gould et al., 2001).

All of the SP-immunoreactive elements seen within the Hp in the three cowbird species appeared to originate from SP-rich beaded fibers entering ventral Hp from the septum. Some of these fibers coursed dorsally, joining a medial fiber bundle running parallel to the medial hippocampal wall (an SP^+ feature first described in the pigeon by Krebs et al., 1991). As they travelled dorsolaterally toward the pial surface, these fibers gave rise to collaterals targeting cells within the medial arm of the trilaminar V. On reaching the dorsomedial region of the hippocampal formation, SP^+ fibers in the medial fiber bundle terminated in a region of intense SP^+ fiber and terminal staining (see Fig. 3A–I), which is similar to the dorsomedial crescent field described in zebra finch by Montagnese and colleagues (1996).

A second, smaller complement of beaded SP^+ fibers originating from the septum took a ventrolateral course, alongside the ventricle wall. Collaterals from this second fiber bundle contacted cells in the lateral arm of the trilaminar V. As the fibers entered a region corresponding to APH, they moved away from the ventricle wall, terminating in a discrete field containing a very dense network of intensely immunoreactive SP^+ fibers studded with large, bouton-like terminals. This striking feature of SP immunoreactivity, which we have termed the *hippocampal SP field*, or SPh, is illustrated for the shiny cowbird in Figure 2D,E and for all three cowbird species investigated in Figure 4 (three rostral-caudal levels).

The progression of SPh throughout the rostral-caudal extent of the Hp is illustrated in Figure 3A–I. SPh first appeared close to the rostral boundary of Hp, originating from beaded fibers extending dorsolaterally from the ventricle wall, resulting in a vertically elongated shape. Caudally, the network of SP-immunoreactive fibers in SPh expanded laterally, resulting in a more circular field. In caudal regions, the density of SP^+ elements and intensity of staining were especially strong in the central region of the terminal field.

The numerous large, intensely staining, bouton-like terminals in SPh appeared to surround the perikarya and proximal dendrites of SP-negative cells (Fig. 2F). To determine the identity of postsynaptic targets for SP^+ boutons in SPh, we used preembedding immunohistochemistry and electron microscopy to investigate the fine structure of the SP^+ terminals (see below).

At the light microscopic level, we observed obvious differences in the prominence of SPh between individual brains. To find out whether these differences were correlated with species or sex, we conducted a quantitative analysis of SPh volume (see below).

SP-immunoreactive elements in other telencephalic areas. In addition to the areas in the dorsomedial telencephalon described above, SP^+ elements were also observed within several dorsal telencephalic regions: the hyperpallium densocellulare (HD), the mesopallium (M; previously hyperstriatum ventrale), nidopallium (N; previously neostriatum), and septum. The entire rostral-caudal extent of HD contained lightly labelled neuropil with sparse SP^+ terminals and fibers as well as numerous lightly staining SP^+ perikarya. More caudally, SP^+ cells appeared at the lateral edge of the mesopallium and of dorsal nidopallium. A clustering of SP^+ cells, terminals, and neuropil was also found in the medial regions of the HD and dorsal M, bordering on the lateral ventricle.

There were relatively few SP^+ elements in the rostral nidopallium. However, in caudal nidopallium, prominent SP^+ neuropil and cells were observed in areas related to the perception and control of vocalization. Field L, caudomedial nidopallium (NCM), and associated auditory areas contained intensely staining SP-immunoreactive neuropil and cells (Fig. 5A,B). Strong neuropil staining was also seen in the song control nucleus HVC.

Within the septum, there was an intense network of SP^+ fibers as well as SP^+ cells. The lateral septum generally displayed a higher intensity of staining than medial regions. This finding is in line with observations in other songbirds (Gould et al., 2001).

In basal telencephalon, a network of SP^+ terminals and fibers occurred throughout the medial striatum (MSt; previously lobus parolfactorius). In ventromedial MSt, a region bordering the ventricle contained a denser network of SP^+ fibers. A second, circular region of more intensely staining neuropil was observed in rostral MSt, corresponding to the song system nucleus, area X (Fig. 5C,D). Caudally, intensely staining SP^+ elements appeared in structures of the basal ganglia—the lateral striatum (StL; previously referred to as paleostriatum augmentatum) and the globus pallidus (GP; previously paleostriatum primitivum; Fig. 5E,F). These basal ganglia regions are known to contain SP^+ fibers and cells in many other avian species (see, e.g., Erichsen et al., 1991; Aste et al., 1995; Gould et al., 2001). For cowbirds, we found SP^+ neuropil, fibers, and cell bodies throughout StL and a denser network of SP^+ fibers and terminals (but few SP^+ cells) in neighboring GP. Fiber tracts, such as FPM and FPL (medial and lateral forebrain bundles), contained large SP^+ fibers as did the septohippocampal tract.

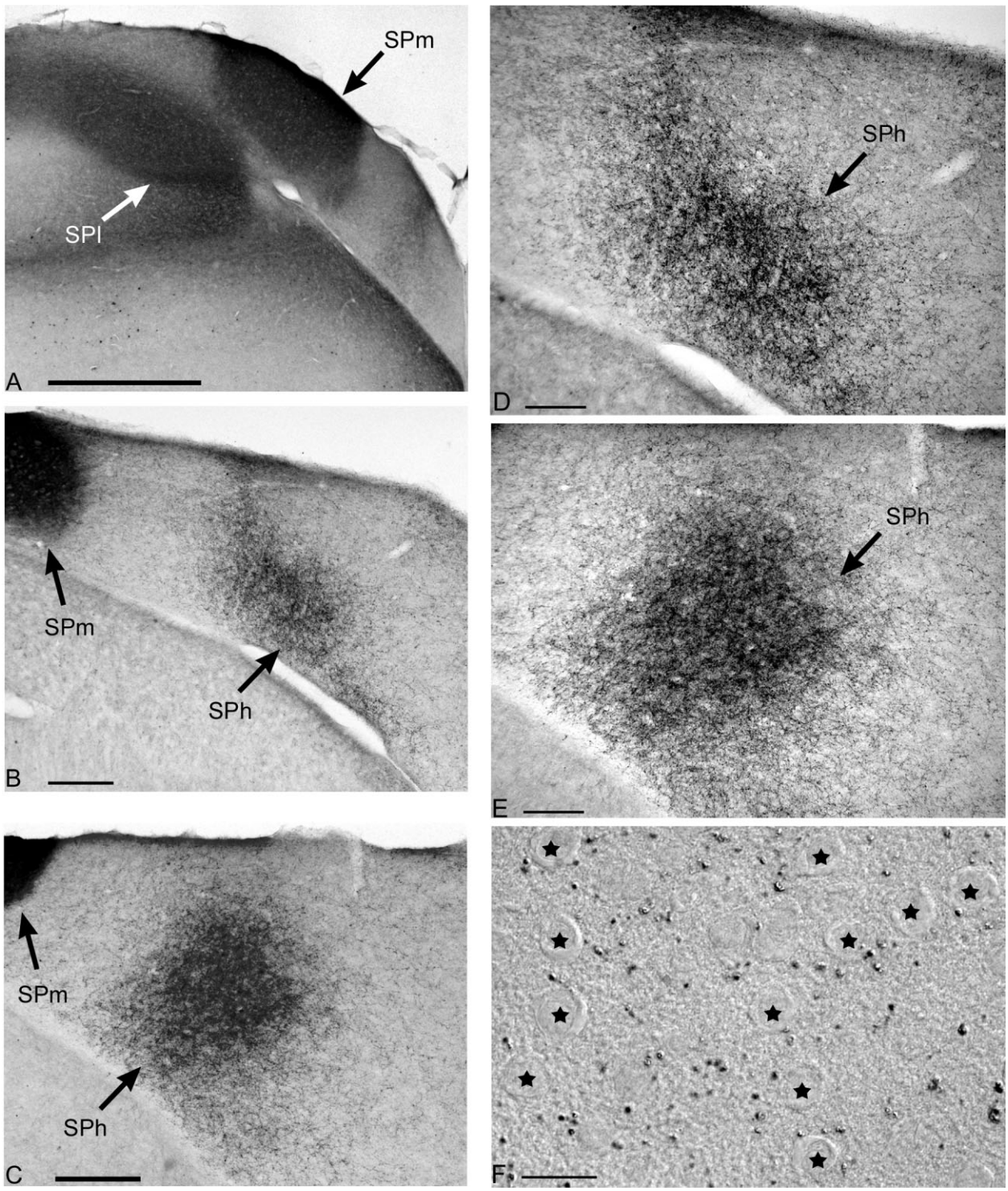

Fig. 2. SP-immunoreactive elements in and around the shiny cowbird hippocampus. **A:** SP-immunoreactive areas in hyperpallium apicale (approximate rostral–caudal level A 4.5). **B,C:** SP immunoreactivity in and around the shiny cowbird hippocampus (B, rostral hippocampus, approximate level A 2.4; C, caudal hippocampus, approximate level AP 0.0). **D,E:** High-power photographs showing SPh in two rostral caudal levels of the shiny cowbird hippocampus (D, approximate level A 2.4; E, approximate level AP 0.0). **F:** Nomarski-enhanced image showing SP-immunoreactive terminals in SPh contacting the cell bodies (stars) and proximal processes of unlabelled neurones. SPl, lateral SP field; SPm, medial SP field; SPh, hippocampal SP terminal field. Scale bars = 1 mm in A; 400 μm in B,C; 200 μm in D,E; 30 μm in F.

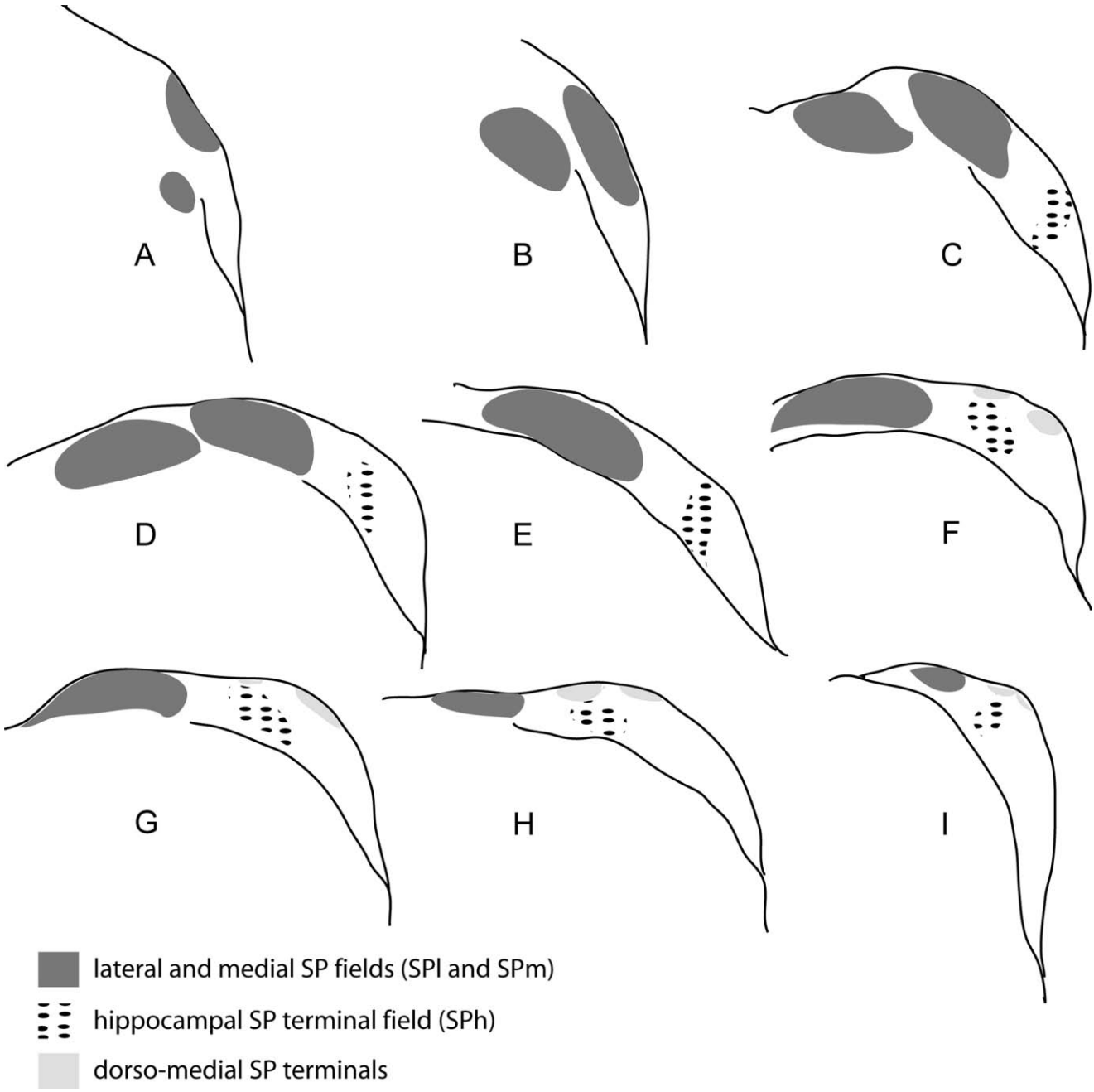

Fig. 3. Rostral-caudal progression and shape of SP-rich areas in and around the shiny cowbird hippocampus. Diagrams illustrating the shape and extent of SP-rich areas in and around the shiny cowbird hippocampus at nine rostral-caudal levels, corresponding approximately to A 6.0 (**A**), A 4.5 (**B**), A 3.5 (**C**), A 3.0 (**D**), A 2.6 (**E**), A 2.2 (**F**), A 1.8 (**G**), A 1.0 (**H**), and P 1.0 (**I**). Stippled areas indicate the extent and shape of the hippocampal SP terminal field, SPh.

Diencephalon and mesencephalon. The distribution of SP in the diencephalon and mesencephalon of the parasitic cowbirds studied here is similar to that reported in other songbirds (Gould et al., 2001). There was a high density of SP-immunoreactive terminals and fibers throughout the hypothalamus, with intense staining in the preoptic nuclei, the paraventricular nucleus, and the posterior hypothalamic nuclei. In lateral hypothalamus, a few small, round SP^+ cells were detected within the background of intensely labelled neuropil and terminals. Caudally, a strong SP^+ fiber input and its termination appeared in lateral hypothalamus and neighboring stratum cellulare externum. In the lateral thalamus, few structures exhibited SP immunoreactivity, with only the tectothalamic tract containing SP^+ fibers. The dorsolateral thalamic nuclei, the nucleus ovoidalis, and the nucleus rotundus were largely negative for SP.

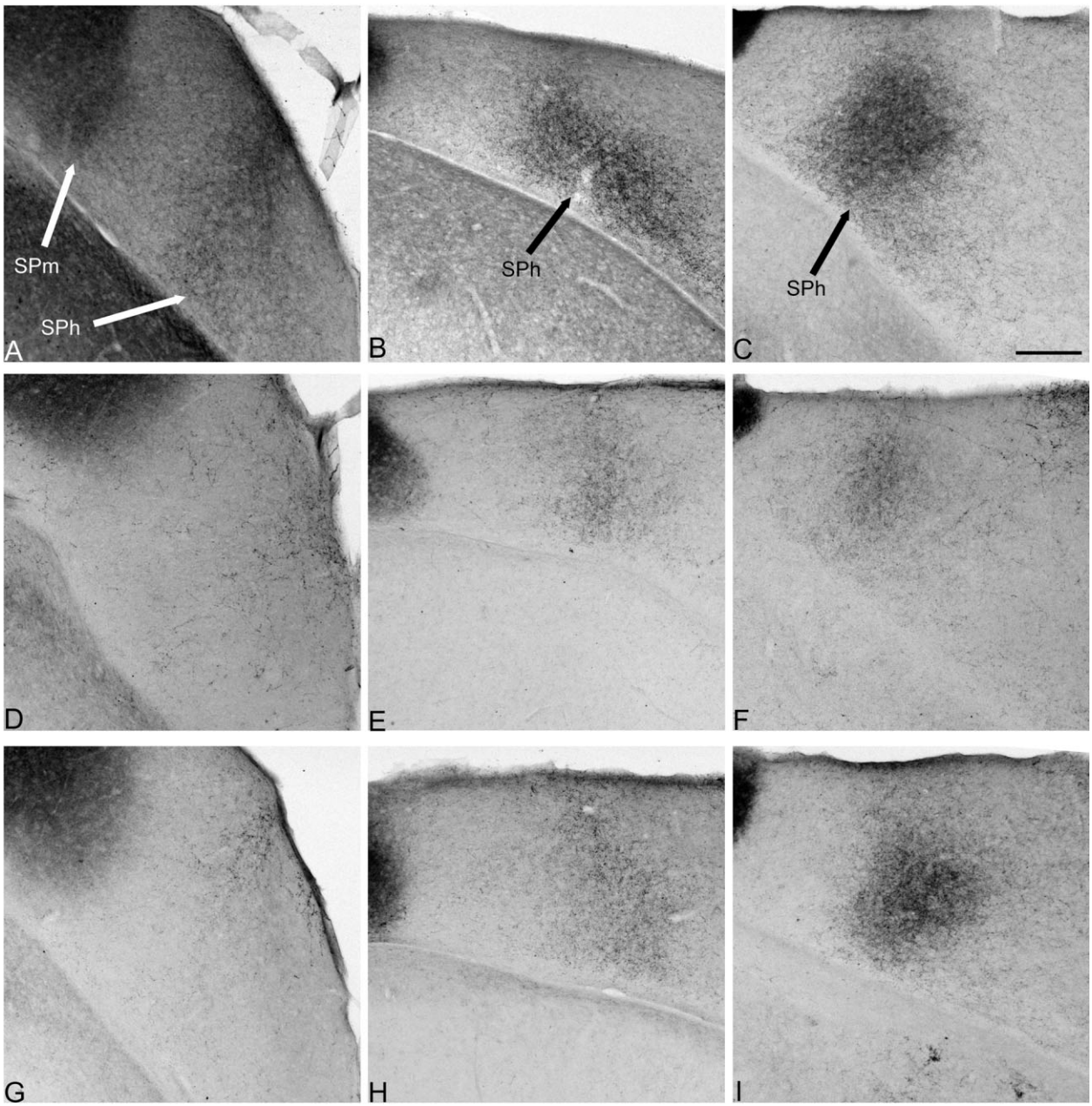

Fig. 4. Comparison of SPh in three cowbird species at three rostral–caudal levels. **A–C:** Shiny cowbird (generalist parasite). A: Rostral hippocampus (approximate level A 4.5). B: Intermediate (approximate level A 2.4). C: Caudal (approximate level AP 0.0). **D–F:** Bay-winged cowbird (nonparasite). D: Rostral. E: Intermediate. F: Caudal. **G–I:** Screaming cowbird (specialist parasite). G: Rostral. H: Intermediate. I: Caudal. SPm, medial SP field; SPl, lateral SP field; SPh, hippocampal SP terminal field. Scale bar = 400 μm in C (applies to A–I).

In the medial mesencephalon, SP immunoreactivity was observed in the neuropil in the AVT (area ventralis of Tsai) and in the interpeducular nucleus (IP). In lateral mesencephalon, intense SP immunoreactivity occurred in the nucleus tegmentipedunculopontinus pars compacta (TPc) and in the nucleus intercollicularis. Throughout the optic tectum, a light level of SP staining was observed following the characteristic laminar pattern reported for other species (Aste et al., 1995; Ehrlich et al., 1987; Gould et al., 2001).

Ultrastructural investigation of synapses within SPh

We used preembedding immunohistochemistry to investigate the fine structure of SP-immunoreactive boutons within SPh. SP^+ terminals were observed throughout SPh in immunostained tissue but not in control sections. SP^+ boutons terminaux as well as boutons en passant were identified by the presence of the electron-dense DAB reaction product, which, at low magnifications, appeared to fill immuno-

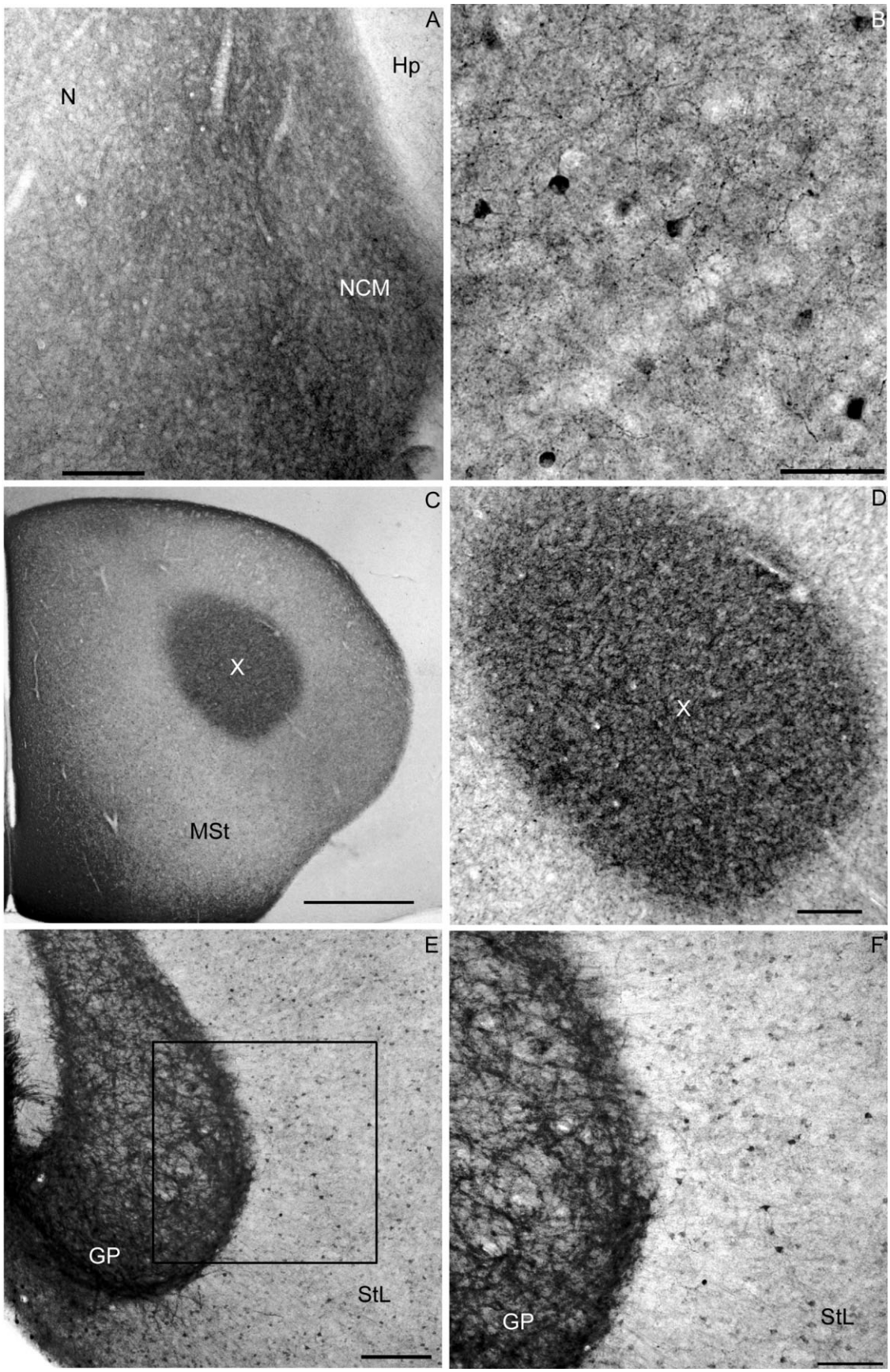

Fig. 5. SP immunoreactivity in the shiny cowbird telencephalon. **A:** SP-immunoreactive fibers in the caudomedial nidopallium (NCM). **B:** SP-immunoreactive cells in field L (caudal nidopallium). **C:** SP-immunoreactive neuropil in the medial striatum (MSt) and in the song control nucleus area X (X). **D:** High-power photograph of SP immunoreactivity in the song control nucleus area X, illustrating dense neuropil staining. **E:** SP immunoreactivity in the globus pallidus and the lateral striatum. **F:** High-power photograph of SP-immunoreactive elements in the lateral striatum (StL) and globus pallidus (GP; corresponding to the region outlined in B). Scale bar = 400 μm in A,D,E; 100 μm in B; 1 mm in C; 200 μm in F.

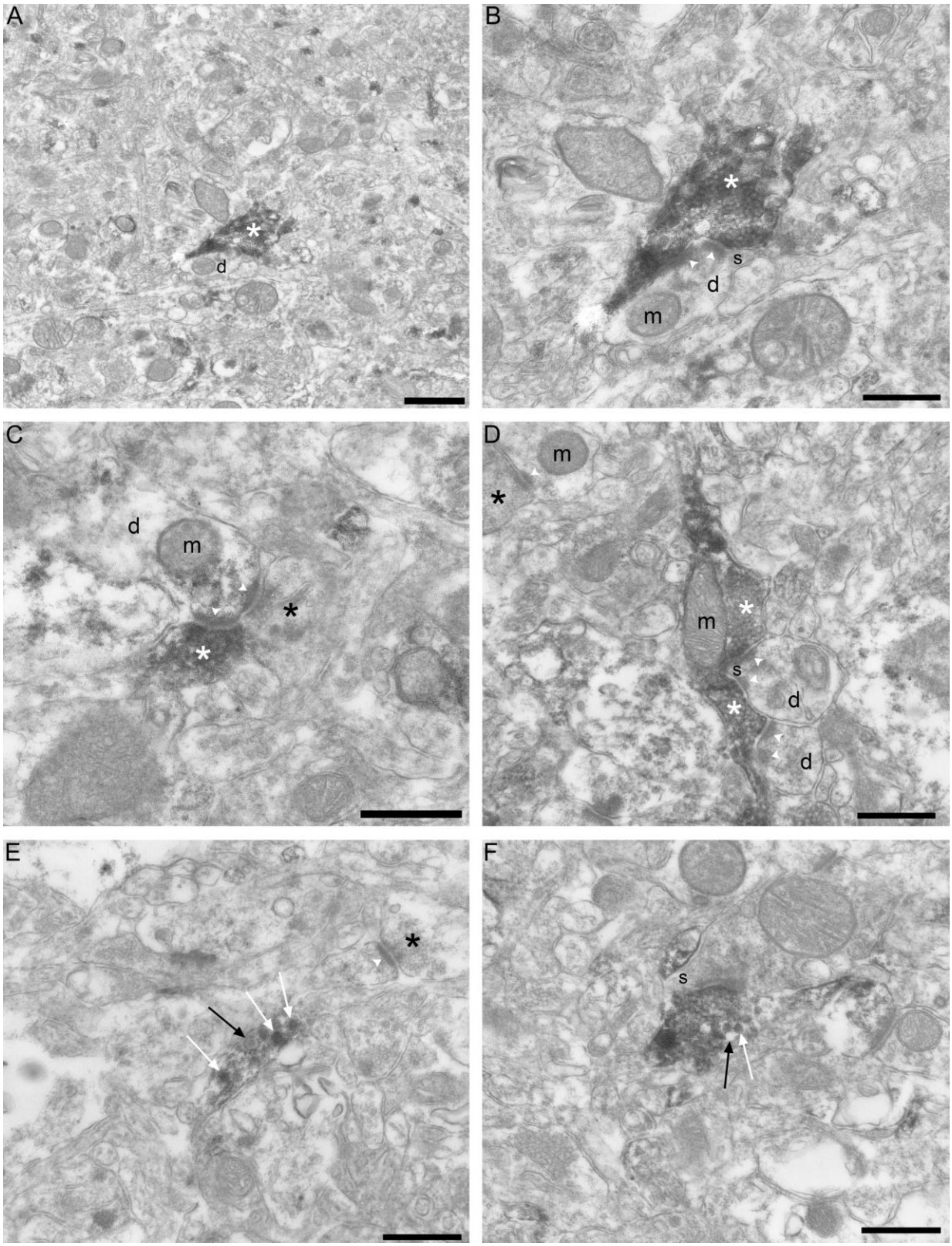

Fig. 6. Ultrastructure of SP-immunoreactive elements in SPh. **A:** Large SP-immunoreactive terminal (asterisk) contacting a dendrite (d). **B:** Immunoreactive terminal (asterisk) and dendrite (d) with mitochondrion (m) from A, at a higher magnification. Arrowhead indicates postsynaptic densities. **C:** Two terminals, one SP-immunoreactive (white asterisk) and the other SP-negative (black asterisk) forming asymmetric synapses with the same dendrite (d). Arrowhead indicates postsynaptic densities. **D:** SP-immunoreactive bouton en passant (white asterisk) forming asymmetric synapses with two dendrites (d), one with a spine (s). Arrowheads indicate postsynaptic densities. The black asterisk (top left) indicates a nonimmunoreactive bouton forming an asymmetric synapse. **E:** Preterminal SP-immunoreactive axon containing SP-filled, large dense-core vesicles (white arrows) and SP-negative dense core vesicles (black arrow). Asterisk (top right) indicates a nonimmunoreactive bouton forming an asymmetric synapse. **F:** SP-immunoreactive terminal containing SP-filled (white arrow) and SP-negative dense-core vesicles (black arrow), contacting a dendritic spine. Scale bar = 1 μm in A; 0.5 μm in B–F.

TABLE 1. Mean Absolute Values for Body Weight and Telencephalon, Hippocampus, SPm, SPl, and SPh Volumes According to Species and Sex

Cowbird species	N	Body weight (g)	Telencephalon (mm^3)	Hippocampus (mm^3)	SPm (mm^3)	SPl (mm^3)	SPh (mm^3)
Bay-winged	6	41.75 (1.44)[1]	644.94 (20.22)	16.29 (0.85)	6.15 (0.26)	3.90 (0.46)	0.82 (0.08)
Males	3	40.50 (2.18)	639.60 (38.30)	17.35 (0.20)	6.14 (0.44)	3.85 (0.44)	0.77 (0.11)
Females	3	43.00 (2.02)	650.20 (23.31)	15.20 (1.55)	6.16 (0.37)	3.96 (0.91)	0.88 (0.13)
Shiny	10	51.80 (1.64)	572.27 (16.14)	14.46 (0.62)	5.76 (0.18)	4.15 (0.36)	1.64 (0.08)
Males	5	53.80 (2.25)	599.60 (15.90)	14.63 (0.58)	5.77 (0.14)	4.48 (0.22)	1.60 (0.06)
Females	5	49.80 (2.24)	544.94 (23.31)	14.29 (1.17)	5.76 (0.35)	3.81 (0.70)	1.68 (0.16)
Screaming	8	53.38 (1.91)	543.27 (20.41)	15.55 (0.71)	5.77 (0.30)	4.10 (0.27)	0.99 (0.08)
Males	4	57.50 (1.54)	583.71 (9.45)	17.41 (0.39)	6.02 (0.32)	4.58 (0.33)	1.08 (0.08)
Females	4	49.25 (1.80)	512.93 (26.42)	14.15 (0.42)	5.58 (0.47)	3.66 (0.30)	0.88 (0.08)

[1]Standard error of the mean is shown in parentheses.

stained terminals homogenously (Fig. 6A). At higher magnifications, it was apparent that most of the DAB product in axon terminals was associated with the surface of clear agranular synaptic vesicles, which were numerous and circular in shape (Fig. 6B–D). Less commonly, DAB product was also seen in and around larger dense-core vesicles (Fig. 6E,F). The presence of this type of secretory vesicle is often considered a hallmark of peptidergic neurons (for review see Hökfelt et al., 1980; Zupanc, 1996).

In some cases, we observed SP-immunoreactive dense-core vesicles and SP-negative dense-core vesicles in the same terminal (see, e.g., Fig. 6E,F), indicating possible colocalization of SP with another neuropeptide. In pigeon, the hippocampal distributions of the peptides SP and Leu-enkephalin are nearly identical (Erichsen et al., 1991), and there is also evidence for colocalization of SP and Leu-enkephalin in neurons and fibers in other areas of the pigeon central nervous system (Erichsen et al., 1982). Certainly, it would be interesting to determine whether colocalization of SP and Leu-enkephalin occurs within SPh in the cowbird.

All SP^+ synapses observed were asymmetric, characterized by prominent postjunctional densities and the presence of many spherical, agranular synaptic vesicles (Fig. 6B,C). Postsynaptic targets consisted exclusively of dendrites (sometimes with spines).We found no evidence for direct axosomatic contacts.

Occasionally, in synapses formed by SP^+ elements, some diffuse DAB reaction product was seen in the postsynaptic compartment (Fig. 6C). Postsynaptic DAB product was always cytoplasmic and was never associated with vesicles or other membrane-bound organelles. This could be an artefact of DAB staining or perhaps diffusion of peptide during tissue processing or, rather, a true physiological occurrence of SP in the postsynaptic compartment. Studies in rodents (peripheral tissues and central neural loci) have shown that binding of SP to its preferred receptor (neurokinin receptor 1) causes a large-scale endocytosis of receptor-bound SP into postsynaptic targets (see, e.g., Bowden et al., 1994; Mantyh et al., 1995). It is theoretically possible that what we detected in the postsynaptic compartment was receptor-bound, internalized SP. However, too little is known about SP–receptor interactions in birds, and, in the absence of evidence for the internalization of SP–receptor complexes in the avian brain, the physiological significance of the observed postsynaptic immunolabelling cannot be established.

Quantitative comparisons

The absolute volumes for telencephalon, Hp, SPm, SPl, and SPh fields were calculated and compared among the three cowbird species (see Table 1 for a summary of mean absolute values). The bay-winged cowbird had a significantly lower body weight than the other two species ($P < 0.001$ in Scheffé-corrected post hoc tests after a two-way ANOVA with body weight as dependent variable and sex and species as independent factors; overall main effect for species $F = 14.87$, $P < 0.001$). In spite of this, the bay-winged cowbird had the largest telencephalon. A two-way ANOVA with telencephalon volume as dependent variable and sex and species as independent factors revealed a main effect of species ($F = 7.4$, $P < 0.01$): the bay-winged cowbird had a larger telencephalon than either shiny cowbird ($P < 0.05$) or screaming cowbird ($P < 0.01$, Scheffé-corrected post hoc tests). We found significant correlations between telencephalon volume and the volumes of hippocampus ($R^2 = 0.23$, $F = 6.68$), SPm ($R^2 = 0.31$, $F = 9.25$), and SPl ($R^2 = 0.31$, $F = 9.91$) from linear regression analyses and scatterplots of the raw data (plots not shown).

Our interspecies comparisons for SPh volume relative to the telencephalon revealed a very robust main effect for species [$F(2,23) = 42.3$, $P < 0.0001$; Fig. 7A,B]. Post hoc tests showed that shiny cowbird had a larger relative SPh than either screaming cowbird or bay-winged cowbird ($P < 0.0001$ for both comparisons, Scheffé test). The relative volume of SPh was also significantly larger in the screaming cowbird than in the bay-winged cowbird although this was a smaller effect ($P < 0.05$, Scheffé test). There was no indication of an overall main effect for sex [$F(1,23) = 0.115$, $P = 0.387$] and no significant interaction between sex and species [$F(1,23) = 1.4$, $P = 0.292$]. Within-species sex comparisons did not reveal any significant differences (Fig. 7C).

After the effect of telencephalon size was taken into account, we found no significant species effects for comparisons of the relative volumes of the hippocampus, SPm, and SPl fields (Fig. 8). The absence of significant species and sex differences for hippocampal volume contrasts with the results of a previous neuroanatomical investigation conducted in the same cowbird comparison group (Reboreda et al., 1996). In this previous study, birds were captured between December and February, earlier in the season than the animals used in our investigation. Our birds were captured in February and might no longer have been engaged in spatial behaviors relating to nest parasitism at the time of capture. Because of the seasonal nature of hippocampal enlargement in the cowbirds (Clayton et al., 1997), this temporal difference could well account for the divergence between our volumetric results and previous findings.

A second difference between our study and previous comparisons was the method used to define the lateral

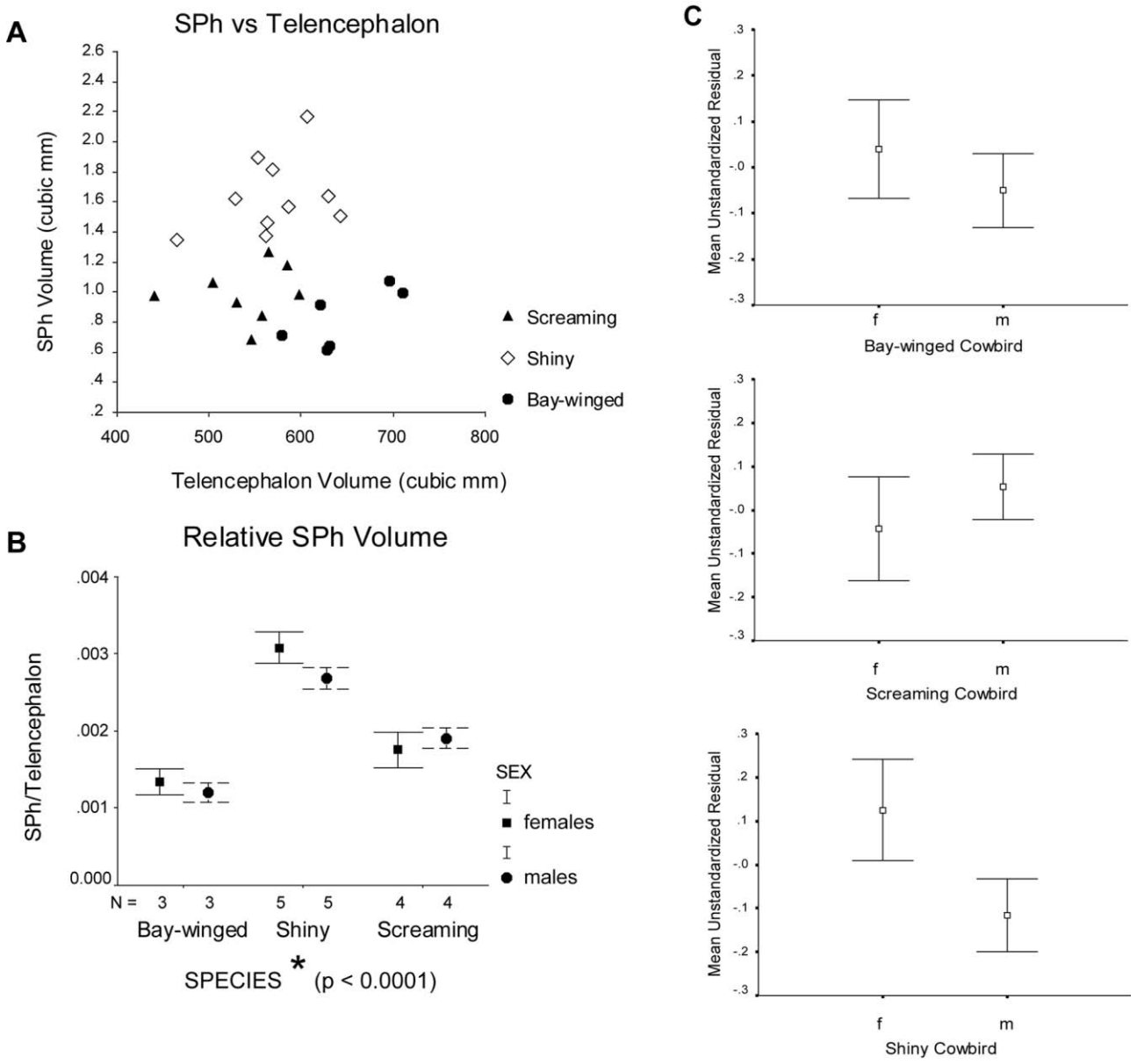

Fig. 7. Inter- and intraspecies comparisons for SPh. **A:** SPh volume plotted against telencephalon volume for the three cowbird species. **B:** Mean relative SPh volume for different combinations of species and sex. Error bars represent the standard error of the mean. There was a significant main effect for species ($P < 0.0001$). **C:** Mean unstandardized residuals from an intraspecies regression analysis of SPh volume vs. telencephalon volume, for males and females. Error bars represent the standard error of the mean. There were no significant differences between males and females.

boundary of the Hp. Previous comparative studies relied on Nissl staining to highlight changes in cell density and size coinciding with the lateral boundary. Our chosen neurochemical marker, SP, reveals a clear, unmistakable separation between lateral Hp and a physiologically and functionally distinct SP-immunoreactive field lying adjacent to the hippocampus in the hyperpallium apicale (Figs. 3, 4). This lateral SP-immunoreactive area, likened to mammalian entorhinal cortex, was first identified as a reliable lateral boundary marker for the avian hippocampal formation by Erichsen and colleagues (1991).

Although certain authors have reported convergence between cytoarchitectonic and cytochemical markers for the lateral boundary of the avian hippocampus (Krebs et al., 1989; Sherry et al., 1993), other reports have highlighted significant differences in the delineation of brain nuclei depending on the type of marker (cytoarchitectonic vs. cytochemical) employed (see, e.g., Gahr, 1997). Variations in the placement of the lateral boundary of the Hp resulting from the use of different delineation markers could possibly account for the differences in volume determinations between our study and previous investigations.

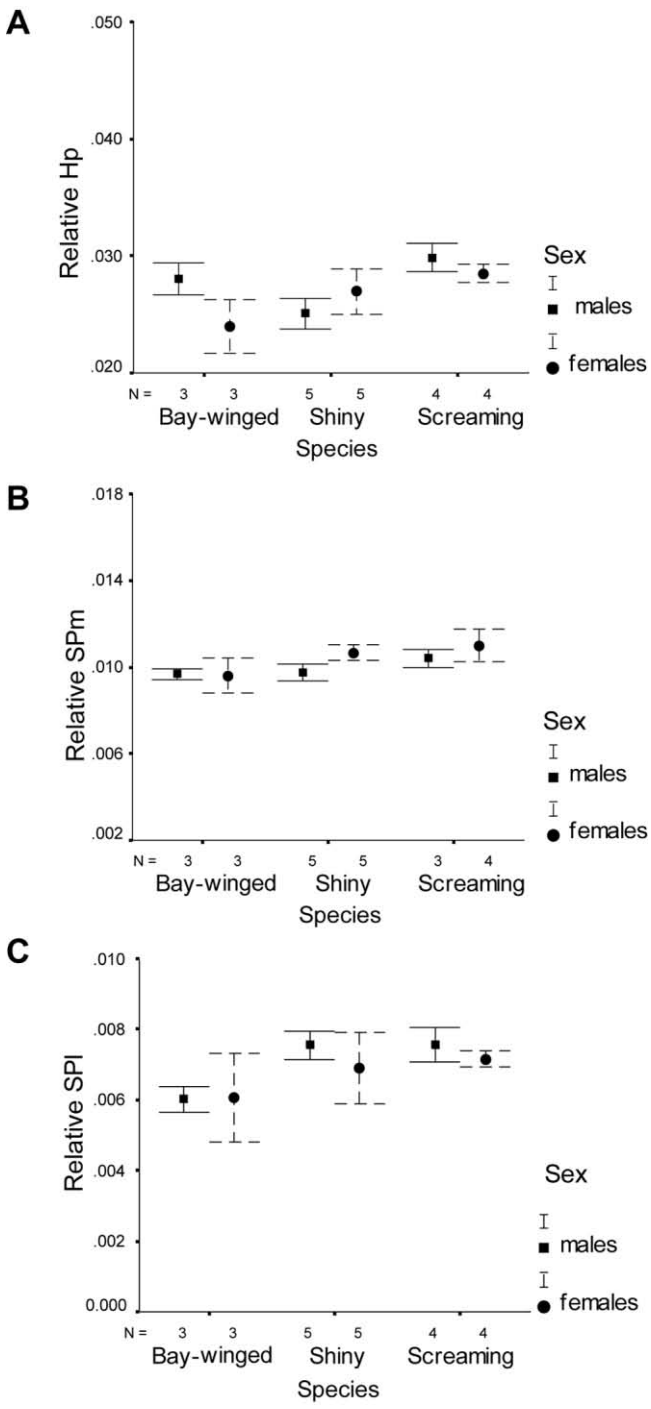

Fig. 8. Inter- and intraspecies comparisons for Hp, SPm, and SPl. **A:** Mean hippocampal volume (relative to the telencephalon). **B:** Mean SPm volume (relative to the telencephalon). **C:** Mean SPl volume (relative to the telencephalon). There were no significant species or sex differences. Error bars represent the SEM.

DISCUSSION

Neuroanatomical comparisons in both mammals and birds have demonstrated an association between hippocampal size and behaviors reliant on spatial memory, such as food hoarding (Krebs et al., 1989; Sherry and Vaccarino, 1989; Krebs, 1990), homing (Bingman, 1992; Gagliardo et al., 1999; Bingman et al., 2003), and brood parasitism (Sherry et al., 1993; Reboreda et al., 1996; Clayton et al., 1997). In the main, such studies have relied on volumetric comparisons and have described hippocampal differences only in terms of gross morphology. Surprisingly little is known about how hippocampal enlargements in species relying heavily on spatial memory translate into anatomical and neurochemical specializations involving the internal structure of the Hp. In this study, we examined the distribution of SP in and around the hippocampal formation of three species of South American cowbird (two parasites and one nonparasite) in order to highlight any special features within this region that might be related to the performance of parasitic behavior.

The most remarkable novel finding emerging from our investigation is the existence of a prominent SP-immunoreactive region in the cowbird Hp that has not previously been described in any other avian species: a discrete area of densely clustered SP-immunoreactive terminals, which we have called the *hippocampal SP terminal field,* or SPh. This intensely stained terminal field occupies a central position within the hippocampal formation and extends throughout almost the entire rostral-caudal extent of this structure (Fig. 3A–I). Electron microscopic analysis (Fig. 6) revealed that the majority of these SP^+ terminals make asymmetric synapses with the proximal dendrites of SP-negative cells. Further immunohistochemical and ultrastructural studies are certainly required to characterize the neurochemistry of the postsynaptic targets for SP innervation within SPh.

Although SPh appears to be a novel area, our analysis also revealed many features of SP staining in and around the cowbird hippocampal formation similar to those previously reported in other avian species. First, two areas of densely staining SP-immunoreactive neuropil found in hyperpallium apicale lateral to the hippocampus (SPm and SPl) are similar to those described for the pigeon and songbirds (Erichsen et al., 1991; Gould et al., 2001). Second, the lack of SP-immunoreactive cell bodies detected within the cowbird Hp is in agreement with published results for three parid species but differs markedly from the findings in pigeon and junco, where numerous SP-labelled cells are seen within the hippocampal formation (Erichsen et al., 1991; Gould et al., 2001). This difference in the detection of SP-positive hippocampal cells between different avian species is paralleled in mammals. Some mammalian species, including humans, monkeys, and guinea pigs, exhibit SP-immunoreactive cell bodies in the Hp (Del Fiacco et al., 1987; Gallagher et al., 1992; Seress and Leranth, 1996), but, in the rat, these are not seen in the absence of colchicine pretreatment (Borhegyi and Leranth, 1997b). Of course, the level of SP in hippocampal cell bodies in rats may be below the threshold for detection by immunohistochemistry, and this could also be true in those avian species studied in which SP-immunoreactive cell bodies have not been found. Rather than a total absence of SP-immunoreactive cells in the Hp of certain avian species, there may simply be a variation in the amount of SP contained by hippocampal cells between species. In any event, the functional significance of this apparent species difference in birds and mammals is not yet clear.

To our knowledge, nothing like the SPh has been reported in any other study of the avian hippocampus. Therefore, we believe that we have identified a new regional subdivision. We briefly review below the major existing schemes of avian hippocampal subdivisions and describe how SPh relates to them.

Subdivision schemes based on immunohistochemistry and Golgi staining

Our new area SPh does not correspond well with any regional subdivision of avian hippocampus previously identified using immunohistochemistry. Immunohistochemical studies in a variety of avian species (for review see Szekely, 1999) have proposed a number of hippocampal subdivision schemes. In the scheme for pigeon Hp established by Erichsen et al. (1991), SPh appears to span areas 3 and 4 but without completely overlapping either. In the layout proposed for the zebra finch (Szekely and Krebs, 1996), SPh falls within the dorsolateral regional subdivision. SPh seems to coincide, at least partially, with the central parahippocampal field (PHc) defined by cell types in a Golgi impregnation study (Montagnese et al., 1996).

Subdivision schemes based on electrophysiology

An electrophysiological characterization of the pigeon Hp (Siegel et al., 2002) highlights two areas that, when taken together, replicate well the shape and rostral-caudal extent of SPh. These two regions, the dorsorostral (DR) and dorsocaudal (DC) subdivisions of the pigeon Hp, are likened to the subiculum and dentate gyrus, respectively, of the mammalian Hp. In fact, cell bodies in both the dentate gyrus and subiculum of the rat are contacted by SP-immunoreactive bouton-like terminals (Borhegyi and Leranth, 1997b), further suggesting a possible homology between SPh in the cowbird and these regions of the mammalian Hp.

Subdivision schemes based on connectivity

Connectivity studies identify several inputs to the avian hippocampal region within which SPh is located, including projections both from within and from outside the Hp (Benowitz and Karten, 1976; Berk and Hawkin, 1985; Casini et al., 1986; Atoji et al., 2002; Hough et al., 2002). Of special interest is a strong projection from the lateral mammillary nucleus of the hypothalamus (ML) to the parahippocampal area in the pigeon (Berk and Hawkin, 1985).

As with the SP^+ fibers we describe here, fibers originating from ML project to the lateral and septal nuclei before entering the Hp. Within the Hp, fibers from the ML travel along the ventral and pial walls of Hp, providing input to both the lateral and the medial arms of the V. ML input also terminates very densely within APH, with a termination field that coincides well with the location, shape, and rostral-caudal extent of the SP-rich terminal field we have identified. The hypothalamic origin of the projection, the lateral mammillary nucleus, is considered the equivalent of the mammalian supramammillary nucleus (SUM), which is the major source of extrinsic SP-rich input to the Hp in many mammals, including cats, monkeys, and rats (Borhegyi and Leranth, 1997a,b; Ino et al., 1988; Leranth and Nitsch, 1994; Nitsch and Leranth, 1994; Yanagihara and Niimi, 1989).

As with the SP^+ boutons that we observed in SPh, the SP-rich terminals of SUM afferents form predominantly asymmetric synapses with hippocampal target neurons in the rat (Borhegyi and Leranth, 1997b). Thus, it seems possible that the SP-rich input to the cowbird Hp that we have described here (i.e., SPh) is the avian equivalent of the mammalian supramammillary-hippocampal pathway. Although we identified some SP^+ cells in the lateral hypothalamus, the very high level of general SP immunoreactivity throughout the hypothalamic region in the cowbird interfered with our ability to detect individual SP-immunoreactive cells within ML. Colocalization experiments, involving pathway tracing and immunohistochemistry, will be required to confirm that the SP input to SPh originates from the lateral mammillary region in the cowbirds.

Functional considerations for SPh

If SPh is indeed the termination of an SP-rich projection from the lateral mammillary nucleus, there may be important implications for the functional organization of the avian Hp. In mammals, the SP-rich projection from the supramammillary nucleus and neighboring structures to the Hp plays a vital role in modulating hippocampal function (McNaughton et al., 1995; Thinschmidt et al., 1995; Leranth et al., 1999; Leranth and Shanabrough, 2001), especially in the context of spatial memory (Rosenstock et al., 1977; Sziklas and Petrides, 1998; Santin et al., 1999; Sziklas and Petrides, 2000; Vann and Aggleton, 2004). Memory deficits generated by mammillary body ablation are strikingly specific to spatial memory. Tasks relying on nonspatial learning and the ability to form associations between locations and arbitrary stimuli are not affected. Deficits appear only during tasks in which the operated animal is required to remember and return to (or avoid) locations it has already visited. For example, damage to the mammillary bodies, including the supramammillary nucleus, specifically disrupts the performance of monkeys, rats, and mice in delayed spatial alternation tasks.

It is not yet known whether the lateral mammillary nucleus, our suggested origin of SP input to SPh, is involved in spatial memory processing as is its proposed mammalian equivalent, the supramammillary nucleus. The discovery of a function in spatial memory processing for the pathway terminating in SPh in the cowbirds would certainly be interesting in light of the species differences that we have uncovered and the putative importance of spatial learning in the context of brood parasitism. Although the exact types of spatial memory tasks involved in host-nest targeting by brood parasites have not yet been elucidated, it is very likely that spatial memory plays a key role in the run up to a parasitic event. Parasites have to learn the location of host nests they have visited during monitoring to return to lay at a later date. When returning to lay, parasites must also remember the location of host nests that have already been targeted, rejecting previously parasitized nests to avoid multiple parasitic events.

Our volumetric comparisons highlighted robust species differences in the relative volume of SPh within our comparison group. There was a ranking in the prominence of SPh, with the two parasitic species being larger than the nonparasitic bay-winged cowbird in terms of SPh volume. For the two parasitic species, SPh was also much larger in the generalist parasite (shiny cowbird) than in the specialist (screaming cowbird), hinting at possible involve-

ment of SPh in behavioral processes related to the exploitation of multiple host species.

Definite conclusions regarding the significance of species differences in the prominence of SPh are not yet possible because of the limited number of species and relatively small sample sizes investigated here. Nonetheless, it is possible to advance some hypotheses based on our present findings. As the only nonparasitic species in our set, the bay-winged cowbird contrasts with the molothrine cowbirds, in which obligate brood parasitism has evolved as the predominant reproductive strategy (for review see Ortega, 1998). Although SPh is present in the nonparasitic bay-winged cowbird, that it is significantly smaller than SPh in the parasitic cowbirds could have arisen because of an association between SPh size and parasitic behavior. However, this possibility should only be considered a working hypothesis, insofar as phylogenetic independence within our comparison group cannot be guaranteed: the two parasitic species are more closely related to each other than to the nonparasitic bay-winged cowbird.

The fact that SPh exists at all in the brain of the nonparasitic cowbird [in contrast to the apparent absence of the SPh area in other heterogeneric nonparasitic songbirds investigated so far (Gould et al., 2001)] may simply reflect their shared taxonomic lineage with the molothrine species. Nonetheless, it is also interesting to speculate that the presence of SPh in the bay-winged cowbird might not simply be a nonfunctional consequence of its phylogenetic relationship with the molothrine brood parasites. Although bay-winged cowbirds incubate and rear their own young, behavioral observations suggest that certain aspects of their nesting are unusual and may reflect a trend toward parasitism (for reviews see Davies, 2000; Johnsgard, 1997; Ortega, 1998). For example, instead of building their own nests, bay-winged cowbirds appropriate nests abandoned by other species (nest parasitism; Friedmann, 1929; Fraga, 1988; Ortega, 1998). Alongside this, there have been reports of conspecific and heterospecific nest parasitism, with bay-winged cowbirds laying eggs in the nests of other birds (Hoy and Ottow, 1964; Ortega, 1998). The emergence of nest parasitism has been suggested as a likely route leading to the evolution of brood parasitism in the parasitic cowbirds (Davies, 2000). Thus, it is conceivable that the existence of SPh in the bay-winged cowbird Hp corresponds to a neural adaptation associated with, and perhaps predisposing toward, the transition from a nonparasitic to a parasitic life-style. It would certainly be interesting to explore this possibility further, perhaps through the examination of Hp anatomy in other nest parasites.

If a more prominent SPh field is indeed associated with the performance of parasitic behavior, the absence of a significant sex difference in the volume of SPh in the shiny cowbird, in which only the female performs host nest targeting, would suggest the lack of a strong selective force for modification of SPh structure in male shiny cowbirds. Further studies investigating sexual dimorphism for SPh may reveal whether there is a clear sexual dimorphism in SPh volume in the shiny cowbird.

Our discovery of a prominent hippocampal SP-containing input with potential involvement in memory processing leaves us with unanswered questions that recommend several avenues for future research. Different methodologies suggest a correspondence between our new hippocampal field SPh and mammalian dentate gyrus or subiculum, which receive significant SP-input from the supramammillary nucleus in many mammals. However, in the absence of sufficiently detailed studies comparing the anatomy and function of projections into the avian and mammalian hippocampal formations, any correspondence between the SP-rich input to SPh and the mammalian supramammillary-hippocampal pathway must remain hypothetical. Further neuroanatomical (especially pathway analysis) and neurochemical comparisons among different brood parasitic and nonparasitic cowbird species encompassing all combinations of species, sex, and season will be required to reveal the true origin and significance of SPh for the performance of parasitic behavior in particular as well as for the operation of general hippocampal spatial memory processes.

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