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Afferent and efferent connections of the sexually dimorphic medial preoptic nucleus of the male quail revealed by in vitro transport of DiI

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Abstract. The medial preoptic nucleus of the Japanese quail is a testosterone-sensitive structure that is involved in the control of male copulatory behavior. The full understanding of the role played by this nucleus in the control of reproduction requires the identification of its afferent and efferent connections. In order to identify neural circuits involved in the control of the medial preoptic nucleus, we used the lipophilic fluorescent tracer DiI implanted in aldheyde-fixed tissue. Different strategies of brain dissection and different implantation sites were used to establish and confirm afferent and efferent connections of the nucleus. Anterograde projections reached the tuberal hypothalamus, the area ventralis of Tsai, and the substantia grisea centralis. Dense networks of fluorescent fibers were also seen in several hypothalamic nuclei, such as the anterior medialis hypothalami, the paraventricularis magnocellularis, and the ventromedialis hypothalami. A major projection in the dorsal direction was also observed from the medial preoptic nucleus toward the nucleus septalis lateralis and medialis. Afferents to the nucleus were seen from all these regions. Implantation of DiI into the substantia grisea centralis also revealed massive bidirectional connections with a large number of more caudal mesencephalic and pontine structures. The substantia grisea centralis therefore appears to be an important center connecting anterior levels of the brain to brain-stem nuclei that may be involved in the control of male copulatory behavior.

Key words: Preoptic area – Sexual dimorphism – Tract tracing – Carbocyanin dyes – Japanese quail

Introduction

Since the discovery of a major sexual dimorphism in the size of the telencephalic song-control nuclei in the zebra finch (some nuclei are 5 to 6-fold larger in males than in females; Nottebohm and Arnold 1976), considerable attention has been focused on the preoptic area (POA) of many vertebrate species in the hope that dimorphic structures causally related to sexually differentiated reproductive behavior would also be discovered there. In rat, a sexually dimorphic nucleus (SDN) has indeed been identified in the medial part of the POA (Gorski et al. 1980), but subsequent studies have in general failed to establish a clear role of the rat SDN in the control of reproductive activities. Specific lesions of the SDN in rat do not affect male copulatory behavior, although larger lesions located in the POA strongly impair this behavior in rat (Arendash and Gorski 1983) and in other species (e.g., goat: Hart 1986). SDN lesions appear, however, to produce deficits in the male-type copulatory behavior displayed by females after treatment with testosterone (T) (Turkenburg et al. 1988) and in the copulatory behavior of inexperienced male rats (De Jonge et al. 1990); nevertheless, the interpretation of this observation remains unclear.

SDNs have also been discovered in various mammalian species including the guinea-pig (Hines et al. 1985), gerbil (Yahr 1985; Commins and Yahr 1984 a), pig (Van Eerdenburg and Swaab 1991), ferret (Tobet et al. 1986) and man (Swaab and Fliers 1985; Allen et al. 1989), but, with one exception, specific lesions of these nuclei have failed to affect male copulatory behavior (De Jonge et al. 1990). In the gerbil, however, lesions of specific parts of the dimorphic area of the POA or of its efferent projections produce clear behavioral deficits indicating a causal involvement in the activation of behavior (Commins and Yahr 1984 b; Yahr and Stephens 1987; Yahr 1993).

Copulatory behavior in quail is sexually differentiated. Mature males immediately mount and copulate with females introduced into their cage,whereas females never show this behavior pattern even after treatment with high doses of T that would activate intense behavior in males (Balthazart et al. 1983). This behavioral dimorphism has led us to investigate the possible presence of a morphological dimorphism in the quail POA ; indeed, morpho-

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metric studies have readily identified an SDN in this area (Viglietti-Panzica et al. 1986). It has been identified as the nucleus preopticus medialis (POM) based mainly on the description of a homologous nucleus in several avian species (see Panzica et al. 1991 for additional discussion); this dinorphism has subsequently been confirmed by Adkins Regan and Watson (1990). Additional morphometric studies have shown that the volume of the POM is T-dependent and that the difference observed between males and females reflects a differential activation by adult steroids, rather than an organizational effect of perinatal hormones (Panzica et al. 1987; 1991).

The role of the POM in the control of the male copulatory behavior has been investigated by placing electrolytic lesions in the medial preoptic area. The activation of the behavior by T in castrated birds is severely disrupted by lesions that destroy a significant part of the POM. Lesions of a similar size placed in the POA but not in the POM have no effect on behavior (Balthazart and Surlemont 1990 a; Balthazart et al. 1992). In addition, stereotaxic T implants activate male sexual behavior in castrated birds only if they are located in or close (less than 200 μm) to the cytoarchitectonic boundary of the nucleus. Implants located elsewhere in the POA are behaviorally ineffective (Balthazart and Surlemont 1990 a; Balthazart et al. 1992). Taken together, these data clearly demonstrate that the POM is a necessary site of T action for the activation of male copulatory behavior (Balthazart 1991; Balthazart and Foidart 1993). Additional studies involving the stereotaxic implantation of synthetic androgens or estrogens, of anti-androgens or anti-estrogens, or aromatase inhibitors into the POM also show that T must be aromatized in this nucleus in order to activate the relevant behavior (Balthazart and Surlemont 1990 b; Balthazart et al. 1990 a).

Several neurochemical markers of the POM have been identified. It is outlined by aromatase-immunoreactive cells (Balthazart et al. 1990 b, 1990 c), by α_2-adrenergic receptors (Ball et al. 1989), and by neurotensin-containing neurons (Absil et al. 1993). Many other peptides including vasotocin, neuropeptide Y, and substance P, are also found in this nucleus (cells and or fibers) but they are present in a larger part of the POA and do not specifically occur at high or low levels in the POM (Panzica et al. 1992).

The complete understanding of the ways in which T action in the POM activates male copulatory behavior requires the identification of the efferent connections of this nucleus. In addition, the presence of a dense peptidergic innervation in the nucleus often associated with a lack of immunoreactive neurons implies that more distant structures project to the POM and modulate its activity. The identification of the afferent connections to POM would also clarify the role played by this nucleus in the control of reproduction.

The present report is a first attempt to reach these goals.We describe here the major afferent and efferent connections of the POM, established by in vitro tracing with the fluorescent marker DiI (*1,1'* dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate), a carbocyanine lipophilic marker with a long carbon chain. DiI has been increasingly used to establish neuronal connections (Honig and Hume 1989); it can be employed in vivo and in vitro. When injected in vivo, DiI either diffuses laterally in the membranes or is incorporated into intracellular vesicles thereby producing granular labeling. These vesicles can be actively transported through the axons at a rate of more than 2 mm per day (Godement et al. 1987). DiI can also be implanted in formalin-fixed tissue; it then exclusively diffuses laterally in the membranes, although this diffusion is much slower (less than 0.4 mm per day) and is limited to the membranes of cells that have incorporated the tracer (Holmqvist et al. 1992).

This in vitro use of DiI has several advantages over other tracing techniques (Holmqvist et al. 1992). Since it is possible to use formalin-fixed tissue, the tracer can be directly implanted with high precision into a specific target. DiI fluorescence is extremely intense and fades slowly compared with other fluorescent tracers. Its in vitro transport is anterograde (from cell bodies toward terminals) and retrograde (from axons to cell bodies), so that afferent and efferent connections of a given nucleus can be determined in a single experiment.

Materials and methods

Subjects

All experiments were carried out on sexually mature male Japanese quail (*Coturnix coturnix japonica*) that were obtained from a local breeder either in Belgium (C. Dujardin, Lierna) or in Italy (Osclli, Torino). Birds were raised in heterosexual groups until they were 4 weeks old and were then isolated in individual cages. In the breeding colony and in the laboratory, animals were exposed to a photoperiod simulating long summer days (16 h light and 8 h dark); this induces rapid testicular development in adult birds (Sachs 1969). Food and water were available ad libitum.

The brains of all quails were perfused with paraformaldehyde-glutaraldehyde. The birds were first injected intravenously with 100 μl heparin (Sigma H-7005; 20 mg, i.e., about 3 300 units/ml). They were then deeply anesthetized with Hypnodil (Janssen Pharmaceutica, Beerse, Belgium; 50 mg/kg body weight) and perfused with a saline solution (NaCl 0.9%; 0.15 M) through the left ventricle until the return blood was clear. The saline was then replaced by a fixative solution (4% paraformaldehyde, 0.1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2) and perfusion was continued for another 10–15 min (about 200–300 ml) until the muscles became completely rigid. The brain was then dissected out of the skull and postfixed overnight in the same fixative at 4°C.

Tracing technique

Three slightly different procedures were used to establish and then confirm the afferent and efferent connections of the POM. Initially, the postfixed brains were blocked (cut) in the coronal plane at the level of the anterior POM or of another nucleus identified as a target of the dimorphic nucleus viz., the tuberal hypothalamus (tuber), area ventralis of Tsai (AVT), substantia grisea centralis (GCt) or nucleus intercollicularis (ICo). No blocking of the brain was made when the dye had to be implanted in the tuber since this structure could be easily reached by a ventral approach. A total of 28 males that were 5–6 weeks old at the age of sacrifice were used in this part of the study. A single crystal of DiI (Molecular Probes, Eugene, Ore., USA) was then implanted into the target tissue by means of a dissecting needle under a dissecting microscope. A coro-

nal cut in the POM, AVT, GCt and ICo separated the brain into two regions and the transport of DiI was studied in the caudal and rostral direction for the POM and GCt but only in the rostral direction from the AVT and ICo.

In a second series of experiments ($n = 3$ male birds, 6–8 weeks old), the brains were blocked in the coronal plane at the level of the caudal POM, and DiI was inserted into the POM either with a dissection needle as described above ($n = 2$) or with the help of an insect pin ($n = 1$) that had been coated with the tracer in the following manner. A saturated solution of DiI in anhydrous alcohol was prepared and a drop of this solution was placed on the tip of the insect pin. After a few seconds, i.e., when the alcohol had evaporated, the procedure was repeated until the tip of the needle was homogeneously covered with microcrystals. The needle was implanted into the POM and the entire brain was stored in fixative overnight. The needle was removed the next day. In one set of additional birds ($n = 3$ male birds, 6–8 weeks old), brains were blocked in the horizontal plane at the level of the ventral portion of the POM. A crystal of DiI was implanted in the POM in a dorsal direction with a dissection needle that was pushed from the ventral cut into the nucleus, as described above. Transport of the dye was then studied in the rostral part of the brain.

In the final series of birds ($n = 4$ male birds, 6–8 weeks old), DiI was implanted directly into the septal region (needle crossing the lateral and medial part of the septum) after the entire telencephalon had been removed to expose the underlying septum. DiI was dissolved in alcohol and implanted in the brain with an insect pin as described above.

Immediately after dye implantation, the brains were included in a mixture of gelatin and egg yolk (2:1) and placed at 4°C until the gelatin had solidified. The blocks containing the brains were then placed in the fixative solution diluted 40 times in 0.1 M phosphate buffer, pH 7.2–7.4, and stored for one month at 33°C, in the dark to prevent fading of the fluorescence. The high temperature was selected to promote faster diffusion of the dye (Godement et al. 1987; Holmqvist et al. 1992). Specimens were then placed at 4°C to reduce the migration and cut within two weeks so that the period of rapid diffusion was similar for all specimens. In preliminary experiments, longer and shorter migration times were also tested, the one-month duration finally being selected as it gave optimal labeling of distant structures (up to 8 mm) without excessive diffusion of the tracer at the implantation site.

Brains and their embedding medium were sectioned in the coronal plane with a Vibratome at a thickness of 50–100 µm. Sections were collected in 0.1 M phosphate buffer, pH 7.3–7.4, and alternate sections were used for observation of the dye under the fluorescence microscope or for Nissl staining with toluidine blue (first series of experiments only). Specimens to be viewed by fluorescence microscopy were mounted in a gelatin solution (12.5%) in phosphate buffer, coverslipped and stored at 4°C in the dark. In the first series of experiments, alternate sections were stained with toluidine blue, dehydrated through a graded series of alcohol, acetone and xylene, and mounted in Eukitt. In the second and third series of experiments, the exact location of fluorescent structures was controlled under darkfield examination. When additional landmarks were needed, the coverslip was removed and the section that had previously been observed under fluorescence optics was counterstained with toluidine blue.

Data analysis

The sections were observed under a fluorescence microscope (Olympus BH-2 or Zeiss Axioplan) equipped with rhodamine filters (green excitation at 545 nm, orange-red emission) allowing visualization of the orange-red fluorescence of DiI. Representative sections were photographed using an automatic light meter leading to exposure times that varied between a few seconds and 8 min. Alternate Nissl-stained sections were observed with the same microscope under brightfield and were used to confirm the anatomical localization of the fluorescent structures. The nomenclature used in this paper is based on previous work on the quail and chicken brain (Baylé et al. 1974; Kuenzel and van Tienhoven 1982; Kuenzel and Masson 1988; Panzica et al. 1991).

All fluorescent structures were drawn on paper with the help of a camera lucida and then their identification was confirmed by reference to the Nissl-stained sections. All observations were summarized in tabular form and on semi-schematic drawings.

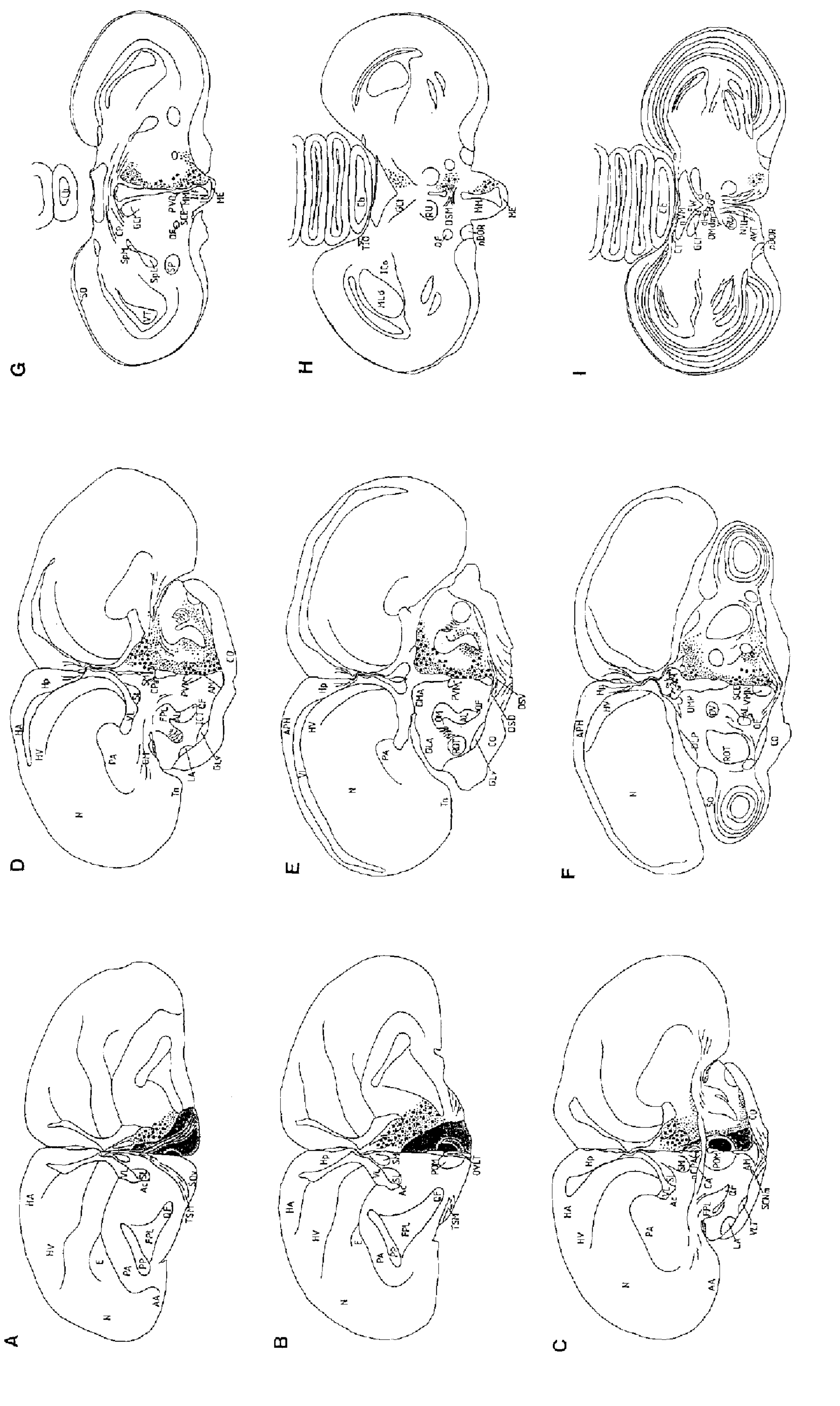
Results

Structures labeled following implantation of DiI into the POM exposed by a coronal cut

In 7 males, a DiI crystal was implanted in the POM that had been exposed by a coronal cut into the nucleus, and the diffusion of the tracer in the structures caudal to POM was analyzed. We only retained 6 birds in which the crystal had been correctly placed in the target structure (placement was too lateral in the last subject). In all 6 subjects, the implantation site covered a large portion of the dimorphic nucleus but a non-specific diffusion zone was also visible in the lateral POA and in areas of the rostral hypothalamus (see Figs. 1A–C, 2 A).

Some individual differences were noted in the placement of the DiI crystal: in some birds, it was located in the rostral portion of the POM, whereas in others, it was placed in the caudal part of the nucleus at the level of the anterior commissure. The fluorescent structures (fibers and cells) were, however, similar in both cases and the minor differences detected from one brain to another could not be related to the position of the implant; thus no further mention of these differences will be made. Details of the structures that were labeled by these implants can be found in Figs. 2 and 3; the reliability of this labeling is indicated in Table 1.

Starting from the implantation site, a large number of labeled fibers were noted moving dorsally and extending into the nucleus septalis lateralis (SL) and medialis (SM; Fig. 1 A–D). Strongly fluorescent cells were also observed in the SL and SM, more cells being labeled in the latter (Fig. 2 B–G). Labeled fibers filled the area around the commissura pallii (CPa) and were also detected just above the commissura anterior (CA) but few or no fibres were detected within these fiber tracts (see Fig. 2 A). In the caudal direction, labeled cells and fibers were found in the ventral part of the diencephalon and mesencephalon, namely in the nucleus paraventricularis magnocellularis (PVN) and in the nucleus anterior medialis hypothalami (AM; Fig. 3A, D). At this level, fibers also extended ventrally and laterally into the regio lateralis hypothalami (LHy; Fig. 3 C) and toward the chiasma opticum (CO), thereby labeling the nucleus ventrolateralis thalami (VLT) and the nucleus intercalatus thalami (ICT). In these nuclei, labeled cells were also observed in 2 out of the 6 birds. At more posterior levels, labeled fibers and cells occurred in the nucleus ventromedialis hypothalami (VMN; Figs.1 F–G, 3 B). The density of the label then decreased toward more caudal areas. Some labeled cells and fibers were, however, found in the caudal part of the tuber and in the AVT (Fig. 1 I). A few cells


A
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and fibers were also detected in the rostral part of the tuberal hypothalamus. The cells extended dorsally into the stratum cellulare externum (SCE; Fig. 1 F–G) and more caudally at the level of the decussatio supramamillaris (DSM; Fig. 1 H). No information could be collected on the median eminence because the structure was always destroyed during Vibratome sectioning.

Further dorsal areas were, however, also labeled. Fibers and cells were present in the nucleus dorsomedialis (DMA, Fig. 3 E–F) and dorsolateralis anterior thalami (DLA). Fluorescent fibers extended more medially between the DLA and the third ventricle (Fig. 1 E). Within DLA, a progressive diminution of the density of the label occurred from more medial to more lateral regions. The labeling extended more caudally in the DMA than in the

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Fig. 1. Semi-schematic drawings of frontal sections of quail forebrain and mesencephalon from rostral to caudal levels illustrating the results from experiments in which the crystals of DiI were implanted in the POM (*front* to *back*). In this and subsequent drawings the implantation site is represented by the *black spot*, the non-specific diffusion area is represented by the *gray shadowing*. *Small dots* indicate the presence of anterogradely labeled fibers, whereas *large dots* indicate retrogradely labeled perikarya. *AA* Archistriatum anterior; *Ac* nucleus accumbens; *AL* ansa lenticularis; *AM* nucleus anterior medialis hypothalami; *AP* area pretectalis; *APH* area parahippocampalis; *AVT* area ventralis (Tsai); *Cb* cerebellum; *CA* commissura anterior; *CO* chiasma opticum; *CP* commissura posterior; *CPa* commissura pallii; *CT* commissura tectalis; *DLA* nucleus dorsolateralis anterior thalami; *DLP* nucleus dorsomedialis posterior thalami; *DMA* nucleus dorsomedialis anterior thalami; *DMN* nucleus dorsomedialis hypothalami; *DMP* nucleus dorsomedialis posterior thalami; *DSD* decussatio supraoptica; *DSM* decussatio supramamillaris; *DSV* decussatio supraoptica ventralis; *E* ectostriatum; *EW* nucleus of Edinger-Westphal; *FPL* fasciculus prosencephali lateralis; *GCt* substantia grisea centralis; *GLv* nucleus geniculatus lateralis, pars ventralis; *HA* hyperstriatum accessorium; *HIP* tractus habenulointerpeduncularis; *Hp* hippocampus; *HV* hyperstriatum ventrale; *ICo* nucleus intercollicularis; *ICT* nucleus intercalatus thalami; *IH* nucleus inferior hypothalami; *IN* nucleus infundibuli hypothalami; *ITC* nucleus intercalatus thalami; *LoC* locus coeruleus; *LA* nucleus lateralis anterior; *LHy* regio lateralis hypothalami; *LLd* nucleus lemnisci lateralis, pars dorsalis; *LPO* lobus paraolfactorius; *ME* eminentia mediana; *MLd* nucleus mesencephalicus lateralis, pars dorsalis; *MM* nucleus mamillaris medialis; *N* neostriatum; *NI* nucleus intermedialis; *NIII* nervus oculomotorius; *nBOR* nucleus opticus basalis; *nCPa* nucleus commissurae pallii; *nVM* nucleus mesencephalicus nervi trigemini; *OM* tractus occipitomesencephalicus; *OMdl* nucleus nervi oculomotorii, pars dorsolateralis; *OMdm* nucleus nervi oculomotorii, pars dorsomedialis; *OMv* nucleus nervi oculomotorii, pars ventralis; *OV* nucleus ovoidalis; *OVLT* organum vasculosum laminae terminalis; *Pap* nucleus papilliformis; *PA* paleostriatum augmentatum; *PM* nucleus pontis medialis; *PMI* nucleus paramedianus internus thalami; *POA* preoptic area; *POM* nucleus preopticus medialis; *PP* paleostriatum primitivum; *PT* nucleus pretectalis; *PVN* nucleus paraventricularis magnocellularis; *PVO* organum paraventriculare; *PVT* paleostriatum ventrale; *QF* tractus quintofrontalis; *ROT* nucleus rotundus; *RPgc* nucleus reticularis pontis caudalis, pars gigantocellularis; *RPO* nucleus reticularis pontis-oralis; *Ru* nucleus ruber; *SpL* nucleus spiriformis lateralis; *SpM* nucleus spiriformis medialis; *SCd* nucleus subcoeruleus dorsalis; *SCv* nucleus subcoeruleus ventralis; *SCE* stratum cellulare externum; *SCNm* nucleus suprachiasmaticus medialis; *SDN* sexually dimorphic nucleus; *SL* nucleus septalis lateralis; *SM* nucleus septalis medialis; *SMe* stria medullaris; *SOv* nucleus supraopticus ventralis; *SO* stratum opticum; *SP* nucleus sub-pretectalis; *T* testosterone; *Tn* nucleus taeniae; *TTO* tractus isthmo-opticus; *TPc* nucleus tegmenti pedunculo-pontinus, pars compacta; *TSM* tractus septomesencephalicus; *VeM* nucleus vestibularis medialis; *VeS* nucleus vestibularis superior; *VL* ventriculus lateralis; *VLT* nucleus ventrolateralis thalami; *VT* ventriculus tecti mesencephali; *VMN* nucleus ventromedialis hypothalami

Table 1. Summary of the fluorescent structures observed in Japanese quail brains that had been implanted with DiI in the POM. *c* Cells; *f* fibers; * does not apply (brain area removed by dissection); *nd* no data, presumably no label; *n* number of animals studied. Abbreviations as given in Fig. 1

	Front to back		Back to front		Bottom-up	
	c	f	c	f	c	f
	$n=6$		$n=3$		$n=3$	
Telencephalon						
SL	6	6	3	3	3	3
SM	6	6	3	3	3	3
PVT	*	*	3	3	1	1
Ventral LPO	*	*	3	3	1	1
nCPa	0	0	*	*	3	3
Tn	0	1	0	0	1	1
Preoptic area						
Anterior PO	*	*	2	3	3	3
POM ant.	*	*	2	3	1	1
Hypothalamus						
AM	6	6	2	2	*	*
SCNm	6	6	0	1	*	*
PHN	6	6	*	*	*	*
LHy	6	6	*	*	*	*
PVN	6	6	*	*	2	3
SCE	5	6	*	*	*	*
VMN	6	6	*	*	*	*
IH	4	5 ($n=5$)	*	*	*	*
IN	2	3 ($n=4$)	*	*	*	*
DMN	5	5 ($n=5$)	*	*	*	*
MM	3	5 ($n=5$)	*	*	*	*
PMM	3	5 ($n=5$)	*	*	*	*
Tu	4	4 ($n=4$)	*	*	*	*
Thalamus						
LA	0	2	*	*	*	*
VLT	2	5	*	*	*	*
ICT	2	6	*	*	*	*
DLA	4	6	*	*	1	0
DMA	6	6	*	*	2	2
HM	1	1	*	*	1	1
ALA	0	6	*	*	*	*
DMP	3	5	*	*	0	0 ($n=1$)
DIP	1	4	*	*	1	1 ($n=1$)
PMI	6	6	*	*	1	2 ($n=2$)
Mesencephalon						
GCt	6	6	*	*	0	0
nI	1	5 ($n=5$)	*	*	*	*
RU			*	*	0	0
ICH	4	6	*	*	*	*
ICo	0	2	*	*	0	0
AVT	4	5	*	*	*	*
IP	1	1	*	*	*	*
TPc	nd	nd	*	*	0	0
Metencephalon						
LoC	nd	nd	*	*	*	*

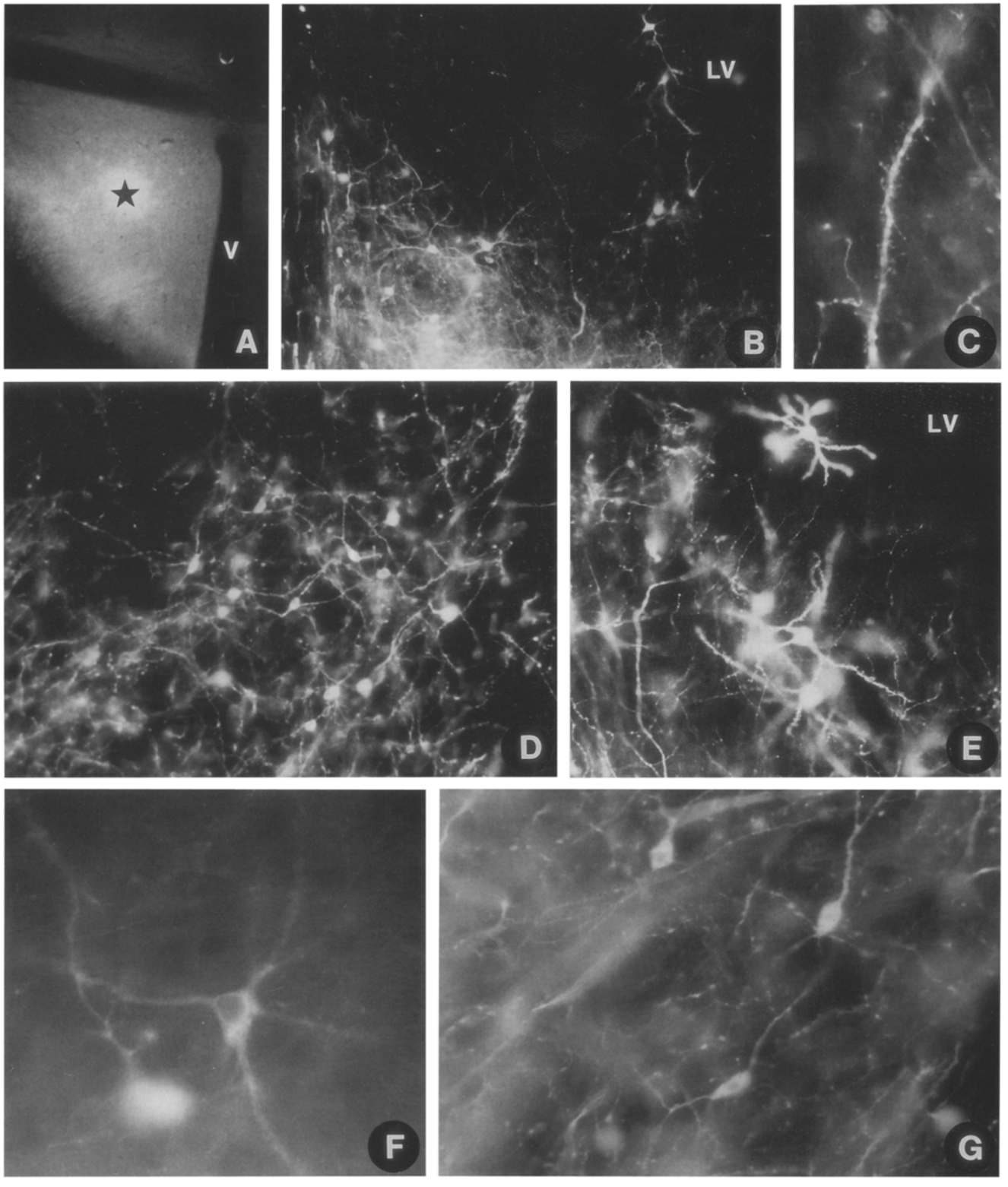

Fig. 2A–G. Photomicrographs of frontal sections of the forebrain illustrating the location of DiI retrogradely labeled neurons, and anterogradely labeled fibers, when the dye was implanted in the POM (*front* to *back*). **A** DiI implantation site (⋆) in the POM. ×25. **B** Low-power enlargement of the septal complex. A large number of neurons are clustered in the SM (*bottom left*), whereas scattered elements are in the SL. ×90. **C** Detail of a dendrite with numerous spines in the SL. ×350. **D** Labeled neurons and fibers in the SM. ×90. **E** Large multipolar neurons in the SL. ×180. **F** Multipolar neuron in the SM. Note the unlabeled nucleus of the cell. ×350. **G** Bipolar and multipolar neurons in the SM. ×280. *V* Third ventricle; *LV* lateral ventricle

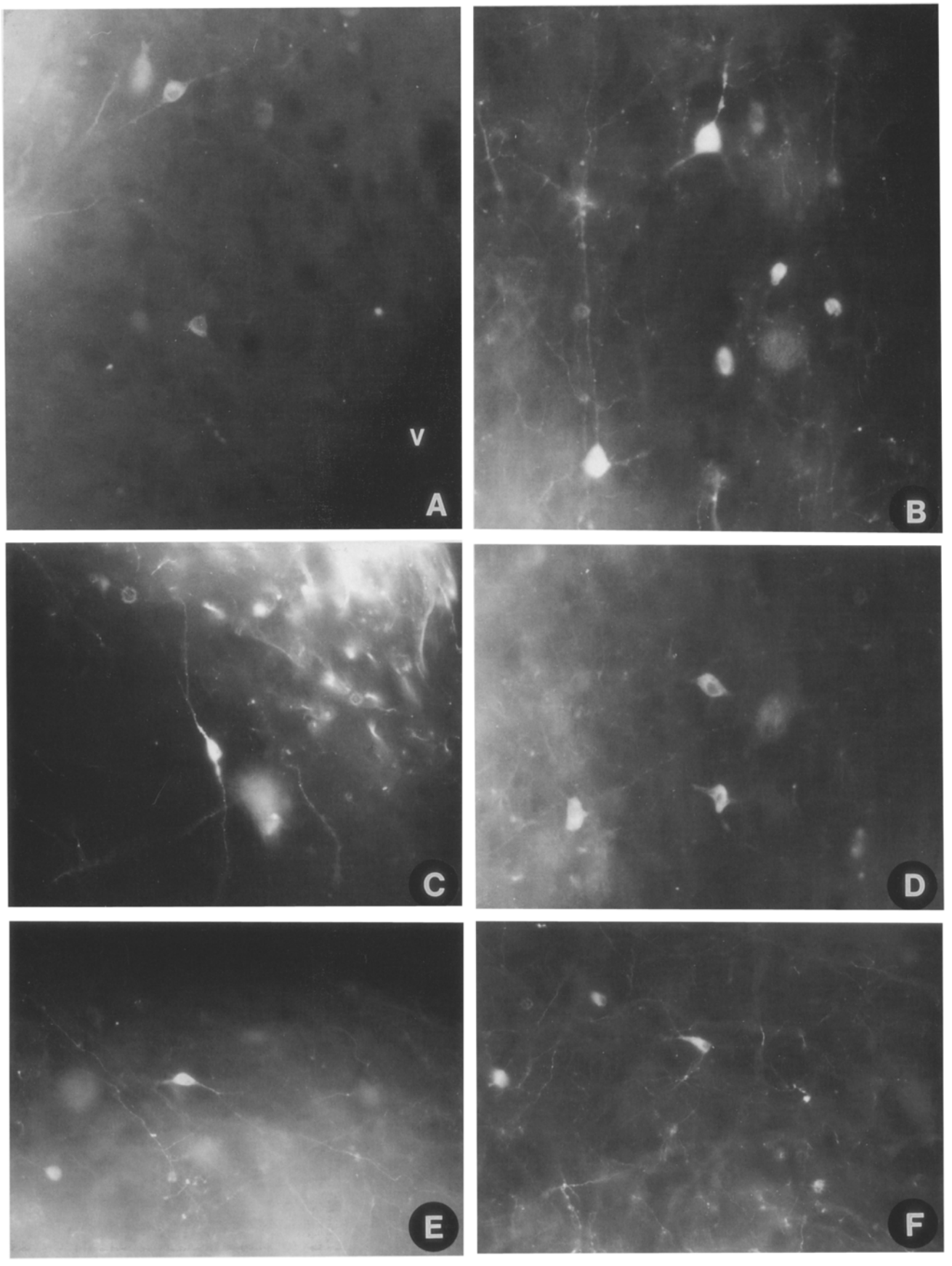

Fig. 3A–F. Photomicrographs of frontal sections of the diencephalon illustrating the location of DiI retrogradely labeled neurons, and anterogradely labeled fibers, when the dye was implanted in the POM (*front* to *back*). **A** Scattered neurons in the AM. **B** Large neurons in the VMN. **C** Bipolar labeled neuron in the LHy. **D** Labeled neurons in the PVN. **E, F** Small bipolar neurons in the DMA. *V* Third ventricle. **A–F** ×300

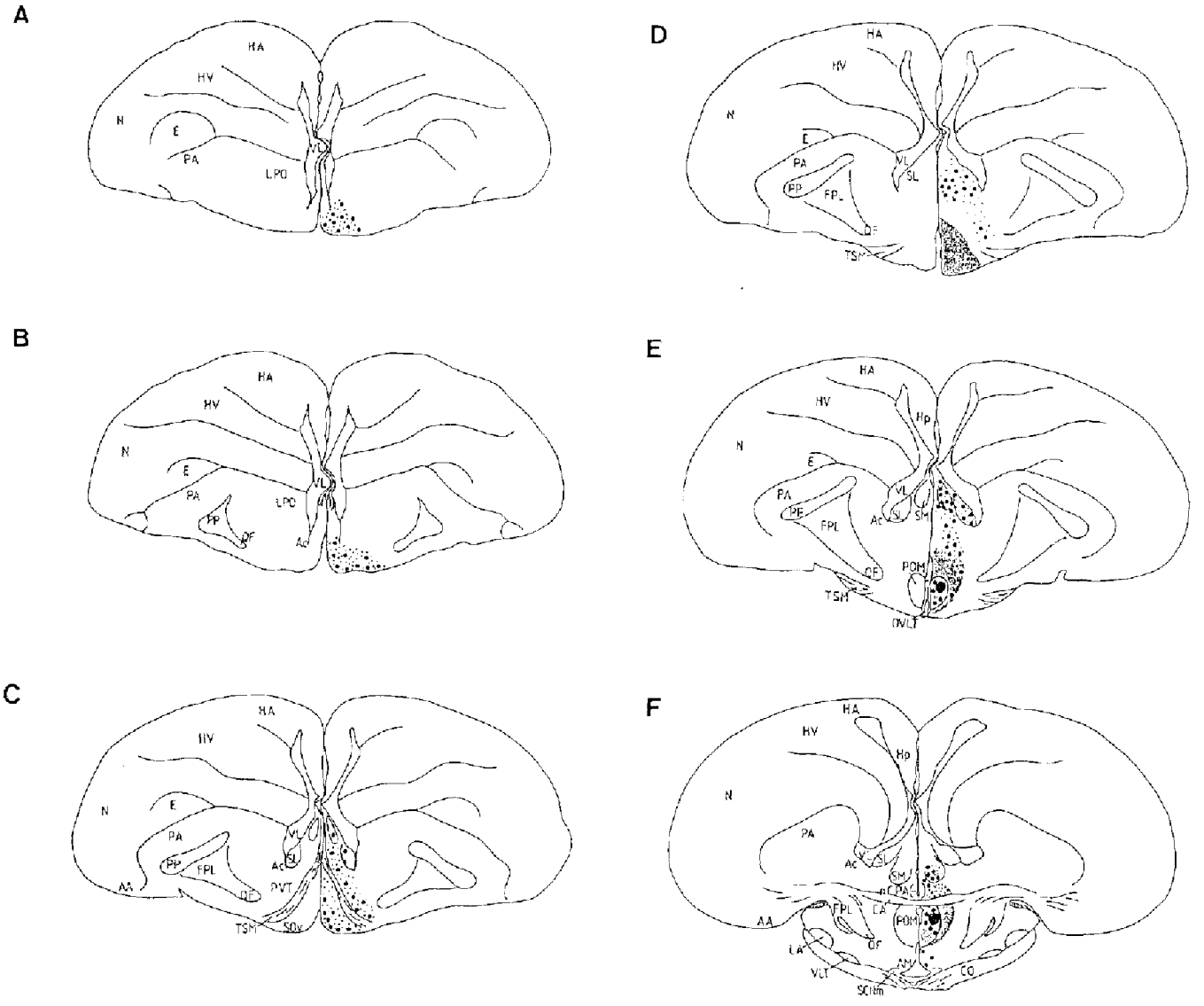

Fig. 4A–F. Semi-schematic drawings of frontal sections of quail forebrain from rostral to caudal levels illustrating the results from experiments in which the crystals of DiI were implanted in the POM (*back* to *front*)

DLA. The density of the fibers originating from the POM then sharply decreased as one reached the nucleus dorsomedialis posterior thalami (DMP; Fig. 1 F). Only a few fibers were observed here and they extended caudally into the GCt where a few labeled cells were also seen (Fig. 1 I).

During this experiment, we also tried to demonstrate possible afferent and efferent connections of the POM in the rostral direction. The brains had been blocked by a coronal cut in the rostral part of the POM. The portion of the brain rostral to this cut was used for these experiments and received a DiI crystal in the anterior part of POM, when it was visible. Little labeling was present in these brains after one month of migration. Only a few fluorescent fibers were detected in the lobus parolfactorius.

Anterior structures labeled following implantation of DiI into the POM from back to front

When DiI was inserted in the POM from the caudal end, dense label was observed in the septal area and in part of the paleostriatum (Fig. 4 C–F). Both the SM and SL contained a high density of fluorescent cells and fibers (Fig. 5 B), but the number of positive cells was much higher in the SM than in SL. This label extended in a ventral and rostral direction into an area located just lateral to the tractus septomesencephalicus (TSM, never labeled) and corresponding to the rostral part of the paleostriatum ventrale, (PVT, Fig. 5 C–D) and, more rostrally, to the ventro-caudal part of the lobus parolfactorius (LPO, Fig. 5 A). Medially, labeled cells could be identified within the rostral POM (these were within the nonspecific diffusion area), and more generally in the entire rostral POA (Fig. 5 A). In two birds, the caudal part of the block included the AM, which contained a few labeled cells and fibers.

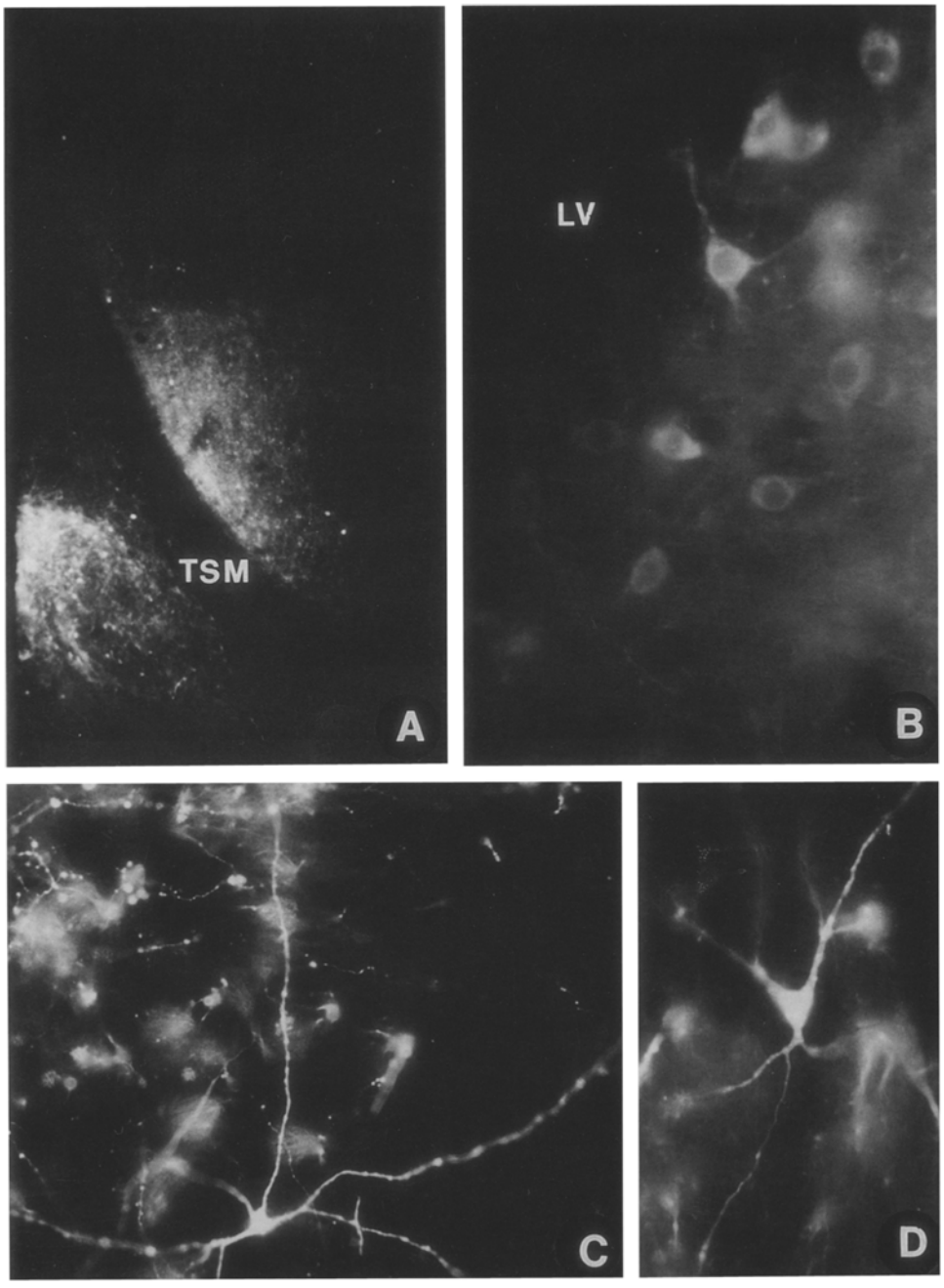

Fig. 5A–D. Photomicrographs of frontal sections of the telencephalon illustrating the location of DiI retrogradely labeled neurons, and anterogradely labeled fibers, when the dye was implanted in the POM (*back* to *front*). **A** Labeling of the anterior preoptic region (*bottom left*), and of the region close to the unstained TSM. This region probably partially corresponds to the medial forebrain bundle. ×25. **B** Labeled perikarya in the anterior septum. ×430. **C, D** Large multipolar neurons labeled in the paleostriatum ventrale. *LV* Lateral ventricle; *TSM* tractus septomesencephalicus. **C** ×180; **D** ×350

Structures labeled following implantation of DiI into the POM exposed by an horizontal cut

The studies described above showed that many brain areas projected to the POM, although this nucleus also sent efferent connections to a large number of structures. The data also suggested that many of these projections traveled through the ventral part of the hypothalamus and mesencephalon. In order to test this idea directly, another set of brains was blocked in the horizontal plane at the level of the ventral POM, and DiI was implanted into the dimorphic nucleus.

In these brains, we observed labeled cells and fibers in telencephalic regions that were also labeled in the other experiments (e.g., SM, SL, PVT, LPO); in addition, fluorescent fibers and cells were present in the nucleus commissurae pallii (nCPa). By contrast, we never detected labeled structures in mesencephalic areas that were listed previously as positive areas. A large part of the ventral hypothalamic target structures could not be studied since they had been removed by the horizontal blocking. A limited number of labeled structures was observed in the dorsal thalamic areas.

These three sets of experiments established the existence of several projections of the POM. In many cases, the areas receiving efferent projections from the POM also contained neurons that projected to the POM. In order to obtain an independent confirmation of these bidirectional links, we tried subsequently to demonstrate the transport of DiI from five POM targets that were

Table 2. Summary of the fluorescent structures observed in Japanese quail brains implanted with DiI in selected nuclei that were shown to receive efferent projections from POM

c Cells; *f* fibers; * does not apply (brain area removed by dissection); *nd* no data, presumably no label; *n* number of animals studied. Abbreviations as given in *Fig. 1*

Nucleus	Tuber		AVT		GCt		ICo		Septum	
	c $n=4$	f	c $n=5$	f	c $n=5$	f	c $n=3$	f	c $n=4$	f
Telencephalon										
SL	0	0	0	0	0	0	0	0	*	*
SM	0	0	0	0	0	1	0	0	*	*
PVT	0	0	0	0	0	0	0	0	1	1
ventral LPO	0	0	0	0	0	0	0	0	0	0
nCPa	0	0	0	0	0	0	0	0	2	3[a]
Tn	0	0	0	0	0	0	0	0	*	*
Preoptic area										
anterior POA	0	0	0	0	0	0	0	0	1	2 ($n=2$)
POM	4	4	3	4	3	4	0	0	4	4
Hypothalamus										
AM	4	4	1	2	4	5	0	0	2	3
SCNm	4	4	0	0	0	0	0	0	0	2
PHN	0	0	0	0	3	5	0	0	1	4
LHy	4	4	4	5	4	5	0	0	1	4
PVN	4	4	5	5	4	5	1	2	4	4
SCE	3	4	0	5	5	5	0	2	3	3
VMN	4	4	4	5	4	5	0	2	1	1
MM	2	2	3	4	2	5	0	0	2	2
PMM	nd	nd	5	5	3	5	0	1 ($n=2$)	0	1 ($n=3$)
Tu	*	*	4	4	3	5	0	0	3	2
Thalamus										
LA	0	0	0	0	0	0	0	0	0	0
VLT	1	1	0	0	0	0	0	0	0	0
ICT	1	3	3	5	1	2	0	0	0	0
DLA	0	0	0	0	0	5	0	3	1	1
DMA	1	3	0	0	3	5	0	2	3	4
HM	1	1	0	0	0	0	0	0	1	3 ($n=3$)
ALA	0	4	0	5	2	5	0	0	0	0
OM	0	4	0	4	3	5	1	3	0	0
DMP	0	0	0	0	1	5	2	2	2	4
DIP	0	0	0	0	0	5	0	0	0	0
PMI	4	4	4	5	5	5	1	3	2	2 ($n=3$)
Mesencephalon										
GCt	2	4	5	5	*	*	3	3	0	2 ($n=3$)
nI	0	0	0	0	0	0	0	0	0	1 ($n=3$)
RU	0	2	3	5	4	5	0	2	2	2[b]
ICH	1	1	0	0	2	4	0	0	0	0
ICo	0	1	4	5	4	5	*	*	0	0
AVT	0	2	*	*	0	3	0	0	2	2 ($n=3$)
IP	0	2	2	2	nd	nd	0	0	0	0
TPc	0	1	3	4	2	3 ($n=3$)	0	0	0	0 ($n=3$)
Metencephalon										
Loc	0	1	3	4	3	3 ($n=3$)	0	0	*	*

[a] Controlateral
[b] Medial to Ru ($n=3$)

selected based on the density of the terminals identified during the first two experiments, and/or on the important role that these areas were presumably playing in the control of reproduction. The results of these implants are summarized in Table 2.

Structures labeled following implantation of DiI into the septal region

Four males were implanted with insect pins coated with DiI in the septal area. Before implantation, the entire telencephalon was removed from the brain to expose the

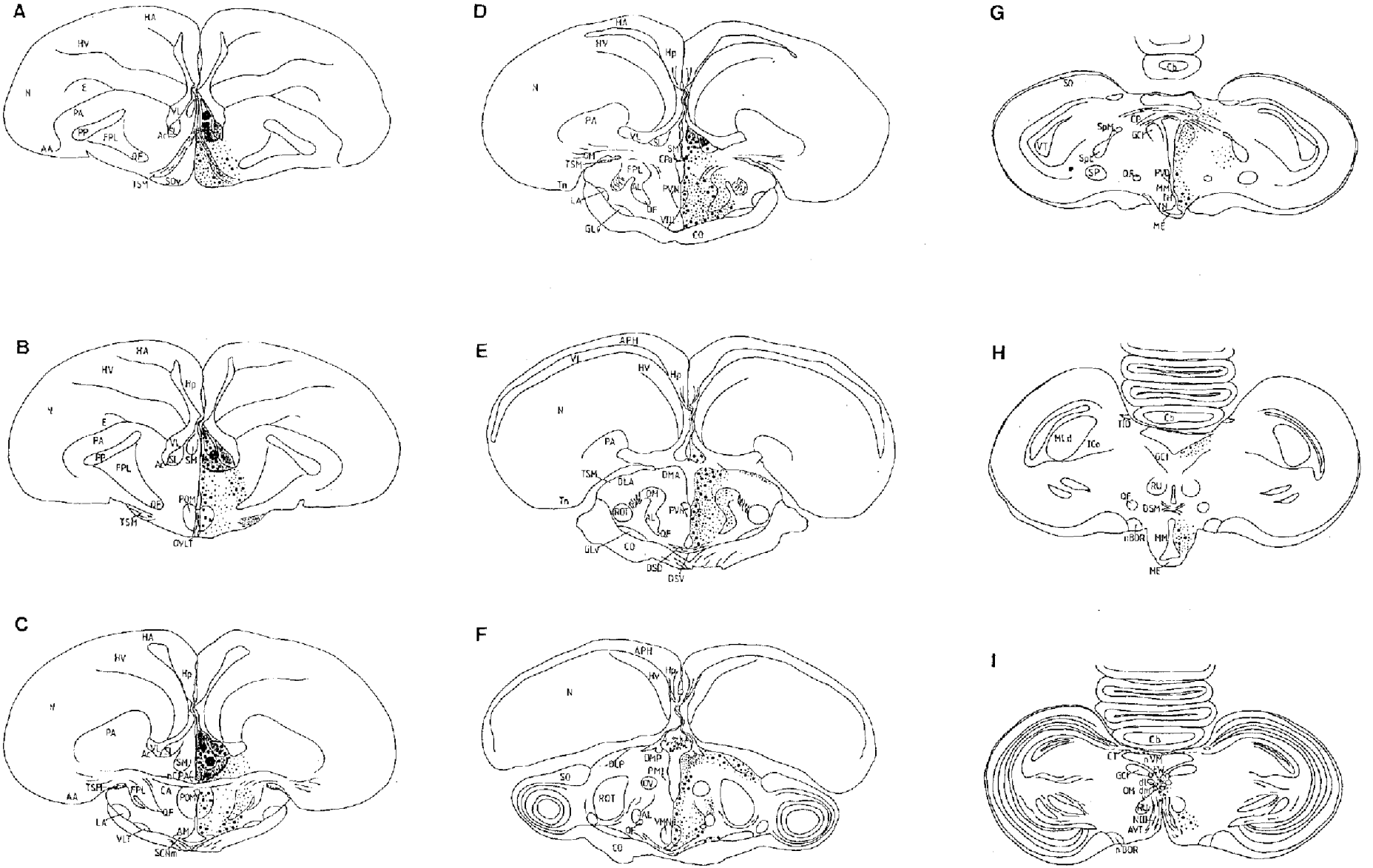

Fig. 6A–I. Semi-schematic drawings of frontal sections of quail forebrain and mesencephalon from rostral to caudal levels illustrating the results from experiments in which the crystals of DiI were implanted in the septum. For these experiments, the telencephalon was removed, preventing a demonstration of the telencephalic connections of the septal region

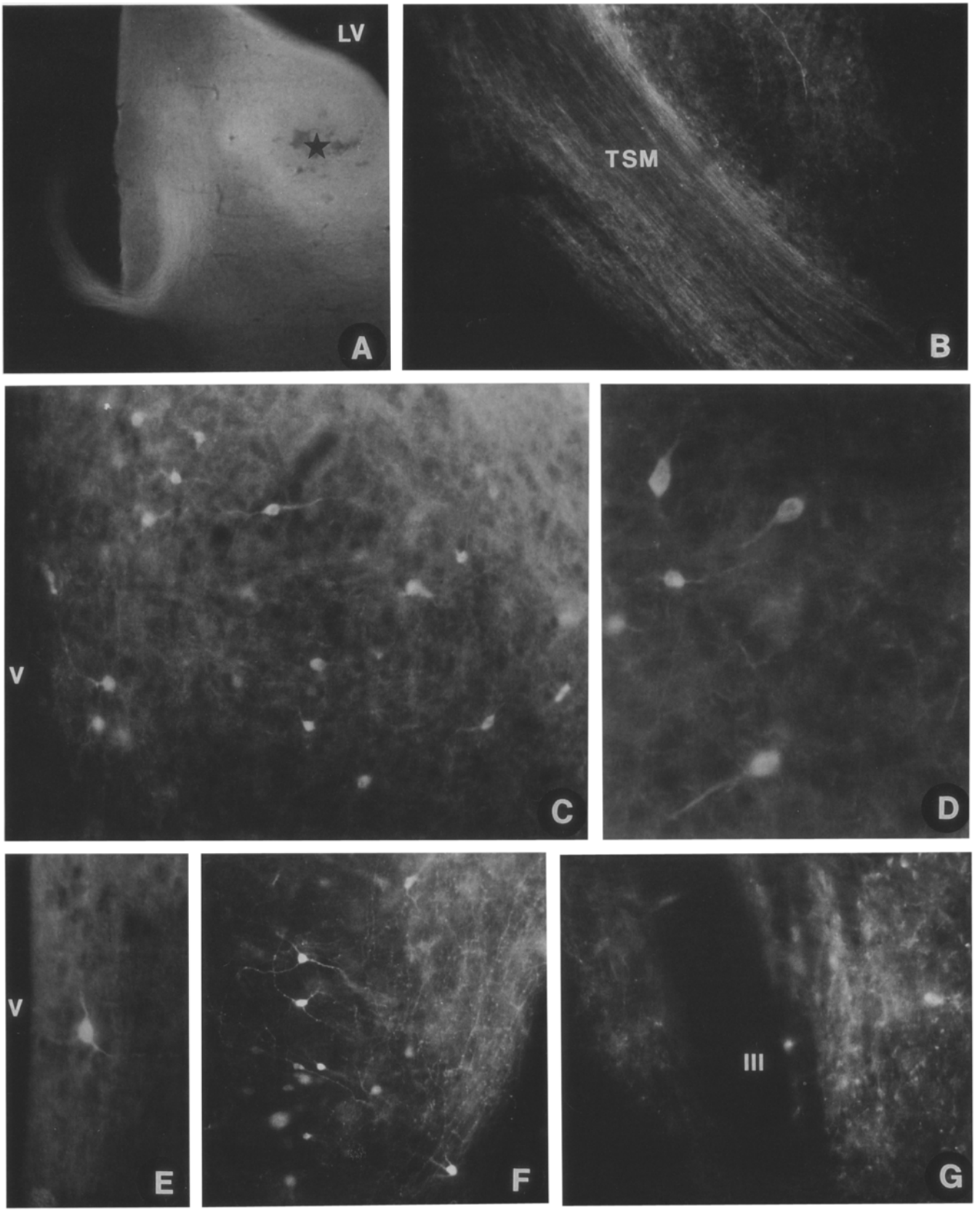

Fig. 7A–G. Photomicrographs of frontal sections of the forebrain illustrating the location of DiI retrogradely labeled neurons, and anterogradely labeled fibers, when the dye was implanted in the septum. **A** Implantation site (*) in the septum. Note the labeling of the pallial commissure. ×25. **B** Labeled fibers traveling along the TSM. ×90. **C** Retrogradely labeled neurons in the POM. ×110. **D** Higher magnification demonstrating bipolar neurons in the lateral part of the POM. ×280. **E** Periventricular neuron in the PVN. ×170. **F** Labeled cells and fibers in the tuber. ×90. **G** Labeled fibers in the AVT, and close to the midline. Note the totally unstained third nerve (*III*) fibers. ×90. *LV* Lateral ventricle; *TSM* tractus septomesencephalicus; *V* third ventricle

septum. This allowed accurate positioning of the implant, but prevented subsequent study of the possible connections of the septal area with more dorsal structures (Fig. 6 A–I). In each subject, the needle was deeply inserted into the septum so that microcrystals were placed both in the lateral and in the medial parts of the area. Extensive non-specific diffusion was observed that covered the entire septum down to the level of the CA. This fiber tract was never fluorescent, although a dense label of the CPa and of the TSM was usually seen (Fig. 7 A–B). The labeling of this last tract was traced to its end in a region close to the DLA, identified as the nucleus of the TSM (Kuenzel and Masson 1988), where scattered positive cells were found (Fig. 6 F). Labeled contralateral cells were seen within the septum, probably reached by fibers coming from the CPa.

Extensive labeling of fibers and scattered fluorescent cells were visible within the anterior preoptic region. More caudally, the POM was always labeled both with fibers and cells, confirming the existence of bidirectional connections between the septal region and this nucleus. The fluorescent elements were detected both in the medial and in the lateral part of the nucleus, and always took the form of small bi- or multipolar neurons (Fig. 7 C–D).

Major diencephalic targets of the septal regions were the PVN (Fig. 7 E) and the DMA, in which both labeled fibers and cells were regularly noted. More laterally, fibers also covered the DLA, reaching caudally the level of the DMP, in which scattered cells were also located. No labeled structures were seen within the ventro-lateral thalamus, although several fibers, and in a few cases cells, were stained in the lateral hypothalamus. The periventricular hypothalamus was always labeled with bundles of fibers lying parallel to the ventricular wall, apparently directed toward the floor of the ventricle. These fibers did not belong to any recognizable fiber tracts that were more laterally located. Scattered cells were also present in the periventricular region. An extensive connection was observed with the tuber, in which both fluorescent cells and fibers were regularly (3 birds out of 4) found (Fig. 7 F). The density of fibers was greatly reduced when reaching the mesencephalon. At this level, they were observed within the GCt, medial to the nucleus ruber (Ru), and in the AVT (Fig. 7 G). In some cases, retrogradely labeled cells were also present (AVT).

Structures labeled following implantation of DiI into the tuber

Four males received a DiI implant in the tuberal hypothalamus. The implantation site overlapped with the median eminence in one case only. In the other 3 birds, the area covered by the crystal was limited to the infundibular region (Fig. 8 A). The territory, in which a high level of undifferentiated fluorescence resulting from non-specific diffusion was present, extended in general to the caudal aspects of the VMN and included parts of the LHy.

Fluorescent cells and fibers were present throughout the rostral to caudal extent of the POM of the 4 birds, demonstrating the existence of anterograde and retrograde transport of the dye between the tuber and the dimorphic nucleus of the POA. The anterograde projections from the tuber extended to the level of the TSM. Labeled fibers were also found in the lateral parts of the POA and along the CA (ventral and dorsal aspects). In 3 out of 4 brains, positive fibers were observed rostrally to POM within the POA, under the TSM.

Dorsal to the implantation site, fluorescent fibers extended to the GCt and labeled the Ru. These fibers continued in a rostral direction to the level of the commissura posterior (CP) without leaving the GCt. Labeled fibers and a small number of labeled cells were found in the SCE and in the nucleus paramedianus internus thalami (PMI), and then more rostrally at the level of the PVN. From the PVN, the fibers extended dorsally and then laterally to enter the DMA. No label was, however, present in the lateral portions of this nucleus.

At the ventral level, fluorescent fibers and cells extended from the implantation site to the VMN (Fig. 8 B) and more laterally into the LHy. Even more rostrally, clear label (cells and fibers) was detected in the AM. Almost no fibers were seen lateral to the implantation point.

Structures labeled following implantation of DiI into the AVT

A total of 5 males were implanted with DiI at various levels in the AVT. As noted previously for the POM, no differences in the labeling could be related to the minor variations in the implantation point; that this low variability (see Table 2) was therefore ignored.

Analysis of the fluorescent structures in these sections revealed anterograde projections from the AVT to the POM in 4 brains out of 5. In 3 of these brains, labeled cells were also observed in POM, thereby confirming the existence of efferent projections from the POM to the AVT. Labeled cells were confined to the caudal portion of the POM (level of the CA) and these were never detected in the more rostral parts of the nucleus. Fluorescent fibers were located along the CA, mostly on its dorsal side.

Dorsal to the implantation spot, labeled cells and fibers were found in the nucleus of Edinger-Westphal (Fig. 8 D). This label was presumably transported through the nervus oculomotorius, which was just at the edge of the implantation site (Fig. 8C), and which was presumably exposed to high levels of the dye. More rostro-laterally, the entire ICo contained labeled fibers and cells. Medially, fibers continued in a rostral direction but were progressively confined to the GCt. Some cells were also found in the caudal but not in the rostral part of GCt. In every bird, the tractus habenulo-interpeduncularis (IIP) was densely labeled by fibers that extended until the level of the nucleus spiriformis lateralis (SpL). Rostral to the HIP, fluorescent cells were noticed close to the stria medullaris (SMe).

In the ventral part of the brain, cells and fibers were observed rostral to the implantation site in the caudal tuber and, from there, labeled cells extended to the level of the VMN. In the five brains, a group of fluorescent

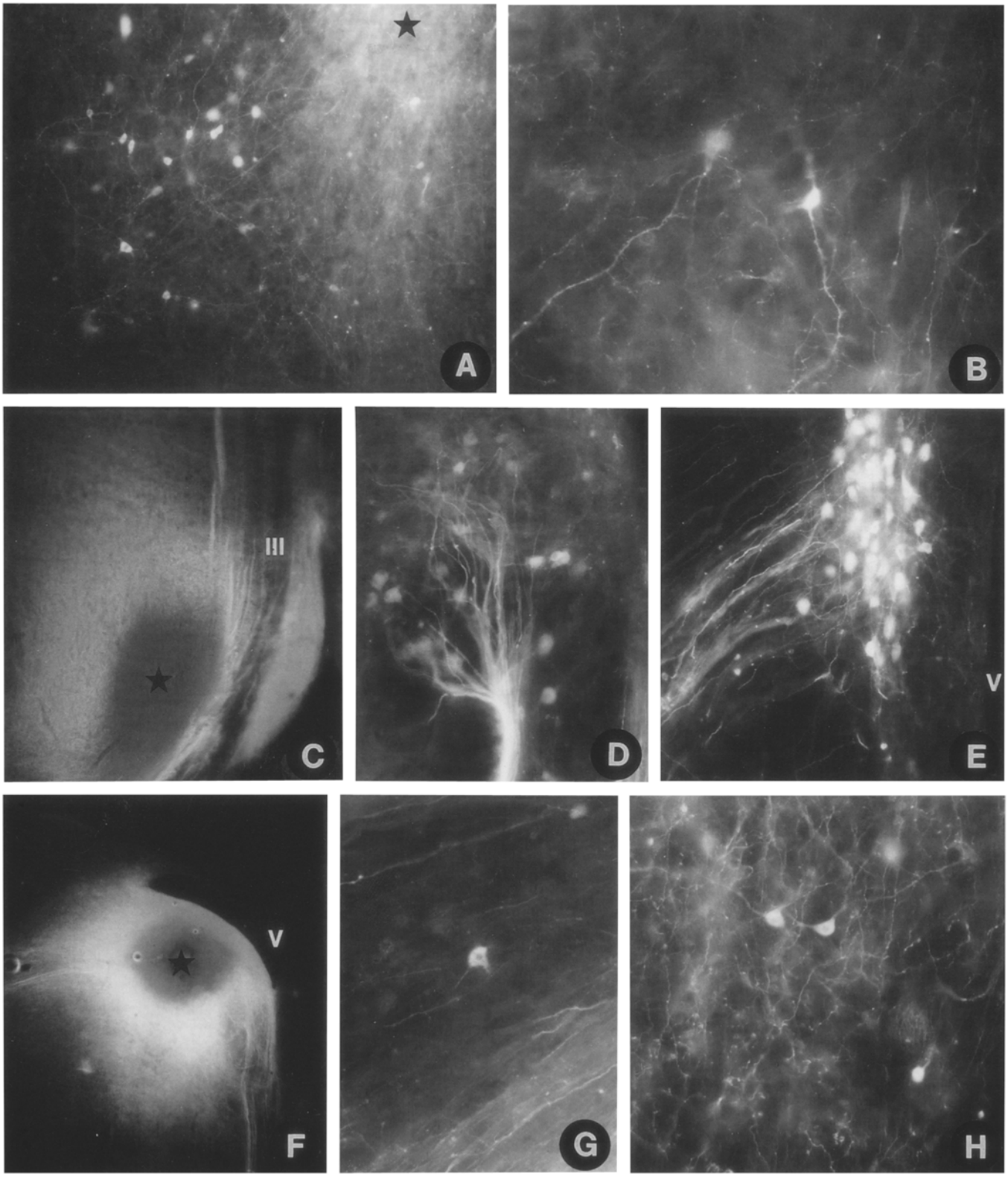

Fig. 8A–H. Photomicrographs of frontal sections illustrating different implantation sites and some of the labeled structures. **A, B** Implant in the tuber; **C–E** implant in the AVT; **F–H** implant in the GCt. **A** Implant of the dye (*). Note the labeled cells surrounding the nonspecific diffusion area. ×100. **B** Multipolar neurons in the VMN. ×300. **C** Implant of the dye (*), close to the third nerve (*III*), which is partially stained. ×60. **D** Retrogradely labeled neurons in the Edinger-Westphal nucleus. ×150. **E** A large cluster of labeled neurons in the PVN. ×150. **F** Implantation site (*). ×60. **G** A labeled neuron in the ICo. ×300. **H** Labeled neurons and fiber network in the AM. ×300. *V* Third ventricle

cells extended from the rostral tuber dorsally to the level of the organum paraventriculare (PVO). More rostrally, cells were also seen in the PMI and in the PVN. This latter nucleus was clearly outlined by labeled cells and the nuclear limits defined in this way corresponded exactly to the cytoarchitectonic boundaries of the nucleus as defined in the Nissl-stained material. (Fig. 8 E). These labeled cells apparently received the fluorescent material through fibers traveling in the medio-ventral part of the fasciculus prosencephali lateralis (FPL). (This fiber tract presumably corresponds to the medial forebrain bundle of mammals although homologies are not clear at this level.) From the PVN, a group of positive fibers extended ventrally toward the tractus quintofrontalis (QF).

Dispersed labeled fibers were seen lateral to the implantation site and at more rostral levels, in the LHy, along the decussatio supraoptica (DS). A dense plexus of positive fibers was present dorsal to the tractus quintofrontalis (QF).

Structures labeled following implantation of DiI into the GCt

Five males received a DiI implant into the GCt and the connections of this nucleus in the rostral direction were analyzed. In 3 subjects, the connections of the GCt in the caudal direction were also investigated. In all cases, the tracer crystal was confined to the GCt but an extensive diffusion zone extended beyond this structure (Fig. 8 F).

In 4 out of 5 subjects, anterograde transport was observed from the GCt to POM (fluorescent fibers in the latter nucleus). Labeled fibers were, in addition, seen in the lateral POA. In 3 of these birds, labeled cells were present in the POM confirming the existence of efferent projections from the POM to GCt. The AM contained both fluorescent fibers and cells (Fig. 8H)

Lateral to the implantation point, dense networks of positive fibers were seen in the ICo. They were found throughout this nucleus; labeled cells were also seen at the rostral end in 4 out of 5 subjects (Fig. 8 G). These labeled fibers extended in a cephalic direction into the DMA but they mostly labeled the lateral part of this area. In the medial part of this nucleus, some positive cells were found in a periventricular position.

In 3 subjects, the implantation site was located in the caudal aspects of GCt, and in these birds, fibers were visible running ventrally to the AVT. More rostrally, fibers were observed in the nucleus inferior hypothalami (IH), the nucleus mamillaris medialis (MM) and the nucleus dorsomedialis hypothalami (DMN), where a few labeled cells were also detected.

During the study of the connections of GCt in the caudal direction (3 brains), a high density of fibers was noted in the caudal aspects of ICo and in the nucleus tegmenti pedunculo-pontinus, pars compacta (TPc) where labeled cells were also detected. More caudally, fibers and cells were observed in a large number of nuclei including the locus coeruleus (LoC), the nucleus subcoeruleus ventralis (SCv), the nucleus reticularis pontis oralis (RPO), the nucleus papillioformis (Pap), the nucleus lemnisci lateralis, pars dorsalis (LLd), and the nucleus vestibularis medialis (VeM). By contrast, only fibers were seen in the nucleus reticularis pontis caudalis, pars gigantocellularis (RPgc), the nucleus pontis medialis (PM) and the nucleus vestibularis superior (VeS).

Structures labeled following implantation of DiI into the ICo

The projections and afferents of the ICo in the cephalic direction were studied in 3 brains. Results obtained in one of these subjects disagreed with those obtained for the other 2; this probably arose because the DiI crystal was placed in a more lateral position in this subject and it therefore overlapped with a large part of the nucleus mesencephalicus lateralis, pars dorsalis (MLd). Unless otherwise mentioned, the description presented below refers to the results in the 2 birds for which the tracer was correctly implanted into the medial portion of ICo. In these individuals, the area of nonspecific diffusion extended outside the cytoarchitectonic boundaries of the nucleus. The specificity of the connections should therefore be confirmed in additional experiments.

No labeled cells or fibers could be detected in the POM of the 3 brains that were analyzed. Dorsal to the CA, a few labeled fibers were found but no other traces of fluorescence could be seen in the POA. No cells occurred in the ventral hypothalamus and only a few rare fibers were noted in the VMN.

Starting from the implantation site, labeled fibers were seen laterally where they extended into the MLd and medially extending into the GCt. At more rostral levels, fluorescent cells and fibers were found in the DMP. Further rostrally, the DMA and the DLA only contained labeled fibers. A network of positive fibers was also observed throughout the PVN but their density progressively decreased in the cephalic direction. The tractus occipitomesencephalicus (OM) was densely labeled by positive fibers from the level of the implantation site to the level of the rostral hypothalamus. In general, few fluorescent cells were detected with the exception of the area pretectalis (AP), the previously mentioned DMP, the GCT, and the area between OM and DMP. Some rare cells were also present around the nucleus pretectalis (PT), the SpL, and the nucleus subpretectalis (SP).

Discussion

Methodological considerations

In order to interpret the results described above, it is important to consider the specificity of the tracing technique that was used and to understand the mechanism underlying the capture and transport of the fluorescent marker. In fixed material, DiI diffuses laterally within the membrane of cells exposed to high concentrations of the dye (Honig and Hume 1989) and therefore produces both anterograde and retrograde labeling of axons, terminals, and cells. Previous experiments have shown that DiI is

not transferred from one cell to another during this transport and that it stays exclusively in the cell in which it was incorporated (Honig and Hume 1989; see, however, Godement et al. 1987 for the description of a few exceptions).

Some nonspecific diffusion of the tracer could take place at two steps during the experiment, viz., during the transport of the tracer (storage of brains at 33°C) and during the sectioning of the blocks; this could confound the results. Thus, all sectioning in the present experiment has been performed on a Vibratome, rather than on a cryostat for which brains would have to be frozen, thereby causing disruption and permeabilization of the cell membranes. During Vibratome sectioning, little structural damage takes place. Moreover, since the section thickness (50–100 μm) is greater than the neuronal size (<15 μm) and *a fortiori* than axonal size, few structures are cut by the Vibratome blade. Observations have always been performed rapidly after cutting to reduce diffusion around the true site of the label. This has resulted in a high level of fluorescence present in cells or fibers and a completely black background.

Three types of fluorescent material have been detected in the sections described here. At the implantation site, the remaining DiI crystal(s) not incorporated into cellular structures produce a red signal visible in normal light (brightfield microscopy), but this is too intense under the fluorescence microscope to permit the identification of any cellular structures. Around the implantation site, a larger area is uniformly covered with a medium level of fluorescence that is not associated with cellular or fiber elements. This area has been called the nonspecific zone of diffusion in the text and its extent has been described and illustrated in some of the drawings and photomicrographs. Finally, in many other remote areas, fluorescent cells and fibers have been detected on a dark background. Sometimes, these anatomically defined labeled structures are also visible in the area of nonspecific diffusion. This paper concentrates on these structures as indicators of afferent and efferent connections of the nucleus under study.

The delineation of the exact area in which cells or fibers picked up the tracer is critical for the interpretation of the present data. Previous work has demonstrated that the incorporation of dye into the cell membranes takes place only in close proximity to the implanted crystals where DiI concentration is high and membranes have been experimentally disrupted (Godement et al. 1987; Honig and Hume 1989; Johnson and Bottjer 1992). The experimental evidence strongly suggests that when a labeled cell or fiber is found in a remote site, it is connected to a structure (respectively fiber or cell) that is located at the DiI implantation site and not in the larger surrounding area of the so-called nonspecific fluorescence. This statement is justified by the following observations. Within the diffusion area, some highly fluorescent cells and fibers are often observed, but these are limited in number. The vast majority of cells in this zone appear as black holes in the sections indicating that they have not accumulated dye in their membrane. When DiI is implanted into the POM, the nonspecific area of diffusion extends laterally and dorsally to cover a number of major fiber tracts, such as the TSM, FPL, CA, and CPa (see Figs. 2 A, 5 A). No fluorescent material has ever been detected in these tracts showing that they cannot assimilate the surrounding dye. More importantly, these tracts never transport any DiI to their known targets. By contrast, when DiI is erroneously implanted into the FPL, CA or CPa, as occurred in some individuals, intense labeling of the fiber tract, of all structures connected to it, and of the bed nucleus of the corresponding tract is found. An example of such an effect is shown in Fig. 8 C, in which the DiI crystal is in contact with part of the third nerve, giving dense labeling of the fibers and corresponding cells in the area of the Edinger-Westphal nucleus. Adjacent fibers of the third nerve that lie in the area of nonspecific diffusion are, by contrast, completely devoid of any fluorescence. Similarly, when the dye is implanted into a nucleus adjacent to the aimed target, this almost always results in a completely different pattern of labeling despite the desired target often being covered by nonspecific fluorescence, i.e., although DiI is present, it is not picked up by the target because it is probably at too low a concentration (see, for example, the case of the dye implanted in MLd rather than ICo). Finally, a completely different pattern of labeling of the telencephalic part of the brain is observed when DiI is implanted into the most rostral tip of POM (coronal cuts made in the anterior part of the nucleus) or in the middle part of the nucleus (coronal cut at the back of the nucleus). In the first case, the area from which the dye has been picked up only overlaps partially with the POM and this results in almost no labeling of the anterior part of the brain. In the second case, the label is mostly in the POM and this provides an intense label of the anterior parts of the septum and of the PVT. These observations indicate that the connections demonstrated in the present tracing experiments originate from the point at which DiI is implanted and not from the surrounding areas.

Anatomical connections demonstrated by DiI tracing

The DiI tracing technique has allowed us to demonstrate that the POM sends long anterograde projections down to the tuber, AVT and GCt. Dense networks of fluorescent fibers are also seen along the third ventricle and in several hypothalamic nuclei, such as the AM, PVN and VMN. A major projection in the dorsal direction has also been observed from the POM toward the SL and the SM.

Because of the retrograde transport of DiI, it has also been possible to investigate the regions from which the POM receives afferent projections. Fluorescent cells have thus been found in the septal region, AM, PVN, VMN, tuber and AVT after the implantation of DiI into the dimorphic nucleus of the POA.

In order to confirm some of these connections, transport of DiI in the opposite direction has been investigated and the bidirectional relationship between the POM on one hand, and the tuber, AVT, GCt, and septum on the other has been corroborated. This provides further support for the specificity of the anatomical origin of

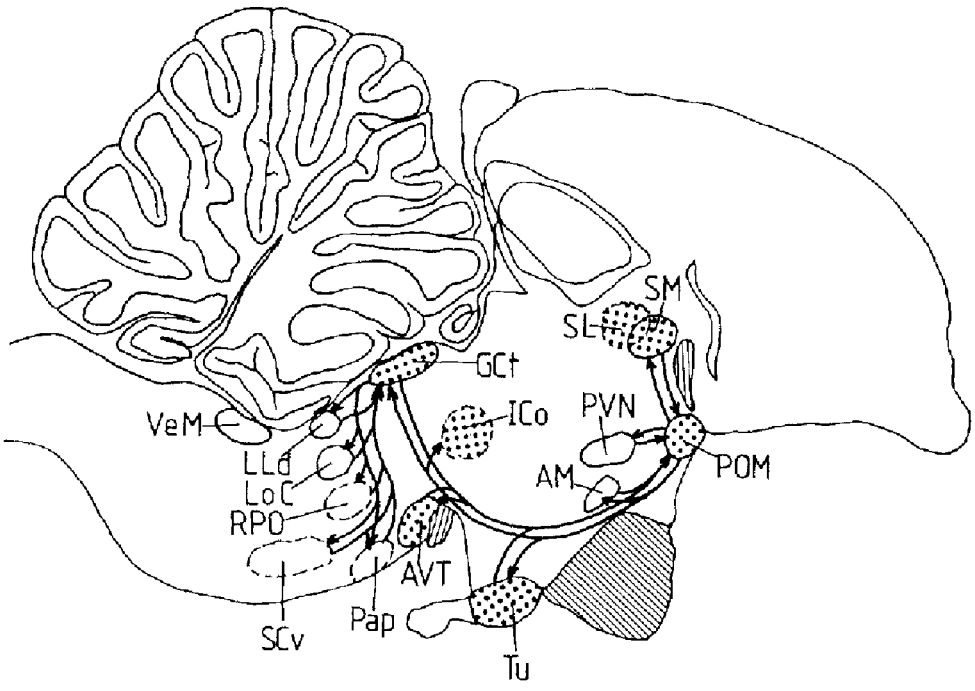

Fig. 9. Schematic drawing illustrating, in a parasagittal plane, the afferent and efferent connections of the POM. *Arrows* indicate afferents to the nuclei. *Dotted areas* indicate sites of DiI implantation

tracer that has been detected in the target. The major findings of this study are summarized semi-schematically in Fig. 9.

One of the major links demonstrated by this study is the extensive bidirectional relationship between the POM and septum, mostly in its medial aspect. Moreover, implantation of DiI into the GCt has revealed massive bidirectional connections with a large number of more caudally located structures in the mesencephalon and pons. Cells and fibers labeled by the dye can be found in the LoC, SCv, the nucleus subcoeruleus dorsalis (SCd), and at more posterior levels in the VeS and in the VeM. The GCt therefore appears to be an important center connecting the posterior parts of the brain to more anterior levels, including the POM. In particular, outputs from the POM could be transmitted via the GCt to the lower brain stem, probably including the medulla (not studied in the present work); this could be a significant part of the circuit originating in the POA, which is implicated in the control of male copulatory behavior (see also below). Furthermore, an important projection from the PVN to the AVT has been identified here; it should be confirmed by studies establishing an anterograde transport from PVN. This efferent is in agreement with previous immunocytochemical observations showing the presence of a vasotocinergic innervation of the AVT in quail (Panzica et al. 1988) and other avian species (Kiss et al. 1987; Voorhuis and De Kloet 1992), an innervation that presumably originates from the PVN (Korf 1984). The POM is also bidirectionally connected to the PVN and this latter nucleus could therefore be an additional important center between the POA and the brain stem (see also below for functional implications of these pathways).

Comparison with previous studies

No study has previously been devoted to the specific analysis of the anatomical connections of the medial preoptic area in quail. Recent work in our laboratories has shown that this region contains a sexually dimorphic (Viglietti-Panzica et al. 1986), steroid-sensitive (Panzica et al. 1987, 1991; Aste et al. 1993) nucleus that is involved in a critical manner in the activation of male copulatory behavior (Balthazart and Surlemont 1990 a, 1990 b). To extend our understanding of the central mechanisms controlling this behavior, it is crucial to establish the anatomical afferents and efferents of this nucleus; this represents one of the major goals of the present work. Comparisons with previously published literature are often limited because other species have been studied and homologies between structures have not always been established. Moreover, detailed information on the anatomical connections of the medial preoptic area are available only for the pigeon (Berk and Butler 1981) and for a few mammals, such as the rat (Chiba and Murata 1985; Simerly and Swanson 1988) and gerbil (De Vries et al. 1988; Yahr and Finn 1990).

Some of the efferent projections of the POM observed here in quail have previously been demonstrated in the pigeon (*Columba livia*) by an autoradiographic procedure identifying the anterograde transport of radioactive amino acids (Berk and Butler 1981); projections of the POM toward the SL and to a lower extent toward the SM have been demonstrated. As in quail, the POM of the pigeon sends projections in a caudal direction into the tuber, GCT and AVT. These fibers mostly travel along the third ventricle.

Similar projections originating in the medial preoptic nucleus have also been reported in the rat. The efferents from the rat medial preoptic area are not limited to short projections. Efferents from this region through the diago-

nal band of Broca, which is probably homologous to the TSM of birds (Crosby and Showers 1969; Reiner et al. 1983), are seen in the septum and the bed nucleus of the stria terminalis (BNST). Projections have also been observed toward a large number of hypothalamic nuclei and more posteriorly in the periventricular central gray, in the ventral tegmental area and in the reticular complex (Conrad and Pfaff 1976; Fahrbach et al. 1986). Projections into homologous areas (tuber, GCt, AVT) have been demonstrated in quail during the present study.

With the help of an anterograde tracer (PHA-L), Simerly and Swanson (1988) identified, in the rat, two types of projections moving on the one hand dorsally toward the telencephalon (SL, nucleus stria terminalis) and on the other hand caudally and ventrally along two major routes: one being located along the walls of the third ventricle, the other traversing the medial forebrain bundle. Structures such as the paraventricular nucleus, the arcuate nucleus and the dorso-medial hypothalamic nucleus receive strong afferents from the periventricular routes. The quantitatively most important projections of the medial POA travel, according to Simerly and Swanson (1988), through the medial forebrain bundle. These fibers end in the dorsomedial and ventromedial hypothalamus, the posterior hypothalamus, and the area of the supramammilar nucleus, with little transport being observed in the anterior part of the hypothalamus. Longer projections in the caudal direction may also use the medial forebrain bundle to reach the ventral tegmental area (Simerly and Swanson 1988).

Homologous areas usually contain fluorescent fibres and punctate structures in quail implanted with DiI in the POM. The same projections have also been identified in the pigeon by studying anterograde transport of tritiated leucine injected into the POM (Berk and Butler 1981). The anatomical data collected in quail (this study) and in pigeon (Berk and Butler 1981) do not permit an exact identification of the pathways followed by the efferent fibers from the POM. However, an intense network of fibers has consistently been observed along the third ventricle and at the ventromedial tip of the lateral forebrain bundle, i.e., in the area of the avian medial forebrain bundle, as described by Crosby and Showers (1969), Reiner et al. (1983), Kuenzel and van Tienhoven (1982), and Kuenzel and Masson (1988). The observations are therefore consistent with the notion that, in quail, the caudal efferent projections of the POM follow the same route as in mammals. This is also supported by the results of the tracing experiments in which DiI is implanted ventrally into the POM after the basal part of the diencephalon has been removed. The horizontal blocking of these brains presumably destroys the medial forebrain bundle, and consequently the labeling in the caudal diencephalon and mesencephalon is abolished.

Additional studies in mammals have also demonstrated connections of the medial preoptic area with the metencephalon and myelencephalon. Simerly and Swanson (1988) have found labeled fibers originating from the medial POA in the periventricular mesencephalon (namely, in the nucleus raphe and in the periaqueductal gray) of the rat. Some of these fibers descend dorsally through the parvocellular reticular nucleus to reach the nucleus of the solitary tract, mostly in its ventral portion. Many other studies have established a large number of often bidirectional connections between the POA and the lower brain stem, in particular with respect to catecholaminergic areas (Cederbaum and Aghajanian 1978; Ricardo and Koh 1978; Rivzi et al. 1992; Ter Horst et al. 1989; Conrad and Pfaff 1976). These observations cannot be directly compared with the present results in quail since, after implantation of DiI in the POM, areas that are more caudal than the nervus oculomotorius (NIII) have never been examined.

Striato-tegmental and striato-nigral projections similar to those observed in mammals have been described in pigeon (Anderson and Reiner 1991; Kitt and Brauth 1981). Neurons located in the paleostriatum augmentatum (PA), the LPO and the nucleus accumbens (Ac) send massive projections toward the TPc, which is the avian homolog of the substantia nigra of mammals. LPO and AC also project to the AVT, the homolog of the ventral tegmental area. These two target areas are the major source of the dopaminergic innervation of the brain (TPc corresponds to area A9 and AVT to area A10 in the nomenclature of Dahlström and Fuxe 1964). These homologies have also been established on the basis of the presence of specific neurotransmitters and neuropeptides (Anderson and Reiner 1991): most cells in the nigro-striatal system contain substance P and enkephalin. It is therefore surprising that no label has been found in the Ac or LPO following implantation of DiI in the AVT. This presumably reflects the technical limitation of the DiI technique: weak label is observed at long distances after one month of migration and much longer migration times or in vivo tracing should be used to identify these long projections.

A major projection originating in the PVN (one of the major vasotocinergic centers in birds) has been detected following the implantation of DiI into the AVT. Most PVN neurons seem to send projections to the AVT. These projections are probably vasotocinergic in nature, at least in part. The presence of a dense plexus of vasotocin-immunoreactive fibers in AVT has indeed been previously reported in several avian species, including the canary (Kiss et al. 1987), zebra finch (Voorhuis and De Kloet 1992), chicken and quail (Panzica et al. 1988). In the pigeon, autoradiographic studies have shown anterograde transport of radioactive amino acids from the PVM (homologous with the quail PVN) to the nucleus of the solitary tract and the nucleus motorius dorsalis nervi vagi; these fibres pass through the AVT (Berk and Finkelstein 1983; Berk 1987). The retrograde transport observed here may therefore be the consequence of the labeling by DiI of these fibres *en passage* in the AVT, suggesting connection between the PVN and lower brain stem in the quail. The present study also demonstrates a strong bidirectional connection between the POM and PVN, confirming and extending similar results obtained in mammals (Chiba and Murata 1985) and in pigeon (Berk and Butler 1981), in which anterograde transport of tracers between the POM and PVN has been shown.

Functional implications

Our previous studies have demonstrated that the POM is a critical site of T action in the activation of male copulatory behavior (Balthazart and Surlemont 1990 a, 1990 b). We show here that this nucleus sends major projections to a large number of brain areas including the septum, PVN, VMN, tuber, GCt, and AVT. Many of these structures have been implicated directly or indirectly in the control of male copulatory behavior in mammals (Sachs and Meisel 1988). A large number of these regions are also steroid-sensitive; their activity could therefore be directly modulated by androgens and/or estrogens. Presumably part of the circuitry identified here plays an important role in the control of male behavior in quail. The exact significance of the different parts of this circuitry should now be established by functional studies, including lesion of the target areas and sectioning of the connecting pathways.

We have recently shown by immunocytochemistry that, in quail brain, aromatase is not only located in the perikarya, but is also present throughout the processes, including the presynaptic endings (Balthazart and Foidart 1993; Naftolin et al. 1990). A large fraction of the aromatase activity in quail brain is accordingly contained in the synaptosomal fraction prepared by differential centrifugation (Schlinger and Callard 1989). The POM contains a large number of aromatase-immunoreactive neurons (Balthazart et al. 1990 b, 1990 c). Some of the projections demonstrated in the present study probably originate from these aromatase-containing cells. It is therefore possible that estrogens are produced locally in the terminal endings of these cells in target nuclei such as the medial and lateral septum, the tuber or the the ICo. regions in which we have identified aromatase-immunoreactive punctate structures (Foidart et al. 1993). These areas are known to contain estrogen receptors (Watson and Adkins Regan 1988; Balthazart et al. 1989). In addition to its autocrine action, aromatase synthesized in the preoptic neurons could therefore act in a paracrine fashion by producing, in terminals, estrogens that might act after diffusion on adjacent cells. Such a mechanism should be tested in functional studies.

The secretory activity of luteinizing hormone-releasing hormone (LHRH) neurons is controlled by estrogens (Kelly et al. 1984, 1989; Condon et al. 1986; Chan et al. 1987; Fink 1988; Ohtsuka et al. 1989; Dornan and Malsbury 1989; Fink et al. 1991), although these cells appear to contain few or no estrogen receptors in mammals (Shivers et al. 1983). The colocalization of estrogen receptors and LHRH has not been investigated in birds, but one study shows the absence of progesterone receptors in the LHRH cells of the hen (Sterling et al. 1984). LHRH-immunoreactive neurons are located in the septal region of the quail (Mikami et al. 1988; Foster et al. 1988; van Gils et al. 1993) to which the POM sends conspicuous afferent projections. Most neurons in the POM contain estrogen receptors (Balthazart et al. 1989, 1991) and many of these cells should be peptidergic (Panzica et al. 1992; Absil et al. 1993). They could represent interneurons that are involved in the control of the LHRH secretion. In mammals, peptides such as NPY, endorphin or neurotensin (review by Dornan and Malsbury 1989) play a key role at this level. The POM could therefore be the primary target of estrogens in the modulation of LHRH, and the projection to the septum demonstrated here would then represent the anatomical substrate underlying the regulation of LHRH neurons by estrogens.

An alternative but less conventional scenario is also possible. The terminals of the projections from the POM to the septum probably contain aromatase (as also suggested by the identification of aromatase-immunoreactive punctate structures in this area; Foidart et al. 1993) and therefore estrogens could possibly be synthesized in close vicinity to the LHRH cells. The steroid could then modulate the secretion of LHRH after binding to the classical steroid receptors of an interneuron located in the septal region or even by a direct membrane effect on the LHRH neuron (for reviews, see Naftolin et al. 1988; Schumacher 1990; Orchinik and McEwen 1993).

The multiple connections of the POM that have been demonstrated here are consistent with the notion that this nucleus plays an important role in the control of reproduction. It must, however, be stressed that the POM is a large structure and is presumably not only involved in the control of this process. The medial preoptic region of mammals has been shown to regulate a variety of endocrine, instinctive (e.g., behavioral), and autonomic functions (for a review, see Simerly and Swanson 1988). The same is probably true for the POM. The connections of this nucleus with the PVN on one hand and with the GCt on the other strongly suggest an involvement in a variety of behavioral and physiological processes. In particular, the organization of the efferent projections of the avian PVN (Berk and Finkelstein 1983; Korf 1984) is in agreement with the concept that the paraventricular nucleus represents an integral component of the neuroendocrine system, which is in turn coupled to the autonomic apparatus (for mammals, see also Swanson and Sawchenko 1983; Kiss 1988). The bidirectional connections of the POM with this nucleus, which plays a crucial (direct or indirect) role in the control of the vegetative system, indicates that POM could be also involved in this control.

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