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Cortical projection patterns of magnocellular basal nucleus subdivisions as revealed by anterogradely transported *Phaseolus vulgaris* leucoagglutinin*

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The present paper deals with a detailed analysis of cortical projections from the magnocellular basal nucleus (MBN) and horizontal limb of the diagonal band of Broca (HDB) in the rat. The MBN and HDB were injected iontophoretically with the anterograde tracer *Phaseolus vulgaris* leucoagglutinin (PHA-L). After immunocytochemical visualization of labeled efferents, the distribution of projections over the cortical mantle, olfactory regions and amygdala were studied by light microscopy. Based on differences in cortical projection patterns, the MBN was subdivided in anterior, intermediate and posterior portions (MBNa, MBNi and MBNp). All subdivisions maintain neocortical projections and are subject to an anterior to posterior topographic arrangement. In the overall pattern, however, the frontal cortex is the chief target. Furthermore, all MBN parts project to various regions of meso- and allocortex, which are progressively more dense when the tracer injection is more anteriorly placed. The most conspicuous finding, however, was a ventrolateral to dorsomedial cortical projection pattern as the PHA-L injection site moved from posterior to anterior. Thus, the posterior MBN projects predominantly to lateral neo- and mesocortex while the anterior MBN sends more fibers to the medial cortical regions. Furthermore, the MBNa is a source of considerable afferent input to the olfactory nuclei and as such should be regarded as a transition to the HDB. The HDB, apart from projecting densely to olfactory bulb and related nuclei, maintains a substantial output to the medial prefrontal cortical regions and entorhinal cortex, as well. Comparison of young vs aged cases indicate that aging does not appear to have a profound influence on cortical innervation patterns, at least as studied with the PHA-L method.

INTRODUCTION

The cholinergic nuclei in the mammalian basal forebrain are localized as rather well-defined cell populations in the MS, VDB and HDB, SI and VP[32,46,54]. Apart from the general interest in the cholinergic (ACh) forebrain systems as a transmitter defined neural circuit[6,11,38], studies of the behavioral effects of drugs blocking acetylcholine receptors specifically in the central nervous system have long implicated cholinergic transmission in cognitive performance[3,9,47]. In addition, examination of the demented human brain provides strong evidence for a significant decrease in cortex and hippocampus of the ACh-related enzymes acetylcholinesterase (AChE) and choline acetyltransferase (ChAT), and concurrent cell death in the magnocellular cholinergic forebrain areas[1,12,13,19,42,51,55]. These biochemical and anatomical deficits in the demented brain led to the hypothesis that the subcortical cholinergic forebrain nuclei and projections constituted a transmitter-specific system that may be intimately involved in the development of senile dementia of the Alzheimer type[1,10,13,55] and probably also to memory deficits in the elderly in general[4]. A number of reports have been published since then on the anatomical organization of ACh af-

* Part of the anterograde tracing data has been reported elsewhere (refs. 33, 49).

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ferentation to the cortex, hippocampal formation, amygdaloid body and olfactory bulb[5,14,24,25,27,34–36,43,44,52,53,56–59]. The majority of these studies were based on retrograde intra-axonal transport methods either alone[14,25,27,52] or combined with histochemical[5,34–36,56] or immunocytochemical[24,35,36,43,53,57] demonstration of cholinergic cell bodies. In summary, these retrograde transport studies, but also anterograde tracing studies[31,44] provide firm evidence that the cerebral cortical mantle is innervated from the SI and VP region, often referred to collectively as the MBN, whereas the MS and VDB is a source of efferents to the hippocampal formation. The OB and limbic cortex were shown to receive cholinergic input from the HDB, while several basal forebrain structures mentioned send projections to the basolateral amygdala. A second major conclusion drawn from retrograde transport studies was the extremely high percentage of cortically projecting MBN cells that are cholinergic (up to 90%)[28]. The percentage of cholinergic cells in MS/VDB and projecting to hippocampus and OB that are cholinergic is much lower, however (45% and 20%, respectively[43,59]).

As a consequence of the methods employed, most of the retrograde transport studies cited above supply information on the source of subcortical cholinergic input, but only allow a limited insight as to the terminal innervation structure within the target area. Such information, however, should be considered of major importance in order to gain understanding of the structural nature of cortical innervation from the basal forebrain nuclei. A detailed mapping of this terminal organization on the light microscopic level would be an important step in elucidating the exact site of cholinergic inputs in cortical circuitry. Such information could also be invaluable in the interpretation of how the MBN interacts with the cortex electrophysiologically.

With respect to nomenclature and topographic organization of the cholinergic forebrain cell groups there is no generally accepted uniformity. For example, Saper[44] collectively labels all cholinergic neurons in the basal forebrain as the magnocellular basal nucleus. Alternatively, Mesulam et al.[35,36] introduced a terminology for central cholinergic cell groups based partly on connectivity patterns and partly on anatomical location, proposing a numerical system of Ch_1-Ch_4, which is largely equivalent for respectively MS, VDB, HDB and MBN. In the present investigation, our interest is aimed at the detailed nature of the cortical projections of the MBN. We will deal here with the study of efferent patterns from magnocellular neurons of the MBN situated in the SI, VP and ansa lenticularis, but also with the HDB as a reported source of input to the cortical mantle. The efferent connectivity patterns of MBN and HDB were studied by the recently developed method of anterograde transport of *Phaseolus vulgaris* leucoagglutinin (PHA-L) and the subsequent immunocytochemical demonstration of the tracer[18,50]. This method allows a precise definition of the tracer uptake area, and an artifact-free display of labeled axons, axonal branching and distribution of terminal presynaptic boutons within the target area.

The determination of the injection coordinates was based on the topographic distribution of MBN neurons as previously described[2,5,7,36,43,46]. Matching of labeled cell bodies at the injection site and forebrain cholinergic neurons was determined by combination of PHA-L procedures and the pharmaco-histochemical regimen for AChE-activity[6,8,45].

The majority of experiments was carried out on 3-month-old juvenile rats. In a number of cases, however, tracing of basal forebrain efferents was performed on aged rats of over 32 months to gain preliminary insight in the possible effects of aging on the structure of cholinergic forebrain efferents.

MATERIALS AND METHODS

In the present study, experiments were carried out on 58 male Wistar rats. Four of these animals were pretreated with di-isopropylfluorophosphate (DFP) and the brains were processed for histological acetylcholinesterase demonstration in order to study the topography of AChE-positive cell bodies. Thirty-seven rats received a PHA-L injection into the MBN/HDB region. The remaining 17 animals received a deposit of PHA-L into the MBN area, as well as subsequent staining for AChE activity following a DFP injection in order to determine the overlap of PHA-L injection spot and AChE-rich neurons. All (except 3) animals were young rats 3 months of age. Three aged specimen of 32–36 months were injected with PHA-L and processed for AChE activity as described in detail below.

PHA-L procedure

For injection of PHA-L animals were anesthetized with 0.4 ml/kg b. wt. Hypnorm (Duphar), i.m., and 30 mg/kg sodium pentobarbital, i.p., and mounted in a Kopf stereotaxic frame adjusted to the coordinate system of Paxinos and Watson[39]. Bevelled glass micropipettes with tip diameters of 15–20 μm were filled with a solution of 2.5% PHA-L (Vector Labs) in Tris-buffered saline (TBS, pH = 7.4). The pipettes were positioned in a brain area defined by the coordinates AP 6.2–8.7; L 1.7–4.5; V 1.0–4.0 (ref. 39) and connected to the positive pole of a Midgard CS 3 constant-current source. The driving force for iontophoretic delivery of the tracer was a current of 5–6.5 μA for 30–40 min in a 7-s on/7-s off cycle. After completion of iontophoresis the glass pipette was left in its injection position for 10 min to prevent leakage of tracer during retraction. The animals survived for 6–9 days. The brain tissue was fixed by transcardial perfusion with a mixture of 2.5% glutaraldehyde, 0.5% paraformaldehyde and 4% sucrose in phosphate buffer (pH 7.4). The brains were dehydrated in 30% sucrose prior to sectioning at 40 μm on a cryostat microtome. Sections were collected in chilled TBS, thoroughly rinsed in this solution for at least 6 h and treated immunocytochemically for PHA-L distribution[18,50]. In all incubations and rinses the solutions were made up of 0.5 M NaCl, 0.5% Triton X-100 and 0.05 M Tris buffer at pH 7.4. The entire procedure was carried out at room temperature in the dark. Incubation with goat anti-PHA-L (Vector Labs) 1:2000 was done for 48 h, rabbit anti-goat IgG (Sigma) 1:200 for 15–24 h and goat peroxidase–anti-peroxidase complex (DAKO) 1:400 for 4 h. The sections were stained for peroxidase in 40 mg DAB (Sigma) in 100 ml Tris buffer at pH 7.4 and 0.9 ml H_2O_2 1.5% for 30–60 min. The sections were mounted and counterstained with Cresyl violet. In all PHA-L experiments, the procedure described above was carried out on every third section.

AChE procedure

For demonstration of AChE activity in basal forebrain nerve cell bodies, a pharmacohistochemical technique was carried out that included pretreatment with DFP as described by Butcher et al.[8]. DFP (Sigma) solved in arachid oil (1:1000) was injected i.m. into the gastrocnemius muscle at a dose of 1.5 mg/kg b. wt., immediately followed by an i.p. injection of 5 mg/kg atropine. An optimal survival time of 5 h[8] was followed by perfusion and fixation of the brain just as described above. Free-floating frozen sections of 40 μm were treated according to the Karnovsky and Roots[26] procedure. Sections were incubated for 1 h at 37 °C in a medium containing 57.9 mg acetylthiocholine iodide and 3.1 mg ethopropazine per 50 ml solvent. This incubation was followed by immersion in 1.25% Na_2S and 1% $AgNO_3$ for 1 min each. After staining the sections were mounted on glass slides and counterstained with Cresyl violet.

In cases of AChE staining of PHA-L injected brains, the sections were treated as described above, but incubated for immunocytochemical processing for the tracer immediately thereafter. In those combined cases, however, alternate sections were stained separately both for PHA-L and for AChE activity as well, since the AChE procedure tends to decrease the intensity of the PHA-L staining.

Of the 54 PHA-L tracing experiments, 8 cases were discarded because of technical failures in the experimental procedure. Eighteen cases produced PHA-L deposits that did not or only partly cover AChE-rich neurons of the basal forebrain nuclei and hence resulted in no or only minor labeling in cortical areas. The latter injections were always localized in the various structures that surround the MBN/HDB complex and some have been indicated in the injection survey figures for reference (Figs. 1–3). The remaining 28 cases were analyzed for anterogradely labeled efferents in the various cortical and amygdaloid target areas and have been listed in Table I. It should be emphasized here again that the description of the result is confined to telencephalic projections only. The often extensive descending brainstem projections will not be dealt within the scope of the present study.

Nomenclature of the cortex

As will become clear from the following description of results, but also from previous investigations, the MBN neurons provide efferents not only to the neocortex and amygdala, but to various other cortical regions. In the present paper, a cortical topographic terminology will be used based on various recent cytoarchitectonic studies[28,29,41,43,44] which also could be identified in the Cresyl violet-stained mate-

rial employed in this investigation. In the neocortex the following regions will be distinguished. The frontal cortex can be subdivided into a PCm[23,45,47], and a PCl[28,29,44], equivalent to the lateral frontal cortex of Rye et al.[43] and Saper[44], and the M sensu stricto. The M is indicated here and by others[43,44] as a narrow strip of cortical tissue situated between PCm and PCl anteriorly, and between PCm and somatic sensory cortex, posteriorly. Zilles et al.[60], however, do not indicate a distinct motor cortex. The motor areas by these authors are considered as large parts of PCm and PCl, which is confirmed by electrophysiological mapping studies[15]. In the parietal cortex, two large somatic sensory fields SS_1 and SS_2 will be distinguished. The homologues of the temporal and occipital cortex will not be further subdivided, and will be referred to as TE and OC, respectively, and include auditory, visual and related association cortices[39,43,44,60]. The remaining cortical regions are divided into meso- and allocortex[41], allowing a separate allocortical classification for rhinencephalic cortex fields. The mesocortex includes most of the prefrontal areas: AC, PL, IL, OF and AI cortices[29,43,44]. The AI is further differentiated into AId, AIv and AIp. Furthermore, the mesocortex comprises the RS and the PR cortex. Several authors do not use the term mesocortex but refer to proisocortex (AC, RS, PL and IL) and periallocortex (AI and PR)[41,44].

The allocortex (as adapted here from Reep[41]) consists of the hippocampal formation, entorhinal cortex — which is subdivided in DLE, VLE and VME parts[30] — and PIR. The rhinencephalic AO, OT and OB[60] are also classified as allocortical structures.

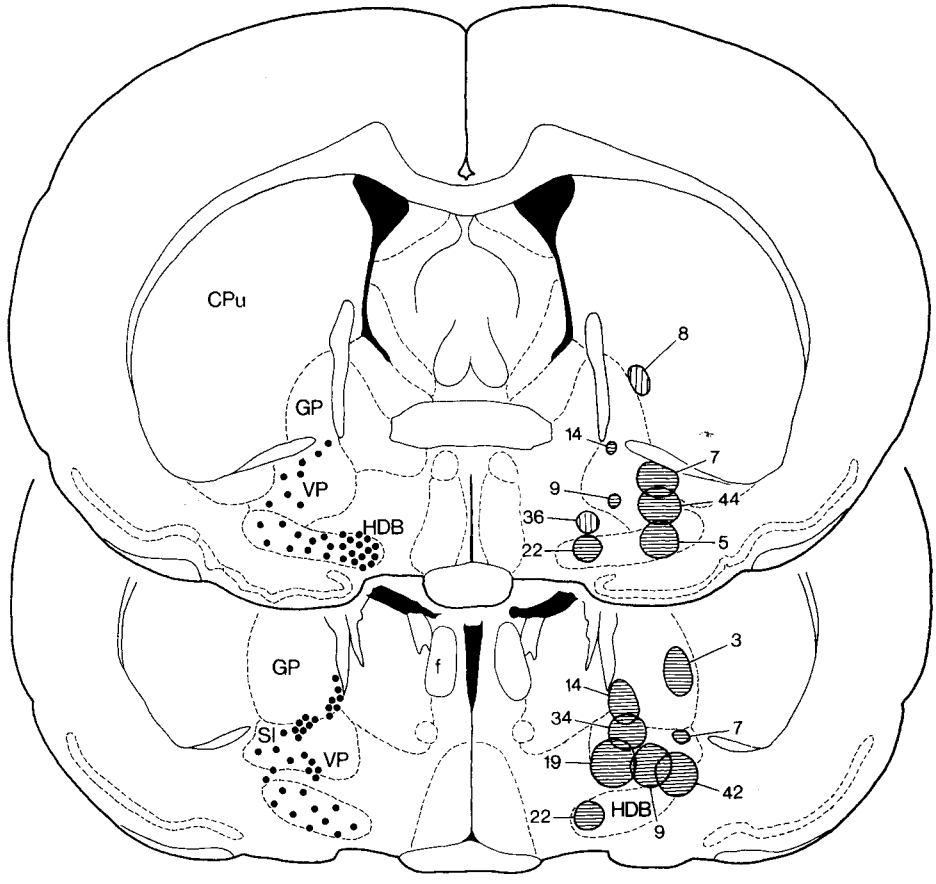


Fig. 1. Transverse section of the anterior forebrain. At the left side of each section in Figs. 1–3, the position and extent of AChE-rich somata is indicated by filled circles. In this figure AChE-rich cells are representing the anterior subdivision of the MBN and the HDB. The PHA-L injection sites in the MBNa region are drawn at the right side of the figure. Horizontal hatched lines represent successful cases for labeling of cortical projecting fibers. Vertical hatched cases were negative for cortical labeling and are given here as reference cases. Numbers correspond to experiments listed in Table I.

RESULTS

Location of the injection sites

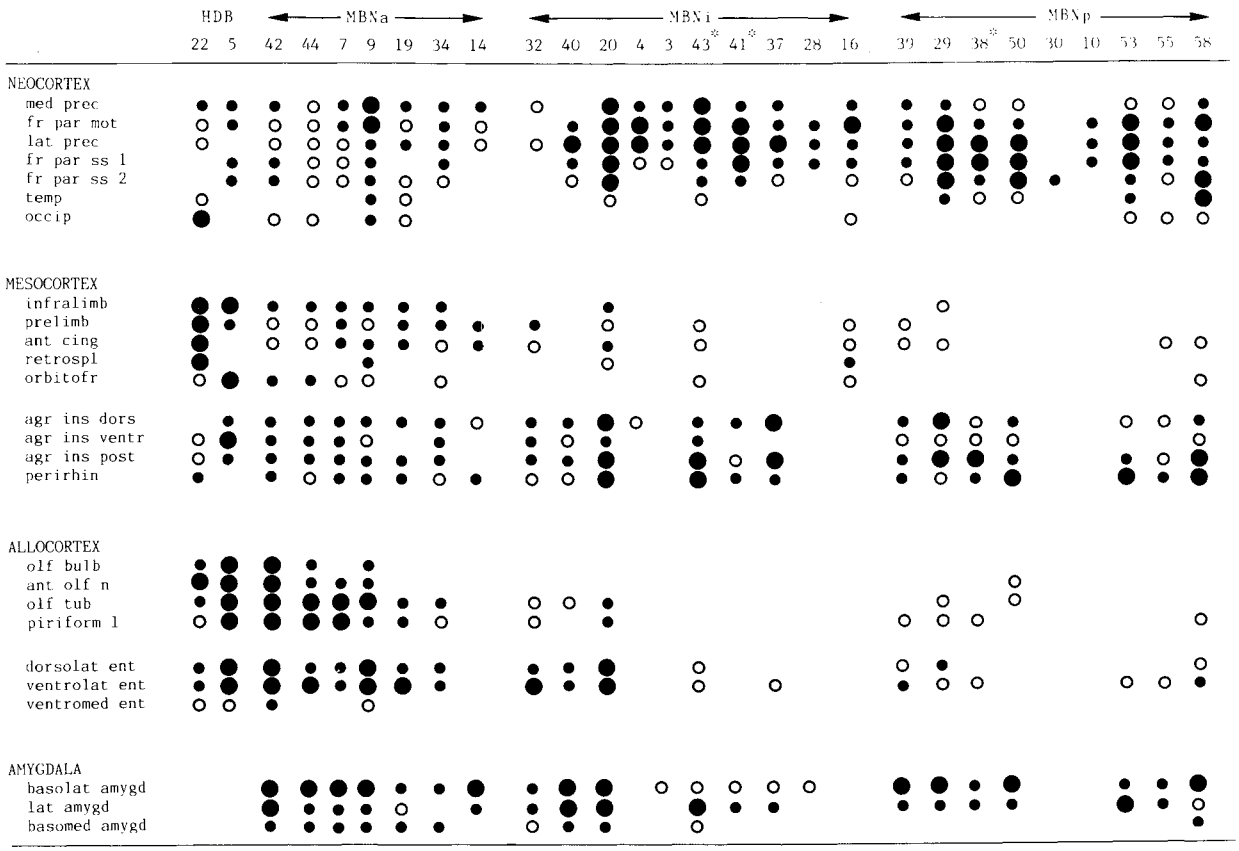
The extension and location of the PHA-L injections described in this report are indicated in Figs. 1–3, comprising one or two coronal sections each. The position of AChE-rich somata is indicated by filled circles in the left half of each section, whereas the PHA-L injection spots are drawn in the corresponding right half of each section. Injections that did result in anterogradely labeled efferents in the cortex are marked with horizontal hatched lines; some non-cortically projecting deposits served as control cases and are indicated by vertical hatching. The numbering of the cases corresponds to the numbers in Table I and to the cases described or illustrated.

Based on differences in cortical projection patterns and topographical position, the MBN was divided into MBNa, MBNi and MBNp portions as illustrated in Figs. 1, 2 and 3, respectively, and as is shown in Table I. The anterior subdivision MBNa comprises AChE-rich cells in those parts of the MBN that parallel the dorsal border of the HDB[17]. With regard to both their anatomical position and their cortical projection pattern the MBNa neurons appear to form a transition to the HDB. The MBNi consists of cells posterior to the HDB and anterior to the rostral pole of the amygdaloid body, so including here the MBN cells in the ventral GP, VP and SI (Fig. 2). MBNp refers to the magnocellular basal forebrain neurons in the posterior parts of the globus pallidus,

TABLE I

Survey of density of anterograde terminal labeling in the various cortical and amygdaloid regions as result of PHA-L injections in the 3 subdivisions of the MBN and HDB area

The numbers on top of the table refer to the experiment numbers and correspond to the numbers in Figs. 1–3. The aged animals are indicated by an asterisk. Open circles: minor labeling; small filled circles: moderate labeling; filled large circles: heavy labeling.



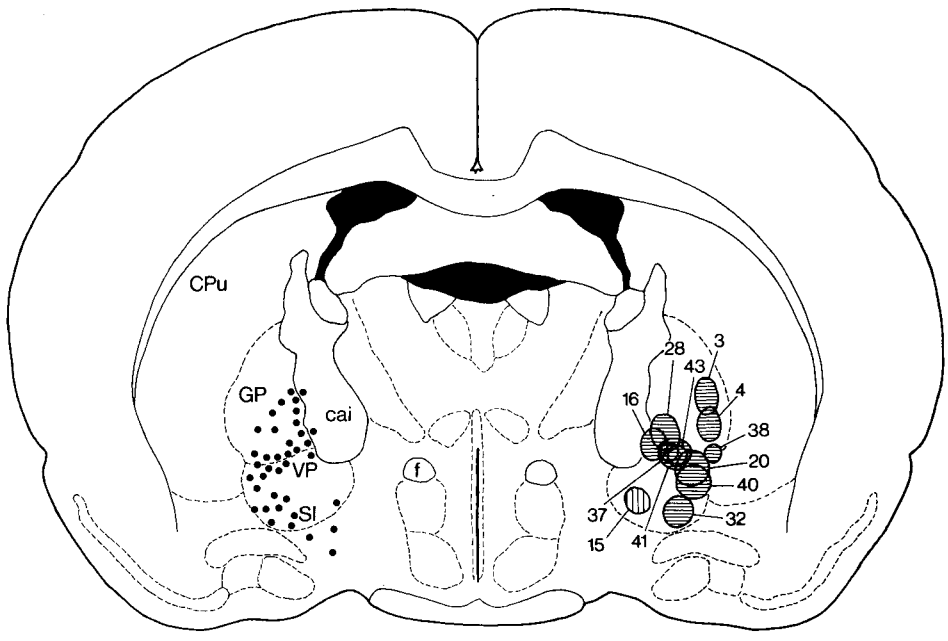


Fig. 2. Coronal section at the level of the intermediate subdivision of the MBN (left). PHA-L injections positive for anterograde tracing of MBNi projections to the cortex are indicated by horizontal hatching in the right side of the figure.

extending dorsolaterally from the nucleus of the ansa lenticularis (Fig. 3). The results of the anterograde labeling of efferents of MBN and HDB will be described on 3 levels. First, a summary will be given of the overall pattern of cortical projections from the various MBN subdivisions. Second, efferent tracing from the site of origin to the cortical target structures will be described in detail, using 3 representative cases. Third, the distribution of terminal innervation within the target areas will be dealt with in greater detail.

Overall pattern of MBN projections to the cortex

A survey of cortical projections from the MBN and HDB found in the PHA-L tracing experiments is given in Table I (and see Fig. 10). The numbers on top of the table refer to the experiment numbers illustrated in Figs. 1–3. The sequence of the experiment numbers is such that from left to right in each subdivision, the injections are given in order of their ventral to dorsal position. The cortical innervation is ranked along the following scale relative to the total fiber projections in each experiment: open circles (sparse innervation), small filled circles (moderate innervation) or large filled circles (rich innervation).

In summary, injections into the MBNa and including here some injections in the HDB resulted in a diffuse, modest labeling of efferents in the neocortical areas that are rather evenly distributed over the frontal and parietal lobes. Temporal and occipital cortical fields are notably less densely innervated by the MBNa. An exception on this general pattern is the relatively rich supply of terminals to more posterior neocortical fields in Expt. 22. Case 22, however, is centered in the medial aspects of the HDB and bears only limited resemblance to the remaining injections in this basal forebrain area, which is also evident from labeling patterns in meso- and allocortical regions.

MBNa maintains rather homogenous efferent projections to the various subdivisions of the mesocortex excluding the RS. The RS appears to be more of a target for the diagonal band nuclei, since RS labeling was only observed after injections partially or entirely centered in the HDB (i.e., Expts. 9 and 22), and also in the VDB (unpublished observations).

Another major observation concerning efferents of MBNa is that both proisocortex and periallocortex are recipients of labeled terminals, which is in contrast with the exclusively periallocortex innervation from MBNi and MBNp. The only exception in this respect again is found after medial HDB injections.

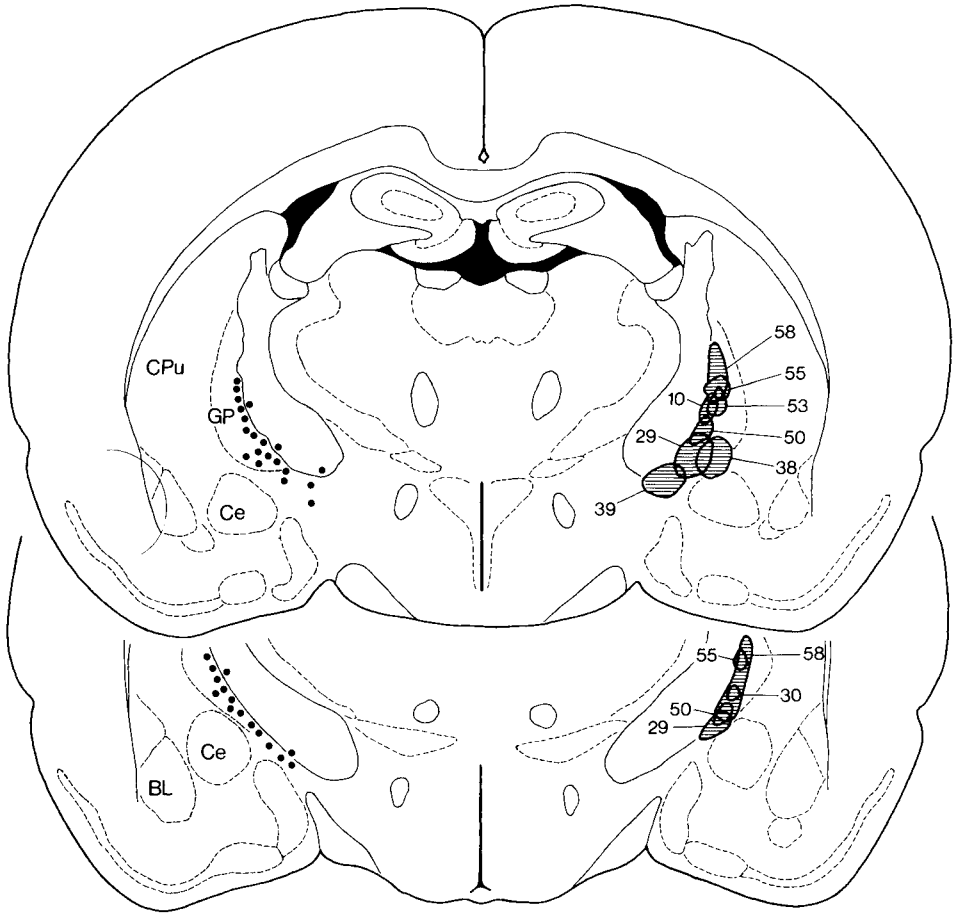


Fig. 3. Sections through the posterior portion of the MBN region with AChE-rich cell bodies left and PHA-L injections at the right side of each figure.

Projections from this area are mainly aimed at proisocortical targets, which emphasizes the particular position of HDB in the magnocellular forebrain systems.

The allocortical structures — apart from the hippocampal formation which appears to be the almost exclusive target of the medial septum and vertical diagonal band nucleus of Broca — in our material are the major aim of efferents from the anterior MBN and HDB. Most injections in this region, notably the more ventral HDB cases, gave rise to dense innervation patterns in olfactory-related structures. Furthermore the nuclei of the lateral zone of the amygdaloid body and in particular the basolateral nucleus are recipients of a well-developed projection from the MBNa region. The amygdala very clearly does not receive any input from the HDB.

PHA-L injections into the intermediate and posterior subdivisions of the MBN resulted in largely comparable patterns of labeling in the cortical mantle. Compared to MBNa, the MBNi and MBNp subdivisions are more specifically aimed at neocortical structures, but the overall weighting of neocortical projections is similar to that seen with MBNa injections: frontal and parietal areas are more richly supplied with efferents than the temporal and occipital cortices. The occipital cortex appeared to receive more innervation from HDB and MBNa than from more posterior regions of the MBN, however.

With respect to projections to the mesocortex, there is a striking partition into a minor innervation of the medial limbic cortex and a relatively dense supply of terminals to the insular and perirhinal areas after PHA-L deposits in MBNi and MBNp. Of the allocortical structures, the entorhinal cortex appears to be the only cortical area that is associated with

MBNi-MBNp cells, mainly from the intermediate subdivision. From the primary olfactory regions the olfactory tubercle and piriform lobe received a modest input from MBNi and MBNp. The lateral and in particular basolateral amygdaloid nucleus are strongly innervated by MBNi and MBNp, as well as by

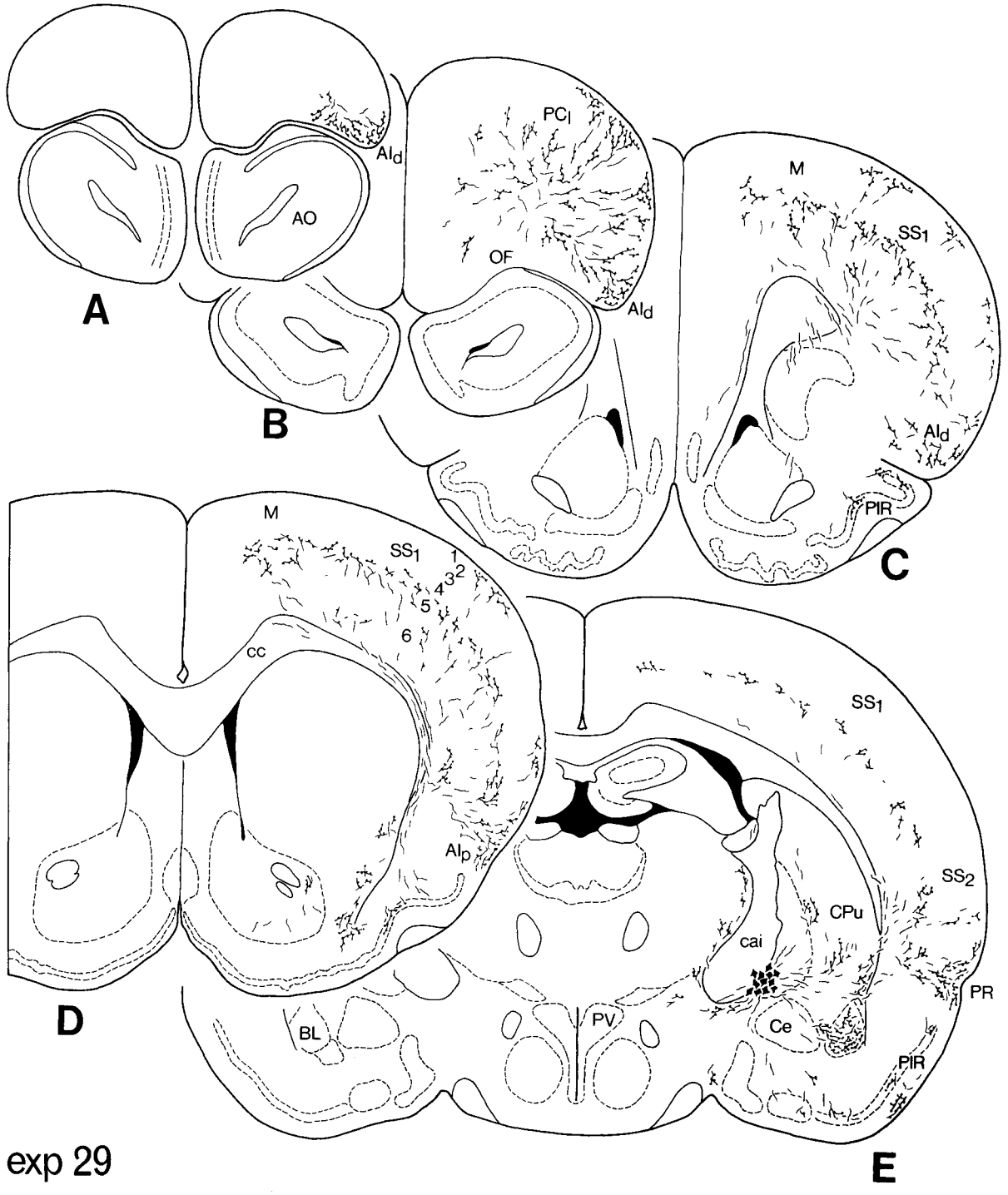


Fig. 4. Series of transverse section of the rat forebrain from anterior (A) to posterior (G) in which an injection of PHA-L in the MBNp region is indicated (Expt. 29), and the anterograde labeling of axons and terminal boutons in the cortex and amygdaloid body.

MBNa. Dorsal injections in general, however, produced only sparse labeling in the amygdaloid body, as compared to the dense projection patterns after ventral MBN injections.

The morphological pattern of cortical innervation from the MBN

A more detailed account of the cortical innervation patterns after PHA-L injections in the MBN cell groups are exemplified by 3 cases. In Expt. 9, efferent connections are demonstrated that originate from the anterior MBN cell population after a PHA-L injection in the anterior SI region. Case 22 is described in order to emphasize some distinct differences in the labeling of efferents that appear after lectin deposits in the HDB, which probably indicates intermingling of neurons projecting to the primary olfactory centers and to the limbic cortex in this nucleus. Expt. 29 will represent the MBNp group of injections. Injections in the MBNi subdivision have been described in greater detail elsewhere[33] and bear largely similar characteristics as the MBNp injection cases.

Projections from MBNi and MBNp

As with the majority of PHA-L injections in MBNi-p, Expt. 29 resulted in a relatively homogenous pattern of terminal labeling in neocortical areas, a well-developed projection to the amygdala, but only moderate labeling in the ventrolateral meso- and allocortical regions (Fig. 4). The neocortical projection pattern is characterized by a distribution over a vast part of the various lobes, except for the occipital visual fields. Temporal, somatic sensory, and parts of the motor and lateral precentral cortex were major recipients of MBNp efferents. In all cases, layer V was found to be most heavily labeled, but in several experiments additional innervation of layers I and II could be observed (notably in SS_1, M and PCl). In general, anterior neocortical regions appeared to have more dense innervation than posterior parts of the cortex. Moreover, in the posterior cortex it was

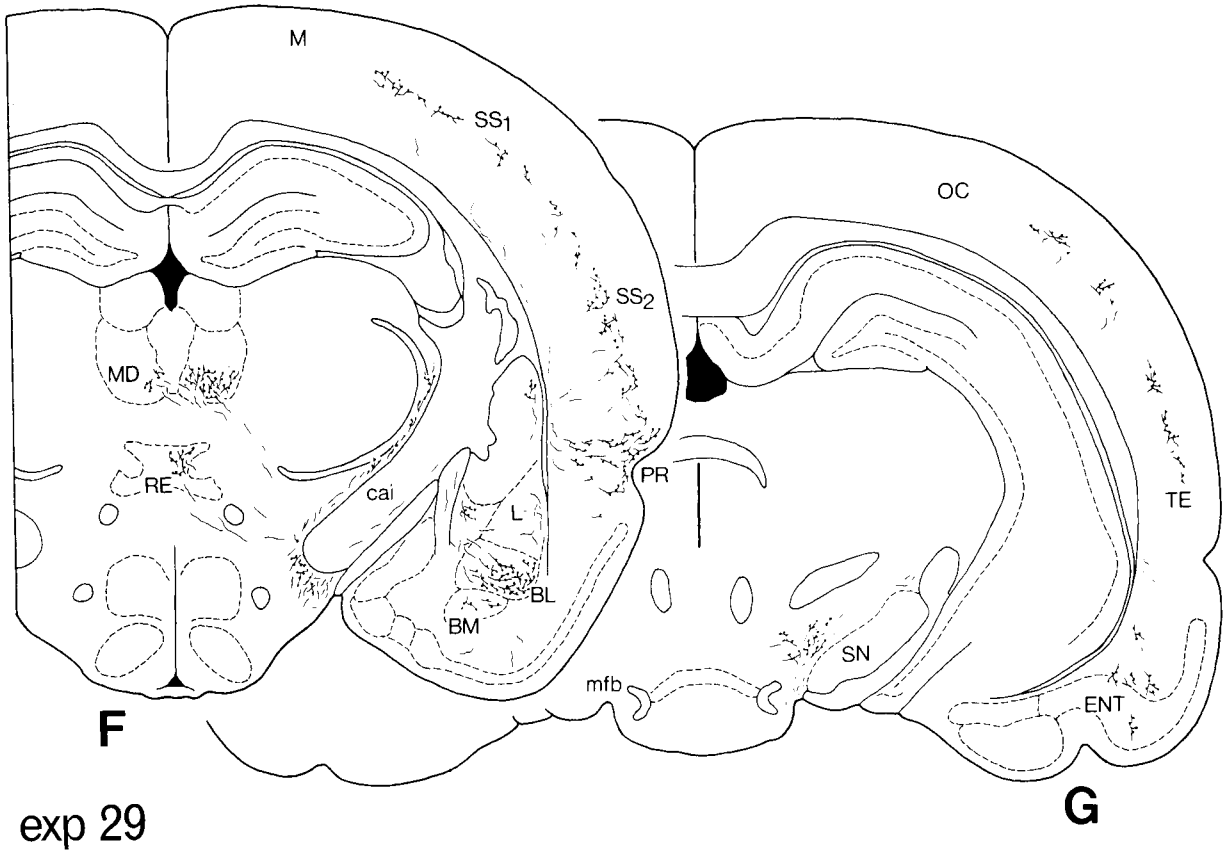


Fig. 4 (continued).

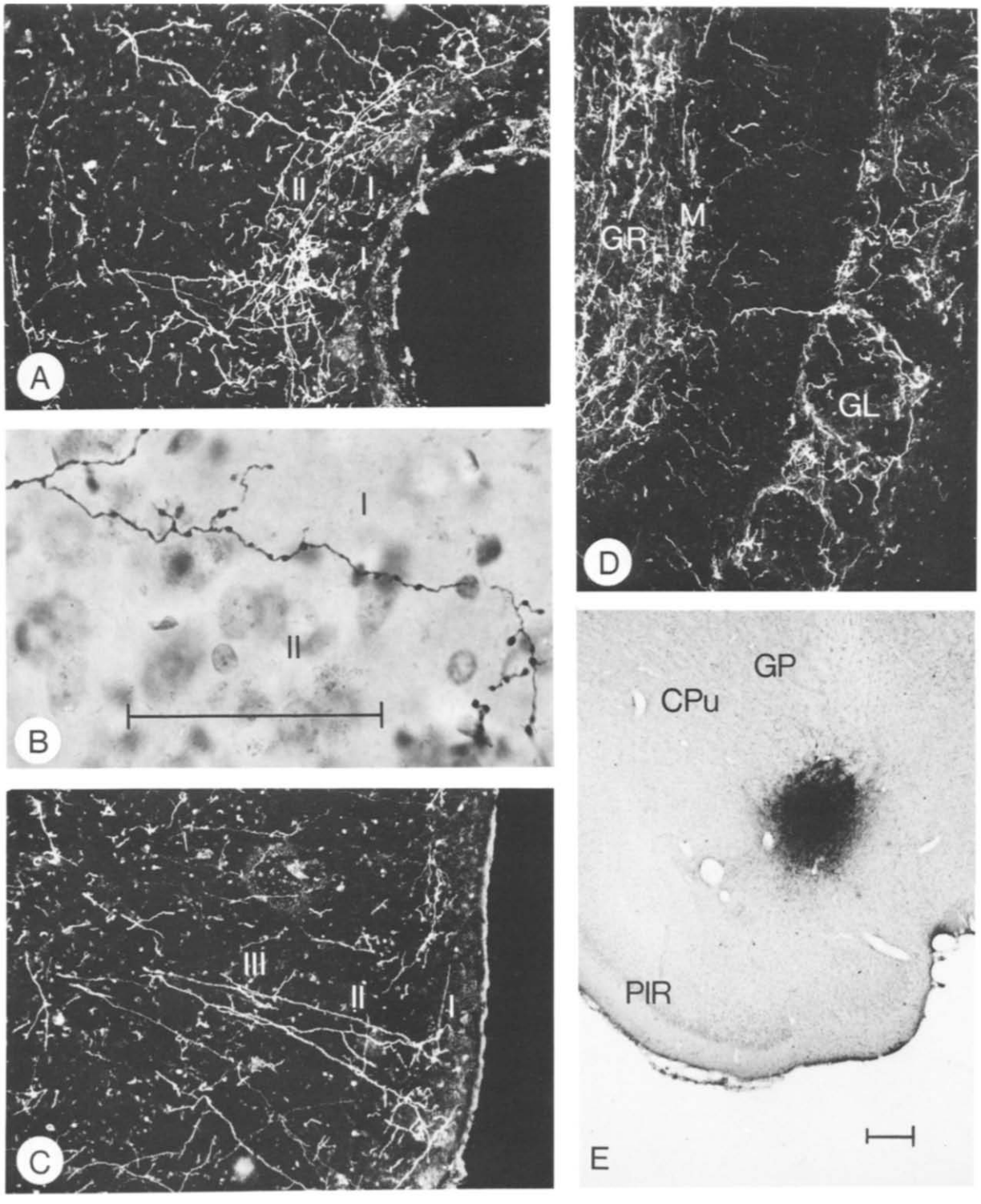


Fig. 5. A, C, and D: dark-field photomicrograph illustrating the pattern of anterogradely labeled terminating fibers in the perirhinal cortex, temporal cortex and olfactory bulb, respectively. B: microphotograph of a PHA-L-labeled terminal axon and boutons in the transition zone of layer I and II of the neocortex. E: example of a PHA-L injection in the MBNa region of the ventral pallidum-substantia innominata. Bar in B = 50 μm; in E = 250 μm.

often observed that labeling occurred in circumscribed, narrow patches in contrast with the more widespread branching patterns in anterior cortical fields.

In almost all PHA-L injections in MBNi-p, the medial cortical zone both from neo- and mesocortical origin, was devoid of any labeled input, which appears to characterize projections that originate from the posterior two-thirds of the MBN complex. In contrast, dense labeling was present in the lateral structures of the mesocortex, most notably in the agranular insular and perirhinal cortices. In case 29, the dorsal and posterior AI are well supplied with MBNp projections, which posteriorly continue into the PR (Fig. 4).

The allocortex was clearly a minor target for efferents from MBNp. Sparse projections were observed to the deep layers of the lateral entorhinal cortex and piriform lobe. As usual in most of the MBN cases, injection 29 was followed by dense terminal labeling particularly in the basolateral amygdaloid nucleus. A number of PHA-L positive terminations were also present in lateral and basomedial amygdala.

With respect to the trajectory of cortical projections there was a consistent pattern in all PHA-L injections in MBNi and p. From the injection locus, fibers coursed in a lateral direction and ran within or adjacent to the callosal fiber systems. In the anterior-posterior plane solitary fibers or small fascicles could be followed into the deep cortical layer (usually layer VI) prior to reaching the site of termination.

Projections from MBNa

While cortical projection patterns from MBNi show considerable similarities to those from MBNp[33], the MBNa gives rise to cortical projections of a different nature. These differences are such that it must be concluded that MBN cells in the VP-SI region cannot be clearly separated from HDB neurons, as defined by their olfactory bulb connections[20,44]. The exact pattern of efferent projections from MBNa can be illustrated by the results of case 9, in which PHA-L was injected in the SI adjacent and dorsal to the lateral HDB (Fig. 6). As a result of this tracer deposit rich labeling of neocortical areas occurred in PCm, PCl and in the predominant anterior aspects of the motor cortex and SS_1 and SS_2. Anterogradely labeled fibers, although much less dense, were also seen in the temporal and occipital regions. In all neocortical target areas mentioned, labeling occurred preponderantly in layer V, but with the exception of SS_1 and SS_2, in superficial layers I and II as well (Fig. 5). Taken together, the labeling patterns after MBNa injections indicate a topographic organization, such that MBNa is aimed at the more medial aspects of neo- and mesocortex. This is manifest in the neocortical projections to M, PCm and PCl, but is likewise present in the medial mesocortical areas. From the injection locus, labeled fascicles can be followed in a medio-anterior direction towards infralimbic and anterior cingulate cortex. At this level, the labeled fibers join the cingulum bundle to terminate in the AC and its posterior counterpart, the retrosplenial cortex. The neocortical occipital lobe is supplied with cholinergic basal forebrain input fibers that run via the cingulum.

Efferents to the mesocortex are aimed at the deeper layers V and VI. In contrast to neocortical projections, there appears to be an equally dense innervation of layer I and II. Apart from the considerable input to the medial mesocortex, MBNa afferents are present to the lateral mesocortical AId, AIp and PR, although these latter areas are less densely innervated than influx from MBNi and p.

The extra-hippocampal allocortex constitutes one of the most dominant targets of the anterior MBN complex. There is a rich supply of projections to the dorsolateral and ventrolateral entorhinal cortex, both to deep, and even more impressively to superficial layers. Topographically, PHA-L injections in the dorsal aspects of the MBN region produced efferent labeling predominantly to deep layers V and VI, whereas more ventral injections were followed by dense labeling patterns in layer IA (Figs. 8A, C; 9). Furthermore, the SI is a strikingly rich source of efferent projections to essentially all primary olfactory structures. Dense projections occur in the lateral olfactory tract nucleus, anterior olfactory nucleus, olfactory bulb, olfactory tubercle and piriform cortex. In the OB, a laminated pattern of terminations is present over the glomerular layer, mitral cell layer and granule cell layers (Fig. 5D). A few cells in the MBNa also appear to send projections to the hippocampal formation (which will be extensively dealt with in a separate report). In the amygdaloid body, abundant labeling of terminal boutons appeared in

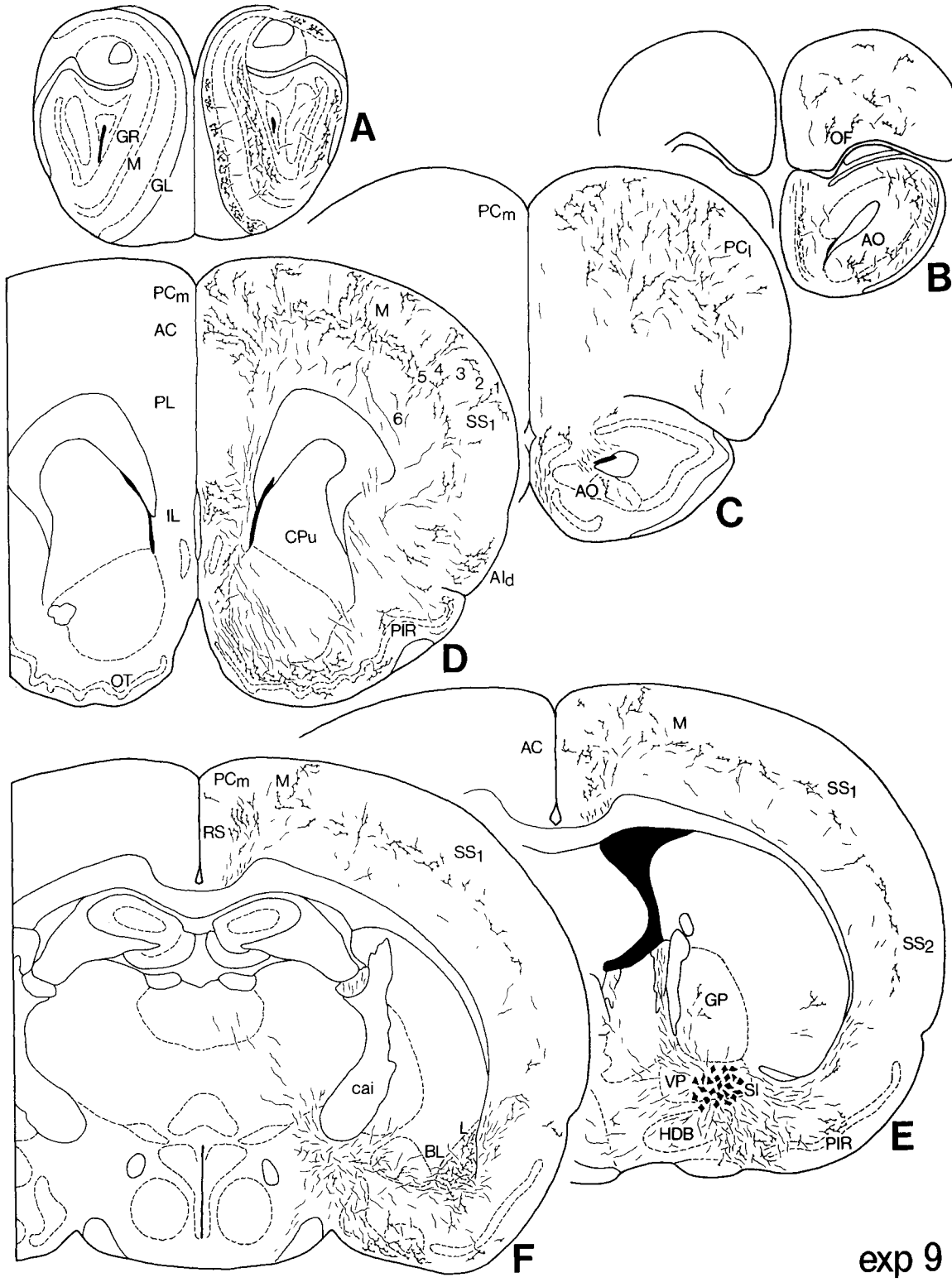


Fig. 6. Coronal sections in an anterior to posterior sequence in which the injection of PHA-L (Expt. 9) is depicted in the MBNa subdivision. The anterogradely labeled fibers in the cortex are shown by fine lines supplied with bud-like thickenings, indicative of terminal presynaptic boutons.

the BL and to a lesser degree in the BM and L. These amygdaloid projections apparently are a consistent feature of output from all subdivisions of the MBN (see Table I).

A final important aspect of cortical projection patterns from the magnocellular basal forebrain is the efferent labeling seen in the cortex after tracer injections in the HDB (Fig. 7). The HDB maintains neocortical projections, which are, however, limited to medial motor cortex and occipital lobe projections. Both cortical target structures are reached by labeled axon bundles that emanate from the site of injection and traverse the septum, where considerable terminations occur, to join the cingulum. At anterior cortical levels, labeled fibers are observed that richly supply input to a mesocortical layer comprising IL, PL and AC. This innervation pattern continues as terminal labeling in the neocortical PCm and the dorsomedial parts of the motor cortex. The labeled fascicles in the cingulum turn caudally over the cc and can be followed over the entire longitudinal extent of AC and RS giving off terminal branches ending in layers I–II and V–VI. PHA-L positive axons at posterior levels move laterally in layer VI of the neocortex to produce fairly dense projections to the visual cortex.

The mesocortical projections originating in the medial HDB are limited to the above-mentioned IL, PL, AC and RS. Agranular insular and perirhinal cortices are only minor recipients of efferent inflow. The projections to allocortical structures are likewise moderate in their extent. There are some efferents towards the superficial layers of the entorhinal cortex, occasional labeling in the hippocampus, but some impressive input to the primary olfactory regions. In particular, the AO appears to be densily innervated.

Detailed structure of efferent projections to the cortex and patterns of lamination

Detailed observation of cortical projection pat-

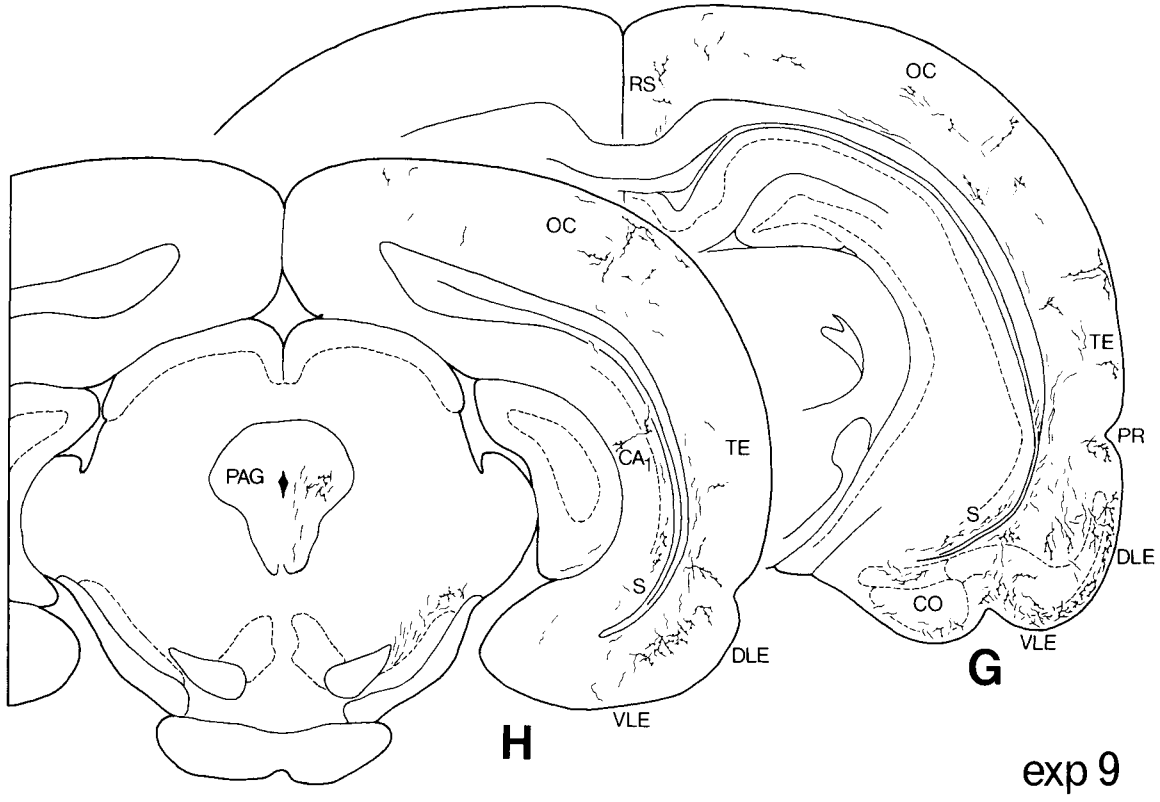


Fig. 6 (continued).

terns reveals a variety of ways in which terminal structures may appear. It can often be seen that labeled fibers are provided with varicosities that are often referred to as boutons 'en passant'. A second type of presynaptic endings occurs as very short side branches from the main fiber trunk, terminating in bud-like thickenings indicative for terminal boutons. This type of termination is a regular feature of long-trajectory axonal systems in the deeper layers of the cortex. The superficial layers often appear as the end

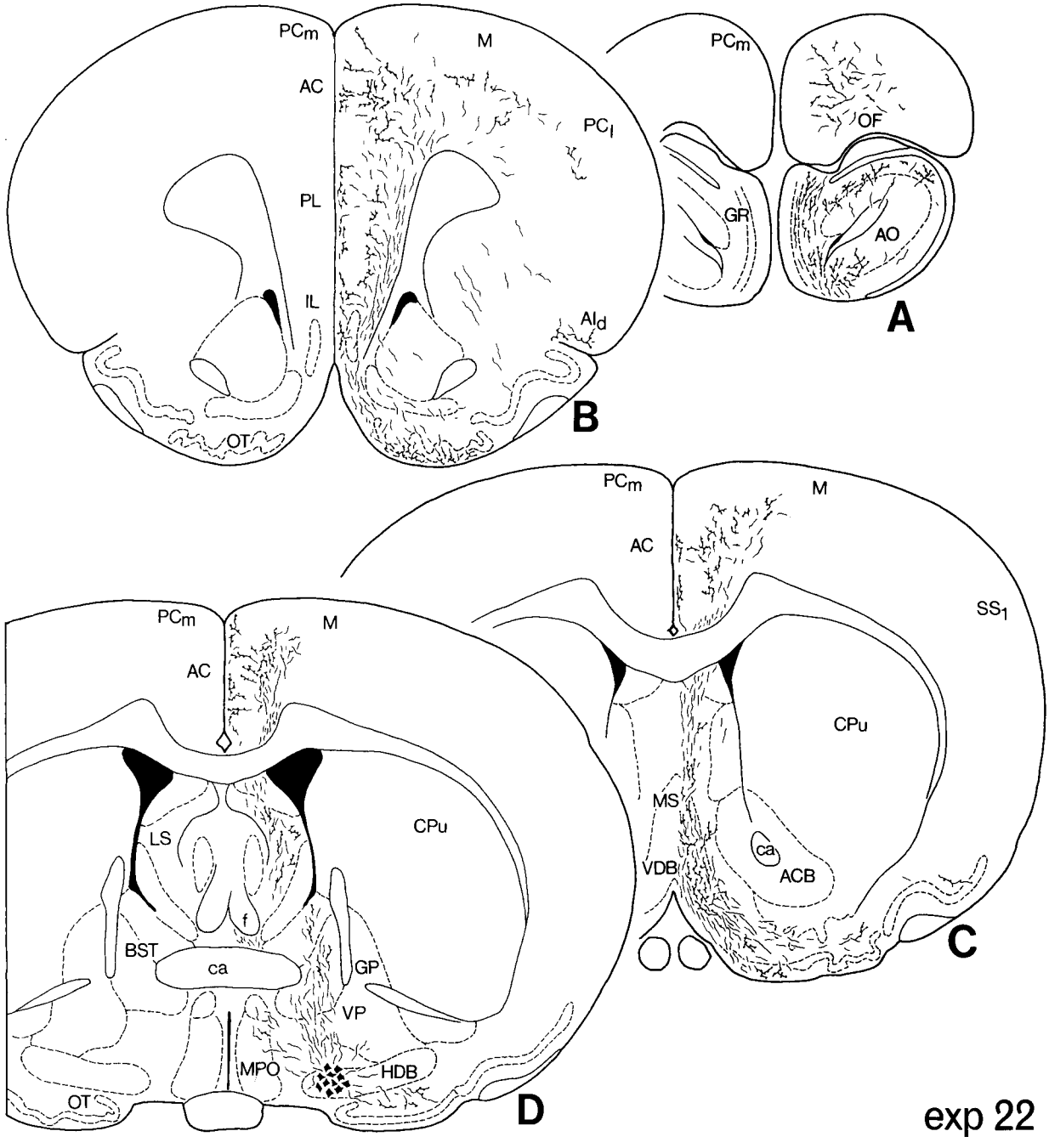


Fig. 7. Transverse charting of PHA-L injection 22 in the medial aspects of the HDB (section D) and the labeling of fibers and endings in the cortex as a result of the tracer injection.

station of long axonal branches after traversing the deeper layers. Here extensive, complicated ramifications are present, carrying large numbers of boutons often arranged as rows of varicosities.

The relation between labeled bouton distribution and cellular arrangements in the target structures can only be related to cell body appearance, due to the nature of the background stain. The greater majority of terminals do not end close to cell bodies, but suggest synaptic contacts with dendritic structures (notably the case in layer I of the cortex). However, in all cortex layers except layer I, intimate contacts appear to exist between terminal boutons and neural somata (Fig. 8B), which suggests intercellular contacts of terminals embracing a receiving cell body.

Apart from interneuronal contacts, there is also evidence of presumed cholinergic terminations onto non-neuronal elements, such as cerebral blood vessels (Fig. 8). The often rich innervation of structures adjacent to the pial surface was commonly observed in the most superficial strata of the entorhinal cortex (Fig. 8).

DISCUSSION

In the present investigation, the projections from the magnocellular basal forebrain areas to cortex and amygdala were studied by anterograde tracing methods with emphasis on the pattern of innervation in the target structures. As a consequence of the nature of the tracing method, a pattern of innervation has appeared with respect to the topographic principles that underlie the MBN-cortical connectivity. In the PHA-L labeled material presented here, two major lines of evidence for topographic organization have become apparent, that also seem to follow certain aspects of cortical evolution.

First, an anterior-posterior differentiation in nucleus basalis projections to the cortex is evident in that the posterior aspects of the MBN provide more efferents to the posterior neocortex than does the anterior MBN region. Similarly the MBNa donates more heavily to the anterior (frontal, parietal) neocortical areas. This topographic arrangement, however, is strongly overshadowed by the fact that all

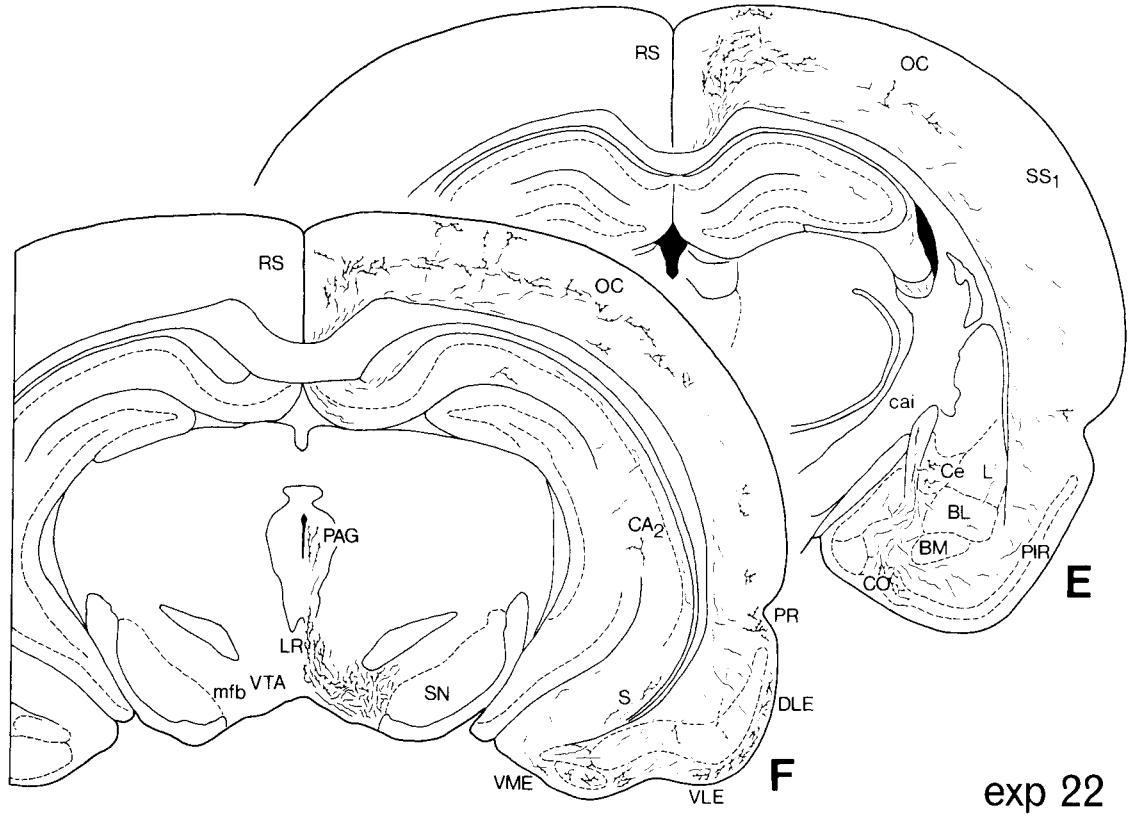


Fig. 7 (continued).

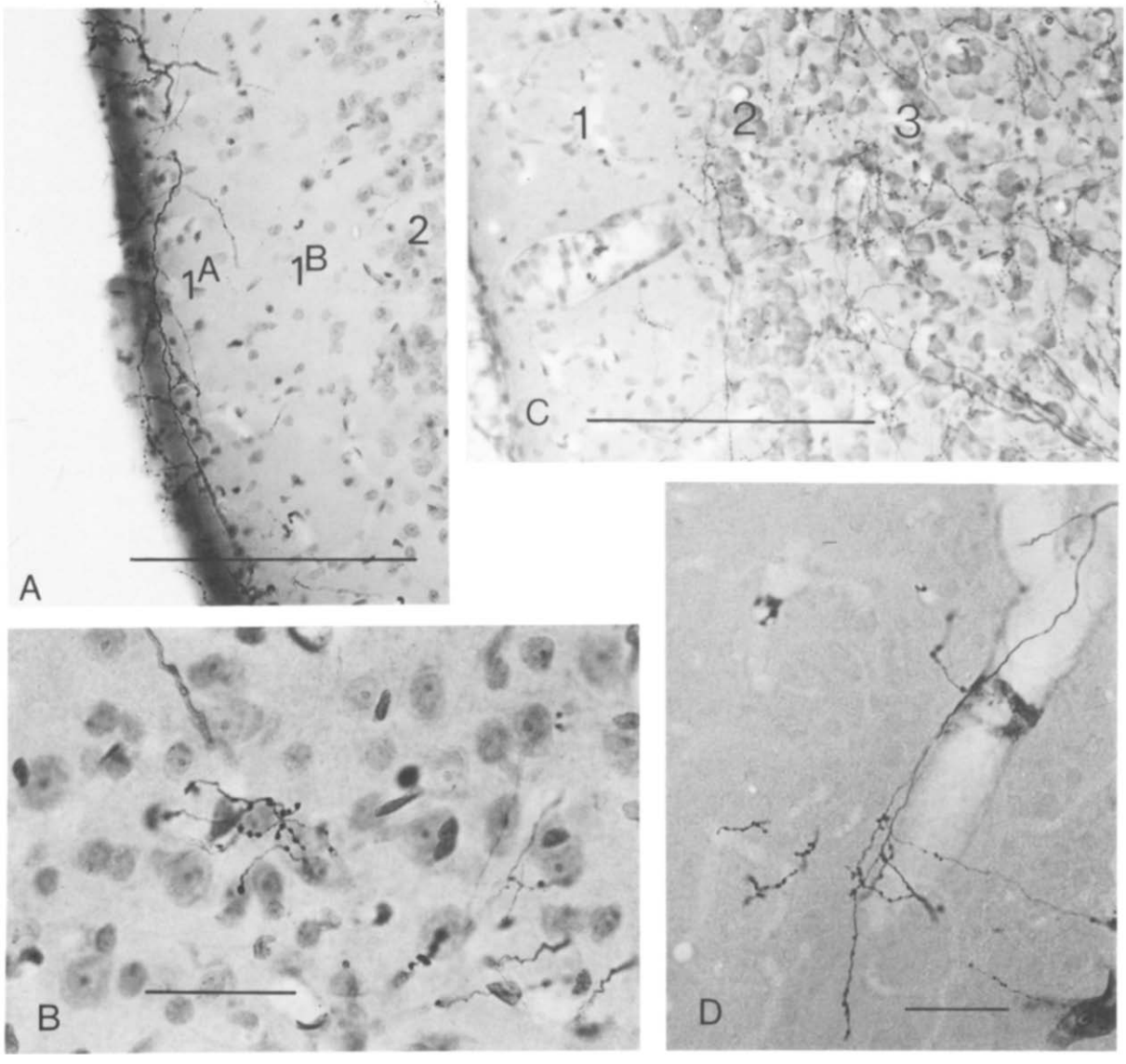


Fig. 8. A: photomicrograph of anterogradely labeled fibers in the most superficial strata of layer I^A in the entorhinal cortex after a PHA-L injection in the ventral SI. B: example of terminal boutons labeling in close contact with a receiving cell body. C: labeling of terminating fibers in layers II and III of the entorhinal cortex after a PHA-L injection in more dorsal aspects of MBNa. D: illustration of PHA-L-positive fibers in close proximity of a blood vessel of the neocortex after a PHA-L deposit in the MBN region. Scale bar in A and C = 200 μm; in B and D = 50 μm.

MBN subdivisions including the MBNp maintain predominant output to frontal-parietal cortices in terms of quantity of anterogradely labeled terminals. In this respect, the present PHA-L data partially conflict with the reported results of retrograde tracer studies. Various studies cite differential labeling in MBNa and MBNp after anterior and posterior cortex injections, respectively[5,34,43,58]. We believe that this discrepancy may be partly explained by differences in the extent of terminal branching of individual neurons to anterior vs posterior cortical areas. It is the impression from our material that single MBN cells projecting to the frontal lobe provide relatively large areas with their presynaptic input. Posterior cortical projections of individual neurons more likely are present as localized columnar patches. This impres-

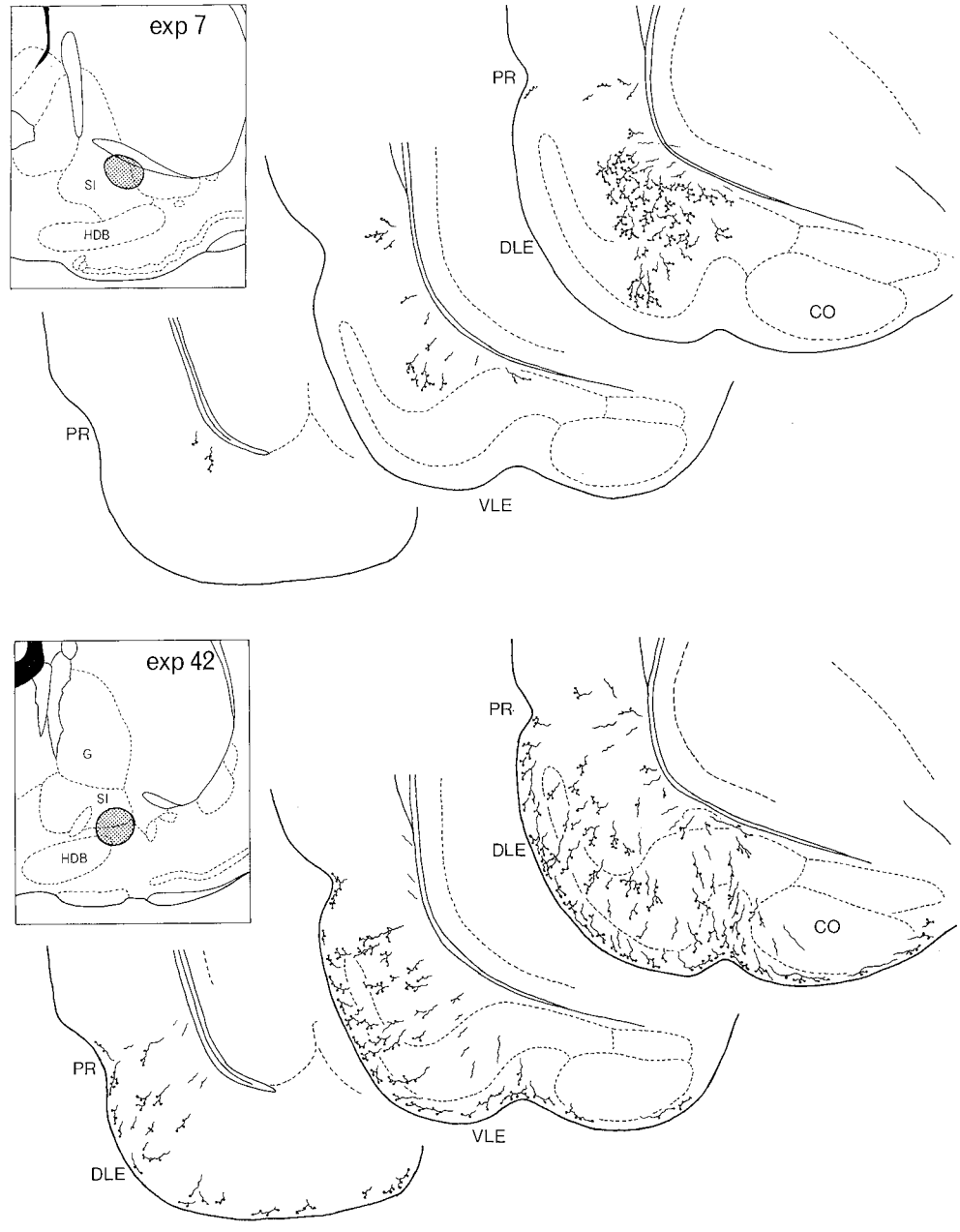


Fig. 9. Differential labeling of efferents in the entorhinal cortex following an injection of PHA-L in the dorsal (Expt. 7) and in the more ventral aspects of the anterior MBN subdivision (Expt. 42).

sion is substantiated by double labeling retrograde tracer experiments that indicate highly localized MBN projections to the more posterior cortex, whereas a number of studies suggest a higher level of collateralization in frontal cortical areas[34,40]. Thus, it is conceivable that the number of MBN neurons involved in innervation of different cortical areas is not related to the extent of cortical projections of the individual cholinergic cell.

Apart from the anterior-posterior topographic arrangement of MBN projections, a second and even more remarkable pattern of topographic organization in the ventrolateral-dorsomedial plane emerges. It was a consistent observation that in all MBNp in-

jections, there was a predominant labeling of efferents in the more lateral aspects both of neo- and mesocortex. Accordingly, there is a rich supply of input from the posterior MBN to PCl, SS_2, SS_1 and TE, and to the mesocortical PR and AI cortices. Moreover, in MBNp cases the medial aspects of M and SS_1 were less densely innervated than in MBNa experiments. The PCm and the medial mesocortical RS, AC, PL and IL are essentially devoid of MBNp input. Anterior MBN injections, on the other hand, result in dense cortical labeling patterns in the more dorsomedial aspects of the cortical mantle. Agranular insular and perirhinal cortex become less densily tracer-positive in favor of the rich innervation of the anterior cingulate, prelimbic, infralimbic and orbitofrontal cortices as the PHA-L injection site moves anteriorly in the MBN. This trend in lateral-medial topography in cholinergic innervation seems to be continued in going from MBNa to HDB cell groups, since the HDB projection is almost exclusively aimed at the medial mesocortex and fails to show a significant neocortical or lateral mesocortical projection.

There are, however, a few exceptions that do not fit into a ventrolateral-dorsomedial topographic pattern. The occipital visual cortex, although localized entirely in the posterior cortex, is not innervated by MBNi or MBNp, but only from the MBNa area and HDB. It is, however, likely that the source of basal forebrain input to the OC originates not only in the HDB, but in the VDB and MS as well which is indicated by labeling of input in OC after PHA-L injections in HDB (Expt. 22), VDB and MS (unpublished observations), which is also reported in previous retrograde tracer studies[5,35,43,44]. Apart from the OC, it is clear that the MBN innervation of the entorhinal cortex is also determined by different organizational principles than those applying to neo- and mesocortex. In spite of its ventral and ventrolateral position, ENT is strongly innervated by MBNi and even more so by MBNa.

Cholinergic vs non-cholinergic cortical projections

The method employed in the present investigation on basal forebrain projections to the cortex does not allow discrimination of cholinergic vs non-cholinergic cortical afferents. Of all basal forebrain neurons in the MBN region that project to the cortical mantle up to 90% appear to be cholinergic, the percentage

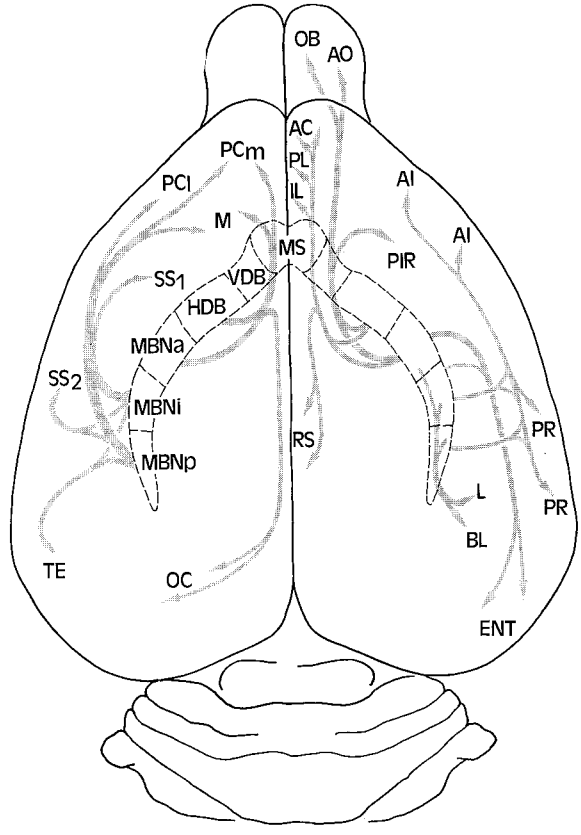


Fig. 10. Diagrammatic survey of the major connections found in this study. At left side of the figure the projections are drawn from the MBN divisions and HDB to the various neocortical areas in a dorsal view of the brain. In the right half of the figure MBN/HDB projections are indicated to the mesocortex, allocortex, olfactory regions and amygdaloid body.

being even higher for the posterior portions of the MBN corresponding to MBNp[36,43]. Consequently, this implies that the anterogradely labeled efferents after PHA-L injections in the MBN region in vast majority are cholinergic in nature. Moreover, the pattern of PHA-L positive efferent fibers and terminals to a considerable extent coincides with the pattern of ChAT immunoreactivity in the rat cortex, notably in the motor areas[21,22]. The ChAT-positive neuritic pattern of subcortical origin in the rodent cortex, however, is obscured by the presence of intrinsic cholinergic interneurons, which may account for the more diffuse arrangement of ChAT in sensory and association areas of the cortex. Comparison of our data with the analysis of Houser et al.[21,22], strongly suggest subcortical MBN terminations in all cortical layers, but with a preference for projections to layers V

and II. In motor areas, the basilar and apical dendrites appear as a major site of termination for cholinergic efferents. The frequently observed axosomatic contacts in our material, do not relate to pyramidal cell bodies but to so far unidentified interneurons of probably stellate type[21]. The differential laminar distribution of MBN projections over superficial vs deep cortical layers, moreover, correlates well with the stratified presence of muscarinic receptors[48]. Superficial layer I–II projections appear to coincide with the presence of M_1 receptors, whereas the deeper layer V and VI projection show an overlap with the distribution of M_2 receptors. This laminar differentiation of cholinergic cortical input and cholinergic receptor distribution may be indicative for a functional differentiation as well, which is subject to further analysis in a behavioral paradigm[49].

Cholinergic terminal innervation of cortical fine vasculature as regularly observed in our tracing material has not been studied or reported by Houser et al.[21,22]. Others[16], however, have described cholinergic innervation of cerebral capillaries and larger vessels which now can be concluded as being at least partly of subcortical MBN origin. Involvement of cholinergic brain circuitry in cerebral vasodilation receives further support from recent data on muscarinic receptor-dependent mechanisms[23].

In several cases described in the present paper the anterograde tracer PHA-L was injected in the MBN region of animals 36 months of age in order to determine the possible effects of aging on the structure of cortical projections. In none of these cases (Table I) however, there was an indication for striking changes in the overall pattern of cortical efferents. Also, on levels of greater detail, the pattern of labeling appeared similar to the innervation in the 3-month-old cases. A more extensive approach in which tracing experiments will be combined with quantitative changes in ChAT-positive labeling of fibers in the cortex and cell bodies in the basal forebrain is currently carried out. Such a combined investigation is thought to be of great importance to study the effects of aging in the subcortical cholinergic innervation of the cortex from the basal forebrain nuclei[37].

Nomenclature of magnocellular basal forebrain neurons

With respect to topographic classification and nomenclature of basal forebrain cholinergic neuronal systems, anterograde tracing of efferents can substantially contribute to the elucidation of questions on anatomical definition. In recent literature on cholinergic forebrain systems, two lines of classification are most in use. First, with reference to the position of cholinergic cell bodies in the basal forebrain, a frequently adapted classification discriminates the MS, VDB and HDB (including a magnocellular preoptic nucleus[7]), and magnocellular nucleus basalis as the major components. This terminology is based both on the topographic position of neuron somata and on connectivity patterns to the cerebral cortex. Based almost solely on the criterion of efferent projections, however, Mesulam et al.[36] proposed an alternative numerical Ch terminology in which the presence of neocortical projections was defined as a prerequisite of Ch4 neurons. In both terminologies, however, it is evident that any classification for magnocellular basal forebrain neurons with respect to their efferent projections to the cortex becomes rather artificial. The fact that both MBNa and HDB maintain projections both to limbic cortical structures and primary olfactory centers indicates that a consistent nomenclature based on efferent projections can not be applied to magnocellular basal forebrain neurons. On the other hand, a mere topographical definition based on more classical cytoarchitectonic criteria can be applied to MBN for the magnocellular neuron contingents in VP and SI, and to HDB for such cells in the horizontal diagonal band and preoptic region. It should be borne in mind then that the latter nomenclature does not hold any meaning for the projection target of its neurons. It has become evident indeed now that magnocellular basal forebrain cell groups from posterior MBN, to MBNa, then to HDB, VDB to MS constitute a more or less continuous cell column in which the output patterns to the cortex gradually shift from neocortex, to meso- (or limbic) cortex, primary olfactory cortex, and finally to allocortex and hippocampal formation (Fig. 10). As a consequence of this gradual change in efferent output targets there are several parts in the magnocellular basal forebrain column that are characterized by a transition in cortical projections. Such a transition zone is clearly present in MBNa and HDB, where the projections gradually shift from limbic cortex to primary olfactory cortex.

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ANATOMICAL ABBREVIATIONS

AC	anterior cingulate cortex
ACB	nucleus accumbens
AId, p, v	dorsal, posterior, ventral agranular insular cortex
AO	anterior olfactory nucleus
BL	basolateral amygdaloid nucleus
BM	basomedial amygdaloid nucleus
BST	bed nucleus of stria terminals
ca	anterior commissure
CA_1/CA_2	cornu ammonis fields 1 and 2
cai	internal capsule
cc	corpus callosum
Ce	central amygdaloid nucleus
CPu	caudate putamen
DLE	dorsolateral entorhinal cortex
ENT	entorhinal cortex
f	fornix
GL	glomerular layer of olfactory bulb
GP	globus pallidus
GR	granular layer of olfactory bulb
HDB	horizontal limb nucleus of diagonal band of Broca
IL	infralimbic cortex
L	lateral amygdaloid nucleus
LR	linear raphé nucleus
LS	lateral septum
M	motor cortex
MBNa, i, p	magnocellular basal nucleus, anterior, intermediate, posterior
MD	mediodorsal thalamic nucleus
mfb	medial forebrain bundle
MPO	medial preoptic nucleus
MS	medial septum
OC	occipital cortex
OF	orbitofrontal cortex
OT	olfactory tubercle
PAG	periaqueductal grey
PCl	lateral precentral cortex
PCm	medial precentral cortex
PIR	piriform cortex
PL	prelimbic cortex
PR	perirhinal cortex
PV	paraventricular hypothalamic nucleus
RE	nucleus reuniens
RS	retrosplenial cortex
S	subiculum
SI	substantia innominata
SN	substantia nigra
SS_1/SS_2	somatosensory cortex 1 and 2
TE	temporal cortex
VDB	vertical limb nucleus of diagonal band of Broca
VLE	ventrolateral entorhinal cortex
VME	ventromedial entorhinal cortex
VP	ventral pallidum
VTA	ventral tegmental area

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