Two Anatomically Specific Classes of Candidate Cholinceptive Neurons in the Rat Olfactory Bulb

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The pharmacohistochemical technique of Butcher (1978) and choline acetyltransferase (ChAT) immunocytochemistry were used to demonstrate the presence, morphology, and differential distribution of 2 classes of acetylcholinesterase (AChE)-positive, ChAT-negative neurons in the rat olfactory bulb. One population of these neurons is located preferentially in a stratum just deep to the mitral cell layer (mcl). These AChE-positive inframitral neurons are significantly larger than the predominant inframitral neuronal type, the granule cell. Inframitral AChE-positive neurons appear to send processes deeper into the granule cell layer and superficially into the external plexiform layer (epi), above the mcl. Neurophysiological experiments reported in a companion article are consistent with the existence of a population of cholinceptive neurons with the location and characteristics of these large inframitral interneurons.

A second class of AChE-positive, ChAT-negative neurons is found exclusively in the glomerular layer. These neurons are located primarily in the periglomerular region and the superficial third of the epi; they are somewhat larger than typical periglomerular cells. Juxtglomerular AChE neurons are smaller than inframitral AChE neurons.

Since there are no neurons in the olfactory bulb that produce ChAT, the synthetic enzyme for ACh, the AChE-positive cells reported here are hypothesized to be cholinceptive neurons for the cholinergic projection from the basal forebrain to the olfactory bulb. Anatomical, physiological, and receptor-ligand binding data are consistent with this interpretation.

All forebrain cortical structures receive projections from a related group of nuclei in the basal forebrain (Mesulam et al., 1983a, b). A significant proportion of the neurons in these projections are cholinergic. Pharmacological blockade of muscarinic transmission results in serious disruption of behavioral function, including loss of spatial memory (Eckerman et al., 1980; Watts et al., 1981); degeneration of the cholinergic component of the projections from basal forebrain to neocortex and hippocampus has been associated with profound cognitive dys-

function and memory loss in human Alzheimer's patients (Whitehouse et al., 1981, 1982; Coyle et al., 1983). These and numerous other observations suggest that cholinergic basal forebrain neurons play an integral role in cortical function. A better understanding of basal forebrain circuitry, therefore, should provide new insights into the principles of cortical function and mechanisms of cortical degenerative diseases.

A significant limitation to our understanding of these systems is that we do not know the synaptic elements upon which basal forebrain afferents terminate in their cortical targets. Chandler and Crutcher (1983) reported that HRP transported from the medial septum is localized in synapses on the cell bodies of granule cells and the somata and dendrites of interneurons, probably basket cells. However, since both cholinergic and GABAergic neurons contribute to the septohippocampal projection (Rye et al., 1984; Amaral and Kurz, 1985; Wainer et al., 1985; Zaborszky et al., 1986), it is not possible to distinguish the targets of cholinergic and GABAergic synapses from such transport studies. Electron microscopic studies of the localization of choline acetyltransferase (ChAT) (Wainer et al., 1984b; Frotscher and Leranth, 1985) demonstrate that cholinergic terminals in the hippocampus and neocortex make synaptic contact with a variety of postsynaptic neuronal types; however, it is not clear from these descriptive studies whether ChAT-positive synapses derive from extrinsic medial septal or from intrinsic cholinergic interneurons.

Physiological studies have provided information about the actions and possible cellular targets of cholinergic synapses in the hippocampus. Application of cholinergic agonists among the dendrites of pyramidal cells inhibits release of transmitter from the terminals of afferent inputs to hippocampus and from hippocampal inhibitory interneurons (Yamamoto and Kawai, 1967; Hounsgaard, 1978; Ben-Ari et al., 1981; Krnjevic, 1981; Krnjevic et al., 1981; Valentino and Dingledine, 1981; Rovira et al., 1983). Application of cholinergic agonists to the pyramidal cell body layer blocks voltage-sensitive K⁺ channels in the pyramidal cells (Krnjevic et al., 1971; Krnjevic and Ropert, 1982; Cole and Nicoll, 1984a, b). Thus, physiological evidence is consistent with the anatomical data in suggesting that the medial septal cholinergic input, at least in part, terminates upon presynaptic terminals of many elements and possibly onto the soma or dendrites of intrinsic hippocampal neurons.

Anatomical and physiological studies to date have not revealed the existence of relatively small numbers of specific neurons which are heavily targeted by cholinergic input, but there is indirect evidence that such cells exist. In the hippocampus, there are interneurons that are strongly stained for acetylcholinesterase (AChE) after lesion of the medial septum or treat-

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ment with di-isopropylfluorophosphate (DFP) (Shute and Lewis, 1967; Zimmer et al., 1983). In the cerebral cortex and hippocampus there are discrete populations of cells that express AChE but not ChAT (Levey et al., 1984).

Thus, it is conceivable that basal forebrain cholinergic systems project primarily or exclusively to specific target neurons in hippocampus and neocortex. However, it has not been easy in these structures to definitely relate candidate cholinergic neurons to other specific cholinergic markers. This is, in part, because both the hippocampus and the neocortex contain intrinsic cholinergic neurons in addition to the extrinsic cholinergic afferents. Because they have not been anatomically related to markers for the cholinergic input, these AChE-positive, ChAT-negative neurons have received surprisingly little attention. The possibility that basal forebrain cholinergic neurons terminate selectively on specific classes of interneurons in their cortical targets, however, has important implications, and further consideration of this hypothesis is warranted.

In the present report we describe 2 populations of neurons in the olfactory bulb that express AChE, but not ChAT. The olfactory bulb receives a projection from the horizontal limb of the diagonal band (HDB) in the basal forebrain that is very similar to the medial septal projections to the hippocampus and basal forebrain magnocellular projections to the neocortex (De Olmos et al., 1978; Macrides et al., 1981; Shipley and Adamek, 1984; Brashear et al., 1986; Zaborszky et al., 1986). Compared with hippocampus and neocortex, the olfactory bulb has a simpler cortical organization (Shepherd, 1970) and its interneuronal connections are better understood. As will be shown in this report, the olfactory bulb, unlike the hippocampus and neocortex, does not contain intrinsic cholinergic neurons. Thus, it is possible to ascribe cholinergic markers to afferents from a single subcortical source. The present report describes 2 anatomically distinct populations of AChE-positive, ChAT-negative interneurons and presents evidence for considering these neurons cholinergic. Based on this evidence, it is worthwhile to consider the possibility that basal forebrain afferents terminate on specific cholinergic neural subpopulations in other cortical structures.

Materials and Methods

AChE histochemistry. To visualize the locations and morphologies of AChE-positive neurons in the olfactory bulb, the pharmacohistochemical method of Butcher (1978) was used. In normal AChE-stained material, the cell bodies and processes of AChE-positive cells are obscured by dense neuropil staining. If, however, the animal is treated with a sublethal dose of an irreversible cholinesterase inhibitor, only residual levels of AChE or AChE synthesized after the cholinesterase treatment will be present when the animal is sacrificed. At an appropriate time after the inhibitor, those cells which contain AChE can be seen clearly without the interference of fiber staining.

The irreversible cholinesterase inhibitor, soman (pinacolylmethylphosphonofluoridate; 1.5 mg/kg), was injected intramuscularly. Survival times ranged from 30 min to 60 d. At the selected survival times, the animals were deeply anesthetized with pentobarbital and perfused intracardially with fixatives. The brain was then processed for AChE histochemistry according to the method of Van Oorteghem and Shipley (1984). A normal brain was always processed in the same reagent solutions as the experimental brain to monitor fluctuations in reaction conditions.

ChAT immunohistochemistry. Cholinergic fibers and terminals were labeled by immunohistochemistry using monoclonal antibodies (Eckenstein et al., 1981; Eckenstein and Thoenen, 1982; Sofroniew et al., 1982; Eckenstein and Sofroniew, 1983; Levey et al., 1984). Rats were perfused with a brief saline flush, followed by a 2 min perfusion with 0.1% glutaraldehyde in 0.1 M phosphate buffer (PB), a 30 min perfusion

with 4% paraformaldehyde, 0.1% glutaraldehyde fixative in PB, and a 30 min perfusion with 4% paraformaldehyde in PB. The brains were removed and placed in the final fixative solution for 1 hr; they were then transferred to 20% sucrose in PB and stored at 4°C for 24–48 hr. The brains were cut at 30 μ m on a freezing microtome and the sections collected in PB. Improved immunocytochemical staining was obtained when solutions and animals were maintained on ice during the perfusion procedures.

To label cell bodies containing ChAT, the sections were processed by the PAP method using a double-bridge technique (Ordonneau et al., 1981). The sections were (1) incubated for 1–4 hr in PBS containing 2% normal goat serum and 0.1–0.25% Triton X-100; (2) rinsed in PBS (2 $\times$ 15 min); (3) incubated in primary antibody (1:25 dilution) overnight on an agitator at 4°C; (4) rinsed in PBS (2 $\times$ 15 min); (5) incubated in antimouse IgG containing 0.0–0.25% Triton X-100; (6) rinsed in PBS (2 $\times$ 15 min); (7) incubated in PAP (Sternberger) conjugates for 1 hr; (8) steps 4–7 were repeated (double bridge), and the sections were then reacted for 10–20 min in 0.05% diaminobenzidine dihydrochloride (Sigma) and 0.04% H₂O₂, then rinsed in PBS (3 $\times$ 15 min). The sections were mounted on gelatin-subbed slides and air-dried. In some cases, an osmium intensification procedure was used to enhance the contrast of the stained cells: The mounted sections were defatted, rehydrated, then dipped for 1 min in 1% OsO₄, rinsed, cleared, and coverslipped.

Results

Normal AChE staining

The normal pattern of AChE staining in the rat is shown in Figure 1. There is heavy staining in the glomerular (gl), external plexiform (epl) and internal plexiform (ipl) layers. In the epl, the staining forms 2 bands: The deepest third of the epl and a narrower band just below the glomerular layer are heavily stained; an intermediate band contains relatively little reactivity. The ipl is heavily stained, and there are intensely stained bands in the granule cell layer (gcl). These bands lie between layers of granule cell soma. AChE staining is heaviest in gl, the superficial and deep portions of epl, and in the ipl. The mitral cell layer (mcl) is notably lacking in staining except for unbranched thin processes passing from the ipl to the epl.

Examination of this material at higher magnification reveals structures suggestive of dendrites, fibers, and terminals, but the density of neuropil staining is too great to allow pre- and post-synaptic structures to be distinguished.

Pattern of ChAT-labeled fibers and absence of ChAT-labeled cell bodies in the olfactory bulb

ChAT activity in the olfactory bulb decreases sharply following lesions of cholinergic neurons in HDB; this suggests that there are no neurons in the olfactory bulb that synthesize this enzyme (Macrides et al., 1981). Such studies cannot, however, rule out the possibility of a small population of ChAT-positive cells. To determine whether such neurons are present, we examined olfactory bulbs stained immunocytochemically with antibodies directed against ChAT. In no case were ChAT-positive neurons found. This is in agreement with other studies that did not report ChAT-positive neurons in the olfactory bulb (Sofroniew et al., 1982; Armstrong et al., 1983; Houser et al., 1983; Rye et al., 1984; Wainer et al., 1984a). Negative immunocytochemical findings must be interpreted with caution since the sensitivity of this method is variable. However, in all our cases, parallel sections through the hippocampus and neocortex reacted at the same time with the same antibodies consistently stained intracortical neurons similar to those reported by others (Sofroniew et al., 1982; Armstrong et al., 1983; Houser et al., 1983; Rye et al., 1984). Thus, our immunocytochemical methods were adequately sensitive to stain known cortical cholinergic neurons. Even with a monospecific polyclonal ChAT antibody that stained

Figure 1. Normal pattern of AChE staining in the olfactory bulb. *A*, Fifty micron section reacted for AChE according to the method of Van Ooteghem and Shipley (1984). Note heavy labeling in the gl and in a layer just below the mcl and the banded appearance of labeling in the gcl. *B*, Higher-magnification view of AChE staining in olfactory bulb layers. Abbreviations: *onl*, olfactory nerve layer; *gl*, glomerular layer; *epl*, external plexiform layer; *mcl*, mitral cell layer; *ipl*, internal plexiform layer; *gcl*, granule cell layer. Scale bars: *A*, 500 µm; *B*, 100 µm.

abundant cholinergic fibers in the olfactory bulb (Shipley et al., in preparation), we were unable to detect ChAT-positive cell bodies. We conclude, therefore, that the olfactory bulb lacks intrinsic cholinergic neurons.

AChE staining after anticholinesterase treatment

Following soman treatment, AChE staining returns to the olfactory bulb over a period of several days. Almost immediately, however, AChE is detectable in cell bodies within the bulb. In a few cases some cells were stained at 1 hr after intoxication. Staining variability after such short survivals probably reflects differences in the quantity of anticholinesterase reaching the brain. By 8 hr after intoxication, in nearly all cases, there are numerous labeled cell bodies scattered chiefly in a stratum below the mitral cells and in the gl. At longer survival times, staining returns to fibers in the inframitral stratum containing the AChE-positive neurons and visualization of individual cell bodies becomes progressively more difficult. By 14 d after treatment, some animals show isolated cell bodies with short processes visible, but in most cases the return of AChE fiber staining is sufficient to obscure cell bodies. By 24 d, the material approaches

the normal staining density, in most cases, and AChE-positive neurons are obscured by extensive fiber labeling.

AChE-positive cells are confined to 2 distinct anatomical layers in the olfactory bulb: (1) One population is present in a relatively discrete band just below the mcl, and we will refer to these as *inframitral AChE neurons*; (2) the second population is confined to the gl and the superficial third of the epl near the gl; we will refer to these as the *juxtglomerular AChE neurons*. AChE-stained neurons were never observed in the middle or deep parts of epl.

In a very few cases, there was a weak, diffuse, silver deposit in the soma of mitral and tufted cells. This deposit was clearly not the typical AChE reaction product, and the cause of this unusual reaction is not known. It may, however, result from staining of damaged or dying cells by the silver-intensification step of the processing, since this procedure is similar to that used to label degenerating cells in classic silver degeneration methods. This artifactual staining may account for earlier reports of AChE activity in olfactory bulb mitral cells (Hunt and Schmidt, 1978a). Whatever the cause of this occasional staining, it is clear that the mitral cells do not express AChE: In our

Figure 2. Photomicrograph of AChE-positive cells below the mcl. Twenty-four hour survival. Scale bar, 50 μ m.

normal AChE material the mitral cells are consistently and notably lacking in AChE staining. This is in accord with the histochemical observations of Carson and Burd (1980) in the mouse, Macrides et al. (1981) in the hamster, and with the microdissection biochemical studies of Godfrey et al. (1980) in the rat.

Inframitral AChE-positive cells

The locations and shapes of the inframitral AChE cells are indicated in Figure 2, which is a photomicrograph of an olfactory bulb section from an animal that had been treated with soman 24 hr prior to sacrifice. It is apparent that the material is lacking AChE activity except for the heavily labeled cell bodies scattered in a stratum deep to the mcl.

To illustrate the range of shapes and sizes of the inframitral cells and their relationships to the mcl, computer-assisted reconstructions of several microscope fields are presented in Figure 3. As can be seen, inframitral AChE cells are relatively uniform in size. They are substantially larger than granule cells: In the cases from which the drawings were made, the mean dimensions of inframitral AChE neurons were 10.9 (range, 6–30; SD 3.9) by 16.3 (range, 9–30; SD 6.30) μ m ($n = 41$). This compares with a mean diameter of 8.3 μ m (range, 5.6–10.8 μ m) reported for the soma of granule cells after intracellular injections of HRP (Mori and Kishi, 1982).

Although a few cells are scattered deeper in the gcl, most of the inframitral AChE-positive cells are located in a relatively narrow band just deep to the ipl. This restricted distribution pattern is illustrated in Figure 4, which is a histogram showing the total number of cells in 8 sections as a function of depth below the mcl. The inset in Figure 4 shows the location of all cells in a line drawing of a typical section. In these sections, the distance from mcl to the ependymal zone was about 300 μ m; thus, the axis shown approximately represents the thickness of the gcl. In dorsal and ventral parts of the bulb, the mcl is further

from the ependymal layer than it is on the medial and lateral sides of the bulb. Thus, the AChE inframitral neurons appeared to maintain their position relative to the mcl rather than to the ependymal layer in all parts of the bulb.

After survival times of several days, when labeled fibers and terminal-like staining had begun to return to the inner part of the epl, it was frequently possible to follow processes traveling vertically from the gcl to the epl; it was occasionally possible to establish continuity between these processes and a particular AChE-stained cell body. Such fiber staining was seen at survival times too short for staining in afferent cholinergic fibers to have returned. This suggests that the stained processes in the epl belong to the inframitral cells.

Juxtaglomerular AChE cells

Cholinesterase staining in the gl usually returned more rapidly than that in deeper layers. In many cases, staining was extensive in the gl when there were no labeled inframitral cells, and no staining in the inner part of epl. In all such short survival time cases, staining in the middle and deep parts of the epl was notably absent. We were never able to trace fibers traversing the epl at such short survival times. These observations strongly suggest that glomerular AChE is not dependent on transport from the inframitral cells or cholinergic afferents; there must be a population of AChE-producing cells in the gl that is responsible for this activity.

We were unable to define the shapes of these juxtaglomerular AChE-positive cells with the same precision as the inframitral cells because the thick dendritic processes of these neurons recovered almost as rapidly as the cell bodies and multiple neurons were stained in each glomerulus (Fig. 5D). Thus, the shapes of the cells were frequently obscured. For this reason we could not determine with certainty whether these neurons represent a morphologically homogeneous group. These neurons comprise only

Figure 3. Computer-assisted tracings of inframitral cells from a typical case (1 d survival). Dashed lines represent approximate top and bottom of mcl; dotted line represents location of ipl.

a subpopulation of the juxtglomerular neurons because in normal, non-AChE-inhibited material, many juxtglomerular neurons lack AChE reaction product.

When juxtglomerular AChE-positive cell bodies could be clearly visualized (Fig. 5C), they were located predominantly in the deep half of the gl, but a few labeled cells were located in the superficial epl. A representative sample of juxtglomerular cells was measured; mean cell size was 14 (SD 4.8) $\times$ 9 (SD 1.4) μm . These dimensions are somewhat larger than reported for periglomerular cells ($10 \times 8 \mu\text{m}$) but are close to the dimensions reported for external tufted cells ($14 \times 11 \mu\text{m}$) and the short axon cells found in the juxtglomerular region (Davis and Macrides, 1983).

Numbers of AChE-positive cells

The number of visible inframitral cells varied appreciably between cases; we assume that this results from variations in both the completeness of the initial AChE inhibition and rate of return of enzyme. In most animals there was a gradation in the intensity of staining of individual neurons in the population, with some neurons staining intensely and some only weakly labeled. This suggests that the number of identifiable cells increases with the extent of recovery, but, as recovery is also accompanied by the return of dense neuropil staining, there is a point beyond which an accurate assessment of the number of AChE-positive cells becomes impossible. For this reason we can give only a lower limit on the number or density of the inframitral AChE neurons. In the case used to construct Figure 4, the mean number of cells per section was 20.7 (8 sections, SD = 7.3). There are up to 150 such sections in the olfactory bulb;

thus, the number of visible inframitral cells in this case would calculate to about 3100. In cases in which there was more substantial recovery of staining there appeared to be a substantially higher density of cell bodies. In another case in which there was more extensive return of AChE staining, the mean number of visible inframitral cells per section was 63.7 (7 sections, SD = 16.9 ; range, 45–87). In this case, the total number of these cells in the bulb would be about 9500. These numbers may be compared with the number of mitral cells, which is about 50,000 (rabbit: Allison and Warwick, 1949; rat: Meisami and Safari, 1981). In a companion electrophysiological article we suggest that inframitral AChE neurons mediate (via granule cells) a profound inhibition of mitral cells. The ratio of one AChE neuron to 5 mitral cells is sufficiently low to make this hypothesis plausible.

The number of juxtglomerular AChE-positive cells is likewise uncertain, but there are probably significantly more of these than the inframitral AChE-positive cells. We estimate that there are 4–8 AChE cells per glomerular profile. There are approximately 80 glomerular profiles in a typical section. Thus, there are 300–600 juxtglomerular AChE-positive cells in a typical section; if there are 150 sections in the bulb, the total number of such cells in the olfactory bulb is in the range of 45,000–98,000.

Discussion

The present results demonstrate that there are interneurons in the olfactory bulb that express AChE but not ChAT. These neurons comprise 2 distinct subpopulations that are restricted to 2 specific sublayers of the main olfactory bulb. Neurons with-

in each subpopulation are morphologically similar to each other but are markedly different from neurons in the other subpopulation.

Comparison of AChE-positive cells with cell types identified by Golgi studies

In the region where the inframitral AChE-positive cells are concentrated, several types of interneuron have been defined by Golgi studies (Schneider and Macrides, 1978). Although it is of interest to speculate that the AChE-positive inframitral cells coincide with one or more of these classes of interneurons, it is difficult to determine which of these classes of neuron is most similar to the AChE-positive cells because delineation of morphological features in our AChE material is not so complete as in Golgi staining. We cannot, for example, see whether the processes of our neurons have spines, nor can we consistently see enough of the dendritic arbors to compare our data with published Golgi profiles. The AChE-positive neurons differ enough in size, morphology, and distribution from granule cells that they are clearly not granule cells. Schneider and Macrides (1978) describe 4 other classes of interneuron which are located in or below the mcl. The laminar distributions of the Golgi-defined inframitral interneurons differ significantly, however. Cajal cells and horizontal cells are restricted to the ipl and part of mcl. Golgi cells occur in part of mcl and throughout most of gcl; Blanes cells are distributed through most of gcl but avoid the mcl. The inframitral AChE-positive cells reported here are mainly, but not exclusively, concentrated in the ipl and the superficial part of the gcl, but were rarely seen in the mcl. This distribution does not exactly match that of any of the Golgi-defined classes, although the Cajal and horizontal neurons are the most reasonable candidates.

The juxtglomerular AChE neurons are larger ($14 \times 9 \mu\text{m}$) than typical periglomerular cells, which are approximately $8 \mu\text{m}$ in diameter (Schneider and Macrides, 1978). The AChE neurons are similar in shape and distribution to external tufted cells, many of which are dopaminergic (Davis and Macrides, 1983; Baker, 1986a, b). The only type of interneuron described by Schneider and Macrides (1978) that might correspond to the juxtglomerular AChE-positive neurons are the superficial short axon cells.

There is good evidence that there are no cells in the olfactory bulb that produce ACh (Macrides et al., 1981; this report); thus, the AChE-positive cells are probably not cholinergic interneurons. It has been suggested (Greenfield, 1984) that AChE may have functions unrelated to cholinergic transmission; however, in the olfactory bulb, the concordance of AChE patterns with other cholinergic markers argues strongly that the primary function of AChE in the olfactory bulb is hydrolysis of acetylcholine. If the neurons described in this report are, in fact, associated with cholinergic transmission in the olfactory bulb, then they are probably postsynaptic to cholinergic terminals. Several lines of anatomical and physiological evidence are consistent with this hypothesis.

The cell bodies of inframitral AChE-positive neurons are located just deep to the mcl. These neurons appear to send processes both deep into the gcl and superficially into the inner third of the epl. This distribution of processes clearly matches the normal distribution of AChE (this report) and of afferent ChAT-positive fibers (Shipley et al., 1986). The histochemical localization of these cholinergic markers is entirely consistent with the elegant microbiobiochemical assay studies of Godfrey et

Figure 4. Distribution of AChE-positive inframitral cells. The distance of all labeled cells from the inner edge of mcl was measured in 8 sections through the olfactory bulb. The histogram represents the total number of cells in those 8 sections. Most cells are between 25 and 100 μm below the mcl. Seven day survival. *Inset*, Tracing of a typical bulb section. The location of all clearly visible inframitral AChE-positive cells in that section is represented.

al. (1980) in which AChE and ChAT levels were biochemically assayed in small laminar and sublaminar samples of tissue microdissected from the rat olfactory bulb. These investigators showed that levels of ChAT and AChE are highly correlated and that both enzymes have very high concentrations in the deep and superficial most parts of the epl and the ipl and moderate levels in the gcl. Tracers injected into the nucleus of the diagonal band, the source of cholinergic inputs to the olfactory bulb, produce strong anterograde labeling in the superficial and deep parts of the epl and the ipl and moderate labeling in the gcl (hamster: Macrides et al., 1981; rat: Shipley et al., 1986).

There is a similar concordance of anatomical and histochemical evidence for the glomerular AChE-positive neurons. There is heavy AChE labeling in the gl (Fig. 1, this report; Carson and Burd, 1980; Van Oorteghem and Shipley, 1984). Biochemically measured levels of both ChAT and AChE are higher in the gl than in any other part of the olfactory bulb (Godfrey et al., 1980). Tracers injected into the nucleus of the diagonal band produce strong anterograde labeling in the gl (hamster: Macrides et al., 1981; rat: Shipley et al., 1986), and there is a high density of ChAT-containing afferent fibers in the glomeruli (Shipley et al., 1986; and unpublished observations).

In the olfactory bulb binding of α -bungarotoxin (α -BT) is confined to the glomerular region (Hunt and Schmidt, 1978a, b; Schwartz et al., 1982; Blaha et al., 1984). The relation between

Figure 5. AChE activity in glomerular region following soman treatment. *A*, Normal Nissl material (50 μ m sections). *B*, AChE in a normal, untreated animal. AChE histochemical reaction is weaker than in Figure 1, allowing visualization of AChE-containing structures within the gl. Fiber structure in this normal animal is similar to that in the treated animals, although less detail can be seen. *C*, AChE-positive cells (arrows) in the gl 24 hr after soman treatment. *D*, AChE stain in the gl 24 hr after AChE inhibition. Recovery in this case is more extensive. Note that cell bodies can be identified, but their morphology is less clear due to the extensive staining of dendrites. Note also the lack of staining in the epl. The nearly simultaneous recovery of AChE in the dendrites and cell bodies of these neurons was a common occurrence (see Results). Scale bars: *A*, 50 μ m; *B*, 25 μ m; *C*, 50 μ m; *D*, 25 μ m.

α -BT and nicotinic receptors in the CNS is not entirely clear (Schwartz et al., 1982). In the glomeruli of the olfactory bulb, however, α -BT binding coincides anatomically with a high concentration of other cholinergic markers and the absence of appreciable levels of scopolamine or QNB binding (Blaha et al., 1984; Blaha and Shipley, 1987). Thus, it is possible that there are nicotinic-like receptors in the glomeruli and that the juxtglomerular AChE-positive neurons have predominantly nicotinic synapses.

By contrast, there is no binding of α -BT in the epl, ipl, and gcl, but there is dense binding of muscarinic agonists (Blaha et al., 1984; Blaha and Shipley, 1987). Thus, the inframitral neurons might have predominately muscarinic synapses. The muscarinic binding sites and other cholinergic markers, however, extend more deeply into the gcl than do the inframitral AChE-positive cells. It is possible that these deeper muscarinic binding sites in the gcl are associated with presynaptic cholinergic terminals or centrifugal afferents that terminate in the gcl since there is considerable physiological evidence for cholinergic presynaptic actions in the gcl (Nickell and Shipley, 1987b, c, and in preparation) as in the hippocampus (Yamamoto and Kawai, 1967; Hounsgaard, 1978; Ben-Ari et al., 1981; Krnjevic, 1981; Krnjevic et al., 1981; Valentino and Dingledine, 1981; Cherubini et al., 1982; Rovira et al., 1983).

Implications for function of cholinergic systems

In summary, the combined results of histochemical, tract tracing, and receptor ligand binding experiments suggest that there are 3 primary sites of cholinergic synaptic action in the olfactory bulb: (1) the gl and the immediately subjacent superficial 10% of the epl, (2) the zone comprising the ipl, the deep part of the epl, and (3) gcl. The 2 classes of AChE-positive, ChAT-negative interneurons and their processes are, thus, well positioned to be the postsynaptic targets of cholinergic synapses in these sublayers. It is also probable that the cholinergic afferents terminate on presynaptic elements in the gcl (Nickell and Shipley, 1987b, c, and in preparation).

The 2 candidate cholinceptive neurons described here are positioned to influence 2 distinct levels of neural processing in the olfactory bulb. The juxtglomerular AChE neurons have rich dendritic arbors in the glomeruli. In this region of the olfactory bulb the major neuronal elements are the terminals of the primary olfactory neurons, the apical dendritic arbors of the mitral and tufted cells, and other periglomerular neurons. Thus, cholinergic inputs to the glomerular layer could influence olfactory processing at the first level of synaptic integration.

Inframitral AChE neurons probably do not appreciably influence physiological activity at the level of the glomeruli because there are no known inframitral interneurons with axons or dendrites that reach the gl. In a companion paper we demonstrate that stimulation of the nucleus of the diagonal band causes a significant synaptic potentiation and completely inhibits the spontaneous firing of mitral cells. Analysis of the HDB field potential suggests that there may be a class of inframitral excitatory interneurons that are excited by the HDB input and activate granule cells, leading to mitral cell inhibition. If this hypothesis is correct, there are both anatomical and physiological grounds for considering that the inframitral neurons are cholinceptive targets of HDB input and that these neurons exert their influence via granule cells. Thus, inframitral AChE neurons may function by operating at the second major level

of synaptic integration in the olfactory bulb—granule-mitral cell interactions.

Conceivably, both classes of AChE neurons influence mitral cells but in different ways. Mitral cells have 2 distinct dendritic branches. The principal apical dendrite extends generally without branching through the epl to ramify specifically in a single glomerulus. AChE-positive juxtglomerular neurons may influence this glomerular dendritic arbor. The secondary dendrites of mitral cells ramify in the epl, where they receive extensive inhibitory synapses from granule cells. HDB excitation of inframitral AChE neurons and their subsequent activation of granule cells would account for the inhibition of mitral cell spontaneous firing (Nickell and Shipley, 1987a, 1988). Therefore, inframitral AChE neurons may influence the activity of mitral cells via activation of granule cell inhibitory synapses on the secondary lateral dendrites of mitral cells and juxtglomerular AChE neurons may influence mitral cells by acting on the primary apical dendrites in the glomeruli. Physiological experiments to determine the potential cholinergic influence of juxtglomerular AChE neurons on mitral cell principal dendrites have not been done, but the present analysis forms the basis for several specific predictions: (1) Cholinergic agonists applied at the gl should modulate the effect of olfactory nerve stimulation on mitral cell activity, (2) this action should be mediated by nicotinic synapses, (3) cholinergic agonists applied in the ipl should excite an interneuron via a muscarinic receptor, and (4) this muscarinic action should modify synaptic events on the lateral, nonglomerular dendrites of mitral cells and thus not directly influence transmission from the olfactory nerve to mitral cells.

In the hippocampus and neocortex, there are also AChE-positive, ChAT-negative neurons that are similar in size and morphology to the inframitral neurons described here; thus, such neurons may be a common feature of other cortical structures innervated by basal forebrain cholinergic neurons. These cells do not appear to be especially numerous; however, their physiological functions might still be significant (Nickell and Shipley, 1987a, 1988). Hence, further evidence for specific classes of cholinceptive target neurons could have important implications for our understanding of cholinergic modulation of cortical activity. The existence of specific classes of cholinceptive target neurons might also have significance for our understanding of the perturbations of cholinergic inputs to cortex in Alzheimer's disease—the loss of cholinceptive target neurons might be an initial event that results, secondarily, in the degeneration or shrinkage of afferent cholinergic neurons (Sofroniew et al., 1987).

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