CHOLINE ACETYLTRANSFERASE AND ACETYLCHELINESTERASE CONTAINING PROJECTIONS FROM THE BASAL FOREBRAIN TO THE AMYGDALOID COMPLEX OF THE RAT

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SUMMARY

The origin of the cholinergic innervation to the amygdaloid complex was investigated with the use of acetylcholinesterase (AChE) histochemistry and choline acetyltransferase (ChAT) assay of microdissected nuclei. Visualization of AChE-positive neurones in the ventral forebrain was facilitated by pretreatment of rats with 1.5 mg/kg di-isopropyl phosphofluoridate (DFP). The AChE-positive neurones in the ventral forebrain are distributed in a continuous system from the septum through the lateral preoptic area to the entopeduncular nucleus caudally. Knife cuts or kainic acid injections (1.5 μg/μl) placed in the lateral preoptic area resulted in substantial depletion of the AChE and ChAT content of the amygdala nuclei. Kainic acid injections (1.5 μg/1 μl) in the diagonal band area or cuts through the stria terminalis dorsally did not significantly modify the AChE staining or ChAT content of the amygdala (although diagonal band injections partially depleted the hippocampus of ChAT). Knife cuts severing both the so-called ventral pathway and the stria terminalis did not produce significantly greater ChAT depletion in the amygdala than those produced by the knife cuts or kainic acid injections in the lateral preoptic area. Parasagittal knife cuts undercutting the lateral pyriform cortex also failed to modify the AChE or ChAT content of the amygdala, but they depleted the undercut cortex of both ChAT and AChE; AChE-positive material accumulated ventrally and medially to the knife cut. It is suggested that the major source of the cholinergic innervation of the amygdala is the magnocellular AChE-positive neurones in the lateral preoptic area and adjacent regions of the ventral forebrain.
INTRODUCTION

Knowledge about the distribution of acetylcholinesterase (AChE) and choline-acetyltransferase (Chat) within the amygdaloid complex of the rat comes mainly from the observations of Shute and Lewis, Jacobowitz and Palkovits, and Ben-Ari et al. The object of this present study was to extend these investigations and, by the use of appropriate lesions combined with microdissection of amygdala nuclei for determination of Chat content and by the use of AChE histochemistry, to determine the origin of the cholinergic projections to the amygdala. In their paper on the ascending cholinergic system, Shute and Lewis suggested that the probable cells of origin of the cholinesterase-positive projections to the amygdala were in the lateral preoptic/anterior amygdala area. In this study we used the technique of Butcher et al., involving di-isopropyl phosphofluoridate (DFP) pretreatment to facilitate visualization of AChE-positive neurones in the ventral forebrain. Once the location of these AChE-positive neurones had been determined, we placed microknife lesions lateral to these cells or injected the neurotoxin kainic acid, in an attempt to interrupt or lesion cholinergic neurones or their axons projecting to the amygdala. The results obtained suggest that AChE-positive neurones in the lateral preoptic area, and globus pallidus, belonging to the magnocellular nuclei of the forebrain (MNFB) (see Divac for review), represent the major sources of the cholinergic innervation of the amygdala.

METHODS

Male albino Wistar rats (280–320 g) were anaesthetized with Equithesin (3.0 ml/kg) and placed in a Kopf stereotactic apparatus. Microknife lesions passing rostro-caudally through the lateral medial forebrain bundle (an area corresponding to part of the ventral amygdofugal pathway) or undercutting the pyriform cortex were made as described in detail by Emson et al. Hemisections through the caudal hypothalamus and MFB were made at 3.0 mm anterior to the interaural line. These cuts, like those undercutting the pyriform cortex or passing through lateral MFB, were made using a knife consisting of a 30-gauge guide cannula bent at one end in such a way that a 0.13 mm diameter tungsten wire forced through the cannula would extend in the direction of the curved end. The knife cut was made by lowering the cannula with the extended wire until the top of the cannula was seen to bend as the wire contacted the bone at the floor of the skull. Then the wire was withdrawn and the cannula lifted out of the brain. As indicated the knife cut designed to undercut the pyriform cortex extends dorsoventrally to the base of the brain (see for example Fig. 2A or B). It separates (undercuts) the bulk of the pyriform cortex, lateral to the cut, from the amygdala; however, a small part of the pyriform cortex medial to the cut is undamaged. Only the pyriform cortex lateral to the knife cut was taken for subsequent biochemical assay. Knife cuts severing the dorsal stria terminalis were made at 6.0 mm anterior to the interaural line. Using a remote control leucotome (Cazard), a complete transection of both the stria terminalis and the so-called ventral pathways (de Olmos) was made. This procedure has been described in detail elsewhere (Ben-Ari et al.). A pre-
amygdaloid frontal hemisection was also performed using the leucotome. This lesion entirely severs the ventral connections between the anterior forebrain (level A 7.3, L 3.0, H 1.3, atlas of Albe-Fessard1) and the amygdala. Kainic acid injections (1.5 µg/1µl of neutral saline) were made into the diagonal band area at co-ordinates LR 0.5, AP 3.0, V 7.5 and into the magnocellular lateral preoptic nucleus at co-ordinates LR 3.0, AP 2.0, V 9.0 according to the atlas of Pellegrino and Cushman21. Placement of the lesions or injections was verified during microdissection of the amygdala or by acetylcholinesterase staining of 40 µm frozen sections through the amygdala and lesioned area.

Microdissections of the amygdala regions were carried out on 0.6–1.0 mm thick sections of fresh chilled amygdala tissue, prepared by hand with an array of razor blades. Individual areas were dissected from these sections with the aid of a binocular microscope fitted with a cooled stage. In another experimental series, microdissection of amygdaloid nuclei was performed using the method of Zigmond and Ben-Ari23. Weighed samples (1–10 mg wet weight) were frozen on dry ice to await homogenization and ChAT assay. The tissues were homogenized in 25 mM sodium phosphate buffer, pH 7.0, containing 0.1 % Triton X-100 and aliquots taken for ChAT assay by the method of Fonnum13. Acetylcholinesterase activity was demonstrated histochemically on frozen sections by the method of Koelle as modified by Lewis17. To facilitate visualization of AChE-positive neurones in the rat forebrain 6 rats were injected with 1.5 mg/kg DFP in anhydrous propyleneglycol as described by Butcher et al.6 and sacrificed between 6 and 24 h after injection.

RESULTS
Effect of lesions on the distribution of AChE in the amygdala

The distribution of AChE-positive material in the normal rat amygdala presently obtained (Fig. 1e–h) agreed well with previous studies3,15,22. As a preliminary to more quantitative studies on the cholinergic innervation of the amygdala based on ChAT determinations, lesions were placed to sever some established projections to the amygdala. Of the lesions used only microknife cuts in the ventral amygdofugal pathway area (parasagittal cuts through the lateral portion of the MFB) and knife cuts separating the pyriform cortex from the amygdala produced histochemically detectable depletions of AChE. Lesions severing the stria terminalis tract or hemisections severing the medial forebrain bundle (MFB) at the level of the caudal hypothalamus failed to modify the staining pattern of AChE in the amygdala.

Parasagittal microknife cuts through the lateral portion of the MFB produced reductions in the intensity of AChE staining in the amygdaloid complex, the nucleus of the lateral olfactory tract and in the overlying pyriform cortex (Fig. 1a–d). Undercutting the pyriform cortex resulted in loss of AChE-positive material from the cortex itself and in rats sacrificed 4 days after lesion AChE-positive material accumulated ventral and medial to the cut (Fig. 2A and B). In one rat a parasagittal knife cut was misplaced and passed through the medial nucleus. In this cut (Fig. 2C contrast with control Fig. 2D) which separated the entopeduncular area, the globus pallidus and
Fig. 1. The effect of a rostrocaudal parasagittal knife cut in the area of the lateral MFB on the acetylcholinesterase (AChE) activity of the amygdaloid complex. Sections a–d represent the lesion side and sections e–h are from the contralateral side of the same rat at different levels through the amygdala. Note the reduced AChE staining of all the amygdaloid nuclei and also the pyriform cortex. Abbreviations: BL, basolateral nucleus; CE, central nucleus; CP, caudate-putamen; LP, lateral-posterior nucleus; M, medial nucleus; PY, pyriform cortex; tol, nucleus of the lateral olfactory tract.
Fig. 2. A and B: the effect of undercutting the pyriform cortex on the AChE staining of the amygdaloid complex (survival time 4 days). Compared with D, note the striking loss of AChE staining in the undercut cortex and enhanced staining of the amygdala suggesting AChE accumulation medial to the knife cut. C and D: effect of a parasagittal knife cut (C), (compare with control D), which isolated the medial amygdaloid nucleus from the rest of the amygdala and also separated the amygdaloid nuclei lateral to the cut from the ventral forebrain nuclei such as the globus pallidus and medial preoptic nucleus. This cut depleted the amygdaloid complex lateral to the cut of AChE staining. There is also an accumulation of AChE staining material in the stria terminalis tract medial to the cut (survival time 4 days). Abbreviations used as in Fig. 1 except ST, stria terminalis tract.
Fig. 3. A–D: schematic representation of the distribution of AChE positive neurone cell bodies (▲) in the ventral forebrain at different rostrocaudal levels (A6670–A3750 according to the atlas of König and Klippel[16]). E and F: AChE staining of the globus pallidus/internal capsule area (E) and the lateral-posterior amygdaloid nucleus (F) after pretreatment of the rat with DFP as described in the methods (survival time 8 h). Note the intensely AChE-positive neurones of the globus pallidus/internal capsule area (E) and the similar AChE positive neurones along the edge of the optic tract (F). In contrast to these areas which have numbers of strongly staining neurones, the AChE staining of the lateral-posterior nucleus and the rest of the amygdaloid complex is mostly in the neuropil (F) and the amygdala as a whole has only a very few strongly staining neurones. Abbreviations used: AD, anterodorsal thalamic nucleus; CP, caudate-putamen; GP, globus pallidus; PI, pyriform cortex; PO, medial preoptic area; ST, bed nucleus of the stria terminalis; VH, paraventricular hypothalamic nucleus; ac, central nucleus; al, lateral nucleus; bl, basolateral nucleus; co, cortical nucleus; lp, lateral-posterior nucleus; m, medial nucleus; and ot, optic tract.
lateral preoptic area from the amygdala, an almost complete depletion of AChE occurred in the amygdala lateral to the cut. In contrast the medial amygdala area central to the cut still retained its AChE activity. In this lesion accumulation of AChE-positive material in the transected stria terminalis tract was also seen.

**Distribution of AChE-positive neurones in the ventral forebrain**

Rats were pretreated with DFP as indicated in Methods. Rats which were killed between 6 and 12 h after DFP treatment gave the clearest results, with the AChE-positive neurones clearly visible against a low level of background staining. The distribution and location of the AChE-positive neurones in the magnocellular forebrain nuclei (including the globus pallidus, diagonal band nucleus and lateral preoptic area) is illustrated in Fig. 3A–F. In general the distribution of AChE-positive neurones in the ventral forebrain agrees with the results previously described by Jacobowit and Palkovits\(^\text{15}\). There are, however, a number of additional forebrain areas which contain strongly staining AChE-positive neurones. These include the nucleus accumbens, nucleus caudatus-putamen and the tuberculum olfactorium. In all these nuclei the dense AChE neuropil staining, which is abolished by DFP treatment, normally obscures the presence of AChE-positive neurones. (This subject is dealt with in much greater detail by Butcher and Bilezikjian\(^\text{5}\) and Butcher et al.\(^\text{6}\)). The amygdala, however, contains only very few weakly staining neurones, located in the medial and lateral-posterior nuclei (Fig. 3F). Six to eight hours after DFP pretreatment the neuropil staining even in the lateral-posterior nucleus, which normally stains very intensely for AChE (Fig. 1A–D), was only faint (Fig. 3F). The neuropil staining in the lateral-posterior nucleus, however, recovers faster than the neuropil staining in the hippocampus, which is innervated from rostral and medial magnocellular forebrain nuclei\(^\text{15}\). This finding suggested that the neurones supplying the AChE-containing fibres to the lateral-posterior nucleus are located closer to the structure they innervate than are those supplying the hippocampus. The most likely origin of this AChE projection seemed to be the AChE-positive neurones in the lateral preoptic area (especially the magnocellular nucleus), the entopeduncular nucleus and in the globus pallidus. All these nuclei contain medium to large multipolar AChE-positive neurones (Fig. 3A–D).

**Effects of lesions on the ChAT content of the amygdala**

As noted previously by Ben-Ari et al.\(^\text{3}\), the distribution of ChAT within the amygdaloid nuclei correlated well with the distribution of AChE demonstrated histochemically (compare Table I with Fig. 1A–C). In an initial effort to investigate the effects of lesions on the ChAT content of the amygdala lesions similar to those described earlier in conjunction with the AChE histochemistry were made. In agreement with the results of AChE histochemistry, only the parasagittal knife cuts through lateral MFB and the cuts separating the pyriform cortex from the amygdaloid nuclei significantly reduced the ChAT content of the amygdaloid nuclei or the pyriform cortex (Table II). The rostrocaudal knife cut through the ventral forebrain (lateral MFB cut) depleted ChAT from all the amygdaloid nuclei and from the
TABLE I
Regional distribution of choline acetyltransferase within the amygdaloid complex

<table>
<thead>
<tr>
<th>Nucleus or region</th>
<th>ChAT (μmol/g wet wt./h)</th>
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</thead>
<tbody>
<tr>
<td>Pyriform cortex</td>
<td>8.21 ± 0.42 (6)</td>
</tr>
<tr>
<td>Anterior amygdala area</td>
<td>7.07 ± 1.02 (5)</td>
</tr>
<tr>
<td>Corticomedial area</td>
<td>6.36 ± 0.81 (6)</td>
</tr>
<tr>
<td>Lateral posterior/basolateral complex</td>
<td>15.85 ± 2.79 (6)</td>
</tr>
<tr>
<td>Central nucleus</td>
<td>5.75 ± 2.27 (5)</td>
</tr>
</tbody>
</table>

pyriform cortex by 50–60%. Furthermore, a complete transection of both the stria terminalis and the so-called ventral pathway produced a similar depletion of ChAT content in both the basolateral nucleus (ChAT content 54.3 ± 6.4%, n=7) and the lateral-posterior nucleus (ChAT content 52.7 ± 7.8%, n=7). On the other hand the frontal hemisection did not significantly affect the ChAT content of any of the amygdaloid nuclei or the pyriform cortex. Undercutting the pyriform cortex produced a similar ChAT depletion of the undercut cortex but did not alter the ChAT content of the other parts of the amygdaloid complex sampled (Table II). The other lesions used, section of the stria terminalis or hemisections through the caudal hypothalamus, did not significantly alter the ChAT content of the amygdaloid complex. None of the lesions produced any detectable change in the ChAT content of the unoperated control side when compared to samples from normal untreated rats.

Unilateral kainic acid injections (1.5 μg/μl) into the area of the rostral diagonal band nucleus produced a substantial depletion of ChAT content in the injection area (Table III) and resulted in a small but significant depletion of the ChAT content of the

TABLE II
Choline acetyltransferase content of the amygdala following lesions to afferent pathways to the amygdala
Rats were killed 8 days after lesion.

<table>
<thead>
<tr>
<th>Unilateral lesion</th>
<th>ChAT content on lesion side as per cent of control side</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Corticomedial area</td>
</tr>
<tr>
<td>(1) Stria terminalis cut</td>
<td>87.5 ± 10.4 (5)</td>
</tr>
<tr>
<td>(2) Lateral MFB cut</td>
<td>46.1 ± 5.0 (5)**</td>
</tr>
<tr>
<td>(3) Hemisection through caudal hypotalamus</td>
<td>94.6 ± 9.02 (4)</td>
</tr>
<tr>
<td>(4) Pyriform cortex undercut</td>
<td>107.5 ± 5.4 (5)</td>
</tr>
<tr>
<td>(5) Ventral pathway/stria terminalis cut</td>
<td>—</td>
</tr>
</tbody>
</table>

* P < 0.05, ** P < 0.01. denotes significantly different from control side (Students t-test).
TABLE III

Effects of kainic acid injections (1.5 µg/1 µl) into the diagonal band area or the lateral preoptic area on the ChAT content of these nuclei and the ChAT content of the amygdala and hippocampus

Units are µmol [14C]ACh formed/g wet weight/h. All values are the means of duplicate determinations on individual samples dissected from at least 4 separate rats. Rats were killed 8 days after kainic acid injection.

<table>
<thead>
<tr>
<th>Nucleus or area</th>
<th>Diagonal band injection</th>
<th>Lateral preoptic area injection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control side</td>
<td>Lesion side</td>
</tr>
<tr>
<td>Diagonal band</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lateral preoptic area (magnocellular nucleus)</td>
<td>29.34 ± 2.51</td>
<td>15.84 ± 2.73**</td>
</tr>
<tr>
<td>Lateral posterior baso-lateral amygdaloid complex</td>
<td>14.60 ± 0.64</td>
<td>10.74 ± 1.95*</td>
</tr>
<tr>
<td>Corticomedial amygdaloid area</td>
<td>15.94 ± 1.44</td>
<td>12.98 ± 1.40</td>
</tr>
<tr>
<td>Hippocampus (dentate gyrus)</td>
<td>12.03 ± 1.15</td>
<td>8.74 ± 0.92*</td>
</tr>
</tbody>
</table>

* P < 0.05, ** P < 0.01 Students t-test.

ipsilateral hippocampus. Kainic acid injections into the diagonal band area, however, did not modify the ChAT content of the amygdaloid nuclei sampled. In contrast kainic acid injections (1.5 µg/1 µl) into the magnocellular lateral preoptic nucleus produced a dramatic depletion of the ChAT content of the amygdaloid nuclei but did not modify the ChAT content of the diagonal band nucleus or the hippocampus (Table III). Histological examination of fixed material indicated that, although considerable parts of the lateral preoptic area or diagonal band area were damaged by the kainic acid, there was no indication that the kainic acid injections directly damaged the amygdala or hippocampus.

DISCUSSION

The results presented here suggest that the major part of the cholinergic innervation of the amygdala originates from cholinesterase-positive neurones in the magnocellular nuclei of the forebrain (MNFB) (see Divac10 for review). The intensely cholinesterase-positive neurones of this system are widely distributed through the ventral forebrain, being found in the nucleus of the diagonal band, the globus pallidus, in the internal capsule along the edge of the optic tract (entopeduncular nucleus), immediately above the supraoptic nucleus and, particularly, in the magnocellular nucleus of the lateral preoptic area. All these AChE-positive neurones, however, form a continuous chain from the diagonal band back to the entopeduncular nucleus and may be classified together. Rostrally these nuclei merge into the nucleus accumbens, nucleus caudatus-putamen and the tuberculum olfactorium, which also contain
AChE-positive neurones\textsuperscript{5}. The AChE-positive neurones of the magnocellular forebrain nuclei differ from those in the basal ganglia in sending long axons to provide the cholinergic innervation to most of the forebrain, whereas the AChE-positive neurones in the striatum and olfactory tubercle seem to represent local interneurones\textsuperscript{18}. Hemisections made through the caudal hypothalamus do not modify the ChAT content of the forebrain nuclei (Fonnum et al.\textsuperscript{14} and this paper) indicating that ascending cholinergic axons do not contribute significantly to the cholinergic innervation of the forebrain. It seems likely that some of the ascending AChE-containing fibres described by Shute and Lewis\textsuperscript{22} in the MFB represent AChE-positive axons originating from the AChE-containing dopamine neurones in substantia nigra which innervate the nucleus caudate-putamen\textsuperscript{5,6}.

The diffuse nature of the magnocellular AChE-positive neurones in the forebrain made a complete lesion of this group of cells virtually impossible. However, knife cuts through the lateral MFB, kainic acid injections into the lateral preoptic area and cuts through the ventral pathway all gave results consistent with a major cholinergic innervation to the amygdala from the ventrolateral parts of the magnocellular forebrain nuclei. These cholinesterase fibres mainly follow the so called ‘ventral pathway’ to the amygdala\textsuperscript{19}. Only a small percentage of these fibres seem to follow the dorsal route via the stria terminalis as interruption of this tract did not modify the ChAT content or the AChE staining pattern of the amygdala (although, in agreement with Shute and Lewis\textsuperscript{22}, we observed AChE positive-fibres running in this tract). The fact that neither knife cuts or kainic acid lesions produced a complete depletion of the ChAT or AChE content of the amygdala presumably reflects the persistence of intact cholinergic neurones either laterally or caudally to these lesions. Contributing to this residual AChE and ChAT may be the small number of AChE-positive neurones within the amygdala.

The depletion of the ChAT of the lateral preoptic and diagonal band nuclei after kainic acid lesions coupled with the disappearance of the AChE-positive neurones in these areas (unpublished observations) indicates that the AChE-positive neurones and ChAT-containing neurones in these areas are probably identical. Furthermore, the data available about the neurotoxicity of kainic acid are consistent with the view that kainic acid damages only neurone cell bodies in the nuclei into which it is injected, but does not affect axons passing through the nucleus from sites distant to the injection\textsuperscript{8}. On this basis one can be reasonably confident that most of the cholinergic projections to the amygdala originate from neurones within the ventrolateral MNFB system. The absence of any decrease in the ChAT content of the amygdala after kainic acid injections into the diagonal band area is consistent with the notion that neurones within the MNFB nuclei are organized to project topographically. The rostral diagonal band/septum area projects to the medial frontal cortex and hippocampus, the rostral and dorsal globus pallidus project to the dorsal and lateral cortex and the caudal and ventral globus pallidus neurones together with those in the lateral preoptic area project to the amygdala, olfactory bulb and ventral cortical areas (see Shute and Lewis\textsuperscript{22}, Emson and Lindvall\textsuperscript{19} for reviews).

The function of the MNFB cholinergic system is unclear; however, the
widespread nature of its connections to areas including the cerebral cortex, amygdala and hippocampus would make it an appropriate system to mediate synchronous activity in these areas. The reported reductions in forebrain cholinergic enzymes in psychiatric disorders such as schizophrenia\(^4\) and senile dementia\(^9\) may also reflect pathological changes in this MNFB cholinergic system.

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