Multiple Cholinergic Markers Are Unexpectedly Not Altered in the Rat Dentate Gyrus following Entorhinal Cortex Lesions

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Since major cholinergic deficits are observed in Alzheimer's disease, the development of models to study possible cholinergic plasticity has generated great interest. In this regard, it has been shown that lesions of the entorhinal cortex, which sends glutamatergic projections to the hippocampus, promote the sprouting and plasticity of presumptive cholinergic septohippocampal fibers in the dentate gyrus, as revealed by AChE histochemistry. This sprouting was reported to be evident at 8 d and up to 30 d postlesion (DPL) and is now widely used as a model of cholinergic neuronal plasticity. In the present study, unilateral lesions of the entorhinal cortex were made in adult rats, and the status of various putative pre- and postsynaptic cholinergic markers was assessed after 2, 4, 8, 14, and 30 DPL. As expected, AChE was increased in the outer molecular layer of the ipsilateral dentate gyrus from 8 to 30 DPL. In contrast, the activity of ChAT, the enzyme responsible for the synthesis of ACh, and the densities of specific binding sites for ³H-AH5183/vesamicol (blocker of the ACh vesicular transport sites), ³H-hemicholinium-3 (blocker of the high-affinity choline uptake sites), muscarinic-M₂ (³H-AF-DX 384 and ³H-ACh), muscarinic-M₁ (³H-pirenzepine), and nicotinic (³H-N-methylcarbamylcholine) cholinergic receptors were not increased on the ipsilateral molecular layer of the dentate gyrus, as compared to their contralateral controls. We conclude that the increase in AChE staining in the molecular layer of the dentate gyrus following entorhinal cortex lesions may be due to changes in noncholinergic neurons.

[Key words: entorhinal cortex lesions, hippocampus, dentate gyrus, muscarinic receptors, nicotinic receptors, high-affinity choline uptake, vesamicol, AChE, sprouting, cholinergic system]

More than ninety years ago, Ramón y Cajal (1901) described an important projection from the entorhinal cortex to the hippocampal formation. This neural system arises from layers II and III of the entorhinal cortex and provides major inputs to

the dentate granule cells and to the pyramidal neurons of the hippocampal formation (Steward and Scoville, 1976). Lesions of this pathway in rodents have become, over the years, a favored model system to study adaptive changes in the CNS. Following entorhinal cortex lesions (ECL), synaptic endings derived from intact fibers in close proximity to the denervated molecular layer of the dentate gyrus grow toward the newly exposed postsynaptic structures. It has been proposed that the sprouting fibers and resulting synapses originate from cholinergic septal neurons (Lynch et al., 1972), glutamatergic commissural-associational pyramidal cells of the CA3/hilus areas (Lynch et al., 1976; Scheff et al., 1980; Geddes et al., 1985), and, to a lesser extent, neurons of the contralateral entorhinal cortex (Steward et al., 1974). The remarkable ability of the dentate gyrus to remodel its circuitry is evident during development and remains in adulthood (Lynch et al., 1972; Cotman et al., 1973; Nadler et al., 1973, 1977; Storm-Mathisen, 1974; Scheff et al., 1980; Hoff et al., 1981; Stanfield and Cowan, 1982).

Marked increases in acetylcholinesterase (AChE) staining or activity in the molecular layer of the dentate gyrus have been observed following disruption of the perforant path (entorhinal-dentate fibers) (Lynch et al., 1972; Cotman et al., 1973; Storm-Mathisen, 1974; Scheff et al., 1980; Stanfield and Cowan, 1982). It was first suggested that the induced increase in AChE staining or activity, following ECL or transection of the perforant path, relies on the integrity of the cholinergic septohippocampal projections, since transection of the fimbria-fornix severely impaired this AChE response (Lynch et al., 1972; Storm-Mathisen, 1974).

Interestingly, a very similar reorganization of dentate synaptic inputs was described in patients suffering from Alzheimer's disease (AD). This neuropathology is characterized by a severe loss of neurons present in layers II and III of the entorhinal cortex (Hyman et al., 1987). Although some studies have reported a clear decrease in AChE activity or AChE staining in hippocampi of AD patients (Davies, 1979; Henke and Lang, 1983), others have observed enhanced AChE staining in the neuropil of some AD patients, especially in the outer two-thirds of the molecular layer of the dentate gyrus (Geddes et al., 1985; Hyman et al., 1987; Senut et al., 1991), a profile similar to that seen following ECL in the rat brain. Increases in AChE staining in the hippocampi of AD patients have been interpreted as evidence for a compensatory sprouting of septohippocampal cholinergic neurons. However, Ransmayr et al. (1989) measured significant reductions in choline acetyltransferase (ChAT)-like immunoreactivity in the molecular layer of the dentate gyrus of AD patients, in accordance with previously reported decrements of

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ChAT activity in the whole hippocampal formation (Reisine et al., 1978; Davies, 1979; Perry et al., 1986; Araujo et al., 1988a; Rinne et al., 1989; Aubert et al., 1992a). It is difficult to explain these apparent inconsistencies but it may be that, as already suggested (Levey et al., 1983, 1984; Small, 1989; Greenfield, 1991; Mesulam and Geula, 1991), AChE-positive fibers are not exclusive to cholinergic neurons.

Similar discrepancies were also observed in the dentate gyrus of the rat brain. For example, it has been shown that selective lesions of the granule cells of the dentate gyrus, mimicking ECL, induced the expected increase in AChE staining in the outer two-thirds of the molecular layer (McKeon et al., 1989). However, this response occurs even in the absence of septal inputs (removed by fimbria-fornix lesions) (McKeon et al., 1989). Following these manipulations, ChAT-like immunoreactivity remains unchanged in the hippocampus, whereas increases in AChE staining were evident (McKeon et al., 1989). It is also of interest that the density of the total population of muscarinic receptors, labeled with ^3H -quinuclidinyl benzilate, did not parallel the increase in AChE staining seen following ECL in the rat brain (Monaghan et al., 1982).

Taken together, these data bring doubts about the cholinergic nature of the sprouting observed in the dentate gyrus following ECL. Therefore, in the present study, we systematically examined key neurochemical markers of hippocampal cholinergic neurotransmission in adult ECL rats. First, the extent of the sprouting was confirmed using AChE staining (Karnovsky and Roots, 1964). Subsequently, the capacity of the newly formed innervation to synthesize acetylcholine (ACh) was evaluated using ChAT activity (Fonnum, 1969; Tucek, 1978), a well-established presynaptic cholinergic marker. We also investigated the integrity of various other pre- and postsynaptic components of the cholinergic synapse using quantitative receptor autoradiography. ^3H -hemicholinium-3 and ^3H -AH5183/vesamicol, which respectively labeled high-affinity choline uptake carrier sites (Rainbow et al., 1984; Quirion, 1985, 1987; Vickroy et al., 1985) and ACh vesicular transport sites (Marien et al., 1987; Parsons et al., 1987; Altar and Marien, 1988; Diebler and Morot Gaudry-Talarmin, 1989), were used as presynaptic markers. As putative presynaptic autoreceptors (Raiteri et al., 1984; Mash et al., 1985; Lapchak et al., 1989; Weiler, 1989), the labeling profile of the muscarinic- M_2 receptors was studied using both ^3H -ACh (Kellar et al., 1985; Quirion and Boksa, 1986; Schwartz, 1986; Quirion et al., 1989) and ^3H -AF-DX 384 (Entzeroth and Mayer, 1990; Miller et al., 1991; Aubert et al., 1992b), while ^3H -*N*-methylcarbamylcholine (^3H -MCC) was used as a marker of putative nicotinic autoreceptors (Abood and Grassi, 1986; Boksa and Quirion, 1987; Yamada et al., 1987; Araujo et al., 1988b, 1989). Postsynaptic muscarinic- M_1 receptor sites were labeled with ^3H -pirenzepine (Watson et al., 1982, 1984; Spencer et al., 1986; Quirion et al., 1989). The results failed to demonstrate the cholinergic nature of the AChE sprouting observed in the dentate gyrus following ECL.

Materials and Methods

Animals and chemicals. Male Fisher-344 rats (250–300 gm) were obtained from Charles River Breeding Farms (St-Constant, Quebec, Canada). ^3H -pirenzepine (70.4–87.0 Ci/mmol), ^3H -AF-DX 384 (87.4–100.8 Ci/mmol), ^3H -choline (85 Ci/mmol), ^3H -hemicholinium-3 (142 Ci/mmol), ^3H -*N*-methylcarbamylcholine (84.0 Ci/mmol), ^3H -AH5183 (48.0 Ci/mmol), and ^{14}C -acetyl coenzyme A (48.8 mCi/mmol) were purchased from New England Nuclear (Boston, MA). Nicotine (free base), atropine sulfate, hemicholinium-3, choline chloride, and eserine hemisulfate were

bought from Sigma Chemical Co. (St. Louis, MO). Acetylcholine chloride was supplied by Hoffmann La Roche (Basel, Switzerland). Unlabeled acetyl coenzyme A was purchased from Boehringer Mannheim (Mannheim, Germany). Tetraphenylboron (sodium salt) and 3-heptanone were from Aldrich Chemical Co. (Milwaukee, WI). Ethyl acetate was bought from American Chemicals Co. (Montreal, Quebec, Canada). Bovine serum albumin (98% fatty acid free) and Ecolite scintillation cocktail were purchased from ICN Biochemicals (Irvine, CA). Triton X-100 (100%) scintillation grade was from Amersham (Arlington, IL). ^3H -Hyperfilm and ^3H -standards were purchased from Amersham (Oakville, Ontario, Canada). Developer (D-19) and fixer (Rapid Fixer) used for ^3H -Hyperfilm processing were from Kodak Chemical Inc. (Montreal, Quebec, Canada). Unlabeled AH5183/vesamicol was provided as a gift by Dr. S. M. Parsons (University of California, Santa Barbara, CA). All other chemicals were from Fisher Scientific (Montreal, Quebec, Canada).

Entorhinal cortex lesions. Adult F344 male rats received multiple electrolytic lesions (30 sec) of the entorhinal cortex area, unilaterally, at three different depths (2, 4, and 6 mm below dura), in three locations (L:3.3, B:0; L:4.3, B:0; L:5.1, B:–1 mm) as described in detail elsewhere (Scheff et al., 1980; Poirier and Nichols, 1991). At various days postlesion (DPL), rats were killed and their brains were rapidly removed from the skull, immersed in 2-methylbutane at -40°C for several seconds, and then stored at -80°C until use. Time course analysis includes controls (unoperated) and 2, 4, 8, 14, and 30 DPL animals.

AChE histochemistry. The internal remodeling of the dentate gyrus following ECL was confirmed by AChE staining as adapted from Karnovsky and Roots (1964). Slides used for ^3H -*N*-methylcarbamylcholine nicotinic receptor autoradiography were next processed for AChE staining. Briefly, they were incubated in a solution containing 2 mg/ml ACh iodide, 13 parts of NaH_2PO_4 (0.1 M), 1 part of Na-citrate (0.3 M), 2 parts of CuSO_4 (30 mM), 2 parts of distilled water, and 2 parts of K-ferricyanide (5 mM) for 5 hr at room temperature. Slides were then dehydrated and mounted for microscopic examination.

Autoradiographic visualization of various cholinergic markers. Coronal frozen sections (15 μm thick) were cut at -18°C , thaw mounted on poly-L-lysine-coated slides (two sections per slide), air dried overnight, and then stored at -80°C until use. Four to 10 animals were used for each point of the time course (control, 2, 4, 8, 14, and 30 DPL).

^3H -*N*-methylcarbamylcholine/nicotinic receptors. Sections were first equilibrated at 4°C and then incubated for 1 hr (4°C) in Tris HCl buffer (pH 7.4) of the following composition (in mM): Tris, 50; NaCl, 120; KCl, 5; CaCl_2 , 2; MgCl_2 , 1; containing 20 nM ^3H -MCC with or without nicotine (10 μM) to determine specific binding. Slides were transferred sequentially through four washes of 2 min each in Tris (50 mM) HCl buffer (pH 7.4) at 4°C before being rapidly dipped in cold deionized water. Sections were dried and apposed to films for 7 months. Films were then developed as described before (Quirion et al., 1981). Specifically bound ^3H -MCC, in layers of the dentate gyrus and CA1 region of the Ammon's horn of the hippocampus, was quantified using ^3H -labeled standards and computerized image analysis (MCID System, Image Research Inc., St. Catharines, Ontario, Canada). For each section, six target fields were taken throughout each lamina of the hippocampus. The variation between target fields was 15% maximum. For each animal, sections were in duplicate. Under these conditions, specifically bound ^3H -MCC represented 40–60% of bound ligand (Araujo et al., 1989).

^3H -hemicholinium-3/high-affinity choline uptake sites. Sections were first equilibrated at 4°C and then incubated for 1 hr at 4°C in Tris (50 mM)-NaCl (300 mM) HCl buffer (pH 7.4) containing 15 nM ^3H -hemicholinium-3 in the presence or absence of unlabeled hemicholinium-3 (20 μM) to determine specific binding. At the end of the incubation, slides were transferred sequentially through six washes of 2 min each in Tris (50 mM) HCl buffer (pH 7.4) at 4°C before being rapidly dipped in cold distilled water to remove ions. Sections were dried and apposed to films for 3 weeks. Films were then developed and analyzed as described for ^3H -MCC. Under these conditions, specifically bound ^3H -hemicholinium-3 represented 80–85% of totally bound ligand (Quirion, 1987).

^3H -AH5183 (vesamicol)/ACh vesicular transport sites. Sections were brought to room temperature and preincubated for 30 min in Tris HCl buffer of the following composition (in mM): Tris, 50; NaCl, 120; KCl, 5; CaCl_2 , 2; MgCl_2 , 1 (pH 7.4) at 22°C ; before being incubated for 1 hr in fresh buffer containing 15 nM ^3H -AH5183 in the absence or presence of unlabeled AH5183 (1 μM) to determine specific binding. Sections were then transferred sequentially through four washes of 30 sec each

Figure 1. Typical photomicrograph of AChE staining in the hippocampus at 30 DPL. The control and lesioned (ECL) sides are illustrated. Anatomical identification is based on the Paxinos and Watson (1982) atlas. CA3, fields CA3 of Ammon's horn of the hippocampus; GrDG, granular layer of the dentate gyrus; L-Mol, lacunosum-molecular layer of the hippocampus; Mol, molecular layer of the dentate gyrus; OrCA1, oriens layer in CA1 field of Ammon's horn of the hippocampus; PyCA1, pyramidal layer in CA1 field of Ammon's horn of the hippocampus; RadCA1, stratum radiatum layer in CA1 field of Ammon's horn of the hippocampus. A similar increase in AChE staining in the molecular layer of the dentate gyrus on the lesion side is also seen at 8 DPL (see Fig. 2) and 14 DPL.

in Tris (50 mM) HCl buffer (pH 7.4) at 4°C followed by a quick dip in cold deionized water. Sections were dried and apposed to films for 3 weeks. Films were then developed and analyzed as described for ^3H -MCC. Under these conditions, specifically bound ^3H -AH5183 represented between 90% and 95% of bound ligand (Marien et al., 1987).

^3H -ACh/muscarinic- M_2 receptors. ^3H -choline was acetylated on the day of the experiment, as described in detail elsewhere (Quirion and Boksa, 1986). Only preparations showing 95% and higher degrees of acetylation were used as radioligands. Sections were equilibrated at 4°C; preincubated for 10 min in Tris HCl buffer of the following composition (in mM): Tris, 50; NaCl, 120; KCl, 5; CaCl_2 , 2; MgCl_2 , 1; diisopropyl-fluorophosphate, 0.1; nicotine, 0.02 (pH 7.4); and incubated for 1 hr at 4°C in fresh buffer containing 20 nM ^3H -ACh, with or without atropine (1 μM) to determine specific binding. Slides were then transferred in two subsequent washes of 5 min each in Tris (50 mM) HCl buffer (pH 7.4) at 4°C followed by a rapid dip into cold distilled water. Sections were dried and apposed to films for 1 month. Films were then developed and analyzed as described for ^3H -MCC. Under those assay conditions, specifically bound ^3H -ACh represented 90–95% of bound ligand and possessed a muscarinic M_2 -like profile (Quirion et al., 1989).

^3H -AF-DX 384/muscarinic- M_2 and ^3H -pirenzepine/muscarinic- M_1 receptors. For both radioligands, a similar protocol was followed as reported previously (Quirion and Boksa, 1986; Quirion et al., 1989; Aubert et al., 1992b). Sections were equilibrated at room temperature (22°C); preincubated for 15 min (22°C) in Krebs buffer of the following composition (in mM): NaCl, 120; MgSO_4 , 1.2; KH_2PO_4 , 1.2; glucose, 5.6; NaHCO_3 , 25; CaCl_2 , 2.5; KCl, 4.7 (pH 7.4); and then incubated at 22°C for 1 hr in fresh buffer containing either 15 nM ^3H -pirenzepine or 2 nM ^3H -AF-DX 384, with or without atropine (1 μM) to determine specific binding. Slides were then transferred sequentially through three rinses (4 min each) in Tris (50 mM) HCl buffer (pH 7.4) at 4°C, followed by a rapid dip in cold distilled water. Sections were next dried under a stream of cold air and tightly juxtaposed to films for 15 d. Films were then developed and layers of the dentate gyrus, CA1, and CA3 regions of the Ammon's horn of the hippocampus were analyzed as described for ^3H -MCC. Under these conditions specifically bound ^3H -pirenzepine and ^3H -AF-DX 384 represented at least 90–95% of bound ligands.

ChAT activity. To determine ChAT activity, dissected ipsilateral hippocampi were homogenized and incubated for 15 min in buffer containing ^{14}C -acetyl coenzyme A as previously described in detail elsewhere (Araujo et al., 1988a) using the method of Fonnum (1969), as modified by Tuček (1978). Three animals were used for each point of the time course (control, 2, 8, and 30 DPL).

Protein content. Protein content of samples used for ChAT activity was determined by the method of Lowry et al. (1951), using bovine serum albumin as standard.

Data analysis. Paired *t* test analysis was used in order to compare the lesion side of each animal with the control side at each given point of the time course. Results were considered significant if $P < 0.01$. ANOVA followed by Newman-Keuls analysis was used to evaluate the effect of the time course on binding parameters in lesioned animals on both lesioned and control sides as compared to control (unoperated) animals.

Results

AChE histochemistry

As reported before by several groups (see introductory remarks), AChE staining is clearly increased in the outer two-thirds of the molecular layer of the dentate gyrus, ipsilaterally to the ECL, from 8 to 30 DPL (Figs. 1, 2).

^3H -MCC/nicotinic receptors

Nicotinic receptor binding sites, labeled with ^3H -MCC, were significantly (paired *t* test, $P < 0.01$) decreased in the molecular layer of the dentate gyrus at 8 and 30 DPL (Figs. 2, 3). However, no significant differences were observed in the CA1 region of the hippocampus at any DPL (Fig. 2).

^3H -hemicholinium-3/high-affinity choline uptake sites

The apparent density of high-affinity choline uptake binding sites, as measured with ^3H -hemicholinium-3, was unchanged in the molecular layer of the dentate gyrus at any DPL (Figs. 2, 4). The granular layer of the dentate gyrus and the oriens, pyramidal, and radiatum laminae of the CA1 region of the hippocampus as well as the lacunosum-molecular layer were also unaltered following ECL (Fig. 2).

^3H -AH5183 (vesamicol)/ACh vesicular transport sites

The apparent density of ^3H -AH5183/vesamicol binding, the blocker of ACh vesicular transport sites, was unchanged in the ipsilateral molecular layer of the dentate gyrus following ECL as compared to the contralateral side (Figs. 2, 4). No differences in ^3H -AH5183 binding were found in the granular layer of the dentate gyrus and other regions of the hippocampus such as the oriens, pyramidal, and radiatum layers of the CA1 region and the lacunosum-molecular layer (Fig. 2). However, a transient but significant bilateral increase (25–30%) in binding was observed at 2 DPL in the molecular layer of the dentate gyrus when compared to control (unoperated animals) (Fig. 4). This significant bilateral increase at 2 DPL was also observed in the granular layer of the dentate gyrus and in different layers of the CA1 region and the lacunosum-molecular layer of the hippocampus (results not shown).

^3H -ACh/muscarinic- M_2 receptors

The apparent density of ^3H -ACh putative muscarinic- M_2 receptor binding sites was not significantly altered in the lesioned

Figure 2. Typical photomicrographs of AChE staining (*AChE*), ³H-MCC/nicotinic receptor (*³H-MCC*), ³H-hemicholinium-3/high-affinity choline uptake site (*³H-HC3*), ³H-AH5183-vesamicol/ACh vesicular transport site (*³H-AH5183*), ³H-ACh/muscarinic-M₂ receptor (*³H-ACh*), ³H-AF-DX 384/muscarinic-M₂ receptor (*³H-AFDX 384*), and ³H-pirenzepine/muscarinic-M₁ receptor (*³H-PZ*) autoradiography in coronal sections of the rat hippocampus in control and 8 and 30 DPL (see Fig. 1 for abbreviations). The control (C) and lesioned (L) sides are illustrated on the left and right, respectively, at 8 and 30 DPL.

Figure 3. Quantitative analysis of ^3H -MCC/nicotinic receptors in the molecular layer of the dentate gyrus. Film autoradiograms (Fig. 2) were analyzed using computerized densitometry. Results are expressed in fmol/mg wet tissue weight and represent the mean $\pm$ SEM of 4–10 animals for each DPL (control, 2, 4, 8, 14, 30). Solid and hatched bars represent, respectively, control and lesioned sides. In *A* and *B*, the outer third and middle third, respectively, of the molecular layer were quantified. * denotes that significant differences were found between the control and lesioned sides at a given DPL if $P < 0.01$ using paired t test analysis.

as compared to contralateral sides (paired t test analysis), at any DPL, in the dentate gyrus (Figs. 2, 4). However, a significant increase (28%) was detected, using ANOVA analysis, in the molecular layer at 30 DPL on the lesioned side as compared to the controls (unoperated animals) (Fig. 4). No significant differences in ^3H -ACh binding were observed in any layers of the CA1 region of the hippocampus (Fig. 2).

^3H -AF-DX 384/muscarinic- M_2 receptors

Muscarinic- M_2 receptor binding, as measured with ^3H -AF-DX 384, was unchanged in the dentate gyrus at any DPL (Figs. 2, 4). Similarly, ^3H -AF-DX 384 binding sites in the oriens, pyramidal, and radiatum laminae of the CA1 and CA3 regions of the hippocampus were unaltered at any DPL (Fig. 2).

^3H -pirenzepine/muscarinic- M_1 receptors

Muscarinic- M_1 receptor binding was unaltered in the dentate gyrus at any DPL (Figs. 2, 4). In addition, no differences in ^3H -pirenzepine binding were observed in any layers of the CA1 and CA3 regions of the hippocampus (Fig. 2). However, a significant (paired t test, $p < 0.01$) increase (30%) in muscarinic M_1 binding sites in the ipsilateral lacunosum-molecular layer of the hippocampus was detected at 30 DPL (Fig. 2).

ChAT activity

ChAT activity was measured in the entire ipsilateral hippocampus and remained unchanged (values ranging between 51 and 63 nmol of ACh formed/mg protein/hr) throughout the whole period following ECL (2, 8, 30 DPL) as compared to control animals (Fig. 5).

Figure 4. Quantitative analysis of ^3H -hemicholinium-3/high-affinity choline uptake sites (^3H /HC-3), ^3H -AH5183-vesamicol/ACh vesicular transport sites (^3H /AH-5183), ^3H -ACh/muscarinic- M_2 receptors (^3H /ACh), ^3H -AF-DX 384/muscarinic- M_2 receptors (^3H /AF-DX 384), and ^3H -pirenzepine/muscarinic- M_1 receptors (^3H /PZ) in the molecular layer of the dentate gyrus. Film autoradiograms (Fig. 2) were analyzed using computerized densitometry. Results are expressed in fmol/mg wet tissue weight and represent the mean $\pm$ SEM of 4–10 animals for each DPL (control, 2, 4, 8, 14, 30). Solid and hatched bars represent, respectively, control and lesioned sides. † denotes that significant differences, using ANOVA/Newman-Keuls analysis, were found between the control or lesioned side at a particular DPL as compared to the controls (unoperated animals represented by the first, single, solid column labeled Control).

Discussion

For the past 20 years, intensification of AChE staining or activity in the outer two-thirds of the molecular layer of the dentate gyrus following ECL has been considered to be a valid dem-

onstration of compensatory cholinergic sprouting in response to deafferentation (see, e.g., Lynch et al., 1972; Nadler et al., 1977; Amaral et al., 1980; Scheff et al., 1980; Fagan and Gage, 1990). It has generally been assumed that alterations in AChE staining or activity are caused by the proliferation of septohippocampal fiber terminals (see introductory remarks). The close relationship between septohippocampal terminals and AChE staining is best exemplified by a marked loss of AChE staining in the molecular layer of the dentate gyrus following transection of the fimbria-fornix in normal and ECL animals (Shute and Lewis, 1963; Lynch et al., 1972; Storm-Mathisen, 1974). However, discrepancies have been reported between AChE staining, ChAT-like immunoreactivity, and tracing of the septohippocampal pathway (Lynch et al., 1978; Stanfield and Cowan, 1982; Baisden et al., 1984; Wainer et al., 1985). These apparent mismatches have been explained by the presence of both cholinergic and noncholinergic septohippocampal or intrinsic projections (Baisden et al., 1984; Wainer et al., 1985), as well as the likely existence of nonseptal cholinergic projections from the supramammillary region (Lasher et al., 1980) and noncholinergic, AChE-positive intrinsic hippocampal innervation (McKeon et al., 1989). Although electron microscopy studies have clearly shown that reactive synaptogenesis occurs in the deafferented zones in which AChE activity increases (Matthews et al., 1976a,b), there has been no *direct* evidence that septal cholinergic projections participate in this response by increasing the number of functional synaptic contacts.

Accordingly, the neurochemical status of the putative cholinergic sprouting was studied using multiple pre- and postsynaptic markers of cholinergic synapses. These failed to show any relationships with the altered hippocampal AChE staining seen post-ECL. Two well-established presynaptic markers, ChAT activity and ^3H -hemicholinium-3 binding to the high-affinity choline uptake sites (Rainbow et al., 1984; Quirion, 1985, 1987; Vickroy et al., 1985), were unaltered in the hippocampal formation at any time postlesions; this was in marked contrast with the increase in AChE staining in the molecular layer of the dentate gyrus. Other putative presynaptic markers of the hippocampal cholinergic synapse such as ^3H -AH5183, a blocker of ACh vesicular storage (Marien et al., 1987; Parsons et al., 1987; Altar and Marien, 1988; Diebler and Morot Gaudry-Talarmain, 1989), and muscarinic- M_2 (Raiteri et al., 1984; Kellar et al., 1985; Mash et al., 1985; Quirion and Boksa, 1986; Schwartz, 1986; Lapchak et al., 1989; Quirion et al., 1989; Weiler, 1989; Entzeroth and Mayer, 1990; Miller et al., 1991; Aubert et al., 1992b) and nicotinic (Abood and Grassi, 1986; Boksa and Quirion, 1987; Yamada et al., 1987; Araujo et al., 1988b, 1989) receptors also failed to be increased as the AChE staining following ECL. In contrast, the significant *decrease* in ^3H -MCC/nicotinic binding in the ipsilateral dentate gyrus at 8 and 30 DPL could be due to the localization of a certain proportion of nicotinic receptors on terminals of the perforant path. Up to now, there is no direct evidence for localization of nicotinic receptors on the terminals of the perforant path. However, Swanson et al. (1987) suggested such a localization in the dentate gyrus using a monoclonal antibody against neuronal nicotinic receptors. Interestingly, the distribution of this monoclonal antibody in the hippocampus (Swanson et al., 1987) is very similar to the labeling pattern of ^3H -ligands for neuronal nicotinic receptors such as ^3H -MCC (Boksa and Quirion, 1987; Yamada et al., 1987; Araujo et al., 1989; present results) and ^3H -nicotine or ^3H -ACh (Clarke et al., 1985; Schwartz, 1986). A localization

Figure 5. Evaluation of ChAT activity in the hippocampal formation for controls and at 2, 8, and 30 DPL. ChAT activity represents the quantity of ACh formed in nmol/mg protein/hr. All assays were performed in duplicate. Data are mean $\pm$ SEM values from three hippocampi for each DPL (control, 2, 8, 30).

of nicotinic receptors on terminals of the perforant path is further supported by the presence of high levels of mRNA for various nicotinic receptor subunits in layer II of the medial entorhinal area, which gives rise to the medial perforant path to the dentate gyrus (Wada et al., 1989). Muscarinic- M_1 receptor binding sites, which are located predominantly postsynaptically (Potter et al., 1984; Quirion, 1985; Spencer et al., 1986), also did not follow the profile of AChE staining intensification in the hippocampal formation after ECL. We also evaluated the density of muscarinic- M_2 (^3H -AF-DX 384) and muscarinic M_1 (^3H -pirenzepine) binding sites up to 64 DPL and no changes were observed in the molecular layer of the hippocampus (I. Aubert, J. Poirier, S. Gauthier, and R. Quirion, unpublished observations). Taken together, these results suggest that the increase in hippocampal AChE staining observed post-ECL is unlikely to relate to a general neurochemical reorganization of cholinergic synapses, at least up to 2 months postlesions.

A direct involvement of the cholinergic septohippocampal pathway in the increased AChE staining seen following ECL is also doubtful. It is well established that dramatic *decreases* in AChE staining or activity are observed in animals with fimbria-fornix lesions (Shute and Lewis, 1963; Lynch et al., 1972; Storm-Mathisen, 1974). However, in animals that received combined lesions of the septum and the entorhinal cortex, an increase of AChE staining in the molecular layer of the hippocampus was still observed (Chen et al., 1983). The source of the axons that are sprouting remains unknown, but in contrast to data reported previously (Lynch et al., 1972), the presence of an intact septohippocampal pathway may not be a prerequisite to increase AChE staining in the molecular layer of the hippocampus following ECL (Chen et al., 1983). Furthermore, animals with a septohippocampal lesion that received in the same surgical session an additional destruction of the granule cells showed a pronounced *increase* in the intensity of the AChE staining in the outer molecular layer of the dentate gyrus (McKeon et al., 1989). However, the increase in AChE staining occurred *without* a corresponding change in ChAT immunoreactivity (McKeon et al., 1989). This is consistent with the data reported here; no correlation was found between AChE staining and ChAT activity at any DPL.

Consequently, the nature of the AChE-positive sprouting fibers observed in the molecular layer of the dentate gyrus post-ECL remains to be elucidated. AChE-positive, *noncholinergic* interneurons are present in several areas of the hippocampus, predominantly in the hilar region (Shute and Lewis, 1966), where perikarya of sprouting commissural–associational fibers are concentrated. A significant portion of these interneurons may be GABAergic and cholinergic, as for interneurons of the rat cerebral cortex (Hallanger et al., 1986). In addition, neurotensin-binding sites have a distribution which resembles that of AChE staining in certain areas of the rat (Moyse et al., 1987) and human (Szigethy et al., 1990) brain. This is of interest since it was proposed that AChE can play a role in the metabolism of certain neuropeptides (Chubb et al., 1980; Small and Chubb, 1988; Checler and Vincent, 1989; Small, 1989) and in the regulation of cellular proliferation and neurite outgrowth (Small, 1990). However, most of these newly proposed roles for AChE are still under debate (Checler and Vincent, 1989; Small, 1989, 1990; Checler, 1990). It will thus be of interest to investigate the status of GABAergic and peptidergic hippocampal reinnervation post-ECL and to correlate any changes with those established for AChE.

Interactions between NGF, its receptors, and cholinergic sprouting in the ECL model also deserve to be discussed. NGF-like growth-promoting activity, NGF immunoreactivity, and NGF receptor immunoreactivity have been shown to increase in the dentate gyrus following ECL (Crutcher and Collins, 1986; Gómez-Pinilla et al., 1987; Conner et al., 1991). This could suggest that NGF can trigger the septal cholinergic sprouting into the dentate gyrus, as cholinergic neurons are known to be highly responsive to NGF (Gage et al., 1990; Hefti et al., 1990). A recent study supports this hypothesis since it shows that antibody to NGF inhibits collateral cholinergic sprouting of septohippocampal fibers following ECL (Van der Zee et al., 1992). However, these authors have characterized the presumptive cholinergic sprouting in the hippocampus solely on the basis of AChE staining. In addition, other types of hippocampal deafferentation such as septal or fimbria-fornix lesions, known to produce drastic decreases in AChE staining in the hippocampus, still caused increases in NGF content or activity in the dentate gyrus (Collins and Crutcher, 1985; Gasser et al., 1986; Weskamp et al., 1986). Therefore, it is more likely that increased NGF-like activity and immunoreactivity following ECL (Crutcher and Collins, 1986; Conner et al., 1991) result from a more general mechanism in which a degenerative process in the hippocampus produces sequential events leading to the secretion of NGF.

In summary, these observations suggest that the increased AChE staining seen in response to deafferentation might be due to the sprouting of noncholinergic, nonseptohippocampal, intrinsic or extrinsic afferents to the molecular layer of the dentate gyrus. While the concomitant shrinkage of the molecular layer seen in ECL animals (Storm-Mathisen, 1974; Lynch et al., 1975; Nadler et al., 1977; Scheff et al., 1980; Stanfield and Cowan, 1982) may have contributed to some alterations in the markers examined, the marked increase in AChE staining is in clear contrast with the absence of significant and consistent pre- and postsynaptic alterations of various cholinergic markers. On the other hand, if AChE-positive sprouting fibers are in fact cholinergic, our data suggest that the newly formed synapses are unlikely to be adequately organized since the cholinergic markers examined were not altered consistently following ECL, even

after 30 DPL. Further experiments are necessary to clarify the nature of the sprouting AChE fibers in the ECL model of synaptic plasticity.

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