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Characterisation of muscarinic autoreceptors in the septo-hippocampal system of the rat: a microdialysis study

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Abstract

The effects of local administration of cholinergic drugs on the release of acetylcholine in the septo-hippocampal system were investigated using intracerebral microdialysis. Dialysis probes were implanted in the cell-body area of septo-hippocampal neurones in the medial septal area, and in the terminal area of the same neurones in the ventral hippocampus. Drugs were administered locally via the dialysis probe. Administration of the mixed muscarinic/nicotinic receptor agonist carbachol caused a decrease, whereas administration of the muscarinic receptor antagonist methyl-atropine caused an increase in the output of acetylcholine in both the hippocampus and the medial septal area. In contrast, perfusion with the same drugs and the acetylcholine esterase inhibitor neostigmine bromide in the septal area had little or no effect on the output of acetylcholine in hippocampus. The results indicate that acetylcholine autoreceptors are localised on nerve terminals in medial septal area and hippocampus, and exert an inhibitory control over acetylcholine release. However, autoreceptors seem to be sparse or absent on dendrites and cell bodies of septo-hippocampal cholinergic neurones.

Keywords: Acetylcholine; Hippocampus; Microdialysis; Medial septum; Muscarinic autoreceptor

1. Introduction

The cholinergic innervation of the hippocampus and cortex has been studied extensively due to its involvement in cognitive processes, such as learning and memory, arousal, and attention (Fibiger et al., 1991; Squire, 1992; Zola-Morgan and Squire, 1993), and the observation that this innervation undergoes progressive degeneration in Alzheimer's disease (Perry et al., 1992). In addition, administration of muscarinic receptor antagonists in animals causes an amnesic effect (Ennaceur and Meliani, 1992) that resembles early symptoms of Alzheimer's disease. These finding have led to a cholinergic hypothesis of Alzheimer's disease (Coyle et al., 1983; Perry et al., 1977). This hypothesis has been supported by many animal studies (Aigner and Mishkin, 1993; Fischer et al., 1991; Lilliquist et al., 1993). Accordingly, the principal therapeutic approach to Alzheimer's disease is the reinstatement of the level of post-synaptic cholinergic stimulation, either directly with muscarinic receptor agonists, or indirectly by elevating the levels of endogenous acetylcholine with acetylcholine esterase inhibitors (Rupiniak, 1992).

Administration of cholinomimetic drugs affects cholinergic neurotransmission at different levels. In addition to stimulating post-synaptic receptors, cholinergic drugs also affect the release of endogenous acetylcholine by activating autoreceptors. Furthermore, some data suggest that cholinomimetic drugs stimulate the release of acetylcholine in hippocampus by activating muscarinic receptors on cell bodies of septo-hippocampal neurones. These neurones are localised in the medial septum and the adjacent vertical limb of the diagonal band of Broca (Gaykema et al., 1990; Mosko et al., 1973). In vivo electrophysiological data show that administration of carbachol in the medial septal area causes excitation in the majority of septo-hippocampal neurones, and induces synchronised field activity in the hippocampus (Lawson and Bland, 1993). Cholinergic nerve terminals are also found in this area, in the vicinity of cholinergic cell bodies (Bialowas and Frotcher, 1987; Milner, 1991). Acetylcholine release from local cholinergic terminals has been measured in

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brain slices of the medial septal area (Metcalf et al., 1988), and in vivo with microdialysis (Moor et al., 1994). Taken together, these data suggest that cholinergic terminals might form excitatory cholinergic/cholinergic synapses in the medial septal area. Consequently, the medial septum might be an additional target by which cholinergic drugs affect acetylcholine release in the hippocampus.

In order to understand the different levels at which cholinergic drugs act on septo-hippocampal cholinergic neurones, the effects of local administration of cholinergic agents in the medial septal area and the ventral hippocampus were investigated in the present study using microdialysis in freely moving rats. In addition, the effects of cholinergic drugs in the medial septal area on acetylcholine release in the ventral hippocampus were studied using the 'dual-probe' design (Santiago and Westerink, 1992).

2. Materials and methods

2.1. Animals and surgery

Adult male albino rats of a Wistar-derived strain (weight, 280–320 g; C.D.L Groningen, Netherlands) were anaesthetised with chloral hydrate (400 mg/kg i.p.) and placed in a stereotaxic frame (Kopf, USA). Dialysis probes were implanted as previously described by Moor et al. (1994). Briefly: one probe was implanted in the right ventral hippocampus, aiming at subfield 3 of Ammon's horn (coordinates: IA: +3.7 mm, lateral: +4.8 mm, and −8.0 mm from dura mater, Paxinos and Watson, 1982), and another probe was implanted in the medial septal area, including the adjacent vertical limb of the diagonal band of Broca (IA: +9.5 mm, lateral: −1.0 mm, −7.0 mm from dura mater, at an angle of 9°). In the diagonal band, the position of the probe was right of the midline because efferent projections to the hippocampus are mainly ipsilateral (Gaykema et al., 1990).

Home-made I-shaped probes, made of a polyacrylonitrile/sodium methyl sulfonate copolymer dialysis tube (inner diameter: 0.22 mm; outer diameter: 0.31 mm, Hospal, Italy) were used. The exposed tips of the dialysis membrane were 4 mm in hippocampus probes and 2.5 mm in septal probes, respectively. Following surgery, rats were housed individually in Plexiglas chambers (25 × 25 × 35 cm) with free access to food and water.

2.2. Microdialysis experiments

Dialysis experiments were conducted at daytime, starting 20 h following surgery. Each rat was used in two dialysis experiments (post-surgery days 1 and 2). In order to prevent interactions between the first and the second experiment, drug treatment was performed only once in each brain area. The probes were perfused with artificial cerebrospinal fluid containing (in mM): NaCl 147, KCl 3.0, $CaCl_2$ 1.2 and $MgCl_2$ 1.2. Perfusion rate was 2.8 μl/min, delivered by a perfusion pump (Braun TUV, Germany). The acetylcholine esterase inhibitor neostigmine bromide was added to the solution in order to obtain detectable quantities of acetylcholine in the dialysate, except in cases where a probe was used exclusively for the administration of drugs. Fifteen-minute samples were collected in a 50 μl loop of an injection valve (Valco, Switzerland) that was automatically activated by an electronic timer. After stabilisation of acetylcholine overflow (five values with maximum 40% variation), drugs were administered in the perfusion fluid. Following the dialysis experiments, rats were killed and the placement of the probes was controlled by visual inspection of brain slices.

2.3. Chemical analysis and drugs

Samples were analysed 'on-line' with high performance liquid chromatography, enzymatic conversion, and electrochemical detection, as previously described by Damsma et al. (1987,1988), with some minor adjustments. Briefly, the samples were injected onto a reverse-phase C18 column preloaded with sodium laurylsulphate. Acetylcholine was converted into hydrogen peroxide and betaine in a post-column enzyme reactor containing immobilised acetylcholine esterase and choline oxidase (Sigma, USA). Subsequently, hydrogen peroxide was detected using a platinum electrode (Antec, Netherlands) set at +500 mV. The mobile phase consisted of a 0.1 M potassium phosphate buffer (pH = 8.0) containing 0.5 mM EDTA and 2 mM tetramethylammonium chloride, deliverd at a rate of 0.5 ml/min. Sensitivity of detection decayed with 10–30% during each experiment. Therefore, an acetylcholine standard was injected before and after each experiment, and the acetylcholine values were corrected for the decay in sensitivity using a standard curve.

Carbachol was obtained from Brocacef (Netherlands), methyl-atropine was obtained from Sigma (USA), neostigmine bromide was obtained from Centrachemie (Netherlands), and kainic acid was obtained from Tocris Neuramin (UK).

2.4. Presentation of data and statistical analysis

All microdialysis data are presented as percentage of control values, i.e. the mean of five samples taken prior to drug administration. The effect of drug administration was tested with analysis of variance with repeated measures (ANOVA –mixed design). Single time points were compared to control using Tukey's pro-

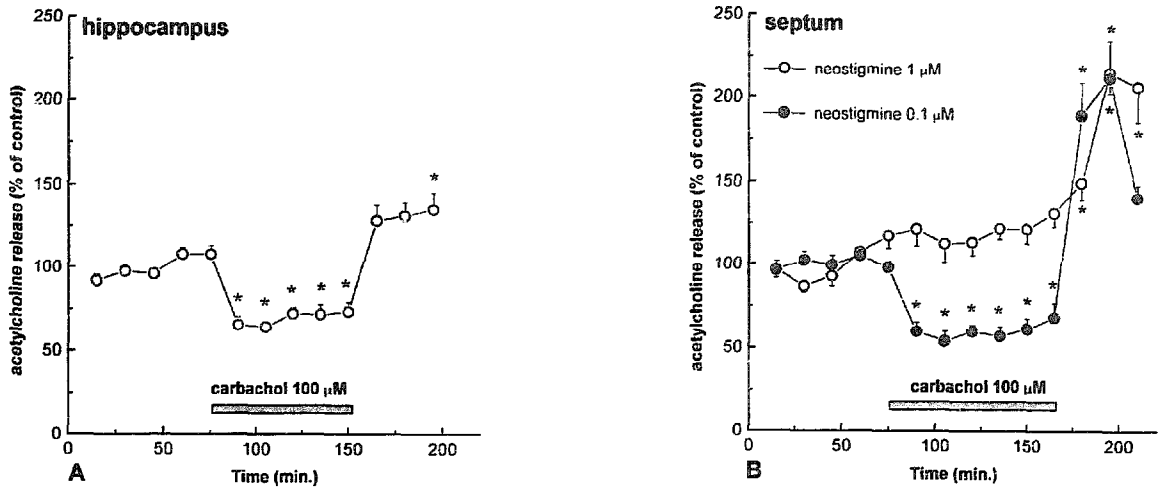

Fig. 1. Acetylcholine release in the ventral hippocampus (A), and the medial septal area (B) during perfusion of 1.0 mM carbachol in the dialysis probe. In B, perfusion of carbachol was performed in the presence of 0.1 μM or 1.0 μM neostigmine bromide in the perfusion fluid. Circles represent mean values of 4–5 animals. Vertical bars represent S.E.M. Asterisks indicate values which are significantly different from pretreatment levels (Tukey's protected *t*-test, $P < 0.01$).

tected *t*-test. Significance level for both tests was set at $P < 0.01$.

3. Results

The average basal (control) levels of acetylcholine in the hippocampus were 61.5 ± 4.23 fmol/min (± S.E.M, $n = 21$), and 23.5 ± 1.95 fmol/min ($n = 4$) in the presence of 1 μM and 0.3 μM neostigmine, respectively. In the medial septal area, the average basal level was 19.7 ± 1.48 ($n = 9$) in the presence of 1 μM neostigmine. The detection limit of the assay was typically 20 fmol per injection.

3.1. Local carbachol and methyl-atropine

In the presence of 1 μM neostigmine, local perfusion of 100 μM carbachol in the hippocampus decreased acetylcholine release to 69% of control values (Fig. 1A, $F(4,36) = 14.27$; $P < 0.0001$). In contrast, local perfusion of carbachol 100 μM in the medial septal area under the same conditions caused a small but significant increase in acetylcholine levels to 116% of control (Fig. 1B, $F(3,30) = 3.425$; $P < 0.001$). However, in the presence 0.1 μM neostigmine, perfusion of carbachol in the medial septal area (Fig. 1B) decreased acetylcholine levels to 60% of control values ($F(3,30) = 12.280$; $P < 0.0001$). Both in experiments with 0.1

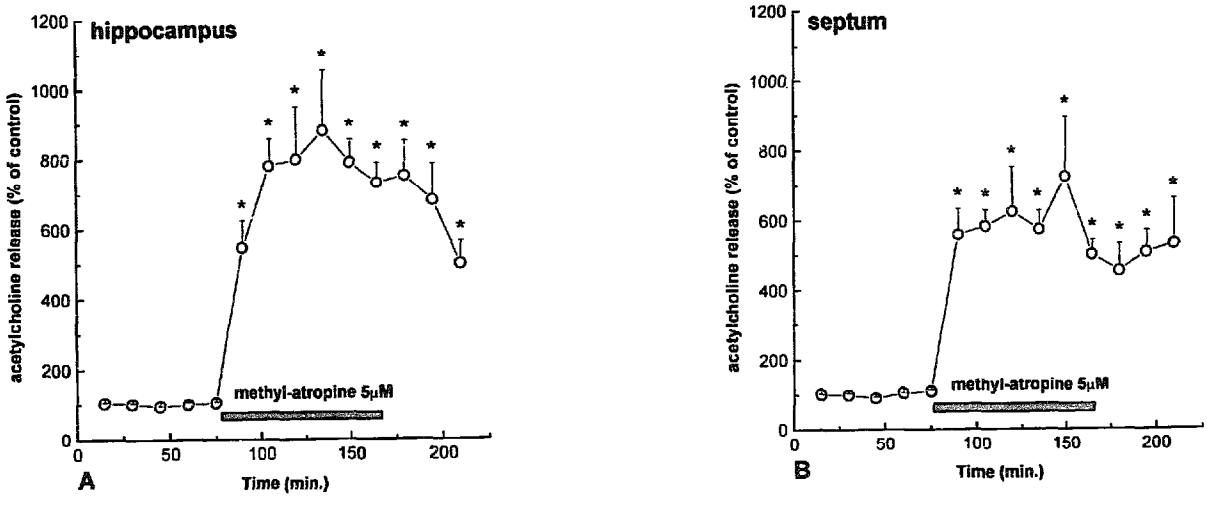

Fig. 2. Acetylcholine release in the ventral hippocampus (A), and the medial septal area (B) during perfusion of 5 μM methyl-atropine in the dialysis probe. Circles represent mean values of five animals. Vertical bars represent S.E.M. Asterisks indicate values which are significantly different from pretreatment levels (Tukey's protected *t*-test, $P < 0.01$).

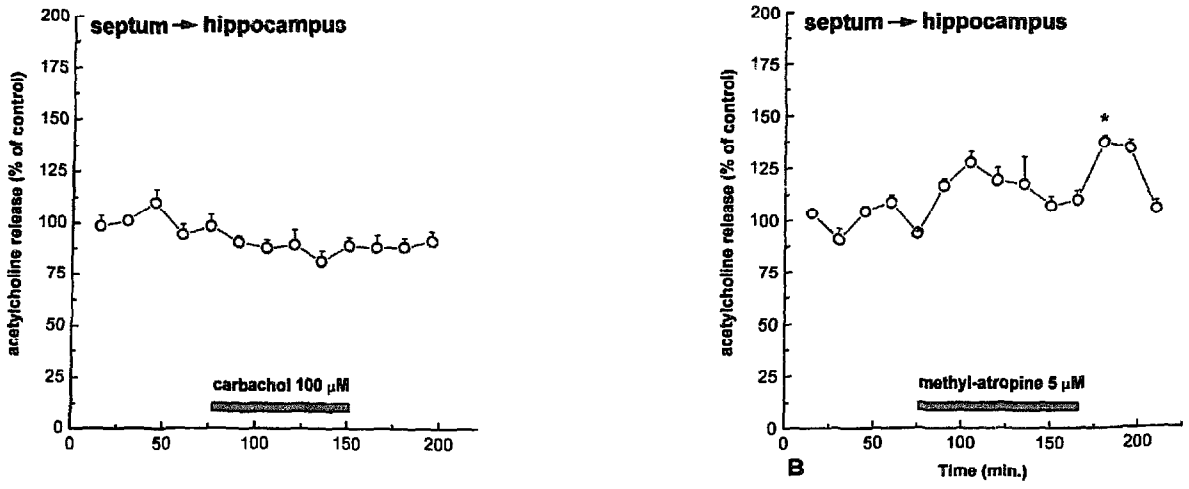

Fig. 3. Acetylcholine release in the ventral hippocampus during perfusion of 1.0 mM carbachol (A) and 5 μM methyl-atropine in a dialysis probe placed in the medial septal area. Data points represent mean values of five animals. Vertical bars represent S.E.M. Asterisks indicate values which are significantly different from pretreatment levels (Tukey's protected *t*-test, $P < 0.01$).

μM and 1 μM neostigmine, acetylcholine levels increased to over 200% of control following carbachol administration in the medial septal area. Although smaller in amplitude, this 'rebound' effect was also observed after local perfusion of carbachol in the hippocampus (131%, Fig. 1A).

Local perfusion with 5 μM methyl-atropine in the hippocampus (Fig. 2A) increased acetylcholine levels to 795% of control ($F(4,40) = 19.651$, $P < 0.0001$). In the medial septal area, local perfusion with 5 μM methyl-atropine (Fig. 2B) increased acetylcholine to 627% of control ($F(4,40) = 16.523$; $P < 0.0001$).

3.2. *'Dual-probe' experiments*

Perfusion with 100 μM carbachol in the medial septal area (Fig. 3A) did not affect the levels of acetylcholine in hippocampus ($F(4,36) = 0.745$; n.s.). Perfusion with 5 μM methyl-atropine in the medial septal area (Fig. 3B) resulted in a relatively small increase in the level of acetylcholine in hippocampus to 118% of control values (maximum increase of 127%). Although the overall drug effect was statistically significant ($F(4,40) = 2.885$; $P < 0.01$), none of the single time points (mean of five animals) proved significant at the set level of 1%.

In order to investigate whether elevated endogenous levels of acetylcholine in the medial septal area affect hippocampal acetylcholine release, artificial cerebrospinal fluid and neostigmine (0.3 μM, 1.0 μM and 3.0 μM, respectively) were perfused in the medial septal area, while acetylcholine was measured in the medial septal area and the hippocampus simultaneously (Fig. 4). Subsequently, kainic acid (5 μM) was perfused in the medial septal area. In a previous study using the same experimental design (Moor et al., 1994), kainic acid was shown to stimulate acetylcholine output in the hippocampus. In the present study perfusion with kainic acid was performed in order to functionally validate the location of the probes. Acetylcholine levels in the medial septal were undetectable during perfusion with cerebrospinal fluid, and increased to 10.6, 27.5, and 42.7 fmol/min during perfusion of 0.3 μM, 1.0 μM and 3.0 μM neostigmine, respectively). Acetylcholine overflow in the hippocampus did not alter significantly during perfusion of neostigmine in the medial septal area ($F(3,60) = 0.822$; n.s.). However, the

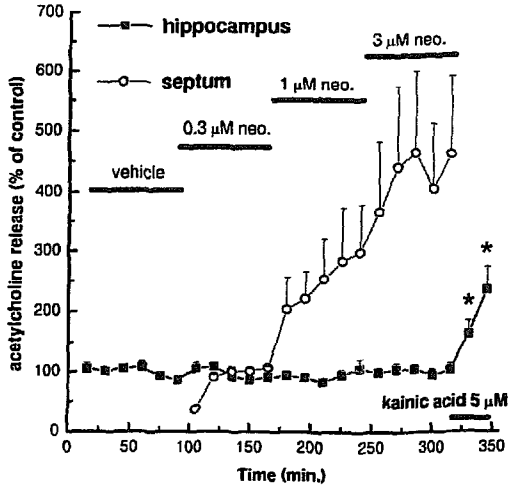

Fig. 4. Simultaneous measurement of acetylcholine release in the medial septal area and in the ventral hippocampus during perfusion of neostigmine bromide (neo.) in the medial septal area. In each experiment, kainic acid (5 μM) was subsequently perfused in the medial septal area in order to validate the placement and function of the probes. Data points represent mean values of four animals. Vertical bars represent S.E.M. Asterisks indicate values which are significantly different from pretreatment levels (Tukey's protected *t*-test, $P < 0.01$).

subsequent perfusion of kainic acid in the medial septal area resulted in a profound increase of hippocampal acetylcholine levels to 312% of control values (Fig. 4).

4. Discussion

4.1. Local carbachol and methyl-atropine

The present results show that the release of acetylcholine in the medial septal area and the ventral hippocampus can be attenuated by autoreceptor stimulation. The output of acetylcholine decreased as a consequence of local administration of carbachol in either brain area. However, perfusion with carbachol failed to decrease the output of acetylcholine in the septal area under routine experimental conditions, with 1 μM neostigmine in the perfusion fluid. In contrast, carbachol decreased the output of acetylcholine when 0.1 μM neostigmine was used. This result indicates that auto-inhibition is maximal in the medial septal area, but not in the hippocampus in the presence of 1.0 μM neostigmine. These differences might reflect either different densities or properties of autoreceptors in these brain areas.

Local administration of carbachol in the medial septal area was followed by rebound of acetylcholine levels: when the perfusion of carbachol was terminated, acetylcholine levels increased to a maximum of over 200% comparing to pre-treatment values. This effect was also observed in the hippocampus, but was much less pronounced. Although this phenomenon is not fully understood, it can be hypothesised that if at a certain degree of autoreceptor occupation receptor desensitisation takes place, the subsequent removal of the drug would result in a lower level of autoinhibition relative to pre-treatment levels. This would cause acetylcholine output to increase beyond the initial level.

In agreement with high levels of autoinhibition, perfusion with methyl-atropine caused a sizeable increase in acetylcholine output in the hippocampus and the medial septal area. Although the effects of methyl-atropine were not significantly different in these brain areas, the increase in the hippocampus (796%) appears somewhat larger than in the medial septal area (627%). Taken together, the results indicate cholinergic nerve terminal in both brain areas have inhibitory muscarinic autoreceptors, although their properties seem to be slightly different.

An interdependency between the effects of muscarinic compounds and the level of acetylcholine esterase inhibition has been previously demonstrated in microdialysis studies. In these studies, muscarinic receptor agonists and antagonists were coadministered with different concentrations of neostigmine (Damsma et al., 1987; DeBoer et al., 1990). In the striatum, the level of autoinhibition appears to be very low when no esterase inhibitor is added to the perfusion fluid, because atropine administration caused only marginal increase in acetylcholine levels. However, when atropine was coadministered with an esterase inhibitor, its effect was much more pronounced. The reverse has been demonstrated with the muscarinic receptor agonist oxotremorine (DeBoer et al., 1990). Considering these results, it should be noted that the effect of atropine in the present study is probably much larger than it would be under physiological conditions.

4.2. 'Dual-probe' experiments

Perfusion with cholinomimetic drugs in the medial septal area did not affect acetylcholine levels in the hippocampus. Perfusion of carbachol in the medial septal area did not affect acetylcholine levels in the hippocampus, and administration of neostigmine failed to affect acetylcholine overflow in the hippocampus, despite the large increase in septal acetylcholine levels.

Perfusion with the muscarinic receptor antagonist methyl-atropine in the medial septal area only caused a small increase in acetylcholine levels in the hippocampus. Thus, the current results imply that muscarinic receptors in the medial septal area are probably insignificant for the modulation of hippocampal acetylcholine release.

Alternatively, it can be argued that the absence of profound effects in the 'dual-probe' experiments is due to methodological problems, such as low concentration of the drugs at the site of the target neurones, or that an insufficient proportion of the neurones were affected by the drug. Nevertheless, local administration of methyl-atropine was extremely potent in elevating acetylcholine release in the hippocampus and medial septal area. Moreover, perfusion of a relatively moderate concentration of kainic acid in the medial septal area resulted in a 2-fold increase in hippocampal acetylcholine release. Thus, the results show that (1) drugs diffuse out of the dialysis probe, and (2) acetylcholine release in the hippocampus can be affected by administration of drugs into the medial septal area. Thus, the absence of an effect of carbachol and neostigmine, and the relatively small effect of methyl-atropine are unlikely to be explained by methodological factors.

In apparent disagreement with the current results, several electrophysiological studies suggest that cholinergic compounds have an excitatory effect on septo-hippocampal neurones. It has been shown that iontophoretic administration of carbachol, acetylcholine, and physostigmine in the septal area increased the discharge rate of the majority of septo-hippocampal

neurones, which were identified by antidromical stimulation of the fornix (Bassant et al., 1988; Dutar et al., 1986; Lamour et al., 1984). In another study, microinjections of carbachol into the medial septal area induced continuous theta field activity in the hippocampus (Lawson and Bland, 1993). The discrepancy between these and the current results might be explained as follows: antidromically stimulated neurones in the electrophysiological studies were not specifically identified as cholinergic neurones. Furthermore, anatomical data suggest that about 50% of all septo-hippocampal neurones are GABA-ergic (Kiss et al., 1990; Wainer et al., 1985). Bialowas and Frotcher (1987) hypothesised that GABA-ergic septo-hippocampal neurones are the major target of cholinergic terminal in the medial septal area. Consequently, the cholinoceptive neurones in these electrophysiological studies might be GABA-ergic. This hypothesis is indirectly supported by the results of a detailed study of the septal complex using immunostaining of choline acetyltranferase (Milner, 1991). In this study, contacts between positive terminals, and negative dendrites and perikarya were 7 times more common than contacts between positive terminals and positive cells. This study shows that although cholinergic/cholinergic synapses in the medial septal area exist, the majority of cholinergic nerve terminals in the medial septal area innervates non-cholinergic cells. Moreover, the effect of carbachol in electrophysiological studies was excitatory. In the present study, methyl-atropine administration in the septal area caused a slight but significant increase in hippocampal acetylcholine release. If this effect is direct and receptor-mediated, it would indicate an inhibitory effect of muscarinic receptor stimulation.

The current results are also supported by a study of medial septum and diagonal band neurones in slices of guinea pig brain (Sim and Griffith, 1991). Intracellular recording obtained from these cells during administration of the muscarinic receptor agonists muscarine and betanechol showed only small and variable changes in membrane potentials. The authors conclude that medial septal neurones are quite insensitive to muscarinic receptor stimulation.

4.3. In conclusion

Muscarinic autoreceptors are located presynaptically on nerve terminals in the hippocampus and the medial septal area. Stimulation of these receptors exerts a powerful inhibitory effect on acetylcholine release. In contrast, muscarinic receptors on cholinergic cell bodies and dendrites are either sparse or absent. Consequently, cholinergic nerve terminals in the medial septal area do not play a significant role in the regulation of acetylcholine release in the hippocampus.

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