Biochemical Pharmacology, Vol. 53, 1379–1382, 1997.

ISSN 0006-2952/97/$17.00 + 0.00
PII S0006-2952(97)00074-9

SHORT COMMUNICATION

Differential Regulation of Expression of Rat Hippocampal Muscarinic Receptor Subtypes Following Fimbria-Fornix Lesion

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ABSTRACT. Quantitative RNase protection assays were performed to determine the levels of muscarinic receptor subtype (m1–m5) mRNAs in rat hippocampi. Results showed that the m1, m3, and m4 subtype mRNAs were expressed at relatively high levels, but the levels of the m2 and m5 subtype were very low. Three weeks following aspiration lesions of the fimbria-fornix to produce cholinergic denervation of the hippocampus, non-M_1 receptors (non-pirenzepine displaceable [^{3}H]quinuclindinyl benzilate binding sites) in the hippocampus were increased significantly, which correlated with increases in the levels of hippocampal m3 and m4 receptor mRNAs (m3: +24% and m4: +41%). These findings indicate that multiple muscarinic receptor subtypes are expressed in the hippocampus with the m3 and m4 subtypes predominantly postsynaptic to the septohippocampal cholinergic terminals. BIOCHEM PHARMACOL 53;9:1379–1382 © 1997 Elsevier Science Inc.

KEY WORDS. muscarinic receptor subtype; hippocampus; fimbria-fornix lesion; gene expression; Alzheimer's disease

Pharmacological studies with the selective antagonist PZ† initially distinguished two major classes of muscarinic cholinergic receptors: M_1 (PZ-sensitive) and M_2 (PZ-insensitive) [1]. However, molecular cloning has revealed that five muscarinic receptor subtypes are expressed in the brain [2, 3]. The heterogeneous distribution of these receptor subtype mRNAs suggests different functional roles for these subtypes in regulating brain function. Central cholinergic neurotransmission has been shown to be involved in learning and memory, and studies have focused, in particular, on the hippocampus [4]. Cholinergic receptor blockade or cholinergic denervation of the hippocampus disrupts learning behaviors [5]. Lesion of the fimbria-fornix results in destruction of the septohippocampal cholinergic input and is accompanied by an early loss of presynaptic muscarinic receptors followed by a later increase in the density of postsynaptic receptors [6]. It has been suggested that PZ-defined M_1 binding sites correspond to the postsynaptic hippocampal muscarinic receptors, while the M_2 (or, more correctly, non-M_1) receptor subtypes may represent the presynaptic autoreceptors on the cholinergic terminals [7]. However, this hypothesis cannot explain the subsequent finding of an M_2 receptor up-regulation following chronic denervation [8]. Recent studies using receptor subtype selective antibodies have reported complex changes in the multiple muscarinic receptor subtypes after hippocampal cholinergic denervation [9, 10]. In the present study, we took a different approach by utilizing sensitive RNase protection assays which provide the first direct evidence that the alterations of muscarinic receptor subtypes following hippocampal cholinergic denervation are due to the differential regulation of receptor subtype gene expression.

MATERIALS AND METHODS

Unilateral and bilateral lesions of the fimbria-fornix of male Sprague–Dawley rats (225–250 g) were performed by aspiration of the fimbria-fornix with overlying cortical tissue. Rats were killed 21 days following the lesion. Whole brains from unilaterally lesioned animals (N = 6) were removed rapidly for receptor autoradiography [8]. Briefly, muscarinic receptors were labeled by incubating tissue sections for 1 hr in a Krebs phosphate buffer (pH 7.4) containing 1 nM [^{3}H]QNB (80.0 Ci/mmol; DuPont NEN, Boston, MA). Nonspecific binding was determined by the addition of 1 μM atropine. The non-M_1 muscarinic receptors were defined indirectly by the addition of 100 nM PZ in the incubation to displace the [^{3}H]QNB from the M_1 sites but leaving binding at non-M_1 sites. The autoradiograms were analyzed quantitatively with an MCID image system (Imaging Research Inc., St. Catharines, Ontario, Canada).

The hippocampi from six individual lesioned animals

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† *Abbreviations:* PZ, pirenzepine; and QNB, quinuclindinyl benzilate.

Received 12 August 1996; accepted 5 December 1996.

and six normal controls were dissected rapidly and homogenized in TRI-reagent (Molecular Research Center Inc., Cincinnati, OH), for RNA analyses. Total cellular RNA was isolated following standard protocols. Rat m1, m2, m3, m4, and m5 cDNAs coding for the receptor peptides, third cytoplasmic loops [2], were a gift from T. I. Bonner. A rat m2 receptor cDNA fragment was also generated by reverse transcriptase-polymerase chain reaction (RT-PCR) from the rat m2 receptor sequence 693 *nt* to 1118 *nt* [11]. Fifteen micrograms of hippocampal RNA was used for the RNase protection assays, which have been described previously [12]. In brief, hippocampal cellular RNA (15 μg) together with m1–m5 muscarinic receptor subtype riboprobes (10^5 cpm each) was mixed in 30 μL of hybridization solution containing 50% formamide, 0.6 M NaCl, 4.5 mM EDTA, 10 mM Tris–HCl (pH 7.4), and 10 mM dithiothreitol. Following denaturation at 95° for 5 min, the hybridization mixture was transferred quickly to 62° overnight. The non-hybridized single-stranded RNAs and excess riboprobes were digested by the addition of 300 μL of RNase mixture containing DNase free RNase A (50 μg/mL and RNase T1 (2 μg/mL) at 30° for 2 hr. Then the double-stranded mRNA hybrids were extracted with phenol–chloroform (1:1) and co-precipitated with yeast tRNA. The sample RNA was separated on a 5% polyacrylamide gel, dried onto 3M filter paper, and exposed to X-ray film (with intensifying screen) at −70° until developed. The dried gel was marked, and the RNA bands were excised by using the X-ray film as a guide. The levels of muscarinic receptor subtype mRNAs were determined by scintillation spectrometry. Standardization of the assay was achieved by producing standard curves with the synthesized sense strand RNA for each muscarinic subtype mRNA assayed. The linear regression correlation coefficients (r^2) of all assay standard curves were > 0.95. Rat β-actin mRNA was also determined in the fimbria-fornix lesion studies to correct for preparation errors.

RESULTS

RNase protection assays showed that all five subtype muscarinic receptors were expressed in the hippocampus but at different levels. The m1 receptor mRNA was the predominant subtype expressed in the hippocampus (25.6 ± 4.5 amol/μg total RNA). The m3 and m4 mRNAs were also expressed in the hippocampus, but their levels were about 20% of that of the m1 subtype (m3 = 6.7 ± 0.8 amol/μg total RNA; m4 = 4.8 ± 0.7 amol/μg total RNA). There were detectable signals for both m2 and m5 mRNAs in the hippocampus, but the levels were very low (m2 = 1.6 ± 0.4 amol/μg total RNA; m5 = 1.9 ± 0.4 amol/μg total RNA).

Following unilateral lesion of the fimbria-fornix, the total population of muscarinic receptor binding as measured by the non-subtype selective ligand [^{3}H]QNB, showed a small but non-significant increase compared with the control side (105 ± 11.6%). However, when 1 nM [^{3}H]QNB binding was quantified in the presence of 100 nM PZ to block the M_1 receptors, the binding sites representing non-M_1 receptors of the hippocampus ipsilaterally to the lesion were increased dramatically (Table 1). The changes were most notable in area CA3 (+31%). RNase protection assays indicated that the increases of non-M_1 receptor binding sites were due to increases in the levels of both m3 and m4 receptor gene expression (m3: +24% and m4: +41%; Table 2). No significant change in the level of the m1 receptor subtype mRNA was detected at 3 weeks post-lesion.

DISCUSSION

The levels of expression of the five muscarinic receptor subtype mRNAs show a good correlation with the levels of receptor proteins, as measured by immunoprecipitation [10] and immunocytochemistry [9]. Both the m1 receptor protein and the m1 mRNA are expressed in the hippocampus at the highest level. The hippocampal m1 receptor protein has been located on pyramidal cells and, perhaps, some other neurons postsynaptic to the septal cholinergic afferents [9]. In contrast, the level of m2 receptor subtype mRNA in the hippocampus is much lower than might be expected from its protein density ([3, 9] and our unpublished observation). This indicates that a large proportion of hippocampal m2 receptors likely reside on the cholinergic terminals and serve as autoreceptors, their mRNA expressed in cell bodies outside of the hippocampus. Indeed, there is a high level of m2 receptor mRNA expression in the medial septum choline acetyl transferase-positive cholinergic cell group ([13] and our unpublished observation). The level of m3 receptor subtype mRNA, like the m1 subtype, was well correlated with the density of m3 receptor protein in the hippocampal region. In fact, an immunocytochemical study has shown a postsynaptic distribution pattern of m3 receptors in the hippocampus [14]. Although this study suggests that the majority of m3 receptors are probably expressed intrinsically by hippocampal neurons rather than being presynaptically localized on the afferent

TABLE 1. Effect of unilateral fimbria-fornix lesion on rat hippocampal non-M_1 muscarinic receptor levels 3 weeks post-lesion

	Non-M_1 receptor binding* (fmol/mg tissue)		
	ULS	LS	% Control
Hipp.†	169.1 ± 5.8	196.0 ± 11.4‡	(116 ± 7)
CA1	201.4 ± 5.3	216.9 ± 8.6	(107 ± 4)
CA3	140.9 ± 4.9	184.4 ± 8.6§	(131 ± 6)
DG†	154.9 ± 8.5	170.8 ± 5.1‡	(115 ± 3)

Values are means ± SEM; N = 6.

* Non-PZ displaceable [^{3}H]QNB binding sites (non-M_1). Abbreviations: ULS, unlesioned side; and LS, lesioned side.

† Hipp. = averaged over whole hippocampus; DG = dentate gyrus.

‡ $P < 0.05$ (paired t-test).

§ $P < 0.01$ (paired t-test).

TABLE 2. Effect of bilateral fimbria-fornix lesion on the levels of rat hippocampal m1, m3, and m4 muscarinic receptor subtype mRNAs 3 weeks post-lesion

	Ratios*		
	Control	Lesioned	% Control
m 1	0.81 ± 0.02	0.89 ± 0.04	(109 ± 4)
m 3	0.21 ± 0.01	0.26 ± 0.01†	(124 ± 7)
m 4	0.15 ± 0.01	0.21 ± 0.01†	(141 ± 8)

Values are means ± SEM; N = 6.
* Levels of muscarinic receptor subtype mRNAs/β-actin mRNA.
† $P < 0.01$ (*t*-test).

cholinergic fibers, there is evidence of m3 receptor expression in the medial septum [13].

A previous study of hippocampal muscarinic receptor densities after fimbria-fornix lesion showed an increase in total hippocampal [^{3}H]QNB muscarinic receptor binding sites [14], [^{3}H]PZ-labeled (M_1) binding sites, and non-PZ displaceable [^{3}H]*N*-methylscopolamine (non-M_1) binding sites [15]. However, other studies failed to observe either the change in total population of the hippocampal muscarinic receptors labeled by [^{3}H]QNB following lesion of fimbria-fornix [16, 17] or M_1 receptor [^{3}H]PZ binding sites after lesion of the medial septum [18]. These discrepancies may possibly be due to technical differences in the methods of analysis. For example, measurement of receptors in homogenates of whole hippocampus would result in the pooling of receptors in this laminated structure, possibly masking differential changes in discrete regions. Likewise, the increase of non-M_1 subtypes would be diluted if the total muscarinic receptor subtype pool was determined [16, 17]. It is now clear that the pharmacologically defined M_1 receptor labeled with [^{3}H]PZ represents primarily the binding sites of the m1 and, perhaps, partially the m4 receptor subtypes. The non-M_1 binding sites visualized with [^{3}H]QNB in the presence of PZ represent the m2, m3, and m5, and partially m4 receptor subtypes. Our results from the RNA analyses suggest that increased transcription of the m3 and m4 receptor genes may be responsible for the changes in the non-M_1 receptor binding seen with receptor autoradiography. Moreover, this increased expression of m3 and m4 receptor genes also indicates that these receptor subtypes are postsynaptic. Recently, using different subtype selective antibodies, two independent research groups have reported that cholinergic denervation of the hippocampus increased hippocampal muscarinic receptor proteins (m1: +14%, m3: +77%, m4: +29% [10] and m1: +29%, m4: +44% [9]). Their results agree well with the increases of m3 and m4 receptor mRNAs reported here. However, we did not detect any change in m1 mRNA 3 weeks after bilateral fimbria-fornix lesion. This may be due to the fact that the changes in m1 receptor density were much smaller and transient (+15% at day 15 [10], or +29% at day 14, and no significant increase at day 28 [9]). The reason for this difference in regulatory pattern is not clear. In both studies [9, 10], a decrease (−25%) in m2 receptor protein was consistently observed following the hippocampal denervation, which suggested that the m2 receptor is localized predominantly on presynaptic cholinergic terminals, although a small population of postsynaptic m2 receptors may also be expressed in the hippocampus ([9, 10] and the present study).

The present data, in conjunction with the work of others, provide evidence for the differential neuronal expression of muscarinic receptor subtypes in the hippocampus. This heterogeneity in the expression of muscarinic receptor subtypes localized both pre- and postsynaptically suggests that major clinical benefits in the treatment of Alzheimer's disease should not be expected when using non-selective muscarinic receptor agonists or antagonists to increase diminished cholinergic tone. For example, non-selective muscarinic receptor agonists may also result in a decreased release of ACh from any remaining cholinergic neurons, by acting at muscarinic autoreceptors. A postsynaptic muscarinic receptor subtype selective agonist or presynaptic autoreceptor antagonist may be a more rational treatment to increase the diminished cholinergic tone of Alzheimer's disease.

This study was supported by MH44211 and NIMH RSA MH00316. We thank Dr. G. Buzsaki for supervising the hippocampal lesions.

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