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Neuronal localization of m1 muscarinic receptor immunoreactivity in the rat basolateral amygdala

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Abstract

Muscarinic cholinergic neurotransmission in the basolateral nuclear complex (BLC) of the amygdala is critical for memory consolidation in emotional/motivational learning tasks. Although knowledge of the localization of muscarinic receptor subtypes in the BLC would contribute to an understanding of the actions of acetylcholine in mnemonic function, previous receptor binding and in situ hybridization studies lacked the resolution necessary to identify which neurons in the BLC express different receptor subtypes. In the present study immunohistochemistry was used to study the neuronal localization of the m1 receptor. The intensity of m1 immunoreactivity varied in different nuclei of the amygdala, and was most robust in the BLC, and in the adjacent posterolateral cortical nucleus. The density and morphology of labeled neurons in the BLC suggested that the m1+ neuronal population included pyramidal cells, the principal neurons in this amygdalar region. In addition, there was dense punctate m1 immunoreactivity in the neuropil of the BLC. Dual labeling immunofluorescence studies of the BLC using antibodies to cell type specific markers were performed to more definitively determine the phenotype of m1-positive (m1+) neurons. An antibody to calcium/calmodulin protein kinase II (CaMK) was used to label pyramidal cells, whereas an antibody to glutamic acid decarboxylase was used to label interneurons. Virtually all of the intensely labeled m1+ neurons of the BLC were CaMK+ pyramidal cells. These data suggest that the ability of M1 receptor antagonists to impair memory consolidation in the BLC is mainly due to blockade of cholinergic influences on the activity of pyramidal neurons.

Keywords

Acetylcholine; Pyramidal cells; Calcium/calmodulin protein kinase II; Immunohistochemistry

Introduction

The basolateral nuclear complex (BLC) of the amygdala, consisting of the lateral, basolateral, and basomedial nuclei, plays an important role in various types of emotional and motivational memory. Several neuromodulators, including acetylcholine, are critical for mnemonic functions performed by the BLC (McGaugh 2004). Thus, posttraining infusions

of muscarinic cholinergic antagonists into the basolateral nucleus, or lesions of the portions of the basal forebrain cholinergic system projecting to the BLC, produce impairments in several types of emotional/motivational learning including inhibitory avoidance, contextual fear conditioning, food reward magnitude learning, conditioned place preference, and drug-stimulus learning (reviewed by Power et al. 2003a). In fact, it has been suggested that the degeneration of the cholinergic projections to the BLC in Alzheimer's disease may be at least as important for the memory disturbances seen in this disorder as the cholinergic projections to the cortex (Kordower et al. 1989; Power et al. 2003a).

Early investigations in the rat had shown that the levels of choline acetyltransferase (ChAT; the synthetic enzyme for acetylcholine) and acetylcholinesterase (the catabolic enzyme for acetylcholine) in the BLC were among the highest in the brain (Ben-Ari et al. 1977). Subsequent studies obtained similar findings in all other mammalian species investigated including human and non-human primates (Girgis 1980; Svendsen and Bird 1985; Hellendall et al. 1986; Amaral and Bassett 1989). Studies combining ChAT immunohistochemistry with retrograde tract tracing demonstrated that the cholinergic basal forebrain, especially the Ch4 group in the substantia innominata, was the main source of these cholinergic inputs to the amygdala in both rodents (Mesulam et al. 1983a; Woolf et al. 1984; Carlsen et al. 1985; Záborszky et al. 1986; Rao et al. 1987) and primates (Mesulam et al. 1983b; Koliatsos et al. 1988; Kordower et al. 1989). Although a small number of ChAT+ interneurons were seen in the rat BLC (Carlsen and Heimer 1986), these were not observed in primates (Amaral and Bassett 1989).

Although cholinergic inputs to the BLC are associated with both nicotinic and muscarinic receptors, most studies of memory consolidation by the BLC utilized muscarinic antagonists (Power et al. 2003a). Pharmacological studies have found at least four muscarinic receptor subtypes (designated by upper case letters as M1–M4), whereas molecular biological techniques have identified five distinct subtypes (designated by lower case letters as m1–m5) (Ehlert et al. 1995). These muscarinic receptor subtypes exhibit a differential distribution in the brain and are characterized by specific signal transduction mechanisms (Richelson 1995). Receptor binding autoradiographic studies in rodents and primates, including humans, demonstrate that the BLC contains both of the two major pharmacologically-defined receptor subtypes, M1 and M2 (Cortés and Palacios 1986; Cortés et al. 1987; Spencer et al. 1986; Mash and Potter 1986; Mash et al. 1988), as well as putative M3 and/or M4 receptors (Smith et al. 1991). In situ hybridization studies using film-based auto-radiography have shown that the m1 receptor was the predominant subtype expressed in the BLC (Buckley et al. 1988).

Power and colleagues (Power et al. 2003b) demonstrated that activation of both M1 and M2 receptors in the BLC are needed for memory consolidation functions performed by this brain region. Although knowledge of the cellular and subcellular localization of these receptors in the BLC is critical for understanding the actions of acetylcholine involved in consolidation of memory, previous receptor binding autoradiographic studies and film-based in situ hybridization studies lacked the resolution necessary to identify which neurons in the BLC express different muscarinic receptor subtypes. Likewise, electrophysiological investigations of neuronal responses to muscarinic drugs in the BLC have been hampered by the lack of

receptor subtype specific agonists and antagonists (Ehlert et al. 1995). However, the development of antibodies to specific muscarinic receptor subtypes has permitted immunohistochemical localization of these receptor proteins at the light and electron microscopic levels (Levey et al. 1991; Mrzljak et al. 1993). An immunohistochemical study of the rat forebrain revealed that m1 was the predominant muscarinic receptor subtype in the amygdala, but no details of the nuclear or neuronal localization of these receptors was provided (Levey et al. 1991). In the present investigation single and dual labeling immunohistochemistry was used to study the neuronal localization of the m1 receptor in the rat amygdala.

Experimental procedures

Tissue preparation

Adult male Sprague–Dawley rats (250–350 g; Harlan, Indianapolis, IN, USA) were used in this study. All experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Use and Care Committee (IACUC) of the University of South Carolina. All efforts were made to minimize animal suffering and to use the minimum number of animals necessary to produce reliable scientific data.

Rats were anesthetized with chloral hydrate (350 mg/kg) and perfused intracardially with phosphate-buffered saline (PBS; pH 7.4) containing 0.5% sodium nitrite (50 ml) followed by 3.0% paraformaldehyde in 0.1 M phosphate buffer at pH 7.4 (500 ml). Following perfusion, brains were removed and postfixed for 2–3 h in 3.0% paraformaldehyde. Brains were then sectioned on a vibratome at a thickness of 50 μ m in the coronal plane. Sections were processed for immunohistochemistry in wells of tissue culture plates. All antibodies were diluted in 0.1 M PBS containing Triton X-100 (0.05–0.4%), 1% normal goat serum, and 1% bovine serum albumin.

Immunoperoxidase experiments

Localization of the m1 muscarinic cholinergic receptor was performed in eight rats using the avidin–biotin immunoperoxidase (ABC) technique. The primary antibody to m1 (1:250; catalog # M-9808; Sigma Chemical Co., St. Louis, MO, USA) was raised in rabbit to a highly purified GST fusion protein of a part of the third intracellular loop of the human m1 receptor corresponding to amino acids 227–353. In each brain 6–10 coronal sections at regular intervals through the rostrocaudal extent of the amygdala were incubated in primary antibody for 48 h at 4°C and then processed for the ABC technique using a Vectastain rabbit ABC kit (Vector Laboratories, Burlingame, CA). Nickel-enhanced DAB (3,3'-diaminobenzidine-4HCl, Sigma) was used as a chromogen to generate a black reaction product (Hancock 1986). Following the immunohistochemical procedures, sections were mounted on gelatinized slides, dried overnight, dehydrated in ethanol, cleared in xylene, and coverslipped in Permount (Fisher Scientific, Pittsburgh, PA, USA). Sections were analyzed using an Olympus BX51 microscope, and digital light micrographs were taken with an Olympus DP2-BSW camera system. Brightness and contrast were adjusted using Photoshop 6.0 software.

Dual labeling immunofluorescence experiments

The results of the immunoperoxidase studies revealed that the BLC was the main portion of the amygdala exhibiting expression of m1 receptors. Previous studies have demonstrated that the rat BLC contains two main classes of neurons that closely resemble their namesakes in the cerebral cortex: (1) glutamatergic pyramidal cells that can be selectively stained using antibodies to the alpha subunit of calcium/calmodulin protein kinase II (CaMK), and (2) GABAergic nonpyramidal interneurons that can be selectively stained using antibodies to GABA, or its synthetic enzyme glutamic acid decarboxylase (GAD) (McDonald 1985, 1992a; Carlsen 1988; Kemppainen and Pitkänen 2000; McDonald et al. 2002). Dual localization of m1 with CaMK or GAD-67 was investigated in four rats to determine if m1 was expressed by pyramidal neurons or nonpyramidal interneurons, respectively.

Sections were incubated for 48 h at 4°C in a cocktail of the rabbit anti-m1 antibody (1:50, Sigma), and either a mouse monoclonal anti-CaMK antibody (1:300; Sigma) or a mouse monoclonal GAD-67 antibody (1:500, Millipore, Billerica, MA, USA). Sections were then rinsed in three changes of PBS (10 min each), and then incubated in a cocktail of goat anti-rabbit Alexa-488 and goat anti-mouse Alexa-546 antibodies (1:200; Invitrogen, Eugene, OR, USA) for 3 h at room temperature. These secondary antibodies were highly cross-adsorbed by the manufacturer to ensure specificity for primary antibodies raised in each species. Sections were then rinsed in three changes of PBS (10 min each) and mounted on glass slides using Vecta-shield mounting medium (Vector Laboratories Inc., Burlingame, CA, USA). Sections were examined with a Zeiss LSM 510 Meta confocal microscope. Fluorescence of Alexa-488 (green) and Alexa-546 (red) dyes was analyzed using filter configurations for sequential excitation/imaging via 488 and 543 nm channels. Digital images were adjusted for brightness and contrast using Photoshop 6.0 software.

In each of the brains processed for dual labeling immunofluorescence, three sections each through the rostral half of the amygdala, where m1 immunoreactivity was greatest (see below), were processed for either m1/CaMK or m1/GAD dual localization. In the m1/CaMK preparations, cell counts of single-labeled m1+ neurons, single-labeled CaMK+ neurons, and double-labeled m1/CaMK+ neurons were performed in the anterior subdivision of the basolateral nucleus (BLa) in the three brains with the most robust m1 immunoreactivity. These counts were made at 400× magnification in five amygdalae of each brain (three on one side, and two on the opposite side of the three sections) after positioning the objective over the center of the nucleus in each section. In the m1/GAD preparations, cell counts of single-labeled GAD+ neurons and double-labeled m1/GAD+ neurons were performed in the BLa in the three brains with the most robust m1 immunoreactivity. These counts were made at 400× magnification in five amygdalae in one or more separate fields in each section until a total of 50 GAD+ neurons in each of the three brains were analyzed (total GAD+ neurons analyzed = 150).

Antibody specificity and controls

The specificity of this antiserum for the m1 receptor has been demonstrated in preadsorption and immunoprecipitation studies in rats (Levey et al. 1991), and in m1 knockout mice (Hamilton et al. 1997). The specificity of the CaMK antibody, which recognizes both

phosphorylated and nonphosphorylated forms of the kinase, has been documented in previous studies (Erundu and Kennedy 1985). The GAD-67 antiserum produced staining in the forebrain, including the amygdala, which was consistent with previous studies using GAD or GABA antibodies (McDonald 1985; Carlsen 1988; Kemppainen and Pitkänen 2000).

A number of method controls for the immunofluorescence dual labeling studies were performed to test the specificity of the secondary antibodies and to determine if there was any “crosstalk” (bleed) between the red and green channels. Incubation of sections in the anti-rabbit secondary antibodies after incubation in the mouse anti-CaMK or mouse anti-GAD primary antibodies produced no labeling. Likewise, incubation of sections in the anti-mouse secondary antibodies after incubation in the rabbit anti-m1 primary antibody also produced no labeling. Examination of sections labeled with the m1/Alexa-488 (green) sequence produced no labeling when examined with the 543 nm (red) channel. Likewise, examination of sections labeled with either the CaMK/Alexa-546 or GAD/Alexa-546 (red) sequence produced no labeling when examined with the 488 nm (green) channel. These results indicate that the secondary antibodies were specific for rabbit or mouse IgGs and that there was no “crosstalk” between the red and green channels.

Results

Immunoperoxidase experiments

The intensity of m1 immunoreactivity varied in different nuclei of the amygdala, and was most robust in the anterior two-thirds of the BLC, and in the adjacent posterolateral cortical nucleus (Fig. 1). In most amygdalar nuclei with significant amounts of m1 immunoreactivity, the great majority of neuronal perikarya, as well as the neuropil, were labeled (Figs. 1, 2). The density of the neuropilar immunoreactivity appeared to be correlated with the intensity of perikaryal staining. The variations in staining intensity in different nuclei were mainly related to the intensity of perikaryal staining, rather than differences in packing density or perikaryal size of the stained neurons (Fig. 1b, d, f).

At rostral levels of the amygdala, the anterior subdivision of the basolateral amygdalar nucleus (BLA) and adjacent portions of the lateral nucleus exhibited the most robust m1 immunoreactivity (Fig. 1a, b). There was also light immunoreactivity in the anterior cortical nucleus (Coa), anteroventral subdivision of the medial nucleus (MeAV), lateral portions of the central nucleus, and the intercalated nuclei (IN). The perikaryal immunoreactivity in neurons of the intercalated nuclei equaled that of the surrounding neuropil, so individual perikarya were difficult to discern (Fig. 1b).

At middle levels of the amygdala, all portions of the BLC, as well as the adjacent posterolateral cortical nucleus, were labeled (Fig. 1c, d). However, the intensity of immunoreactivity was much less in the ventrolateral subdivision of the lateral nucleus (Lvl; Fig. 1d) and the basomedial nucleus (BM) compared with other portions of the BLC. There was also very light labeling in the posteroventral subdivision of the medial nucleus (MePV) and lateral subdivision of the central nucleus.

At caudal levels of the amygdala, all portions of the BLC, the Copl, as well as the adjacent posteromedial cortical nucleus (Copm) and amygdalohippocampal area (AHA), were labeled (Fig. 1e, f). However, the intensity of m1 immunoreactivity was light to moderate compared with more rostral parts of the amygdala.

In all portions of the BLC (and also in the Copl, Copm, and AHA) the density of perikaryal labeling indicated that the m1+ neuronal population included the principal neurons of these nuclei, the pyramidal cells (McDonald 1992a, b). The pyramidal or piriform perikarya of these neurons were 15–20 μm in diameter and often exhibited a thick apical dendrite (Fig. 2). m1 reaction product was particulate and filled the perikaryal cytoplasm and proximal dendrites, but not the nucleus of m1+ neurons. In addition, there was dense punctate m1 immunoreactivity in the neuropil of all nuclei of the BLC (Fig. 2).

Dual labeling immunofluorescence experiments

m1/CaMK dual localization—The pattern of m1 immunoreactivity in the dual-labeling immunofluorescence experiments was identical to that seen in the immunoperoxidase preparations (Figs. 3, 4). Likewise, the CaMK staining in the BLC was identical to that seen in previous studies (McDonald et al. 2002). Thus, a high density of large pyramidal or piriform CaMK+ perikarya were observed. In addition, the neuropil contained a large number of CaMK+ processes of varying diameters. Examination of all portions of the BLC in the double-stained sections revealed that virtually all CaMK+ pyramidal cells exhibited robust m1 immunoreactivity (Fig. 3). Cell counts in the BLA found that among 406 CaMK+ and/or m1+ neurons analyzed, 95.6% (388/406) were m1+/CaMK+ double-labeled neurons, 2.2% (9/406) were single-labeled CaMK+ neurons, and 2.2% (9/406) were single-labeled m1+ neurons. All of the latter exhibited large pyramidal or piriform perikarya, suggesting that they were pyramidal cells. In general, there was overlap of the two labels in the perikaryal cytoplasm of double-labeled cells. However, in some double-labeled cells portions of the perikaryal cytoplasm were stained with only one of the two antibodies (see neurons indicated with arrowheads in Fig. 3a2, b2, c3). Because of the use of low Triton X-100 levels required for CaMK staining, there was little penetration of immunoreagents into the section. Thus staining for both CaMK and m1 was restricted to the surface of each section. As a result, only thin slivers of the perikaryal cytoplasm of each cell were stained. It therefore seems likely that many of the single-labeled m1+ or CaMK+ neurons observed could have been created by the section plane intersecting a portion of the perikaryal cytoplasm that contained only one of the two labels.

m1/GAD dual localization—The GAD staining in the BLC in the dual-labeling immunofluorescence experiments was identical to that seen in previous immunohistochemical studies (Carlsen 1988). Thus, most GAD+ neurons had small to medium-sized ovoid or spherical perikarya, and 2–4 primary dendrites (Fig. 4). In addition, GAD+ axonal varicosities were observed surrounding the pyramidal or piriform perikarya of m1+ neurons (Fig. 4b, c). Cell counts in the BLA found that only 2 of the 150 GAD+ neurons analyzed (1.3% of GAD+ neurons) had significant levels of m1 immunoreactivity (i.e., above neuropil levels; Fig. 4c). However, it was not uncommon to find GAD+

perikarya that had a few immunoreactive particles in the perikaryal cytoplasm. These particles, however, were never seen in the nucleus of these cells (Fig. 4b).

Discussion

The results of the present investigation indicate that in the rostral two-thirds of the amygdala, its lateral portions (including the BLC and Copl) exhibit intense m1 immunoreactivity, while more medial portions of the amygdala are very lightly labeled. At caudal levels of the amygdala, behind the centromedial nuclear complex, moderate levels of immunoreactivity extend throughout the entire nuclear complex. These data demonstrate that the cortex-like portions of the amygdala (McDonald 1992a), characterized by glutamatergic principal neurons and GABAergic interneurons, are the main amygdalar regions that exhibit significant levels of m1 immunoreactivity. In fact, the distribution and relative density of m1 immunoreactivity in the amygdala is remarkably similar to that of vesicular glutamate transporter 1 (VGLUT1) mRNA revealed by in situ hybridization (Poulin et al. 2008). Our immunohistochemical findings appear to be identical to those obtained with in situ hybridization studies of m1 mRNA localization (see Fig. 2d1, e1 of Buckley et al. 1988), and in M1 receptor binding autoradiographic studies of the rat amygdala (see Fig. 1e, f of Spencer et al. 1986). Although there is evidence that the M1 subtype of muscarinic receptor identified using pharmacological procedures corresponds to both m1 and m4 subtypes identified using molecular biological techniques, the expression of m4 is much lower than m1 in the amygdala (Bonner et al. 1987; Levey et al. 1991).

BLC cell types expressing m1 immunoreactivity

Previous receptor binding autoradiographic studies and film-based in situ hybridization studies did not have sufficient resolution to identify which neurons in the BLC expressed M1 or m1 receptors, respectively. This was accomplished for m1 receptors in the present investigation using immunohistochemistry. The great majority of the intensely labeled m1+ neurons in the BLC had the morphology of pyramidal cells and expressed the pyramidal cell marker CaMK. Likewise, pyramidal cells in the neocortex and hippocampus are also m1+ (Mrzljak et al. 1993; Levey et al. 1995). Because only light microscopic studies were performed in the present investigation, we could not ascertain the subcellular distribution of m1 immunoreactivity in BLC pyramidal cells. Electron microscopic studies in the neocortex, however, indicate that perikaryal m1 immunoreactivity in pyramidal cells is mainly localized to the Golgi apparatus and endoplasmic reticulum and that there is also m1 immunoreactivity in the cytoplasm of the dendrites of pyramidal cells (Mrzljak et al. 1993). It is of interest that neuropilar m1 immunoreactivity in the neocortex was mainly located in the cytoplasm and postsynaptic densities of pyramidal cell dendritic spines receiving asymmetrical synaptic contacts formed by putative glutamatergic axon terminals. In addition, m1 immunoreactivity was observed postsynaptically in dendritic shafts contacted by presumptive cholinergic axon terminals forming symmetrical synapses (Mrzljak et al. 1993).

Because of the many similarities of BLC and cortical pyramidal cells (McDonald 1992a; Muller et al. 2006), it seems likely that much of the punctate neuropilar m1

immunoreactivity observed in the present study may be located in pyramidal cell dendrites. The m1 immunoreactivity observed in the cell bodies and proximal dendrites of BLC pyramidal cells most likely represents m1 receptor protein that is in the process of being transported to distal dendrites and spines. Similar to the neocortex, distal dendrites and spines of pyramidal cells are the main postsynaptic targets of cholinergic inputs to the BLC from the basal forebrain (Carlsen and Heimer 1986; Muller et al. 2008) as well as glutamatergic inputs from the thalamus, cortex, and neighboring BLC pyramidal neurons (see Muller et al. 2006 for a review of the literature).

Since the m1 receptor is the main muscarinic cholinergic receptor subtype in the BLC (Buckley et al. 1988; Levey et al. 1991), and most of the m1 immunoreactivity is found in pyramidal cells, it seems likely that many of the muscarinic responses observed in BLC pyramidal cells are mediated by this receptor subtype. One of the main effects of muscarinic agonists and/or stimulation of cholinergic afferents to the amygdala is an increase in excitability of BLC pyramidal cells due to the suppression of several potassium currents, including the muscarine sensitive M-current (I_M), a voltage-insensitive leak current (I_{Leak}), the calcium-activated slow after hyperpolarization current (sI_{AHP}), and a hyperpolarization-activated inward rectifier current (I_Q) (Washburn and Moises 1992; Womble and Moises 1992, 1993; Yajeya et al. 1999). In addition, a recent study has demonstrated that activation of muscarinic cholinergic receptors activates small conductance calcium-activated potassium (SK) channels in BLC pyramidal cells, following the release of calcium from intracellular stores via activation of the phosphatidylinositol signal transduction pathway (Power and Sah 2008). Since the M1-family of receptors is known to activate the phosphatidylinositol system (Richelson 1995), m1 receptors in BLC pyramidal cells may mediate this response.

Van der Zee and colleagues labeled a subpopulation of neurons in the BLC using a monoclonal antibody (M35) that appears to recognize a unique conformational epitope shared by all five muscarinic receptor subtypes (Van der Zee and Luiten 1999). The nonpyramidal morphology of M35+ neurons in the BLC, as well as their low density, suggests that they are interneurons (see Fig. 7 of Van der Zee and Luiten 1999). In addition, the high density of asymmetrical synapses on the dendrites of M35+ neurons seen at the ultrastructural level is also typical of BLC interneurons (Smith et al. 1998). It is not clear why pyramidal cells were not labeled with this antibody.

Similar to the results of studies of the rat neocortex and hippocampus (Levey et al. 1995; Volpicelli and Levey 2004), very few GAD+ interneurons in the BLC were intensely stained in the present investigation. However, the fact that a few particles of m1 immunoreactivity were observed in the perikaryal cytoplasm of many GAD+ interneurons, but not in the nucleus, leaves open the possibility that many GABAergic interneurons may express low levels of the receptor. The rapid transport of the receptor protein to dendrites could deplete perikaryal levels. On the other hand, these very low levels of m1 immunoreactivity could represent non-specific background. Electron microscopic studies have demonstrated that the somata and dendrites of GABAergic BLC interneurons are innervated by cholinergic inputs (Carlsen and Heimer 1986; Nitecka and Frotscher 1989; Muller et al. 2008), although the types of postsynaptic receptors associated with these inputs were not identified. Likewise,

there is electrophysiological evidence for muscarinic activation of BLC interneurons, but the receptor subtypes were not determined (Washburn and Moises 1992; Yajeya et al. 1997). Future studies using in situ hybridization techniques may help clarify this issue.

Functional implications

The main function associated with the muscarinic cholinergic innervation of the BLC is memory consolidation in a wide variety of learning tasks including inhibitory avoidance, contextual fear conditioning, food reward magnitude learning, and conditioned place preference (see Power et al. 2003a for a review). Power and co-workers demonstrated that both M1 and M2 pharmacological receptor subtypes in the BLA were important for memory consolidation of inhibitory avoidance (Power et al. 2003b). Given the high levels of m1 immunoreactivity in the BLA, it seems likely that this receptor subtype is critical for the M1 effects.

It is thought that the BLC enhances mnemonic function by promoting interactions between hippocampal regions and neocortical memory storage sites (Paré 2003). There is evidence that this involves synchronization of atropine-sensitive theta oscillations (4–8 Hz) in the BLC with oscillations in structures of the medial temporal lobe memory system (Seidenbecher et al. 2003; Pape et al. 2005). It is of interest in this regard that mice with a null mutation of the gene encoding the m1 receptor have impairments in memory consolidation in contextual fear conditioning during a time period corresponding to that in which these memories are thought to become transferred from the hippocampus to the neocortex (Anagnostaras et al. 2003). The finding of the present investigation that BLC pyramidal cells, the projection neurons of the BLC, express high levels of m1 receptor protein suggests that these neurons may contribute to the generation of these theta oscillations. BLC pyramidal cells have intrinsic conductances, including conductances sensitive to muscarinic cholinergic agonists that produce theta (Paré et al. 1995; Pape et al. 1998). It has been suggested that muscarinic cholinergic reduction of the slow AHP current may facilitate these theta oscillations in BLC pyramidal cells (Pape et al. 2005).

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Abbreviations

AHA	Amygdalohippocampal area
BLA	Anterior subdivision of the basolateral nucleus
BLp	Posterior subdivision of the basolateral nucleus
BLC	Basolateral nuclear complex
BM	Basomedial nucleus
BMA	Anterior subdivision of the basomedial nucleus

BMP	Posterior subdivision of the basomedial nucleus
CAMK	Calcium/calmodulin protein kinase II
ChAT	Choline acetyltransferase
CL	Lateral subdivision of the central nucleus
CM	Medial subdivision of the central nucleus
Coa	Anterior subdivision of the cortical nucleus
Copl	Posterolateral subdivision of the cortical nucleus
Copm	Posteromedial subdivision of the cortical nucleus
CPu	Caudatoputamen
IN	Intercalated nucleus
L	Lateral nucleus
Ldl	Dorsolateral subdivision of the lateral nucleus
Lvl	Ventrolateral subdivision of the lateral nucleus
Lvm	Ventromedial subdivision of the lateral nucleus
m1	m1 subtype of the muscarinic receptor (molecular designation)
M1	M1 subtype of the muscarinic receptor (pharmacological designation)
MeAD	Anterodorsal subdivision of the medial nucleus
MePD	Posterodorsal subdivision of the medial nucleus
MePV	Posteroventral subdivision of the medial nucleus
Pir	Piriform cortex

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Fig. 1. m1 immunoreactivity in the rat amygdala. **a, c, and e** Low power digital photomicrographs of m1 immunoreactivity at rostral (**a**), middle (**c**), and caudal (**e**) levels of the amygdala (Bregma levels taken from the atlas by Paxinos and Watson 1997). **b, d, and f** Higher power photomicrographs of the basolateral amygdala at rostral (**b**), middle (**d**), and caudal (**f**) levels of the amygdala. Scale bars 400 μ m in **e** (**a, c** are at the same magnification) and 200 μ m in **f** (**b, d** are at the same magnification). AHA amygdalohippocampal area, BLa anterior subdivision of the basolateral nucleus, BLp posterior subdivision of the basolateral nucleus,

BM basomedial nucleus, *BMA* anterior subdivision of the basomedial nucleus, *BMP* posterior subdivision of the basomedial nucleus, *CL* lateral subdivision of the central nucleus, *CM* medial subdivision of the central nucleus, *Coa* anterior subdivision of the cortical nucleus, *Copl* posterolateral subdivision of the cortical nucleus, *Copm* posteromedial subdivision of the cortical nucleus, *CPu* caudatoputamen, *IN* intercalated nuclei, *L* lateral nucleus, *Ldl* dorsolateral subdivision of the lateral nucleus, *Lvl* ventrolateral subdivision of the lateral nucleus, *Lvm* ventromedial subdivision of the lateral nucleus, *MeAD* anterodorsal subdivision of the medial nucleus, *MePD* posterodorsal subdivision of the medial nucleus, *MePV* posteroventral subdivision of the medial nucleus, *Pir* piriform cortex

Fig. 2. m1 immunoreactivity in the rat basolateral amygdala: **a** BLa, **b** rostral Lvm, **c** BLa, **d** caudal BLp (i.e., at the level shown in Fig. 1e, f). There is m1 immunoreactivity in numerous pyramidal or piriform perikarya, as well as punctate labeling in the neuropil. *Scale bars* 40 μ m in **b** (**a** is at the same magnification); 20 μ m in **d** (**c** is at the same magnification)

Fig. 3. Dual localization of m1 with CaMK in the BLC using confocal laser scanning microscopy. **a1–a2** colocalization of m1 and CaMK in the BLA. **a1** m1+ perikarya in the BLA (*green*). **a2** merged image of m1 (*green*) and CaMK (*red*) immunoreactivity in the same field shown in **a1**. *Yellow* indicates colocalization of m1 and CaMK. All intensely-labeled m1+ neurons are CaMK+. **b1–b2** Colocalization of m1 and CaMK in the dorsolateral subdivision of the lateral nucleus (*Ldl*). **b1** m1+ perikarya in the *Ldl* (*green*). **b2** Merged image of m1 (*green*) and CaMK (*red*) immunoreactivity in the same field shown in **b1**. *Yellow* indicates

colocalization of m1 and CaMK immunoreactivity. Note that all intensely-labeled m1+ neurons are CaMK+. **c1–c3** Higher power photomicrographs of colocalization of m1 and CaMK in the BLA. **c1** seven m1+ perikarya in the BLA (*green*). Note intense m1 immunoreactivity in the somatic cytoplasm, but none in the nucleus of these neurons. Also obvious is a moderate level of immunoreactivity in the neuropil. **c2** CaMK-immunoreactivity in the same field as **c1** (*red*). Note immunoreactivity in somata, thick proximal dendrites, and thinner distal dendrites. **c3** Merged image of m1+ (*green*) and CaMK+ (*red*) in the same field as **c1/c2**. *Yellow* indicates colocalization of m1 and CaMK immunoreactivity. Note that the somata of all seven of the intensely labeled m1+ neurons are CaMK+ and that the m1 immunoreactivity is more robust in portions of the cytoplasm adjacent to the nucleus. m1 Immunoreactivity is also associated with many dendrites, at least three of which are thick proximal dendrites (*arrows*). *Arrowheads* in **a**, **b**, and **c** indicate neurons that have areas of perikaryal cytoplasm that contain only one of the two labels, and could be misidentified as single-labeled cells in other planes of section. *Scale bars* 20 μm in **b** (**a** is at the same magnification); 20 μm in **c**

Fig. 4.

Dual localization of m1 with GAD-67 in the BLC using confocal laser scanning microscopy.

a1–a2 Dual localization of m1 and GAD in the ventromedial subdivision of the lateral nucleus (Lvm). **a1** m1+ perikarya in the Lvm (*green*). **a2** Merged image of m1 (*green*) and GAD (*red*) immunoreactivity in the same field shown in **a1**. Note that none of the intensely labeled m1+ neurons are GAD+. **b1–b2** Dual localization of m1 and GAD in the BLA. **b1** m1 immunoreactivity in the BLA (*green*). **b2** Merged image of m1 (*green*) and GAD (*red*) in the same field shown in **b1**. *White arrows* in **b1** and **b2** indicate the position of the GAD+ neuron shown in **b2**. *Yellow* indicates colocalization of m1 and GAD immunoreactivity.

Note that the perikaryal cytoplasm of the GAD+ neuron has at least two m1 puncta (*yellow*) located just above the nucleus, but there are no m1+ puncta in the GAD-negative nucleus of this neuron. Since this image was obtained using a $\times 63$ oil immersion objective and a thin “optical section” (1.67 μm) through the center of the GAD+ soma, it is likely that these two m1 puncta are actually located in the perikaryal cytoplasm. **c1–c3** Dual localization of m1 and GAD in the BLA in a section used for the quantitative analysis depicting one of two GAD+ neurons that also exhibited m1 immunoreactivity. **c1** m1 immunoreactivity in the BLA (*green*). **c2** GAD immunoreactivity in the same field as C1 (*red*). **c3** Merged image of m1 (*green*) and GAD (*red*) in the same field as **c1/c2**. *White arrows* indicate a GAD+ neuron that exhibits m1 immunoreactivity. *Yellow* indicates colocalization of m1 and GAD immunoreactivity in **C3**. *Scale bars* 20 μm in **a**, **b**, and **c**