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Evidence for M₂ muscarinic receptor modulation of axon terminals and dendrites in the rodent basolateral amygdala: an ultrastructural and electrophysiological analysis

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Abstract

The basolateral amygdala receives a very dense cholinergic innervation from the basal forebrain that is important for memory consolidation. Although behavioral studies have shown that both M₁ and M₂ muscarinic receptors are critical for these mnemonic functions, there have been very few neuroanatomical and electrophysiological investigations of the localization and function of different types of muscarinic receptors in the amygdala. In the present study we investigated the subcellular localization of M₂ muscarinic receptors (M₂Rs) in the anterior basolateral nucleus (BLA) of the mouse, including the localization of M₂Rs in parvalbumin (PV) immunoreactive interneurons, using double-labeling immunoelectron microscopy. Little if any M₂R-immunoreactivity (M₂R-ir) was observed in neuronal somata, but the neuropil was densely labeled. Ultrastructural analysis using a pre-embedding immunogold-silver technique (IGS) demonstrated M₂R-ir in dendritic shafts, spines, and axon terminals forming asymmetrical (excitatory) or symmetrical (mostly inhibitory) synapses. In addition, about one-quarter of PV+ axon terminals and half of PV+ dendrites, localized using immunoperoxidase, were M₂R+ when observed in single thin sections. In all M₂R+ neuropilar structures, including those that were PV+, about one-quarter to two-thirds of M₂R+ immunoparticles were plasma-membrane-associated, depending on the structure. The expression of M₂Rs in PV+ and PV-negative terminals forming symmetrical synapses indicates M₂R modulation of inhibitory transmission. Electrophysiological studies in mouse and rat brain slices, including paired recordings from interneurons and pyramidal projection neurons, demonstrated M₂R-mediated suppression of GABA release. These findings suggest cell type specific functions of M₂Rs and shed light on organizing principles of cholinergic modulation in the BLA.

Keywords

cholinergic; electron microscopy; immunohistochemistry; whole-cell recording; IPSC

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INTRODUCTION

Cholinergic neurons in the basal forebrain innervate several major structures in the forebrain including the neocortex, hippocampus, and amygdala. Although the neocortex and hippocampus have been the focus of most anatomical and electrophysiological studies of the basal forebrain cholinergic system, the anterior basolateral amygdalar nucleus (BLA) is actually the region that receives the densest cholinergic innervation from the basal forebrain (Mesulam et al., 1983a, b; Carlsen et al., 1985; Rao et al., 1987; Kordower et al., 1989). Acetylcholine is important for memory consolidation by the basolateral amygdala (McGaugh, 2004). Injections of muscarinic cholinergic antagonists into the basolateral amygdala, or lesions of the basal forebrain cholinergic neurons projecting to the amygdala, produce impairments in several kinds of learning related to emotion and motivation including fear conditioning and inhibitory avoidance (Power et al., 2003a; Wilson and Fadel, 2016).

Activation of both M₁ and M₂ muscarinic receptors (M₁Rs and M₂Rs) in the BLA is required for memory consolidation by the amygdala (Power et al., 2003b). However, almost nothing is known about muscarinic mechanisms and their role in mnemonic function in the amygdala. It is clear that basic immunohistochemical and electrophysiological studies of muscarinic receptor subtypes in the BLA will be an important first step in clarifying their involvement in amygdalar memory circuits. In general, M₁Rs are excitatory and M₂Rs are inhibitory (Richelson, 1995; Ehler et al., 1995). Although it is well established that excitatory inputs to pyramidal projection neurons (PNs) of the BLA are important for associative learning, including fear conditioning, recent studies indicate that inhibition of GABAergic interneurons (INs) may also play a key role by providing disinhibition of PNs (Letzkus et al. 2015; Wolff et al., 2014; Tovote et al., 2015). The initial hypothesis of the present study, which was confirmed in our electrophysiological studies in brain slices in both rat and mouse, was that inhibitory M₂Rs in the axon terminals of GABAergic INs of the BLA disinhibit PNs by suppressing GABA release, as they do in hippocampus (Szabo et al., 2010).

However, like the hippocampus and neocortex, the BLA contains several distinct GABAergic IN subpopulations that can be identified on the basis of their expression of calcium binding proteins and neuropeptides (Spampanato et al., 2011). INs expressing the calcium binding protein parvalbumin (PV) are the predominant IN subpopulation in the BLA, constituting about 40% of the total IN population (McDonald and Betette, 2001; Mascagni et al., 2003). As in the hippocampus and neocortex, many of the PV+ INs in the BLA are basket cells or axo-axonic chandelier cells (Muller et al., 2006; Rainnie et al., 2006; Vereczki et al., 2016; Veres et al., 2017). Since PV+ INs in the hippocampus express M₂Rs in their axon terminals that suppress GABA release (Hajos et al., 1998; Szabo et al., 2010), we hypothesized that the terminals of BLA PV+ INs would also express M₂Rs. To test this second hypothesis we used dual-labeling immunoelectron microscopy in mouse brain. PV was localized using an immunoperoxidase (IP) technique whereas M₂Rs were localized using a high-resolution pre-embedding immunogold-silver (IGS) technique. The use of the IGS technique for M₂R permitted differentiation of membrane-associated versus cytoplasmic M₂Rs. In addition, since this is the first study of M₂Rs in the BLA using the high-resolution IGS method, we

also analyzed the extent of membrane-associated M₂Rs in PV-negative structures in the BLA, including dendritic shafts, spines, axon terminals forming asymmetrical (excitatory) synapses, and axon terminals forming symmetrical (inhibitory/neuromodulatory) synapses.

EXPERIMENTAL PROCEDURES

Animals

Experiments were performed using either male B6129SF2/J mice (Jackson Laboratory, Bar Harbor, Me) or male Sprague Dawley rats (Envigo; Indianapolis, IN). All animals were housed on a 12-h light/dark cycle with ad libitum access to food and water. 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.

Brain slice electrophysiology

Mice (5 weeks old) or rats (18- to 28-d-old) were decapitated under deep isoflurane anesthesia. The brain was removed and coronal brain slices (300 μ m thick) were prepared in ice-cold, oxygenated ACSF (artificial cerebrospinal fluid) using a Leica vibratome (VT1000S). Brain slices were incubated for 60 minutes in warmed (32–34°C), bubbled ACSF containing (in mM) 120 NaCl, 3.3 KCl, 1.0 NaH₂PO₄, 25 NaHCO₃, 10 glucose, 0.5 CaCl₂, and 5 MgCl₂ and bubbled with a 95% O₂/5% CO₂ gas mixture at pH 7.4. Osmolarity was 301–308 mOsm. For recording, slices were transferred to a recording chamber and continuously perfused with warmed (32–34°C), oxygenated ACSF, pH 7.4, containing 2.0 mM CaCl₂ and 1.0mM MgSO₄. Whole-cell recording was performed using borosilicate glass electrodes (6–8 M Ω) as described previously (Mott et al., 1997). Slices were individually transferred to a recording chamber maintained at 32–34°C and continuously perfused with oxygenated ACSF, pH 7.4, containing 2.0 mM CaCl₂ and 1.0mM MgSO₄. Whole-cell recordings were obtained using borosilicate glass electrodes (6–8 M Ω). The intracellular solution used for PNs contained (in mM) 130 Cs-gluconate, 5 KCl, 10 HEPES, 0.5 EGTA, 2 MgCl₂, 2 MgATP, and 0.3 NaGTP, pH 7.3. Whole-cell patch-clamp recordings were made from the BLA, identified in the slice based on its position between the external capsule (located lateral to the BLA) and the intermediate capsule (located medial to the BLA). Voltage-clamp recordings were made at a holding potential of –70 mV. Series resistance was 10–25 M Ω , and recordings in which series resistance changed significantly were discarded. Monosynaptic GABA_A IPSCs were evoked by stimulation in the BLA near the recording electrode. Stimuli were 0.1 ms, cathodal, monophasic, rectangular constant current pulses (10e100 mA) delivered through monopolar, platinum-iridium stimulating electrodes (FHC Inc, Bowdoin, ME). IPSCs were isolated by bath application of CNQX (20 μ M, Abcam, Cambridge, MA) and D-APV (50 μ M, Abcam).

For paired IN – PN recordings the intracellular solution used for INs contained (in mM) 130 K-gluconate, 5 KCl, 10 HEPES, 0.5 EGTA, 2 MgCl₂, 2 MgATP, and 0.3 NaGTP, pH 7.3. INs were held in current-clamp mode at a membrane potential of –65 mV, and stimulated by

brief current pulses (1.5 ms, 1–2 nA). Only stable recordings from pairs of synaptically connected cells were included in the study. Muscarinic receptors were activated by bath application of muscarine chloride (10 μ M; Sigma Chemical St. Louis, MO) in the perfusion media. AF-DX 116 (100 nM, Sigma), a selective M₂R antagonist, was bath applied with muscarine. Responses were recorded using a Multiclamp 700B amplifier and filtered at 1 kHz. Responses were digitized by a Digidata 1440A analog-to-digital (A-D) board (Molecular Devices, Sunnyvale, CA) in a Windows-based computer using pClamp 10 software. Analysis was performed using pClamp 10 (Molecular Devices) and Origin (MicroCal, Northampton, MA) software packages. Statistical comparisons were performed using the Student's t test or ANOVA with post hoc tests. Values are given as mean $\pm$ SEM.

Tissue preparation for light and electron microscopy

Ten mice (7–10 weeks old) were used in immunohistochemical experiments at the light and electron microscopic level. For electron microscopy mice were deeply anesthetized with ketamine-xylazine 1:1 (1.0 mL/kg, IP) and perfused intracardially with PBS (pH 7.4) containing 0.5% sodium nitrite, followed by ice-cold fixative containing 4% paraformaldehyde, 0.05% glutaraldehyde, and 15% (v/v) saturated picric acid in 0.1 M phosphate buffer (PB, pH 7.4). The glutaraldehyde was not included in the fixative mixture for brains prepared for light microscopy. After perfusion, brains were removed and immersed in the perfusate for 2 h. Tissue blocks were rinsed thoroughly in 0.1M PB. Coronal sections (40 μ m for light microscopy and 60 μ m for electron microscopy) were cut on a vibratome (Leica VT 1200; Leica, Nussloch, Germany). Amygdalar sections were placed in tissue culture chamber slides for immunohistochemical processing.

M₂R immunohistochemistry for light microscopy

Light microscopic immunohistochemistry for the m2 muscarinic receptor was performed in 3 mice using immunoperoxidase methods (Lujan et al., 1996). Four muscarinic receptor subtypes have been identified using pharmacological techniques (designated by upper case letters M₁–M₄), whereas five distinct subtypes have been identified using molecular biological techniques (designated by lower case letters m1–m5) (Ehlert et al., 1995). In the present study we performed light and electron microscopic analyses of the mouse BLA using an m2 receptor subtype specific antibody. For convenience, this receptor will be abbreviated “M₂R”, with the understanding that it is actually the m2 molecular subtype that was localized. Previous studies have shown that there is excellent correspondence in the amygdala and other brain regions between the m2 receptor protein localized using immunohistochemistry, and the M₂ receptor localized using receptor binding autoradiography (Levey et al., 1991).

Briefly, sections (40 μ m-thick) through the BLA were incubated for 1 h in 10% normal goat serum (NGS) diluted in 50 mM Tris buffer (pH 7.4) containing 0.9% NaCl (TBS) with 0.2% Triton X-100. Sections were then incubated in a rat monoclonal antibody to the m₂ muscarinic receptor (1:700; Millipore, Billerica, MA) for 48 hours at 4°C and then incubated in biotinylated goat anti-rat secondary antibody for 2 h at room temperature (1:500; Jackson ImmunoResearch, West Grove, PA). Sections were then processed for the avidin–biotin immunoperoxidase technique using a Vectastain Elite ABC Kit (Vector Laboratories,

Burlingame, CA) with DAB as a chromogen. All antibodies were diluted in 1% NGS/TBS. Finally, sections were air-dried and coverslipped in DPX before observation using an Olympus (Tokyo, Japan) BX51 microscope. Digital light micrographs were taken with an Olympus DP2-BSW camera system.

M₂R/PV double-labeling immunohistochemistry for electron microscopy

Immunohistochemical reactions at the electron microscopic level were carried out in 6 mice using a pre-embedding double-labeling immunoperoxidase/immunogold-silver technique (Fajardo-Serrano et al., 2013). The first 3 mice were used in preliminary studies to determine optimal antibody dilutions, and the last 3 mice were used for quantitative studies (see below). PV immunoreactivity was visualized by the immunoperoxidase technique and M₂R immunoreactivity was visualized by the silver-intensified immunogold technique. Briefly, sections (60 µm-thick) through the BLA (bregma levels –0.9 to –1.4 in the atlas by Paxinos and Franklin, 2013) were first incubated for 1 h in 10% NGS diluted in 50 mM TBS. Sections were then incubated in a cocktail of the rat monoclonal antibody to M₂R (1:50) and a rabbit polyclonal antibody to PV (1:15,000; Swant Inc., Marly, Switzerland) for 48 hours at 4°C. Sections were then incubated for 2 h at room temperature in a secondary cocktail consisting of goat anti-rat antibody coupled to 1.4 nm gold (1:100; Nanoprobes, Yaphank, NY) and biotinylated goat anti-rabbit antibody (1:200; Vector Laboratories). Sections were then post-fixed in 1% glutaraldehyde and washed in double distilled water, followed by silver enhancement of the gold particles with a HQ Silver kit (6 m; Nanoprobes Inc.). Sections were then processed for the avidin–biotin immunoperoxidase technique using a Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA) with DAB as a chromogen. All antibodies were diluted in 1% NGS/TBS. In addition, one mouse brain was processed for single-label M₂R staining using the IGS technique.

The sections were then treated with osmium tetroxide for 30 m (1% in 0.1M PB), block-stained with uranyl acetate, dehydrated in graded series of ethanols and flat-embedded on glass slides in Durcupan (Sigma). Sections were cut at 70 nm on an ultramicrotome (Ultracut R, Leica, Germany) and collected on formvar-coated copper slot grids. Ultrastructural analyses were performed using a JEOL-1400 Plus Transmission electron microscope (JEOL, Tokyo, Japan).

Quantification of immunogold labeling at the electron microscopic level

Quantification procedures were similar to those used previously (Lujan et al., 1996; Fajardo-Serrano et al., 2013). To minimize false negatives, ultrathin sections were cut close to the surface of each block, as immunoreactivity decreased with depth, especially using the IGS technique. Areas near the tissue/resin interface were photographed and quantified from 2–3 vibratome sections from each of three brains that were dual-labeled for M₂R (IGS) and PV (IP). Since the IGS technique typically produces non-specific labeling at the tissue/resin interface, areas within 1 µm from the interface were not included in the quantitative analysis. The criteria used to identify M₂R+ structures was identical to that used in a previous IGS study to identify structures labeled for the NR1 glutamate receptor and D1 dopamine receptor in the BLA (Pickel et al., 2006): one immunoparticle in the plasma membrane, or two total, including particles in the cytoplasm.

Three separate quantitative analyses were performed: (1) determination of the percentages of different M₂R+ structures in the BLA, and the percentage of membrane versus cytoplasmic immunoparticles in each type of structure; (2) determination of the extent of M₂R/PV colocalization in dendrites and axon terminals forming symmetrical contacts, and (3) determination of the spatial relationship of membrane-associated immunoparticles to the presynaptic active zone of PV+ axon terminals and PV-negative axon terminals forming symmetrical synapses.

For the first analysis all M₂R+ structures, including both PV+ and PV-negative structures, were analyzed. PV+ structures were not counted separately (see below for analysis of PV+/M₂R+ terminals and dendrites). Small-caliber dendritic shafts (< 1 µm wide), large-caliber dendritic shafts (> 1 µm wide), spines, axons, and axon terminals forming asymmetrical or symmetrical synapses or synapse-like contacts were identified using established morphological criteria (Peters et al., 1991). “Synapse-like contacts” were defined as presumed synapses that appeared, in the single thin-section examined, to be synapses that were sectioned obliquely through the synapse or cut through the edge of the synapse. The following determinations were made from immunoparticle counts: (1) the percentage of M₂R+ structures that each type of M₂R+ structure represents, (2) the percentage of the total number of immunoparticles in M₂R+ structures that each type of structure contains, and (3) the percentage of plasma membrane-associated immunoparticles in each type of M₂R+ structure. Immunoparticles were considered membrane-associated if they were touching or within 15 nm of the plasma membrane.

For the second analysis, the determination of the extent of M₂R/PV colocalization in axon terminals and dendrites, we sampled from the same ultrathin sections used for the first analysis. However, to minimize false negatives due to unequal penetration of antibodies, only thin sections with both PV+ and M₂R+ structures were analyzed for PV/M₂R colocalization. This situation was found in about three-quarters of the fields used in the first analysis. A total of 155 PV+ terminals that formed synapses or synapse-like contacts with cell bodies or dendrites were counted and the percentage that exhibited M₂R/PV co-expression was determined. All PV+ axon terminals making synaptic contacts formed symmetrical synapses as in previous studies (Muller et al., 2006, 2007). In addition, the number of M₂R+ axon terminals forming symmetrical synapses or synapse-like contacts that were PV-negative was also counted in the same fields used for determining PV/M₂R colocalization. A total of 37 PV+ dendrites were found in the material analyzed for PV+ terminals, and the percentage of these dendrites that expressed M₂R-ir was determined. We also determined the percentage of plasma membrane-associated immunoparticles in all M₂R+/PV+ axon terminals and dendrites.

For the third analysis, the determination of the spatial relationship of membrane-associated immunoparticles to the presynaptic active zone of axon terminals forming symmetrical synapses, we sampled from the same ultrathin sections used for the first analysis. Only membrane-associated immunoparticles from axon terminals with clearly defined presynaptic active zones were quantified (PV+ terminals: 21 particles in 21 terminals; PV-negative terminals forming symmetrical synapses: 27 particles in 16 terminals). The distance between the center of each particle and the closest edge of the presynaptic active zone was measured

using NIH Image J software (see schematic diagram in Fig. 7). The number of immunoparticles was expressed as the relative frequency in bins corresponding to 60 nm segments of plasma membrane.

Statistical analyses for the semi-quantitative electron microscopic studies were performed using IBM SPSS statistics for windows version 24.0 (Armonk, NY) and statistical significance of the distribution of gold particles and distances to the active zone of gold particles among samples, defined as $P < 0.05$, were assessed with the Shapiro-Wilk nonparametric tests. Because differences in the distribution of gold particles among samples were not statistically significant ($P > 0.05$), data from the 3 brains was pooled for all quantitative analyses.

Antibody specificity

The rat monoclonal M₂R antibody (Millipore, catalog #mAB367, RRID: AB_94952) was raised against a highly purified GST fusion protein of a part of the third intracellular loop of the human M₂ receptor corresponding to amino acids 225–359. This antibody recognized the M₂R fusion protein in immunoblots, and showed no cross-reactivity to other muscarinic receptor subtypes (Levey et al., 1995). No immunostaining was observed in M₂R knockout mice (Jositsch et al., 2009). The pattern of M₂R immunohistochemical labeling with this antibody in the rat brain, including the amygdala (McDonald and Mascagni, 2011), is identical to that seen with other M₂R antibodies developed by the Levey laboratory (Levey et al., 1995).

The rabbit polyclonal parvalbumin (PV) antibody (Swant Inc., catalog #PV27, RRID: AB_2631173) was produced against recombinant rat parvalbumin. It recognizes the antigen at 12 KDa after SDS-gel electrophoretic separation of brain extracts. The labeling of PV immunoreactive neurons in the amygdala appeared to be identical to that obtained in previous studies using other PV monoclonal and polyclonal antibodies (Muller et al., 2006, 2007). No PV immunoreactivity was observed in PV knockout mice (manufacturer's studies).

RESULTS

Brain slice electrophysiology: M₂Rs inhibit IPSCs in BLA pyramidal neurons

To investigate the functional effect of M₂Rs on inhibitory transmission we recorded monosynaptic IPSCs from BLA PNs in mouse brain slice preparations. In the presence of CNQX and D-APV to block AMPA/kainate and NMDA receptors, electrical stimulation near the recorded PN evoked a monosynaptic GABA_A IPSC. This IPSC was significantly inhibited ($p < 0.05$, $n = 5$) by bath application of a saturating concentration of muscarine (10 μ M, Fig. 1A). The selective M₂R antagonist, AF-DX 116 (1 μ M) reversed this effect of muscarine, indicating that the suppression of GABAergic IPSCs was mediated by M₂Rs. Similar results were found in both mouse and rat (Fig. 1 B,C). The inability of muscarine to completely inhibit the evoked IPSC at saturating concentration suggests that either M₂Rs are unable to produce complete suppression of the IPSC or that they are present on only a subpopulation of inhibitory terminals. To examine these possibilities we recorded from

connected IN-PN cell pairs in the BLA. Depolarization of the presynaptic interneuron evoked an action potential in that cell and a corresponding unitary IPSC in the connected postsynaptic PN (Fig. 1D). Bath application of muscarine (10 μ M) blocked this unitary IPSC, but not the action potential in the presynaptic IN (Fig 1E, F), indicating that muscarine acted on presynaptic muscarinic receptors (mAChRs) on the inhibitory terminal. Furthermore, application of muscarine was accompanied by an increase in spontaneous IPSCs in the PN, demonstrating that muscarine enhanced IPSCs from some INs, while inhibiting it from others. Taken together, these findings indicate the presence of M₂Rs on a subpopulation of inhibitory terminals in the BLA and are in line with studies in hippocampus which have reported differential muscarinic suppression of GABA release from distinct IN subpopulations (Hajos et al., 1998; Fukudome et al., 2004; Neu et al., 2007; Szabó et al., 2010).

Light microscopy: dense M₂R neuropilar expression including perisomatic axon terminals

The electrophysiological findings suggested that inhibitory axon terminals in the BLA should be among the structures exhibiting immunoreactivity for M₂R (M₂R-ir). Light microscopic analysis revealed that the BLA exhibited the most intense M₂R-ir in the mouse amygdala. There was moderate to strong immunoreactivity in the lateral nucleus, but the central nucleus had much lighter M₂R-ir, especially in its lateral portions (Fig. 2A). M₂R-ir in the BLA was very dense in the neuropil, but virtually all cell bodies appeared unstained as in previous studies in the rat (McDonald and Mascagni 2011, Muller et al. 2016) (Fig. 2B). In some cases small M₂R+ punctate structures resembling axon terminals were seen making contact with the cell bodies of presumptive PNs (Fig. 2B, inset). It is well established that only GABAergic inhibitory axon terminals target the cell bodies of PNs (Muller et al., 2006).

Electron microscopy: M₂Rs are expressed in dendrites and axon terminals, including dendrites and terminals of PV+ interneurons

A semi-quantitative electron microscopic analysis of M₂R+ immunoparticles in sections processed for M₂R(IGS)-PV(IP) double-labeling revealed that M₂Rs are expressed in dendrites and axon terminals. PV+ structures were not counted separately in this first analysis (see below for analysis of PV+/M₂R+ terminals and dendrites). Thin dendritic shafts (< 1 μ m wide) were the most numerous M₂R+ structures (>50% of all M₂R+ structures; Figs. 3A,C,E, 4A). Although no formal counts were performed it appeared that most dendritic shafts in the BLA were M₂R+. Dendritic spines constituted 13.7% of M₂R+ structures (Fig. 3A, B-D, 4A). There were approximately equal percentages of M₂R+ axon terminals forming asymmetrical synapses/contacts (7.2% of M₂R+ structures) or symmetrical synapses/contacts (8.3% of M₂R+ structures) (Fig. 3B-D, 4A). The former mainly targeted dendritic spines, many of which were also M₂R+ (Fig. 3B-D), while the latter mainly targeted unlabeled perikarya, M₂R+ dendritic shafts (Fig. 3C), and in one case an M₂R+ axon initial segment (Fig. 3B). Consistent with light microscopic observations, only a few scattered immunoparticles were seen in cell bodies. Thin dendrites contained by far the greatest percentage of the total number of M₂R + immunoparticles (Fig. 4B). Only 13% of M₂R+ immunoparticles were plasma membrane-associated in thick dendrites, but

one-third to two-thirds of M₂R+ immunoparticles were plasma membrane-associated in the other structures of the neuropil (Fig. 4C).

Since PV+ INs are the predominant GABAergic IN subpopulation in the BLA, and are one of the main cell types providing powerful perisomatic inhibitory inputs to PNs, we used M₂R/PV double-labeling electron microscopy to determine if some of the M₂R+ perisomatic terminals seen at the light microscopic level (Fig. 2), and other M₂R+ terminals, express PV. A total of 155 PV+ terminals that formed synapses or synaptic-like contacts with cell bodies or dendrites were analyzed for M₂R co-expression. 23.3% of these PV+ terminals (36/155) were M₂R+ (Table 1; Fig. 5). 23.3% (7/30) of PV+ terminals targeting the somata of putative PNs were M₂R+ (Fig. 5D), and 23.8% (29/122) of PV+ terminals targeting dendritic shafts were M₂R+ (Fig. 5B, C). In PV+/M₂R+ terminals targeting somata, 53.3% of immunoparticles were membrane-associated, whereas 30.6% of immunoparticles were membrane-associated in PV+/M₂R+ terminals targeting dendritic shafts. PV+/M₂R+ terminals constituted 58% (36/62) of all M₂R+ terminals forming symmetrical synapses or synaptic-like contacts. Of the 42% (26/62) of these M₂R+ terminals that were not PV+, almost all targeted dendritic shafts (96%, 25/26). Only one targeted a cell body. In controls in which the M₂R antibody was omitted only a few scattered silver grains were seen (Fig. 6). In controls in which the PV antibody was omitted no peroxidase staining was observed.

Analysis of the subsynaptic distribution of membrane-associated immunoparticles in M₂R+/PV+ terminals and M₂R+/PV-negative terminals forming symmetrical synapses revealed that the majority of immunoparticles were in the presynaptic active zone and adjacent perisynaptic zone (Fig. 7). Thus, 29% of immunoparticles in M₂R+/PV+ terminals were in the presynaptic active zone and 33% were in the perisynaptic zone, defined as the extrasynaptic membrane segment within 180 nm of the active zone. Similarly, 33% of immunoparticles in M₂R+/PV-negative terminals were in the presynaptic active zone and 38% were in the perisynaptic zone.

The great majority of PV+ axon terminals observed were small to medium-sized (0.5–1.0 µm in diameter) terminals that belong to INs, but two of the terminals appeared to correspond to the larger (>1.0 µm in diameter) PV+ terminals of basal forebrain GABAergic afferents to the BLA (McDonald et al., 2011). Both terminals had one M₂R+ immunoparticle that contacted the membrane (Fig. 5C). These two terminals also had two other distinguishing features of basal forebrain GABAergic afferents: (1) many mitochondrial profiles, and (2) large numbers of synaptic vesicles.

Finally, a total of 37 PV+ dendrites were observed in the sections used for the PV+ terminal analysis. Nineteen of these were M₂R+ (51.0%; 19/37), and 22% of the immunoparticles in these PV+/M₂R+ dendrites were plasma membrane-associated (Fig. 8).

DISCUSSION

This investigation includes the first light and electron microscopic studies of M₂Rs in the mouse basolateral amygdala. These studies demonstrated that the neuropil of the mouse BLA, like that of the rat (Muller et al., 2016), has a very high density of M₂R+ structures,

including dendritic shafts, dendritic spines, axon terminals forming asymmetrical (glutamatergic) synapses, and axon terminals forming symmetrical (GABAergic or neuromodulatory) synapses. In all of these structures approximately 20–70% of the M₂R+ immunoparticles were associated with the plasma membrane. The ubiquity and high levels of M₂R-ir in the neuropil of the BLA at the ultrastructural level of analysis is consistent with our light microscopic observations, and with autoradiographic receptor binding studies (Mash and Potter, 1986; Spencer et al., 1986). We have also shown that about one-quarter of axon terminals of PV+ INs are M₂R+, which is consistent with our electrophysiological studies in amygdalar slices showing that IPSCs in pyramidal neurons were suppressed via an M₂R-mediated mechanism.

M₂R+ dendrites and spines in the BLA: M₂R-mediated postsynaptic modulation

More than 50% of the M₂R+ structures in the BLA neuropil were thin dendritic shafts, and about 14% were spines. In fact, about three-quarters of the total M₂R+ immunoparticles in the neuropil of the BLA were contained in dendritic shafts and spines. The prominent expression of M₂R in these dendritic structures was also seen in the rat BLA using immunoperoxidase (IP) techniques, where it was found that over 90% of dendritic shafts and over 50% of dendritic spines were M₂R+ (Muller et al., 2016). In the rat BLA using the IP technique it was observed that label for M₂R was sometimes found adjacent to or contacting the plasma membranes of dendrites and spines, including at the edges of cholinergic synapses, suggesting that the excitability of dendrites could be modulated via M₂R (Muller et al., 2016). However, since the extent to which the IP reaction product could diffuse from the antigenic binding site was not clear, the precise localization of M₂R-ir to the plasma membrane of dendrites could not be determined with certainty. The present study used a high-resolution pre-embedding IGS technique to confirm plasma membrane-associated M₂R in both dendritic shafts and spines, indicating that the excitability of dendrites and spines may be modulated through M₂R-mediated mechanisms after release and diffusion of acetylcholine from cholinergic terminals. Cytoplasmic M₂R-ir in dendritic shafts most likely represents receptor protein that is in the process of being transported to or from the plasma membrane of dendritic shafts and spines.

Since PNs comprise about 85% of BLA neurons, and tend to have longer and more extensively branched dendrites than most INs, the finding that many dendritic shafts are M₂R+ indicates that the majority of M₂R+ dendritic shafts seen in this study belong to PNs. Likewise, the great majority of all dendritic shafts in the rat BLA appeared to be M₂R+ (Muller et al., 2016). Since most dendritic spines in the BLA arise from PN distal dendrites (Muller et al., 2006), the great majority of M₂R+ spines observed in this study probably belong to PN dendrites.

In the present study we also observed M₂R-ir in about half of the dendrites of PV+ INs. These data are consistent with our IP findings in the rat BLA, which found light M₂R-ir in aspiny dendrites that received multiple inputs from axon terminals forming asymmetrical synapses, typical of PV+ IN dendrites (Muller et al., 2011). Dendrites of PV+ INs in the rat BLA receive synaptic inputs from cholinergic terminals (Muller et al., 2011), providing an anatomical basis for cholinergic regulation of interneuron excitability.

One paradoxical finding of the present study was that whereas PN dendrites and PV+ IN dendrites (as well as PV+ axon terminals, see below) were M₂R+, little or no M₂R-ir was observed in cell bodies in the BLA. In our previous study of the rat BLA we found that M₂R-ir was actually seen in the cell bodies of BLA neurons, but was confined to the trans-Golgi network of the Golgi complex (Muller et al., 2016). However, there was very dense M₂R-ir in the primary dendrites and axon initial segments arising from these cell bodies. This suggests that M₂Rs are quickly transported from the Golgi complex to dendritic and axonal processes of BLA neurons (Nathanson, 2008). It is of interest that a similar situation appears to exist in the hippocampus where no cell bodies of PV+ INs were M₂R+, but M₂R-ir was seen in the axon terminals of most PV+ INs (Hajos et al., 1998).

M₂R+ axon terminals in the BLA: M₂R-mediated presynaptic modulation of glutamatergic and GABAergic axon terminals

M₂R+ axon terminals in the mouse BLA formed asymmetrical and symmetrical synapses. Our IP study of M₂Rs in rat reported that some reaction product was associated with the plasma membrane of axon terminals (Muller et al., 2016), and the present IGS study in mouse more definitively confirmed this, suggesting M₂R modulation of glutamate or GABA release at these synapses. Previous studies of the basolateral amygdala have shown that axon terminals forming asymmetrical synapses represent glutamatergic inputs from the cerebral cortex and thalamus, as well as from amygdalar PNs (e.g., Carlsen and Heimer, 1988; Smith and Paré, 1994; Brinley-Reed et al., 1995). Washburn and Moises (1992) found that muscarinic agonists reduced the amplitude of synaptically-evoked EPSPs in PNs of the BLA via a presynaptic mechanism, but the subtype of muscarinic receptor was not investigated. Using M₂R antagonists Yajeya and coworkers determined that approximately 30% of the reduction in the amplitude of synaptically-evoked glutamatergic EPSPs in BLA PNs with external capsule stimulation was mediated by presynaptic M₂Rs (Yajeya et al., 2000). Since many of the postsynaptic targets of M₂R+ axons forming excitatory asymmetrical synapses in the present study were M₂R+ spines or distal dendrites, this receptor may actually mediate both pre- and postsynaptic effects at these synapses.

Many of the symmetrical synapses in the BLA are formed by axon terminals of several distinct subpopulations of GABAergic INs, including PV+ INs (Carlsen, 1988; Aylward and Totterdell, 1993; Smith et al., 2000; McDonald et al., 2002; Muller et al., 2003, 2006, 2007). However, symmetrical synapses are also formed by monoaminergic inputs from the brainstem (Asan, 1998; Muller et al., 2007, 2009; Farb and LeDoux, 2010; Zhang, 2013) and cholinergic inputs from the basal forebrain (Carlsen and Heimer, 1986; Muller et al., 2011). Our dual-labeling IP study in rat demonstrated that at least some cholinergic terminals in the BLA express M₂Rs (Muller et al., 2016), suggesting an autoreceptor role at these terminals, as in the hippocampus (Rouse et al., 2000).

In the present study we have focused on M₂R modulation of GABA release. We observed M₂Rs, including plasma membrane-associated M₂Rs, in PV+ and PV-negative axon terminals forming symmetrical synapses or synapse-like contacts with perisomatic (somata, proximal dendrites, and axon initial segments) and dendritic targets, suggesting that M₂Rs regulate GABA release in these axon terminals. These findings are consistent with our

electrophysiological studies showing that IPSCs in PNs were suppressed through an M₂R-mediated mechanism. It is well established that acetylcholine, acting via muscarinic receptors can modulate GABAergic inhibition in the hippocampus (Ben-Ari et al., 1981; Behrends and ten Bruggencate, 1993; Szabo et al. 2010). However, few studies have examined its regulation of GABAergic inhibition in amygdala. Washburn and Moises (1992) reported that muscarinic agonists reduced synaptically-evoked IPSPs in PNs of the BLA via a presynaptic mechanism, although the subtype of muscarinic receptor was not determined. Sugita et al (1991) found that muscarine reduced GABA release in the lateral nucleus of the amygdala and suggested that this occurred through presynaptic M₁Rs and M₃Rs. We have previously reported the expression of M₂Rs in terminals forming symmetrical synapses (Muller et al., 2016). However, the present study is the first to report that muscarinic suppression of GABA release in BLA occurs through presynaptic M₂Rs on GABAergic terminals. Moreover, consistent with regulation of neurotransmitter release at the active zone of these synapses, the use of the high-resolution IGS technique to localize M₂Rs in the present study demonstrated that the majority of these receptors in PV+ terminals and PV-negative terminals forming symmetrical (presumptive inhibitory) synapses were in the presynaptic active zone and surrounding perisynaptic zone.

Paired PN-IN recordings revealed that whereas muscarine suppressed the IPSC, it also had a direct excitatory effect on GABAergic interneurons. This finding is in agreement with previous studies in hippocampus showing that acetylcholine can regulate the membrane properties of inhibitory neurons as well as modulate GABA release (Behrends and ten Bruggencate, 1993; Lawrence, 2008). The identity of the muscarinic receptor responsible for modulation of interneuron excitability differs depending upon the cell type. For example, the firing frequency of hippocampal CCK+ interneurons is controlled by M₃Rs, whereas excitability of hippocampal PV+ interneurons is regulated by M₁Rs (Cea-del Rio et al., 2010). Comparable studies of muscarinic regulation of interneurons in BLA have not been reported. In this study we have identified M₂R expression in dendrites of BLA interneurons. However, it seems unlikely that these dendritic M₂Rs are responsible for the observed increase in spontaneous IPSCs in PNs, as studies in CA1 interneurons have reported dendritic M₂Rs to be inhibitory (Seeger and Alzheimer, 2001; Zheng et al., 2011). Further studies are needed to identify the mechanism underlying muscarinic excitation of interneurons in BLA. Overall, the ability of released acetylcholine to enhance interneuron firing, while selectively suppressing GABA release from specific M₂R+ inhibitory terminals provides a powerful mechanism to alter amygdalar network dynamics.

In addition to increasing interneuronal excitability, muscarine strongly suppressed the monosynaptic IPSC in PNs. This suppression was blocked by the M₂R antagonist, AF-DX 116. At the concentration used in the present study, AF-DX 116 is highly selective for M₂Rs, suggesting that other muscarinic receptors play little role in regulating the IPSC. This differs from hippocampus, where postsynaptic M₁R/M₃Rs can produce an indirect suppression of GABA release through endocannabinoid signaling (Fukudome et al., 2004; Neu et al., 2007). Muscarine suppressed the monosynaptic IPSC by approximately 50%, consistent with the expression of presynaptic M₂Rs on terminals of only a subset of interneurons. The extent of muscarinic inhibition of the IPSC in BLA is somewhat less than the 71% reported in hippocampus using carbachol (Behrends and ten Bruggencate, 1993),

but similar to that reported in entorhinal cortex using muscarine (Apergis-Schoute et al., 2007). In hippocampus, M₂R_s are preferentially expressed on terminals of PV⁺ basket cells, where they play a powerful role in regulating IPSCs in PNs (Hájos et al. 1998; Fukudome et al. 2004; Lawrence et al., 2015). Similarly, in BLA we found that a majority (58%) of the M₂R_s are on PV⁺ terminals.

There appear to be at least 3 subpopulations of PV⁺ INs in the BLA: (1) PV⁺ basket cells innervating the somata and dendrites of PNs, (2) PV⁺ axo-axonic (chandelier) cells innervating axon initial segments of PNs, and (3) PV⁺ dendrite-targeting cells that innervate distal dendrites of PNs (McDonald and Betette, 2001; Rainnie et al., 2006; Muller et al., 2006; Woodruff and Sah, 2007a; Bienvenu et al., 2012; Vereczki et al., 2016). In the present study 23% of terminals of PV⁺ basket cells targeting the cell bodies of putative PNs were M₂R⁺, and 24% of PV⁺ terminals targeting dendritic shafts were M₂R⁺. In addition, in one brain that was only stained for M₂R, an M₂R⁺ axon terminal was observed forming a symmetrical synapse with an axon initial segment (AIS); since only PV⁺ terminals form contacts with AISs (Vereczki et al., 2016), this terminal most likely belonged to a PV⁺ axo-axonic cell. The percentage of PV⁺ terminals that were M₂R⁺ in the present study represents an underestimate of the actual percentage of PV⁺/M₂R⁺ terminals because the counts were obtained from single 70 nm thin sections rather than from serial sections through each terminal. Since most PV⁺ terminals are 500–1000 nm in diameter many PV⁺ terminals in our study that did not meet criteria for being considered M₂R⁺ in a single thin section might have met criteria if analyzed in a series of 7–14 thin sections.

The findings of the present study in the mouse BLA regarding M₂R⁺/PV⁺ terminals forming perisomatic contacts are consistent with those of our earlier IP study in the rat BLA which found that many of the M₂R⁺ terminals forming symmetrical synapses contacted somata (Muller et al., 2016). Similar results were obtained in the hippocampus, where it was shown that 62% of PV⁺ terminals (contacting somata, AISs, and dendrites) were M₂R⁺, and GABA release by both PV⁺ basket cells and PV⁺ axo-axonic cells was modulated by M₂R activation (Szabo et al. 2010). Since the perisomatic region is where sodium-mediated action potentials are generated, M₂R-mediated modulation of GABAergic PV⁺ inputs to this region of BLA PNs would be expected to regulate the firing of these neurons and contribute to network oscillations (Woodruff and Sah, 2007b). Since virtually all of the M₂R⁺ terminals forming perisomatic contacts in the present study were PV⁺, these findings suggest that the other main IN subpopulation forming perisomatic contacts, CCK⁺ basket cells (Vereczki et al., 2016; Veres et al., 2017), do not express M₂R_s.

As in our previous IP study of the rat BLA (Muller et al., 2016), M₂R⁺ terminals forming symmetrical synapses in the present study often contacted thin dendritic shafts, many of which were M₂R⁺. The large number of thin dendrites contacted suggests that most of these are distal dendrites of PNs rather than INs (Muller et al., 2006). In the present study we demonstrated that some of these M₂R⁺ terminals belong to dendrite-targeting PV⁺ INs. However, many of the M₂R⁺/PV⁻ terminals that targeted dendritic shafts in the present study probably contain somatostatin (SOM) or neuropeptide Y (NPY), since in our previous immunofluorescence study in the rat many M₂R⁺ axon terminals in the neuropil exhibited SOM-ir or NPY-ir (McDonald and Mascagni, 2011). It is well established that

SOM+ axon terminals mainly synapse with distal dendrites and spines of PNs (Muller et al., 2007; Wolff et al., 2014). Cholinergic modulation of GABA release from both dendritic and perisomatic-targeting inhibitory terminals in the BLA could be important for regulating synaptic plasticity during fear conditioning (Wolff et al., 2014).

Finally, in the present study two M₂R+/PV+ axon terminals were very large and had numerous mitochondrial profiles and synaptic vesicles, characteristic of type 1 axon terminals of PV+ GABAergic basal forebrain (BF) inputs to the BLA (McDonald et al., 2011). This is consistent with our recent IP study in the rat BLA where morphologically similar M₂R+ axon terminals were observed (Muller et al., 2016). However, in that study PV immunohistochemistry was not performed along with the M₂R staining. Amygdalopetal PV +/GABA+ projection neurons in the BF are intermingled with cholinergic neurons in this region, where they constitute about 10–15% of all BF amygdalopetal neurons (Mascagni and McDonald, 2009). The innervation of the BLA by M₂R+ GABAergic axons from the BF is in agreement with studies which have shown that non-cholinergic BF neurons express M₂R protein and mRNA (Vilaro et al., 1992; Levey et al., 1995). The expression of M₂R-ir in these terminals suggests that release of acetylcholine by BF cholinergic axons, controlled by M₂R+ autoreceptors, might be followed by a rapid reduction of GABA release from type 1 BF axons via M₂R+ heteroreceptors. This coordinated regulation of neurotransmitter release from BF cholinergic and GABAergic axons may play a role in regulating oscillatory activity in the BLA, as in the hippocampus (Freund and Buzsaki, 1996; Borhegyi et al., 2004).

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Highlights

- Electrophysiological studies show suppression of IPSCs in the basolateral amygdala (BLa) by M₂ muscarinic receptors (M₂Rs)
- Dual cell recording indicated that M₂Rs suppressed IPSCs generated by only a subpopulation of BLa interneurons
- M₂R immunoreactivity was found in 23% of axon terminals of parvalbumin (PV) interneurons targeting somata and dendrites
- Many PV-negative inhibitory axon terminals, excitatory axon terminals, dendrites, and spines are also M₂R+ in BLa
- One-quarter to two-thirds of M₂Rs were plasma membrane-associated in M₂R + structures in BLa, depending on the structure

Figure 1. Differential suppression of IPSCs by mAChRs

(A) Evoked monosynaptic IPSCs are inhibited by muscarine. In both mouse (B) and rat (C) IPSC suppression by muscarine is relieved by AF-DX 116, a selective M2R antagonist. (D) Paired recording from an IN→PN cell pair. Ten superimposed sweeps (grey) are shown for the PN with the averaged response in black. (E) Muscarine suppressed the IPSC evoked by the presynaptic IN, but increased sIPSCs in the PN. (F) Averaged suppression of the evoked IPSC in 3 connected cell pairs (* $p < 0.05$, ** $p < 0.01$). Suppression of the IPSC was seen in all 3 pairs.

Figure 2. Light micrographs of M₂R-ir in the mouse BLA

(A) Low power photomicrograph of a section through the BLA and adjacent amygdalar nuclei at the bregma -1.28 level. There is dense staining in the BLA and adjacent portion of the lateral nucleus (Lat), and lighter staining in the main intercalated nucleus (IN), lateral central nucleus (CL), and caudatoputamen (CP). Square indicates field shown at higher power in B. (B) High power photomicrograph of the BLA. There is dense neuropilar staining and little or no M₂R-ir in neuronal cell bodies (asterisks). Also note that there are M₂R+ puncta in apparent contact with some of the cell bodies (inset shows 10 such puncta [arrowheads] contacting the cell body of the neuron indicated by an arrow). Scale bars = 100 μm in A, 20 μm in B.

Figure 3.

Electron micrographs illustrating the localization of M_2R+ immunoparticles in the plasma membrane versus cytoplasm of M_2R+ structures in the mouse BLA. In A and C-E crossed arrows indicate plasma membrane-associated immunoparticles, and non-crossed arrows indicate cytoplasmic particles. Because of the large number of particles in B, only membrane-associated particles are indicated (non-crossed arrows). All micrographs are from sections stained for M_2R (IGS) and PV (IP) except B, which was from a section single-labeled for M_2R . However, there are no PV+ structures in any of these fields. (A) An M_2R+ dendrite (Den) and an M_2R+ spine (s). The spine receives asymmetrical synaptic input from an M_2R -negative axon terminal (at). (B) Two M_2R+ axon terminals (at₂ and at₄) form

symmetrical synapses with an M₂R+ axon initial segment (Ais), identified on the basis of its dense plasma membrane undercoating. Although all axon terminals forming synapses in the BLA with axon initial segments are PV+ (Vereczki et al., 2016), this section was not stained for PV, and was not part of the quantitative survey. In the upper left and lower left corners two M₂R+ axon terminals (at₁ and at₃) form asymmetrical synapses with spines (s). The upper left spine is M₂R+. (C) A field that contains two M₂R+ dendrites (Den) and two spines (s) that receive asymmetrical synapses from axon terminals (at). The spine in the upper right corner is M₂R+ and is postsynaptic to an axon terminal (at₁) that is M₂R-negative (only 1 cytoplasmic immunoparticle). The spine in the lower left corner is M₂R-negative but is postsynaptic to an axon terminal that is M₂R+ (at₂). In addition, an M₂R+ axon terminal (at₃) forms a symmetrical synapse with one of the M₂R+ dendrites (asterisk). (D) An M₂R+ spine (s) receives an asymmetrical synapse from an M₂R+ axon terminal (at) that exhibits one membrane-associated immunoparticle located away from the synapse. The spine and axon terminal are both in contact with an M₂R+ process (Den) that may be a very thin dendrite or a spine. (E) Two M₂R+ dendrites (Den). Scale bars = 600 nm in A, C; 400 nm in B; 500 nm in D; 800 nm in E.

Figure 4. Bar graphs showing percentages of different types of M_2R+ structures

(A), percentage of total immunoparticles in different types of M_2R+ structures (B), and the percentage of membrane-associated immunoparticles in different types of M_2R+ structures (C) in the BLA. Total immunoparticle counts in all 3 brains = 4019. Total M_2R+ structures analyzed in all 3 brains ($n = 1439$): Thin dendritic shafts (Thin Den) = 776; thick dendritic shafts (Thick Den) = 15; spines = 197; axon terminals forming asymmetrical synapses or synapse-like contacts (Asym term) = 104; axon terminals forming symmetrical synapses or

synapse-like contacts (Sym term) = 119, axons or terminals not forming synapses or
synapse-like contacts (Axon) = 228.

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Figure 5. PV+ terminals that are either M₂R-negative (A) or M₂R+ (B-D; crossed arrows indicate plasma membrane-associated immunoparticles). (A) Two M₂R-negative PV+ terminals (Pt) contact an M₂R+ dendritic shaft (Den). The lower terminal forms a symmetrical synapse (asterisk) with the dendritic shaft. (B) An M₂R+/PV+ axon terminal (Pt) forms a symmetrical synapse (asterisk) with an M₂R+ dendritic shaft (Den). (C) A large M₂R+/PV+ axon terminal (Pt) forms a synapse-like contact (asterisk) with a dendritic shaft (Den). The large size of this terminal as well as its numerous mitochondria and synaptic vesicles suggests that it is a GABAergic afferent axon from the basal forebrain. (D) M₂R+/PV+ axon terminal (Pt) forms a synapse-like contact with a soma (Som). The presence of the immunoparticles at the site of contacts in C and D suggests M₂R-mediated regulation of GABA release. Scale bars = 600 nm in A, B, and D; 500 nm in C.

Figure 6. Representative control section in which the M2R antibody was omitted

Very few silver grains were seen in each of the ultrathin sections of the controls (arrows).

Abbreviations: at, unlabeled axon terminals; Den, unlabeled dendritic shaft; Pt, PV+ axon terminal; s, unlabeled dendritic spine. Scale bars = 500 nm.

Figure 7. Quantitative analysis of the proximity of M2R+ immunoparticles to the presynaptic active zone in PV+ and PV-negative axon terminals forming symmetrical synapses
 (A) Schematic diagram demonstrating the quantification procedure in axon terminals (at). Distances of immunoparticles were measured from the edge of the active zone (AZ). Distances of particles located within the active zone were given negative values whereas distances of particles located in the surrounding zone (SZ) were given positive values. Values were allocated to 60-nm-wide bins and expressed as relative frequencies for each of the two types of terminals. (B) Histogram showing the spatial distribution of immunoparticles for PV+ terminals (gray bars) and PVnegative axon terminals forming symmetrical synapses (white bars).

Figure 8.

A PV+ dendrite (Den) exhibits M₂R-ir (arrows indicate cytoplasmic immunoparticles; crossed arrow indicates a single membrane-associated immunoparticle). Note multiple axon terminals (at) forming asymmetrical synapses (asterisks) with the dendrite, typical of PV+ dendrites. Scale bar = 500 nm.

Table 1

Percentages of PV+ axon terminals in the BLA that were M2R+, and their postsynaptic targets.

Brain	Postsynaptic Targets	Total PV+ Terminals	PV+/M₂R+ Terminals	Percentage of PV+ terminals that were M₂R+
M5	Cell bodies	18	5	27.7% (5/18)
	Dendritic shafts	57	16	28.1% (16/57)
	Spines	3	0	0% (0/3)
	Total	78	21	27.0% (21/78)
M14	Cell bodies	4	1	25.0% (1/4)
	Dendritic shafts	33	6	18.9% (6/33)
	Spines	0	0	0% (0/0)
	Total	37	7	18.9% (7/37)
M15	Cell bodies	8	1	12.5% (1/8)
	Dendritic shafts	32	7	21.9% (7/32)
	Spines	0	0	0% (0/0)
	Total	40	8	20% (8/40)
	Combined total	155	36	23.3% (36/155)