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Mu Opioid Receptor Localization in the Basolateral Amygdala: An Ultrastructural Analysis

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Abstract

Receptor binding studies have shown that the density of mu opioid receptors (MORs) in the basolateral amygdala is among the highest in the brain. Activation of these receptors in the basolateral amygdala is critical for stress-induced analgesia, memory consolidation of aversive events, and stress adaptation. Despite the importance of MORs in these stress-related functions, little is known about the neural circuits that are modulated by amygdalar MORs. In the present investigation light and electron microscopy combined with immunohistochemistry was used to study the expression of MORs in the anterior basolateral nucleus (BLA). At the light microscopic level, light to moderate MOR-immunoreactivity (MOR-ir) was observed in a small number of cell bodies of nonpyramidal interneurons and in a small number of processes and puncta in the neuropil. At the electron microscopic level most MOR-ir was observed in dendritic shafts, dendritic spines, and axon terminals. MOR-ir was also observed in the Golgi apparatus of the cell bodies of pyramidal neurons and interneurons. Some of the MOR+ dendrites were spiny, suggesting that they belonged to pyramidal neurons, while others received multiple asymmetrical synapses typical of interneurons. The great majority of MOR+ axon terminals (80%) that formed synapses made asymmetrical (excitatory) synapses; their main targets were spines, including some that were MOR+. The main targets of symmetrical (inhibitory and/or neuromodulatory) synapses were dendritic shafts, many of which were MOR+, but some of these terminals formed synapses with somata or spines. All of our observations were consistent with the few electrophysiological studies which have been performed on MOR activation in the basolateral amygdala. Collectively, these findings suggest that MORs may be important for filtering out weak excitatory inputs to pyramidal neurons, allowing only strong inputs or synchronous inputs to influence pyramidal neuronal firing.

Keywords

mu opioid receptor; basolateral amygdala; immunohistochemistry; electron microscopy; pyramidal neurons; interneurons

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INTRODUCTION

The endogenous opioid system plays an important role in the process of stress adaptation by attenuating or terminating stress responses (Drolet et al., 2001). Endogenous opioid peptides including enkephalin, dynorphin and beta-endorphin, produce their effects via three major types of G-protein coupled opioid receptors: mu (MOR), delta (DOR), and kappa (KOR). Considerable evidence indicates that MORs in the basolateral nuclear complex of the amygdala (BLC) are involved in stress-related hypoalgesia (Helmstetter et al., 1995; Helmstetter et al., 1998; Shin and Helmstetter, 2005; Finnegan et al., 2006). Although BLC neurons do not directly project to portions of the bulbospinal descending antinociceptive pathway such as the periaqueductal gray (PAG), the BLC has extensive projections to the central amygdalar nucleus which has dense reciprocal interconnections with the PAG (Hopkins and Holstege, 1978; Rizvi et al., 1991; Harris, 1996). Additionally, MORs in the anterior subdivision of the basolateral nucleus of the BLC (BLA) are involved in memory consolidation; the opiate antagonist naloxone has been found to enhance retention of inhibitory avoidance, and this effect can be reversed by the MOR agonist DAMGO (Introini-Collison et al., 1995, McGaugh, 2004).

Autoradiographic receptor binding studies have found that the density of MORs in the BLA is among the highest in the brain (Mansour et al., 1987). Despite the fact that MOR activation in the BLA is critical for the regulation of the stress response and memory consolidation, little is known about the neural circuits in this brain region that are modulated by MORs. Knowledge of the ultrastructural localization of MORs should contribute to a better understanding of how opioids modulate BLA circuits. In the present investigation electron microscopy combined with a sensitive immunoperoxidase technique was used to study the expression of MORs in the BLA.

EXPERIMENTAL PROCEDURES

Tissue preparation

Six adult male Sprague-Dawley rats (250–350g; Harlan, Indianapolis, IN) were used in this study. Three rats were used for light microscopy and three rats were used for electron microscopy. 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 sodium pentobarbital (50 mg/kg), or a mixture of ketamine (85mg/kg), xylazine (8mg/kg), and acepromazine (4mg/kg,) and perfused intracardially with phosphate buffered saline (PBS; pH 7.4) containing 1% sodium nitrite, followed by 2% paraformaldehyde-3.75% acrolein in phosphate buffer (PB; pH 7.4) for 1 minute, followed by 2% paraformaldehyde in PB for 20 minutes. Sodium pentobarbital was used to anesthetize the rats used for light microscopy, whereas the ketamine/xylazine/acepromazine mixture was used to anesthetize the rats used for electron microscopy. This change in anesthesia was due to our inability to procure pharmaceutical-grade pentobarbital midway

through the study. After perfusion all brains were removed and postfixed in 2% paraformaldehyde for one hour. Brains were sectioned on a vibratome in the coronal plane at 50 μ m for light microscopy and 60 μ m for electron microscopy. Sections were rinsed in 1.0% borohydride in PB for 30 min and then rinsed thoroughly in several changes of PB for 1 hr. All sections were processed for immunohistochemistry in the wells of tissue culture plates.

Light microscopic immunohistochemistry

Light microscopic MOR localization was performed in rats using a rabbit antibody to MOR (catalog #24216; ImmunoStar, Hudson, WI). All antibodies were diluted in PBS containing 0.4% Triton X-100 and 1% normal goat serum. Sections were incubated in the MOR antibody (1:1000) overnight at 4°C and then processed using a rabbit ABC kit with DAB (diaminobenzidine 4HCl, Sigma Chemical Co., St. Louis, MO, USA) as a chromogen to generate a brown reaction product. After the reactions, 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.

Electron microscopic immunohistochemistry

Immunoperoxidase methods were used to observe the ultrastructural localization of MOR in the anterior subdivision of the basolateral nucleus (BLa; bregma levels -2.1 through -2.6; Paxinos and Watson, 1997). Sections were incubated in the MOR antibody (1:1000) overnight at 4°C after 30 minutes in a blocking solution (PBS containing 3% normal goat serum, 1% BSA and 0.02% Triton X-100). All antibodies were diluted in the blocking solutions. Sections were then processed using a biotinylated goat anti-rabbit antibody (1:500, Jackson ImmunoResearch, West Grove, PA) and a Vectastain Standard ABC kit (Vector Laboratories, Burlingame, CA). MOR immunoreactivity was then visualized using a Vector-VIP (Very Intense Purple) peroxidase substrate kit (Vector Laboratories). This step produced a reaction product that appeared purple in the light microscope and granular in the electron microscope. In the smallest structures, such as spines and small axon terminals, the criterion for calling a structure “labeled” was the observation of at least 2 granules of reaction product in the structure. For all other structures the criterion was three or more granules. Omission of the primary antibody resulted in no staining when sections were examined by either light or electron microscopy (see Fig. 4B).

Sections were then postfixed in 2% osmium tetroxide in 0.16 M sodium cacodylate buffer for one hour, dehydrated in ethanol and acetone, and flat embedded in Polybed 812 (Polysciences, Warrington, PA) in slide molds between sheets of Aclar (Ted Pella, Redding, CA). Areas in the BLa were remounted onto resin blanks. Silver thin sections were collected on formvar-coated slot grids, stained with uranyl acetate and lead citrate, and examined with a JEOL-200CX electron microscope. Micrographs were taken with an AMT XR40 digital camera system (Advanced Microscopy Techniques, Danvers, MA).

Ultrastructural Analysis

One representative vibratome section from each of the 3 rats was selected for thin sectioning and analysis. From these thin sections, areas that were judged to have the best ultrastructural preservation and uniform immunohistochemical staining for MOR were chosen for quantitative analysis. Areas that were very close to the tissue/plastic interface exhibited poor ultrastructure whereas areas that were too deep in the vibratome section had diminished MOR staining. The areas selected for quantitative analysis appeared to be midway between the latter two extremes. About 3600 μm^2 from each section was used for quantitation. Profiles were identified as somata, large-caliber dendritic shafts ($>1\mu\text{m}$), small-caliber dendritic shafts ($<1\mu\text{m}$), spines, axons, axon terminals, and glial processes according to established morphological criteria (Peters et al., 1991). Since the proportions of MOR+ structures were very similar in all three rats, the data were pooled and yielded a total of 2,384 MOR+ profiles.

The BLA contains pyramidal projection neurons (PNs) and nonpyramidal interneurons that are very similar to those in the cerebral cortex (McDonald, 1992a; Sah et al., 2003; McDonald, 2003; Pape and Pare, 2010; Spampinato et al, 2011). In the absence of specific markers for these neurons, putative pyramidal cell perikarya were identified by their large size, round nuclei, and synaptic inputs from terminals forming only symmetrical synapses (Muller et al., 2006). Dendrites exhibiting spines were considered to be candidate pyramidal cell dendrites. Putative nonpyramidal cell perikarya are smaller, often have infolded nuclei, and receive both symmetrical and asymmetrical synapses (Muller et al., 2005, 2011). Their dendritic shafts could be followed a significant length without a visible spine, and they often have a high density of asymmetrical synapses.

MOR-positive (MOR+) terminals were followed in 3 to 13 serial thin sections. Serial sections were helpful for verifying label in small and/or lightly immunoreactive structures, and for determining synaptic contacts. MOR+ terminals were considered to form synapses if they showed the following characteristics: (1) parallel presynaptic and postsynaptic membranes exhibiting membrane thickening, (2) clustered vesicles associated with the presynaptic membrane, and (3) a synaptic cleft containing dense material. Asymmetrical and symmetrical synapses were identified based on the presence or absence of a prominent postsynaptic density and the widths of their synaptic clefts. Asymmetrical synapses usually have wider synaptic clefts (20 nm) than symmetrical ones (12 nm) (Peters et al., 1991). The postsynaptic targets of these terminals, as well as whether they were MOR-positive or negative, were noted. Since only MOR+ axon terminals and their postsynaptic structures were followed in serial sections, some dendrites that appeared to be MOR-negative when examined in a single thin section may have actually been MOR+ (i.e., false negatives).

Antibody Specificity

The rabbit polyclonal MOR antiserum (ImmunoStar, catalog # 24216) used in this study was raised against a synthetic peptide corresponding to amino acids 384–398 of the carboxyl terminus of the cloned rat MOR1 coupled to bovine thyroglobulin with glutaraldehyde. Specificity of this antibody has been well documented in previous studies using Western blotting, epitope-expressing cell lines, and adsorption controls (Arvidsson et al., 1995). It

does not recognize the cloned DOR (Arvidsson et al., 1995) or the MOR splice variants MOR-1A-E (Abbadie et al., 2000). Preadsorption studies of the MOR antiserum with its antigenic peptide (35 µg/ml) in our lab eliminated all immunostaining, and cross-adsorption of the antiserum with a DOR-specific peptide sequence did not diminish immunostaining (Wilson et al., 2002).

RESULTS

Light microscopic observations

At the light microscopic level, MOR immunoreactivity (MOR-ir) in the forebrain, including the amygdala, was identical to that described in previous studies (Arvidsson et al., 1995; Ding et al., 1996). Thus, MOR-ir was in the “patches” of the striatum, and was light to moderate in interneurons in the neocortex and hippocampus. In the amygdala at the level of the BLA the intercalated nuclei were intensely stained, the central nucleus was moderately stained, and the BLA was very lightly stained, as in previous studies (Ding et al., 1996) (Fig. 1A). A small number of small to medium-sized nonpyramidal cell bodies (10–15 µm in diameter) with light to moderate MOR-ir was observed in the BLA (2–5 per section; Fig. 1B). These neurons were the size and shape of BLA interneurons and resembled the MOR+ interneurons seen in the hippocampus. The neuropil of the BLA contained lightly-stained dendritic processes, as well as small puncta that were the size of axon terminals (0.5–1.0 µm in diameter) (Fig. 1B). A similar pattern of MOR-ir, including the staining of a small number of nonpyramidal interneurons, was also observed in the lateral nucleus of the BLC, but neuropilar staining was lighter than in the BLA (Fig. 1A).

Electron microscopic observations

At the electron microscopic level, light to moderate MOR-ir was seen in various structures including somata, dendritic shafts, spines, axon terminals, thin unmyelinated or myelinated axons, and glial processes (Fig. 2). Only six somata were seen in areas sampled for quantitative analysis (Table 1). Two of these somata appeared to be MOR+ interneurons. One of these interneuronal somata (EM09-1) was identified as an interneuron because it received both asymmetrical and symmetrical synapses; it only had granules of reaction product in the vicinity of its Golgi apparatus (Fig. 3B). Only a small portion of the soma of the other MOR+ candidate interneuron was seen (EM09-2). There were only 2 synapses with the soma of neuron EM09-2 in the thin sections available for analysis. Although both synapses were symmetrical, this neuron was considered a candidate interneuron because it contained diffuse MOR-ir typical of interneurons seen at the light microscopic level. Four somata were identified as PNs because they only received symmetrical synapses (Table 1). Two of these PNs were MOR+ (EM03-1, EM05-1), with granules of reaction product only found in the vicinity of the Golgi apparatus (Fig. 3A, Table 1). The other two PNs (EM03-2, EM05-2) appeared to be MOR-negative.

The most frequently labeled structures in the neuropil were dendritic structures. Counts of MOR+ profiles revealed that large-caliber dendrites, small-caliber dendrites, and spines comprised 11.1% (265/2384), 50.3% (1200/2384) and 10.8% (258/2384) of all MOR+ profiles, respectively (Figs. 2, 4–6). The presence of spines on some MOR+ dendrites

suggests that they may belong to pyramidal cells (Figs. 5B, 5C) (McDonald, 1992a; Muller et al., 2006). Several dendrites were observed that received multiple asymmetrical synapses (Fig. 4C), a characteristic of interneuronal dendrites in the BLA (Muller et al., 2011). MOR-ir in these labeled dendrites and spines was mainly cytoplasmic, and was sometimes found contacting microtubules in dendrites, but MOR+ labeling near the plasma membranes was observed in some cases as well (Figs. 5, 7). It was not uncommon to observe MOR-negative spines arising from MOR+ dendritic shafts (Figs. 5B, 5C).

MOR-ir was also seen in a few small unmyelinated or myelinated axons, and was commonly observed in axonal terminals, constituting 21.1% (502/2384) of MOR+ profiles (Fig. 2). MOR-ir in these MOR labeled terminals was associated with synaptic vesicles near the active zone, or randomly in the cytoplasm of the varicosities (Figs. 3–5, 7, 8). About 40.2% (202/502) of MOR+ terminals formed synapses. MOR+ terminals formed synapses with both labeled and unlabeled profiles, and their postsynaptic targets included somata, large and small dendritic shafts, and spines (Figs. 3A, 4C, 5A, 6, 7, 8; Tables 1 and 2). Asymmetrical synapses (80.2%, 162/202) were more common than symmetrical synapses (19.8%, 40/202) (Fig. 9; Tables 1 and 2). The main postsynaptic targets of MOR+ terminals forming asymmetrical synapses were spines, the great majority of which were MOR-negative (Fig. 9; Table 2). In fact, MOR-negative spines constituted 72.3% (117/162) of the total number of postsynaptic targets of asymmetrical synapses formed by MOR+ terminals (Fig. 9; Table 2). The main targets of MOR+ terminals forming symmetrical synapses were MOR+ small-caliber dendrites, which comprised 35.0% (14/40) of the total number of postsynaptic targets of symmetrical synapses formed by MOR+ terminals (Fig. 9; Table 2).

Approximately 4.5% (9/202) of MOR+ terminals forming synapses contacted the 6 somata identified in the quantitative study (Tables 1 and 2, Figs. 3A, 7A, 8A). Seven of these nine MOR+ terminals formed symmetrical synapses with PNs, constituting 28.0% (7/25) of PN axo-somatic synapses. Of the 18 MOR-negative terminals forming axo-somatic synapses with PNs, two had a unique morphology. Both of the latter terminals formed deep invaginations into the PN plasma membrane, and appeared to form symmetrical synapses on each side of the invagination (Fig. 8B).

DISCUSSION

Light microscopic analysis of MOR-ir in the BLA indicated that there was a small subpopulation of interneurons, as well as in some processes and puncta in the neuropil, that exhibited light to moderate levels of MOR-ir. Electron microscopic analysis revealed that light to moderate MOR-ir was present in diverse neuronal profiles, and some glial processes as well. The most frequently labeled structures were small-caliber dendrites and terminals. MOR+ terminals formed synapses with both labeled and unlabeled profiles. The main postsynaptic targets of MOR+ terminals forming asymmetrical synapses were unlabeled spines, while the main targets of MOR+ terminals forming symmetrical synapses were MOR+ small-caliber dendritic shafts. The ubiquitous distribution of MOR-ir in the BLA at the ultrastructural level of analysis is consistent with previous autoradiographic MOR binding studies in the amygdala which revealed extremely dense MOR binding in the BLA (Mansour et al., 1987).

Although MOR-ir in our preparations was mainly found in the cytoplasm, it was also found near the plasma membrane in many cases as well. MOR has to be inserted into membrane to be functional. Although particulate Vector-VIP reaction product may not represent the precise location of the antigen due to diffusion of the peroxidase reaction product, it is highly possible that the MOR-ir near the plasma membrane may reflect membrane-associated receptors, while MOR-ir associated with cytoplasmic organelles may represent a pool of MORs undergoing transport to or from the plasma membrane in other parts of MOR + neurons. The fact that Vector-VIP particulate reaction product was found apposing membranes of the Golgi apparatus in the present study suggests that these particles are deposited at or very near the location of the receptor. Likewise, in a previous ultrastructural study of muscarinic type 1 receptor localization performed in this lab, particulate Vector-VIP reaction product was found in high concentrations just beneath the synaptic active zone in presynaptic axon terminals (Muller et al., 2013). Whereas future studies using immunogold-silver techniques are required to provide a more precise knowledge of the exact subcellular distribution of MORs in the BLA (e.g., Wang et al., 1996; Drake and Milner, 1999), one advantage of the Vector-VIP immunoperoxidase technique for initial studies of receptor localization is its very high sensitivity. Experience with this technique in our lab has shown that it is much more sensitive than DAB-based immunoperoxidase techniques (unpublished observations), and it is well documented that the latter are much more sensitive than immunogold-silver techniques. It is also much easier to identify Vector-VIP particles versus light flocculent DAB reaction product in lightly labeled structures at the ultrastructural level.

MORs are expressed in somatodendritic compartments of pyramidal cells and interneurons in the BLA

In the present study light MOR-ir was found associated with the Golgi apparatus, but not along the plasma membrane of the cell body, in neuronal somata that appeared to be PNs on the basis of their morphology and synaptology. This suggests that MORs in this location are in the process of being packaged for transport to dendritic and/or axonal processes of PNs. Since PNs constitute the great majority (85%) of neurons in the BLA (McDonald, 1992b), the finding of MOR+ PN somata agrees with in situ hybridization studies which demonstrated that many neurons in the BLA express MOR mRNA (Poulin et al., 2006). PNs in the BLA also have more complex dendritic arborizations than interneurons and are spine-dense (McDonald, 1982). Therefore, our finding that MOR+ dendrites were found in high density and that many exhibited spines suggests that most of these dendritic shafts belong to PNs. Similarly Glass and co-workers, in an immunoperoxidase electron microscopic study that mainly focused on the central nucleus, observed that some dendrites in the BLA were MOR+ (Glass et al., 2005). Collectively these data indicate that MORs are synthesized and packaged for transport in the somata of BLA PNs, but instead of becoming incorporated into the plasma membrane of PN somata they are transported to dendritic shafts and spines of these neurons (as well as axon terminals; see below). This is consistent with electrophysiological studies which have shown that the selective MOR agonist DAMGO activates a voltage-dependent potassium current in dendrites, but not in the somata, of PNs (Faber and Sah, 2004).

At the light microscopic level we observed a small number of putative interneurons in both the BLA and the lateral nucleus that exhibited light to moderate MOR-ir. The cell bodies of two interneurons (Fig. 3B), MOR+ dendrites of putative interneurons (Fig. 4C), as well as MOR-negative interneuronal dendrites (Fig. 7C), were observed in the BLA at the electron microscopic level. Collectively these data indicate that some interneuronal subpopulations in the BLA and lateral nucleus are MOR+. These findings are consistent with electrophysiological studies which have shown that activation of MORs hyperpolarize some interneurons in the lateral nucleus of the BLC (Sugita and North, 1993a, b). Since the great majority of BLC interneurons use the inhibitory neurotransmitter GABA (McDonald and Mascagni, 2001, 2002; Mascagni and McDonald, 2003) and innervate PNs (Muller et al., 2003, 2006, 2007), MOR-mediated inhibition of these interneurons should have a disinhibitory (excitatory) effect on pyramidal projection neurons in the BLA. We also observed MOR+ interneurons in the hippocampus and neocortex, in agreement with previous studies of these areas. Discrete subpopulations of hippocampal and neocortical interneurons were found to express MORs (Drake and Milner, 1999, 2002; Taki et al., 2000). Since distinct interneuronal subpopulations in the BLA innervate discrete PN compartments (Muller et al., 2003, 2006, 2007), it will be important in future studies to determine which interneurons in the BLA express MORs.

MORs are expressed in axon terminals forming asymmetrical or symmetrical synapses in the BLA

Our finding that some axon terminals in the BLA are MOR+ is consistent with observations in an immunoperoxidase electron microscopic study that mainly focused on the central nucleus (Glass et al., 2005). In the present study 80% of the synapses formed by MOR+ terminals were asymmetrical, and their main postsynaptic targets were spines of presumptive PNs, some of which were also MOR+. These features are characteristic of glutamatergic inputs to the BLA that arise from the cerebral cortex (Hall, 1972; Smith and Paré, 1994; Brinley-Reed et al., 1995; Farb and LeDoux, 1999; Smith et al., 2000, Pinard et al., 2010), midline/intralaminar thalamus (Carlsen and Heimer, 1988; LeDoux et al., 1991), and internuclear/intranuclear amygdalar connections arising from basolateral amygdalar PNs (Stefanacci et al., 1992; Smith and Paré, 1994; Paré et al., 1995; Smith et al., 2000). Our findings are consistent with a recent study which found MOR-mediated modulation of glutamate release in the BLC (Yang et al., 2014). Interestingly, distinct effects were associated with DAMGO or morphine. DAMGO decreased the frequency of evoked EPSCs in BLC PNs, whereas morphine increased the frequency of evoked EPSCs, by two separate MOR-Gi signaling pathways (Yang et al., 2014). Since not all terminals forming asymmetrical (excitatory) synapses were MOR+, it will be of interest to determine if MOR-ir is only associated with some of the glutamatergic inputs to the BLA. Both DAMGO and enkephalin, but not DOR or KOR agonists, inhibit glutamate release in the central nucleus upon BLA stimulation (Zhu and Pan, 2005). This suggests that the axons of BLA PNs projecting to the central nucleus contain MORs. It would therefore not be surprising if some of the MOR+ terminals seen in the present study belong to the intranuclear arborizations of BLA PNs. These findings also suggest that the MOR-ir associated with membranes of the Golgi apparatus and endoplasmic reticulum of BLA PNs is transported to axons, as well as dendrites (see above), of these neurons.

Approximately one-fifth of the synapses formed by MOR+ terminals were symmetrical. Many of the symmetrical synapses in the BLA are formed by several distinct subpopulations of GABAergic interneurons (Carlsen, 1988; Aylward and Totterdell, 1993; Smith et al., 2000; McDonald et al., 2002; Muller et al., 2003, 2006, 2007). Additional GABAergic inputs to the BLA arise from the intercalated nuclei (Marowsky et al., 2005; McDonald and Augustine, 1993), basal forebrain (Mascagni and McDonald, 2009; McDonald et al., 2011), and parahippocampal cortices (McDonald and Zaric, 2015). Moreover, monoaminergic projections from the brainstem (Asan, 1998; Muller et al., 2007, 2009; Pinard et al., 2010; Farb and LeDoux, 2010; Zhang, 2013) and cholinergic projections from the basal forebrain (Carlsen and Heimer, 1986; Muller et al., 2011) also form symmetrical synapses.

Dual-labeling ultrastructural studies in the hippocampus have demonstrated MOR-ir in axon terminals expressing GABA or neuronal markers for several GABAergic interneuronal subpopulations, including parvalbumin and somatostatin containing subpopulations (Drake and Milner 1999, 2002). Terminals of parvalbumin-positive (PV+) basket cells, but not cholecystokinin-positive (CCK+) basket cells, formed symmetrical synapses with pyramidal cell perikarya in the hippocampus (Drake and Milner 2002). PV+ and CCK+ basket cells are also the main interneuronal subpopulations forming symmetrical synapses with PN perikarya in the BLA (Muller et al, 2006; Yoshida et al., 2011). Axon terminals of CCK+ basket cells in the BLA form a distinct invaginating type of contact with PN somata that exhibits symmetrical synapses on each side of the invagination (Yoshida et al., 2011). Significantly, the two CCK-like invaginating terminals seen in the present study (Fig. 8B) were MOR-negative, suggesting the possibility that CCK+ basket cells in the BLA, like those in the hippocampus, are not modulated by MORs. This also suggests that the MOR+ terminals forming symmetrical synapses with PNs in the present study belong to PV+ basket cells. In both the BLA (Sugita and North, 1993b; Finnegan et al., 2005) and hippocampus (Wagner and Chavkin, 1995) MOR agonists disinhibit pyramidal cells by inhibiting presynaptic GABA release.

It is also likely that some of the MOR+ axon terminals forming symmetrical synapses are noradrenergic. Previous studies have shown that the release of norepinephrine in the BLA during stress is inhibited by opioids (enkephalin and beta-endorphin) and opiates (morphine) that activate MORs, and that this effect is blocked by naloxone (Quirarte et al., 1998; Tanaka et al., 2000). The great majority of synapses formed by noradrenergic terminals in the BLA are symmetrical, and they mainly target dendrites of PNs (Asan, 1998; Zhang et al., 2013).

Functional Implications

This study demonstrates that light to moderate MOR-ir is seen in various structures in the BLA including somata, dendritic shafts, spines, and axon terminals. This ubiquitous distribution of MOR-ir in the BLA at the ultrastructural level is consistent with autoradiographic MOR binding studies which revealed extremely dense MOR binding in the BLA (Mansour et al., 1987), and with electrophysiological data on the effects of MOR activation in these neuronal structures (see above). Enkephalin or enkephalin-like peptides are the most likely activators of MORs, since the BLA receives an extremely sparse

innervation by beta-endorphin containing axons (Gray et al., 1984), the other main opioid peptide associated with MORs. Preproenkephalin mRNA is found in PNs and interneurons in the BLA (Poulin et al., 2008), and might also be associated with extrinsic inputs.

Excitatory glutamatergic inputs, conveying information regarding stimuli and events in the external world, mainly target dendritic spines of BLA PNs via asymmetrical synapses. If it is assumed that most effects of MOR activation in the BLA involve inhibitory Gi protein signaling pathways (Wagner and Chavkin, 1995), the finding that some of these presynaptic excitatory inputs and/or their postsynaptic spines are MOR+ suggests that these inputs could be selectively filtered via MORs. One possible function of this inhibitory filtering might be to filter out weak or asynchronous inputs, and allow only strong and/or synchronous excitatory inputs to influence PN firing.

MOR-ir was found in both the somata and presumptive axon terminals of some interneurons in the BLA, where it has been shown that MOR activation has a disinhibitory effect on PN activity (see above). The ratio of MOR+ terminals forming symmetrical (mainly inhibitory) synapses versus asymmetrical excitatory synapses increases as the distance to the cell body of MOR+ PNs decreases along the dendritic tree (Fig. 9), and some MOR+ terminals form symmetrical synapses with PN somata. As a result, it would appear that it is mainly the perisomatic compartment of PNs that is disinhibited. Since the firing of BLA PNs is tightly controlled by GABAergic interneurons (Ehrlich et al., 2009), this disinhibitory effect of MORs should have a profound influence on PN firing. It would therefore appear that once EPSPs related to strong emotionally-salient excitatory inputs to PN spines reach the cell body, their ability to fire these PNs would be greatly facilitated due to the disinhibitory effect of MORs in interneurons. Thus, the overall effect of MOR activation at excitatory and inhibitory synapses with PNs in the BLA may be to increase the signal to noise ratio. Future electrophysiological, pharmacological, and behavioral studies will be required to test this hypothesis.

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Highlights

- MOR-ir was seen in the Golgi apparatus of pyramidal neurons (PNs) and interneurons
- Many dendritic shafts and spines were MOR+
- Diffuse MOR-ir was seen in the perikarya and dendritic shafts of some interneurons
- MOR+ axon terminals forming asymmetrical synapses mainly contacted spines
- MOR+ terminals forming symmetrical synapses contacted PN somata and dendrites

Fig. 1.

Light micrographs of MOR-ir in the amygdala. (A) Low power photomicrograph of a section through the BLA. Note light MOR-ir in the BLA, intense MOR-ir in the surrounding intercalated nuclei (two of which are indicated by asterisks), and moderate MOR-ir in the central nucleus (Ce). Additional abbreviations: La, lateral nucleus; CP, caudatoputamen. (B) Higher power photomicrograph of a section through the BLA. There are two presumptive interneurons with moderate MOR-ir in this field (larger arrows). Asterisks indicate pyramidal cell nuclei surrounded by perikarya with MOR-ir that is slightly above background. Smaller arrows indicate some of the puncta in the neuropil that may represent axon terminals. In the upper left quadrant of this field are vertically-oriented MOR+ processes with light MOR-ir that resemble dendrites. Scale bars = 200 μ m in A, 10 μ m in B.

Fig. 2. Histogram showing the number of each MOR+ structure observed in the quantitative analysis of the BLA. So: Somata; LD: large-caliber dendrites ($\geq 1\mu\text{m}$); SD: small-caliber dendrites ($<1\mu\text{m}$); Sp: spines; T: terminals; Ax: axons; G: glial processes.

Fig. 3.

Electron micrographs of MOR+ somata in the BLA. Arrowheads indicate examples of granular MOR-ir. (A) A MOR+ soma (M-So; neuron EM03-1 of Table 1) is contacted by a MOR+ terminal (M-t), forming a symmetrical synapse (arrow). MOR-ir is present near the Golgi apparatus in the soma. This soma only received symmetrical synapses suggesting that it is the cell body of a pyramidal neuron. (B) A MOR+ soma (M-So; neuron EM09-1 of Table 1) receiving asymmetrical synaptic contacts (arrows) from two unlabeled terminals (U-t). The presence of asymmetrical synapses suggests that this is the cell body of an interneuron. Scale bars = 0.5 μ m.

Fig. 4.

Electron micrographs of MOR+ dendrites. Arrowheads indicate examples of granular MOR-ir. (A) A large-caliber MOR+ dendrite (M-LD) and two nearby small-caliber MOR+ dendrites (M-SD). (B) A control section in which the primary MOR antibody was omitted during immunohistochemical processing. Note total absence of granular label in large and small caliber dendrites (LD and SD), spines (sp), and terminals (t). The cisternae with dark membranes (arrows) are easily distinguished from the granular Vector-VIP labeling of MORs shown in A and C. These cisternae may be endosomes. (C) A longitudinally-

sectioned large-caliber MOR+ dendrite receives multiple asymmetrical synapses (arrows) from either MOR+ (M-t) or unlabeled (U-t) terminals. The presence of multiple asymmetrical synapses suggests that this dendrite may belong to an interneuron. Scale bars = 0.5 μ m.

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Fig. 5.

Electron micrographs of MOR+ small caliber (<1μm) dendrites. Arrowheads indicate examples of granular MOR-ir. (A) A long MOR+ small-caliber dendrite (M-SD) and nearby MOR+ (M-t) or unlabeled (U-t) axon terminals which both form asymmetrical synapses with a MOR+ spine (M-Sp). There is a large-caliber dendrite (M-LD) nearby. (B) Two small-caliber MOR+ dendrites (M-SD), one of which has an unlabeled spine (U-Sp). (C) A small-caliber MOR+ dendrite (M-SD) with an unlabeled spine (U-Sp). Scale bars = 0.5 μm.

Fig 6.

Electron micrographs of MOR+ spines. Arrowheads indicate examples of granular MOR-ir. (A) A MOR+ spine (M-Sp) receives an asymmetrical synaptic contact (arrow) from an unlabeled terminal (U-t). (B) A MOR+ spine receives an asymmetrical synaptic contact from an unlabeled terminal (arrow). Three unlabeled spines (asterisks) also receive asymmetrical synaptic contacts from unlabeled terminals. Scale bars = 0.5 μm.

Fig. 7.

Electron micrographs of various postsynaptic targets of MOR+ terminals. Arrowheads indicate examples of granular MOR-ir. (A) A MOR+ terminal (M-t) forms a symmetrical synapse (arrow) with an unlabeled soma (U-So; neuron EM03-2 of Table 1)). There are two MOR+ small-caliber dendrites (M-SD) nearby. (B) A MOR+ terminal (M-t) form a symmetrical synapse (arrow) with a MOR+ small-caliber dendrite (M-SD). (C) Two MOR+ terminals (M-t) form asymmetrical synapses (arrows) with an unlabeled small-caliber dendrite (U-SD), which also receives an asymmetrical synapse (arrow) from an unlabeled

terminal (U-t). Multiple asymmetrical synapses are indicative of interneuronal dendrites. (D) A MOR+ terminal (M-t) forms an asymmetrical synapse (arrow) with a MOR+ spine (M-Sp). (E) A MOR+ terminal (M-t) forms an asymmetrical synapse (arrow) with an unlabeled spine (U-Sp). Scale bars = 0.5 μ m.

Fig. 8.

(A) Electron micrograph of a MOR+ axon terminal (M-t) with 3 MOR-ir granules forming a "flat" symmetrical synapse (arrow) with a MOR+ soma (M-So; neuron EM03-1 of Table 1); the MOR-ir was only associated with the Golgi apparatus in this soma, and is not seen in the field shown. To the right of the axon terminal is a glial process with several granules of reaction product. (B) Electron micrograph of an unlabeled axon terminal (U-t) forming an "invaginated" contact with a MOR+ soma (M-So; neuron EM05-1 of Table 1). There are contacts (arrows) on both sides of the invagination that appear to be obliquely-sectioned symmetrical synapses, identified as such due to their association with clusters of synaptic vesicles directly beneath the contacts, and the presence of densities that appear to be of the intraleft type. Scale bar = 0.5μm for both A and B.

Fig. 9.

Histogram showing the number of symmetrical (black) or asymmetrical (dark gray) synapses of MOR+ terminals with various MOR+ (+) or MOR-negative (-) structures. So: somata; LD: large-caliber dendrites ($\geq 1\mu\text{m}$); SD: small-caliber dendrites ($<1\mu\text{m}$); Sp: spines. See Table 2 for exact numerical counts.

Table 1

Number of synaptic inputs and types of synapses (symmetrical versus asymmetrical) of MOR+ and MOR- axon terminals onto MOR+ and MOR- somata.

Somata		Symmetrical Synapses		Asymmetrical Synapses	
Neuron #	Neuron Type	Type of Somatic MOR-ir	MOR+ Terminals	MOR- Terminals	MOR- Terminals
EM03-1	PN	Golgi Apparatus	3	2	0
EM03-2	PN	None (MOR-)	1	2	0
EM05-1	PN	Golgi Apparatus	0	5 ²	0
EM05-2	PN	None (MOR-)	3	9	0
PN Total			7	18	0
EM09-1	IN	Golgi Apparatus	1	2	4
EM09-2	IN?	Diffuse	0	2	0
IN Total			1	4	4

¹ There were only 2 synapses with the soma of neuron EM09-2 in the thin sections available for analysis. Although both were symmetrical this neuron was considered a candidate interneuron because it contained diffuse perikaryal MOR-ir typical of interneurons seen at the light microscopic level.

² Two of the five MOR-negative terminals forming symmetrical synapses made invaginating contacts (see Fig. 8B).

Table 2

Postsynaptic targets of MOR+ terminals in the BLA.

Synapse Type of MOR+ Terminals	MOR+ Postsynaptic Structures				MOR- Postsynaptic Structures			
	So+	LD+	SD+	Sp+	So-	LD-	SD-	Sp-
Asymmetrical	1 (0.6%)	2 (1.2%)	11 (6.8%)	20 (12.3%)	0 (0%)	3 (1.9%)	8 (4.9%)	117 (72.3%)
Symmetrical	4 (10.0%)	7 (17.5%)	14 (35.0%)	5 (12.5%)	4 (10.0%)	0 (0%)	2 (4.9%)	4 (10.0%)

Abbreviations: So+: MOR+ somata; LD+: MOR+ large dendrites (diameter >1µm); SD+: MOR+ small dendrites (diameter <1µm); Sp+: MOR+ spines; So-: unlabeled somata; LD-: unlabeled large dendrite (diameter >1µm); SD-: unlabeled small dendrite (diameter <1µm); Sp-: unlabeled spine.