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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 (PNs) and interneurons. Some of the MOR-positive (MOR+) dendrites were spiny, suggesting that they belonged to PNs, 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 PNs, allowing only strong inputs or synchronous inputs to influence pyramidal neuronal firing.

Key words: mu opioid receptor, basolateral amygdala, immunohistochemistry, electron microscopy, pyramidal neurons, interneurons.

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, 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 (250–350 g; Harlan, Indianapolis, IN, USA) were used in this study. Three rats were used for light microscopy and three rats were used for electron microscopy. All experiments were carried

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Abbreviations: BLa, basolateral nucleus; BLC, basolateral nuclear complex; CCK+, cholecystokinin-positive; DAB, diaminobenzidine; DOR, delta opioid receptor; KOR, kappa opioid receptor; MOR, mu opioid receptor; MOR+, MOR-positive; MOR-ir, MOR-immunoreactivity; PAG, periaqueductal gray; PB, phosphate buffer; PBS, phosphate-buffered saline; PV+, parvalbumin-positive.

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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 (85 mg/kg), xylazine (8 mg/kg), and acepromazine (4 mg/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 min, followed by 2% paraformaldehyde in PB for 20 min. 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 1 h. Brains were sectioned on a vibratome in the coronal plane at 50 μm for light microscopy and 60 μm 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 h. 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, USA). 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 min 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, USA) and a Vectastain Standard ABC kit (Vector Laboratories, Burlingame, CA, USA). MOR-immunoreactivity (MOR-ir) 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 two 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 1 h, dehydrated in ethanol and acetone, and flat embedded in Polybed 812 (Polysciences, Warrington, PA, USA) in slide molds between sheets of Aclar (Ted Pella, Redding, CA, USA). 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, USA).

Ultrastructural analysis

One representative vibratome section from each of the three 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 ($\geqslant 1$ μm), small-caliber dendritic shafts (< 1 μm), 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 2384 MOR+ profiles.

The BLa contains pyramidal projection neurons (PNs) and nonpyramidal interneurons that are very similar to those in the cerebral cortex (McDonald, 1992a; McDonald, 2003; Sah et al., 2003; Pape and Pare, 2010; Spampanato 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.