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ULTRASTRUCTURAL RELATIONSHIP BETWEEN *N*-METHYL-D-ASPARTATE-NR1 RECEPTOR SUBUNIT AND MU-OPIOID RECEPTOR IN THE MOUSE CENTRAL NUCLEUS OF THE AMYGDALA

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Abstract—The central nucleus of the amygdala (CeA) is an important neuroanatomical substrate of emotional processes that are critically involved in addictive behaviors. Glutamate and opioid systems in the CeA play significant roles in neural plasticity and addictive processes, however the cellular sites of interaction between agonists of *N*-methyl-D-aspartate (NMDA) and μ-opioid receptors (μOR) in the CeA are unknown. Dual labeling immunocytochemistry was used to determine the ultrastructural relationship between the essential NMDA-NR1 receptor subunit and μOR in the CeA. It was found that over 80% of NR1-labeled profiles were dendrites while less than 10% were axons. In the case of μOR-labeled profiles, approximately 60% were dendritic, and over 35% were axons. Despite their somewhat distinctive patterns of cellular location, numerous dual-labeled profiles were observed. Approximately 80% of these were dendritic, and less than 10% were axonal. Moreover, many dual-labeled dendritic profiles were contacted by axon terminals receiving asymmetric-type synapses indicative of excitatory signaling. These results indicate that NMDA and μORs are strategically localized in dendrites, including those receiving excitatory synapses, of central amygdala neurons. Thus, postsynaptic co-modulation of central amygdala neurons may be a key cellular substrate mediating glutamate and opioid interaction on neural signaling and plasticity associated with normal and pathological emotional processes associated with addictive behaviors.

Key words: addiction, glutamate, opioids, synaptic plasticity.

A critical coordinator of emotion and behavior (Lang and Davis, 2006), the central nucleus of the amygdala (CeA) is also emerging as a key neuroanatomical substrate of substance abuse (Koob, 2008). Glutamate transmission in the central amygdala may play an essential role in these processes. The CeA receives sensory information via glutamatergic afferents from areas of the thalamus, prefrontal cortex, and basolateral nucleus of the amygdala (BLA), as well as information from memory systems in the hippocampal formation. The CeA integrates these signals, and in turn modulates the activity of brain areas in the extended amygdala, hypothalamus, and brain stem that are involved in autonomic, endocrine, and behavioral processes (LeDoux, 2000; Knapska et al., 2007).

Many of the important actions of glutamate in the CeA are mediated by the ionotropic glutamate receptors, particularly the *N*-methyl-D-aspartate (NMDA) receptor subtype. NMDA receptors are tetrameric heteromers formed by the essential NMDA-NR1 (NR1) receptor subunit along with NMDA-NR2 subunits (Dingledine et al., 1999). Neurons in the CeA express the NR1 gene (Sato et al., 1995) and protein (Petralia et al., 1994), as well as NMDA ligand binding sites (Monaghan and Cotman, 1985). A critical feature of the NMDA receptor is its high permeability to Ca^{2+} and ability to activate numerous intracellular signaling cascades (Dingledine et al., 1999). These properties appear to have profound effects on cellular and behavioral plasticity (Tsien, 2000), including long-term potentiation (LTP) and long-term depression (LTD) (Kullmann et al., 2000), as well as spatial memory (Shapiro and Eichenbaum, 1999), and emotional learning (Walker and Davis, 2002), respectively. Behavioral neuropharmacological evidence also indicates that NMDA receptors in the CeA play an important role in emotional learning and memory. For example, blockade of NMDA receptors in the CeA attenuates conditioned instrumental learning (Andrzejewski et al., 2004), as well as acquisition of conditioned auditory or contextual fear (Goosens and Maren, 2003).

The central amygdala may be an important neuroanatomical substrate for NMDA and μ-opioid receptor (μOR) interactions in opioid addictive behaviors. The CeA contains intrinsic neurons and axon terminals that contain opioid peptides (Fallon and Leslie, 1986; Cassell and Gray, 1989; Poulin et al., 2006). Moreover, the μOR is expressed in neurons in the CeA (Mansour et al., 1988, 1995; Poulin et al., 2006). Pharmacological antagonists of NMDA receptors (Watanabe et al., 2002), as well as spatial-temporal deletion of NR1 (Glass et al., 2008) have been shown to inhibit the conditioned aversive properties of opioid withdrawal. Although glutamate and opioid signaling in the amygdala have important roles in behavioral processes, the synaptic organization of NMDA and mu-opioid receptors in the CeA is unclear.

Electrophysiological studies suggest that stimulation of NMDA and mu-opioid receptors can have significant interactive effects on neuronal activity in the central amygdala.

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Abbreviations: Acb, nucleus accumbens; BLA, basolateral nucleus of the amygdala; BSA, bovine serum albumin; CeA, central nucleus of the amygdala; EPSP, excitatory postsynaptic potential; GC, Golgi complex; IgG, immunoglobulin G; LTP, long-term potentiation; NMDA, *N*-methyl-D-aspartate; NR1, NMDA-NR1 subunit; PB, phosphate buffer; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; μOR, mu-opioid receptor.

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There are distinct classes of CeA neurons, one of the major types being low threshold bursting neurons (Schiess et al., 1999; Dumont et al., 2002; Zhu and Pan, 2004; Chieng et al., 2006). Most of these neurons are inhibited by μOR agonists, while a subpopulation of these neurons is excited by NMDA receptor activation (Zhu and Pan, 2004; Chieng et al., 2006). Some evidence indicates that μOR activation has direct postsynaptic inhibitory effects on excitatory postsynaptic signaling in CeA neurons, possibly involving G-protein-regulated inwardly rectifying potassium (GIRK) channels (Zhu and Pan, 2004). Alternatively, activation of μOR has also been shown to decrease spontaneous excitatory postsynaptic potentials (EPSPs) without affecting EPSP amplitude, suggesting that opioid receptor activation inhibits presynaptic glutamate release, possibly involving phospholipase A2–activated AP-4-sensitive potassium channels (Zhu and Pan, 2005). Similar ambiguity exists with respect to the synaptic mechanisms of NMDA receptor–mediated neural plasticity (Shindou et al., 1993) in the CeA. Thalamic stimulation produces LTP in CeA neurons, which may be dependent on presynaptic NMDA receptors (Samson and Paré, 2005). However, it has also been shown that corticotropin releasing factor (CRF)–dependent LTP in the BLA–CeA pathway is potentiated 2 weeks after cocaine withdrawal, a process that may involve postsynaptic NMDA receptors (Pollandt et al., 2006). Thus, the electrophysiological studies suggest diverse and competing synaptic models of NMDA and μOR signaling. These include the presence of μOR and/or NMDA receptors on functional plasma membrane sites in somatodendritic profiles contacted by excitatory axon terminals, or exclusively on excitatory axon terminals. To date, however, there is no direct ultrastructural evidence that would discriminate between these possibilities.

The exquisite spatial resolution provided by electron microscopic immunocytochemistry provides a powerful and unique tool to test specific synaptic models of interaction between related receptor systems. This can be achieved within the framework of dual labeling immunocytochemistry employing visually distinct immunoperoxidase and immunogold markers. Thus, we used this approach to characterize the ultrastructural relationship between NMDA and mu-opioid receptors in the CeA. Given the significance of genetic models involving the mouse in neurobiological studies of addiction and other psychiatric syndromes involving the amygdala (Glass et al., 2008), the analysis was performed in the CeA of this species.

EXPERIMENTAL PROCEDURES

Subjects

The experimental protocols 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 Care and Use Committees at Weill Medical College of Cornell University. All efforts were made to minimize the number of animals used and their suffering. Male C57/BL/6 mice ($n=9$) weighing 20–30 g were housed in groups of three to five animals per cage and maintained on a 12-h light/dark cycle (lights out 18:00 h). All mice had unlimited access to water and rat chow in their home cages.

Tissue preparation

Mice were anesthetized with pentobarbital (150 mg/kg i.p.), and their brains were fixed by aortic arch perfusion sequentially with: (a) 15 ml of normal saline (0.9%) containing 1000 U/ml of heparin, (b) 40 ml of 3.75% acrolein in 2% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4), and (c) 100 ml of 2% paraformaldehyde in PB, all delivered at a flow rate of 100 ml/min. The brains were removed and post-fixed for 30 min in 2% paraformaldehyde in PB. Coronal sections (40 μm) from the forebrain at the level of the CeA, according to the atlas of Hof et al. (2000), were cut with a vibrating microtome. Tissue sections were next treated with 1.0% sodium borohydride in PB and washed in PB. To enhance tissue permeability, sections were immersed in a cryoprotectant solution (25% sucrose and 2.5% glycerol in 0.05 M PB) for 15 min, followed by freeze–thawing in liquid Freon and liquid nitrogen. Sections were next rinsed in 0.1 M Tris-buffered saline (TBS, pH 7.6) and then incubated for 30 min in 0.5% bovine serum albumin (BSA) to minimize nonspecific labeling.

Antisera and dual labeling immunocytochemical procedures

Brain sections containing the amygdala were processed for dual labeling of NR1 and μOR using polyclonal rabbit (cat #: AB1516, lot #: 0611044491) and guinea-pig (cat #: AB1774, lot #: 0507003792) antipeptide antisera, respectively (Chemicon, Temecula, CA, USA). Immunohistochemical detection of the NR1 antiserum corresponds to sites of NMDA labeling found with receptor autoradiography, and its specificity has been determined by Western immunoassay and adsorption controls (Aicher et al., 1999). Moreover, amygdala NR1 labeling with this antiserum is significantly reduced following conditional NR1 deletion in the CeA (Glass et al., 2008). The μOR antiserum was raised against amino acids 384–398 of the cloned rat μOR. Immunolabeling of this receptor is abolished by preadsorption with the antigenic peptide (Drake and Milner, 2002), and significantly attenuated in mice with a knockout of exon 1, 2/3, or 11 of the μOR gene, respectively (Jaferi and Pickel, 2009).

Tissue sections were processed for dual labeling immunocytochemistry as previously described (Leranth and Pickel, 1989; Chan et al., 1990). Briefly, sections were incubated for 48 h in a primary antiserum cocktail including NR1 (peroxidase: 1:400; gold: 1:100) and μOR (peroxidase: 1:400; gold: 1:100), where peroxidase and gold markers were reversed in adjacent brain sections. After incubation, separate sections were rinsed in TBS and prepared first for peroxidase identification. Sections were incubated in anti-rabbit or anti-guinea-pig immunoglobulin G (IgG) conjugated to biotin, rinsed in TBS, and then incubated for 30 min in avidin–biotin–peroxidase complex (1:100, Vectastain Elite Kit, Vector Laboratories, Burlingame, CA, USA) in TBS. The bound peroxidase was visualized by reaction for 5–6 min in a 0.2% solution of 3,3′-diaminobenzidine and 0.003% hydrogen peroxide in TBS, followed by several washes in TBS. In preparation for immunogold labeling, sections were rinsed in 0.01 M phosphate-buffered saline (PBS) (pH 7.4), and blocked for 10 min in 0.8% BSA and 0.1% gelatin in PBS to reduce non-specific binding of gold particles. Sections then were incubated for 2 h in anti-guinea-pig or anti-rabbit IgG conjugated with 1 nm gold particles (1:50, AuroProbeOne, Amersham, Arlington Heights, IL, USA), then rinsed in 0.5% BSA and 0.1% gelatin in PBS, and then PBS. In order to investigate possible cross-reactivity, tissue was processed with omission of one or the other primary antisera followed by incubation with the secondary antisera corresponding to the alternate species. As previously shown, there was little detectable