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Research Report

Distribution of NMDA and AMPA receptor subunits at thalamo-amygdaloid dendritic spines

Jason J. Radley^{a,d,*}, Claudia R. Farb^c, Yong He^{a,d}, William G.M. Janssen^a,
Sarina M. Rodrigues^c, Luke R. Johnson^{c,d}, Patrick R. Hof^{a,b,d},
Joseph E. LeDoux^{c,d}, John H. Morrison^{a,b,d}

^aDepartment of Neuroscience, Mount Sinai School of Medicine, New York, NY 10029, USA^bDepartment of Geriatrics and Adult Development, Mount Sinai School of Medicine, New York, NY 10029, USA^cCenter for Neural Science, New York University, New York, NY 10003, USA^dNIMH Center for the Neurobiology of Fear and Anxiety, New York, NY, USA

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ABSTRACT

Synapses onto dendritic spines in the lateral amygdala formed by afferents from the auditory thalamus represent a site of plasticity in Pavlovian fear conditioning. Previous work has demonstrated that thalamic afferents synapse onto LA spines expressing glutamate receptor (GluR) subunits, but the GluR subunit distribution at the synapse and within the cytoplasm has not been characterized. Therefore, we performed a quantitative analysis for α -amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptor subunits GluR2 and GluR3 and N-methyl-D-aspartate (NMDA) receptor subunits NR1 and NR2B by combining anterograde labeling of thalamo-amygdaloid afferents with postembedding immunoelectron microscopy for the GluRs in adult rats. A high percentage of thalamo-amygdaloid spines was immunoreactive for GluR2 (80%), GluR3 (83%), and NR1 (83%), while a smaller proportion of spines expressed NR2B (59%). To compare across the various subunits, the cytoplasmic to synaptic ratios of GluRs were measured within thalamo-amygdaloid spines. Analyses revealed that the cytoplasmic pool of GluR2 receptors was twice as large compared to the GluR3, NR1, and NR2B subunits. Our data also show that in the adult brain, the NR2B subunit is expressed in the majority of in thalamo-amygdaloid spines and that within these spines, the various GluRs are differentially distributed between synaptic and non-synaptic sites. The prevalence of the NR2B subunit in thalamo-amygdaloid spines provides morphological evidence supporting its role in the fear conditioning circuit while the differential distribution of the GluR subtypes may reflect distinct roles for their involvement in this circuitry and synaptic plasticity.

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* Corresponding author. Laboratory of Neuronal Structure and Function, Salk Institute for Biological Studies, La Jolla, CA 92037, USA. Fax: +1 858 4538104.

E-mail address: radley@salk.edu (J.J. Radley).

Abbreviations: B, basal nucleus of the amygdala; Ce, central nucleus of the amygdala; ec, external capsule; LA, lateral nucleus of the amygdala; MGd, medial geniculate, dorsal division; MGm, medial geniculate, medial division; MGv, medial geniculate, ventral division; PIN, posterior intralaminar nucleus; SG, supragenulate body

1. Introduction

In recent years, considerable progress has been made in elucidating the neural circuits that underlie Pavlovian fear conditioning, a behavioral task in which a neutral conditioned stimulus (CS), often an auditory tone, acquires the ability to elicit fear responses following its association with an aversive unconditioned stimulus (US), typically footshock. Evidence from different kinds of studies points to the lateral amygdala (LA) as an important site of plasticity in auditory fear conditioning (LeDoux, 2000; Maren, 2000; Sah et al., 2003; Tsvetkov et al., 2004). The LA is the primary recipient of convergent inputs from auditory thalamic and neocortical areas that process the CS. Lesions of the LA, or pharmacological disruption of neural activity in this region, prevent fear conditioning (LeDoux et al., 1990; Nader et al., 2001; Wilensky et al., 1999, 2000; Bailey et al., 1999). Neurons in LA are responsive to both CS and US (Romanski et al., 1993) and their responses to the CS changes after CS-US pairing (Quirk et al., 1995; Rogan et al., 1997; McKernan and Shinnick-Gallagher, 1997; Repa et al., 2001; Pare and Collins, 2000; Goosens and Maren, 2002). Moreover, induction of long-term potentiation by electrical stimulation of the auditory thalamus enhances CS-evoked responses in LA (Rogan and LeDoux, 1995). Together, these findings suggest that thalamo-amygdaloid synapses are facilitated in response to Pavlovian fear conditioning (LeDoux, 2000; Maren, 2001).

Auditory thalamic inputs into LA exert their effects through the postsynaptic glutamate *N*-methyl-*D*-aspartate (NMDA) and α -amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptors (Li et al., 1995; Farb and LeDoux, 1997; Humeau et al., 2003). Blockade of NMDA receptors in LA prevents fear conditioning (Miserendino et al., 1990; Walker and Davis, 2000; Rodrigues et al., 2001), while facilitation of AMPA receptors, and in turn NMDA receptors, enhances fear conditioning (Rogan et al., 1997).

Increasing evidence suggests that NMDA and AMPA receptors are capable of modulating synaptic plasticity through shifts in their receptor subunit stoichiometry (Shi et al., 2001; Rumpel et al., 2005; Barria and Malinow, 2005). While some information has been gleaned regarding the glutamate receptor subunit composition in identified auditory pathways to LA (Farb and LeDoux, 1997, 1999; Rodrigues et al., 2001; Mead et al., 2006), the subcellular organization of glutamate receptors at thalamo-amygdaloid synapses is not known. Therefore, the aim of the present study was to characterize and quantify the synaptic and non-synaptic distribution of the GluR2, GluR3, NR1, and NR2B glutamate receptor (GluR) subunits within dendritic spines that receive synapses from the auditory thalamus. Since receptor trafficking is a candidate mechanism underlying memory formation (Shi et al., 2001; Baudry et al., 1980), differences between the cytoplasmic and synaptic distribution of these GluR subtypes may reflect distinct roles in synaptic plasticity. Thalamic afferents to the LA were identified by anterograde tracer injection of biotinylated dextran amine (BDA) into the auditory thalamus (medial subdivision of the medial geniculate nucleus and posterior intralaminar nucleus, MGm/PIN) and the GluRs were visualized using postembedding immunogold.

2. Results

2.1. Thalamic inputs to LA

Injections of BDA into MGm/PIN produced labeling of cell bodies, axons, and processes at the injection site and in adjacent locally projecting regions (Figs. 1A, B). A dense plexus of anterogradely labeled BDA fibers was evident throughout the LA, particularly within the dorsal LA, and in the striatum and amygdala-striatum transition areas, regions immediately dorsal and medial to the amygdaloid complex. (Fig. 1C). Higher magnification (Fig. 1D) revealed the presence of many labeled fibers and boutons. No labeled cell bodies or dendrites in the LA were observed. Electron microscopic verification of BDA-labeling was performed on ultrathin sections prior to the immunogold analysis. Thalamo-amygdaloid-labeled terminals were comprised of loosely packed clear, round vesicles, and sometimes contained dense-core vesicles. Some BDA-

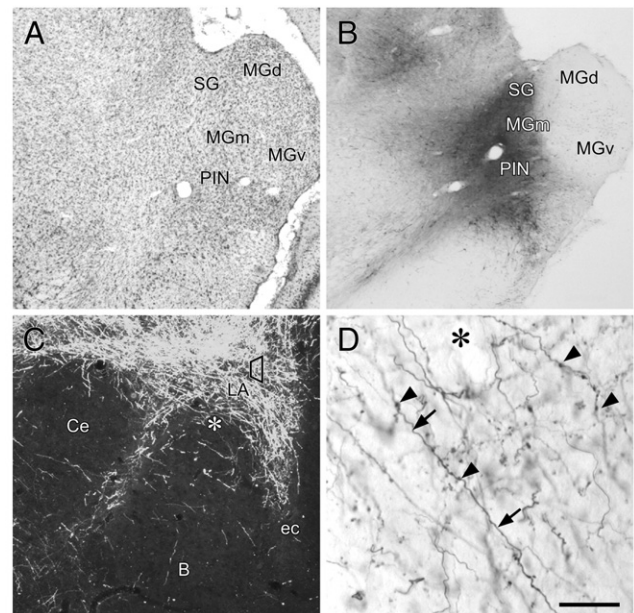


Fig. 1 – Low-power photomicrographs showing BDA injections in the auditory thalamus and corresponding anterograde transport to the amygdala. (A) Nissl-stained coronal section, approximately 6.0 mm caudal to bregma, containing the subregions of the auditory thalamus. (B) Representative BDA injection site into the MGm/PIN. Local anterograde and retrograde transport in nearby regions is also shown. (C) Dark field photomicrograph of anterograde transport to LA following a BDA injection in MGm/PIN. The trapezoid in the LA represents the approximate region sampled for electron microscopic examination. The asterisk corresponds to the region of higher magnification shown in panel D. (D) High-power magnification using Nomarski optics reveals the presence of BDA-labeled axons (arrows) and boutons (arrowheads) in the LA. Cell bodies and dendrites are not seen in the LA. Asterisk denotes region shown at lower power in panel C. Scale bar = 500 μ m in panels A and B, 100 μ m in panel C, and 25 μ m in panel D.

labeled terminals did not have any identifiable postsynaptic contacts or made synapses that were not obviously asymmetric (i.e., thin postsynaptic density). Since serial section analysis (unpublished observations) revealed that thalamo-amygdaloid terminals with these characteristics often form asymmetric synapses in other planes of sectioning, our analysis was restricted to those labeled terminals that formed asymmetric synapses. We analyzed 526 BDA-labeled terminals and found that the vast majority (74%; 391/526) of these formed synapses onto spines. The remaining labeled terminals (26%; 135/526) formed asymmetric synapses onto small and large dendritic shafts. These findings are consistent with previous observations about thalamo-amygdaloid synapses (LeDoux et al., 1991).

2.2. Distribution of glutamate receptors at thalamic auditory spines in LA

Given that the vast majority of thalamic afferents to the LA target dendritic spines, the quantitative analysis for GluR subunits in this study was limited to thalamo-amygdaloid spines with identifiable synaptic structures, as previously defined. Thus, within a randomly selected grid square, every BDA-labeled terminal forming an asymmetric synapse (Gray's Type I) onto a spine was analyzed. Spines were considered to be GluR labeled if they contained two or more gold particles within its cytoplasm or within 60 nm of the PSD or synaptic cleft. The pattern of immunogold localization within thalamo-amygdaloid spines was similar for NR1, NR2B, and GluR3, with the most abundant labeling in compartments within or subjacent to the synaptic cleft and postsynaptic density (Fig. 2A) and less labeling within the cytoplasmic compartment. By contrast, GluR2 had a higher proportion of immunogold labeling in the cytoplasmic compartment than in the synaptic compartment. Though GluR subunits are usually associated with endomembranous structures, damage caused by the postembedding immunogold processing and the high sensitivity of endomembranes to such treatment prevented us from observing this relationship.

The compartmental distribution of immunogold labeling for each subunit in thalamo-amygdaloid spines is shown in

Fig. 2 – (A) Electron micrographs show thalamic afferents labeled with BDA form asymmetric synapses onto LA spines that contain immunogold labeling (arrows) for glutamate receptor (GluR) subunits. Scale bar=0.25 μ m. (B) Histogram showing the percentages of thalamo-amygdaloid spines that contained immunogold labeling for each GluR subunit examined. The vast majority of these spines were immunopositive for the glutamate receptors GluR2, GluR3, and NMDAR1 (>80%), while fewer spines expressed NR2B (59%). (C) Histogram showing the cytoplasmic: synaptic ratios for each subunit in thalamo-amygdaloid spines. The ratio of cytoplasmic: synaptic labeling was 2.2-fold higher for GluR2 than for GluR3, NR1, and NR2B. The inset illustrates how the synaptic bins were established for the assignment of gold particle locations in the spines analyzed. Asterisks in panels B and C denote a significant difference from the other 3 subunits; $P<0.001$.

Table 1. Across four animals, a total of 701 spines were quantified, such that an average of 175 spines per subunit was counted. For each animal, approximately 44 thalamo-amygdaloid spines per subunit were examined. The proportion of thalamo-amygdaloid spines in LA expressing immunogold-labeling was $82.9\pm 1.3\%$ (136/164) for GluR2, $80.1\pm 0.6\%$ (157/196) for GluR3, and $82.8\pm 1.0\%$ for NR1 (101/122). By contrast, a significantly smaller proportion of spines expressed the NR2B

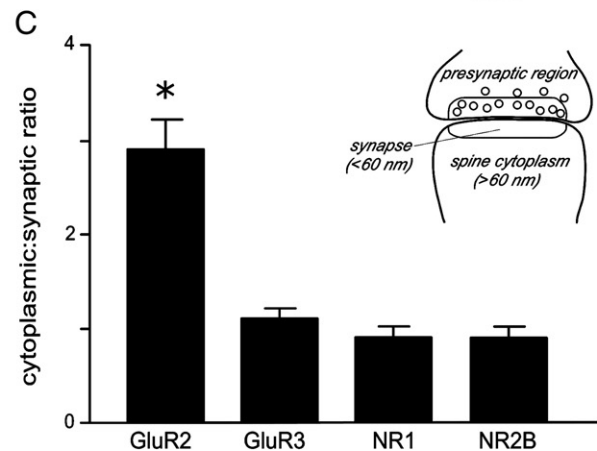
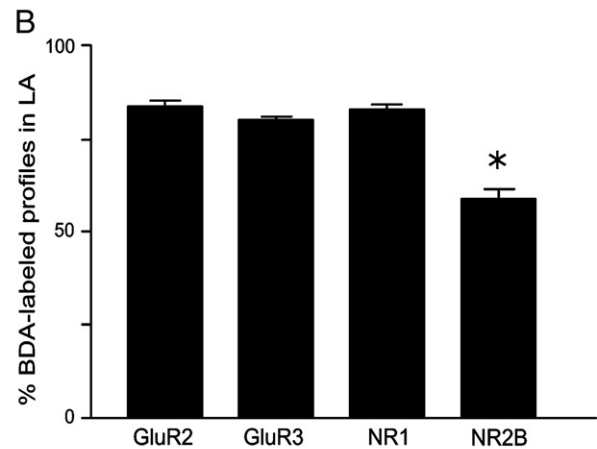
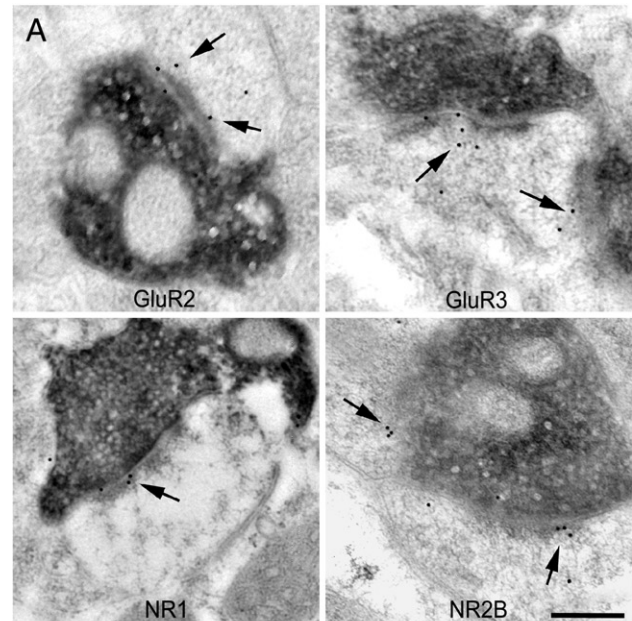


Table 1 – Mean values for each compartment analyzed in thalamo-amygdaloid axospinous synapses

Bin description	GluR2	GluR3	NR1	NR2B
Percentage of BDA+immunogold labeling	83.5	80.1	82.3	58.9*
Postsynaptic				
Synaptic (cleft+0–60 nm)	1.76	2.38	1.93	2.29
Spine cytoplasmic (>60 nm)	3.86	2.68	1.79	2.00
Cytoplasmic: synaptic ratio	2.2*	1.1	0.9	0.9
Presynaptic				
0–60 nm	0.17	0.34	0.10	0.20
>60 nm	0.46	0.11	0.46	0.36

Asterisk indicates significant difference ($P < 0.01$); comparisons made across subunits.

subunit, $58.9 \pm 2.5\%$ (128/219), as compared to the other GluR subunits examined ($F_{(3,12)} = 58.3$, $P < 0.0001$; Fig. 2B). Examination of the cytoplasmic: synaptic gold particle ratios within thalamo-amygdaloid spines revealed the largest difference with the GluR2 subunit, 2.2:1 (2.2 ± 0.2 ; $F_{(3,12)} = 13.1$; $P < 0.0005$), compared to GluR3, 1.1:1 (1.1 ± 0.1 ; $P < 0.001$), NR1 0.9:1 (0.9 ± 0.1 ; $P < 0.001$), and NR2B 0.9:1 (0.9 ± 0.1 ; $P < 0.001$; Fig. 2C). In all cases, immunogold particles were also occasionally located presynaptically (Table 1).

3. Discussion

This is the first study that characterizes the ultrastructural distribution of the glutamate receptor subunits GluR2, GluR3, NR1, and NR2B within thalamo-amygdaloid spines. Our results show that within the LA: (1) the GluR subunits GluR2, GluR3, NR1, and NR2B are present in the majority spines that are postsynaptic to auditory thalamic afferents; (2) NR2B was present in 59% of thalamo-amygdaloid spines, a value that is considerably larger than expected based on studies in the rodent forebrain (Monyer et al., 1994; Lopez de Armentia and Sah, 2003); (3) twice as much GluR2 is expressed in the spine cytoplasm as compared to the synapse. This ratio is two times greater than that of the GluR3, NR1, and NR2B subunits, where approximately the same number of receptors is present in the cytoplasm as compared to the synapse. As discussed below, the prevalence of NMDA receptor subunits in synapses is consistent with past anatomical and physiological studies demonstrating the involvement of both AMPA and NMDA receptors in synaptic transmission in the thalamo-amygdaloid pathway. Further, the prevalence of the NR2B subunit within thalamo-amygdaloid spines in adult rats is consistent with the emerging view that NR2B subunits are expressed in adult as well as young animals. These findings also support the idea that cytoplasmic pools of AMPA receptors retained in thalamo-amygdaloid spines may be available for insertion into the synapse relative to NMDA receptors.

NMDA receptors are heteromeric assemblies composed of NR1 subunits in combination with at least one form of NR2 (A–D). Our observation that NR1 is present in a larger proportion of thalamo-amygdaloid spines (83%) than NR2B (59%) is

consistent with its role as an obligatory subunit in the receptor heteromer. The role of NR2B is of particular interest in the thalamo-amygdaloid circuit, since the infusion of its antagonist into LA has been shown to block the acquisition of fear conditioning (Rodrigues et al., 2001) and synaptic plasticity (Bauer et al., 2002). The NR2B subunit has a smaller time constant than NR2A and NR2C subunits (Vicini et al., 1998), resulting in a slower attenuation of excitatory postsynaptic currents (Monyer et al., 1994). In addition, the NR2A–NR2B complex is characterized by a slower activation and deactivation and longer rise and decay time course than the NR1–NR2A complex (Chen et al., 1999). This conductive property of NR2B might provide a means for coincidence detection in the enhancement of synaptic efficacy (Tsien, 2000) and may play an integral role in learning and plasticity.

A number of reports have shown that the NR2B subunit mRNA and protein are expressed at peak levels neonatally in the forebrain, and then decline throughout development (Monyer et al., 1994; Lopez de Armentia and Sah, 2003), presumably replaced by NR2A subunit (Vicini et al., 1998). Our results, showing that 59% of thalamo-amygdaloid spines are positive for NR2B, are in agreement with past studies showing that the NR2B subunit is maintained into adulthood in LA (Rodrigues et al., 2000), and other brain regions (Jin et al., 1997; Charton et al., 1999; Khan et al., 2000), and provide anatomical evidence that is consistent with behavioral and physiological findings for the role of NR2B in fear learning. Direct infusion of an NR2B antagonist into LA disrupts the acquisition of fear conditioning *in vivo* (Rodrigues et al., 2001) and blocks LTP produced by prolonged tetanic stimulation *in vitro* (Bauer et al., 2002). Blocking NR2B receptors *in vitro* also decreases the response properties of LA pyramidal neurons and reduces EPSCs in lateral amygdala but not hippocampus (Lopez de Armentia and Sah, 2003). Collectively, these data support the possibility that the role of the NR2B subunit in the LA in the adult brain has been hitherto underestimated.

The receptor subunit stoichiometry and composition of AMPA receptors is more heterogeneous than for NMDA receptors, giving rise to a more complex set of functional properties that may also be relevant for LA plasticity and fear conditioning. One prominent feature of AMPA receptors is that they are dynamically regulated, undergoing frequent insertion into, and removal from synapses (Luscher et al., 1999; Shi et al., 2001; Kauer and Malenka, 2006). Several studies suggest that a maximum of two receptor subunits may be present in the AMPA receptor tetrameric complex (Ayala and Stern-Bach, 2001; Mansour et al., 2001; Greger et al., 2003). These are comprised either of GluR2/3 or GluR1/2, together with a small proportion of GluR1 homomeric receptors (Wenthold et al., 1996; Shi et al., 2001). Whereas GluR2/3-containing AMPA receptors are important for their constitutive expression and cycling (Shi et al., 2001), trafficking of GluR1-containing AMPA receptors into activated synapses has been shown to underlie increases in synaptic efficacy (Shi et al., 1999, 2001). Recent evidence suggests that GluR1 receptor trafficking into LA synapses may underlie auditory fear conditioning (Rumpel et al., 2005). Nonetheless, these findings do not necessarily preclude a role of GluR2 in plasticity in learning, as studies using GluR2-knockout mice

demonstrated an essential role for the GluR2 subunit in several different amygdala-dependent operant conditioning tasks (Mead and Stephens, 2003; Mead et al., 2006). In this context, our data showing that GluR2 and GluR3 have a spine cytoplasmic to synaptic ratio of 2:1 and 1:1, respectively, suggest that GluR3 may form a complex with a subpopulation of total GluR2, leaving the remaining pool of spine cytoplasmic GluR2 unassembled. Thus, while the entire population of GluR2 may be representative of both types of AMPA receptors, which are inserted in an activity-dependent and constitutive manner, the GluR3 immunogold profile may represent GluR2/3 AMPA receptors that are constitutively cycled. Related ongoing studies investigating the synaptic distribution of GluR1 in thalamo-amygdaloid spines will help to further clarify this issue.

4. Conclusions

Our anatomical findings provide a framework with which to understand the role that different glutamate receptor subunits play in synaptic plasticity and learning. Our results are consistent with the ideas that the NR2B subunit is involved in learning, while differences between the cytoplasmic and synaptic distribution of these GluR subtypes may reflect distinct roles for their involvement in synaptic plasticity and fear conditioning.

5. Experimental procedures

5.1. Animals

All procedures related to the care and treatment of animals were conducted in accordance with the National Institutes of Health *Guidelines for the Care and Use of Experimental Animals* using protocols approved by the Institutional Animal Care and Use Committee at Mount Sinai School of Medicine and New York University. Male Sprague–Dawley rats (Hilltop Laboratories, Scottsdale, PA) weighing 300–400 g were housed on an alternating 12-h light/dark cycle (lights on 7 AM, lights off 7 PM).

5.2. BDA injections

Animals ($n=4$) were anesthetized with a combination of ketamine (Ketaset, 120 mg/kg, i.p.), xylazine (Xyla-Jet, 6.0 mg/kg, i.p.), and medetomidine hydrochloride (Domitor, 0.5 mg/kg, i.p) and placed in a stereotaxic apparatus. Lysine-fixable biotinylated dextran-amine (BDA) (10%, dissolved in 10 mM phosphate buffer, pH 7.25; Molecular Probes, Eugene, OR) or BDA conjugated to tetramethylrhodamine (micro-ruby, Molecular Probes) was iontophoretically delivered (5 μ A pulsed DC current, 7 s on, 7 s off) for 15–20 min to the medial subdivision of the medial geniculate nucleus (MGm) and posterior intralaminar nucleus (PIN) through glass micropipettes (tip diameter 12–15 μ m). Following BDA injections, the wound was closed and animals recovered in the surgery area before returning to the animal facility.

5.3. Fixation and BDA histochemistry

After a 3- to 5-day survival period, animals were deeply anesthetized with sodium pentobarbital (120 mg/kg) and transcardially perfused with 1% paraformaldehyde (PFA) made in phosphate buffer (PB; pH 7.4), followed by a mixture of 0.125% glutaraldehyde and 4% PFA made in PB. Brains were removed, blocked, and postfixed in the same fixative overnight at 4 °C. Blocks were sectioned on a Vibratome (50 μ m), freeze-thawed as previously described (Farb and LeDoux, 1997), and incubated in avidin–biotin horseradish peroxidase substrate (ABC Elite Kit; Vector Laboratories, Burlingame, CA) overnight. The following day, sections were rinsed and reacted with 3,3'-diaminobenzidine tetrachloride (DAB; Sigma-Aldrich, St. Louis, MO) for 10–15 min. To optimize BDA staining, sections were incubated a second time in ABC for 2 h and then reacted with DAB a second time. The LA was identified at low magnification on the light microscope, dissected, and processed for postembedding immunogold.

5.4. Postembedding immunogold

The freeze substitution and low-temperature embedding of the specimens were performed as described previously with several modifications (Chaudhry et al., 1995). Briefly, sections were cryoprotected by immersion in 4% D-glucose, followed by incubations of increasing concentrations of glycerol in PB (10%, 20%, and 30%) and were plunged rapidly into liquid propane (180 °C) cooled by liquid nitrogen in a Universal Cryofixation System KF80 (Reichert-Jung, Vienna, Austria). The samples were immersed in 1.5% uranyl acetate (for en bloc fixation) made in anhydrous methanol (90 °C, 24 h) in a cryosubstitution Automated Freeze Substitution unit (Leica, Vienna, Austria). The temperature was increased in steps of 4 °C/h from 90 °C to 45 °C. The samples were washed with anhydrous methanol and infiltrated with Lowicryl HM20 resin (Electron Microscopy Sciences, Fort Washington, PA) at 45 °C, with a progressive increase in the ratio of resin to methanol for 1 h each, followed with pure Lowicryl overnight. Polymerization was performed with ultraviolet light (360 nm) at 45 °C for 48 h, followed by 24 h at room temperature. Ultrathin sections of the LA were cut by diamond knife on a Reichert-Jung ultramicrotome (Vienna, Austria) and collected on nickel mesh grids. Grids containing the ultrathin sections were initially treated with a saturated solution of sodium hydroxide in absolute ethanol, rinsed, treated in 0.1% sodium borohydride and 50 mM glycine for 5 min, followed by treatment in Tris-buffered saline containing 2% normal human serum for 10 min. The immunogold procedure was carried out by incubating ultrathin sections in primary antibodies to GluR2 (mouse monoclonal, 5 μ g/ml; Pharmin-gen, San Diego, CA; Vissavajhala et al., 1996), GluR3 (mouse monoclonal, 1.0 μ g/ml; Chemicon, Temecula, CA; Moga et al., 2003), NR1 (rabbit polyclonal, 2 μ g/ml; Chemicon, Temecula, CA; Huntley et al., 1997), and NR2B (rabbit polyclonal, 10 μ g/ml; Novus, Littleton, CO; Adams et al., 2004) overnight. On the following day, sections were incubated in 1:40 dilution of either goat anti-mouse IgG conjugated to 10-nm gold particles (GluR2 and GluR3; Electron Microscopy Sciences, Fort, Washington, PA) or goat anti-rabbit IgG conjugated to 10-

nm gold particles (NR1 and NR2B; Electron Microscopy Sciences). Ultrathin sections were counterstained with uranyl acetate and Reynolds lead citrate, and viewed at 80 kV on a Jeol 1200EX electron microscope (Tokyo, Japan). Images were captured at 10,000 \times using the Advantage CCD camera (Advanced Microscopy Techniques Corporation, Danvers, MA). The specificities for these primary antibodies have been previously demonstrated in our laboratory (GluR2, Vissavajhala et al., 1996; GluR3, Moga et al., 2003; NR1, Siegel et al., 1994; NR2B, Adams et al., 2004). In summary, Western blot analyses across a panel of different glutamate receptor subunit rat cDNAs transfected into HEK cells showed that each antibody recognized only a single band corresponding to its predicted molecular weight. When each antibody was preadsorbed to its corresponding protein, staining in brain tissue was abolished. Secondary antibody specificity was established by verifying that no immunogold labeling was observed when the primary antisera were omitted from the immunocytochemical protocol.

5.5. Data analysis

The immunogold particle density and distribution were analyzed using software developed in our laboratory (SynBin; Adams et al., 2001) and is based upon the concept of particle localization relative to its proximity to the pre- and postsynaptic membrane structures (Blackstad et al., 1990; Ruud and Blackstad, 1999). The program analyzes the resulting data map and objectively assigns each gold particle to a specific bin, with bin sizes and synaptic domains established prospectively. The designated bin width was 60 nm from the PSD since this width readily accommodates the theoretical limit of lateral resolution for gold particle localization at the ultrastructural level (He et al., 2000). Thus, for postsynaptic structures, particles located within 60 nm bin of the cleft were considered “synaptic” and those outside the 60-nm bin width were considered “cytoplasmic”. The information from each labeled spine was stored in a database from which the average number of gold particles per bin can be determined.

LA spines were quantified when there was clear visualization and delineation of the classic synaptic structures such as pre- and postsynaptic membranes, a synaptic cleft, presynaptic vesicles, and a postsynaptic density. Thalamo-amygdaloid spines without these features or those that were obliquely cut were excluded from the quantitative analysis. The cytoplasmic to synaptic gold particle ratios within thalamo-amygdaloid spines were computed to allow for the comparison across subunits since this ratio minimizes qualitative differences that may arise from different antibodies (e.g., specificity, epitope location). Statistical comparisons were performed with a one-way analysis of variance followed by a post hoc tests (Bonferroni), and data were expressed as mean \pm SEM.

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