Depolarization of hippocampal neurons induces formation of nonsynaptic NMDA receptor islands resembling nascent postsynaptic densities

Nonsynaptic NMDA receptor islands resemble PSDs

Jung-Hwa Tao-Cheng\(^1\), Rita Azzam\(^1\), Virginia Crocker\(^1\), Christine A Winters\(^2\) and Tom Reese\(^2\)

\(^1\)NINDS Electron Microscopy Facility, National Institute of Neurological Diseases and Stroke, National Institutes of Health, Bethesda, MD, 20892
\(^2\)Laboratory of Neurobiology, National Institute of Neurological Diseases and Stroke, National Institutes of Health, Bethesda, MD, 20892

DOI: 10.1523/ENEURO.0066-15.2015

Received: 16 June 2015
Revised: 15 October 2015
Accepted: 3 November 2015
Published: 18 November 2015


Funding: NINDS intramural funds;

Conflict of Interest: The authors declare no competing financial interests.

NINDS intramural funds.

Corresponding author: Jung-Hwa Tao-Cheng, NIH, bldg. 49, Room 3A50, Bethesda, MA 20892. Tel: 301 496 0579; Email: chengs@ninds.nih.gov

Cite as: eNeuro 2015; 10.1523/ENEURO.0066-15.2015

Alerts: Sign up at eneuro.org/alerts to receive customized email alerts when the fully formatted version of this article is published.
Depolarization of hippocampal neurons induces formation of nonsynaptic NMDA receptor islands resembling nascent postsynaptic densities
Depolarization of hippocampal neurons induces formation of nonsynaptic NMDA receptor islands resembling nascent postsynaptic densities

Abbreviated Title: Nonsynaptic NMDA receptor islands resemble PSDs

Authors: Jung-Hwa Tao-Cheng¹, Rita Azzam¹, Virginia Crocker¹, Christine A Winters², and Tom Reese²

1. NINDS Electron Microscopy Facility, National Institute of Neurological Diseases and Stroke, National Institutes of Health, Bethesda, MD, 20892
2. Laboratory of Neurobiology, National Institute of Neurological Diseases and Stroke, National Institutes of Health, Bethesda, MD, 20892

Corresponding author: Jung-Hwa Tao-Cheng
Address: NIH, bldg. 49, Room 3A50
Bethesda, MA 20892
Tel: 301 496 0579
Email: chengs@ninds.nih.gov

Number of pages: 31, Number of figures: 8, Number of tables: 3
130 words in abstract, 356 words in Introduction, 957 words in Discussion.

ACKNOWLEDGEMENT Funded by NINDS intramural funds. We thank Dr. Ayse Dosemeci for helpful discussions and critical reading of the manuscript.

The authors declare no competing financial interests.
ABSTRACT

Depolarization of neurons in three week-old rat hippocampal cultures promotes a rapid increase in the density of surface NMDA receptors (NR), accompanied by transient formation of nonsynaptic NMDA receptor clusters, or NR islands. Islands exhibit cytoplasmic dense material resembling that at postsynaptic densities (PSD), and contain typical PSD components including MAGUKS, GKAP, Shank, Homer and CaMKII detected by pre-embedding immunogold electron microscopy. In contrast to mature PSDs, islands contain more NMDA than AMPA receptors, and more SAP102 than PSD95, features shared with nascent PSDs in developing synapses. Islands do not appear to be exocytosed or endocytosed directly as preformed packages because neurons lacked intracellular vacuoles containing island-like structures. Islands form and disassemble upon depolarization of neurons on a time scale of 2-3 min, perhaps representing an initial stage in synaptogenesis.

SIGNIFICANCE STATEMENT

Islands of extrasynaptic NMDA receptors populate the plasma membranes of hippocampal neurons. The receptor Islands also contain many typical postsynaptic density proteins and thus resemble nascent PSDs. NMDA receptors appear to be exocytosed only individually or in small groups rather than in concentrated clusters, so islands must form by clustering of individual NMDA receptors already in the neuronal plasma membrane. Additional islands rapidly form and resolve when neurons are depolarized during a 2-3 min window. These findings provide possible insight into one of the mechanisms of synapse formation.
INTRODUCTION

Two types of glutamate receptors (AMPA and NMDA receptors) support most excitatory synaptic transmission at mammalian central nervous system synapses. It is well established that AMPA receptors undergo dynamic, activity-dependent changes in distribution at synapses (Anggono and Huganir, 2012). More recently, activity-dependent plasticity of synaptic (Lau and Zukin, 2007; Rebola et al, 2010; Dupuis et al., 2014) as well as extrasynaptic NMDA receptors (Rao et al., 1997) has been recognized. Extrasynaptic NMDA receptors (NR) carry functional implications different from synaptic ones (Hardingham and Bading, 2010; Papouin and Oliet, 2014), and often form distinctive clusters (Petralia et al., 2010). Based on examination of brain by electron microscopy (EM), these clusters appear as “free PSDs”, “nonsynaptic surface specializations” or “bare densities” (Blue and Parnavelas, 1983; Steward and Falk, 1986; Fiala et al., 1998). Extrasynaptic NR clusters also label for SAP102 and PSD95 (Sans et al., 2000), two scaffold proteins associated with the postsynaptic density (PSD). The present study focuses on the extrasynaptic clusters of NMDA receptors, which we refer to as nonsynaptic NMDA receptor islands, or NR islands, in dissociated hippocampal neuronal cultures where experimental conditions can be easily manipulated. To determine whether neuronal activity affects the formation of NR islands, the numbers of islands are compared under control, high K⁺ and recovery conditions, showing the time course of their formation and disassembly. We also looked for associated evidence of exocytosis and endocytosis indicative of trafficking of NMDA receptors.
Because NR islands include a cytoplasmic dense material resembling that at the PSD, we analyzed their composition by EM immunolabeling for seven PSD-associated proteins, and found that many of these proteins localized to NR islands. The PSD complex is composed of a network of specialized proteins that are involved in signal transmission and modulation (Sheng and Kim, 2011). These proteins have a layered distribution within PSDs in mature synapses (Valtschanoff and Weinberg, 2001) as well as during development (Petralia et al., 2005). In order to determine the degree of similarity between NR islands and PSDs, labeling frequencies, intensities, and the laminar distribution of PSD proteins at islands were compared with those at PSDs.

MATERIALS AND METHODS

1.1 Antibodies

Mouse monoclonal antibodies against NR2B (clone N59/36, at 1:100), SAP102 (clone N19/2 at 1:50), GKAP (clone N127/31 at 1:100) and Shank2 (clone N23B/6 at 1:200) were from NeuroMab (Davis, CA); mouse monoclonal antibody against NR1 (clone R1JHL at 1:100) was from Calbiochem (San Diego, CA); rabbit polyclonal antibody against NR2A/B (catalog # AB1548, at 1:100) was from Chemicon (Temecula, CA); mouse monoclonal antibodies against GluR2 (clone 6C4 at 1:100) and α-CaMKII (clone 6G9(2) at 1:100) were from Millipore (Billerica, MA); rabbit polyclonal antibody raised to residues 290–307 of PSD-95 (at 1:500) was custom made by New England Peptide (Gardener, MA, USA); mouse monoclonal antibody against Homer 1 (clone 2G8, at 1:200) was from Synaptic Systems (Göttingen, Germany).
1.2 Dissociated hippocampal neuronal cultures and treatments

The animal protocol was approved by the NIH Animal Use and Care Committee and conforms to NIH guidelines. Hippocampal cells from 21-day embryonic Sprague-Dawley rats of either sex were dissociated and grown on a feeder layer of glial cells for 19 – 21 days. During experiments, culture dishes were placed on a floating platform in a water bath maintained at 37°C. Control incubation medium was: 124 mM NaCl, 2 mM KCl, 1.24 mM KH₂PO₄, 1.3 mM MgCl₂, 2.5 mM CaCl₂, 30 mM glucose in 25 mM HEPES at pH 7.4. High K⁺ solution contained 90 mM KCl with osmolarity compensated by reducing the concentration of NaCl in control medium. Cell cultures were washed with control medium and treated for 2 min with control or high K⁺ media. For recovery experiments, samples were treated with high K⁺ for 2 min and then washed with control medium (5 times within 2 min), then either fixed at 2-3 min after washout of high K⁺ or left in control medium for 30 min. One set of sister cultures treated with different conditions is counted as one experiment. Cells were fixed with 4% paraformaldehyde (EMS, Fort Washington, PA) in PBS for 30-45 min, and thoroughly washed before immunolabeling.

1.3 Pre-embedding immunogold labeling and electron microscopy

Fixed samples were washed, and most were then blocked and made permeable with 5% normal goat serum and 0.1% saponin in PBS for 30-60 min. Some samples for labeling with the NR1, NR2B and GluR2 antibodies were permeabilized with 50% ethanol for 10 min, and then treated with 5% normal goat serum in PBS for 20-30 min. All steps were carried out at room temperature unless otherwise indicated. Samples
were incubated with primary and secondary antibodies (Nanogold, Nanoprobes, Yaphand, NY) for 1 hr, fixed with 2% glutaraldehyde in PBS for 30 min, then held at 4°C, washed in water, and silver enhanced (HQ kit, Nanoprobes), treated with 0.2% osmium tetroxide in 0.1M phosphate buffer at pH 7.4 for 30 min on ice, en block stained with 0.25-0.5% uranyl acetate in acetate buffer at pH 5.0 for 1 hr at 4°C, dehydrated in graded ethanols, and embedded in epoxy resin. Controls for immunolabeling include omitting the primary antibody or comparison between different primary antibodies for specific labeling for different structural entities.

1.4. Sampling of synapses, nonsynaptic NMDA receptor islands and morphometry

Asymmetric synapses were identified by clusters of synaptic vesicles in the presynaptic terminal, the dense material underneath the postsynaptic membrane, and by the rigidly apposed pre- and post-synaptic membranes forming a synaptic cleft.

Every synaptic profile encountered within randomly selected grid openings was photographed with a bottom-mounted digital CCD camera which collects 2.6 K x 2.6 K pixels (AMT XR-100, Danvers, MA, USA). Only cross-sectioned synaptic profiles with clearly delineated postsynaptic membranes were included for measurement. There was no difference in structure or pattern of labeling by various antibodies between dendritic and somal asymmetric synapses, so measurements were pooled from synapses located on dendrites and somas. Since there were many more dendritic than somal asymmetric synapses, the great majority of synapses sampled here were dendritic. To measure labeling intensity at the PSD, every gold particle that lay within the PSD complex was counted. PSD complexes were outlined by the postsynaptic membrane,
two parallel lines dropped perpendicular to the postsynaptic membrane, and an arbitrary border 120 nm deep to the postsynaptic membrane (cf. Tao-Cheng et al., 2015). Total amount of label within this designated PSD complex was then divided by the length of the PSD as an index of labeling intensity (number of particles / μm length of PSD).

**Nonsynaptic NMDA receptor islands** are defined as patches of neuronal plasma membrane at nonsynaptic locations showing clusters of label for NMDA receptors and displaying a characteristic dense material on the cytoplasmic side of the membrane (Fig. 1).

Islands may be apposed by other cellular processes such as glia, dendrites or axons (Petralia et al., 2010). However, the presence of an apposing axon could indicate a nascent synapse in formation, and the presence of other cellular processes may indicate interactions between the two elements. Thus, in order to study the formation of these islands without the influence of other cellular elements, we limited sampling of islands to those that lacked any apposing elements.

In dissociated hippocampal cultures, neuronal somas are typically plump in shape and situated on top of a layer of glia. The glia cells are flat and spread out on the substrate with their cellular processes intermingled with axons and dendrites. Thus, the surface of neuronal somas facing the culture medium is typically not covered by cellular elements. Thin sections were cut en face near these unapposed surfaces of neuronal somas. No distinction in NMDA receptor distribution between neuronal somal and dendritic plasma membrane was apparent. However, because multiple dendrites can
arise from one soma, we limited sampling of labeling density to somas to assure that each data point was from a different neuron. Every unapposed neuronal somal profile encountered was photographed for measuring the overall labeling intensity of NMDA receptors. The total number of gold particles on somal plasma membrane, including those clustered in islands, was measured and divided by the length of the plasma membrane.

For the same statistical rationale, islands for quantitation were also sampled only from somas. The frequency of islands was measured in two different ways, and the particular method is specified in each figure or table: (1) The total number of islands was pooled and divided by the pooled total length of somal plasma membrane to arrive at # islands / 100 µm plasma membrane. (2) The total number of islands and the total number of somal profiles were counted and the final number normalized to express number of islands per 100 somas. After verifying that the two methods yielded comparable results, the majority of data were analyzed by the second method because it is more efficient to gather a large data set without photographing the entire length of plasma membrane of every neuronal soma encountered.

NR islands are readily detectible due to the clustered gold particles (Fig. 1). Once we learned how to recognize the NR islands, islands could be identified based on the structural characteristics of the cytoplasmic density beneath the plasma membrane. Since not all islands label for all the antibodies, percent of island labeled was calculated for each antibody. Every neuronal somal profile encountered was scored for presence of islands based on the characteristic dense material underneath the plasma membrane (arrows in Fig. 2A, B). Every island was photographed regardless of whether the island
was labeled for any particular antibody. The percent of islands that were labeled from each experiment was then calculated for each antibody.

Labeling intensity of islands was calculated among labeled islands by counting the total number of particles within the marked area, then divided by the length of the island and expressed as # particles / running µm island (Fig. 1C). A ratio of labeling intensities between islands and PSDs within each experiment was calculated for each antibody. The laminar distributions of labels at islands were compared to those at the PSD. Because the laminar distribution of many of the PSD proteins was skewed, a non-parametric test (Wilcoxon test) was used to compare the medians. Finally, since somal and dendritic plasma membranes are continuous, it seemed reasonable to compare data gathered from somal islands to data from dendritic synapses.

RESULTS

2.1. Distribution of NMDA receptors in dissociated hippocampal neurons

Labeling patterns for NR1 and NR2B, two subunits of NMDA receptors that often coexist in a functional tetrameric complex (Wenthold et al., 2003), were similar in three week-old cultures. Because the NR2B antibody provided better labeling, most micrographs and all measurements are from samples labeled for NR2B. Two structural entities stood out due to their high labeling intensities: asymmetric synapses (arrowheads in Fig. 3A, B) and nonsynaptic NR clusters (arrows in Fig. 3A, C). Both
entities existed throughout somal and dendritic locations, and there was no structural
distinction for either entity whether they are located on soma or dendrites. The synaptic
distribution of NMDA receptors under different experimental conditions will be examined
separately; here the focus is on the nonsynaptic NR clusters. We will refer to these
clusters hereafter as nonsynaptic NMDA receptor islands, or NR islands. These islands
are additionally defined by a cytoplasmic density (arrow in Fig. 3C), which resembles a
postsynaptic density (PSD, arrowhead in Fig. 3B).

[Please place Fig. 3 here, column width 8.5 cm]

The NR2B antibody used here is against an extracellular epitope, so particles
representing silver-enhanced gold labels were located, as expected, in the synaptic cleft
(Fig. 3B) or on the extracellular surface of the plasma membrane of an island (Fig. 3C).
Scattered individual particles (small arrows in Fig. 3A, D) were also present along
somal/dendritic plasma membranes at nonsynaptic locations, and the density of these
extrasynaptic labels was similar whether they are on somas or on primary dendrites
extending from these somas. This observation is consistent with the observation from
single particle tracing using live light microscopy that extrasynaptic NMDA receptors are
freely diffusible on plasma membranes (Groc et al., 2009).

Specificity of the NR2B antibody was also verified by comparing the labeling
pattern on somal/dendritic plasma membrane with that on samples processed without
primary antibody as well as with samples labeled with other antibodies such as GKAP,
Shank and CAMKII. Compared to a labeling count for extrasynaptic NMDA receptors of
4.86 ± 0.41 labels per somal/dendritic profile (5 exp), there was negligible labeling on
the extracellular side of the plasma membrane from these control samples (0.13, 0.05, 0.03 and 0.1 labels per somal/dendritic profile, respectively; P<0.0001 in all cases, images not shown).

2.2. Characterization of nonsynaptic NMDA receptor islands

Islands consistently and prominently labeled with three different antibodies to NMDA receptors: NR2B (Fig. 4A); NR1 (Fig. 4B); and NR2A/B (Fig. 4C). Virtually all structurally identifiable islands showed tightly clustered labels. Islands viewed in single sections appeared heterogeneous in size (80-400 nm), with clusters of label for NR2B at densities of 20-80 labels/µm. Serial thin section analyses (e. g., Fig. 3D1-D3) from more than 50 islands (41 on soma, and 10 on dendrites) confirmed that there are no structural distinctions between somal and dendritic islands, that label for NR is always closely associated with the underlying cytoplasmic densities (arrows in 4A, B, D2, D3), and that label as well as the densities are limited by sharp borders.

[Please place Fig. 4 here, column width 7.5 cm]

2.3. Overall concentration of NMDA receptors and the number of NR islands both increased after depolarization with high K+ 

The overall labeling density of nonsynaptic NMDA receptors (number of gold particles per unit length of plasma membrane) on neuronal somas was measured in areas of the plasma membrane unopposed by processes of other cells, and counts included both individual particles and particles clustered in islands. The amount of label for receptors consistently increased after high K+ treatment (2 min, 90 mM) in five
experiments (Fig. 5A), and the mean labeling density increased by 25%. This result suggests that additional receptors were inserted into the plasma membrane upon depolarization with high K\(^+\), and that these additional receptors were probably exocytosed onto the plasma membrane during depolarization.

The average number of NR islands per unit length of somal plasma membrane increased to 2.5 fold of controls after depolarization with high K\(^+\) (Fig. 5B), indicating that islands formed within 2 min upon depolarization. NR islands appear similarly induced to form in dendrites, as number of NR islands per unit length of dendritic plasma membrane increased to ~3 fold of control values upon depolarization (2.48 ± 0.24 islands/ 100 µm dendritic plasma membrane in control vs. 7.23 ± 0.42 in high K\(^+\), 3 exp).

To test for the possibility that the epitope for NR antibody may have become more accessible upon depolarization, numbers of islands were counted in identically treated samples labeled with other antibodies such as CaMKII, Shank, Homer and GKAP. Because not all islands labeled for these other antibodies, Islands were scored based on their structural characteristics (cf. Fig. 2 in Methods). Number of islands consistently increased upon high K\(^+\) treatment in ten sets of samples (13.3 ± 1.9 islands/100 somas in control vs. 66.1 ± 6.6 in high K\(^+\) samples, P<0.0001, paired t-test).

It was interesting that the average length of the NR-labeled islands was smaller in high K\(^+\) samples (130 ± 5 nm, range 80-300 nm, n=76) than in controls (175 ± 12 nm,
range 75-255 nm, n=19; P<0.001, paired t-test, 5 exp). Also, there are many more small islands in the high K⁺-treated samples than in controls. These small islands cannot all be the result of breakdown subunits from larger islands because the sum of the lengths of all islands pooled from control samples was only about 40% of that in high K⁺ samples (3.32 µm from 89 soma for control samples, and 10.08 µm from 117 soma for high K⁺ samples). Thus, there were indeed more small islands formed de novo after high K⁺ treatment. These newly formed islands could result from direct insertion of a preformed cluster of receptors into the plasma membrane, or they might assemble quickly from individual receptors.

2.4. NR islands are not exocytosed as a preformed package

A search for evidence that islands are inserted into neuronal plasma membrane as a preformed package revealed no intracellular vacuoles containing concentrated NMDA receptors. Occasionally, vacuoles contained a few labels (small arrow in Fig. 6C), but these vacuoles never had associated cytoplasmic densities (cf. arrowhead in Fig. 6A) and thus differed from the NR islands on the plasma membrane. In contrast, patches of clustered AMPA receptors (large arrow in Fig. 6B) appear to be directly inserted into the plasma membrane as packages of concentrated receptors (small arrow in Fig. 6D; Tao-Cheng et al., 2011). These AMPA receptor patches (Fig. 6B) lack the cytoplasmic density that characterizes NR islands (arrowhead in 6A). Thus, AMPA receptor patches and NR islands are different structural entities.

[Please place Fig. 6 here, column width 8.5 cm]
2.5. Dynamic disassembly of NR islands.

To explore whether NR islands disassemble or persist after stimulation, cells were treated for 2 min with high K⁺ and then left to recover for 2-3 min or 30 min in control medium. The numbers of NR islands on somal plasma membrane increased significantly after 2 min depolarization with high K⁺, quickly decreased to near control levels after 2-3 min of recovery, and stayed at that level after 30 min of recovery (Table 1). These results indicate that most NR islands disappear from surface membrane within 2-3 min of ending stimulation. Receptors clustered in Islands could either scatter on plasma membrane as individual receptors, or they could be internalized as a package.

In order to see whether NR islands are endocytosed as a package, we searched for vacuoles containing island-like cytoplasmic densities that could represent the aftermath of endocytosed islands. NMDA receptors are internalized by clathrin-mediated endocytosis (Roche et al., 2001; Nong et al., 2003; Petralia et al., 2003; Washbourne et al., 2004). Indeed, some clathrin-coated pits (Fig. 7A, B) and vesicles (Fig. 7C) contained a few labels for NR2B, but many clathrin-coated pits did not label for NMDA receptors (Fig. 7D, E). Occasionally, NR islands were present on plasma membranes immediately adjacent to coated pits (arrow in Fig. 7E) reminiscent of peri-PSD endocytic profiles (Petralia et al., 2003; Rácz et al., 2004), but islands with a cytoplasmic density were never present at a coated pit or vesicle.
2.6. Nonsynaptic NMDA receptor islands contain many PSD proteins

Immunogold labeling of a set of proteins associated with PSDs was used to compare the composition of islands to that of PSDs. Because more islands appeared after treatment with high K⁺, we analyzed high K⁺-treated samples. Although virtually all islands labeled for NMDA receptors, not all islands labeled for all of the PSD proteins (Fig. 8).

Ranking from high to low in percentage of islands labeled, NMDA receptors and CaMKII were detected in almost all islands. Shank, Homer and GKAP were detected in 60-70%, GluR2 and SAP102 in 35-45%, and PSD95 in only 18% of islands defined by structural criteria (Table 2, column A). In contrast, virtually all PSDs in high K⁺-treated samples labeled for most PSD-associated proteins (Tao-Cheng et al., 2010, 2011, 2014, 2015) except for SAP102, which only labeled in about half of them.

2.7. Differential distribution of PSD proteins at islands

Labeling intensities of PSD proteins at islands and PSDs were measured as number of labels per µm of island or PSD, and the ratio of labeling intensities of island to PSDs from the same sample was calculated for each antibody (Table 2, column B). Numbers lower than one indicate that labeling intensity at islands is lower than that at PSDs.
NMDA receptors (NR2B) was much more prominent in islands than AMPA receptors (GluR2). Essentially all islands labeled for NR2B, but only about one third labeled for the GluR2 subunit of AMPA receptors (P<0.0001, t test). Between the two members of the membrane-associated guanylate kinases (MAGUK) family, SAP102 had a significantly higher presence at islands than PSD 95, both in percent labeling (P<0.01, t test) and in ratio of labeling intensity (P<0.05, t test).

Among the next three PSD scaffold proteins, GKAP, Shank and Homer all showed similar labeling at islands that was consistently lower than that at PSDs, both in percent of islands labeled and in labeling intensity (Table 2). Interestingly, CaMKII (calcium/calmodulin-dependent kinase) had a strong presence at islands in high K⁺-treated samples where all islands labeled at the same intensity as that at PSDs (Table 2).

2.8. Layered distributions of PSD proteins at islands are similar to those at PSDs

Distances of label from the plasma membrane were measured to assess the laminar distribution of PSD proteins at islands. Measurements were taken from high K⁺-treated samples where many more islands were present. Because some of the proteins (Shank2 and CaMKII) redistribute upon high K⁺ treatment, and the degree of redistribution is variable in different experiments, comparisons were only made within each experiment. Values for median instead of mean were used for a nonparametric statistical test between islands and PSDs because the distributions were typically skewed. There was no statistical difference in distances of labels between islands and PSDs in any experiments (Table 3).
Different proteins were localized within different layers at islands in an order similar to that at PSDs: SAP102, PSD95 and GKAP were located in a narrow band close to the plasma membrane, while Shank, Homer and CaMKII were in a broad band in the more distal part of the PSD complex (Fig. 7; Sans et al., 2000; Valtschanoff and Weinberg, 2001; Petralia et al., 2005; Yang et al., 2011; Tao-Cheng et al., 2014; 2015). [Please place Table 3 here]

DISCUSSION

The present study uses preembedding immunogold electron microscopy to explicate the depolarization-induced redistribution of NMDA receptors (NR), focusing on NR clusters at nonsynaptic locations. We refer to these structures as nonsynaptic NR islands, and present evidence that they are not the immediate product of exocytosis of NMDA receptors, but form subsequent to arrival of receptors on neuronal surfaces. Islands then incorporate PSD-associated proteins to eventually form a pre-assembled PSD-like entity. The formation and disassembly of islands provoked by depolarization is dynamic on a time scale of 2-3 min. This activity-dependent induction of NR islands provides a window during which nascent synapses could form after neuronal activity.

AMPA receptors tagged with a pH-sensitive fluorescence probes are exocytosed in concentrated packages giving rise to intense puffs of fluorescence when their acidic vesicular lumens are exposed to the neutral extracellular milieu (Yudowski et al., 2007). Similar exocytic events occur with other receptors including transferrin receptors (Shen et al., 2014) but, so far, none are reported for NMDA receptors (Groc et al., 2009).
Electron microscopy verified the presence of intracellular vacuoles containing concentrated label for AMPA and transferrin receptors (Tao-Cheng et al., 2011), but not for NMDA receptors. Thus, we speculate that NMDA receptors, unlike the AMPA receptors, may be exocytosed at low concentrations that are not readily detectible by direct, live, light microscopy via the pHfluor method.

The differential recruitment of various PSD-associated proteins into islands mirrors that at developing synapses. NMDA receptors are much more prevalent than AMPA receptors at islands, consistent with early recruitment of NMDA receptors and late arrival of AMPA receptors at both nascent synapses and nonsynaptic NR clusters (Rao et al., 1998; Petralia et al., 1999; Pickard et al., 2000; Washbourne et al., 2002; Shiraishi et al., 2003; Gerrow et al., 2006). All islands in high K⁺-treated samples labeled intensely for CaMKII, consistent with early recruitment of CaMKII to PSDs during development (Swulius et al., 2010).

SAP102, a member of the MAGUK family, which includes PSD95, is recruited to PSDs early during synapse development, later to be replaced by PSD95 (Sans et al., 2000). Thus, developing PSDs, like islands, contain more SAP102 than PSD95 (Petralia et al., 2010). Our results suggest that PSD95 is incorporated into islands relatively late, consistent with observations by light microscopy at nascent synapses during PSD development (Washbourne et al., 2002; Barrow et al., 2009; Swulius et al., 2010).

GKAP, Shank and Homer, three PSD scaffold proteins (Sheng and Kim, 2011), are present in ~55-65% of NR islands, consistent with light microscopy observations that these proteins are at some but not all nonsynaptic NR clusters (Rao et al., 1998;
Shiraishi et al., 2003; Gerrow et al., 2006). Although labeling intensities of these scaffold proteins are lower at islands than at PSDs, their laminar distributions at islands are identical to those at PSDs. Altogether, our data indicate that NR islands contain a set of PSD proteins expected in nascent PSDs.

Preassembled specializations of pre- and postsynaptic proteins can form independently in early development, and both can initiate synapse formation (McAllister AK, 2007). Preformed PSD scaffold complexes are prevalent at nonsynaptic sites in young cells at 7 days in vitro (Gerrow et al., 2006), and some of them are mobile, but whether these complexes are at cell surfaces or are intracellular transport packets is unclear with light microscopy. By electron microscopy, it is clear that nonsynaptic NR islands reported here are at cell surfaces, but no intracellular entity representing preformed PSD complexes is evident in the three week-old cultures.

Nonsynaptic NR clusters, perhaps corresponding to the NR islands studied here, are common at surfaces of young cells by live cell imaging (Washbourne et al., 2004), and these may eventually be recruited to synaptic sites (Washbourne et al., 2002). Axonal processes occasionally contacted small clusters of NMDA receptors on dendrites, resulting in an entity resembling an incipient. Alternatively, these axons may be retracting from incipient synapses, leaving the postsynaptic elements to become islands as remnants of transient contacts (Petralia et al., 2010).

Nonsynaptic NR islands form rapidly upon 2 min depolarization with high K⁺. Because measurements of number of islands was made on surfaces of neuronal somas facing the culture media, with no axons nearby, the increase in number of islands cannot be caused by retracting axons. Although the high K⁺ treatment is not
physiological, it provides a convenient and consistent experimental level of stimulation for EM examination of structural changes at the synapses. Furthermore, depolarization with high K\(^+\) does not severely damage hippocampal neurons in culture, as cells recover fully (Dosemeci et al., 2001; Tao-Cheng et al., 2011).

Because islands can form on surfaces unapposed by other cellular processes, it is possible that the neuronal soma alone is capable of forming islands without direct interaction from other cellular elements. Alternatively, since high K\(^+\) likely induces release of glutamate and/or other modulators from neurites and glia, islands might also be induced to form as a result of such releases from nearby processes. Regardless of the mechanism, three week-old hippocampal neurons are capable of forming islands rapidly upon induction. Whether this capability is age-related awaits further investigation. Interestingly, islands are much more frequently seen in developing rats than in adults (Petralia et al., 2010), but whether islands are induced in developing brain via similar mechanisms as in neuronal cultures remains untested.

Our EM observations of three week-old neurons suggest that NMDA receptors are exocytosed and endocytosed as individuals or in small numbers. Once on the surface of the neuron, they cluster and become associated with other PSD proteins to form islands. Islands can be induced to assemble by depolarization of the neuron and spontaneously disassemble within minutes upon recovery. Within this short window, islands may be poised to attract nearby axons to form nascent synapses.
REFERENCES


Steward O, Falk PM (1986) Protein-synthetic machinery at postsynaptic sites during...


**FIGURE LEGENDS**

**Fig. 1. NR island.** NR islands are defined by patches of somal/dendritic plasma membrane that label for NMDA receptors (NR2B), and are associated with cytoplasmic dense material (arrow). Scale bar=0.1 µm.

**Fig. 2. Morphometry of islands.** Islands can be identified by their characteristic cytoplasmic density (arrows in A, B) without immunogold labeling. Many islands are not labeled with GluR2 (A) or PSD95 (B). To measure label that is located on the cytoplasmic side of islands (e.g., CaMKII in C), two vertical lines extending 120 nm into the cytoplasm are drawn to mark the area for measurement as in the case of the PSD complex (Tao-Cheng et al., 2015). The distance between the two vertical lines represents the length of island. Distance of label was measured from the center of the particle to the outer edge of the plasma membrane (white arrows in C) for all the particles within the marked area. All islands in this figure were from soma. Scale bar=0.1 µm.

**Fig. 3. Distribution of NR2B on plasma membrane of hippocampal neurons.** Label for NR2B in three week-old dissociated hippocampal neurons prepared by pre-embedding immunogold labeling. A. NR2B is concentrated at asymmetric synapses (arrowhead in A and at high mag in B), but is also present in nonsynaptic parts of the plasma membrane where it can be either in clusters (large arrows in A and C) associated with a cytoplasmic density, or as single individual grains (small arrows in A, D). A and B were sampled from dendrites, C and D from soma. Scale bar = 0.1 µm.
Fig. 4. Nonsynaptic NR islands contain cytoplasmic densities (arrows) mimicking the structure of PSDs. Islands label for different subunits of NMDA receptors—NR2B in A, NR1 in B, and NR2A/B in C, the first two at extracellular epitopes. Serial thin section analysis (D1-D3) shows that labels are closely associated with an underlying density manifesting sharp borders. All islands in this figure were sampled from dendrites. Scale bar = 0.1 µm.

Fig. 5. Labeling density of NMDA receptor and NR islands on somal plasma membrane after depolarization with high K⁺.
(A) After high K⁺ (2 min, 90 mM), overall labeling intensity for NR2B in somal plasma membranes increases to 126 ± 5% (*) of control values (five experiments, P< 0.01, paired t-test). (B) After high K⁺, the density of NR islands increases to 249 ± 34% (^) of control values (five experiments, P< 0.05, paired t-test).

Fig. 6. Comparison of NR islands and AMPA receptor patches. NR2B labeled islands (A) have a distinct cytoplasmic density (arrowhead) which is lacking in AMPA receptor-labeled patches (B, GluR2 antibody). AMPA receptor patches are thought to be exocytosed from cytoplasmic vacuoles containing concentrated receptors (small arrow in D). In contrast, NR2B labels are typically at low concentrations, if present, in clear-membraned vacuoles (small arrows in C). A, C, D were samples from dendrites, and B was from soma. Scale bars = 0.1 µm.
Fig. 7. Endocytosis of NMDA receptors. Label for NR2B is sometimes located in clathrin-coated pits (A, B) and the lumens of coated vesicles (C). Many clathrin-coated pits are not labeled for NR2B (D, E). NR-labeled islands (arrow in E) may lie immediately adjacent to a coated pit, but are not endocytosed as an island. Scale bars = 0.1 µm.

Fig. 8. Islands and PSDs labeled for various PSD-associated proteins. Antibodies and percent of islands labeled are listed on left. Images are arranged based on their laminar distribution at the PSD. Glutamate receptors at the top (NR2B and GluR2) are integral membrane proteins, here labeled with antibodies with extracellular epitopes. Extending successively deeper into the cytoplasm are the MAGUKs (SAP102 and PSD95) immediately adjacent to the postsynaptic membrane; then GKAP, a binding partner of both PSD95 and Shank; and a broad band of Shank and Homer, two scaffold proteins that bind to each other. Additionally, CaMKII, a kinase that can bind to NR2B or self-aggregate, is distributed throughout the PSD complex. The laminar localization of these proteins at islands mirrors that at PSDs. Images of islands and PSDs are all from the same sample, and samples are from experiments treated with 2 min high K⁺. All islands were sampled from soma, and all PSDs were sampled from dendrites. Scale bars = 0.1 µm.
Table 1. Effects of stimulation on number of non-synaptic NMDA receptor islands in neuronal plasma membranes

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>2 min high K⁺</th>
<th>2 min K⁺ + 2-3 min recovery</th>
<th>2 min K⁺ + 30 min recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exp 1</td>
<td>11.8 (17)</td>
<td>38.5 (26)</td>
<td>12.5 (40)</td>
<td></td>
</tr>
<tr>
<td>Exp 2</td>
<td>13.0 (23)</td>
<td>35.0 (20)</td>
<td></td>
<td>16.0 (25)</td>
</tr>
<tr>
<td>Exp 3</td>
<td>10.3 (29)</td>
<td>68.1 (47)</td>
<td>18.5 (27)</td>
<td>18.2 (22)</td>
</tr>
<tr>
<td>Exp 4</td>
<td>5.0 (20)</td>
<td>57.7 (26)</td>
<td>11.5 (26)</td>
<td>16.2 (37)</td>
</tr>
<tr>
<td>Mean ± SEM</td>
<td>10.0 ± 1.8</td>
<td>49.8 ± 7.9**</td>
<td>14.2 ± 2.2</td>
<td>16.2 ± 0.7</td>
</tr>
</tbody>
</table>

(n) = Number of neuronal somal profiles scored, and number of islands is normalized to per 100 somal profiles.

**Number of islands is significantly higher in high K⁺: P<0.0005 vs. control, P<0.005 vs. 2 min K⁺+2-3 min recovery and 2 min K⁺+30 min recovery; ANOVA with Tukey's post test.
Table 2. Percent of NR islands labeled for PSD proteins and the ratios of labeling intensity at islands to that at PSDs

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>A. % islands labeled (n=number of islands scored)</th>
<th>B. Ratio of labeling intensity (islands / PSDs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NR2B</td>
<td>97.5 ± 2.5 (4 exp, 42)</td>
<td>1.76 ± 0.13 (3 exp)</td>
</tr>
<tr>
<td>GluR2</td>
<td>36.3 ± 0.6 (2 exp, 33)</td>
<td>1.07 ± 0.24 (2 exp)</td>
</tr>
<tr>
<td>SAP102</td>
<td>44.7 ± 4.8 (6 exp, 113)</td>
<td>1.02 ± 0.15 (4 exp)</td>
</tr>
<tr>
<td>PSD95</td>
<td>18.3 ± 4.8 (3 exp, 46)</td>
<td>0.39 ± 0.08 (3 exp)</td>
</tr>
<tr>
<td>GKAP</td>
<td>55.7 ± 15.9 (3 exp, 38)</td>
<td>0.70 ± 0.15 (3 exp)</td>
</tr>
<tr>
<td>Shank2</td>
<td>67.5 ± 12.5 (2 exp, 44)</td>
<td>0.60 ± 0.12 (2 exp)</td>
</tr>
<tr>
<td>Homer1</td>
<td>63.8 ± 6.4 (5 exp, 105)</td>
<td>0.59 ± 0.06 (3 exp)</td>
</tr>
<tr>
<td>CaMKII</td>
<td>100</td>
<td>1.0 ± 0.14 (3 exp)</td>
</tr>
</tbody>
</table>

Column A: Percent of islands labeled at neuronal somas in three week-old dissociated hippocampal cultures after depolarization with high K⁺. Islands were first identified by structural characteristic of the cytoplasmic density, and then scored for presence of immunogold labeling with the various antibodies.

Column B: Ratio of labeling intensities for each antibody as number of labels per running µm of island compared to that at PSDs in the same experiment.
Table 3. Median distances of label to plasma membrane at islands and PSDs measured from high K⁺-treated samples.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Island (n) = # particles measured</th>
<th>PSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAP102</td>
<td>exp 1 26.7 (19)</td>
<td>26.7 (30)</td>
</tr>
<tr>
<td></td>
<td>exp 2 26.7 (17)</td>
<td>28.3 (18)</td>
</tr>
<tr>
<td>PSD95</td>
<td>exp 1 28.3 (14) (pooled from 3 exp)</td>
<td>26.7 (158)</td>
</tr>
<tr>
<td></td>
<td>exp 2</td>
<td>26.7 (130)</td>
</tr>
<tr>
<td>GKAP</td>
<td>exp 1 35.0 (12)</td>
<td>33.3 (215)*</td>
</tr>
<tr>
<td></td>
<td>exp 2 30.0 (14)</td>
<td>30.0 (72)</td>
</tr>
<tr>
<td>Shank 2</td>
<td>exp 1 56.7 (56)</td>
<td>50.0 (345)*</td>
</tr>
<tr>
<td></td>
<td>exp 2 43.3 (53)</td>
<td>46.6 (483)*</td>
</tr>
<tr>
<td>Homer 1</td>
<td>exp 1 53.3 (49)</td>
<td>56.7 (120)</td>
</tr>
<tr>
<td></td>
<td>exp 2 50.0 (36)</td>
<td>53.3 (209)</td>
</tr>
<tr>
<td>CaMKII</td>
<td>exp 1 53.3 (97)</td>
<td>53.3 (113)</td>
</tr>
<tr>
<td></td>
<td>exp 2 56.7 (84)</td>
<td>60.0 (135)</td>
</tr>
</tbody>
</table>

* data from Tao-Cheng et al., 2015

Statistical analysis by Wilcoxon test.