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NMDA receptor surface mobility depends on NR2A-2B subunits

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The NR2 subunit composition of NMDA receptors (NMDARs) varies during development, and this change is important in NMDAR-dependent signaling. In particular, synaptic NMDAR switch from containing mostly NR2B subunit to a mixture of NR2B and NR2A subunits. The pathways by which neurons differentially traffic NR2A- and NR2B-containing NMDARs are poorly understood. Using single-particle and -molecule approaches and specific antibodies directed against NR2A and NR2B extracellular epitopes, we investigated the surface mobility of native NR2A and NR2B subunits at the surface of cultured neurons. The surface mobility of NMDARs depends on the NR2 subunit subtype, with NR2A-containing NMDARs being more stable than NR2B-containing ones, and NR2A subunit overexpression stabilizes surface NR2B-containing NMDARs. The developmental change in the synaptic surface content of NR2A and NR2B subunits was correlated with a developmental change in the time spent by the subunits within synapses. This suggests that the switch in synaptic NMDAR subtypes depends on the regulation of the receptor surface trafficking.

Results

Specific Detection of NR2A and NR2B Subunits. To selectively track surface NR2A- and NR2B-containing NMDARs, polyclonal antibodies directed against extracellular epitopes of NR2A subunit were developed, and a previously described antibody directed against NR2B subunit were used (20, 21). As shown in Fig. 1a, the peptide sequences used for antibody production correspond to amino acid sequences in the N-terminal domain of the NR2 subunits. Importantly, an alignment of the two peptide sequences, peptide NR2A versus full-length NR2B and peptide NR2B versus full-length NR2A, show no amino acid sequence similarity. To test the specificity of the NR2A antibody, HEK293 cells were transfected with NR1/NR2B subunit cDNAs, total cell homogenates were prepared and analyzed by immunoblotting with either anti-NR2A (44–58) antibodies (Fig. 1b), anti-NR2A antibodies (1454–1464) (Fig. 1c) that recognize both NR2A and NR2B subunits, or anti-NR2B antibodies (1307–1323) (Fig. 1c) that recognize both NR2C and NR2D subunits (22).

The authors declare no conflict of interest.

NMDA receptors (NMDARs) are heterotetrameric cation channels composed of NR1 and NR2/3 subunits (1). NMDARs are assembled early in the endoplasmic reticulum, and both NR1 and NR2 subunits are necessary for their association and their successful cell surface targeting (2). In addition to glutamate and glycine, NMDARs require membrane depolarization to open with high probability (3), making this receptor a pre- and postsynaptic activity coincident detector involved in the induction of Hebbian synaptic plasticity. The functional properties of NMDARs depend also on the subunit composition, and such subunit heterogeneity of synaptic NMDARs is thought to play an important role during synaptic development, maturation, and plasticity processes (4). During synaptic development, the subunit composition of synaptic NMDARs changes from heterodimers containing predominantly NR2B subunits at early stages to heterodimers containing NR1/NR2B, NR1/NR2A, and NR1/NR2A/NR2B subunits at mature stage (1, 5–14). This change often is associated with the refinement of neuronal connections within cortical areas, although this model has been challenged and, thus, is likely incomplete (4). The pathways by which neurons differentially traffic NR2A- and NR2B-containing NMDARs remain, however, an open question of crucial importance to understand the shaping of synaptic maturation and plasticity.

Changes in NR2 subunit composition of NMDARs within synapses can be triggered by mechanisms that include differences in insertion (15), internalization (16, 17), and/or lateral diffusion. Interestingly, NMDARs diffuse laterally at the neuronal surface (18, 19). In immature neurons, synaptic NMDARs are replaced rapidly by extrasynaptic ones through lateral diffusion (18), suggesting that surface mobility of NMDARs may be involved in shaping mature NMDAR synaptic components. In this study, we investigated the surface mobility of NR2A- and NR2B-containing NMDARs by using single-particle and single-molecule approaches. To selectively discriminate between these NMDAR types, we used antibodies directed against specific extracellular epitopes of these two subunits. Our results indicate that the surface mobility of NR2A-containing NMDARs is much smaller than that of NR2B-containing ones. During neuronal maturation, the decreased contribution of synaptic surface NR2B-containing NMDARs correlated with decreases in synaptic stabilization of the more mobile NR2B-containing NMDARs.

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Abbreviations: IQOR, interquartile range; NMDAR, NMDA receptor; QD, quantum dot.

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with 0.25% Triton X-100, and then antibody was added for incubation. The same conclusion regarding antibody specificity was reached (Fig. 1f). Finally, we tested the specificity of antibodies by labeling live HEK cells double-transfected with the NR1 coupled to yellow fluorescent protein and either NR2A or NR2B subunit cDNAs. The presence of membrane NR2A subunits then was represented by extended line trajectories. (c) Scattered plot distributions of the instantaneous diffusion coefficient of NR2A- and NR2B-containing NMDARs in the extrasynaptic (Left) and the synaptic area (Right). The bar in each group represents the median value. (d) Superimposed distribution histograms of the instantaneous diffusion coefficient of NR2A- (filled bars) and NR2B- (hatched gray bars) containing NMDARs. The first point of the distribution corresponds to the percentage of immobile receptors (bin size = 0.0075 μm²/s). Note the higher percentage of immobile synaptic NR2A- (83%) when compared with NR2B-containing (59%) NMDARs.

**Fig. 1.** Characterization of anti-NR2A and anti-NR2B antibodies. (a) Peptide sequences from the N-terminal domain of NR2A (44–58) and NR2B (42–60) subunits used to raise antibodies. (b) HEK293 cells were transfected with either NR1/NR2A, NR1/NR2B, NR1/NR2C, or NR1/NR2D subunit cDNAs. Cells were analyzed by immunoblotting with an anti-NR2A (44–58) antibody. Note the specific detection of NR2A subunit (arrow). (c) Using the same method as in b, NR2A, NR2B, NR2C, or NR2D subunit was detected by using either an anti-NR2A antibody (1454–1464) or anti-NR2B antibody (100 kDa, right lower arrowhead) and NR2D (M, 180 kDa) subunits (left arrowhead in lanes 1 and 2) or anti-NR2D antibody (1307–1323) that recognizes both NR2C (M, 135 kDa, right lower arrowhead) and NR2D (150 kDa, right upper arrowhead) (lanes 3 and 4). The positions of molecular mass standards (kDa) are shown on the right. (d) P2 fractions were prepared from whole brain (15 μg of wet weight tissue applied per gel lane) of either wild-type (WT) or NR2A (hatched gray bars) mice and analyzed by immunoblotting with anti-NR2A (44–58) or anti-NR2B (1454–1464) antibodies. In lane 1, note that the anti-NR2A (44–58) antibody does not recognize an immunoreactive species in the P2 fraction prepared from NR2A (hatched gray bars) mice. In lane 2, note that both NR2B (M, 180 kDa) and NR2D (M, 180 kDa) antibodies recognize both NR2C (M, 135 kDa) and NR2D (150 kDa) subunits.

**Differential Surface Diffusion of NR2A- and NR2B-Containing NMDARs.** Because NR2A- and NR2B-containing NMDARs have different surface distributions, we then measured and compared the surface mobility of both NR2A- and NR2B-containing NMDARs by using two approaches: (i) single-particle tracking based on the detection of quantum dots (QDs) and (ii) single-molecule tracking based on the detection of the single organic fluorophores, i.e., cyanine 3. QD-based tracking provides a unique tool for long-term recording of receptor surface diffusion because QDs are more photostable than organic dyes (19, 23). However, as reported in ref. 19, QD-based tracking may be biased to some extent within a confined space, so we also used the single-molecule approach to track synaptic receptors. To differentiate synaptic versus extrasynaptic receptors, synapses were labeled with the active mitochondria marker, Mitotracker (rhodamine derivative), which was shown to colocalize with the presynaptic synaptotagmin clusters (19, 24).

Representative summed trajectories recorded at days in vitro 15 and over a 60-s period time (reconstruction from image series acquired at 30 Hz rate), are shown in Fig. 2 a and b. The NR2A-containing NMDAR summed trajectories (a single red trace) show that subunits are immobile or very slowly mobile in both synaptic (green spots) and extrasynaptic compartments (Fig. 2a). In contrast, the NR2B-containing NMDAR summed trajec-
Native NR2A and NR2B Subunit Surface Distributions and Overdevelopment. To investigate the developmental changes of NR2A and NR2B-containing NMDAR surface diffusion, we first performed live immunostainings of native NR2A- or NR2B-containing NMDARs at the surface of dendritic arbors (somatic staining was not considered) of live hippocampal cultured neurons at two different developmental stages: D7–9, referred as “D8” and D14–16, referred as “D15.” In agreement with previous reports, surface NR2A and NR2B subunit immunostainings were different: (i) the dendritic surface NR2A subunit staining significantly increased during this period, whereas the surface density of NR2B subunit slightly, but not significantly, decreased during this period, and (ii) the percentage of NR2A subunit staining that colocalize with the presynaptic marker, synaptotagmin, significantly increased during this period, whereas the opposite trend was observed for NR2B subunit staining (Fig. 6, which is published as supporting information on the PNAS web site). Moreover, respective to function, we recorded NMDAR-mediated miniature excitatory postsynaptic currents (mEPSCs) at these two developmental stages (Supporting Methods). The NMDAR mEPSC decay time decreased from D8 to D15 neurons (D8 : two exponential fit: \( \sigma_1 = 20 \pm 4 \) ms and \( \sigma_2 = 196 \pm 38 \) ms, \( n = 5 \); D15 : \( \sigma_1 = 9 \pm 4 \) ms and \( \sigma_2 = 91 \pm 17 \) ms, \( n = 5 \)), suggesting a functional switch in the NR2 subunit composition of synaptic NMDAR from NR2B-containing receptors to NR2A-containing ones, as previously reported by numerous studies with cultured neurons (5, 9, 12, 14, 25–27).
Based on these findings, we then asked whether such developmental change correlated with changes in surface diffusion of NR2B-containing NMDAR between D8 and D15. The diffusion of NR2B-containing NMDARs decreases significantly from D8 to D15, mostly because of a higher proportion of immobile receptors (first point in the cumulative curves) (Fig. 4a). We further tested whether surface diffusion and distribution of NR2B-containing NMDARs are modulated by changes in global neuronal activity. To determine the NR2B-containing NMDAR surface distribution, the relative content of synaptic, perisynaptic (300-nm annulus around the synapse), and extrasynaptic detected molecules was quantified. In control conditions, 21 ± 4% of molecules were synaptic, 15 ± 3% perisynaptic, and 64 ± 6% extrasynaptic (n = 23 dendritic fields). After a chronic incubation of neurons from D9 to D15 with an NMDAR antagonist (50 µM AP-5), a GABAA receptor channel blocker (100 µM picrotoxin), or a sodium channel blocker [1 µM tetrodotoxin (TTX)], NR2B-containing NMDAR surface distribution did not significantly change (P > 0.05 in all conditions) (Fig. 4b Middle). Moreover, the surface diffusion of NR2B-containing NMDARs remains unaffected by these treatments (Fig. 4b). All together, these results indicate that the surface distribution and diffusion of NR2B-containing NMDARs are developmentally regulated in an activity-independent manner.

**Differential Stability of NR2A- and NR2B-Containing NMDARs Within Synapses Overdevelopment.** The decreased content of surface synaptic NR2B-containing NMDAR overdevelopment could come from several processes, i.e., a restriction of NR2B-containing NMDAR to enter laterally the synapse, a lack of stabilization of the receptor within the postsynaptic membrane, or a change in the cycling rate of NR2B-containing NMDAR between intracellular and membrane pools. To test these possibilities, we first measured the exchange rate of the NR2B-containing NMDARs that alternate between the extrasynaptic and synaptic membranes (Fig. 5a). The percentage of exchanging NR2B-containing NMDARs at D15 (29%) was slightly higher than at D8 (22%) and than for NR2A-containing NMDARs at D15 (22%). The exchange rate, defined as the number of compartment changes over a time period (60 s), and the synaptic dwell time, defined as the mean time spent by exchange receptor within the synaptic area, were calculated. The exchange rate of NR2B-containing NMDARs was not significantly different at D8 and D15, remaining at ~0.6 Hz (~36 compartment changes per min) (Fig. 5b), ruling out a potential restriction of NR2B-containing receptors to laterally enter mature synapses. We then measured the residency time of exchanging NR2B-containing NMDARs within synapses to estimate receptor stabilization within the postsynaptic membrane. Interestingly, the residency time was significantly decreased by a factor of three from D8 to D15 (Fig. 5c), indicating a higher surface stabilization of NR2B-containing NMDAR in early synapses when compared with more mature ones. It can be noted that at D15 the residency time of NR2A-containing NMDAR was significantly higher than that of NR2B-containing NMDAR, indicating a better stabilization of NR2A-containing NMDAR within mature synapse. We thus propose that the relative decreased content of NR2B-containing NMDAR within mature synapse is due to instability of the surface receptor within the postsynaptic membrane. Finally, the observed decreased content of surface synaptic NR2B-containing NMDAR overdevelopment that would come from an increased internalization of the receptor is unlikely because NR2B-containing NMDAR internalization remains constant overdevelopment (from D5 to D12) (17). Along this line, we measured the internalization rate of all NMDARs by using an anti-NR1 antibody directed against an extracellular epitope (19) (Supporting Methods) and found that the internalization of NMDARs during development is significantly decreased (D8: 32 ± 3% of internalized NR1-containing NMDARs, n = 5; D15: 10.5 ± 5%, n = 5; P < 0.05). Thus, the decreased contribution of synaptic NR2B-containing NMDARs is not due to an increased internalization of NMDAR overdevelopment.

**Discussion**

In the present study, we show that part of the surface mobility of NMDARs depends on the NR2A-2B subunit subtype, NR2A-containing NMDARs being more stable than NR2B-containing ones. The synaptic composition of NMDARs changed over maturation with an increase in the NR2A/NR2B subunit ratio. Interestingly, the developmental switch in the synaptic NR2A- and NR2B-containing NMDAR surface distribution correlates with developmental changes in the time spent by subunits within synapses without any change in the lateral exchange of the receptors (Fig. 5f). These data shed light on how surface NR2A- and NR2B-containing NMDARs can be differentially trafficked and they propose a developmental model in which the regulation of synaptic NMDAR subtypes depends on the synaptic surface stabilization of the receptors.

Our current knowledge of the differential distribution of NR2A and NR2B-containing NMDARs at the neuronal surface has come from either electrophysiological approaches or detection of genet-
iewed protein 102 (SAP-102) and postsynaptic density 95 (PSD-95), membrane-associated guanylate kinases (MAGUKs), synapse associ-
state of the NR subunits, and including binding affinity to scaffold proteins, phosphorylation
NMDARs are likely the result of multiple cellular processes,
from differences in cycling processes (outside synapse) between
temporary stabilized. Such a result suggests that the surface distri-
extrasynaptic membrane and synapse, in which they were only
exchanging NR2B- and NR2A-containing NMDARs between the
in the extrasynaptic membrane, we found a similar proportion of
cycling processes (outside synapse) between

Differences in surface diffusion of NR2A- and NR2B-containing NMDARs are likely the result of multiple cellular processes, including binding affinity to scaffold proteins, phosphorylation state of the NR subunits, and/or extracellular factors. Two mem-
membrane-associated guanylate kinases (MAGUKs), synapse associ-
protein 102 (SAP-102) and postsynaptic density 95 (PSD-95), which both contribute to form a scaffold for ionotropic glutamate
receptors at the postsynaptic density, indeed have been proposed to
play a role in the NMDAR subunit switch during development (4).
Schematically, a preference of certain MAGUKs for different
NMDAR subtypes suggest that different NMDAR scaffolding
proteins could affect the trafficking and synaptic localization of
NR2A- and NR2B-containing NMDARs during synaptic develop-
ment (11, 32, 33). In this model, NR2A-containing NMDARs are
synaptically incorporated, PSD95 is inserted into the center of
the postsynaptic density and displaces the NR2B subunit–SAP102
complexes, which were initially located at the postsynaptic density,
to the perisynaptic and extrasynaptic membranes (32, 34, 35). Our
current data on the surface mobility of NR2A/B subunits support
this hypothesis and further indicate that the lateral shift of the
subunits observed by electrophysiological means likely results from
differences in lateral mobility and stabilization of the subunits.
Indeed, surface NR2A-containing NMDARs are more stable than
NR2B-containing ones within mature synapses, possibly due to a
high proportion of PSD-95 over SAP-102 in the postsynaptic
density. Interestingly, the domains on the C-terminal tail are critical
to retain NR2A- (36) and NR2B-containing NMDARs (30) within
synapses and the binding of NMDARs to PDZ proteins is a
regulated process, depending on kinase activation (37), suggesting
that NR2A/B surface mobility is indeed dynamically regulated by
intracellular interactions. To The synaptic retention of NMDARs
also depends on extracellular factors such as the EphB receptor,
which interacts with NMDARs through N-terminal extracellular
domains (38), cell-adhesion molecules (e.g., integrins) (39), and
proteins of the extracellular matrix (e.g., reelin) (40). Interestingly,
the type of presynaptic neuron is a critical determinant of the
subunit composition of NMDARs expressed at synapses (41),
suggesting that appropriate expression of molecules in both
pre- and postsynaptic compartments is necessary for NMDAR
maturation.

In conclusion, the surface mobility of NMDARs depends, in part,
on the NR2A versus 2B subunit composition. The presence of
triheteromeric structure (NR2A and NR2B subunits) or other NR
subunits such as NR3A early in development (42) is also likely to
play a role in determining surface mobility of NMDARs. Our
results unravel a way to differentially traffic NR2A- and NR2B-
containing NMDARs at the neuronal surface and indicate that the
maturation of excitatory glutamate synapses is accompanied by
changes in the stability of specific NMDAR subtypes.

Methods
Cell Culture, Synaptic Live Staining, and Protein Expression in Neurons.
Preparation of the cultured neurons for single molecule/ particule staining has been done as described in refs. 19 and 24.

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Schematically, hippocampal neurons from 18-day-old rat embryos were cultured on glass coverslips by following the Banker technique. To label synapses, neurons were incubated for 1–2 min at 20°C with 1 nM Mitotracker (Deep Red-Fluorescent Mitotracker; Molecular Probes, Amsterdam, The Netherlands). For protein expression, days in vitro 10–15 hippocampal neurons were transferred 24–36 h before experiment by using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA). SEP-NR2A and SEP-NR2B cDNAs were constructed by fusing the supererectile pHluorin (enhanced mutant of pH-sensitive GFP) to the N terminus of rat NR2A and NR2B subunits, respectively. For transfection, culture coverslips were incubated with ~1 μg cDNA for 40 min at 37°C. The superf elective pHluorin allow the specific visualization of surface SEP-NR subunits (43, 44), which ensure that the overexpressed proteins were well targeted to the plasma membrane.

**Immunocytochemistry.** Surface NR2A or NR2B subunits were stained specifically by using the newly developed rabbit polyclonal antibodies against NR2A or NR2B subunits for 30 min. The stained neurons were visualized by using the newly developed rabbit polyclonal antibodies directed against NR2A or NR2B subunits for 30 min. The primary antibodies were revealed by using anti-rabbit Alexa 568 (Molecular Probes, Eugene, OR) directed against NR2A or NR2B subunits. All neurons, which are mainly excitatory ones, were measured within only dendritic field (soma excluded from quantification, the average total intensity and the pixel area were measured). For the surface fluorescence quantification, the average total intensity and the pixel area were measured within only dendritic field (soma excluded from analysis). For the colocalization measurement, the pixel area of synaptotagmin and NR2 subunit staining were compared, and the percentage of overlap between the two was calculated. The fluorescence analysis was realized by using Metamorph software (Universal Imaging, Downingtown, PA).

**Single-Molecule and Particle (QD) Tracking.** Cyanine 3 was coupled to the affinity-purified rabbit polyclonal anti-NR2A or anti-NR2B antibodies that are both directed against extracellular epitopes of NR2 subunits. All neurons, which are mainly excitatory ones, were incubated for 10 min at 37°C with the respective cyanine–antibody complexes. As described in ref. 19, all recording sessions were acquired within 30 min after primary antibody incubation to minimize receptor endocytosis. Single-molecule detection was realized as described in refs. 19 and 24. Briefly, a custom wide-field single-molecule fluorescence inverted microscope equipped with a ×100 oil-immersion objective was used. The samples were illuminated for 30 msec at a wavelength of λ = 532 nm by a frequency doubled YAG laser (Coherent, Les Ulis, France) at a rate of 15 Hz. Appropriate filter combinations (DCLP550, HQ600/75; Chroma Technology, Brattleboro, VT) allowed the detection of individual fluorophore by a CCD camera system (Micromax; Princeton Instruments, Trenton, NJ). Using the same excitation path, Red Deep Mitotracker (Molecular Probes) was excited with the λ = 633 nm line of a He-Ne laser (JDS Uniphase, Manteca, CA) at an illuminating intensity of 7 ± 1 kW/cm². We imaged and resolved discrete fluorescence spots (45). Fluorescence spots exhibit one-step photobleaching and not gradual decay as for ensemble photobleaching. We calculated the instantaneous diffusion coefficient, D, for each trajectory, from linear fits of the first four points of the mean-square-displacement versus time function by using MSD(t) = <r²(t) − r²(0) > / 6D. The 2D trajectories of single molecules in the plane of focus were constructed by correlation analysis between consecutive images by using a Vogel algorithm. For QD tracking, QD 655 Goat F(ab’)2 anti-Rabbit IgG (0.1 μM; Ozyme, Paris, France) first were incubated for 30 min with the polyclonal antibodies against NR2A (1 μg) and NR2B subunits (1 μg). Nonspecific binding was blocked by additional cassein (Vector Laboratories, Paris, France) to the QD 15 min before use. Neurons were incubated for 10 min at 37°C in culture medium with precoated QD (final dilution 0.1 nM). QDs were detected by using a xenon lamp (excitation filter HQ500/20X (Chroma Technology; Mitotracker) 560RDFF55 (Omega, QD) and appropriate emission filters [respectively, HQ560/80M (Chroma Technology), and 655WB20; Omega Filters]). Images were obtained with an integration time of 50 msec with respect to with 1200 consecutive frames. Signals were detected by using a CCD camera (Cascade; Princeton Instruments). QD-labeled NR2 subunits were followed on randomly selected dendritic regions for up to 30 min. The trajectory reconstruction was carried out as for single-molecule tracking (see above).

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