The Effects of NMDA Receptor Activation on Synaptogenesis

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Introduction

The fundamental units of the central nervous system, neurons, communicate with each other through synapses, informing, for instance, neurons in the visual cortex about stimuli experienced by photoreceptors in the retina. Synaptogenesis, the formation of these synapses, begins during development, in conjunction with other events of embryonic development, and continues even after the organism is fully developed. Synapses are formed and broken throughout the organism's lifetime, giving the brain a physiological flexibility, called plasticity, with the connections that are maintained between different neurons.

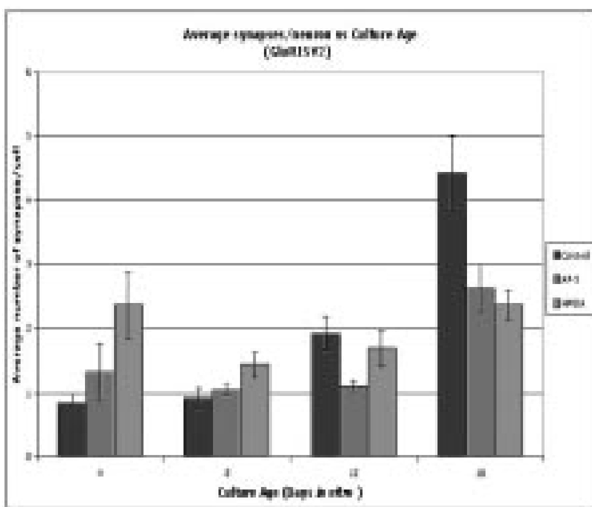

Figure 1. Average number of synapses/neuron vs. Culture Age. (a) Cultures labeled for the NMDA receptor and Synaptophysin. (b) Cultures labeled for the GluR1 receptors and Sv2. As the cultures become older, the average number of synapses per cell under all three treatments increases, but the NMDA treated cultures had fewer synapses/neuron than the control in cultures labeled for the NMDA receptor and those labeled for the AMPA receptor. In the AP-5 cultures, there are fewer synapses/cell compared to the control, but the number of synapses in the GluR1SV2 cultures at 16DIV is lower than that in the NMDAR/Synaptophysin cultures.

The level of glutamate neurotransmission at a particular synapse determines whether it will persist once it is formed. Glutamate, an excitatory neurotransmitter, mediates this selection through two of its ionotropic receptors: the N-methyl-D-aspartate (NMDA) and the α-amino-3-hydroxy-5-methyl-4-izoxazole-propionic acid (AMPA) receptors.[1] NMDA receptors are unique because they are both ligand and voltage gated; immediately after glutamate binds to NMDA receptors, magnesium molecules bind as well, blocking the ion channel. The magnesium block is removed in response to an increase in membrane potential.[2] However, The AMPA receptors only require glutamate for activation and can therefore provide the depolarization necessary for NMDA receptor activation.[1] Upon glutamate binding, AMPA receptors permit sodium and potassium ion influx. NMDA receptors also require glycine, an amino acid, in order to open their ion channel.[3] Once activated, NMDA receptors allow the influx of calcium ions into the cell, affecting dendritic spine formation and various calcium-dependent signal transduction pathways.[4,5]

Because NMDA receptors rely on AMPA receptors for activation, synapses that contain purely NMDA receptors are electrophysiologically silent.[6] Indeed, many of the synapses formed initially during neural development are called "silent synapses" because they only contain NMDA receptors and cannot initiate action potentials in response to presynaptic glutamate release.[7] However, perhaps due to a high glutamate affinity and temporary insensitivity to the magnesium block, young NMDA receptors can be easily activated by the spontaneous opening of calcium channels. The calcium influx will displace the magnesium block,

depolarizing the neurons.[8,9,10] This activation is sufficient to recruit AMPA receptors to the synapse, creating functional connections between neurons.[10]

The goal of the present study is to determine how the NMDA receptor's function affects synapse formation. We manipulated the activity of the NMDA receptor in *Xenopus laevis* primary neuronal cultures by treating them with AP-5, an NMDA receptor antagonist, or NMDA, an agonist. We studied the effects of these drugs on cultures of four different ages (4, 8, 12, and 16 days in vitro) labeled for the NR1 subunit of the NMDA receptor or the GluR1 subtype of the AMPA receptor or Glutamic Acid Decarboxylase (GAD65), the biosynthetic enzyme of the inhibitory neurotransmitter, γ-amino-butyric-acid (GABA), and a presynaptic marker. We examined the effects of the different treatments on synapses with inhibitory neurons because these neurons also receive excitatory glutamatergic input.

We found, in agreement with previous studies, that treatment with AP-5 decreased the number of AMPA receptors containing synapses while not affecting the number of NMDA receptors containing synapses. The NMDA-treated cultures, however, had fewer AMPA and NMDA receptor synapses, but more GABA synapses, suggesting that as the NMDA receptors at a synapse are activated, some of the NMDA receptors are internalized to reduce the calcium influx associated with glutamate signaling.

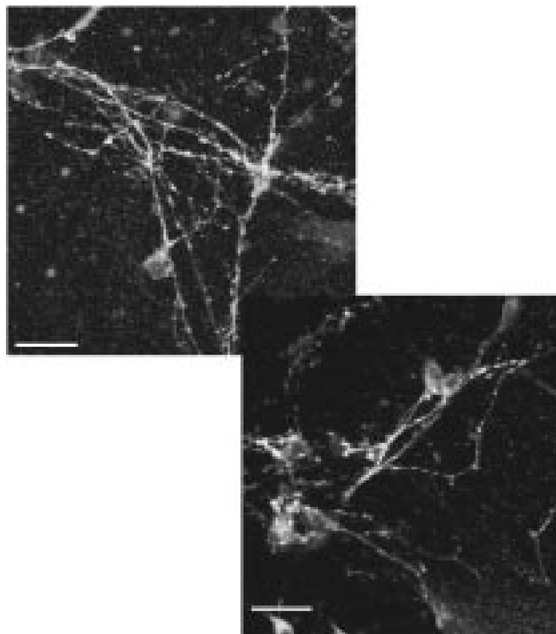

Figure 2. Examples of control and NMDA treated 16DIV cultures, labeled for GluR1 and SV2. (Top) A 16 DIV Xenopus laevis primary neuronal culture labeled for GluR1 subtype of the AMPA receptor and the SV2, a presynaptic molecule. (Bottom) An NMDA treated culture, also at 16DIV, with fewer synapses/neuron than the control culture. (Scale bar = 5 μm)

Materials and Methods

Tectal culture

The tectal tissue used for the dissociated cultures was prepared from *Xenopus laevis* anesthetized in 0.1% MS222 (tricant methanesulfonate) prior to dissection. Once the tecta were removed from the animals' skulls, they were placed in HBSS (58mM NaCl, 0.7mM KCl, 0.3mM $CaCl_2$ $2H_2O$, 0.083mM $MgSO_4$, 4.6mM HEPES, pH 7.4), and any remaining blood vessels and membranes were removed manually. The cleaned lobes were transferred to 2ml of trypsin diluted with HBSS and incubated for 30 minutes. The trypsin was then removed by aspiration and the tissue rinsed three times with 2ml HBSS. After the last rinse, the tissue was titurated with fire polished pipettes in 2ml of Frog Culture Media, which was 50% L-15 medium (Life Technologies), 10% fetal calf serum, 5% JSFH salts (240mM NaCl, 9mM KCl, 21mM $CaCl_2$, 21mM $MgSO_4$, 400mM HEPES and 40mM $NaCO_3$), 2% penicillin/streptomycin, 0.1% gentamycin and 40μl/100ml of 25mg/ml insulin/trasnferrin sodium selenite (Boehringer Mannheim), and 20μl of 0.1mg/ml DNAse I. The uniformly mixed suspension was centrifuged briefly at 2600 rpm and the FCM removed. This was repeated twice. The second time, the supernatant was removed, except for approximately 10μl of FCM, and an additional volume of 30μl of FCM/coverslip was added. The cells were plated in the center of 22mm coverslips and allowed to adhere for 20 minutes. Finally, 30μl of 10mM AP-5 or 3μl of 100mM NMDA were added along with 3ml of FCM to each coverslip. The cells were incubated at 18° C.

Immunohistochemistry

When the cultures reached the appropriate age (4, 8, 12, and 16 Days in vitro), they were fixed with 4% paraformaldehyde and then washed twice for 10 minutes each in 1x PBS (320g NaCl, 8g KCl, 57.6g Na_3PO_4, 4.6g KH_2PO_4). The cultures were permeabilized with 0.1% Saponin in PBS and blocked with Bovine Serum Albumin (Sigma) (0.4% BSA, 0.01% NaN_3 in PBS). The antibodies used to label the two different receptors and presynaptic molecules were diluted in BSA and used at the following concentrations: GluR1 (Upstate) 1:100, SV2 (Developmental Studies Hybridoma Databank)1:20, 1:1000 amti-glutamic acid decarboxylase (Sigma), anti-NR1 (Pharmingen) 1:100, and Synaptophysin (Zymed) 1:100. Once the coverslips were incubated for an hour with each of these antibodies, they were washed three times for 10 minutes in 0.1% Tween20 in PBS and incubated with secondary antibodies from Jackson Labs at the following concentrations: Cy-3 goat anti-rabbit 1:2000, FITC donkey anti-mouse 1:100, biotin goat anti-mouse 1:500, and Cy-5 streptadivin 1:300. After three more washes in 0.1% Tween20 in PBS, they were mounted on slides and photographed using a Nikon Eclipse E-600FN upright confocal microscope.

Synaptic Quantification

In order to determine how the NMDA receptor's activity affects the formation of synapses, the number of synapses formed under different treatments were

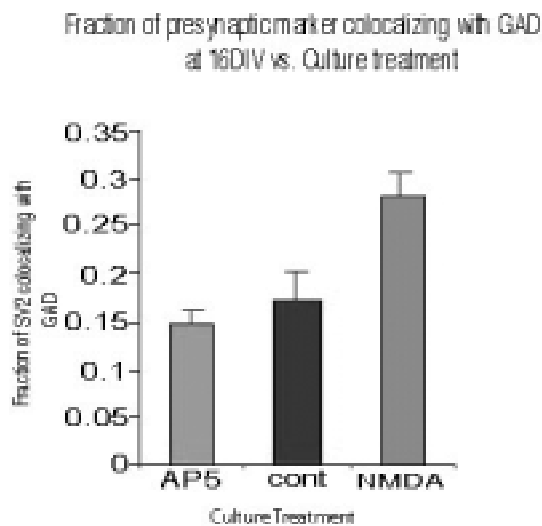

Figure 3. Fraction of presynaptic marker that was colocalized with the GAD65/67 at 16DIV cultures vs. culture treatment. At 16DIV, NMDA treated cultures had a significantly higher fraction of colocalization between SV2, a presynaptic marker, and GAD65/67, a marker for GABA, compared to the control and AP-5 treated cultures.

counted. Because the number of neurons in the field varied from image to image, we normalized the number of synapses by the total number of neurons in each image. The images were analyzed using NIH Image with custom-designed macros to provide a consistent, reproducible counting mechanism. Images were thresholded such that everything except 3% of the brightest pixels were eliminated in order to remove noise, or nonspecific staining. Afterward the cell bodies were "cut" out of the image because of excessive staining. Synapses were defined as an overlap between $0.21\mu m^2$ to $4.20\mu m^2$ of pre- and post-synaptic elements. ANOVA Tukey post-hoc tests were used to ascertain statistical significance.

Electrophysiology

The slides with the 8, 12, and 16DIV (days in vitro) cultures were incubated in an extracellular recording solution that contained 117mM NaCl, 2mM $CaCl_2$, 10mM Glucose, and 10mM HEPES (pH=7.3), but no magnesium. In order to prevent mistaking GABA receptor currents with NMDA receptor currents, the extracellular medium was also infused with 50µM picrotoxin, a noncompetitive GABA receptor antagonist. The pipettes used to record the movement of ions contained 110mM K-Gluconate, 10mM KCL, 5mM NaCl, 1.5mM $MgCl_2$, 0.5mM EGTA, 20mM HEPES, and 200mg/ml Amphotericin B at a pH of 7.3. The Amphotericin B created perforations in the cell membrane and was diluted in dimethyl sulfoxide immediately prior to use. At a membrane potential of either +10 or +15mV, an Axopatch 200B amplifier (Axon Instruments) was used to record the spontaneous currents discernible under whole-cell patch-clamp conditions. AP-5 (100mM) or CNQX (10mM), an AMPA receptor antagonist, was included in the bathing medium and washed out after each experiment to allow the cultures to recover.

Results

As the cultures grew older from 4DIV to 16DIV, the average number of synapses per cell increased in the control, the AP-5 and the NMDA-treated cultures, as expected during development (Figures 1a,b). However, at 16DIV, the NMDA-treated cultures labeled for GluR1 or the NR1 subunit of the NMDA receptor had significantly fewer synapses that contained NMDA (p=0.006, Tukey post-hoc test) and AMPA (p=0.002) receptors than the control cultures, respectively. Figure 2 compares a control to an NMDA treated culture, labeled for GluR1 and SV2, a presynaptic molecule, at 16DIV. The AMPA receptors are shown in red and the presynaptic molecules in green. The synapses formed between the two are in yellow. In order to compensate for density differences among cultures, we calculated the average number of synapses per neuron.

Blocking the NMDA receptors, by treating the cultures with AP-5, decreased the formation of synapses between presynaptic markers and GluR1 (Figure 1a). At 16DIV, the AP-5 cultures labeled for GluR1 had significantly fewer synapses (p=0.007, Tukey post-hoc test) than the control cultures. This reduction in the number of GluR1 synapses in AP-5 treatment confirms that, indeed, activity in the NMDA receptors is required for recruitment of AMPA receptors to synaptic sites. However, because the number of synapses in the AP-5-treated cultures labeled for the NR1 subunit of the NMDA receptor is not significantly lower than the control culture's, we can conclude that the receptors' activity does not determine whether they are clustered at synapses during development (Figure 1b). Additionally, chronic activation of the NMDA receptors at 16DIV increased the number of inhibitory synapses present compared to control cultures (p=0.014), without changing the number of GABAergic neurons (Figure 3). NMDA-receptor blockade, on the other hand, did not significantly alter the number of GABAergic synapses.

In the 8DIV control cultures examined electrophysiologically, the spontaneous excitatory postsynaptic currents (EPSCs) recorded had long decay times, characteristic of NMDA-receptor activation. When these cultures were treated with AP-5, currents were still seen but with shorter decay times, indicating that AMPA receptor currents were also present at this age. The NMDA-treated 8DIV cultures exhibited an even larger decrease in decay times (p=0.033, Tukey post-hoc test)) than the AP-5 treated cultures. This decrease mirrored that seen in 12 and 16DIV cultures across treatment groups. Like the control and AP-5-treated cultures, the NMDA-treated cultures at 12 and 16 DIV also had decreased decay times.

Discussion

NMDA- and AMPA- receptor activation influences synaptic strength during development. Through long-term potentiation (LTP) and long-term depression (LTD), these receptors also mediate the efficacy of connections formed as organisms learn and form memories.[11] LTP is

the process by which synapses are strengthened through repetitive, high-frequency stimulation of NMDA receptors and LTD, the process by which synapses are weakened through repetitive, low-frequency stimulation of the NMDA receptors.[12] In response to LTP- or LTD-inducing stimuli, additional AMPA receptors are recruited to or withdrawn from synapses.[13] The transport of AMPA receptors in LTP and LTD is similar to that seen during development, also as a consequence of NMDA-receptor activation. This connection between the plasticity of the adult brain and development makes it essential to establish how the level of activity of the NMDA receptor affects synapse formation. Our results indicate that chronic activation of the NMDA receptors reduces the number of NMDA receptors and consequently AMPA receptors at synapses.

While the number of synapses per cell in all three cultures increased steadily as the cultures aged, the AP-5-treated cultures and the NMDA-treated cultures had fewer GluR1-containing and NMDA-containing synapses per cell than the control. The reduction of GluR1-containing synapses seen in AP-5-treated cultures is consistent with the withdrawal of AMPA receptors in response to low or no NMDA receptor activation. The chronic activation of the NMDA receptors by NMDA treatment may have prompted internalization of some of the receptors, reducing the number of NMDA and AMPA receptors present at synaptic sites.

A previous study from our laboratory showed that activation of the NMDA receptors suppresses process formation.[8] The current data, which suggests a reduction in number of NMDA receptors at the synapses of NMDA-treated cultures, is in agreement with this finding. The 8DIV NMDA-treated cultures had EPSCs with shorter decay times like the control cultures at later ages, suggesting that NMDA treatment induced a premature shift to-AMPA receptor currents. After AMPA receptors are recruited to the synapse, the number of NMDA receptors on the surface may be reduced to compensate for the increased level of intracellular calcium.

We also saw a significant decrease in the number of synapses containing GluR1 formed in the AP-5- and NMDA-treated cultures compared to the control cultures. The lack of NMDA receptor activity, in the AP-5 cultures, resulted in fewer AMPA-containing synapses, confirming that NMDA receptors have to be active in order to recruit GluR1 receptors to synapses. The internalization of some synaptic NMDA receptors caused by their chronic activation would explain the observed decrease in the number of AMPA-receptor-containing synapses.

NMDA-receptor activation also has a bearing on the formation of GABAergic synapses. We found that chronic activation of the NMDA receptors resulted in the formation of more inhibitory synapses, suggesting that developmental mechanisms compensate for chronic activation. Indeed, a previous in vivo study conducted by our laboratory in the rat superior colliculus showed that chronic NMDA-receptor stimulation increased the efficacy of GABA receptors at synapses. Hence, increased activity at excitatory synapses appears to potentiate GABAergic synapses, limiting the exposure to glutamate and potential overexcitation as development takes place.[14]

References

1. Constantine-Paton, M., and H.T.Cline (1998). LTP and Activity-Dependent Synaptogenesis: the more alike they are, the more different they become. *Current Opinion in Neurobiology* 8(1):139–148.
2. Burnashev, N. (1998). Calcium permeanility of ligand-gated channels. *Cell Calcium* 24:325–332.
3. Mothet, J-P., A.T.Parent, H.Wolosker, et al. (2000). D-Serine is an endogenous ligand for the glycine site of the *N*-methyl-D-aspartate receptor. PNAS 97:9 4926–4931.
4. Wu, G.Y., K. Deisseroth, and R.W.Tsien (2001). Spaced stimuli stabilize MAPK pathway activation and its effects on dendritic morphology. *Nature Neuroscience* 4:151–8.
5. Lee, H.K., K.Takamiya, J.S. Han, H.Man, C.H.Kim, G.Rumbaugh, S.Yu, L.Ding, C.He, R.S. Petralia, R.J Wenthold, M.Gallagher, R.L.Huganir (2003). Phosphorylation of the AMPA receptor GluR1 subunit is required for synaptic plasticity and retention of spatial memory. *Cell* 112(5):631–43.
6. Malenka, R.C. (2003). Synaptic Plasticity and AMPA Receptor Trafficking. Annals of New York Academy of Science 1003: 1–11.
7. Issac, J.T., R.A. Nicoll, and R.C. Malenka (1995). Evidence for Silent Synapses: Implications for the expression of LTP. u 15: 427–34.
8. Miskevich, F., W. Lu, S-Y Lin, and M. Constantine-Paton (2002). Interaction between Metabotropic and NMDA Subtypes of Glutamate receptors in Sprout Suppression at Young Synapses. *J. of Neuroscience* 22(1):226–38.
9. Liao, D., R.H.Scannevin and R. Huganir (2001). Activation of Silent Synapses by Rapid Activity-Dependent Synaptic Recruitment of AMPA Receptors. *J. of Neuroscience* 21(16):6008–17.
10. Takahashi, T., D.Feldmayer, N.Suzuki, et al. (1996). Functional correlation of NMDA receptor e subunit expression with the properties of single channel synaptic currents in the developing cerebellum. *J. of Neuroscience* 16:4376–82.
11. Dudek and Bear (1992). Homosynaptic Long-Term Depression in Area CA1 of Hippocampus and Effects of N-Methyl-D-Aspartate Receptor Blockade. PNAS 89:4363–7.
12. Malenka, R. (1994). Synaptic Plasticity in the Hippocampus: LTP and LTD. *Cell* 78 (4): 535–538.
13. Hayashi, Y., S.H. Shi, J.A. Esteban, et al. (2000). Driving AMPA receptors into synapses by LTP and CaMKII: requirement for GluR1 and PDZ domain interaction. *Science* 287: 2262–2267.
14. Aamodt S.M., J.Shi, M.T.Colonnese, W.Veras, and M.Constantine-Paton (2000). Chronic NMDA exposure accelerates development of GABAergic inhibition in the superior colliculus. *J. of Neurophysiology* 83 (3):775–781.