

# Ethanol Enhances Neurosteroidogenesis in Hippocampal Pyramidal Neurons by Paradoxical NMDA Receptor Activation

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Using an antibody against 5 $\alpha$ -reduced neurosteroids, predominantly allopregnanolone, we found that immunostaining in the CA1 region of rat hippocampal slices was confined to pyramidal neurons. This neurosteroid staining was increased following 15 min administration of 60 mM but not 20 mM ethanol, and the enhancement was blocked by finasteride and dutasteride, selective inhibitors of 5 $\alpha$ -reductase, a key enzyme required for allopregnanolone synthesis. Consistent with a prior report indicating that *N*-methyl-D-aspartate (NMDA) receptor (NMDAR) activation can promote steroid production, we observed that *D*-2-amino-5-phosphonovalerate (APV), a competitive NMDAR antagonist, blocked the effects of 60 mM ethanol on staining. We previously reported that 60 mM ethanol inhibits the induction of long-term potentiation (LTP), a cellular model for memory formation, in the CA1 region. In the present study, LTP inhibition by 60 mM ethanol was also overcome by both the 5 $\alpha$ -reductase inhibitors and by APV. Furthermore, the effects of ethanol on neurosteroid production and LTP were mimicked by a low concentration of NMDA (1  $\mu$ M), and the ability of NMDA to inhibit LTP and to enhance neurosteroid staining was reversed by finasteride and dutasteride, as well as by APV. These results indicate that ethanol paradoxically enhances GABAergic neurosteroid production by activation of unblocked NMDARs and that acute LTP inhibition by ethanol represents a form of NMDAR-mediated metaplasticity.

## Introduction

During acute ethanol intoxication, individuals can perform complex acts for which they have no recollection, a state referred to as a “blackout” (White, 2003). Understanding how this dense amnesia occurs is important for understanding alcoholism as a neurocognitive disorder. Ethanol is thought to impair memory by inhibiting long-term potentiation (LTP), a form of synaptic plasticity associated with memory processing (Bliss and Collingridge, 1993; Martin et al., 2000). Although ethanol is a partial *N*-methyl-D-aspartate (NMDA) receptor (NMDAR) antagonist (Izumi et al., 2005), ethanol’s effects on LTP involve enhanced GABA-mediated inhibition via 5 $\alpha$ -reduced neurosteroids, including 3 $\alpha$ -hydroxy-5 $\alpha$ -pregnan-20-one (allopregnanolone) (Izumi et al., 2007). It is presently unclear which neural cells are responsible for neurosteroidogenesis and how ethanol promotes steroid production.

In this study, we pursued the hypothesis that ethanol’s effects on LTP represent a form of metaplasticity involving unblocked NMDARs (Izumi et al., 1992a,b). We based these studies on the findings that ethanol alters extracellular glutamate levels (Moghaddam and Bolinao, 1994; Chefer et al., 2011) and is a partial

NMDAR antagonist in the hippocampus (Izumi et al., 2005). We found that both the enhancement of neurosteroid levels and inhibition of LTP by ethanol in CA1 hippocampal pyramidal neurons involve NMDAR activation, not inhibition, and that a low micromolar concentration of NMDA mimics the effects of ethanol on neurosteroidogenesis and LTP.

## Materials and Methods

**Animals.** Protocols for animal use were approved by the Washington University Animal Studies Committee in accordance with the NIH guidelines for care and use of laboratory animals.

**Hippocampal slice preparation.** Hippocampal slices were prepared as described previously (Zorumski et al., 1996) from postnatal day 30–32 male Sprague Dawley rats purchased from Harlan. Rats were anesthetized with isoflurane and decapitated. Slices were cut transversely into 500  $\mu$ m slices using a rotary slicer in artificial CSF (ACSF) containing the following (in mM): 124 NaCl, 5 KCl, 2 MgSO<sub>4</sub>, 2CaCl<sub>2</sub>, 1.25 NaH<sub>2</sub>PO<sub>4</sub>, 22 NaHCO<sub>3</sub>, and 10 glucose, bubbled with 95% O<sub>2</sub>/5% CO<sub>2</sub> at 4–6°C. Acutely prepared slices were placed on nylon mesh in 10 ml beakers containing gassed ACSF and maintained for at least 1 h at 30°C before experiments.

**Immunohistochemistry.** Hippocampal slices used for immunohistochemistry were initially screened by electrophysiology to diminish slice-to-slice variability in staining for 5 $\alpha$ -reduced neurosteroids. Immunostaining was performed as described previously (Tokuda et al., 2010). Slices were incubated with various reagents in separate 10 ml beakers. For experiments (including electrophysiology) in which we examined the role of 5 $\alpha$ -reductase in the effects of ethanol or NMDA, inhibitors of the enzyme were administered for at least 15 min before the other agents. This was done to allow time for the enzyme inhibitors to access their intracellular sites of action. Following drug treatment, slices

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were fixed in fresh 4% paraformaldehyde in PBS for 30 min. Samples were then washed with PBS and incubated in blocking solution (1% donkey serum/PBS) for 2 h at 25°C. Slices were incubated with a primary antibody raised in sheep against 5 $\alpha$ -reduced neurosteroids diluted 1:2500 in blocking solution for 48 h at 4°C. The polyclonal antibody against 5 $\alpha$ -steroids has minimal cross-reactivity with other neurosteroids in rats (Bernardi et al., 1998).

After incubation with primary antibody, slices were rinsed with PBS and incubated with a secondary antibody, Alexa Fluor 488 donkey anti-sheep IgG (diluted 1:500), for 2 h at 25°C. After staining, slices were washed with PBS and mounted onto microscope slides with Fluoromount-G (Southern Biotech).

Confocal images were obtained using a 60 $\times$  objective (1.4 numerical aperture), a C1 laser scanning confocal microscope, and Z-C1 software (Nikon Instruments). All parameters were kept constant within an experiment. Digital images were analyzed, and the average intensity of the tissue was measured using MetaMorph software (Universal Imaging).

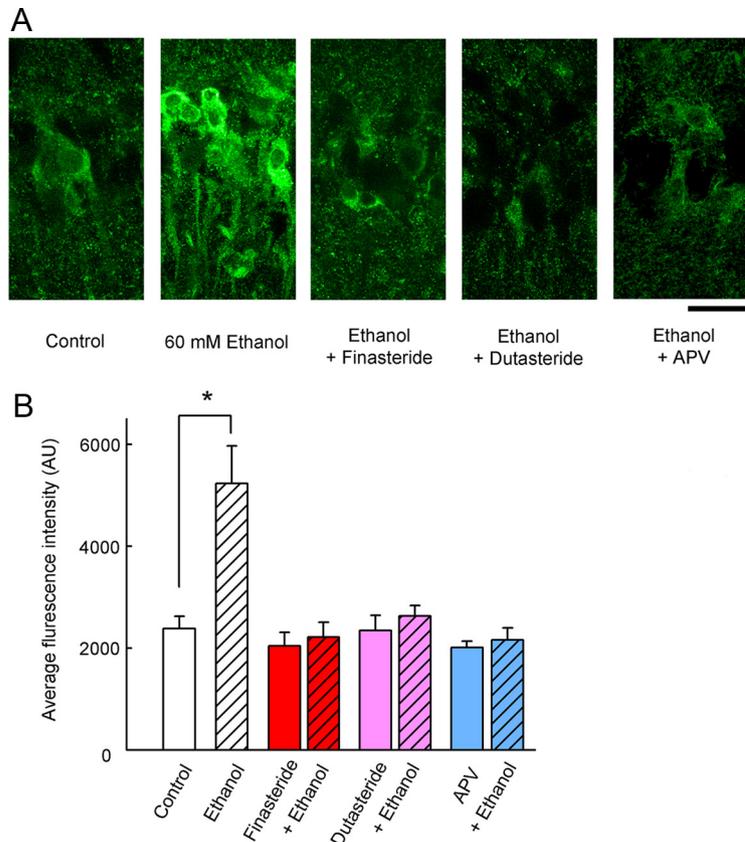
**Extracellular field potential recording.** For electrophysiology, slices were incubated in a submerged recording chamber with continuous bath perfusion of oxygenated ACSF at 2 ml/min at 30°C. Extracellular recordings were obtained from the apical dendritic layer of the CA1 region elicited with 0.1 ms constant current pulses through a bipolar stimulating electrode (Rhodes Medical Instruments) in stratum radiatum. LTP was induced by applying a single 100 Hz  $\times$  1 s high-frequency stimulation (HFS) using a 50% maximal stimulus. Input–output curves were repeated 60 min following HFS for statistical comparisons of changes in EPSP slopes at the half-maximal point. Signals were digitized and analyzed using PCLAMP software (Molecular Devices).

**Statistical analysis.** All data are expressed as mean  $\pm$  SEM. Student's *t* test was used for comparisons between two groups. If an equal variance test failed, the nonparametric Mann–Whitney rank sum test was applied. For multiple comparisons, ANOVA followed by *post hoc* Holm–Sidak test was used. Statistical analyses were performed using commercial software (Sigma Stat 3.11; Systat Software). *p* values of <0.05 were considered statistically significant.

**Materials.** Finasteride was obtained from Steraloids. Dutasteride was obtained from AK Scientific. All other chemicals were purchased from Sigma. Anti-allopregnanolone antisera were purchased from Dr. Robert Purdy, University of California–San Diego, La Jolla, CA. Alexa Fluor 488 was purchased from Invitrogen.

## Results

We initially examined the effects of ethanol on neurosteroid immunostaining in the CA1 region of rat hippocampal slices by using an antibody against 5 $\alpha$ -reduced steroids, predominantly allopregnanolone. Consistent with earlier reports (Saalman et al., 2007; Tokuda et al., 2010), we found that steroid staining was largely, if not exclusively, confined to CA1 pyramidal neurons. Ethanol increased neurosteroid staining in a concentration-dependent fashion, with significant effects following 15 min administration of 60 mM (244.7  $\pm$  39.5% vs control, *n* = 5, \**p* < 0.001) (Fig. 1*A,B*) but not 20 mM ethanol (108.6  $\pm$  7.0%) (data not shown). The enhanced staining was blocked completely by 1  $\mu$ M finasteride and 1  $\mu$ M dutasteride, selective inhibitors of 5 $\alpha$ -reductase, a key enzyme required for neurosteroid synthesis



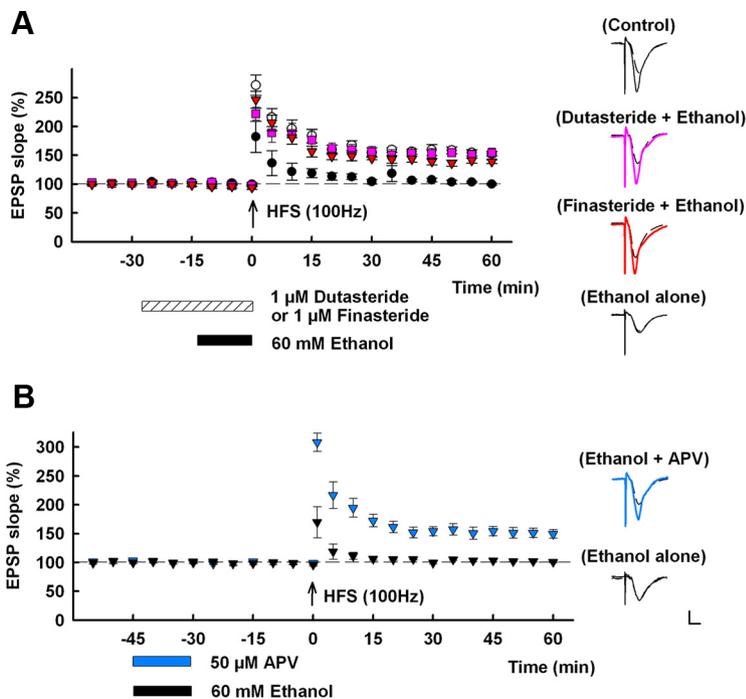
**Figure 1.** Ethanol-mediated neurosteroidogenesis results from activation of NMDARs and 5 $\alpha$ -reductase. **A**, Immunostaining against 5 $\alpha$ -reduced neurosteroids was observed in cell bodies of pyramidal neurons in naive hippocampal slices. The neurosteroid staining was enhanced by 15 min incubation with 60 mM ethanol but blocked by 1  $\mu$ M finasteride, 1  $\mu$ M dutasteride, and 50  $\mu$ M APV. Scale bar, 25  $\mu$ m. **B**, Summary of immunostaining studies shows fluorescence intensity (arbitrary units) as mean  $\pm$  SEM. *p* values are calculated by Holm–Sidak *post hoc* test, *n* = 5; \**p* < 0.001.

(97.4  $\pm$  16.1% and 107.5  $\pm$  14.7%, *n* = 5, respectively) (Fig. 1*A,B*) (Aggarwal et al., 2010).

We subsequently sought to understand how ethanol enhances neurosteroidogenesis. Based on reports indicating that NMDAR activation promotes steroid production in the hippocampus (Kimoto et al., 2001) and that ethanol promotes release of glutamate (Chefer et al., 2011), we examined the effects of NMDAR inhibition on neurosteroid staining. Administration of 50  $\mu$ M D-2-amino-5-phosphonovalerate (APV), a broad spectrum competitive NMDAR antagonist, did not alter baseline staining (93.0  $\pm$  11.4% vs control, *n* = 5) (Fig. 1*B*) but completely prevented the effects of 60 mM ethanol (88.9  $\pm$  12.9%, *p* = 0.008 vs 60 mM ethanol alone) (Fig. 1*A,B*).

We also found that 60 mM but not 20 mM ethanol inhibited LTP induction (Izumi et al., 2005, 2007), and this LTP inhibition was overcome by 1  $\mu$ M finasteride (EPSP change: 139.2  $\pm$  3.4% of baseline, *n* = 5) (Fig. 2*A*) and 1  $\mu$ M dutasteride (EPSP change: 154.0  $\pm$  8.1% of baseline, *n* = 5) (Fig. 2*A*). Exploiting an earlier observation that ethanol's block of LTP persists for over an hour after drug washout (Izumi et al., 2005), we also found that effects on LTP were reversed by coadministration of 50  $\mu$ M APV (EPSP change: 149.5  $\pm$  7.6% of baseline, *n* = 5) (Fig. 2*B*). Administration of APV alone 30 min before HFS followed by washout had no effect on LTP induction (EPSP change: 157.7  $\pm$  9.2% of baseline, *n* = 5, data not shown).

Because these results indicate that ethanol's effects on neurosteroid production and LTP involve activation of NMDARs, we



**Figure 2.** Ethanol-mediated LTP inhibition involves activation of NMDARs and  $5\alpha$ -reductase. **A**, In control slices, LTP is readily induced (white circles) by a 100 Hz  $\times$  1 s high-frequency stimulation (HFS, arrow). Fifteen minute administration of 60 mM ethanol (black bar) inhibited LTP induction (black circles), while finasteride (red triangles) or dutasteride (pink squares) overcame LTP inhibition. **B**, Ethanol's inhibition of LTP (black triangles) persisted for at least 30 min after washout (black bar). This inhibitory effect was prevented by coadministration of APV (blue bar) with ethanol (blue triangles). Traces depict EPSPs before (dashed lines) and 60 min after HFS (solid lines). Calibration, 1 mV, 5 ms.

also examined the effects of a low concentration of NMDA (Izumi et al., 1992a,b). When administered at  $1 \mu\text{M}$ , NMDA, like ethanol, enhanced neurosteroid staining in pyramidal neurons ( $244.2 \pm 22.8\%$  vs control,  $n = 5$ ,  $*p < 0.001$ ) (Fig. 3A,B). Also similar to ethanol, the enhancement of neurosteroid staining by NMDA was reversed by finasteride and dutasteride ( $102.3 \pm 8.0\%$  and  $110.6 \pm 6.6\%$ ,  $n = 5$ , respectively) (Fig. 3A,B). Furthermore,  $1 \mu\text{M}$  NMDA administered before tetanic stimulation inhibited the induction of LTP (EPSP change:  $100.2 \pm 8.5\%$  of baseline,  $n = 5$ ) (Fig. 4A), and this effect was also overcome by finasteride and dutasteride (EPSP change:  $157.6 \pm 10.9\%$  of baseline,  $153.4 \pm 5.8\%$  of baseline,  $n = 5$ , respectively) (Fig. 4A). Finally, NMDA also had persistent effects on LTP, lasting at least 30 min following washout (EPSP change:  $101.3 \pm 2.8\%$  of baseline,  $n = 5$ ) (Fig. 4B). Again, similar to ethanol, APV coadministration with NMDA blocked the LTP inhibition (EPSP change:  $157.7 \pm 9.2\%$  of baseline,  $n = 5$ ) (Fig. 4B).

## Discussion

Ethanol has previously been shown to increase neurosteroid levels in the isolated hippocampus (Sanna et al., 2004), although it has remained unclear which cells are responsible for steroid production and how this effect occurs. Pyramidal neurons in the CA1 region express the machinery for cholesterol trafficking and neurosteroid synthesis (Kimoto et al., 2001; Agís-Balboa et al., 2006), and these neurons are the principal if not exclusive cells in the region that are immunopositive for allopregnanolone and other  $5\alpha$ -reduced neurosteroids under physiological conditions (Saalman et al., 2007; Tokuda et al., 2010). We found that CA1 pyramidal neurons are also the cells that show enhanced neurosteroid staining following exposure to concentrations of ethanol that impair LTP. Interestingly, the acute effects of ethanol on

neurosteroidogenesis, like effects on LTP, require high concentrations with no effect at 20 mM but significant effects at 60 mM. Thus, if neurosteroids are involved in the negative effects of ethanol on cognitive function, it is likely that only high concentrations of ethanol acutely produce these effects because of the apparent threshold for neurosteroid synthesis. Based on the observation that both enhanced steroid production and LTP inhibition are prevented by  $5\alpha$ -reductase inhibitors (Izumi et al., 2007), our results provide further support for the hypothesis that high concentrations of ethanol impair hippocampal LTP and presumably cognitive functions through neurosteroidogenesis.

The present studies also provide insights into how ethanol alters neurosteroid levels and LTP. While it is widely recognized that ethanol antagonizes NMDARs acutely, its effects are only partial even at concentrations of 50–100 mM (Lovinger et al., 1989). We previously found that at concentrations up to 60 mM ethanol partially depresses NMDAR-mediated EPSPs in the CA1 region. These effects are mimicked and occluded by ifenprodil, a selective NR1/NR2B antagonist (Izumi et al., 2005), suggesting that ethanol preferentially antagonizes a subtype of NMDARs in this region. Interestingly, CA1 LTP is not blocked by ifenprodil (Liu et al., 2004; Izumi et al., 2006), and partial (~50%) inhibition of NMDAR EPSPs does not depress LTP induction (Izumi et al., 2006). Thus, it is unlikely that ethanol blocks LTP induction solely through effects on NMDARs. Consistent with this, ethanol's block of LTP is prevented by inhibition of GABA<sub>A</sub> receptors (Izumi et al., 2005).

We previously found that administration of low concentrations of NMDA before tetanic stimulation dampens LTP induction via a metaplastic effect (Izumi et al., 1992a,b), indicating that low-level but sustained NMDAR activation paradoxically inhibits an NMDAR-dependent form of synaptic plasticity. Based on studies showing that ethanol can alter extracellular glutamate (Moghaddam and Bolinao, 1994; Chefer et al., 2011), we postulated that ethanol may produce its effects on LTP by a similar metaplastic mechanism. How ethanol influences extracellular glutamate levels remains uncertain. Based on our prior work, it does not appear that ethanol acutely increases glutamate release at CA1 synapses, because ethanol does not alter EPSP paired-pulse plasticity in hippocampal slices (Murayama et al., 2006). It is possible that ethanol alters extracellular glutamate via release from glia, as has been shown in hippocampal astrocytes (Salazar et al., 2008), or by effects on glutamate uptake (Othman et al., 2002; Melendez et al., 2005).

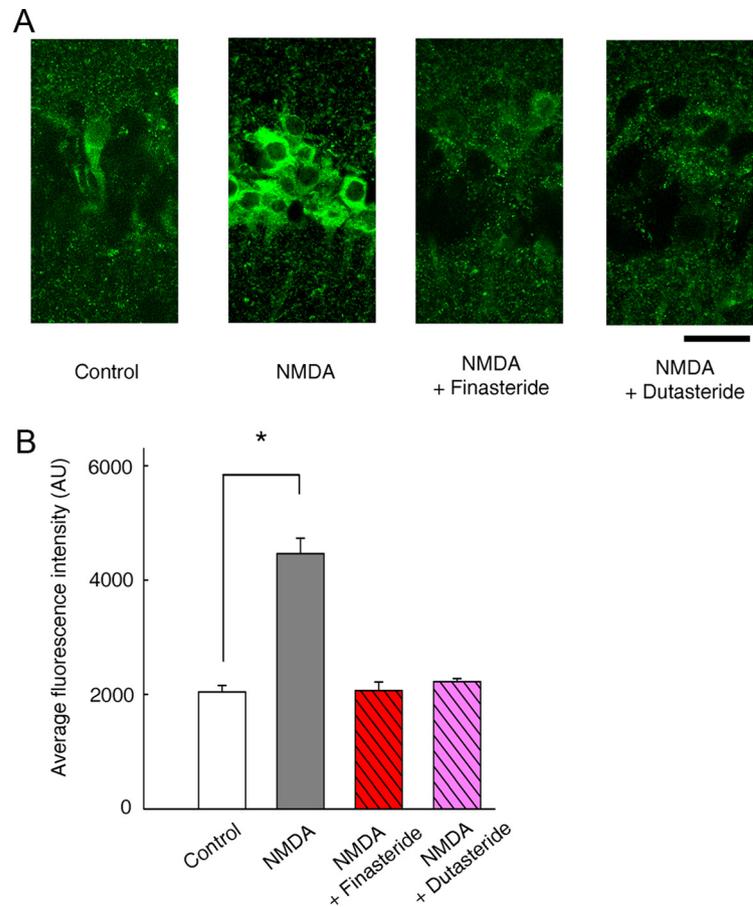
The present results support the idea that ethanol's effects on both neurosteroidogenesis and LTP result from activation of unblocked NMDARs. Indeed, we found that both effects of ethanol were prevented by complete NMDAR inhibition. Moreover, we observed that brief administration of  $1 \mu\text{M}$  NMDA in the presence of extracellular magnesium is sufficient to facilitate neurosteroidogenesis in CA1 pyramidal neurons and block LTP. While we did not examine the concentration–response for NMDA's

effects on steroid immunostaining, we found that low-level NMDAR activation is sufficient to increase neurosteroid staining in pyramidal neurons. Both effects of NMDA are also reversed by NMDAR antagonism (Izumi et al., 1992a,b) and 5 $\alpha$ -reductase inhibitors, providing strong parallels to the effects of ethanol. These results further suggest that low-level but persistent NMDAR activation is an important regulator of local neurosteroid production and synaptic plasticity, acting through an as yet unknown signaling pathway that could include calcineurin, nitric oxide synthase, and p38 MAP kinase, molecules previously linked to NMDAR-mediated LTP inhibition (Izumi et al., 2008). We note, however, that a recent study found that ethanol facilitates neurosteroid production *in vivo* through release of adrenocorticotrophic hormone and synthesis of steroidogenic acute regulatory protein (Boyd et al., 2010). Thus, mechanisms other than direct regional synthesis via NMDAR activation contribute to ethanol's overall effects on neurosteroid generation. Furthermore, ethanol alters release of other signaling molecules, including GABA, and these agents could also contribute to modulation of LTP (Siggins et al., 2005). Additionally, the effects of ethanol on LTP take longer than NMDA (~15 min compared to 5 min), and thus there are some differences between these agents in their metaplastic actions.

We conclude that ethanol, despite being a partial NMDAR antagonist (Izumi et al., 2005), inhibits LTP induction via enhancement of neurosteroidogenesis triggered by activation of unblocked NMDARs in CA1 hippocampal pyramidal neurons. Thus, acute ethanol-induced LTP inhibition appears to represent a form of NMDAR-mediated metaplasticity (Izumi et al., 1992a,b). These results suggest novel strategies to prevent the cognitive impairment resulting from acute ethanol intoxication and perhaps to prevent the longer-term cognitive consequences of alcoholism.

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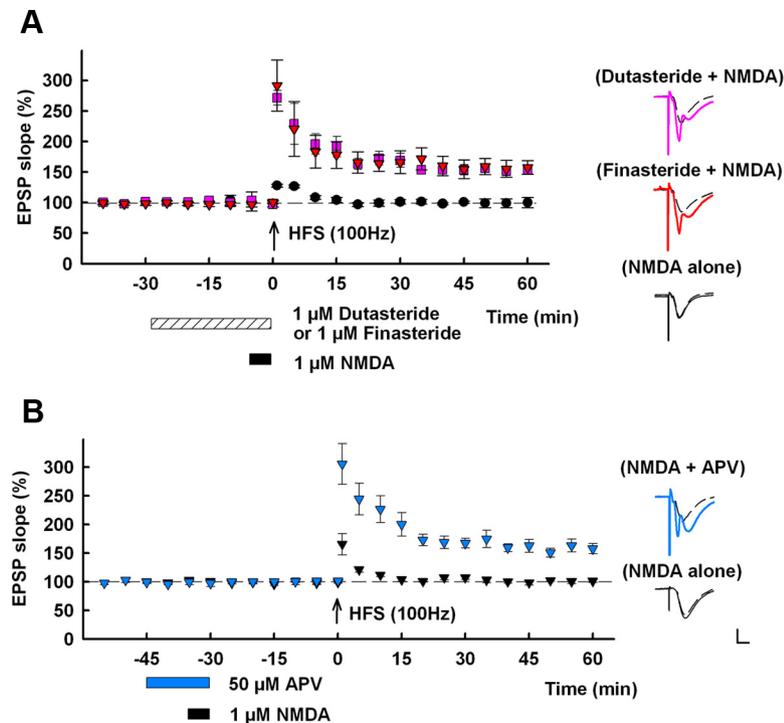
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**Figure 3.** NMDA promotes neurosteroidogenesis and inhibits LTP via effects on neurosteroids. **A**, In naive slices, cell bodies of CA1 pyramidal neurons were diffusely positive for 5 $\alpha$ -reduced steroids. The staining was enhanced when incubated with 1  $\mu$ M NMDA for 5 min but was inhibited by pretreatment with 1  $\mu$ M finasteride or with 1  $\mu$ M dutasteride. Scale bar, 25  $\mu$ m. **B**, Summary of immunohistological studies shows fluorescence intensity as mean  $\pm$  SEM ( $n = 5$ ).  $p$  value was calculated by Holm–Sidak *post hoc* method; \* $p < 0.001$ .

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**Figure 4.** NMDA-mediated LTP inhibition involves neurosteroidogenesis. **A**, Five minute treatment with 1  $\mu$ M NMDA (black bar) before a high-frequency stimulation (HFS, arrow) was sufficient to block CA1 LTP (black triangles). This LTP inhibition was overcome by 1  $\mu$ M finasteride (red triangles) or 1  $\mu$ M dutasteride (pink squares). **B**, The effects of 1  $\mu$ M NMDA on LTP inhibition lasted at least 30 min following washout (black triangles). Coadministration of APV (blue bar) overcame NMDA's inhibition of LTP (blue triangle). Traces depict EPSPs before (dashed lines) and 60 min after HFS (solid lines). Calibration, 1 mV, 5 ms.

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