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The involvement of the nicotinic acetylcholine system in early LTP consolidation

DISSERTATION

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DOCTOR OF PHILOSOPHY

in Biological Sciences

by

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ABSTRACT OF THE DISSERTATION

The involvement of the nicotinic cholinergic system in early LTP consolidation

By

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Doctor of Philosophy in Biological Sciences

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Professor Katumi Sumikawa, Chair

Long-term potentiation (LTP) shows memory-like consolidation and thus becomes increasingly stable over time. LTP quickly becomes resistant to disruption by non-LTD-inducing paradigms such as low frequency stimulation (LFS). However, it is known that nicotine application during LFS uniquely depotentiates consolidated LTP. Here, we investigated how nicotine allows LFS to disrupt stabilized LTP. We found that selective removal of hippocampal cholinergic inputs allowed for depotentiation of consolidated LTP in the absence of nicotine, implying that nicotine depotentiates consolidated LTP by preventing endogenous ACh signaling. Moreover, that α7 nicotinic acetylcholine receptor (nAChR) antagonists mimicked the effect of nicotine, indicating that nicotine's effect occurs through α7 nAChR desensitization. Furthermore, nicotine-induced depotentiation does not involve long-term depression mechanisms and requires the activation of GluN2A-containing NMDARs. We further examined whether nicotine-induced depotentiation involves the reversal of LTP mechanisms. Phosphorylation of Ser-831 on the GluA1 subunit of AMPARs increases single-channel conductance. LTP causes increased phosphorylation of Ser-831. This phosphorylation remained unchanged after nicotine-facilitated

depotentiation. LTP involves the insertion of new AMPARs into the synapse; additionally, the internalization of AMPARs is associated with dephosphorylation of Ser-845 on GluA1 and caspase-3 activity. Nicotine-induced depotentiation occurred without dephosphorylation of the Ser-845 site and in the presence of a caspase-3 inhibitor. LTP is also accompanied by increased filamentous actin (F-actin) and spine enlargement. Nicotine-induced depotentiation was prevented by jasplakinolide, which stabilizes F-actin, suggesting that nicotine depotentiates consolidated LTP by destabilizing F-actin. Our results demonstrate a new role of nicotinic cholinergic system for protecting potentiated synapses from depotentiation via the interaction of α7 nAChR- and GluN2A-NMDAR-mediated signaling for modulating actin destabilization.

INTRODUCTION

Ribot's law of retrograde amnesia states that brain damage impairs recently formed memories to a greater extent than older memories (Ribot, 1881). These findings gave birth to the idea that memories are at first fragile and become increasingly stable over time through the process of consolidation. In 1900, Müller and Pilzecker found that new learning disrupts previously learned information. This disruption was greater when the time interval between the two learning bouts was minimized. These findings provided the first experimental support for the idea of memory consolidation. For more than 100 years, the notion that memories consolidate has gained experimental support (reviewed in McGaugh, 2000; Nadel et al., 2012). While memories are cognitive phenomena, they are believed to be created and stored by changes in synaptic strength (Barnes et al., 1994; Abel et al., 1998; Malleret et al., 2001; Matynia et al., 2002; Martin and Morris, 2002; Lee et al., 2003; Gruart et al., 2007; Madroñal et al., 2007, Keifer and Zheng, 2010; Martin and Morris, 2002).

Synaptic strength can be increased, known as potentiation, or decreased, referred to as depression. Long lasting forms of synaptic plasticity are known as long-term potentiation (LTP) and long-term depression (LTD). These long-term changes play important roles in learning and memory (Bliss and Collingridge, 1993; Martin and Morris, 2002; Malenka and Bear, 2004; Whitlock et al., 2006; Gruart and Garcia., 2007; Madroñal et al., 2007). In particular, LTP has several of the key features required to be a molecular correlate of learning and memory: LTP can be induced by learning, inhibiting LTP disrupts learning, and enhancing LTP enhances some forms of memory (Barnes et al., 1994; Abel et al., 1998; Malleret et al., 2001; Matynia et al., 2002; Lee et al., 2003; Guart et al., 2006;

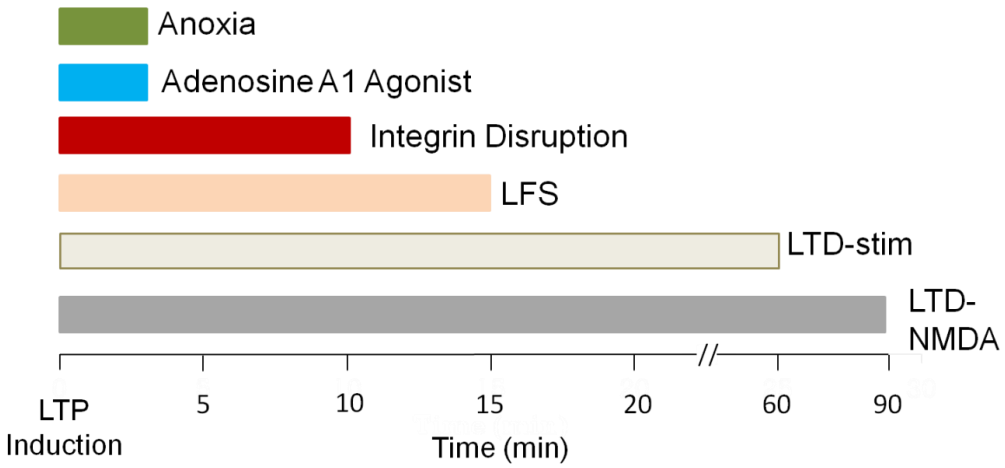
Whitlock et al., 2006; Gruart et al., 2007; Shukla et al., 2007). Importantly, LTP can last for hours in-vitro (Sajikumar and Frey, 2004) and up to months in-vivo (Abraham et al., 2002). Although optimal conditions allow LTP to persist for prolonged periods, LTP is vulnerable to disruption immediately after its induction (O'Dell and Kandel, 1994; Stäubli and Chun, 1996). Like memory, LTP goes through a process of consolidation, becoming increasingly difficult to disrupt over time.

Synaptic strength is measured by analyzing the response of one neuron to another. When a presynaptic neuron releases glutamate, it causes the opening of glutamatergic AMPA receptors (AMPARs). Once open, AMPARs allow an influx of Na^+ ions into the postsynaptic dendrite. These Na^+ ions cause a depolarization known as an excitatory-post-synaptic potential (EPSP). Changes in synaptic strength cause changes to AMPAR responses. LTP induction leads to increased kinase activity, which results in increased AMPAR current, increased AMPAR synaptic expression, and increased spine size (Benke et al., 1998; Hayashi et al., 2000; Yuste and Bonhoeffer, 2001; Malenka and Bear., 2004; Matsuzaki et al., 2004; Makino and Manilow, 2009). These changes are believed to be key mediators of LTP expression.

LTP has at least two consolidation phases. The initial consolidation, which does not require protein synthesis, protects LTP from removal by several treatments including anoxia, low-frequency stimulation, integrin disruption, and adenosine A1 receptor agonists (Arai and Lynch, 1990; Stäubli and Chun., 1996; Huang and Hsu, 1999; Fujii and Kato, 1991; Huang et al., 2001; Kramár et al., 2006). These paradigms, which are relatively weak, do not induce LTD in a naive slice, but can cause depression in a previously potentiated slice (Stäubli and Chun., 1996; Yang et al., 2008). The depression of a previously potentiated

slice is referred to as depotentiation (DP). In order for non-LTD-inducing treatments to cause DP, they must be applied early after LTP induction (E-DP) (O'Dell and Kandel, 1994; Stäubli and Chun, 1996). As consolidation occurs, each of these paradigms gradually become less effective (Fig. 1). Within 30 minutes of LTP induction, these disruptive methods become completely ineffective (Arai et al., 1990; Fujii et al., 1991; Larson et al., 1993; Stäubli et al., 1998; Huang et al., 1999; Fujii et al., 1991; Guan et al., 2006). Thus, the first consolidation phase reaches completion within 30 minutes.

Although LTP becomes more difficult to disrupt during the first consolidation phase, consolidated LTP can still be disrupted by at least two separate methods. Inhibiting protein synthesis will cause LTP to decay to baseline within a few hours of LTP induction (Fonseca



Adapted from:
Arai et al., 1990; Huang et al., 1999; Stäubli et al., 1998; Yang et al., 2008; Massey et al., 2004

Fig. 1. LTP becomes resistant to disruption by non-LTD-inducing paradigms within 30 minutes. Each bar represents the maximum time after LTP induction that a given treatment can be applied to successfully induce depotentiation. Anoxia (green) and adenosine A1 receptor agonist (blue) can only induce depotentiation if applied less than five minutes after LTP induction. Integrin disruption (red) occurring within 10 minutes after LTP induction leads to depotentiation. LFS (salmon) applied up to 15 minutes after LTP induction can cause DP. LTD-inducing paradigms such as LTD-stimulation (light grey) or chemical LTD (dark grey) can cause DP even 30 minutes after LTP induction.

et al., 2004; Zhang et al., 2009). However, the need for new protein synthesis during LTP has recently been challenged (Lynch et al., 2014). Stimulation can also be used to induce depotentiation of consolidated LTP. The strength of the stimulation is what dictates whether consolidated LTP will be disrupted. Stronger paradigms, which induce LTD in naive slices, can depotentiate consolidated LTP (LTD-DP) (Lee et al., 2000; Fujii and Sumikawa, 2001; Jouvenceau et al., 2003) (Fig. 1). Non-LTD-inducing stimulation can only induce DP if applied within 30 minutes of LTP induction, while LTD-inducing stimulation can lead to DP when given 30 minutes after LTP induction.

What are the changes occurring during consolidation that prevents non-LTD inducing stimulation from causing depotentiation? Studies comparing the effect of E-DP inducing stimulation before and after the consolidation phase have rarely been done; when done, these studies have focused primarily on morphological changes (Kramár et al., 2006; Yang et al., 2008; Rex et al., 2009). LTP induction is accompanied by an increase in spine size (Yuste and Bonhoeffer, 2001; Yang et al., 2008; Bellot et al., 2014; Bosch et al., 2014). LFS applied minutes after LTP induction, which induces E-DP, causes a reduction in spine size (Yuste and Bonhoeffer, 2001; Yang et al., 2008; Rex et al., 2009; Bellot et al., 2014; Bosch et al., 2014). However, the same LFS applied after the completion of LTP consolidation, which does not induce E-DP, does not affect spine size (Yang et al., 2008; Rex et al., 2009). These findings indicate that E-DP reduces spine size and that consolidation prevents the morphological changes associated with E-DP. The effect of LTD-DP on morphological changes has not been directly investigated. Aside from consolidation possibly preventing spine shrinkage, it is currently not known what other changes occur during LTP consolidation.

Neuromodulators such as acetylcholine (ACh) can enhance or inhibit memory (Power et al., 2003; Boccia et al., 2010; Blake et al., 2014; Grogan et al., 2015; Osborne et al., 2015; Reichenbach et al., 2015). The cholinergic system is divided into the muscarinic and nicotinic (nACh) subgroups. The muscarinic system is composed of G protein-coupled receptors, while the nicotinic system contains several subtypes of ligand-gated ion-channels. Both the nicotinic and muscarinic systems play a role in memory and synaptic plasticity (Levin and Torry, 1996; Parent and Baxter, 2004; Deiana et al., 2011; Robinson et al., 2011; Blake et al., 2012). However, I will focus on the effects of the nACh system, as it is known to have effects on LTP consolidation.

Nicotinic acetylcholine receptors (nAChR) are located presynaptically and postsynaptically on GABAergic and glutamatergic neurons (Seguela et al., 1993; Fabian-Fine et al., 2001; Berg and Conroy, 2002; Fayuk and Yakel, 2005). The effect of nAChR on synaptic plasticity depends on the location of nACh activation. nACh activation of CA1 pyramidal neurons simultaneously with a short-term potentiation-inducing stimulation (STP) can produce LTP (Ji et al., 2001). The boost from STP to LTP only occurs when nACh activity leads to a threshold pyramidal neuron depolarization. These results indicate that nACh-induced depolarization of pyramidal neurons can contribute to synaptic plasticity. The activation of presynaptic nAChRs on interneurons increases GABA release (Ji et al., 2001; Sharma and Vijayaraghavan, 2002; Nakamura and Jang, 2010; Marchi and Grilli, 2010; Zappettini et al., 2011). nAChR-induced increase of GABA release is capable of reducing LTP to STP (Ji et al., 2001). As nAChR activity in CA1 pyramidal neurons cause their depolarization and nAChR activation on interneurons increases the probability of

GABA release (Reviewed in McKay et al., 2007), nAChR activity generally controls system excitability. These changes in excitability can enhance or inhibit plastic events.

The nicotinic system modulates synaptic plasticity differently depending on its temporal coordination with neuronal firing. Ge and Dani, 2005 found that nACh stimulation facilitates LTP when provided either one to five seconds prior to stimulation or one second after stimulation. However, if the nACh activity occurred within one second of stimulation LTD was induced (Ge and Dani, 2005). Additionally, the endogenous ACh release can directly induce LTP or LTD depending on millisecond differences between ACh stimulation and presynaptic stimulation. The tightly controlled ACh dependent synaptic plasticity required nAChRs activation (Gu and Yakel 2011). Together, these studies demonstrate the precision and breath of effects the nicotinic system has on the most well studied forms of plasticity.

The nicotinic system can also modulate depotentiation. Nicotine, an agonist of nicotinic acetylcholine receptors (nAChRs), facilitates the loss of consolidated LTP by LFS (Nic-DP) (Guan et al., 2006). Nic-DP is the only method that, when given after the completion of consolidation, allows for depotentiation by a non-LTD inducing stimulation. Due to the wide array of effects, the nACh system can have on synaptic plasticity; it is unknown how similar Nic-DP is to either E-DP or LTD-DP. The mechanisms utilized during Nic-DP that allow for the reversal of consolidated LTP are largely unknown. The major aim of our work is to further the understanding of both upstream and downstream mechanisms utilized by Nic-DP. The results provide insight into the role of ACh in synaptic plasticity as well as in the changes occurring during consolidation and the requirements of depotentiation.

Chapter 1

The hippocampus is heavily innervated by cholinergic neurons. The medial septum provides up to 90 percent of cholinergic afferents to the hippocampus (Dutar et al., 1995). At least a portion of the cholinergic system is preserved in the hippocampal slice preparation, as endogenous ACh is both spontaneously released (Alkondon et al., 1998) and released in response to stimulation (Frazier et al., 1998; Hefft et al., 1999). The endogenous ACh, either released spontaneously or evoked by stimulation, is capable of affecting nAChR located on GABAergic interneurons (Alkondon et al., 1998) and glutamatergic neurons (Hefft et al., 1999). Inhibiting nAChRs in hippocampal slices, modify both LTP and LTD, demonstrating that endogenous ACh is involved with plastic events.

Although nicotine is an agonist of nAChRs, nicotine's effect on nAChRs activity is complex. Short exposure to nicotine causes nAChRs to open and pass current. Prolonged exposure to nicotine causes nAChRs to enter a desensitized state. Once desensitized, receptors no longer increase conductance in response to ligands (Alkondon et al., 2000; Quick and Lester, 2002; Dani and Bertrand, 2007). Via its agonist activity or desensitizing properties, nicotine could enhance or inhibit nAChRs. However, it is not clear if nicotine enhances or inhibits nAChRs during Nic-DP.

Nicotinic receptors are pentameric complexes composed of various subunits (Wada et al., 1989; Alkondon and Albuquerque, 1993; Sargent 1993; Dani and Bertrand, 2007; Yakel, 2014). The nicotinic system contains two major subtypes of receptors, the homomeric α7 (α7 nAChRs) and the heteromeric non-α7 (non-α7 nAChRs) -containing receptors (Alkondon and Albuquerque, 1993; Sargent 1993; Dani and Bertrand, 2007; Shen

and Yakel, 2009). The predominate hippocampally expressed non-α7 nAChR is the α4β2 receptor (Dani and Bertrand, 2007; Placzek et al., 2009). All nAChRs permit cation passage and thus cause depolarization; however, different subtypes of neuronal nAChRs are differentially permeable to Ca^{2+} (Sands and Barish, 1991; Fucile, 2004; Shen and Yakel, 2009). In particular, α7 nAChRs are highly permeable to Ca^{2+}, while most non-α7 nAChRs are less so (Khiroug et al., 2003; Fucile, 2004; Fayuk and Yakel, 2005). The Ca^{2+} influx from α7 nAChRs allows α7 nAChR to modulate signaling in ways that non-α7 nAChRs cannot (Shen and Yakel, 2009).

High Ca^{2+} permeability allows α7 nAChRs to alter internal Ca^{2+} concentrations in three ways (Fig. 2). α7 nAChR can directly allow significant Ca^{2+} influx into the cell (Shoop et al., 2001; Zappettini et al., 2011; Zhong et al., 2013). α7 nAChRs activity can also trigger the opening of voltage-gated-calcium channels (VGCC) via depolarization (Shoop et al., 2001; Fayuk et al., 2005; McKay et al., 2007). Finally, the Ca^{2+} from α7 nAChRs or VGCC can lead to calcium-induced calcium release (CICR) from internal stores (McKay et al., 2007; Barrio et al., 2011; Zhong et al., 2013). Non-α7 nAChRs cause depolarization and can facilitate VGCC opening (Zappettini et al., 2011); however, they do not increase internal Ca^{2+} to the same level as α7 nAChRs and do not directly cause CICR (Dickinson et al., 2007).

Depending on their location, nAChR activity can employ these mechanisms to alter neuronal excitability and molecular signaling in different ways (Fig. 2). Presynaptically located nAChRs are well known for enhancing vesicle release (Berg and Conroy, 2002; Dani and Bertrand, 2007; McKay et al., 2007). Synaptic vesicle release both requires and is induced by Ca^{2+}. Normally, presynaptic depolarization occurring during action potentials opens VGCC allowing Ca^{2+} influx and subsequent vesicle release. Ca^{2+} influx directly from

presynaptically located α7 nAChRs, but not from non- α7 nAChRs, can add to this Ca^{2+}, leading to increased vesicle release (Berg and Conroy, 2002). α7 nAChR-dependent increases in vesicle release occur in both GABAergic interneurons and glutamatergic neurons (Alkondon et al., 1998; Arnaiz-Cot et al., 2008; Marchi and Grilli, 2010). Perisynaptically and postsynaptically expressed nAChRs can also alter intracellular signaling. Synaptically expressed α7 nAChR can directly modulate NMDAR activation and signaling by increasing the NMDA open probability via depolarization (McKay et al., 2007). The additional Ca^{2+} from the α7 receptor or CICR can modulate the signaling pathways triggered by Ca^{2+} entry through NMDARs (Li et al., 2013). Additionally, depolarization from α7 and non-α7 nAChRs can increase intracellular Ca^{2+} concentrations by facilitating VGCCs opening (Shoop et al., 2001; Fayuk et al., 2005; McKay et al., 2007). Presynaptic VGCC opening increases vesicle release and postsynaptic VGCC can lead to CICR (Vijayaraghavan et al., 1992; Sharma and Vijayaraghavan, 2002).

Nic-DP and several other stimulation-induced forms of DP show a dependence on NMDAR activation (Huang et al., 2001; Guan et al., 2006; Li et al., 2007; Zhang et al., 2009). NMDA receptors are tetrameric complexes containing a combination of two GluN1 and two GluN2 subunits. There is a single GluN1 isoform while four GluN2 subunit isoforms (GluN2A, GluN2B, GluN2C, and GluN2D) exist. The GluN2A- and GluN2B-containing NMDA receptors (GluN2A-NMDA and GluN2B-NMDA, respectively) are the most highly expressed in the

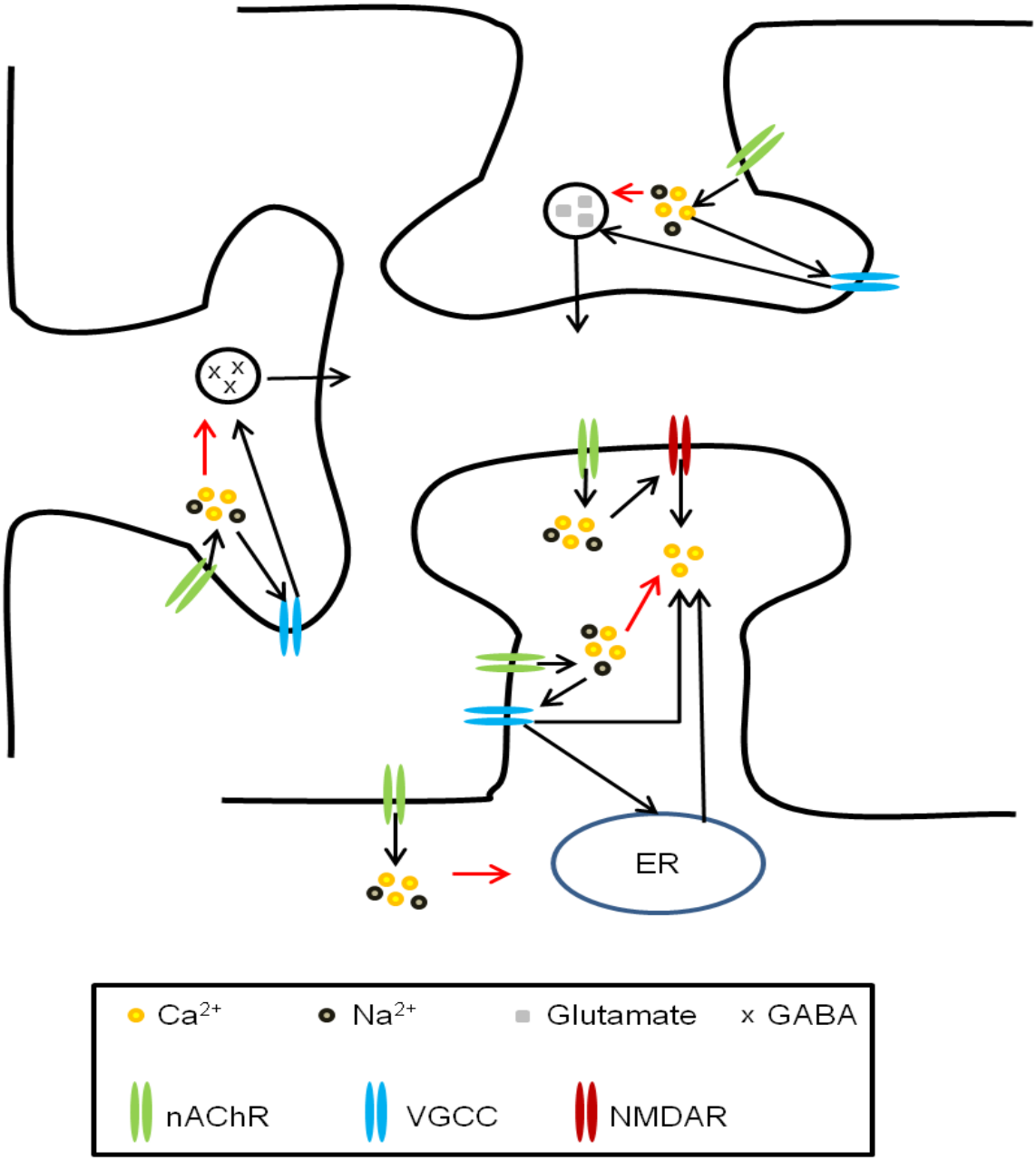


Fig. 2. The modulation of intracellular Ca^{2+} by nAChRs. The opening of nAChRs allows for cation influx. The ratio of Na^{2+} to Ca^{2+} varies by receptor subtype. Black indicates pathways both α7 and non- α7 nAChRs utilize. The cation influx through nAChRs leads to depolarization and allows for increased activity of voltage-sensitive channels such as VGCC and NMDARs. Presynaptic VGCC activity induces vesicle release. Postsynaptically VGCC and NMDAR allow for changes in Ca^{2+} concentration that can alter intracellular signaling. α7 nAChRs allow for a large amount of Ca^{2+} influx. The changes caused Ca^{2+} influx allows for additional signaling (shown by red arrows), which include increased vesicle release, CICR, and directly increasing intracellular Ca^{2+} levels.

hippocampus (Kohr et al., 2003; Liu et al., 2004; Massey et al., 2004; Bartlett et al., 2007; Zhang et al., 2009; Foster et al., 2010). The GluN2 composition of the receptor determines some important receptor properties. The GluN2A-NMDA and GluN2B-NMDA receptors have different channel properties. GluN2A has a higher open probability (Chen et al., 1999; Erreger et al., 2005) and faster kinetics (Monyer et al., 1994; Erreger et al., 2005) than its GluN2B counterpart does. In most cases, the elongated open time leads to GluN2B-NMDA receptors transferring more charge than GluN2A-NMDA receptors (Erreger et al., 2005; Yashiro and Philpot, 2008). Additionally, a larger portion of the charge transferred by GluN2B-NMDA receptors is Ca^{2+} (Sobczyk et al., 2005). The differences in receptor properties, in conjunction with stimulation rate, affect the Ca^{2+} contributions of each receptor subtype. During faster stimulations, such as high-frequency stimulation, the higher channel open probability and faster kinetics allow GluN2A-NMDA receptors to transfer a larger charge than GluN2B-NMDA receptors (Erreger et al., 2005). The relatively slow, low-frequency stimulation, leads to larger current transfer through GluN2B-NMDA receptors (Erreger et al., 2005). The properties of each NMDA receptor allow differential Ca^{2+} contributions during the stimulation that is associated with potentiation and depression.

GluN2A and GluN2B-containing receptors also differ in their associated proteins. GluN2B-NMDARs are associated with CaMKII (Leonard et al., 1999; Strack and Colbran, 1998; Strack et al., 2000), RasGRF1 (Krapivinsky et al., 2003), ERK (Krapivinsky et al., 2003), and Rap1. In general, GluN2B subunits are associated with proteins involved with LTP. The GluN2A subunit is mainly associated with β-catenin and Rap2 (Liu et al., 2004; Barria and Manilow, 2005; Zhu et al., 2005; Yashiro and Philpot, 2008; Fetterolf and Foster, 2011). β-catenin acts as a transcription factor during synaptic activity (Wisniewska, 2013) and Rap2 leads to the internalization of AMPARs (Zhu et al., 2001; Zhu et al., 2005; Yang et al., 2011). The proteins associated with each NMDA subtype make it likely that GluN2A-

Table 1. Comparison of α7 nAChR and non-α7 nAChR signaling properties

	α7 nAChR	Non-α7 nAChR	
Calcium Permeability	High	Low	Seguela et al., 1993; Fabian-Fine et al., 2001; Berg and Conroy, 2002; Fayuk and Yakel, 2005; Jia et al., 2010; Wallace and Porter, 2011
Sodium Permeability	High	High	
Depolarization	Yes	Yes	Berg and Conroy, 2002; Quick and Lester, 2002; McKay et al., 2007
Ability to cause CICR	High	Low	Berg and Conroy, 2002; Zhong et al., 2012; Shen and Yakel, 2009; Sharma and Vijayaraghavan, 2003
Ability to induce VGCC	Yes	Yes	Berg and Conroy, 2002; Barrio et al., 2011; Dickinson et al., 2007

NMDAR activation is involved with depression and GluN2B-NMDA activity with potentiation.

Credited to their differences, the two NMDA subtypes are associated with different forms of synaptic plasticity. While, both GluN2A-NMDARs and or GluN2B-NMDARs contribute to LTP, LTD specifically requires GluN2B-NMDA receptor activation (Massey et al., 2004; Barria and Manilow, 2005; Li et al., 2007; Jin and Feig, 2010). Although LTD-stimulation given to a naive slice requires GluN2B-NMDAR activity to reduce synaptic strength, when LTD-inducing stimulation is given to a previously potentiated slice, the DP induced requires GluN2A-NMDA but not GluN2B-NMDA activity (Massey et al., 2004). This change in specificity likely occurs due to differences in the signaling cascades required for LTD and LTD-DP.

The nicotinic system can modulate synaptic plasticity through changes in neuronal excitability or directly altering signaling cascades. In order to determine the possible molecular pathways utilized during Nic-DP, we must first understand which receptors are key to Nic-DP. It is currently known that Nic-DP involves both nicotinic receptors and NMDA receptor activity (Guan et al., 2006). However, the receptor subtype of nAChR or NMDA involved in Nic-DP is not known. Nicotinic and NMDA receptor subtypes have important differences in their signaling capabilities. The aim of chapter 1 is to further understand nicotinic and NMDA receptor subtype involvement during Nic-DP.

Table 2. Comparison of GluN2A- and GluN2B-NMDA receptor properties and associated proteins

	NR2AR	NR2BR	
Open probability	High	Low	Chen et al., 1999; Erreger et al., 2005
Activation/Deactivation Kinetics	Fast	Slow	Monyer et al., 1994; Erreger et al., 2005
Charge transfer (general)	Low	High	Erreger et al., 2005; Yashiro and Philpot, 2008
Charge transfer (LFS)	Low	High	Erreger et al., 2005
Charge transfer (HFS)	High	Low	Erreger et al., 2005
CaMKII binding	Weak	Strong	Leonard et al., 1999; Strack and Colbran,1998; Strack et al., 2000
RasGRF1	Weak	Strong	Krapivinsky et al., 2003
Rap2	Strong	Weak	Liu et al,. 2004; Barria and Manilow, 2005; Zhu et al., 2005; Yashiro and Philpot, 2008; Fetterolf and Foster, 2011
Plasticity	LTP/DP	LTD	Massey et al., 2004; Barria and Manilow, 2005; Li et al., 2007; Jin and Feig, 2010

Materials and methods

All animal procedures were conducted in accordance with the National Institute of Health Guide for the care and use of laboratory animals and with protocols approved by the Institutional Animal Care and Use Committee at the University of California, Irvine.

Hippocampal Slice Preparation

Transverse hippocampal slices (375 μm) were prepared from P30-50 male Sprague-Dawley rats anesthetized with isoflurane. The brains were harvested and cut in ice-chilled cutting solution containing (in mM): NaCl 85, KCl 2.5, NaH_2PO_4 1.25, $MgSO_4$ 4, $CaCl_2$ 0.5, $NaHCO_3$ 24, sucrose 75 and glucose 25), and maintained at 30–32 °C in oxygenated artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl 124, KCl 5, NaH_2PO_4 1.25, $MgSO_4$ 2, $CaCl_2$ 2.5, $NaHCO_3$ 22, and glucose 10) for at least 1 hour before recording.

Electrophysiology

Slices were placed in a recording chamber, submerged, and continuously perfused with oxygenated ACSF at 30 °C. A NiCr bipolar stimulating electrode was placed to stimulate the Schaffer collateral/commissural pathway. Field EPSPs (fEPSP) were recorded from the stratum radiatum of the CA1 region using glass electrodes filled with 2M NaCl. Stimuli were short current pulses (0.2 ms duration) delivered every 20 seconds. The strength of the stimulus was adjusted to elicit fEPSPs that were 30–50% of the maximum response. The intensity and duration of each stimulus pulse remained invariant thereafter. Recorded signals were amplified (A-M Systems, Sequim, WA, USA), digitized, stored on a computer and analyzed using NAC 2 software (Thetaburst Corp., Irvine, CA, USA). Baseline responses were recorded for at least 15 minutes to establish the stability of slices. LTP was then induced by theta-burst stimulation (TBS, 10 theta bursts with each burst containing 4 pulses at 100 Hz, individual bursts were separated by 200 ms); pulse duration was doubled during TBS. Depotentiation was induced either 6 or 30 minutes after LTP induction by low frequency stimulation (LFS, 5 Hz train for 1 min; three trains were used with the interval

between trains set at 1 min). The baselines were calculated by averaging the final ten minutes of responses before TBS. LTP magnitudes were taken by comparing baseline averages with those from 20-30 min or 55-60 min. The levels of LTP reversal were calculated by comparing the magnitudes of LTP between 20–30 min and 55–65 min (50-60 min in the case of slices used for western blotting) from each slice and expressed as follows: % LTP remaining = (% potentiation after LFS − 100) × 100/(% potentiation before LFS − 100).

Surgery

Rats (P30) were anesthetized with ketamine (60 mg/kg) and Xylene (10 mg/kg), placed in a stereotaxic instrument, and 0.4 μl 192-Saporin (192-Sap; Advanced Targeting Systems; 300) or phosphate-buffered saline (PBS) was infused into the medial septum by four 0.1 μl infusions (from bregma: P 0.5 mm, L ± 0.45 mm, V 7.3 or 6.0 mm). Animals were allowed to recover and maintained for at least 14 days before used in electrophysiology experiments.

Drugs Application

Nicotine (Sigma), the GluN2A-NMDAR selective antagonist NVP (Novartis Pharma) and the GluN2B-NMDAR selective antagonist ifenprodil (Sigma) were dissolved in ACSF and used at concentrations of 1 μM, 50 nM, and 3 μM respectively. Unless stated otherwise, drugs were bath-applied 10 min before and during LFS.

Statistics

Data are expressed as the mean ± SEM. Statistical analysis using one-way ANOVA and a Student's t-test was applied. The overall ANOVA was followed by post-hoc Tuckey HSD test to identify which groups were significantly different.

Nicotine facilitates the depotentiation of consolidated LTP with a non-LTD inducing stimulation

Before investigating the mechanisms of Nic-DP, we first wanted to confirm that our stimulation paradigms successfully induce LTP and do not produce LTD. LFS applied to non-potentiated slices caused a transient reduction in fEPSP. After thirty minutes, responses from slices receiving LFS were no different from those only receiving baseline stimulation (Fig. 3D; % of baseline fEPSP slope after 40-50 minutes recording; baseline (BL) alone, 95.3 ±14.2, n=4 vs. LFS, 100.9 ±5.6, n=5, p=0.7). Thus the LFS used during our experiments does not induce LTD. In order to qualify as LTP, the increase in response must last at least one hour. Theta-Burst stimulation (TBS) induced a potentiation lasting at least 60 minutes (Fig. 3C; % of baseline fEPSP slope 50-60 minutes after TBS; 155 ±9.8% Baseline EPSP). These experiments confirm that the LFS we used does not induce LTD and that LTP is successfully induced by TBS.

We next wanted to properly define the consolidation window and confirm the ability of bath applied nicotine with LFS to disrupt consolidated LTP. LFS applied thirty seconds or six minutes after the TBS lead to a nearly complete depotentiation (Fig. 4A, B; % of baseline fEPSP slope 20-30 minutes after LFS; 30-second LFS, 107.7 ±9.8%, n=5; six minutes, 113.4 ±5.3%, n=6). The same LFS applied thirty minutes after LTP induction caused a short depression that returned to a potentiated state within 20 minutes (Fig. 4C;

% of baseline fEPSP slope 20-30 minutes after LFS; 175.7 ±7.3%); confirming that consolidation is completed with thirty minutes after TBS is applied. The addition of nicotine (1 μM) ten minutes prior to and during LFS, permitted a significantly greater reduction of LTP than LFS alone

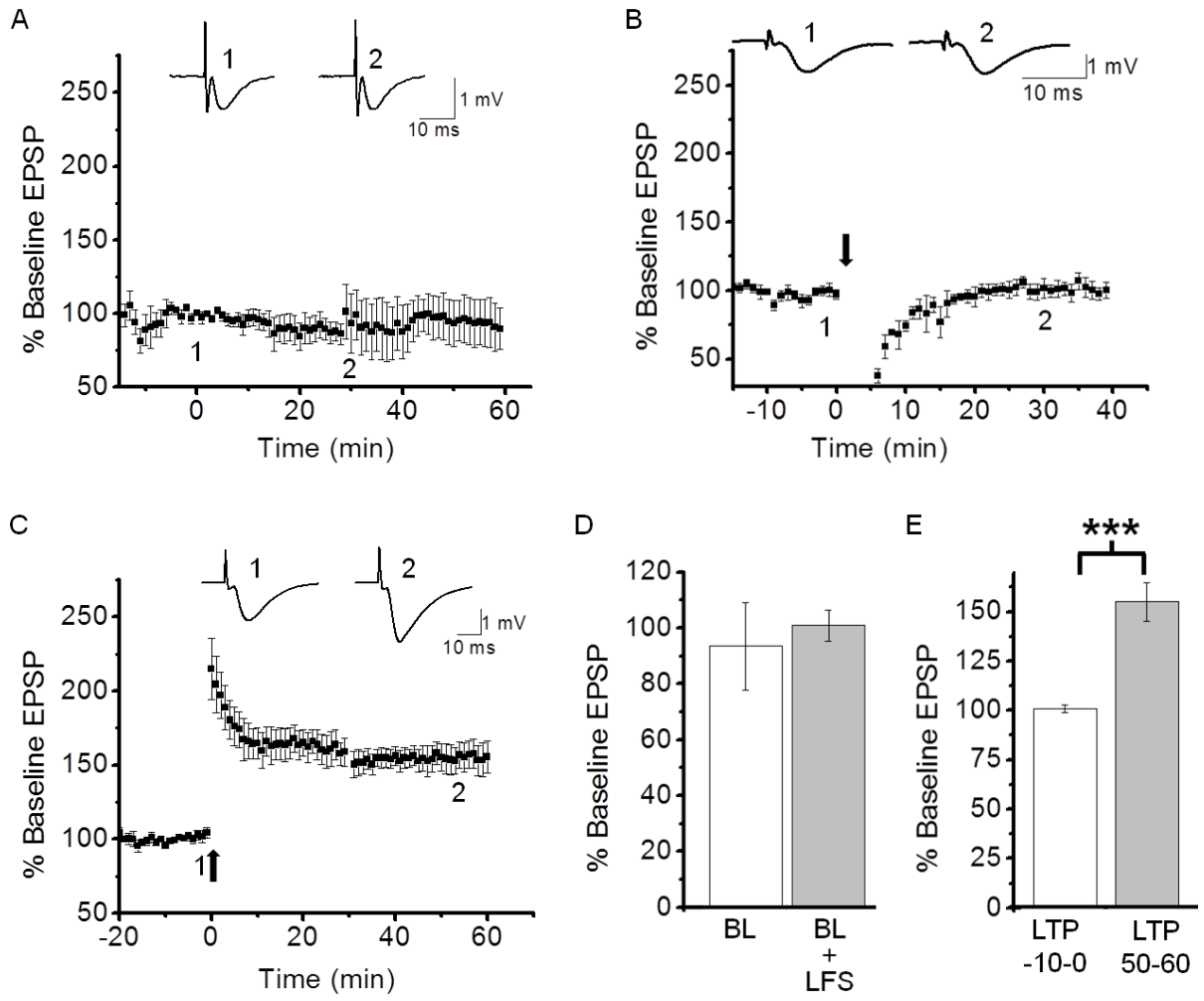


Fig. 3. LFS and LTP induction A. Recordings can be held consistent for at least 75 minutes. B. Delivery of LFS did not result in LTD in the naive hippocampal CA1 region. C. Delivery of TBS resulted in potentiation lasting for one hour. D. Summary data comparing the fEPSP magnitudes of the baseline stimulated and LFS treated slices. E. Summary data comparing the baseline and potentiated states of slices receiving TBS. In this figure and the following figures, traces above each graph are representative waveforms recorded at the time indicated by the number. LTP-inducing TBS and LFS were delivered at the time indicated by the upward and downward arrows, respectively, *P<0.05

(Fig. 4F; % of LTP remaining; LFS alone, 84.1 ±6.6%, n=5 vs. LFS + nicotine, 22.0 ±8.9%; P=0.0005). These results corroborate the previous finding that co-application of nicotine with LFS allows for the depotentiation of consolidated LTP (Guan et al., 2006).

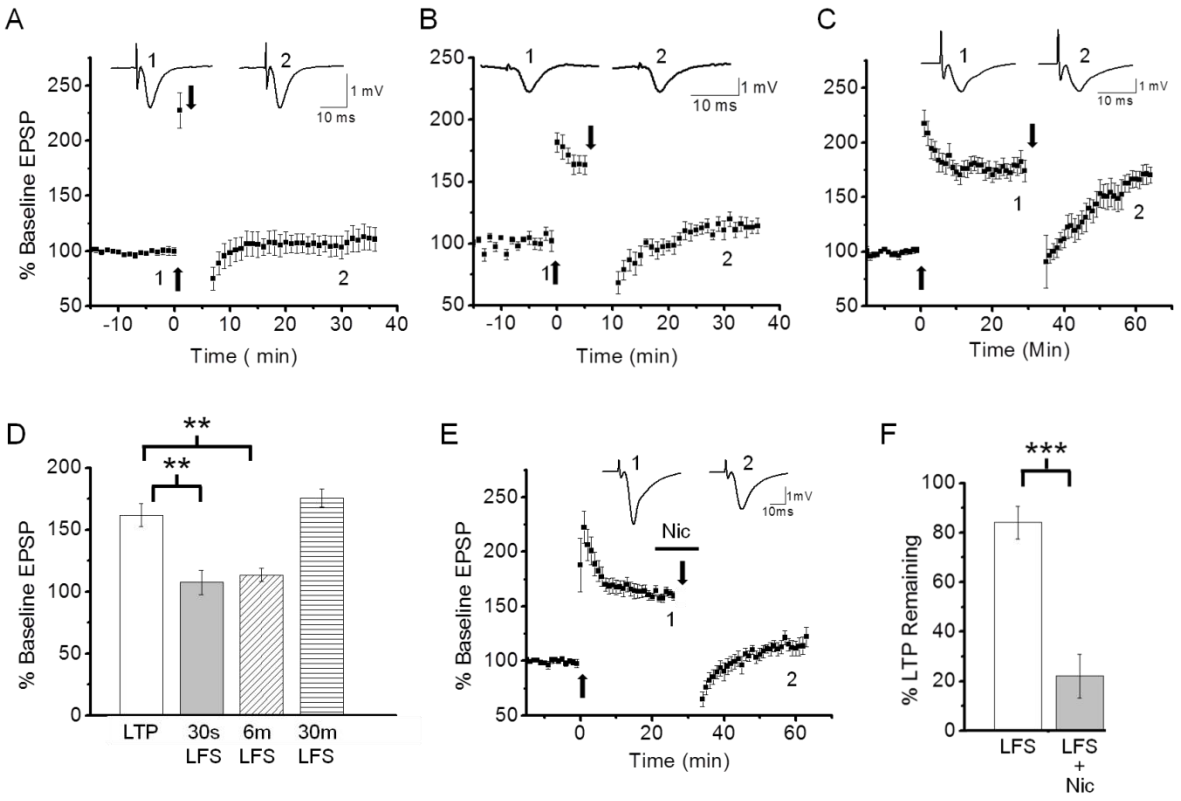


Fig. 4. Nicotine facilitates the loss of consolidated LTP. A-B. Potentiation was lost if LFS was applied either 30 seconds or 6 minutes after LTP induction. C. LFS applied 30 minutes after LTP induction returned to a potentiated state. D. Summary data comparing the magnitudes of potentiation remaining after LFS application 30 s, 6 min, or 30 min after TBS with slices receiving only TBS. E. Nicotine application during LFS depotentiated consolidated LTP. F data comparing LTP remaining after LFS in the absence and presence of nicotine. *P<0.05

Nic-DP is mimicked by antagonism of α7 nAChRs

We next investigated the involvement of nicotinic receptor subtypes during Nic-DP. Because endogenous ACh is present, nicotine may act to desensitize nAChRs, thereby inhibiting endogenous ACh-induced nAChR activity. Thus Nic-DP could occur due to nicotine activating or desensitizing all or a subset of nicotinic receptors. Previous findings

from our lab, indicate that antagonism of α4-containing nAChR, which includes the highly expressed α4β2, using DHβE [100 nM] in conjunction with LFS, did not destabilize consolidated LTP (Guan et al., 2006). Additionally, a DHβE and nicotine cocktail allowed for the loss of consolidated LTP (Guan et al., 2006). These findings indicate that neither nicotine-induced activation, nor inactivation of α4-containing nAChR is required for Nic-DP and suggest that Nic-DP requires changes to α7 nAChR activity. Mecamylamine was used at concentrations that inhibit several nAChR subtypes, including α3β4, α4β2, α3β2, and α7, to determine if general nicotinic antagonism can allow for DP of consolidated LTP. Mecamylamine (mec) [3μM] treated slices depotentiated to a greater extent than LFS alone (Fig. 5A, D; % of LTP remaining; LFS+ mec, 44.5 ±6.0, n=5 vs. LFS alone, 84.1 ±6.6, n=5, p=0.04). Although mec trended towards decreased DP, mec and nicotine treated slices had similar levels of LTP remaining (% of baseline fEPSP slope; LFS + nicotine, 113.4 ±5.82%, n=7 vs LFS + mec, 126.1 ±2.0, n=5, p=0.9). This corroborates previous findings where non-specific nAChR antagonism facilitated the loss of consolidated LTP (Guan et al., 2006). These results indicate that antagonizing the nicotinic system can facilitate DP of consolidated LTP and support the notion that Nic-DP occurs due to decreased nAChR activity.

To test if decreasing α7 nAChR activity is specifically involved with DP of consolidated LTP, two selective α7 nAChRs antagonists (MLA or α-bungarotoxin), were applied ten minutes prior to and during LFS application. Either MLA or α-bungarotoxin (α-BuTx) allowed for greater loss of consolidated LTP than LFS alone (Fig. 5B-D; % of LTP remaining; LFS alone, 84.1 ±6.6, n=5 vs LFS + MLA, 33.7 ±9.7%, n=6, p=0.01 or LFS + α-

BuTx, 27.3 ±9.3%, n=5, p=0.005). Together, these experiments provide evidence that nicotine is likely facilitating depotentiation by inhibiting α7 nAChR activity.

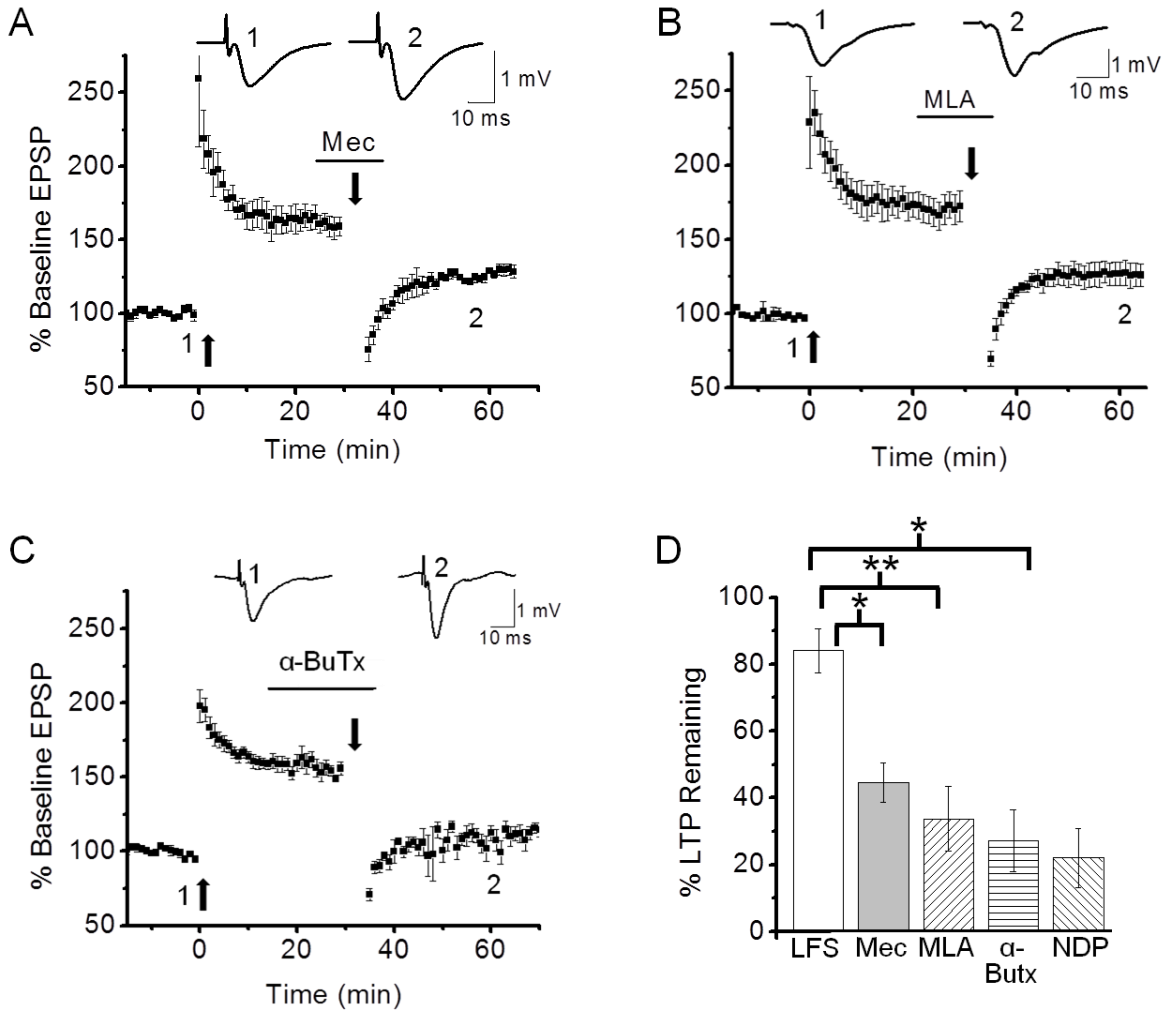


Fig. 5. Antagonists of α7 nAChR facilitate the loss of consolidated LTP. A. The non-selective nAChR antagonist, Mecamylamine, allowed LFS to reverse consolidated LTP. B,C. The selective -α7 nAChR antagonists MLA (B) and α-bungarotoxin (c) facilitated depotentiation by LFS. D. Summary data comparing the amount of LTP remaining after LFS with or without nAChR antagonist. *P<0.05

Endogenous ACh is involved with preventing DP

The pharmacological data indicate that the additional stability achieved during consolidation involves α7 nAChR activity. If this is true, then after consolidation, endogenous ACh release is required for consolidated LTP's elevated resistance to

depotentiation. Thus, slices lacking the major afferent ACh connection should remain vulnerable to disruption 30 minutes after LTP induction. We next tested this possibility. The medial septum, which provides up to 90 percent of cholinergic input to the hippocampus (Dutar et al., 1995), was targeted with the selective cholinergic immunotoxin 192-Sap or PBS (control). 192-Sap is a selective cholinergic toxin that is directed to $p75^{NTR}$, which is a cell-surface antigen only expressed at high levels on cholinergic neurons. Thus, 192-Sap only targets cholinergic neurons while leaving other neuron types in the medial septum unharmed. Once inserted into cholinergic neurons 192-Sap shuts down protein synthesis occurring in ribosomes leading to cell death. Removal of cholinergic afferents was confirmed by the loss of staining for the cholinergic axonal marker acetylcholinesterase (AChE) in hippocampal slices (Fig. 6C-E). Following lesion of the cholinergic projection to the hippocampus, we examined whether LFS alone induces the reversal of consolidated LTP. We found that similar magnitudes of LTP were induced in hippocampi from the PBS-infused and 192-Sap-infused rats (Fig. 7A-C; % of baseline fEPSP slope 20-30 minutes post TBS; PBS, 167.9±5.9%, n=9 vs. 192-SAP, 158.1±8.8%, n=5, p=0.35). However, LFS delivered 30 min after LTP induction reversed LTP in 192-SAP-treated hippocampi, but not PBS-injected control hippocampi (Fig. 7D; % of LTP remaining; PBS, 74.7±6.5%, n=9 vs. 192-SAP, 21.6±11.7%, n=5, p=0.00098). Taken together, our findings strongly suggest that ACh released during LFS protects potentiated synapses from depotentiation via α7 nAChR activation and, that nicotine prevents endogenous ACh signal by inducing α7 nAChR desensitization.

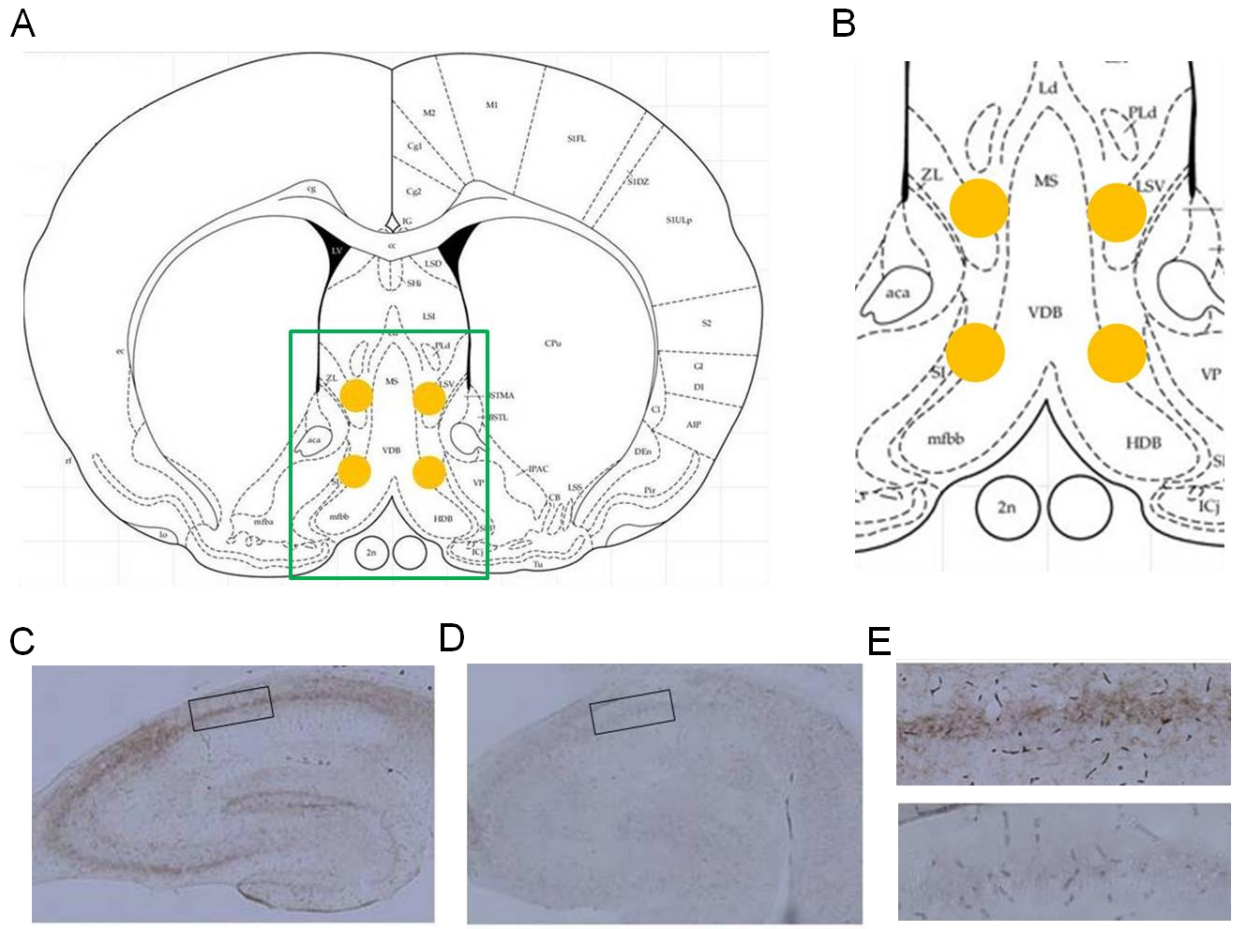
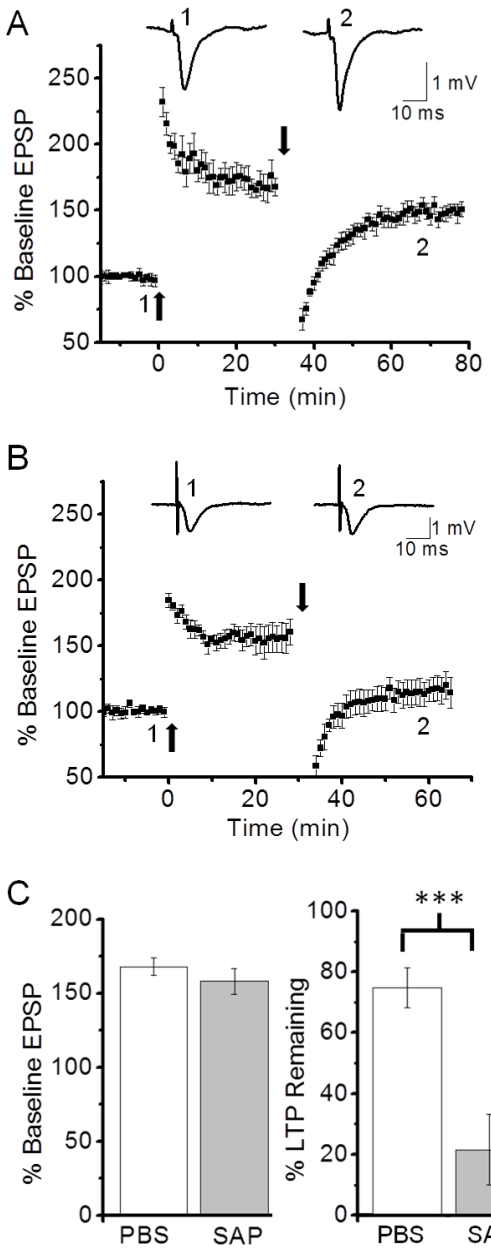


Fig. 6. 192-Saporin infusion animals had reduced hippocampal ACh innervation. A. Schematic illustrating the infusion locations. B. E. Enlarged view of the targeted infusions. B,D Representative images of AChE staining in the hippocampus from PBS (C) or 192-SAP (D) infused rats. E. Enlarged images of AChE staining in the hippocampus region indicated by the black box in (C) and (D) are shown in (E, top) and (E, bottom) respectively.

NMDAR subtype specificity of depotentiation

All activity-dependent forms of depotentiation are dependent on NMDAR activation (Huang et al., 2001; Massey et al., 2004; Zhu et al., 2005; Guan et al., 2006; Li et al., 2007; Zhang et al., 2009). GluN2A-NMDARs and GluN2B-NMDARs are the major subtypes of



NMDAR expressed in the hippocampus (Monyer et al., 1994) and pharmacological evidence

Fig. 7 Endogenous ACh is involved with preventing depotentiation. A. Reversal of consolidated LTP did not occur in the hippocampus from PBS-infused rats. B. LTP and depotentiation of consolidated LTP were induced in the hippocampus from 192-SAP-infused rats. C. Summary data comparing the magnitudes of LTP induced in slices from PBS and 192-SAP infused rats. D. Summary data comparing the effect of LFS on consolidated LTP in hippocampal slices from PBS and 192-SAP infused rats. $P<0.05$

indicates that LTD-DP requires GluN2A-NMDAR activation (Massey et al., 2004; Liu et al., 2004). However, it is largely unknown whether E-DP and Nic-DP also require common GluN2A-NMDAR-mediated signaling. To investigate whether NMDAR-mediated signaling for depotentiation is altered during LTP consolidation, we first investigated the involvement of GluN2A-NMDAR and GluN2B-NMDA receptors in E-DP. In our hands, LFS also did not induce DP ten minutes after LTP induction. However, LFS was effective at inducing DP up to six minutes after LTP induction (Fig. 4B) This six minute gap provided the necessary incubation time for NVP and Ifenprodil, which take effect in seconds to minutes. LFS-induced depotentiation was prevented by NVP (Fig. 8A, C; % of LTP

remaining; LFS alone, 113.4±5.3%, n=5 vs. LFS + NVP, 145.4±6.2%, n=5, p=0.007) but not by Ifenprodil (Figs. 8B, C; % of baseline fEPSP slope; LFS alone, 113.4±5.3%, n=5 vs. LFS + ifenprodil, 115.9% ±5.5, n=5, p=0.99). The present results indicate that the E-DP specifically requires GluN2A-NMDAR activation.

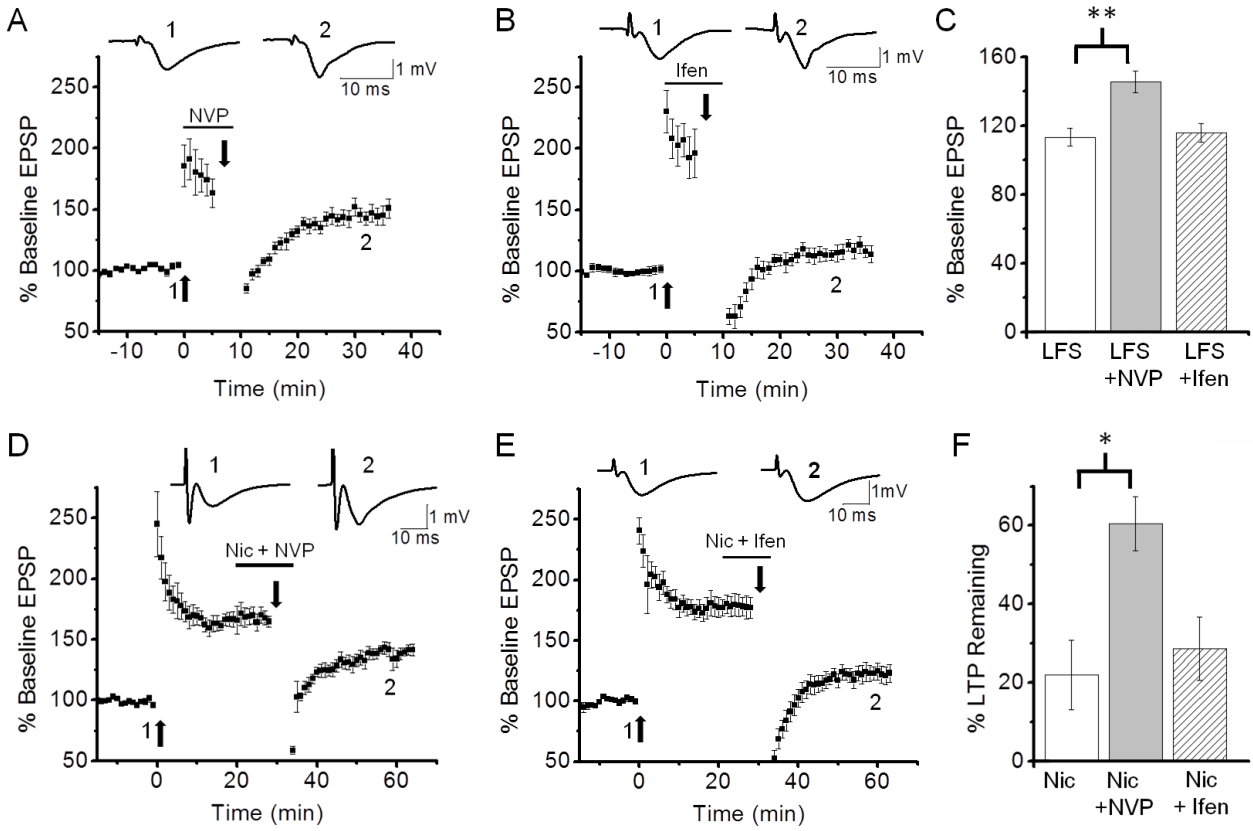


Fig. 8. Depotentiation of unconsolidated and consolidated LTP required GluN2A-NMDAR activation. A-C. LFS-induced depotentiation of unconsolidated LTP was prevented by the GluN2A-NMDAR selective antagonist NVP, but not the GluN2B-NMDAR selective antagonist ifenprodil. LFS was delivered 6 minutes after LTP induction in the presence of NVP (A) or ifenprodil (B). C. Summary data comparing the effects of the antagonists on LFS-induced depotentiation. D-F. Nicotine-induced depotentiation of consolidated LTP was blocked by NVP (D), but not ifenprodil (E). C. Summary data comparing the effects of the antagonists on nicotine-induced depotentiation. *P<0.05.

Nic-DP is blocked by the NMDAR antagonist 2-amino-5-phosphonopentanoate, but not ifenprodil (Guan et al., 2006), indicating the requirement of either GluN2A-NMDAR signaling or the Ca^{2+} influx from either GluN2A- or GluN2B-NMDARs is sufficient for Nic-

DP. To determine the role of GluN2A-NMDAR activation in Nic-DP, we next investigated the effects of a GluN2A antagonist on the nicotine-facilitated reversal of LTP. NVP, which specifically inhibits GluN2A-NMDARs, prevented Nic-DP (Figs. 8D, F; % of LTP remaining; LFS-nicotine alone, 22.0±8.9%, n=7 vs. LFS-nicotine + NVP, 60.5±6.8%, n=5, p=0.025). Additionally, we confirmed that ifenprodil had no significant effect on the reversal of potentiation (Figs. 8E, F; % of LTP remaining; LFS-nicotine alone, 22.0±8.9%, n=7 vs. LFS-nicotine + ifenprodil, 28.6% ±8.1%, n=6, p=0.99). These results provide evidence that Nic-DP specifically requires GluN2A-NMDARactivity.

Discussion

Acetylcholine is an important modulator of many forms of synaptic plasticity and memory (Parent and Baxter, 2004; Deiana et al., 2011; Kenney et al., 2011). Applied alone, LFS does not lead to the DP of consolidated LTP. However, if nicotine is present, LFS can depotentiate consolidated LTP (Guan et al., 2006). We found that Nic-DP is mimicked by antagonism of α7 nAChRs. Furthermore, removing the majority of endogenous ACh afferents to the hippocampus prevented the completion of consolidation within thirty minutes of LTP induction. These findings indicate that nicotine facilitates depotentiation primarily by decreasing α7 nAChRs activity via desensitization. Additionally, we demonstrated that E-DP and Nic-DP requires GluN2A-NMDAR activity. Combined with previous work on LTD-DP (Massey et al., 2004; Zhu et al., 2005; Li et al., 2007), our findings suggest that GluN2A-NMDAR activity is specifically required for stimulation-induced DP. After consolidation, α7 nAChR activity prevents GluN2A-NMDAR-induced depotentiation;

nicotine reduces α7 nAChR activity, allowing for LFS to induce DP via GluN2A-NMDAR signaling.

α7 nAChRs are located dendritically, presynaptically, and perisynaptically on GABAergic and glutamatergic neurons and have high permeabilities to Ca^{2+} and Na^{+} (Seguela et al., 1993; Fabian-Fine et al., 2001; Berg and Conroy, 2002; Fayuk and Yakel, 2005; Wallace and Porter 2011). Regardless of their location, α7 nAChRs modulate both excitability and downstream signaling. Many α7 nAChRs effects are capable of modulating NMDA receptor activity. In order to further understand how α7 nAChRs and GluN2A-NMDA interact during Nic-DP, the location of the α7 nAChRs responsible for Nic-DP must be elucidated.

Our findings suggest that GluN2-NMDAR activity is specifically required for stimulation-induced DP. Why might GluN2A containing NMDARs be particularly important for DP? The GluN2A c-terminal is associated with RAP2 (Liu et al., 2004; Barria and Manilow, 2005; Zhu et al., 2005; Yashiro and Philpot, 2008; Fetterolf and Foster, 2011). RAP2 is theorized to be involved with AMPAR endocytosis, an important mechanism for decreasing synaptic strength (Zhu et al., 2005; Kielland et al., 2009). It is possible that GluN2A-NMDA receptors are important for all forms of stimulation-induce DP because it specifically activates RAP2. However, all studies that have investigated the NMDA specificity during DP have been done in adult animals, which adds a possible experimental confound. As rats age the ratio of GluN2A to GluN2B containing NMDA receptors increases (Monyer et al., 1994; Yashiro and Philpot, 2008). Li et al., 2007, demonstrated that in rats P12-18 an GluN2A-NMDAR antagonist reduced 70-80% of NMDA mediated EPSPs. Li et al., 2007, further found that inhibiting GluN2A-NMDA receptors completely prevented LTP

induction; indicating that the GluN2B-NMDARA receptor activity was not sufficient to induce LTP. However, amplifying GluN2B-NMDAR responses by decreasing Mg^{2+} allowed GluN2B-NMDAR activity alone to induce LTP. Amplifying NMDA responses likely alters Ca^{2+} spread and the bath application of NMDA used in this experiment activates non-synaptic NMDARs, which in themselves can alter synaptic plasticity (Massey et al., 2004; Li et al., 2007; Yashiro and Philpot, 2008; Ai et al., 2011). These issues aside, the authors interpreted their findings as evidence that the receptor that Ca^{2+} passes through is not important only the amount of Ca^{2+} transferred is important.

The idea that only the amount of Ca^{2+} transferred is important is supported by the previous finding that individual GluN2A-NMDARs transfer more charge during high-frequency stimulation, while GluN2B-NMDARs has a higher charge transfer during low-frequency stimulation (Erreger et al., 2005). Remember that GluN2A-NMDARs are important to LTP induction and GluN2B-NMDARs are important to LTD induction, which required high- and low-frequency stimulation, respectively. In young animals, when GluN2B is more prevalent, GluN2B-NMDA is sufficient to induce LTP (Yashiro and Philpot, 2008; Foster et al 2010). GluN2B-NMDA activity is no longer required for LTP in slices from older animals (Foster et al., 2010). Together, these findings provide evidence that the magnitude of Ca^{2+} entering the dendrite affects synaptic plasticity.

However, synaptic plasticity is not dictated purely on the amount of Ca^{2+} entering the dendritic spine. Intracellular binding partners and locations of the NMDA subtypes are also involved with changes in synaptic strength. NMDA binding partners largely depend on the GluN2A or GluN2B c-terminals (Sprengel et al., 1998; Fetterolf and Foster, 2011). Pharmacological evidence has indicated GluN2A-NMDAR activity is sufficient for adult LTP

induction (Massey et al, 2004; Zhang et al., 2009). However, Foster et al., 2010, found that when GluN2B is genetically removed LTP could not be induced. GluN2B reintroduced without the proper c-terminal did not rescue LTP, while chimera GluN2A subunits containing GluN2B c-terminals did recover LTP. This finding provides strong evidence that the GluN2B c-terminal is critical to LTP. This is likely due to the GluN2B c-terminals well-known association with CaMKII (Strack and Colbran, 1998; Strack et al., 2000; Li-Min et al., 2014). CaMKII is well known as a key kinase for LTP induction (Reviewed in Lisman, 2002). When given an enhanced CaMKII interaction, GluN2A subunits can induce LTP in GluN2B-KO animals (Barria and Malinow, 2005). Additionally, mice expressing NMDARs with no the intracellular C-terminal have plasticity issue similar to mice lacking the entire receptor (Sprengel et al., 1998). These findings indicate that the binding partners and interactions with other proteins, which are unique to NMDAR subtype, have an important role in synaptic plasticity.

Studies have indicated that GluN2B-NMDA receptors in adult rats can induce synaptic plasticity (Massey et al., 2004; Fox et al., 2006). Nic-DP was prevented by a GluN2A-NMDA receptor antagonist, but was not affected by a GluN2B-NMDA antagonist. As GluN2A-NMDA accounts for the majority of NMDARs in adult rats decreased Ca^{2+} entry occurring by inhibiting GluN2A-NMDA receptors is greater than that of GluN2B-NMDA inhibition. Thus, the percentage of Ca^{2+} lost could partially account for the subtype specificity. However, the subtype specificity could also be due to GluN2A binding partners. In either case, in the adult rat depotentiation, including Nic-DP requires, GluN2A-NMDA receptor activity.

Chapter 2

Synaptic strength is highly modulated by posttranslational modifications such as phosphorylation. The induction of LTP requires the increased activity of several kinases (Reviewed in Kennedy et al., 2005; Baudry et al., 2014). These kinases phosphorylate a variety of proteins culminating in a persistent increase of AMPA receptor current, synaptic AMPA receptor (AMPAR) expression and an enlarged dendritic spine (Benke et al., 1998; Hayashi et al., 2000; Yuste and Bonhoeffer, 2001; Malenka and Bear., 2004; Matsuzaki et al., 2004; Makino and Manilow, 2009) (Fig. 9). Together, these three changes underlie early LTP expression.

The three changes maintaining LTP expression all affect AMPA receptors. AMPARs are the class of glutamatergic receptor responsible for mediating the majority of post-synaptic excitatory current occurring during synaptic transmission (Lüthi et al., 2004; Kennedy et al., 2005). AMPARs are tetrameric proteins composed of subunits ranging from GluA1 to GluA4. Each subunit first forms dimmers with itself, then two dimmers combine to form a functional AMPAR (Shepherd and Huganir, 2007; Traynelis et al., 2010). Thus AMPAR are composed of no more than two receptor subunits. GluA1/4 AMPARs are expressed in the hippocampus at high levels in young rodents (Luchkina et al., 2014; Cantanelli et al., 2014). However, with age the prevalence of GluA1/4 type AMPAR decreases dramatically (Luchkina et al., 2014). In adult rats, AMPARs are predominately heteromeric with GluA2 forming complexes with either GluA1 (GluA1/2) or GluA3 (GluA2/3) (Wenthold et al., 1996). The presence of the GluA2 subunit is of particular

importance as it prevents AMPARs from passing Ca^{2+}. In adults, the GluA1/2 and GluA2/3 AMPARs mediate depolarization primarily by allowing the entrance of Na^{+}.

AMPAR subunits differ functionally in their intracellular c-terminus. GluA1 and GluA4 contain long C-terminals while GluA2 and GluA3 have a short c-terminus (Bredt et al., 2003; Jiang et al., 2007; Shepherd and Huganir, 2007; Traynelis et al., 2010). These c-terminals contain distinct motifs allowing for different intracellular binding partners; altering trafficking and function of the receptor.

The GluA1 c-terminus contains several phosphorylation sites, including the Ser-831, Ser-845, and Ser-818 sites (Roche et al., 1996; Barria et al., 1997; Mammen et al., 1997, Keifer and Zheng, 2010). Phosphorylation of Ser-831, which is a substrate of CaMKII and PKC, causes an increase in the single-channel conductance of GluA1 containing receptors (Barria et al., 1997; Mammen et al., 1997; Derkach and Soderling, 1999). Increases in both phosphorylation of Ser-831 and an increase in GluA1/2 channel conductance can be seen with LTP induction and maintenance (Benke et al., 1998; Lee et al., 2000). These studies demonstrate that increased phosphorylation of the GluA1 Ser-831 site is associated with LTP induction and imply that the elevated AMPAR conductance occurring during LTP is in part due to the phosphorylation of the Ser-831.

AMPARs are constantly being trafficked into and out of the membrane. AMPAR trafficking involves AMPAR insertion into the membrane, diffusion into the synapse, retention in the synapse, and endocytosis from the membrane (Zhu et al., 2001; Oh et al., 2006; Hanley, 2008; Yang et al., 2008; Makino and Manilow, 2009; Patterson et al., 2010) (Fig. 9). This means that the number of membrane expressed AMPARs can be changed by synaptic activity in at least three ways; synaptic activity can alter the insertion, retention,

and removal of AMPARs. GluA1/2 and GluA2/3 are differentially regulated by synaptic activity. GluA2/3 receptors are involved with basal synaptic transmission (Passafaro et al., 2001; Shi et al., 2000; McCormack et al., 2005). LTD is due at least in part to decreased retention and increased removal both GluA1/2 and GluA2/3 receptors (Beattie et al., 2000; Ahmadian et al., 2004; Holman et al., 2007; He et al., 2011). LTP causes increases in the membrane expression of the GluA1/2 receptor subtype (Heynen et al., 2000; Shi et al., 2000; Zhu et al., 2001; Sheng et al., 2002; Makino and Manilow, 2009). The elevated membrane-bound AMPAR occurring during LTP induction is due to an increased insertion rate, enhanced retention, and decrease removal of GluA1/2 following LTP induction (Heynen et al., 2000; Shi et al., 2000; Zhu et al., 2001; Sheng et al., 2002; Makino and Manilow, 2009; Keifer and Zheng, 2010).

LTP induces an enlargement of the dendritic spine (Yuste and Bonhoeffer, 2001; Yang et al., 2008; Bellot et al., 2014; Bosch et al., 2014) and spine shrinkage is considered a major factor leading to the decrease in synaptic responses occurring during LTD (Okamoto et al., 2004; Yang et al., 2008). Actin is the major cytoskeletal unit composing dendritic spines. Actin exists in either a polymerized filamentous state (F-actin) or a depolymerized globular state (G-actin) (Lang et al., 2004; Bramham, 2008). Increased F-actin is involved with increased spine size while decreasing F-actin shrinks spines (Fukazawa et al., 2002; Zhou et al., 2004; Bosch et al., 2014). The state of actin is believed to control the size of the dendritic spine. Integrin disruption, which destabilized F-actin, and actin polymerization inhibitors cause the destabilization of LTP (Stäubli et al., 1998; Kramár et al., 2006; Yang et al., 2008; Rex et al., 2009; Kim and Lisman, 1999; Ramachandran and Frey, 2009).

Together, these findings indicate F-actin is important for managing spine size and the stability of F-actin is involved with LTP maintenance.

E-DP (O'Dell and Kandel, 1994; Huang et al., 2001; Zhuo et al., 1999; Yang et al., 2008;), LTD-DP (Lu et al., 1996; Lee et al., 2000; Jouvenceau et al., 2003; Jouvenceau et al., 2006;) and Nic-DP (Guan et al., 2006) all require protein phosphatase activity. In opposition to kinase activity, protein phosphatase activity leads to dephosphorylation (O'Dell and Kandel, 1994; Huang et al., 2001; Jouvenceau et al., 2003). Because early LTP expression depends on changes in phosphorylation and activity-dependent depotentiation requires dephosphorylation, it is possible that activity-dependent DP is reversing the changes induced during LTP induction. If the sites phosphorylated during LTP induction are the same as those dephosphorylated during DP, DP would reverse some of the LTP-induced changes returning the slice to a naive state.

Evidence that E-DP and LTD-DP reverse aspects of LTP, come primarily from investigations into individual LTP expression mechanisms. E-DP can reduce spine size (Yuste and Bonhoeffer, 2001; Yang et al., 2008; Rex et al., 2009; Bellot et al., 2014; Bosch et al., 2014) and inhibiting spine depolymerization prevents E-DP and LTD-DP (Yang et al., 2008, Rex et al., 2009; Peng et al., 2010). These findings implicate spine shrinkage as a possible mechanism underlying E-DP and LTD-DP. Both E-DP and LTD-DP are associated with dephosphorylation of Ser-831 (Lee et al., 2000; Huang et al., 2001; Lüthi et al., 2004 Kramár et al., 2006) and LTD-DP has been shown to reduce AMPAR expression (Heynen et al., 2000). AMPAR endocytosis has yet to be directly investigated during E-DP. However, E-DP induction involves pathways that can result in AMPAR endocytosis (Zhu et al., 2005; Yang et al., 2011). LTP induction leads to increases in spine size, AMPAR current and

synaptic AMPAR expression; the above findings provide evidence that E-DP and LTD-DP can reverse the majority LTP-induced changes.

The reversal of LTP mechanisms returns that mechanism to its naive state. Synapses, which have been depotentiated due to a reversal of LTP mechanisms, can be repotentiated by a second LTP inducing stimulation (O'Dell and Kandel, 1994; Stäubli and Chun, 1996). For example, LTP induces AMPAR phosphorylation at the Ser-831 site (Huang et al., 2001; Lu and Roche, 2012). If depotentiation reverses this phosphorylation, returning the site to a dephosphorylated state, then a second LTP-inducing stimulation can phosphorylate Ser-831 again. Depotentiating paradigms that do not reverse LTP mechanisms are unable to be repotentiated (Delgado and O'Dell, 2005).

Although depotentiation is associated with the reversal of LTP mechanisms, depotentiation can occur due to a separate form of depression (Delgado and O'Dell, 2005; Yamazaki et al., 2011). Very few studies have investigated these forms of DP, so they are not well understood. One form of DP, which does not affect LTP mechanisms, is dependent on protein phosphatase (PP) activity and CICR (Yamazaki et al., 2011). The nicotinic system is known to affect CICR (McKay et al., 2007; Barrio et al., 2011; Zhong et al., 2013) and Nic-DP requires PP activity (Guan et al., 2006). It is unknown if Nic-DP reverses LTP mechanisms.

Very little is known about the pathways controlling receptor trafficking during E-DP or LTD-DP. p38 MAPK is involved with E-DP (Liang et al., 2008) as well as with endocytosis of AMPAR during both metabotropic glutamate receptor dependent long-term depression (mGluR-LTD) (Huang et al., 2004) and stimulation-induced LTD (Zhu et al., 2001). p38 MAPK particularly leads to the endocytosis of GluA2/3 containing AMPARs (Zhu et al.,

2001). Although p38 MAPK is involved with E-DP and AMPAR endocytosis, it is unclear if p38 MAPK acts to induce reduce membrane-bound AMPARs during E-DP. Similarly, Rap2 and JNK have demonstrated some involvement with both DP (Zhu et al., 2005; Yang et al., 2011) and AMPAR endocytosis (Zhu et al., 2005). Although Rap2 and JNK have been shown to affect both AMPAR trafficking and depotentiation separately, neither Rap2 nor JNK has been shown to alter AMPAR trafficking during depotentiation. MAPK, Rap2, and JNK are all valid targets for studying DP. However, these proteins may affect AMPAR trafficking, or they could be changing other aspects allowing for depression.

Because it is unknown what mediates changes in AMPAR trafficking during DP, the study of proteins that are specifically involved with AMPAR endocytosis is an appealing option. LTD is accompanied by AMPAR endocytosis (Lee et al., 2000; Lee et al., 2010; He et al., 2011) via dephosphorylation of the AMPAR GluA1 subunit at the Ser-845 site (Man et al., 2007; He et al., 2011) and caspase-3 activity (Li et al., 2010; D'Amelio et al., 2010; Han et al., 2013). Dephosphorylation of Ser-845 decreases GluA1/2 membrane insertion and stability (Boehm et al., 2006; Oh et al., 2006; He et al., 2011). Caspase-3 activity increases during NMDA activity (Keifer and Zhen et al., 2010) and leads to Gap43 dependent AMPAR endocytosis (Han et al., 2013). The involvement of Ser-845 and caspase-3 activity, specifically in AMPAR trafficking makes them good targets for the study of AMPAR trafficking during Nic-DP.

Nicotine facilitates the depotentiation of consolidated LTP with a stimulation that alone would not. Although Nic-DP uses the same electrical stimulation as E-DP, this does not mean that the stimulus will induce a similar form of DP. LTD stimulation given to either a naive or previously potentiated slice lead to the dephosphorylation of two separate sites

on the AMPAR, Ser-845 and Ser-831 respectively (Lee et al., 2000) and required different phosphatase activities (Jouvenceau et al., 2003). Moreover, LTD is accompanied by endocytosis of GluA1 and GluA3 containing AMPAR, while LTD-DP specifically reduces GluA1 containing AMPAR (Heynen et al., 2000). Together, these studies demonstrate that stimulation paradigms can lead to different cellular cascades depending on the synaptic state. Nic-DP, which uses a weak stimulation on a consolidated slice, may decrease synaptic strength by methods distinct from either E-DP or LTD-DP. Additionally, the reduction in α7 nAChR activity occurring during Nic-DP may also influence intracellular signaling (et al., 2001; Zappettini et al., 2011; Zhong et al., 2013). Here, we investigate the major mechanisms of LTP affected by Nic-DP.

Materials and methods

All animal procedures were conducted in accordance with the National Institute of Health Guide for the care and use of laboratory animals and with protocols approved by the Institutional Animal Care and Use Committee at the University of California, Irvine.

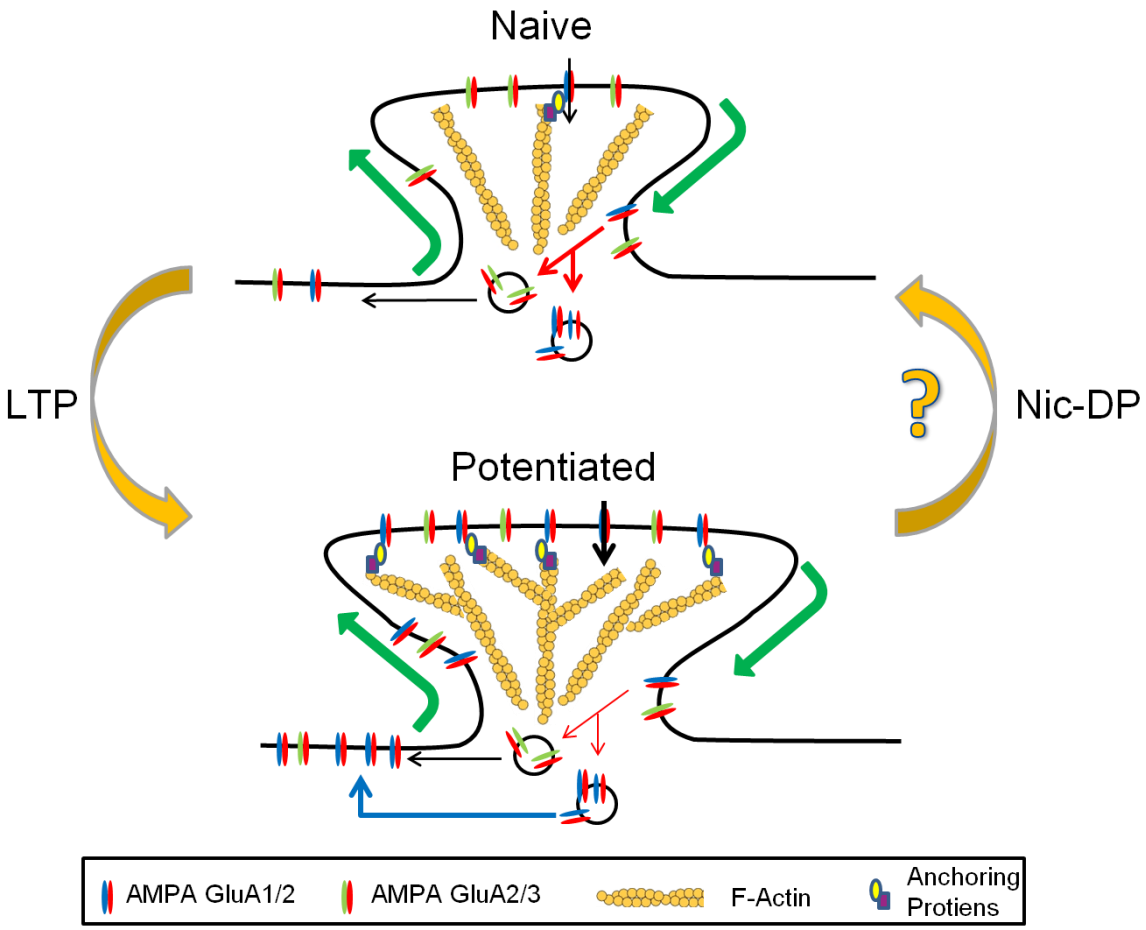


Fig. 9. Summary of LTP mechanisms. Through changes in AMPAR associated proteins and AMPAR phosphorylation LTP increases GluA1/2 AMPA receptor current and synaptic expression. LTP induction is accompanied by an increase in F-actin and dendritic spine size. It is unknown which if any of these changes are reversed during Nic-DP induction.

Hippocampal Slice Preparation

Transverse hippocampal slices (375 μm) were prepared from P30-50 male Sprague-Dawley rats anesthetized with isoflurane. The brains were harvested and cut in ice-chilled cutting solution containing (in mM): NaCl 85, KCl 2.5, NaH2PO4 1.25, MgSO4 4, CaCl2 0.5, NaHCO3 24, sucrose 75 and glucose 25), and maintained at 30–32 °C in oxygenated artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl 124, KCl 5, NaH2PO4 1.25, MgSO4 2, CaCl2 2.5, NaHCO3 22, and glucose 10) for at least 1 hour before recording.

Electrophysiology

Slices were placed in a recording chamber, submerged, and continuously perfused with oxygenated ACSF at 30 °C. A NiCr bipolar stimulating electrode was placed to stimulate the Schaffer collateral/commissural pathway. Field EPSPs (fEPSP) were recorded from the stratum radiatum of the CA1 region using glass electrodes filled with 2M NaCl. Stimuli were short current pulses (0.2 ms duration) delivered every 20 seconds. The strength of the stimulus was adjusted to elicit fEPSPs that were 30–50% of the maximum response. The intensity and duration of each stimulus pulse remained invariant thereafter. Recorded signals were amplified (A-M Systems, Sequim, WA, USA), digitized, stored on a computer and analyzed using NAC 2 software (Thetaburst Corp., Irvine, CA, USA). Baseline responses were recorded for at least 15 minutes to establish the stability of slices. LTP was then induced by theta-burst stimulation (TBS, 10 theta bursts with each burst containing 4 pulses at 100 Hz, individual bursts were separated by 200 ms); pulse duration was doubled during TBS. Depotentiation was induced either 6 or 30 minutes after LTP induction by low frequency stimulation (LFS, 5 Hz train for 1 min; three trains were used with the interval between trains set at 1 min). The baselines were calculated by averaging the final ten minutes of responses before TBS. LTP magnitudes were taken by comparing baseline averages with those from 20-30 min or 55-60 min. The levels of LTP reversal were calculated by comparing the magnitudes of LTP between 20–30 min and 55–65 min (50-60 min in the case of slices used for western blotting) from each slice and expressed as follows: % LTP remaining = (% potentiation after LFS − 100) × 100/(% potentiation before LFS − 100).

Drugs Application

Nicotine (Sigma) was dissolved in ACSF and used at concentrations of 1 μM. Jasplakinolide (200 nM; Tocris) was added immediately after TBS and remained present until the end of the experiment. The caspase-3 inhibitor Z-DEVD-FMK (R&D Systems) was dissolved in DMSO and added to the holding chamber at a concentration of 2 μM for at least two hours. Z-DEVD-FMK was not bath perfused during electrophysiological recordings. Unless stated otherwise, drugs were bath-applied 10 min before and during LFS.

Western Blot Analysis

Sixty minutes after LTP induction, CA1 regions were isolated and immediately placed in 1x SDS solution (1x Tris-Glycine SDS sample buffer + 5% 2-mercaptoethanol) and heated to 95°C. Each CA1 slice was then homogenized by pipette and frozen at -80°C. Homogenates were later thawed and centrifuged for 5 minutes. Samples were loaded into SDS-PAGE gels (4-12% Bis-Tris gel) and run. The resulting gels were transferred onto PVDF membranes. Membranes were blocked for 1 hour in 5% BSA and 0.1% TBST then immersed in anti-phospho-GluA1-S831 (Upstate), anti-phospho-GluA1-S845 (Upstate), or anti-phospho-GluA1-S845 (Cell Signaling). The appropriate secondary antibodies coupled to horseradish peroxidase were used, and immunoreactive bands were visualized using the Pierce Super-signal chemiluminescent substrate (Pierce) and analyzed using Kodak Image Station 400MM Pro with Molecular Imaging Software. Blots were then stripped using a solution of 2% glycogen with 1% SDS (pH 2.0), and reprobed for the total-GluA1 with anti-GluA1 antibody (Upstate). The relative amount of GluA1 phosphorylation was determined by

calculating the ratio of signals (the phosphorylation site-specific-antibody signal/the total GluA1 signal). The ratio was then used for statistical analysis.

Statistics

Data are expressed as the mean ± SEM. Statistical analysis using one-way ANOVA and a Student's t-test was applied. The overall ANOVA was followed by post hoc Tuckey HSD test to identify which groups were significantly different.

Nicotine-induced depotentiation involves the reversal of LTP mechanisms

Depotentiation could occur either by reversing LTP mechanisms or by utilizing a form of depression that does not involve LTP mechanisms. E-DP is known to reverse changes induced by LTP such as AMPAR phosphorylation and shrinking spine size (Huang et al., 2001; Lüthi et al., 2004 Kramár et al., 2006). Because little is known about Nic-DP, we first investigate if nicotine, applied during LFS-induced E-DP, utilizes additional depressive mechanisms not normally employed during E-DP. E-DP can occur if LFS is applied within six minutes after LTP induction (Fig. 4B, D). If nicotine utilizes depressive mechanisms not evoked during LFS alone, then a combination of nicotine and LFS six minutes after LTP induction will induce a greater level of depression than LFS does alone. Six minutes after LTP induction, nicotine applied with LFS did not induce a greater reduction of synaptic strength than LFS alone (Fig. 10A, B; % of baseline fEPSP slope; LFS alone, 113.4±13.0%, n=5 vs. LFS + nicotine, 106.8±4.3%, n=5, p=0.93). The results imply that nicotine facilitates the utilization of the same depressive mechanisms involved with E-DP.

Synapses expressing LTP can be depotentiated by treatments, which reverse LTP mechanisms, and such synapses can be repotentiated by the same LTP mechanisms (O'Dell and Kandel, 1994; Stäubli and Chun, 1996). However, synapses depotentiated by a treatment, which does not reverse LTP mechanisms, cannot be repotentiated (Delgado and Kandel, 2005). If Nic-DP is reversing aspects of LTP, then after undergoing Nic-DP slices receiving a second LTP-inducing stimulation will cause the slice to repotentiate. Application of nicotine (1 μM) during LFS reversed consolidated LTP (Fig. 10C; % of LTP remaining; LFS alone, 84.1 ±6.6%, n=5 vs. LFS + nicotine, 22.0 ±8.9%, n= 7, p=0.0003). We then reapplied LTP-inducing TBS and found that repotentiation was induced to a level similar to the initial potentiation (Fig. 10C, D; % of baseline fEPSP slope; first LTP, 162.2 ±2.9% vs. second LTP, 158.4 ±7.4%, n=5, p=0.64). To ensure that the maximum potentiation was induced by a single TBS, a second TBS was given thirty minutes after the first. We found that the magnitudes of the first and the second LTP were very similar (Fig. 10E, F; % of baseline fEPSP slope; first LTP, 177.1 ±15.7% vs. second LTP, 182.7 ±12.3%, n=5, p=0.79), indicating that the first TBS induced a saturated level of LTP. Cumulatively, these experiments provide evidence that Nic-DP is due to a reversal of LTP mechanisms.

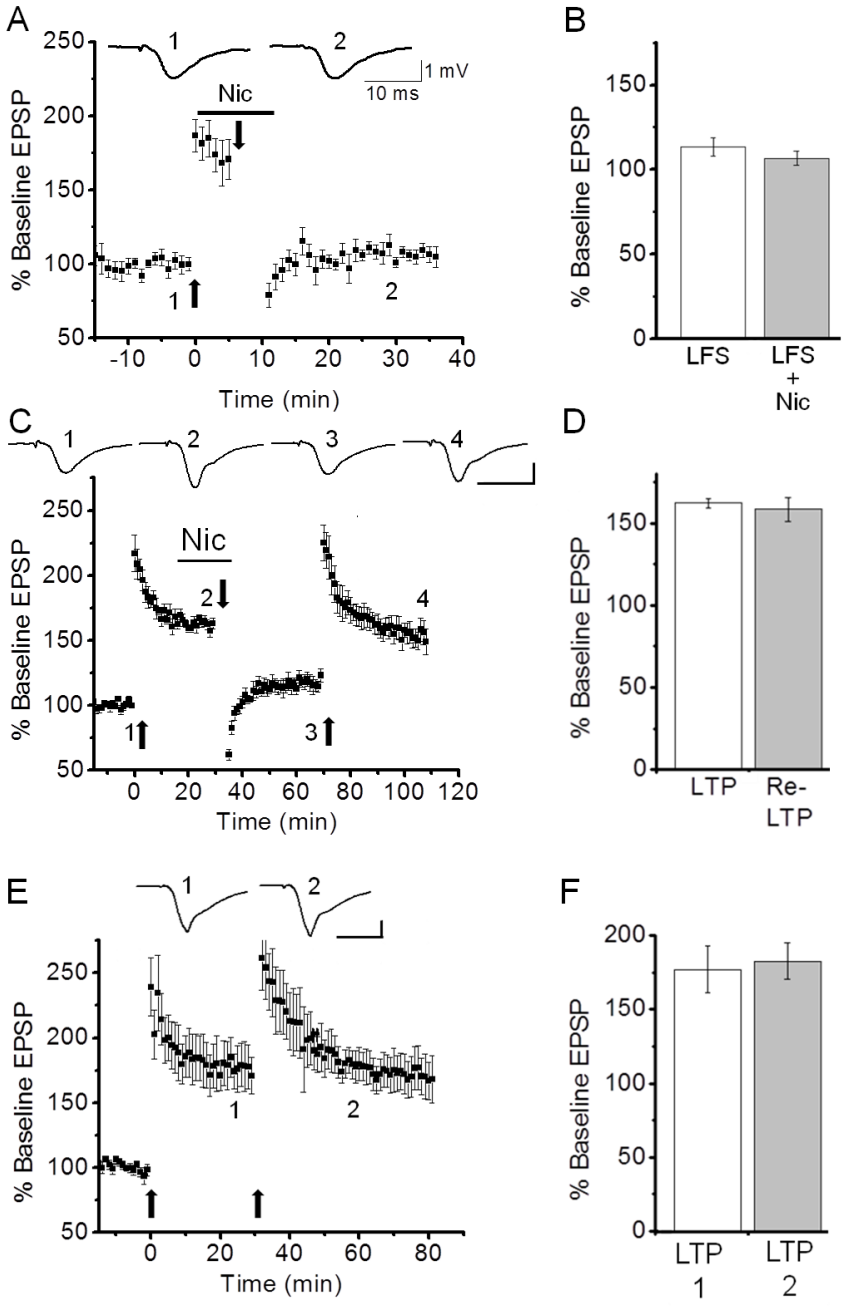


Fig. 10. Nicotine-induced depotentiation occurred by reversing LTP mechanisms . Bath application of nicotine had no effect on LFS-induced reversal of unconsolidated LTP. Six minutes after LTP induction by TBS, LFS was applied in the absence or presence of nicotine. C. LFS had no effect on consolidated LTP. Thirty minutes after LTP induction, LFS was delivered. D. Nicotine application during LFS depotentiated consolidated LTP. E. Summary data comparing LTP remaining after LFS in the absence and presence of nicotine. F. After nicotine-induced depotentiation, reapplication of LTP-inducing TBS caused repotentiation. G. Summary data comparing the magnitudes of the first LTP and the second LTP induced following depotentiation. H. Delivery of the first TBS-induced a saturated LTP. The second TBS application had no further effect on the magnitude of LTP. I. Summary data comparing the magnitudes of the first LTP and the second LTP induced without depotentiation.

Nicotine-induced depotentiation occurs without dephosphorylation of Ser-831 on GluA1 of AMPARs

LTP induction is accompanied by an increase in the single-channel conductance of AMPARs, which is mediated by CaMKII-dependent phosphorylation of Ser-831 on GluA1 of AMPARs. This is thought to be one of LTP expression mechanisms (Huang et al., 2001; Lu and Roche, 2012).

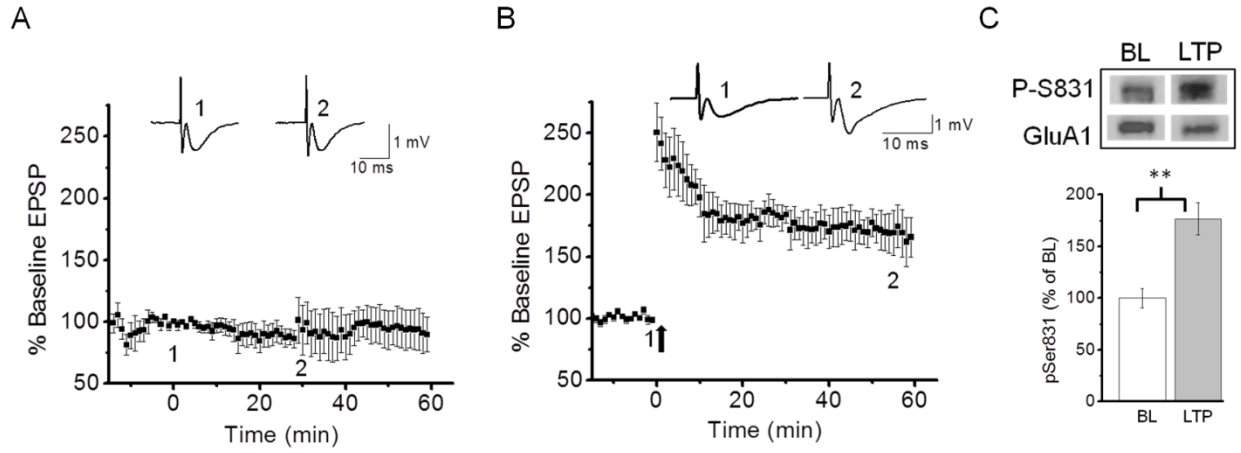


Fig. 11. LTP increases phospho-Ser831 levels. A. Recording from slices receiving only Baseline stimulation. B. Recording from potentiated slices. C. Western blot analysis with anti-GluA1 phospho Ser-831 antibody and anti-GluA1 antibody was carried out with protein samples from CA1 slices, which received baseline stimulation only (BL) or induced LTP (LTP). Representative phospho-Ser-831 (P-S831) and GluA1 bands are shown (top). Phosphorylation at Ser-831 was analyzed by normalizing the signal from phosphorylation site-specific antibody to the total amount of GluA1 measured using anti-GluA1 antibody. Summary data are presented (bottom). $P<0.05$

Because both LTD-DP and E-DP are associated with dephosphorylation of Ser-831 (Lee et al., 2000; Huang et al., 2001), we next investigated whether Nic-DP is also mediated through dephosphorylation of Ser-831. To monitor changes in phosphorylation of Ser-831 after LTP and nicotine-induced depotentiation, we used western blot analysis with anti-GluA1 phospho Ser-831 antibody (pSer-831) and anti-GluA1 antibody. In agreement with

previous studies (Lee et al., 2000; Huang et al., 2001), we found that samples taken from potentiated CA1 slices showed a significant increase in Ser-831 phosphorylation as compared to CA1 slices that received baseline stimulation only (Fig. 11A-C; % change relative to baseline slices; baseline slices, 100±9.4%, n=4 vs. LTP slices, 176.7±15.3%, n=4, p=0.005). These findings indicate that like other forms of LTP, our LTP stimulation induces the phosphorylation of Ser-831.

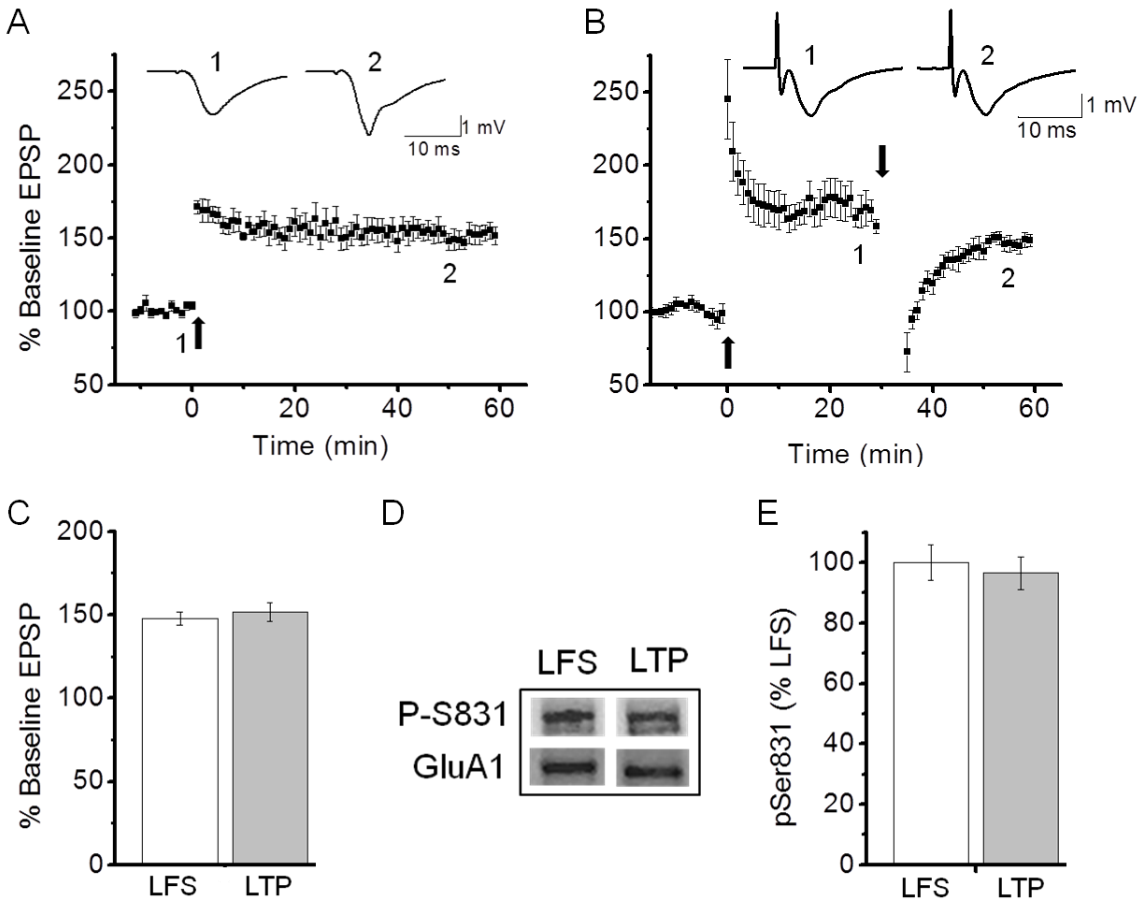


Fig. 12. LFS delivered to potentiated slices did not alter the level of Ser-831 phosphorylation. A. TBS-induced potentiation . B. LFS delivered to potentiated slices did not alter the level of potentiation. C. . LFS had no effect on consolidated LTP. Thirty minutes after LTP induction, LFS was delivered. D. Representative phospho-Ser-831 (P-S831) and GluA1 bands are shown. E. Summary data comparing the ratio of phospho-Ser-831 of LFS and LTP samples

LFS applied within minutes of LTP induction results in DP and is known to reduce Ser-831 phosphorylation (Lee et al., 2000; Huang et al., 2001; Lüthi et al., 2004 Kramár et al., 2006). It is unknown whether consolidation, which prevents LFS from inducing depotentiation, prevents the dephosphorylation of Ser-831 by LFS. If consolidation protects Ser-831 from dephosphorylation, then LFS applied after the consolidation window should not reduce pSer-831. Potentiated slices and those receiving LFS displayed similar levels of potentiation (Fig. 12A-C; % of baseline fEPSP slope 50-60 minutes post TBS; TBS + LFS, 147.8 ±3.8, n= 4 vs. TBS alone, 151.6 ±5.8, n=5, p=0.63). Increased levels of Ser-831 phosphorylation in potentiated (LTP) slices were not altered following LFS (Fig. 12D, E; %

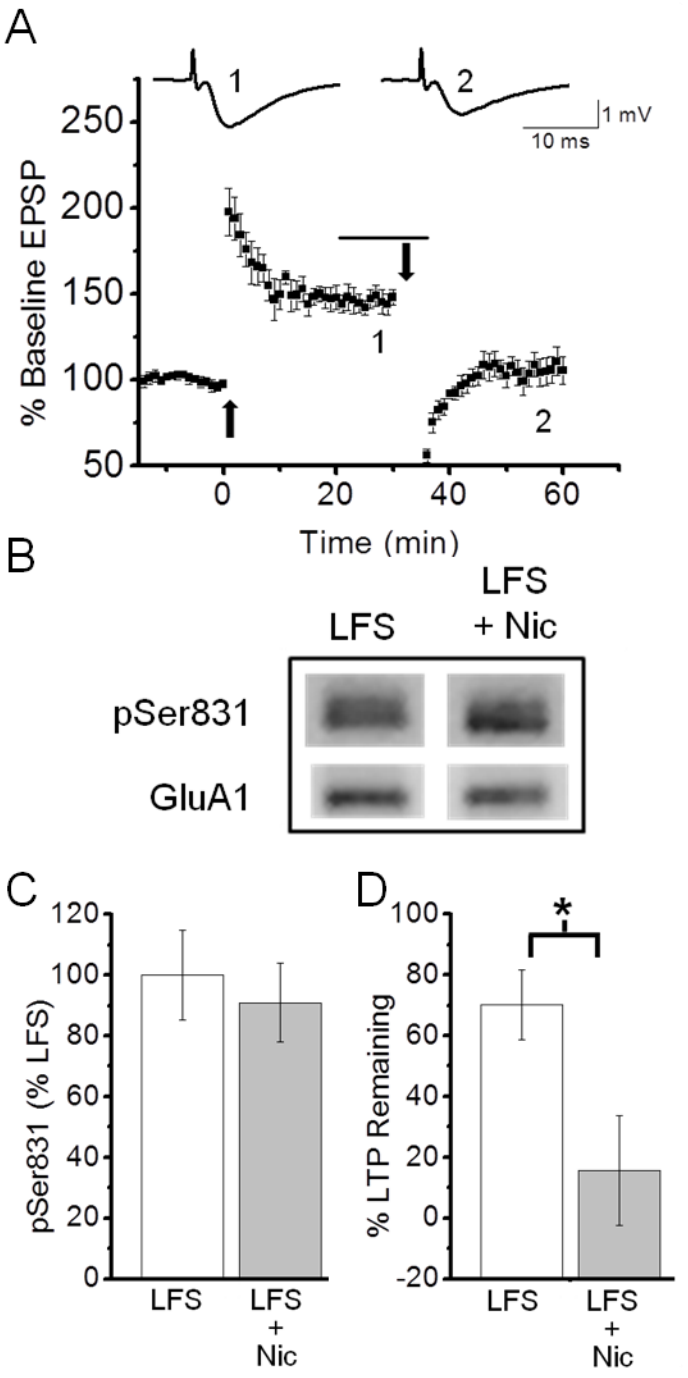


Fig. 13. Nicotine-induced depotentiation was not accompanied by dephosphorylation of Ser-831 on GluA1 of AMPARs. A. LFS and nicotine-induced depotentiation. B. Representative protein bands from LFS alone slices (LFS) and LFS + nicotine slices (Nic-DP) are compared. C. Nicotine-induced depotentiation did not change the level of Ser-831 phosphorylation. D. Summary data comparing the effects of LFS with and without nicotine on LTP. P<0.05

changes relative to LFS slices (LFS slices, 100 ±5.9%, n=4 vs. LTP slices, 96.5 ±5.5%, n=5, p=0.68). These data indicate that LFS is no longer able to dephosphorylate Ser-831 or induce DP once consolidation has occurred.

Nicotine allows LFS to induce depotentiation after LTP consolidation has occurred. If dephosphorylation of S-831 is required for depotentiation, then Nic-DP should decrease pSer-831. When applied 30 minutes after LTP induction, LFS paired with nicotine reduced potentiation to a greater extent than LFS alone (Fig. 13A, D; % of LTP remaining; LFS-alone, 70% ± 11.5, n=4 vs. LFS + Nicotine, 15.6% ±18, n=5, p=0.048, P<0.05); however, their levels of pSer-831 were similar (Fig. 13 B, C; % change relative to LFS alone slices; LFS alone slices, 100±2.1%, n=4 vs. LFS + nicotine, 98.7±10%, n=5, p=0.92). These results strongly suggest that nicotine-induced depotentiation is not mediated by dephosphorylation of Ser-831.

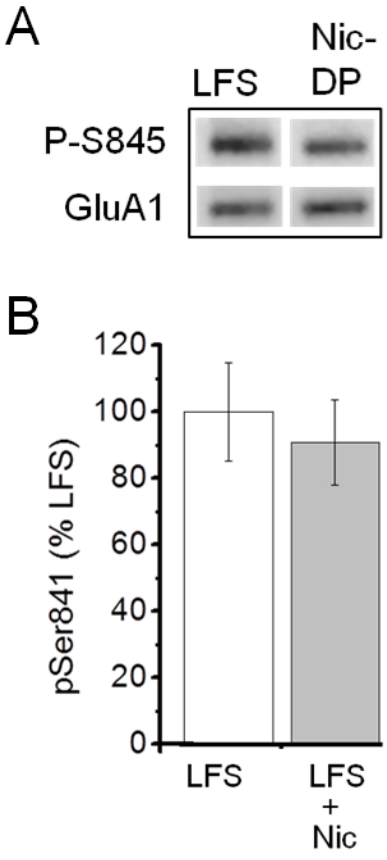


Fig. 14. Nicotine-induced depotentiation occurred without Ser-845 dephosphorylation. A. Representative phospho-Ser-845 (P-S845) and GluA1 bands are shown B. Summary data of phospho-Ser-845 analysis.

Nicotine-induced depotentiation does not involve AMPAR internalization via dephosphorylation of Ser-845 on GluA1 or caspase-3 activity

Dephosphorylation of Ser-845 is associated with AMPAR internalization, which is required for NMDA-dependent LTD (Lee et al., 2000; Lee et al.,

2010; He et al., 2011). Although both LTD-DP and E-DP occur without altering phosphorylation levels of Ser-845 (Lee et al., 2000; Huang et al., 2001), we investigated the possibility that Nic-DP involves dephosphorylation of Ser-845 by western blot analysis with anti-GluA1 phospho Ser-845 antibody and anti-GluA1 antibody. We found that there was no difference in phosphorylation levels of Ser-845 between slices received LFS alone following LTP and those depotentiated by application of nicotine during LFS (Fig. 14A, B; % change related to LFS alone; LFS alone, 100±14.8%, n=5 vs. LFS + nicotine, 91.0±12.9%, n=5, p=0.66). These findings indicate that Nic-DP, like E-DP and LTD-DP, does

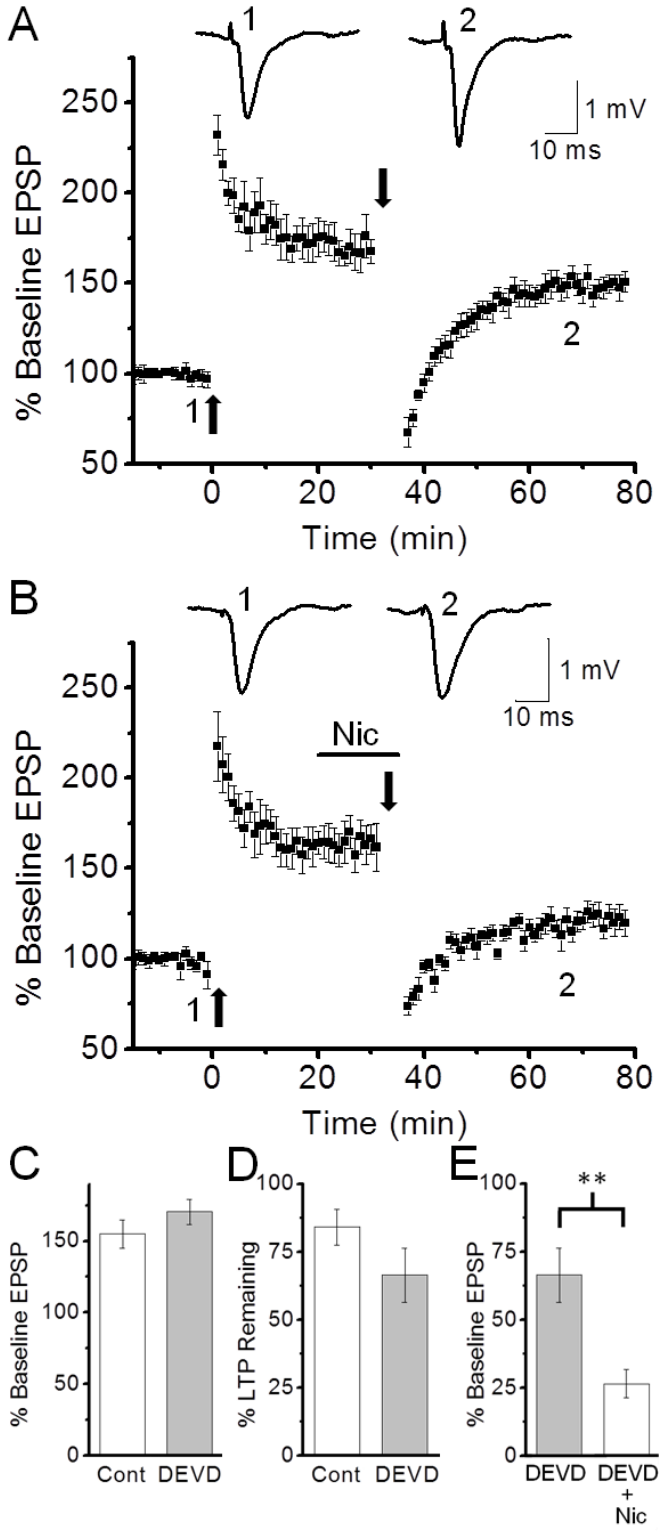


Fig. 15. Nicotine-induced depotentiation does not require caspase-3 activity A. DEVD treated slice became resistant to LFS within 30 minutes. B. Nicotine and LFS caused depotentiation in DEVD treated slice. C. Summary data comparing the magnitudes of LTP induced in untreated and DEVD-treated slices. D. Summary data comparing LTP remaining between untreated and DEVD-treated slices 20-30 minutes after LFS. E. Summary data comparing LTP remaining in DEVD-treated slices after LFS in the absence or presence of nicotine. *P<0.05

not require dephosphorylation of Ser-845 on GluA1.

Nic-DP may induce AMPAR internalization by utilizing a caspase-3 dependent process. To determine whether Nic-DP utilizes the caspase-3-dependent process, we examined the effect of the irreversible caspase-3 inhibitor DEVD on the depotentiation. Preincubation with the DEVD did not inhibit LTP induction or maintenance (Fig. 15 A, C; % of baseline fEPSP slope; untreated, 156.7±8.6%, n=5 vs. DEVD-treated, 170.3±8.7%, n=5,

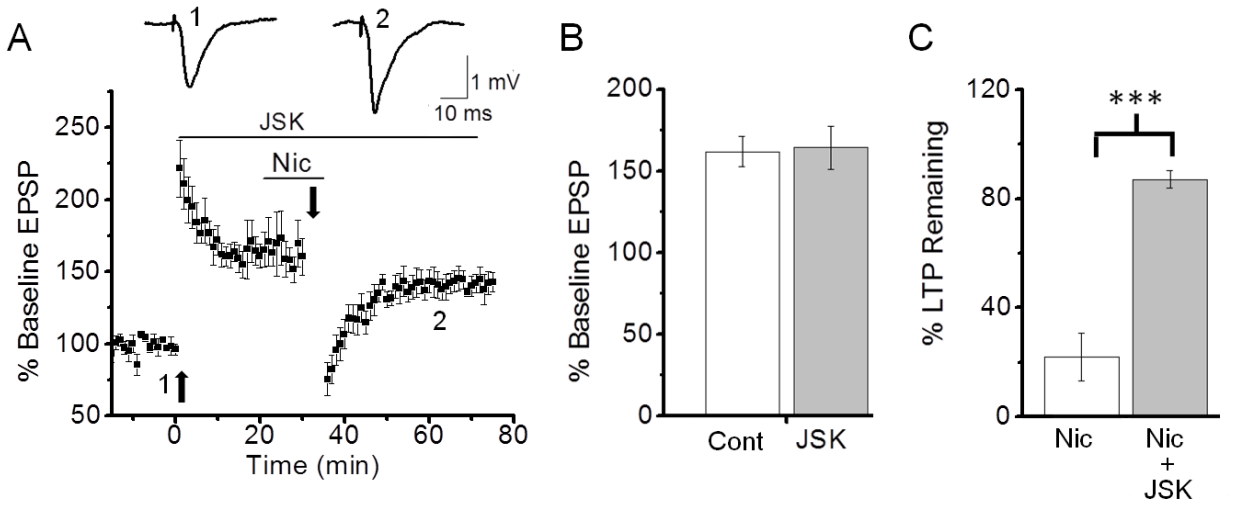


Fig. 16. Inhibition of actin depolymerization blocked nicotine-induced depotentiation A-C. Jasplakinolide (JSK), which stabilizes F-actin, had no effect on LTP induction (B), but prevented nicotine-induced depotentiation (C). A. TBS and LFS were delivered in the presence of JSK (200 nM) as indicated by the horizontal bar. Nicotine (1 μM) was also present during LFS. B. Summary data comparing the magnitudes of LTP induced in slices in the absence and presence of JSK. C. Summary data comparing LTP remaining in unexposed and JSK-exposed slices after LFS in the presence of nicotine. *P<0.05

p=0.58). Furthermore, DEVD treated slices underwent normal LTP consolidation, becoming resistant to LFS-induced depotentiation (Figs. 15A, C; % of LTP remaining; LFS alone, 84.1±6.6%, n=5 vs. LFS + DEVD, 66.4±9.9%, n=5, p=0.18). In addition, the treatment did not block Nic-DP (Figs. 15B, D; % of LTP remaining; LFS + nicotine, 22.0±8.9%, n=7 vs. LFS + nicotine +DEVD, 26.5±5.3%, n=6, p=0.69). The results provide evidence that Nic-DP does not decrease synaptic strength through caspase-3 dependent processes.

Nicotine-induced depotentiation requires destabilization of F-actin

Increases in F-actin correlate with increased spine size while decreasing F-actin induces spine shrinkage (Fukazawa et al., 2002; Zhou et al., 2004; Bosch et al., 2014). Because inhibiting actin depolymerization prevents LTD-DP and E-DP (Yang et al., 2008, Rex et al., 2009; Peng et al., 2010), we examined whether blocking actin depolymerization also prevents Nic-DP. If Nic-DP requires spine shrinkage, then preventing actin depolymerization will prevent the loss of synaptic strength occurring during Nic-DP. JSK, which stabilizes filamentous actin (F-actin), added after LTP induction did not significantly alter the strength of LTP (Fig. 16A, B; % of baseline fEPSP slope; untreated, 161.8±9.1%, n=5 vs. JSK-treated, 164.2±13.3%, n=5, p=0.89). However, JSK treated slices did not depotentiate to the same level as slices receiving only nicotine and LFS (Figs. 16A, C; % of LTP remaining; LFS + nicotine, 22.0±8.9%, n=7 vs. LFS + nicotine + JSK, 87.0±3.2%, n=5, p=0.00024). These results indicate that Nic-DP involves spine shrinkage.

Discussion:

Nic-DP is the only known form of depotentiation that utilizes a non-LTD inducing stimulation to reverse consolidated LTP. The major aim of this chapter was to determine the downstream targets being affected during Nic-DP. We found that Nic-DP reverses at least one LTP mechanism. Nic-DP did not require the dephosphorylation of Ser-831, which is a component of LTP maintenance, and was not dependent on altered AMPAR trafficking through dephosphorylation of Ser-845 or caspase-3 activity. However, inhibiting actin destabilization during Nic-DP prevented the loss of LTP. Together the findings indicate that

actin destabilization is a key mechanism for Nic-DP and supports the notion that DP requires actin destabilization.

We found that Nic-DP did not require dephosphorylation of Ser-831. There is one previous case where dephosphorylation of Ser-831 was not required for depotentiation (Lüthi et al., 2004). However, dephosphorylation of Ser-831 was not required only if the initial LTP did not elevate Ser-831 phosphorylation. Thus, in all previous studies, when LTP was associated with increased levels of pSer-831, depotentiation induction led to the reversal of that phosphorylation. Our LTP induction was associated with increased Ser-831 phosphorylation (Fig.11). Ours is the first case where a stimulation-induced form of DP did not reverse the LTP-induced increase in phosphorylated Ser-831.

Although our findings indicate that decreasing pSer-831 is not required for DP, dephosphorylation of Ser-831 may become more difficult during consolidation. Depotentiation with LFS prior to consolidation leads to the reversal of elevated pSer-831 levels (Huang et al., 2001). Our findings demonstrate that after consolidation, LFS alone no longer reduces pSer-831 or LTP. However, pSer-831 levels could be reduced if LTD-DP was administered 50-70 minutes after LTP induction (Lee et al., 2000). The discrepancy between our two studies likely has to do with the difference in duration (3 vs. 15 min) between our depotentiating stimuli. The dephosphorylation of Ser-831 may become more difficult following consolidation, requiring a more intense LTD-inducing stimulation. Together with this previous data, our results provide evidence that consolidation increases the intensity of stimulation that is required to induce dephosphorylation of Ser-831. Dephosphorylation of Ser-831 is not mandatory for depotentiation but the protection of pSer-831 could decrease the likelihood of depotentiation by less aggressive manipulations.

How might elevated pSer-831 decrease the likelihood of LTP disruption? The interactions between the multiple phosphorylation sites that are present on AMPAR might provide some insight. Mice with single mutations of Ser-831 had normal LTP and LTD (Lee et al., 2010), while mutations of Ser-845 had impaired LTD and normal LTP (Lee et al., 2010). These results demonstrate that either Ser-831 or Ser-845 can support LTP. Mice with double knock-in mutations of Ser-831 and Ser-845 had impaired LTP and LTD (Lee et al., 2003). Thus, while either Ser-831 or Ser-845 can support LTP, phosphorylation of one is necessary for proper LTP induction. Additionally, Ser-831 and Ser-845 were shown to work in concert with an additional phosphorylation site, Ser818, to increase AMPAR membrane incorporation (Boehm et al., 2006). Preventing Ser-831 dephosphorylation could support membrane incorporation, increasing the likelihood that an increased number of AMPAR would be present at the synapse.

pSer-831 and pSer-845 are not dephosphorylated by Nic-DP, indicating that there are elevated levels of membrane-bound receptors. Synaptic strength could be reduced if these membrane-bound receptors were not synaptically expressed. Nic-DP could be disturbing anchoring proteins required for AMPARs to remain in the synapse. AMAPA receptors are constantly being trafficked into and out of the membrane (Bredt and Nicoll, 2003; Oh et al., 2006; Groc and Choquet, 2006; Man et al., 2007; Glebov et al., 2015). Once in the membrane, AMPAR diffuse into the synapse. Once in the synapse, anchoring proteins interact and retain AMPAR (Borgdorff and Choquet, 2002; Groc and Choquet, 2006; Makino and Malinow, 2009). The time they spend in the synapse largely depends on the anchoring proteins present (Zhou et al., 2001; Opazo et al., 2010; Hanley et al., 2014). Actin has been shown to interact with anchoring proteins (Zhou et al., 2001; Earnshaw and Bressloff,

2008; Hanley et al., 2014) and spine size is correlated with post-synaptic density size (Bosch et al., 2014). The spine shrinkage occurring during Nic-DP is capable of decreasing synaptic strength without reducing Ser-831.

Nic-DP could still be reducing AMPAR currents. Phosphorylation of Ser-831 occurring in GluA1/2 AMPAR is alone not capable of increasing the current passed by AMPARs (Oh and Derkach, 2005). However, when accompanied by transmembrane-regulatory proteins such as stargazin, phosphorylation of Ser-831 is capable of increasing AMPAR receptor conductance (Kristensen et al., 2011). Nic-DP could be reducing AMPAR current by altering the localization of transmembrane-regulatory proteins.

Although nicotine can facilitate LTD induction (Fujii and Sumikawa, 2001), we found that Nic-DP did not result in decreased pSer-845 or require caspase-3 activity, both of which are required for LTD induction (Li et al., 2010; Lee et al., 2010; He et al., 2011). While changes to Ser-845 phosphorylation or caspase-3 are specifically involved with AMPAR trafficking during LTD, AMPAR trafficking is complex and may be affected by other pathways not studied here. Our findings provide evidence that during Nic-DP, nicotine does not facilitate the loss of synaptic strength via LTD mechanisms. p38 MAPK (Liang et al., 2008) and the Rap2-JNK pathway (Zhu et al., 2005; Yang et al., 2011) have been implicated in AMPAR trafficking (Zhu et al., 2001; Huang et al., 2004; Zhu et al., 2005; Thomas et al., 2008). Inhibitors of p38 MAPK, Rap2, or JNK all impair either E-DP or LTD-DP (Zhu et al., 2005; Liang et al., 2008; Yang et al., 2011). Whether these proteins lead to reduced synaptically expressed AMPAR during any form of depotentiation has yet to be determined. Nic-DP could involve the removal of membrane-bound AMPAR via a process dependent on p38 MAPK or Rap2-JNK. p38 MAPK and Rap2-JNK should both be investigated as a possible

mechanism of Nic-DP-induced alteration to AMPAR trafficking. As neither p38 MAPK nor Rap2-JNK have direct evidence for reducing AMPARs during DP, the direct monitoring of AMPAR synaptic expression must be performed in such a study to confirm that either p38 MAPK or Rap-JNK is affecting AMPAR trafficking and not any other aspect of synaptic strength.

Filamentous actin (F-actin) is the primary structural component of spines. LTP is accompanied by enlarged spines and increased levels of F-actin (Yuste and Bonhoeffer, 2001; Yang et al., 2008; Bellot et al., 2014; Bosch et al., 2014). In opposition to LTP, E-DP leads to decreased F-actin and spine size (Kramár et al., 2006; Yang et al., 2008). The LTP-induced increase in F-actin stabilizes in roughly 30 minutes (Yuste et al., 2001; Matsuzaki et al., 2004; Lang et al., 2004; Kramár et al., 2006; Chen et al., 2007). The actin/integrin hypothesis proposes that the increase in spine size accompanying LTP takes time to stabilize and that this stabilization is responsible for LTP's initial consolidation phase (Lynch et al., 2006; Honkura et al., 2007). In support of the actin/integrin hypothesis, we found that destabilization of the actin cytoskeleton is required for Nic-DP.

Nic-DP, like E-DP, was dependent on GluN2A-NMDAR activity and could be repotentiated. Additionally, Nic-DP does not act in a summative fashion with E-DP. Together, these findings provide evidence that nicotine is reducing synaptic strength specifically by reversing the mechanisms maintaining LTP expression. LTP expression involves increased AMPAR current and synaptic expression as well as increases in spine size (Benke et al., 1998; Hayashi et al., 2000; Yuste and Bonhoeffer, 2001; Malenka and Bear, 2004; Matsuzaki et al., 2004; Makino and Manilow, 2009). We only found evidence

that Nic-DP is reversing the increased spine size associated with LTP. However, the reduction in spine size can interact with other potentiation mechanisms.

Actin can increase the time an individual AMPAR spends in the synapse by increasing the cytoskeletal size, density, and AMPAR interacting proteins (Schwechter and Tolias et al., 2013; Rudy, 2014). In particular, the AMPAR -> Stargazin -> PSD-95 -> GKAP/SPAR/Shank/Cortactin -> F-actin association complex increases following LTP and is known to retain AMPAR (Newpher and Ehlers, 2008). As discussed above, the stargazin could also assist in modifying AMPAR current (Cuadra et al., 2004; Bats et al., 2006; Kessels et al., 2009; Opazo et al., 2010). Moreover, preventing depolymerization has been shown to prevent AMPAR internalization (Zhou et al., 2001). The interactions discussed here are only a brief display of how actin can alter both AMPAR trafficking and current. Many of the actin dependent interactions are currently being investigated and are not well understood (what we know about Actin interactions are reviewed in Bellot et al., 2014;; Hanley, 2014; and Lynch et al., 2014). Of the mechanisms test, we found only that actin was involved with Nic-DP. However, due to actins involvement with other LTP mechanisms, actin associated changes in receptor current or trafficking are likely occurring.

Closing Statement

Acetylcholine is a well-known modulator of memory (Power et al., 2003; Boccia et al., 2010; Blake et al., 2014; Grogan et al., 2015; Osborne et al., 2015; Reichenbach et al., 2015). The cholinergic system's effects on memory are complex and are currently being investigated. Here, we studied the effects of the nicotinic system on depotentiation. We confirmed that nicotine facilitates the loss of consolidated LTP by LFS (Guan et al., 2006). Our major aim was then to determine the mechanisms utilized by the nicotinic system as it disrupts consolidated LTP. Nicotine acts as an agonist to all nicotinic receptors. However, prolonged activation of nicotinic receptors causes the receptors to enter a desensitized state. Once desensitized, nAChR will not respond to any agonist, including ACh. Whether Nic-DP occurred due to nicotine-induced activation or desensitization of nAChRs, and which receptor populations are important for Nic-DP, was still in need of clarification. We found that α7 nAChR antagonism or disruption of endogenous ACh mimicked Nic-DP. This finding indicates that Nic-DP is likely due to the inhibition of α7 nAChR activity normally occurring during LFS. α7 nAChR activity changes can modify both system excitability and intracellular signaling in several ways (Fig. 9). Because the effect of α7 nAChR varies depending on its location, we are unable to decipher the exact changes occurring due to decreased α7 nAChR activity. Determining the location of the α7 nAChR should be one goal of future experiments.

Inhibiting α7 nAChRs can directly alter GABAergic inhibition, presynaptic vesicle release, postsynaptic depolarization, and internal calcium levels. Changes to any or all of these systems would affect NMDA receptor activity. Nic-DP is known to require NMDAR activity (Guan et al., 2006). Inhibiting the GluN2B-NMAR receptors did not affect Nic-DP

(Guan et al., 2006). Currently, there is evidence supporting the idea that LTP, LTD, and DP depend on activation of specific NMDA subtypes. However, there is also evidence that only the amount of Ca^{2+} entering the postsynaptic neuron is important in dictating synaptic plasticity. Thus Nic-DP could specifically require GluN2A-NDMA signaling, or the Ca^{2+} influx from either GluN2A or GluN2B could be sufficient to induce Nic-DP. We found that blocking GluN2A-NMDARs prevents Nic-DP, indicating that Nic-DP requires GluN2A-NMDAR specific signaling.

Nic-DP requires PP1, PP2A, and PP2B (calcineurin) activity (Guan et al., 2006). The Ca^{2+} entering through the NMDA receptors can increase the activity of all three PP (Mulkey et al., 1994; Malenka and Bear, 2004; Yang et al., 2008). Phosphorylated Ser-831 is an important regulator of AMPAR insertion and conductivity (Barria et al., 1997; Mammen et al., 1997; Derkach and Soderling, 1999). E-DP and LTD-DP are accompanied by a reduction of Ser-831 phosphorylation (Lee et al., 2000; Huang et al., 2001; Lüthi et al., 2004 Kramár et al., 2006); calcineurin is involved with E-DP, Nic-DP, and LTD-DP and can dephosphorylate Ser-831 (Zhuo et al., 1999; Jouvenceau et al., 2003; Huang et al., 2001). Unlike E-DP and LTD-DP, the loss of pSer-831 did not occur during Nic-DP. Additionally, Nic-DP was not dependent on dephosphorylation of Ser-845 or caspase-3 activity. Both Ser-845 and caspase-3 are required for AMPAR internalization that occurs during LTD induction. These data suggest that Nic-DP does not require changes in AMPAR phosphorylation. Nicotine-facilitated depotentiation required destabilization of the actin cytoskeleton. The actin cytoskeleton is known to be regulated by the actin severing/depolymerizing factor cofilin. PP1 and PP2A activity is required for Nic-DP and are capable of activating cofilin and thus destabilizing F-actin (Tomasella et al., 2014; Huet

et al., 2013). It is possible that, during Nic-DP, protein phosphatase activity is altering F-actin levels via increase cofilin activity. However, how phosphatases and actin interteract are far from clear, there may be other yet to be identified factors mediating the destabilization of actin during Nic-DP. Cumulatively, our findings indicate that ACh, via activation of α7 nAChRs, is involved with preventing LTP reversal by inhibiting spine

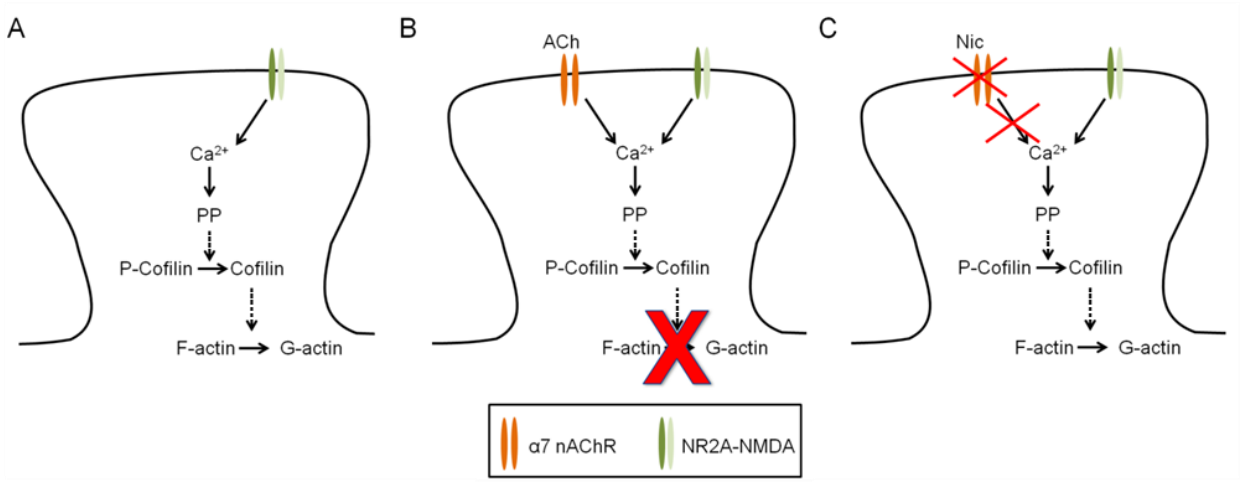


Fig. 17. Porposed mechanism of Nic-DP. A. NR2A-NMDA signalling during LFS induces actin destabilization. B. α7 nAChR activity, induced by ACh released during LFS, alters NR2A-NMDA dependent signaling. Preventing NR2A-NMDA signaling from inducing actin destabilization. C. Nicotine inhibits α7 nAChR activit, allowing for NR2A-NMDA signaling to induce actin destabilization.

destabilization at post-consolidated synapses. Nicotine inhibits α7 nAChRs activity, allowing for destabilization of the actin cytoskeleton via a GluN2A-NMDA, PP1, PP2A, and PP2B dependent fashion. The destabilization of the actin cytoskeleton would allow for the loss of LTP (Fig. 17).

DP has been hypothesized to be important for a variety of functions including forgetting (Staubli and Lynch, 1990; Huang et al., 1999; Kang-Park et al., 2003; Lynch et al., 2003; Liang et al., 2008; Yang et al., 2011; Qi et al., 2012), sharpening of memory traces and increasing of cellular flexibility (Staubli and Chun, 1996; Jouvenceau et al., 2003; Zhou and

Poo, 2004; Vyazoviskiy et al., 2008; Chung et al., 2009). DP occurs in awake behaving animals (Lin et al., 1998; Qi and Rowan, 2012). In particular, theta frequencies, which occur during exploration and running, appear to induce DP (Lin et al., 1998; Qi and Rowan, 2012). Although depotentiation occurs normally in behaving animals, evidence supporting only one of the proposed functions, forgetting, has been collected. Navabi et al., 2014, successfully used optogenetic stimulation to replace the tone in tone+shock fear conditioning. They found that, after training, an LTP like increase in AMPAR current occurred. If LTD stimulation, which would induce DP, was given later, then the rat did not demonstrate memory. If LTP stimulation was given after LTD stimulation, then the memory returned. These authors demonstrated that DP could be involved with forgetting.

If DP were a cellular correlate of forgetting, how would our results fit in with the role of ACh in memory consolidation? Hippocampal ACh levels are elevated between 130-200% for at least 15 minutes after the learning of several hippocampal-dependent behavioral tasks, after which, ACh levels decrease in both rodents and cats (Marrosu et al., 1995; Orsetti et al., 1996; Ragozzino et al., 1996; Reviewed in Deiana et al., 2011). It is believed that increased ACh allows for attention and encoding and that lower levels facilitate memory consolidation (Hasselmo and McGaughy, 2004; Micheau and Marighetto, 2011; Pepeu and Giovannini, 2010). Indeed, elevating cholinergic activity during consolidation disrupt declarative memory in humans and spatial memory in rats (Gais and Born, 2004; Bunce et al., 2004). However, inhibiting nicotinic activity in the hippocampus post-training, during consolidation, also inhibits later performance in inhibitory avoidance (Arthur and Levin, 2002; Addy et al., 2003; Barros et al., 2004). Although required for consolidation, only low ACh concentrations facilitate consolidation. Elevating or

decreasing ACh from baseline levels, during consolidation, inhibits later performance. Our findings implicate actin instability as a possible mechanism for why ACh is required during memory consolidation. The lack of ACh could allow for spine instability, allowing for DP, which is then expressed behaviorally as a lack of memory.

The malfunctioning of the cholinergic system occurs in several neurological disorders, including Alzheimer's disease (AD), Parkinson's disease, and schizophrenia (Schliebs and Arendt, 2006; Wallace and Porter, 2011). The α7 nAChR loss occurring during AD is correlated with memory impairment (Guan et al., 2000; Schliebs and Arendt, 2006; Kadir et al., 2006). Treatments that increase α7 nAChR activity increase cognitive factors in AD patients (Wallace and Porter, 2011). Our work indicates that the loss of cholinergic projections to the hippocampus that are seen in Alzheimer's disease patients could have profound impacts on LTP consolidation, leading to the loss of LTP and thereby affecting memory formation.

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