UNIVERSITY OF CALIFORNIA, SAN DIEGO

Nicotinic Control of Glutamate Receptor Trafficking

A dissertation submitted in partial satisfaction of the requirements for the degree of Doctor of Philosophy

in

Biology

by

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The Dissertation of Andrew William Halff is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

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DEDICATION

To my parents and brother for their love and support.
Without them this would not have been possible.

EPIGRAPH

Success is the ability to go from one failure to another with no loss of enthusiasm.

Winston Churchill

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Chapter 1, in full is currently being prepared for submission for publication of the material. David Gómez-Varela and Darwin K. Berg are co-authors on chapter 1. The dissertation author was the primary investigator and author of this material.

David Gómez-Varela and Darwin K. Berg are co-authors on chapter 2, which may be prepared as part of a future publication. The dissertation author was the primary investigator and author of this material.

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ABSTRACT OF THE DISSERTATION

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Nicotinic cholinergic signaling acting through nicotinic acetylcholine receptors influences numerous cognitive functions including learning and memory. Critical for these higher brain functions are basic attributes of excitatory transmission that depend on proper trafficking and lateral mobility of glutamate receptors to and from synapses. The link between nicotinic signaling and glutamate receptor trafficking, however, remains unclear. Here we measure the effect of nicotine on the surface expression and lateral

mobility of AMPA receptors on hippocampal neurons in culture. We find that a short exposure to nicotine for only a few hours leads to the stabilization and accumulation of GluR1-containing AMPA receptors on dendritic spines. This process occurs through direct action on postsynaptic nicotinic acetylcholine receptors, independent of coincident fast, excitatory synaptic transmission, and results in increased synaptic efficacy. The pathway relies on intracellular calcium signaling, PDZ interactions, and the lateral diffusion, but not exocytosis, of GluR1-containing AMPA receptors. Prolonging the nicotine exposure by a few days, however, leads to the destabilization of AMPA receptors on spines. This is measured as an increase in the mobility of GluR2-containing AMPA receptors. Surprisingly, we find that nicotinic agonists alone, in the absence of nicotine, have the same effect on GluR2-containing AMPA receptor mobility, suggesting a non-canonical nicotinic mechanism. Our results demonstrate that varied nicotinic manipulations can have profound effects on glutamate receptor trafficking and offer insight into the possible mechanisms underlying the cognitive effects of nicotinic signaling.

INTRODUCTION TO THE DISSERTATION

Nicotinic cholinergic signaling has profound and pervasive behavioral effects in the mammalian central nervous system, yet the underlying cellular and molecular mechanisms are not fully understood. The plant alkaloid nicotine, through its action on nicotinic cholinergic signaling, mediates addiction to tobacco, a leading cause of preventable death in the world (Danaei et al., 2009; WHO, 2012). Furthermore, nicotinic signaling plays a role in a variety of neurological disorders such as schizophrenia, epilepsy, autism, Alzheimer's Disease, Parkinson's disease, and dementia (Newhouse et al., 1997; Zoli et al., 1999; Picciotto and Zoli, 2002; Raggenbass and Bertrand, 2002; Teper et al., 2007; Tizabi, 2007). It also participates in a number of higher order behaviors including learning and memory, attention, and cognition (Peeke and Peeke, 1984; Meyer et al., 1997; Levin et al., 1998; Kenney and Gould, 2008; Thiel et al., 2008). This dissertation, in the interest of improving public health and adding to our collective knowledge of the natural world, presents novel investigations into the cellular and molecular mechanisms thought to subserve the diverse behavioral effects of nicotinic signaling.

Nicotinic signaling is mediated by a class of ligand-gated, cation-selective ion channels termed nicotinic acetylcholine receptors (nAChRs). As their name suggests, these receptors are activated endogenously by the neurotransmitter acetylcholine (ACh) and exogenously by the drug nicotine (Albuquerque et al., 2009; Millar and Gotti, 2009; Taly et al., 2009). Mammalian neuronal nAChRs are pentameric transmembrane proteins. They can exist as homopentamers comprised of α7-10 subunits, or as heteropentamers

containing variable combinations of at least one α2-6 and β2-4 subunit (Decker at al., 1995; Jones et al., 1999; McGehee, 1999; Hogg et al., 2003). The most abundant and widely expressed receptor subtypes in mammalian central nervous system are the homopentameric α7-containing nAChR (α7-nAChR) and the heteropentameric β2-containing nAChR (β2*-nAChR), of which α4β2-nAChR is the most common (Margiotta and Pugh, 2004; Albuquerque et al., 2009; Millar and Gotti, 2009; Taly et al., 2009). The particular subunit combinations of the receptors confer upon them different functional attributes. When compared to α4β2-nAChRs, α7-nAChRs have fast activation kinetics, a high conductance, and enter a desensitized state rather quickly, but also demonstrate a low affinity for agonist activation by ACh and Nicotine (Zhang et al., 1996; Fenster el al., 1997; Cordero-Erausquin et al., 2000; Papke et al., 2001; Berg and Conroy, 2002; Wooltorton et al., 2003). Most importantly for intracellular signaling and synaptic plasticity, α7-nAChRs are known to have a high calcium permeability (Séguéla et al., 1993; Zhang et al., 1994; Castro and Albuquerque, 1995; Dajas-Bailador and Wonnacott, 2004). They can flux calcium at levels comparable to and exceeding that of NMDA receptors (Bertrand et al., 1993; Patrick et al., 1993; Uteshev, 2012), and can do so at resting and hyperpolarized potentials when NMDA receptors are blocked (Mayer et al., 1984; Albuquerque et al., 1995; Broide and Leslie, 1999).

Of particular interest is the mammalian hippocampus for its role in mediating learning and memory (Bird and Burgess, 2008; Kenney and Gould, 2008). The hippocampus receives cholinergic input from the medial septum and diagonal band of broca via the fimbria-fornix, which terminates in all regions of the hippocampus as both synaptic and large volume-release sites (Wainer et al., 1984; Frotscher and Léránth,

1985; Woolf, 1991; Dutar et al., 1995; Umbriaco et al., 1995; Descarries et al., 1997). Furthermore, a limited amount of nicotinic input arises from local cholinergic interneurons (Frotscher et al., 1986, 2000). The hippocampus expresses high levels of nAChRs, and *in situ* hybridization studies reveal that the most highly expressed subunits are α7 and β2 (Wada et al., 1989, Dineley-miller and Patrick, 1992; Séguéla et al., 1993). For the purposes of this dissertation we will focus on the α7-nAChRs, which ultrastuctural analysis of immuno- and toxin-labeled sections reveals is present at the majority of hippocampal synapses, as well as many non-synaptic locations (Fabian-fine et al., 2001). Interneurons express the highest levels of functional α7-nAChRs in the hippocampus (Zarie et al., 1999; Sudweeks and Yakel, 2000; Khiroug et al., 2003; Fayuk and Yakel, 2007). Though they are also present in the principal excitatory pyramidal cells of the hippocampus, the low level of surface expression and fast desensitization kinetics have made recording postsynaptic α7-nAChR currents difficult and hard to reproduce consistently (Hefft et al., 1999; Sudweeks and Yakel, 2000; Ji et al., 2001; Khiroug et al., 2003; Mielke and Mealing, 2009). The recent development of positive allosteric modulators, however, has confirmed that pyramidal neurons in the CA regions of the hippocampus express functional somatic and dendritic α7-nAChRs (Kalappa et al., 2010; Uteshev, 2012).

The prevalence of presynaptic receptors on axon terminals and their ability to modulate glutamate release underlie the majority of the nicotinic effects on glutamatergic synapse plasticity (Wonnacott, 1997; MacDermott et al., 1999; McKay et al., 2007; Placzek et al., 2009). Acute challenge with nicotinic agonists increases the probability of glutamate release (McGehee et al., 1995; Gray et al., 1996; Radcliffe and Dani, 1998)

and has been shown to unsilence pre-synaptically silent synapses in the developing hippocampus (Maggi et al., 2003). It is through these presynaptic mechanisms that chronic nicotine exposure is thought to lower the threshold for the induction of long-term potentiation (LTP; Fuiji et al., 1999; Kenney and Gould, 2008). Furthermore, acute nicotinic regulation of lasting glutamatergic synaptic plasticity is quite finally tuned; only milliseconds separate the difference between coincident glutamatergic and nicotinic induction protocols leading to long-term depression (LTD) or changes in threshold and magnitude of LTP (Ge and Dani, 2005; Gu and Yakel, 2011; Gu et al., 2012; Yakel, 2012).

These nicotinic influences on neuronal plasticity are believed to underlie nicotine-induced effects on hippocampal-dependent behaviors (Martin et al., 2000; Whitlock et al., 2006; Kenney and Gould, 2008; Placzek et al., 2009). In humans and non-human primates, nicotinic agonists improve learning and memory (Levin et al., 2006). Rodent behavioral testing reveals that nicotinic manipulation alters performance on spatial memory in the Morris water maze, working memory in the radial arm maze, and contextual learning in fear conditioning assays (Bernal et al., 1999; Gould and Wehner, 1999; Levin et al., 2002; Kenney and Gould, 2008; Placzek et al., 2009).

A critical component of the synaptic plasticity events underlying these behaviors is the proper trafficking of glutamate receptors to and from synapses (Sheperd and Huganir, 2007; Rusakov et al., 2011). The majority of fast, excitatory synaptic transmission in the mammalian brain is mediated by the neurotransmitter glutamate binding to AMPA receptors (AMPARs) located on specialize postsynaptic structures called dendritic spines (Alvarez and Sabatini, 2007; Bourne and Harris, 2008). One

mechanism by which neural circuits are thought to store information is through the persistent increase in synaptic efficacy known as LTP (Bliss and Collingridge, 1993; Whitlock et al., 2006). The primary mechanism for LTP at hippocampal synapses is through the addition of AMPARs to the postsynaptic site (Bredt and Nicoll, 2003; Kopec et al., 2006; Tanaka and Hirano, 2012). AMPARs are ionotropic receptors structured as tetrameric transmembrane proteins. They are formed by different subunits, GluR1-4, which are assembled as a dimer of dimers (Tichelaar et al., 2004; Greger et al., 2007). In the hippocampus the most prevalent subunits are GluR1, GluR2 and GluR3 (Lynd-balta et al., 1996; Lu et al., 2009). Subunit composition determines the particular receptor function in signal transduction and synaptic plasticity. For example, GluR2-lacking receptors flux calcium and rectify at positive potentials (Iino et al., 1990; Bowie, 2012). Furthermore, a widely accepted mechanism for LTP involves the activity-dependent addition of GluR1-containing AMPARs to synapses followed by their replacement with GluR1-lacking/GuR2-containing AMPARs in a non-activity dependent manner (Liao et al., 1995; Hayashi et al., 2000; Malinow and Malenka, 2002; Tanaka and Hirano, 2012). The preponderance of these studies were performed with the overexpression of single GluR subunits which tends to form homomeric AMPARs (Shi et al., 2001). Some controversy exists over the presence of endogenous homomeric receptors at synapses (Wenthold et al., 1996; Plant et al., 2006; He et al., 2009; Lu et al., 2009). A recent study in vivo, however, suggests that this general model for LTP holds true for both homomeric and heteromeric recombinant AMPARs (Makino and Malinow, 2011).

Original theories suggested that AMPARs were added and removed from the postsynaptic density through direct exo- and endocytosis (Carroll et al., 2001; Park et al.,

2004; Gerges et al., 2006). A growing body of evidence, however, indicates that the lateral diffusion of receptors already on the surface of the cell is a critical step in the process of receptor trafficking (Makino and Malinow, 2009; Petrini et al., 2009; Opazo and Choquet, 2011). Lateral mobility of surface AMPARs is important for a number of glutamatergic signaling processes. The degree of lateral mobility can be an indication of synaptic incorporation, with receptors moving faster outside of synapses than inside (Borgdorff and Choquet, 2002; Triller and Choquet, 2005; Ehlers et al., 2007; Makino and Malinow, 2009, 2011). Free exchange of previously desensitized synaptic AMPARs with un-desensitized extrasynaptic receptors through lateral diffusion is critical for maintaining high frequency synaptic transmission (Heine et al., 2008). In LTP, the source of newly added synaptic AMPARs arises from extrasynaptic surface receptors (Makino and Malinow, 2009). This process involves the local exocytosis of AMPARs at extra/perisynaptic sites proximal to the site of potentiation followed by the subsequent trapping of diffusing surface receptors by postsynaptic scaffold slots through the calcium-dependent phosphorylation of TARP AMPAR auxiliary subunits (Chen et al., 2000; Hayashi et al., 2000; Bats et al., 2007; Ehlers et al., 2007; Petrini et al., 2009; Opazo et al., 2010, 2011; Mondin et al., 2011; Opazo and Choquet, 2011).

The overarching aim of this dissertation is to determine how nicotinic signaling participates in the regulation of glutamate receptor trafficking, a critical component of neurological function. Chapter 1 describes a novel mechanism in hippocampal neurons by which nicotinic activity through post-synaptic α7-nAChRs drives the synaptic incorporation of GluR1-containing AMPARs independent of coincident fast glutamatergic transmission. In Chapter 2, preliminary results suggest that prolonged

nicotinic manipulation in hippocampal neurons can lead to an increase in the mobility of GluR2-containing AMPARs through a possibly unconventional molecular mechanism.

CHAPTER 1

A NOVEL MECHANISM FOR NICOTINIC INDUCTION OF GLUTAMATERGIC PLASTICITY

By

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SUMMARY

Nicotinic cholinergic signaling influences a number of cognitive functions. Critical for these higher functions are basic attributes of excitatory transmission that depend on proper synaptic trafficking of glutamate receptors. The link between nicotinic signaling and glutamate receptor trafficking, however, remains unclear. To examine this we measured the effect of nicotine on the mobility and distribution of recombinant AMPA receptors on the surface of hippocampal neurons in culture. Our results demonstrate a novel mechanism by which nicotinic signaling, acting through α7-containing nicotinic receptors on the postsynaptic cell, leads to the stabilization and accumulation of GluR1-containing AMPA receptors at spines. This occurs independent of coincident fast glutamatergic transmission and results in increased synaptic strength. It relies on intracellular calcium signaling, PSD-95 family member PDZ interactions, and GluR1 receptor lateral diffusion but not exocytosis. These findings define a new avenue by which nicotinic signaling modulates synaptic mechanisms thought to subserve learning and memory.

INTRODUCTION

Signaling through neuronal nicotinic acetylcholine receptors (nAChRs) has profound behavioral effects including its role in mediating addiction to nicotine (Dani et al., 2001; Mansvelder and McGehee, 2002; O'Dell et al., 2007) and its impact on cognitive functions such as attention, learning, and memory (Peeke and Peeke, 1984; Levin et al., 1998; Kenney and Gould, 2008; Thiel et al., 2008). Conversely, impairments in proper nicotinic signaling have been implicated in a number of neurological disorders

such as Alzheimer's disease, schizophrenia, and Parkinson's disease (Newhouse et al., 1997; Zoli et al., 1999; Picciotto and Zoli, 2002; Tizabi, 2007). Despite these pervasive actions, the mechanisms by which nicotinic input affects learning and memory are poorly understood.

The hippocampus is widely recognized as a critical brain region in the formation of memories (Bird and Burgess, 2008; Kenney and Gould, 2008), and it expresses high levels of the two major neuronal nAChR subtypes, the α7-containing homopentamers (α7-nAChR) and α4β2-containing heteropentamers (α4β2-nAChR; Wada et al., 1989; Zarei et al., 1999; Fabian-Fine et al., 2001). Nicotinic activity affects excitatory transmission in the hippocampus through diverse mechanisms. It regulates the proper integration of neurons into developing circuits (Campbell et al., 2010, Lozada et al., 2012a,b) and modulates synaptic plasticity by enhancing long-term potentiation (LTP) or reducing the threshold for LTP induction (Fuiji et al. 1999; Ji et al., 2001; Kenney and Gould, 2008; Placzek et al., 2009; Gu et al., 2012; Yakel, 2012). Critical for these fundamental aspects of excitatory transmission is the proper trafficking of glutamate receptors to and from the synapse (Rusakov et al., 2011). Links between nicotinic signaling and glutamate receptor trafficking, however, remain to be determined.

Activation of AMPA receptors (AMPARs) located on dendritic spines by the neurotransmitter glutamate accounts for most of the fast excitatory synaptic transmission in the brain, and recruitment of these receptors to synapses underlies synaptic strengthening in most examples of LTP (Bredt and Nicoll, 2003; Kopec et al., 2006; Alvarez and Sabatini, 2007; Bourne and Harris, 2008; Tanaka and Hirano, 2012). Though AMPARs were originally thought to be added directly to synapses from internal pools

(Carroll et al., 2001; Park et al., 2004; Gerges et al., 2006), it is now clear that potentiation involves extrasynaptic receptors moving laterally to the postsynaptic site (Makino and Malinow, 2009; Petrini et al., 2009; Opazo and Choquet, 2011). The mobility of surface AMPARs is not only critical for synaptic plasticity but also for proper maintenance of synaptic transmission (Choquet, 2010; Heine et al., 2008). These features make AMPAR trafficking a likely target for nicotinic modulation.

Here we explore possible effects of nicotinic signaling on the lateral mobility and trafficking of AMPARs at hippocampal synapses. We report a novel, and likely cell-autonomous effect. Nicotine acting on postsynaptic α7-nAChRs promotes the accumulation and stabilization of GluR1-containing AMPARs (GluR1s) at spine independent of coincident fast glutamatergic signaling. This pathway relies on an increase in intracellular calcium, the availability of PDZ-binding scaffold proteins, and the lateral mobility of surface AMPARs. The outcome is strengthened synaptic transmission. Our findings offer insight into the role nicotinic signaling plays in shaping synaptic efficacy as a possible mechanism underlying its effects on higher level cognitive function.

RESULTS

Nicotine reduces GluR1 mobility at spines

To determine how nicotinic activity might affect the postsynaptic trafficking of AMPARs, we used fluorescence recovery after photobleaching (FRAP) to monitor the movement of fluorescently-tagged AMPARs on the cell surface of neurons. For this purpose, hippocampal slices were transferred to organotypic (OT) culture and, after 8

days, were biolistically transfected with constructs expressing super ecliptic pHluorin (SEP) tagged GluR1 and red fluorescent protein (RFP). The GluR1-SEP subunit of AMPARs fluoresces only when on the cell surface because of a pH dependence (Fig. 1A; Miesenböck et al., 1998; Kopec et al., 2006). After 2 weeks, the slices were treated with 1 μM nicotine for 2-3 hrs and then analyzed by FRAP for GluR1-SEP on the spines of basal dendrites of CA1 pyramidal neurons. This concentration of nicotine is within the upper limit of concentrations found in smokers (Rose et al., 2010). Pre-bleach baseline fluorescence was measured at spines, followed by photo-bleaching with high intensity light. FRAP was assessed by repeated imaging over the next 31 min (Fig. 1B,C). The signal was normalized both to the RFP spine signal to account for changes in spine size, and to a separate section of the neuron to correct for incidental bleaching (see Experimental Procedures). Under control conditions the GluR1-SEP signal completely recovered to pre-bleach levels within 31 min, but in cultures treated with nicotine the recovery levels were significantly reduced (Fig. 1D). These results demonstrate that recombinant GluR1 is relatively mobile at spines as reported previously (Makino and Malinow, 2009, 2011), but appears to become stabilized by nicotinic stimulation.

Nicotinic control of AMPAR mobility is GluR subunit-specific and associated with receptor enrichment on spines

As seen with slices, nicotine treatment (1 μM, 1-3 hrs) of neurons in dissociated hippocampal cell culture significantly reduced GluR1-SEP mobility at spines (Fig. 2A,B,D). In these cultures a 16 min protocol was sufficient for 100% FRAP at spines in control cells. The reduction in mobility appeared slowly during the 2-hr incubation with

nicotine (Fig. S1). In contrast, the mobility of GluR2-SEP-containing receptors was unchanged by nicotine (Fig. 2C,E). Furthermore, GluR2-SEP receptors appear more stable at spines than GluR1-SEP, as previously reported (Makino and Malinow, 2009, 2011).

To determine whether the observed FRAP represented lateral movement of surface GluR1-SEP rather than receptor cycling with intracellular pools, we depleted the potential reserve pool of fluorescent surface receptors by bleaching an extended region of the dendrite. The result was a marked attenuation in the extent of recovery for both control and nicotine-treated cells, making them equivalent (Fig. 3A-C). This suggests that the difference observed in spine FRAP experiments represents a change in mobility of pre-existing surface receptors.

To test if nicotine increased the number of receptors at spines we analyzed the baseline images taken prior to bleaching in the FRAP experiments. Cytosolic-RFP signal was used to measure spine size and revealed no difference between control and nicotine (Fig. 3D,E). Measuring the ratio of spine to dendrite GluR1-SEP intensity, however, revealed a significant enrichment of GluR1-SEP at spines on nicotine-treated cells (Fig. 3F,G). Analysis of a larger area of the dendritic arbor revealed a shift in the population distribution of GluR1-SEP spine enrichment in nicotine treated cells, with a subset of spines showing a high level of enrichment (Fig. S2). As with the reduction in GluR1-SEP mobility, spine enrichment of GluR1-SEP occurred gradually over a 2-hr exposure to nicotine (Fig. S3). No change was seen in spine size or in SEP enrichment at spines for GluR2-SEP (Fig. S4). These results indicate that nicotinic activity selectively immobilizes and enriches GluR1s on spine without affecting spine dimensions.

Nicotine-induced enrichment and stabilization of GluR1s on spines rely on postsynaptic α7-nAChRs, not on AMPARs and NMDA receptors

The two major subtypes of nAChRs found in the hippocampus are α7-nAChRs and β2-containing nAChRs (β2*-nAChRs), with α4β2-nAChRs being the most common version of the latter (Wada et al., 1989; Zarei et al., 1999). We used specific antagonists to selectively block individual receptor subtypes as a means of assessing their roles in mediating the nicotine effect on GluR1 trafficking. The α7-nAChR-specific antagonist methyllycaconitine (MLA, 100 nM) prevented nicotine from altering GluR1-SEP mobility (Fig. 4A) and from enriching GluR1-SEP on spines (Fig. 4B). Treatment with the α4β2-nAChR-specific antagonist dihydro-β-erythroidine (DHβE, 1 μM), failed to prevent either nicotinic effect (Fig 4C,D). These results indicate that nicotine acts through α7-nAChRs, not α4β2-nAChRs, to elicit the effects on GluR1 trafficking.

In the hippocampus, α7-nAChRs are found both pre- and postsynaptically at most synapses (Fabian-fine et al., 2001). Presynaptic receptors are known to facilitate neurotransmitter release (McGehee et al., 1995; Gray et al., 1996; Maggi et al., 2003). To test for the possibility that nicotine acts indirectly through glutamatergic transmission to affect postsynaptic AMPAR trafficking, we used the antagonists APV (50 μM) and NBQX (20 μM) to block activity through NMDA receptors (NMDARs) and AMPARs, respectively. Surprisingly, we found that the antagonists were unable to prevent either the nicotine-induced reduction of GluR1-SEP mobility (Fig. 4E) or the GluR1-SEP enrichment on spines (Fig. 4F). This indicates that glutamatergic signaling through AMPARs and NMDARs is not necessary for the effects, raising the possibility that postsynaptic α7-nAChRs act in a cell-autonomous manner.

To determine if postsynaptic α7-nAChRs mediate nicotinic control of GluR1 trafficking, we utilized a short-hairpin RNA (shRNA) targeted against the rat and mouse α7-nAChR sequences (Campbell et al., 2010; Lozada et al., 2012a). Sparse transfection conditions, together with RFP labeling, allowed us to avoid spines contacted by transfected axons, thereby ensuring that only the postsynaptic cell expressed the shRNA (Fig. S5). In neurons lacking α7-nAChRs, nicotine had no effect on the mobility of GluR1-SEP (Fig. 4G), nor could it increase the enrichment of GluR1-SEP at spines (Fig. 4H). A scrambled shRNA had no such effects in similarly treated sister cultures (Fig. 4G,H). The shRNA results indicate a novel mechanism by which nicotine, most likely in a cell-autonomous manner, can drive synaptic plasticity at glutamatergic synapses independent of coincident excitatory glutamate transmission.

Nicotinic signaling requires calcium and PDZ-scaffold interactions to induce GluR1 enrichment and stabilization on spines

Local rises in intracellular calcium can significantly reduce AMPAR mobility (Borgdorff and Choquet 2002; Heine et al., 2008), and ligand-induced activation of α7-nAChRs can elevate intracellular calcium (Séguéla et al., 1993; Dajas-Bailador and Wonnacott, 2004). To determine if elevation of intracellular calcium is necessary for the nicotine effects on AMPAR trafficking, we treated cultures with BAPTA-AM, a calcium chelator that only buffers calcium intracellularly. BAPTA-AM (10 μM) added 30 min prior to the 1-3 hr incubation with 1 μM nicotine prevented the decrease in GluR1-SEP mobility, rendering it equivalent to vehicle-treated controls (Fig. 5A). BAPTA-AM also prevented the increase in the GluR1-SEP enrichment at spines (Fig. 5B), indicating that

α7-nAChR stimulation acts through calcium to decrease AMPAR mobility and position more of the receptors on spines.

The canonical mechanism by which AMPARs are held at synapses involves interactions between their TARP auxiliary subunits and PDZ-scaffold proteins (Chen et al., 2000; Bats et al., 2007; Opazo et al., 2010, 2011; Opazo and Choquet, 2011). To determine if PDZ interactions with scaffold proteins are necessary for nicotine-induced immobilization of GluR1 at spines, we utilized a form of the protein CRIPT which exerts a dominant negative effect, blocking PDZ3 binding of all PSD-95 family members (Passafaro et al., 1999; Conroy et al., 2003). Neurons expressing CRIPT showed no change in GluR1-SEP mobility when treated with nicotine (Fig. 5C) and did not display increased numbers of GluR1-SEP at spines (Fig. 5D). The results indicate that a PDZ-scaffold is likely to be required for nicotinic stabilization of AMPARs at synapses. This is consistent with current models for synaptic potentiation in which passively diffusing surface receptors are trapped by postsynaptic scaffold anchoring slots (Opazo et al., 2010, 2011; Mondin et al., 2011).

Nicotine-induced effects on GluR1 trafficking depend on lateral diffusion, not exocytosis

To determine if the nicotinic effect uses existing surface GluR1s to increase receptor density on spines, we restricted the lateral diffusion of GluR1-SEP by antibody cross-linking. This procedure greatly hinders the lateral surface mobility of AMPARs while leaving exocytosis largely unaffected (Ashby et al., 2006). Indeed, cross-linking GluR1-SEP with an anti-GFP antibody prior to and throughout the nicotine treatment

almost completely abolished GluR1-SEP FRAP, indicating a marked reduction in the mobility of surface GluR1s (Fig. 6A). Under these conditions nicotine was unable to induce the enrichment of GluR1-SEP at spines (Fig. 6B), demonstrating that lateral diffusion of GluR1-SEP is required for this effect.

To test the possibility that exocytosis is needed to replenish extrasynaptic AMPARs as a source of receptors for the nicotinic effect, we blocked exocytosis of internal receptors by treating cells with 10 μg/ml Brefeldin-A (BFA; Galan et al., 2004). In this case nicotine was still able to reduce the mobility of GluR1-SEP and increase receptor density at spines (Fig. 6C,D). This suggests that contrary to other forms of synaptic potentiation, exocytosis of AMPARs to replenish surface-receptor reserve pools is not required for the nicotinic effects (Kopec et al., 2006; Makino and Malinow, 2009; Petrini et al., 2009; Opazo and Choquet, 2011).

Nicotine-stabilized GluR1s are synaptically incorporated

To determine whether the nicotine-induced increases in AMPARs on spines are relevant for synaptic signaling, we compared miniature excitatory postsynaptic currents (mEPSCs) in control and nicotine-treated cells. The experiments were performed on untransfected cells to test if the nicotinic effect applied to endogenous native receptors. The mEPSCs were recorded in the presence of 1 μM tetrodotoxin (TTX) to block action potentials, and the cells were clamped at -70 mV (Cl^- reversal potential) to eliminate miniature inhibitory postsynaptic currents (mIPSCs). The mEPSCs were fully blocked by 50 μM APV plus 20 μM NBQX (data not shown), confirming they represented glutamatergic events. Nicotine applied at 1 μM for 1-3 hrs increased both the frequency

and size of mEPSCs as seen in cumulative histograms of inter-event intervals and event amplitudes (Fig. 7A-C). The increase in mEPSC amplitude is likely to reflect the additional AMPARs located at postsynaptic sites after nicotine treatment, while the increase in mEPSC frequency could reflect both pre- and postsynaptic effects.

To confirm that the nicotine-induced shift in mEPSC amplitude was due to increased numbers of GluR1s at synapses via lateral diffusion, we tested the effects of antibody cross-linking. As reported above, antibody cross-linking of surface GluR1-SEP prevented lateral diffusion and, as a consequence, the nicotine-induced enrichment of the receptors at spines. We treated untransfected cultures with an antibody that recognizes an extracellular epitope on native GluR1. In these cultures nicotine exposure did not increase the number of large-amplitude events though it still increased mEPSC frequencies, albeit to a lesser extent (Fig. 7D-F). Accordingly, the change in amplitude is likely the result of additional AMPARs being incorporated into postsynaptic sites, while the increase in frequency is, at least in part, due to a presynaptic effect such as an increase in probability of release.

DISCUSSION

The results demonstrate a novel mechanism by which nicotinic activity can drive synaptic potentiation. Stimulation of α7-nAChRs stabilizes GluR1s at postsynaptic sites on spines, drawing them from the surface pool of mobile extrasynaptic receptors. The stabilization is calcium-dependent, requires functional PSD-95 family members, and reflects both an increase in the number of such receptors on spines and a decrease in their mobility. The outcome is an increased signaling capacity for the synapse, reflected by

larger mEPSCs. Remarkably, the changes rely upon the activation of postsynaptic α7-nAChRs and do not require coincident signaling through AMPA and NMDARs, suggesting a cell-autonomous mechanism (Fig. 8). As a result, extended nicotinic activity could promote synaptic plasticity at multiple synapses across a region independent of concomitant rapid synaptic transmission at those synapses.

Nicotinic cholinergic modulation of glutamatergic synaptic plasticity in the hippocampus has frequently been ascribed to the pre- or postsynaptic enhancement of glutamatergic transmission, enabling it to produce long-term changes in synaptic efficacy (Fuiji et al., 1999; Ji et al., 2001; Kenney and Gould, 2008; Placzek et al., 2009; Gu et al., 2012; Yakel, 2012). The present results differ from the foregoing in two respects. First, the enhancement did not require rapid glutamatergic transmission, which, in combination with the necessity of α7-nAChRs located on the postsynaptic cell, suggests a cell-autonomous mechanism. Cell-autonomous actions have been proposed for nAChRs in regulating proper network integration and synapse formation in developing neurons (Campbell et al., 2010; Lozada et al., 2012b). Second, the time course of nicotine-induced changes was intermediate between acute and chronic treatments, requiring 1-2 hrs of continued nicotine exposure to become significant. This amount and duration of exposure mimic the systemic administration and brain tissue accumulation of nicotine that occur during smoking (Rose et al., 2010). It is also consistent with persistent ambient levels of acetylcholine (ACh) thought to occur in the CNS as a result of volume transmission (Umbriaco et al., 1995; Descarries et al., 1997). As a result, the nicotinic mechanisms found here may be relevant not only for nicotine exposure but also for endogenous nicotinic cholinergic signaling via ACh.

A widely accepted model of LTP is that GluR1s are inserted into the plasma membrane, freely diffuse to the synaptic site, and become tethered to a PSD-95 scaffold due to an increase in binding affinity elicited by a calcium-dependent, activity-driven process. GluR1-lacking receptors subsequently replace the GluR1 place-holders in a constitutive, non-activity-dependent manner (Liao et al., 1995; Hayashi et al., 2000; Malinow and Malenka, 2002; Makino and Malinow, 2009, 2011; Opazo et al., 2010, 2011; Opazo and Choquet, 2011; Tanaka and Hirano, 2012). Consistent with this view are the findings here that the nicotine-driven increase in AMPAR localization and stabilization on spines is specific for GluR1s, calcium-dependent, requires PDZ interactions of the PSD-95 family, and relies on the free lateral diffusion of GluR1s. No increase was seen in spine size with nicotine, though increases are often, but not always, associated with synaptic potentiation (Kopec et al., 2006; Makino and Malinow, 2009, 2011). This scaffold-dependent receptor entrapment may, therefore, account for both the increase in GluR1 number on spines and their simultaneous immobilization there. Our results, however, cannot rule out the possibility that nicotine leads to an increase in the total number of PDZ scaffold sites at spines rather than an increase in the affinity of GluR1 for them. Consistent with this are studies showing that overexpression of PSD-95 can lead to synaptic potentiation (Ehrlich and Malinow, 2004; Elias et al., 2006). The excess of PSD-95 molecules at spines as compared to AMPARs under normal conditions and the non-specific nature of such an increase, however, make this an unlikely physiological scenario for synaptic plasticity (Sheng and Hoogenraad, 2007; Opazo et al., 2011). Notably, α7-nAChRs have been shown to interact with members of the PSD-95 scaffold family (Farías et al., 2007; Gómez-Varela et al., 2012). The reliance on PDZ

interactions, therefore, may also reflect a requirement for α7-nAChR anchoring and localization at postsynaptic sites.

For LTP, the tethering of AMPARs to the postsynaptic scaffold usually results from a calcium-dependent phosphorylation of their TARP auxiliary subunits (Chen et al., 2000; Bats et al., 2007; Opazo et al., 2010, 2011; Opazo and Choquet, 2011). The calcium-dependence of the nicotine-induced effects reported here may reflect a similar mechanism. The slow time course of the nicotinic effect, however, is also consistent with a requirement for early transcriptional events (Greenberg et al., 1992). Activation of α7-nAChRs by prolonged exposure to nicotine is known to alter gene transcription (Chang and Berg, 2001; Dajas-Bailador and Wonnacott, 2004) and signaling through α7-nAChRs on hippocampal neurons has been shown to activate the transcription factor CREB in a manner at least partly independent of NMDA and AMPARs (Hu et al., 2002).

The FRAP experiments indicated a reduction in the mobility and a 50% increase in the amount of GluR1-SEP on spines following the nicotine treatment, suggesting the receptors became synaptically incorporated (Makino and Malinow, 2009, 2011). Consistent with this, patch-clamp recording indicated an increase in the fraction of mEPSCs having large amplitudes. Importantly, the recorded mEPSCs were generated by native AMPARs, and like the nicotine-induced increases in GluR1-SEP on spines, the increase in mEPSC amplitude was prevented by cross-linking AMPARs on the neuron surface. The amplitude increase, however, was less dramatic than the increase in GluR1-SEP accumulation on spines. Part of the reason for this may be that no change was seen in GluR2s over this time frame, yet the majority of synaptic AMPARs contain GluR2, presumably contributing to a large portion of synaptic transmission (Sans et al., 2003; Lu

et al., 2009). A second reason, however, may be that the nicotine treatment recruited GluR1s, at least in part, to synapses with an insufficient number of AMPARs to generate mEPSCs, i.e. so-called "silent synapses" (Isaac et al. 1995; Liao et al., 1995). Consistent with this, cross-linking AMPARs prevented some of the increase in mEPSC frequency induced by nicotine, as though part of this increase represented the postsynaptic unsilencing of previously silent synapses. These mEPSCs, if small in size, would detract from the net increase in mean mEPCS amplitude recorded for nicotine-treated cells.

Nicotine is known to enhance contextual learning as evidenced by the role cued and context-evoked cravings play in nicotine addiction and relapse behavior (Kenney and Gould, 2008, Placzek et al., 2009). It is likely to do so, at least in part, by Hebbian plasticity as documented for activity-dependent nicotinic enhancement of synaptic plasticity (Dani et al., 2001; Mansvelder and McGehee, 2002; Kenney and Gould, 2008; Placzek et al., 2009; Yakel, 2012). Unexpectedly, the present results demonstrate a new form of nicotine-driven synaptic plasticity at glutamatergic synapses, namely one that occurs more slowly and does not require coincident fast, excitatory transmission at those same synapses. Such a mechanism raises the prospect of nicotinic signaling, either from systemic nicotine or from endogenous cholinergic input, affecting large numbers of synapses in a region without being constrained by patterns of ongoing activity. A different possibility, however, is that the nicotinic effect may be confined to synapses having specific properties, e.g. sufficient GluR1s in reserve pools on the membrane surface which can be rate-limiting (Makino and Malinow, 2009; Petrini et al., 2009). This possibility is suggested by the fact that the nicotinic effect occurs independent of exocytosis and relies instead on pre-existing reserve pools of surface receptors. A further

constraint may be the specific cellular location and levels of α7-nAChRs, which are known to be limited and subject to activity-dependent regulation (Zarei et al., 1998; Fabian-Fine et al., 2001; Kawai et al., 2002; Fernandes et al., 2010; Gómez-Varela et al., 2012). Consistent with both scenarios is the observed change in population distribution of spines based on their levels of GluR1-SEP. The apparent shift in population distribution is consistent with a cell-wide effect, but the appearance of a subset of highly enriched spines suggests that the nicotinic effect may be more prominent on specific subsets of synapses that were primed by prior events.

The findings presented here describe a novel and robust mechanism by which nicotinic signaling can modulate the cellular and molecular mechanisms thought to underlie learning and memory. These and related studies contribute to the understanding of how nicotinic signaling shapes behavior. Knowledge of this process is likely to be fundamental for developing effective treatments for addiction and nicotinic-related neurological disorders, as well as for providing insight into how basic cellular function can lead to complex behavior and thought.

EXPERIMENTAL PROCEDURES

Experiments were conducted according to the National Institutes of Health guidelines for animal research and were approved by the Institutional Animal Care and Use Committee at the University of California, San Diego.

DNA Constructs. The pCI-GluR1-SEP and pCI-GluR2-SEP constructs were generous gifts from Roberto Malinow and were cloned as described (Kopec et al., 2006). The RNAi-C-RFP and pHcRED-CRIPT vectors were generated as described for other

constructs (Conroy et al., 2003; Neff et al., 2009). FUGW-GluR1-SEP was constructed by removing GFP from FUGW (Addgene plasmid 14883; Lois et al., 2002) with the restriction enzymes AgeI and BsrG1 (New England BioLabs [NEB]) and inserting the PCR-amplified GluR1-SEP from pCI-GluR1-SEP with SgrAI- and BsiWI-cut (NEB) sticky ends. Sequence integrity for the GluR1-SEP was confirmed using primer-walk sequencing (Integrated DNA Technologies). In FUGW, GluR1-SEP is expressed under a ubiquitin promoter which allows for higher expression levels in neurons than pCI-GluR1-SEP.

The α7-shRNA and scrambled Scr-shRNA used the sequences 5'-AGGCAGATATCAGCAGCTATA-3' and 5'-GAGAGTACGCTAAGATCCTAA-3', respectively (Campbell et al., 2010; Lozada et al., 2012a), contained within the plasmid FG12 (Addgene plasmid 14884; Qin et al., 2003). The H1-shRNA cassette from these constructs was excised using the restriction enzymes PacI and SnaBI (NEB) and ligated into a similarly cut FUGW-GluR-SEP to generate constructs driving shRNA expression under the promoter H1 and driving GluR1-SEP expression under a ubiquitin promoter.

Rat hippocampal dissociated cell cultures. Dissociated hippocampal cultures were prepared from embryonic day 18-19 Sprague-Dawley rat embryos (Harlan) of both sexes as described (Kawaii et al., 2002; Gómez-Varela et al., 2012). Briefly, cells were plated on a 12 mm glass coverslip in 500 µl of medium at 10^6 cells per well in a 24-well plate (Falcon), maintained in a humidified tissue culture incubator at 37°C with 5% CO_2, and fed twice weekly by replacing half the volume with fresh medium. All cultures were used between day-in-vitro (DIV) 14-17, and when necessary, transfected on DIV6-7 using a calcium-phosphate precipitation kit (Clontech; Goetze et al., 2004). SEP and RFP

constructs were co-transfected at a ratio of between 3:1 and 12:1. In some cases SEP constructs were transfected alone. The pHcRED-CRIPT construct was co-transfected with FUGW-GluR1-SEP and RNAiC-RFP constructs at a ratio of 5:10:3, respectively.

Mouse hippocampal slice cultures. Postnatal day 1-2 mouse pups (C57BL/6 Charles River) were quickly decapitated and their brains were rapidly excised into ice-cold sucrose-buffered artificial cerebral spinal fluid (sucrose-ACSF) saturated with 95% O_2/5% CO_2, containing (in mM): 110 sucrose, 2.5 KCl, 1.25 NaH_2PO_4, 7 $MgCl_2$, 2.5 $CaCl_2$, 25 $NaHCO_3$, 10 glucose. Transverse hippocampal slices including a portion of the entorhinal cortex were made at 300 μm using a vibratome (Series 1000 Plus; Technical Products International). Slices were kept in culture as described (Stoppini et al., 1991; Lozada et al., 2012b). Briefly, four slices were plated per Millicell insert (Millipore) and fed once weekly by replacing half the volume with fresh medium. Cultures were biolistically transfected (Gene-gun, Bio-Rad) on DIV8 as described (Woods and Zito, 2008), and used for experiments on DIV14-16. Transfection parameters were: 4-5 mg of Au, 35 μg of pCI-GluR1-SEP or FUGW-GluR1-SEP, and 15 μg of RNAiC-RFP per round of bullets made. Standard barrels were covered with a fine mesh (Sefar Nitex: CMN-0090-D; 90 μm opening, 30 μm thread diameter, 48.5% open area), and slices were shot using 80-100 PSI of Helium.

Imaging. FRAP experiments were performed on a Leica SP5 confocal microscope using the FRAP-Wizard Leica software plugin (LAS-AF version: 2.6.0.7266). Time-lapse images were taken as simultaneously scanned, multichannel single panes in the z dimension. Excitation and absorption parameters were designed to maintain signal ranges below saturation and were tested to minimize crosstalk between

channels. The following settings were used for OT slice image acquisition: 40% Argon laser power with 13% of 488 nm excitation; 15% 543 nm HeNe excitation; PMT1 absorption 500-525 nm, gain 1100, offset -1%; PMT2 absorption 588-683; gain 700-100, offset -1%; 63× (numerical aperture, 0.9) water immersion objective; Pinhole at 1 AU; 512×512 pixels; 700Hz; 15× zoom; 2× frame average; 32.1 nm × 32.1 nm pixel size; 16.4 μm × 16.4 μm image size. The OT slice FRAP protocol was as follows: 2 baseline images per 10 secs; two zoom-in bleach images for a total of 3 secs with 543 at 0% and 458, 476, and 488 at 100%; 5 recovery images/30secs; manually-refocused recovery images taken 2, 4, 9, 14, 19, and 29 min after the automatic image acquisition. Slices were continuously perfused for a maximum of 2 hrs at 33°C in 95% O_2/5% CO_2-saturated ACSF containing (in mM): 119 NaCl, 2.5 KCl, 1 NaH_2PO_4, 1.3 $MgSO_4$, 2.5 $CaCl_2$, 26 $NaHCO_3$, 11 glucose..

Unless otherwise noted, images of dissociated hippocampal cultures were acquired using similar parameters with the following differences: 30-40% Argon laser power; pinhole opened to 4AU; PMT1 absorption 500-530, gain 1000, offset -10%; PMT2 absorption 571-683 offset -5%. The FRAP protocol included 4 baseline images per 10 secs; 2 zoom-in bleach images for a total of 3 secs with 543 at 0% and 458, 476, and 488 at 100%; 6 recovery images/30secs; 7 recovery images/120 secs. Dissociated cultures were imaged at 33°C in continuously perfused HEPES-buffered saline designed to mimic the Neurobasal-E (Invitorgen) growth medium (Mock NB) containing (in mM): 51.72 NaCl, 26.19 Na-gluconate, 0.906 NaH_2PO_4, 5.33 KCl, 0.814 $MgCl_2$, 1.8 $CaCl_2$, 10.92 HEPES, 25 glucose, pH 7.4 with NaOH. Once on perfusion, cultures were imaged for no longer than 1.5 hrs.

Drug and antibody treatments. All stock solutions were dissolved in double-distilled water and diluted in recording/growth medium to the final concentration unless otherwise indicated. The stock and final concentrations for drugs were: BAPTA-AM (Invitrogen B6769) 10 mM stock (in 50/50 DMSO/Pluronic F-127, Invitrogen P3000MP), 10 μM final; BFA (Tocris 1231) 5 mg/ml in DMSO, 10 μg/ml (35.67 μM); DHβE (Sigma D149) 10 mM, 1 μM; DL-AP5 (APV; Tocris 0105) 10 mM, 50 μM; MLA (Sigma M168) 10 mM, 100 nM; NBQX (Tocris 0373) 20 mM in DMSO, 20 μM; nicotine (Sigma N3876) 100 mM, 1 μM; TTX (Tocris 1069) 1 mM, 1 μM. Antibodies used for cross-linking were: rabbit anti-GFP-alexa647-conjugate (A31852), 2 mg/ml stock in provided solution, 0.4 μg/ml final; rabbit anti-GluR1 (Calbiochem PC246) designed against the n-terminus of the receptor subunit, 100 μg/ml stock in provided solution; 2 μg/ml final.

For FRAP experiments in slices nicotine was added to the medium below the filter coincident with a feeding, and slices were submerged in 20-50 μl of the nicotine-containing medium. Cultures were then kept in the incubator for 1 hr before being transferred to the perfusion chamber also containing nicotine where they were imaged for no longer than 2 hrs.

For FRAP experiments in dissociated cultures, unless otherwise stated nicotine was added directly to the culture medium. Cells were then incubated with nicotine under growing conditions for 1-1.5 hrs. Coverslips were transferred to a perfusion chamber containing nicotine and imaged for no longer than 1.5 hrs. The antagonists MLA, DHβE, and APV + NBQX were added just prior to nicotine. BAPTA-AM, BFA and the anti-GFP antibody were added 30 min prior to nicotine treatment. All were present throughout

incubation and imaging, except the antibody which was washed out prior to imaging. In this case imaging was limited to 30 min. All electrophysiological experiments were limited to 30 min in the perfusion chamber and were performed in the presence of drugs but not antibodies. Controls were always performed on day-matched sister cultures that were handled and treated with vehicle in the same manner as the corresponding experimental groups.

Electrophysiology. For mEPSC recordings, dissociated cell cultures (DIV14-15) were immersed in HEPES-buffered saline containing (in mM): 0.001 TTX, 125 NaCl, 2 KCl, 4 $MgCl_2$, 1 $CaCl_2$, 10 HEPES, 20 glucose, pH 7.4 with NaOH. Recordings were done at 20-23°C in a perfusion chamber mounted on a Zeiss Axiovert. Microelectrodes (2-5 MΩ) were pulled from thin-walled glass capillaries (Warner Instruments: G86150T-4) with a P-97 pipette puller (Sutter Instrument) and contained (in mM): 130 $CsMeSO_4$, 3 CsCl, 10 HEPES, 1 EGTA, 10 NA_2-phosphocreatine, 2 MgATP, 0.3 NaGTP, pH 7.25 with CsOH. Whole-cell recordings were made with an Axopatch 200A and Clampex 8.2 software (Molecular Devices) using the whole-cell patch-clamp configuration in voltage-clamp mode held at -70 mV. Data were acquired at 5 kHz and filtered at 2 kHz at a gain of 5×. Whole-cell capacitance was canceled in all recordings, and only recordings with a series resistance of $\leq$ 25MΩ were used. Average resistance for each experimental group was 16-17 MΩ with no significant difference between groups (data not shown). Recordings were taken between 1-10 min after going whole-cell.

Data analysis. Images were analyzed using ImageJ software with the MBF plugin bundle (http://www.macbiophotonics.ca/imagej/). For FRAP experiments, x-y drift was corrected for using a rigid body transformation. Fluorescence signal intensity within a

region of interest (ROI) was measured as the optical density, the sum intensity of all the pixels divided by the number of pixels. Fluorescence recovery was calculated as a fraction of pre-bleach baseline fluorescence and was normalized to both the RFP spine signal to account for changes in spine size, as well as a separate section of the neuron to correct for incidental bleaching. Background fluorescence was measured from three separate areas with no cellular process and subtracted from the SEP signal:

$$Signal = \frac{(SEP - BG)}{RFP} \qquad and \qquad R_t = \frac{(E_t/E_0)}{(C_t/C_0)}$$

where *Signal* at an ROI was calculated as the *SEP*-channel optical density (*SE*P) minus background optical density (*BG*) over the corresponding *RFP*-channel optical density (*RFP*). R_t is the relative fluorescence recovery at spines for any given time point t. E_t is the *Signal* at the experimental spine; C_t is the *Signal* at a control spine or region of dendrite; E_0 and C_0 are the corresponding baseline *Signals* calculated as the average of all the pre-bleach frames. All FRAP experiments were analyzed in this manner except Fig. 4A,E and S1A,B because the low transfection efficiency of pCI-GluR1-SEP did not allow for RFP co-transfection in dissociated cultures. In these cases spine data were discarded if the spine substantially changed in size during the recovery. In those FRAP experiments where the images went out of focus during the automatic acquisition, a manually-focused image was obtained at the end to measure the last recovery point; all out-of-focus images were discarded.

The first baseline image from a corresponding FRAP series was used to measure spine enrichment. An ROI was drawn tightly around the SEP signal of a spine and

another around the entire length of the dendrite. The SEP optical density of the spine was divided by the SEP optical density of the dendrite to yield the enrichment value.

For mEPSC analysis, 100 events were counted from each cell. Events were detected using Mini Analysis by Synaptosoft. The detection parameters were as follows: amplitude threshold was four times the root mean square of the noise, area threshold was 10-20, and complex peak detection was turned on. Automatic detection was manually checked for accuracy.

Statistics. All data are presented as means ± SEMs, except for cumulative frequency plots. The mean values for each FRAP plot were fit with a double-exponential curve (IGOR Pro; WaveMetrics). Statistical significance (p value) was assessed for final FRAP points, enrichment values, and mean mEPSC values using an unpaired t-test (GraphPad Prism 4) between matching control and experimental groups. When necessary, Welch's correction was used for unequal variances and noted in the figure legend as WC. Unless otherwise stated, experiments were repeated ≥ 3 times with each time representing cultures from a separate plating; n represents the number of spines, with 1-3 spines being taken from a single cell. The two-sample Kolmogorov-Smirnov test (KS; Mini Analysis, Synaptosoft) was used to compare cumulative frequency distributions. Grubbs' outlier test (GraphPad QuickCalcs) was used on all mean mEPSC amplitude and frequency data. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$

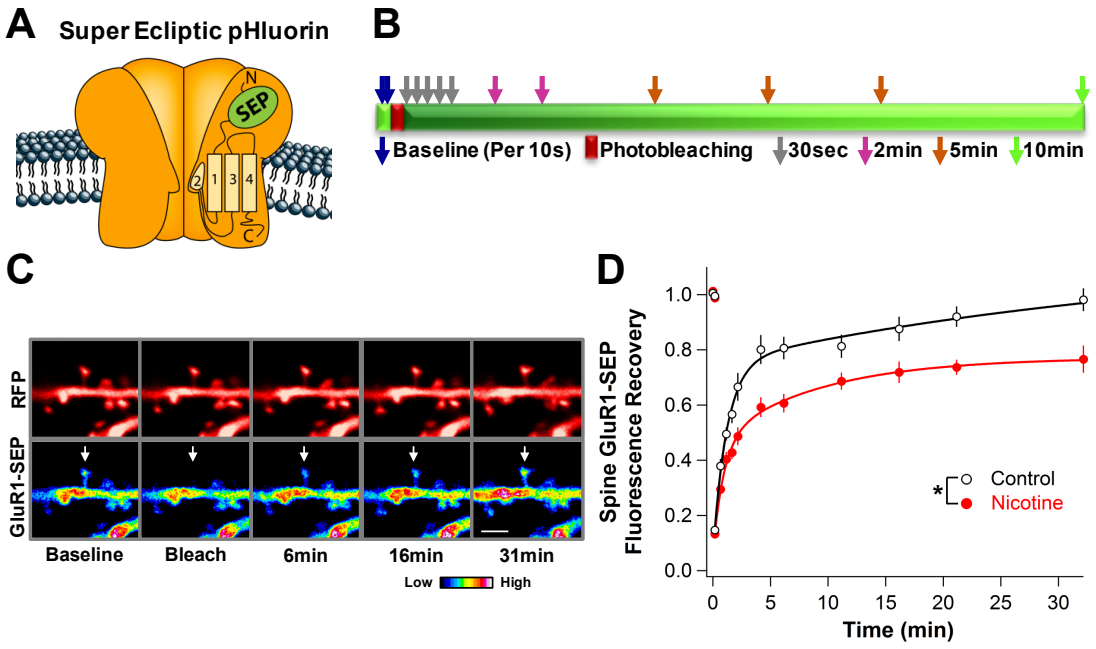


Fig. 1 Nicotine increases the immobile fraction of GluR1-SEP at spines of CA1 pyramidal neurons in slice culture. **(A)** A depiction of an AMPAR containing a transgenic GluR subunit tagged with Super Ecliptic pHluorin (SEP), a pH sensitive GFP that only fluoresces while on the surface of the cell. **(B)** FRAP protocol schematic with 31 min imaged for recovery. Two pre-bleach baseline images taken 10 sec apart (blue arrows), then a series of recovery images taken at the indicated interval, i.e. fast recovery images taken every 30 sec (gray arrows). **(C)** Images taken from the basal dendrites of CA1 pyramidal neuron depicting GluR1-SEP FRAP under control conditions with accompanying cytosolic-RFP images. Arrows indicate bleached spine. Scale bar: 2 µm. **(D)** In cultures treated with 1 µM nicotine for 2-3 hrs, GluR1-SEP fluorescence at spines recovers to a lesser extent in 31 min following photobleaching than corresponding controls, indicating decreased mobility (Ctrl vs Nic: 98 ± 4 vs 77 ± 5%; n = 12,14; $p < 0.05$).

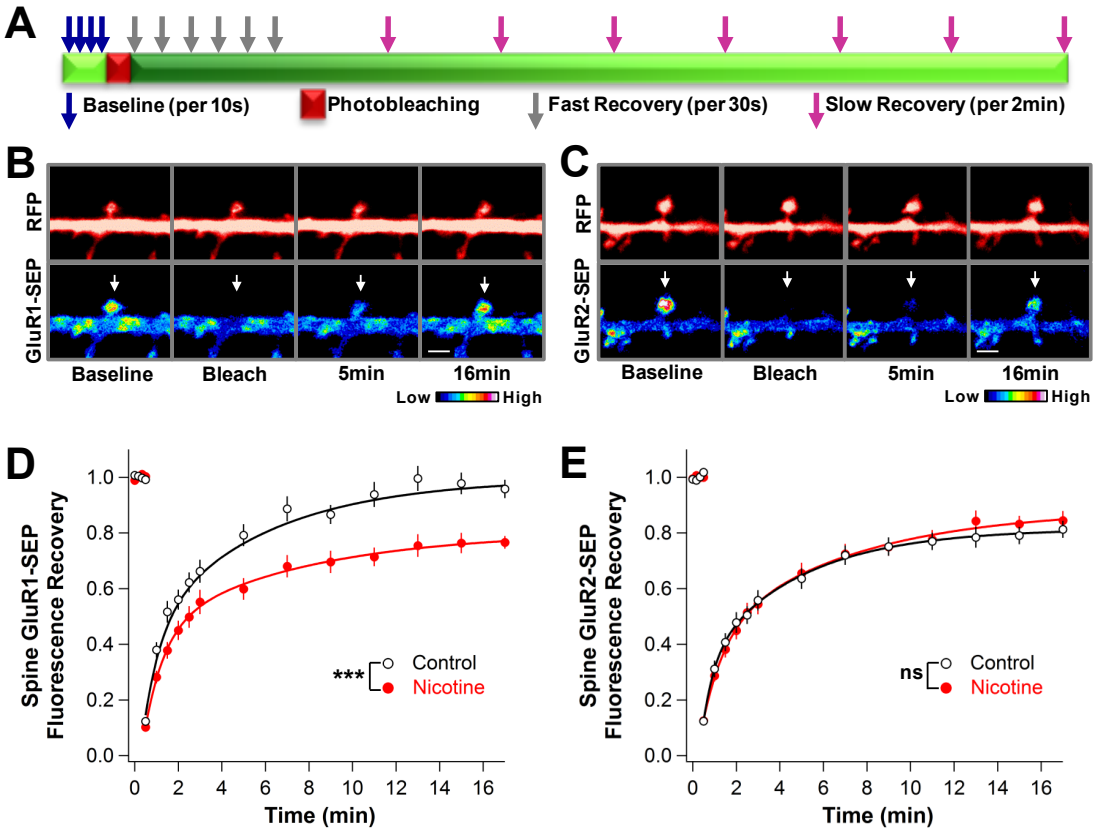


Fig. 2 Nicotine-induced stabilization of AMPARs is specific to GluR1, not GluR2, receptors. **(A)** FRAP protocol schematic for dissociated culture with 16.5 min imaged for recovery. Four pre-bleach baseline images taken 10 sec apart (blue arrows), six images taken 30 sec apart (gray arrows), and seven images taken 2 min apart. **(B)** Control condition images of GluR1-SEP and **(C)** GluR2-SEP FRAP in dissociated culture with accompanying cytosolic-RFP images. Arrows indicate bleached spine. Scale bar: 2 µm, here and below. **(D)** In dissociated cultures treated with 1 µM nicotine for 1-3 hrs GluR1-SEP fluorescence at spines recovers to a lesser extent in 16.5 min following photobleaching than corresponding controls, indicating decreased mobility (Ctrl vs Nic: 96 ± 3 vs 77 ± 2%, n = 24,24; $p < 0.001$, WC). **(E)** Nicotine has no effect on GluR2 mobility, yielding a recovery equivalent to control (Ctrl vs Nic: 81 ± 3 vs 84 ± 3%; n = 15,17; $p = 0.49$).

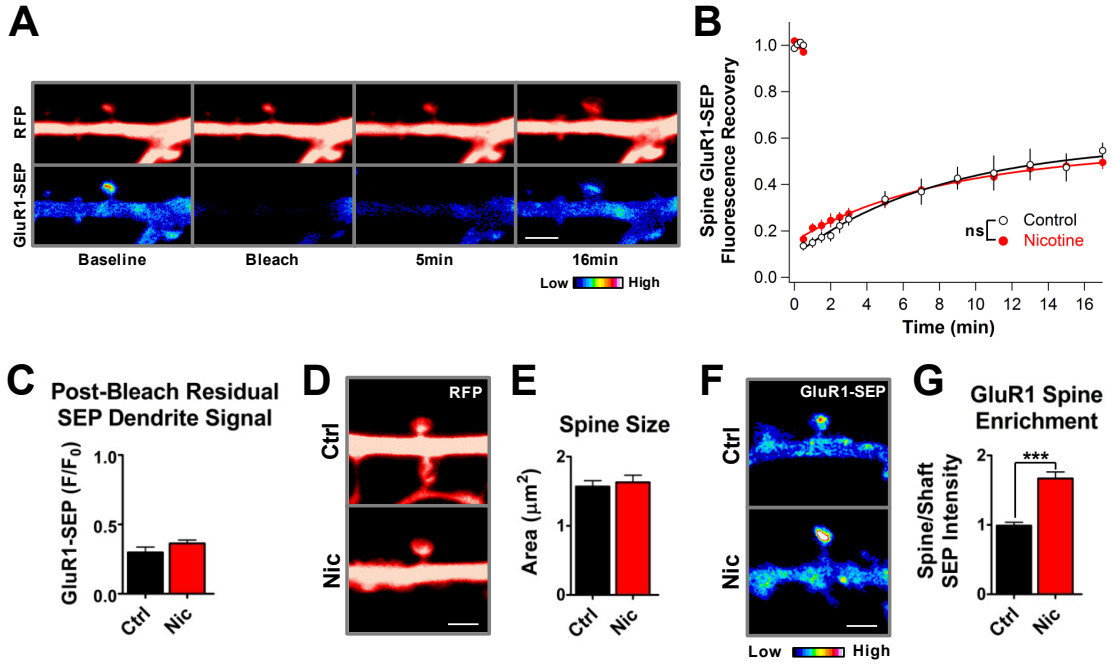
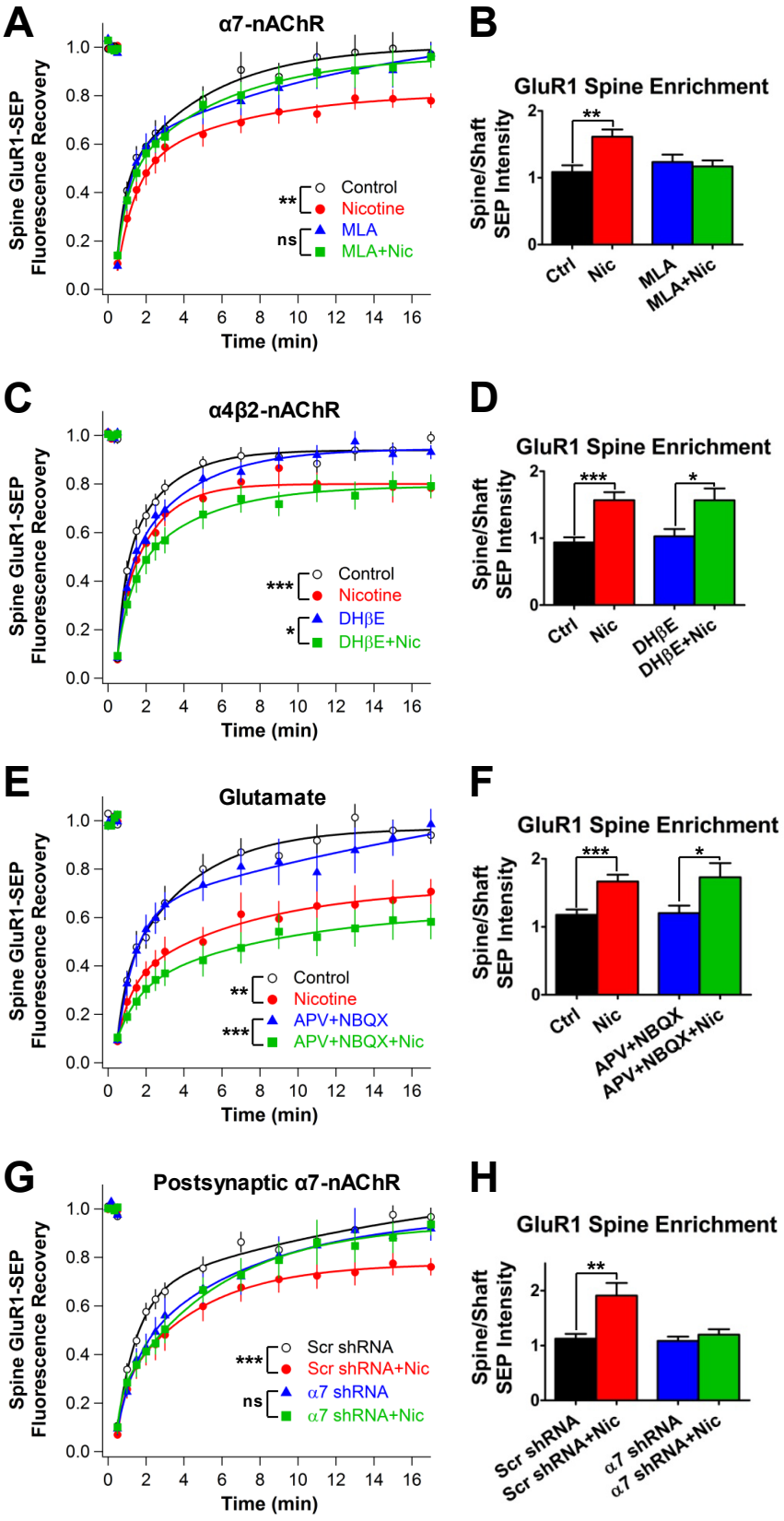


Fig. 3 The nicotine effect on GluR1-SEP FRAP represents a change in the rate of exchange between spine and dendrite surface receptors, and correlates with an increase in receptor density at spines. **(A)** Representative images depicting bleach of GluR1-SEP signal along 10 μm of the dendrite surrounding a bleached spine. Scale bar: 3 μm. **(B)** When the reserve pool of surface fluorescent receptors is depleted, GluR1-SEP recovery at the spine is greatly attenuated, suggesting that GluR1-SEP FRAP requires dendritic surface receptors. There is no measurable difference between the recoveries for control and nicotine-treated cells, indicating that the previously measured difference represented a reduction in the exchange rate between spine and dendrite receptors (Ctrl vs Nic: 55 ± 3 vs 50 ± 3%; n = 12,12; p = 0.28). **(C)** The extent of GluR1-SEP dendrite bleach. A 15-sec bleach protocol produces 65-70% bleach of the dendritic SEP signal for both control and nicotine-treated cells (Ctrl vs Nic; 30 ± 4 vs 36 ± 3%, n = 12,12; p = 0.18). **(D)** Representative pre-bleach images from FRAP experiments from which spine area was measured using the RFP signal. **(E)** Quantification of spine area yielded no significant difference between control and nicotine-treated cultures, suggesting that the nicotine-induced reduction in GluR1-SEP mobility was not accompanied by a change in spine size (Ctrl vs Nic: 1.57 ± 0.08 vs 1.63 ± 0.11 μm^2; n = 33,33; p = 0.66). **(F)** Representative pre-bleach images from FRAP experiments demonstrating nicotine-induced enrichment of GluR1-SEP at spines. **(G)** Quantification of GluR1-SEP enrichment on spines by measuring spine/shaft optical density yielded a significant increase in cells exposed to nicotine, suggesting nicotine leads to the accumulation of receptors at spines. (Ctrl vs Nic: 0.99 ± 0.05 vs 1.67 ± 0.1; n = 29,28; p < 0.001, WC).

Fig. 4 Antagonist blockade and shRNA knockdown reveal that nicotine acts through postsynaptic α7-nAChRs, not α4β2-nAChRs, to enrich and stabilize GluR1 at spines independent of activity through AMPA and NMDA receptors. All experiments are shown with day-matched controls. **(A)** Blocking α7-nAChRs with 100 nM MLA during 1-3 hrs of nicotine treatment prevents the reduction in GluR1-SEP mobility at spines (MLA vs MLA + Nic: 97 ± 5 vs 96 ± 5%, n = 12,15; p = 0.86; Ctrl vs Nic: 97 ± 5% vs 78 ± 3%, n = 14,14; p < 0.01, WC). **(B)** MLA also prevents nicotine-induced GluR1-SEP spine enrichment, suggesting that this too relies on signaling through α7-nAChRs (MLA vs MLA + Nic: 1.23 ± 0.11 vs 1.17 ± 0.09; n = 16,16; p = 0.66; Ctrl vs Nic: 1.09 ± 0.10 vs 1.61 ± 0.11; n = 16,16; p < 0.01). **(C)** Blocking α4β2-nAChRs with 1 μM DHβE during 1-3 hrs of nicotine treatment fails to prevent the reduction in GluR1-SEP mobility at spines (DHβE vs DHβE + Nic: 93 ± 3 vs 79 ± 5%, n = 11,12; p < 0.05, WC; Ctrl vs Nic: 99 ± 3 vs 78 ± 4%, n = 12,11; p < 0.001), **(D)** DHβE does not prevent nicotine-induced GluR1-SEP spine enrichment, suggesting that signaling through α4β2-nAChRs is not necessary for this effect (DHβE vs DHβE + Nic: 1.03 ± 0.11 vs 1.57 ± 0.18; n =1 2,12; p < 0.05; Ctrl vs Nic: 0.94 ± 0.08 vs 1.57 ± 0.12; n = 12,12; p < 0.001). **(E)** Antagonizing AMPARs and NMDARs with 50 μM APV and 20 μM NBQX, respectively, during 1-3 hrs of nicotine treatment fails to block the reduction in GluR1-SEP mobility at spines (APV + NBQX vs APV + NBQX + Nic: 98 ± 6 vs 58 ± 7%, n = 10,11; p < 0.001; Ctrl vs Nic: 94 ± 4 vs 71 ± 5%, n = 10,11; p < 0.01). **(F)** APV and NBQX also fail to block GluR1-SEP spine enrichment, indicating that this process acts independent of fast, excitatory glutamatergic transmission (APV + NBQX vs APV + NBQX + Nic: 1.2 ± 0.11 vs 1.73 ± 0.21; n = 11,11; p < 0.05, WC; Ctrl vs Nic: 1.18 ± 0.08 vs 1.67 ± 0.1; n = 11,11; p < 0.001). **(G, H)** Nicotine has no effect on GluR1-SEP trafficking in neurons expressing α7-shRNA. This suggests that nicotine acts directly through α7-nAChRs located on the postsynaptic neurons to stabilize and enrich GluR1-SEP at spines (α7-shRNA vs α7-shRNA + Nic, FRAP: 92 ± 5 vs 94 ± 5%, n = 12,12; p = 0.79; Enrichment: 1.08 ± 0.08 vs 1.2 ± 0.1; n = 12,12; p = 0.37; Scr-shRNA vs Scr-shRNA + Nic, FRAP: 97 ± 4, vs 76 ± 4%, n = 11,12, p < 0.001; Enrichment: 1.13 ± 0.09 vs 1.91 ± 0.23, n = 11,12, p < 0.01, WC).


A
α7-nAChR
Spine GluR1-SEP Fluorescence Recovery
Time (min)
Control
Nicotine
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B
GluR1 Spine Enrichment
Spine/Shaft SEP Intensity
Ctrl
Nic
MLA
MLA+Nic
C
α4β2-nAChR
Control
Nicotine
DHβE
DHβE+Nic
***
*
D
GluR1 Spine Enrichment
Ctrl
Nic
DHβE
DHβE+Nic
E
Glutamate
Control
Nicotine
APV+NBQX
APV+NBQX+Nic
F
GluR1 Spine Enrichment
G
Postsynaptic α7-nAChR
Scr shRNA
Scr shRNA+Nic
α7 shRNA
α7 shRNA+Nic
H
GluR1 Spine Enrichment

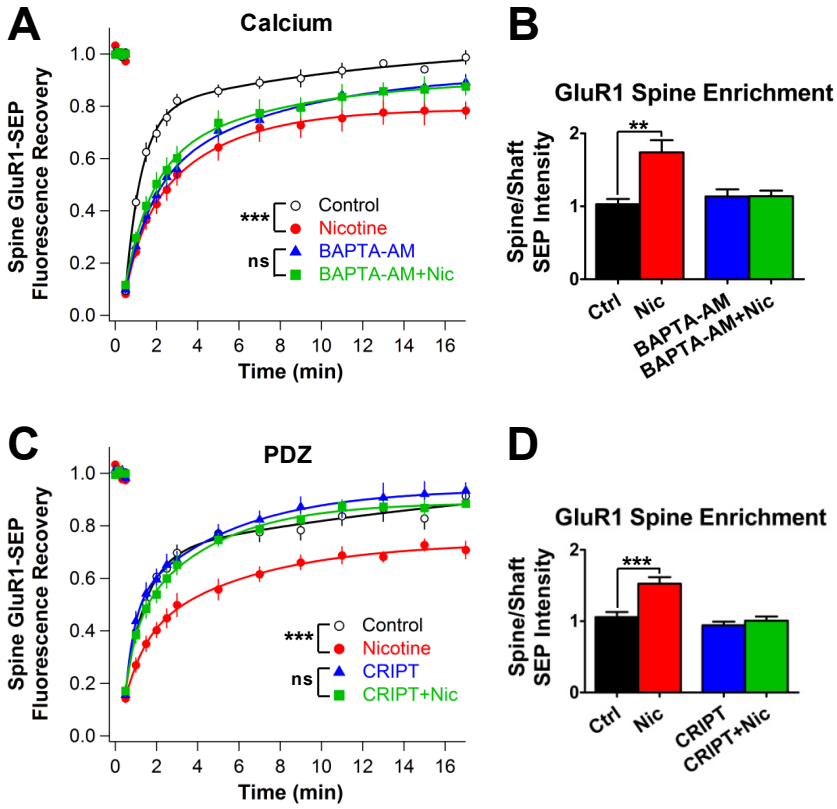


Fig. 5 Both intracellular calcium signaling and PSD-95 family member PDZ-interactions are necessary for the nicotine-induced effects on GluR1 trafficking. **(A, B)** Exposure to the calcium chelator, BAPTA-AM beginning 30 min prior to and throughout the 1-3 hrs of nicotine prevents the effects on GluR1-SEP trafficking, suggesting that they rely on intracellular calcium signaling (BAPTA-AM vs BAPTA-AM + Nic, FRAP: 89 ± 3 vs 88 ± 3%, n = 15,15; p = 0.75; Enrichment: 1.14 ± 0.1 vs 1.14 ± 0.08; n = 15,15; p = 0.98; Ctrl vs Nic, FRAP: 99 ± 3, vs 78 ± 3%, n = 14,13, p < 0.001; Enrichment: 1.03 ± 0.07 vs 1.74 ± 0.18, n = 14,13, p < 0.01, WC). **(C, D)** Nicotine had no effect on GluR1-SEP trafficking in neurons transfected with CRIPT, a dominant negative protein that disrupts PDZ-interactions with PSD-95 family members, suggesting that PDZ binding is critical for nicotine-induced enrichment and stabilization of GluR1-SEP at spines (CRIPT vs CRIPT+Nic, FRAP: 93 ± 3 vs 88 ± 2%, n = 14,13; p = 0.18, WC; Enrichment: 0.94 ± 0.05 vs 1.01 ± 0.06; n = 14,14; p = .42; Ctrl vs Nic, FRAP: 91 ± 3, vs 71 ± 4%, n = 12,12, p < 0.001; Enrichment: 1.06 ± 0.07 vs 1.53 ± 0.09, n = 12,12, p < 0.001).

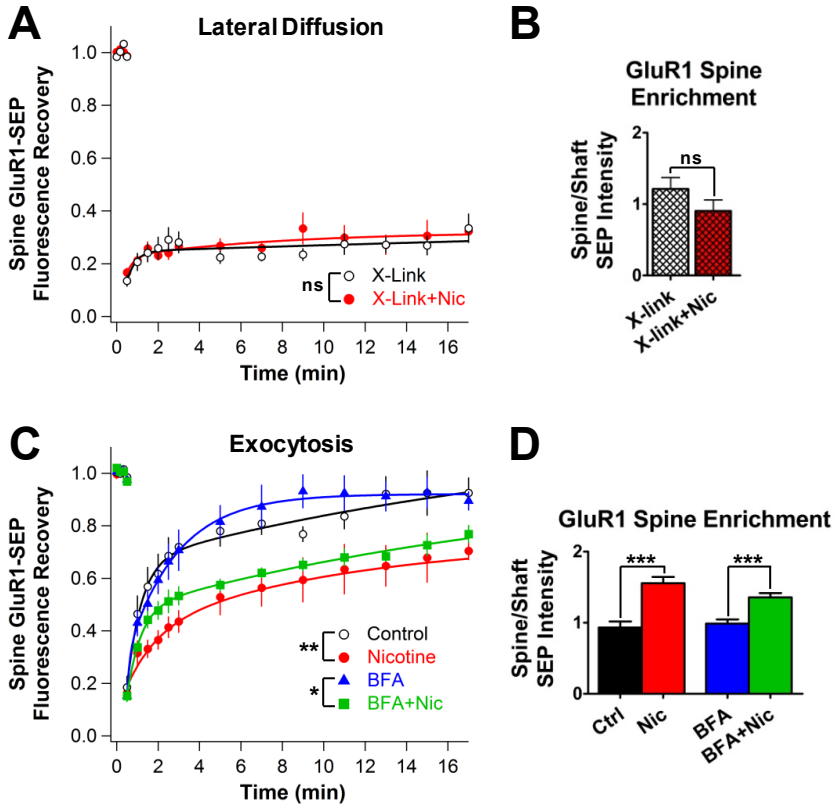
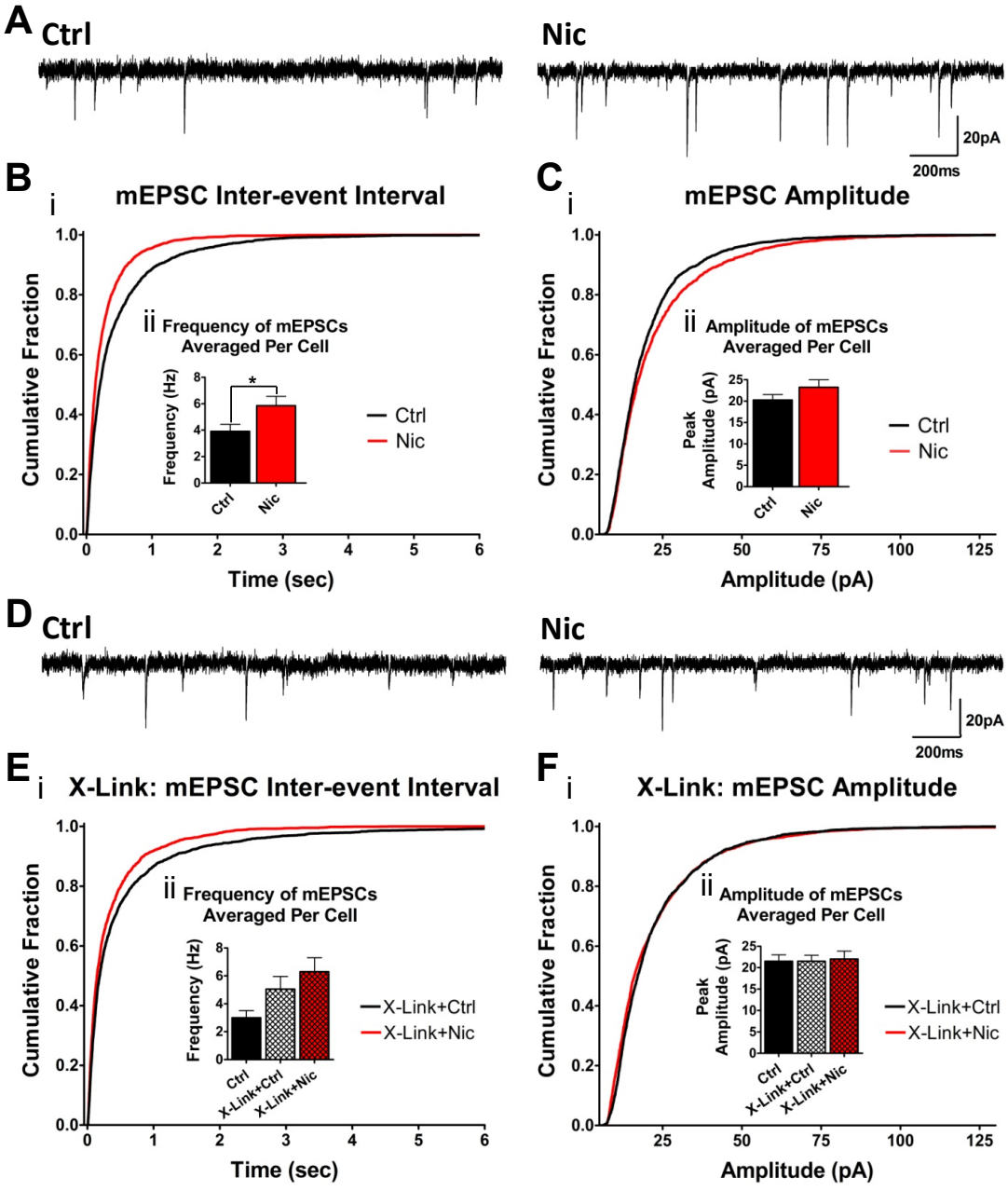


Fig. 6 Restricting the lateral mobility of AMPARs with antibody cross-linking (x-linking) prevents the nicotine effects on GluR1 trafficking, but blocking exocytosis does not. **(A)** Treating cultures with a GFP-specific antibody prevents normal GluR1-SEP FRAP, indicating that these receptors are now largely immobilized (x-link vs x-link+Nic; 33 ± 6 vs 32 ± 4%, n = 10,10; p = 0.88). **(B)** Antibody x-linking 30 min prior to and throughout 1-3 hrs of nicotine treatment prevents the enrichment of GluR1-SEP at spines, suggesting that the free diffusion of GluR1s is necessary for this effect (x-link vs x-link + Nic; 1.21 ± 0.16 vs 0.9 ± 0.15, n = 10,10; p = 0.19). **(C, D)** Blocking exocytosis with BFA 30 min prior to and during the 1-3 hrs of nicotine treatment does not prevent the enrichment and stabilization of GluR1-SEP at spines, suggesting that existing surface receptors are sufficient for these effects without the need for ongoing exocytosis (BFA vs BFA+Nic, FRAP: 90 ± 4 vs 77 ± 4%, n = 13,12, $p < 0.05$; Enrichment: 0.99 ± 0.06 vs 1.36 ± 0.06, n = 13,13, $p < 0.001$; Ctrl vs Nic, FRAP: 93 ± 6, vs 70 ± 4%, n = 10,10, $p < 0.01$; Enrichment: 0.93 ± 0.08 vs 1.56 ± 0.09, n = 10,10, $p < 0.001$).

Fig. 7 Nicotine treatment increases mEPSC frequency and amplitude generated by native AMPARs, and the amplitude change is blocked when GluR1-enrichment is prevented with antibody cross-linking (x-linking). **(A)** Representative traces revealing an increase in mEPSC frequency and amplitude in cells treated with nicotine for 1-3 hrs as compared to sister-culture controls. **(Bi)** The distribution of mEPSC inter-event intervals is shifted because events occur more frequently in cells treated with nicotine (Ctrl vs Nic, n = 2800 events for both, $p < 0.001$, KS). **(Bii)** Data presented as mean frequency averaged per cell reveal a significant increase in nicotine treated cells as compared to control (Ctrl vs Nic, 3.92 ± 0.53 vs 5.86 ± 0.7 Hz, n = 28,28, $p < 0.05$). **(Ci)** A comparison of the distributions of mEPSC amplitudes from control and nicotine-treated cells reveals a significant increase in the number of large amplitude events in cells treated with nicotine (Ctrl vs Nic, n = 2800 events for both, $p < 0.001$, KS). **(Cii)** Data presented as cell-averaged amplitudes reveal a trend for larger mean amplitudes in nicotine-treated cells (Ctrl vs Nic, 20.26 ± 1.27 vs 23.23 ± 1.76 pA, n = 28,28; p = 0.18, WC). **(D)** Representative traces from control and nicotine-treated cells which had their GluR1s x-linked prior to treatment. **(E)** Antibody x-linking of surface GluR1s does not fully prevent the increase in mEPSC frequency, suggesting that this is, at least in part, not due to GluR1 enrichment at spines (Ei: Ctrl vs Nic, $p < 0.001$, n = 2700, 2600 events; KS; Eii: Ctrl, 3 ± 0.51 Hz, n = 15; x-link+Ctrl vs x-link+Nic, 5.05 ± 0.91 vs 6.3 ± 1 Hz, n = 27,26, p = 0.36). **(F)** Antibody x-linking completely prevents the increase in amplitude induced by nicotine, indicating that the GluR1 enrichment at spines likely represents the synaptic incorporation of these receptors (Fi: Ctrl vs Nic, n = 2600, 2800 events; $p<0.001$, KS; Fii: Ctrl, 21.5 ± 1.51 pA, n=16; x-link+Ctrl vs x-link+Nic, 21.49 ± 1.4 vs 22.02 ± 1.8 pA, n = 26,28; p = 0.82). (The distribution of event amplitudes from x-link + Nic differs from that of x-link + Ctrl because the former has more small events. This is apparent in the rising phase of the graph and is in the opposite direction from the difference in Ci.)


A
Ctrl
Nic
20pA
200ms
B
i
mEPSC Inter-event Interval
Cumulative Fraction
Time (sec)
ii Frequency of mEPSCs Averaged Per Cell
Frequency (Hz)
Ctrl
Nic
C
i
mEPSC Amplitude
Cumulative Fraction
Amplitude (pA)
ii Amplitude of mEPSCs Averaged Per Cell
Peak Amplitude (pA)
Ctrl
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i
X-Link: mEPSC Inter-event Interval
Cumulative Fraction
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ii Frequency of mEPSCs Averaged Per Cell
Frequency (Hz)
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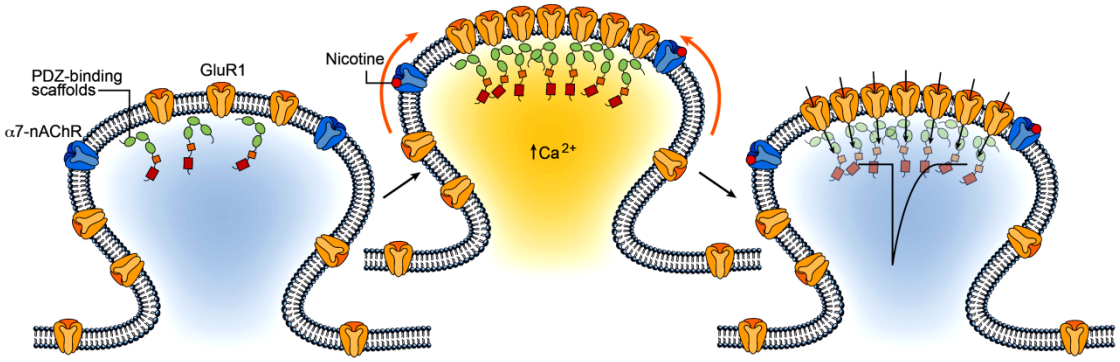

Fig. 8 Under basal conditions most GluR1s are not stabilized at spines and therefore not synaptically incorporated. Nicotine acts on postsynaptic α7-nAChRs to affect AMPAR trafficking independent of fast, excitatory glutamatergic transmission. It does so by elevating intracellular calcium and increasing the available PDZ-binding scaffold slots. As a consequence, GluR1-AMPARs diffusing on the cell surface become trapped at spines. This enrichment and stabilization of GluR1-AMPARs represents synaptic incorporation of the receptors, strengthening synaptic transmission.

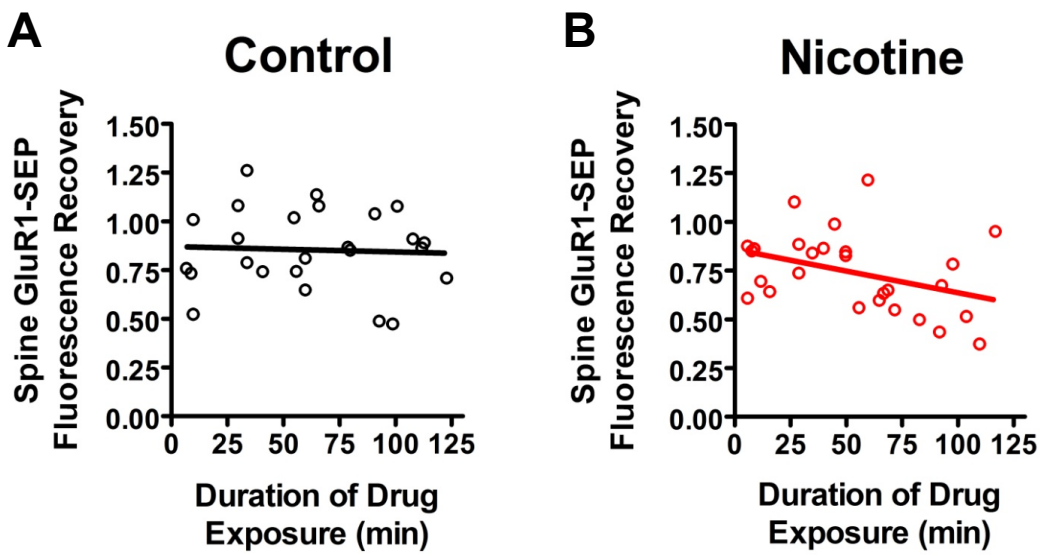

Fig. S1 The nicotine effect on GluR1-SEP FRAP occurs gradually over two hours. Cultures were transferred into a perfusion chamber with 1 μM nicotine and imaged for 2 hrs. **(A, B)** Data points represent the final recovery point from FRAP experiments. Values were fit with a linear regression demonstrating a trend in nicotine-treated cultures, but not control cultures (Control: slope -0.03 ± 0.12%, y-intercept 87.18 ± 8.42%, n = 25, r^2 = 0.0026, p = 0.8087; Nicotine: slope -0.22 ± 0.10%, y-intercept 85.85 ± 6.86%, n = 27, r^2 = 0.1429, p = 0.0519).

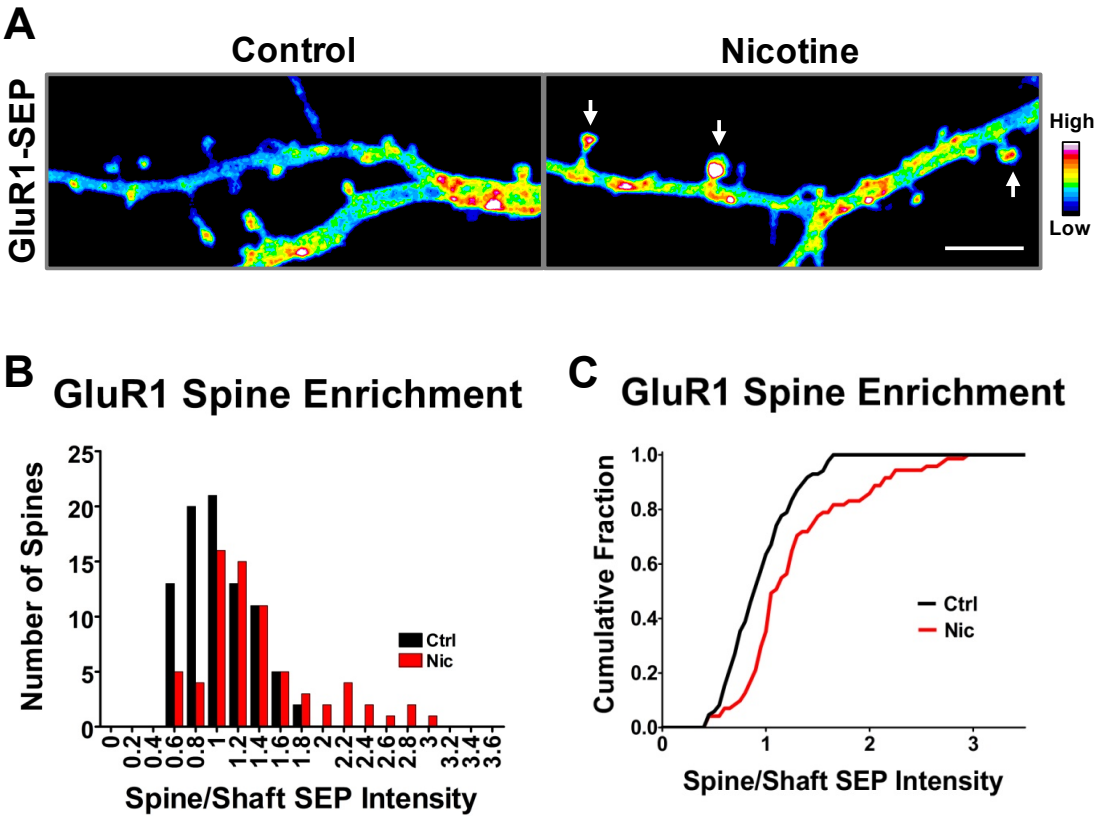

Fig. S2 Population distribution of GluR1-SEP spine enrichment on dendritic arbors. **(A)** Representative images depicting the effect of nicotine on GluR1-SEP spine enrichment. Images were taken at 5× zoom (enrichment images from all other experiments were at 15×) to allow for the visualization and quantification of multiple spines from a single dendrite. Z-stacks were collapsed as a sum of all slices in ImageJ. Arrows indicate enriched spines. Scale bar: 5 µm. **(B)** Frequency histogram of GluR1-SEP enrichment at spines from multiple neuronal dendritic arbors revealing a population of heavily enriched spines in nicotine treated cells (Ctrl n = 85 spines from 5 cells; Nic n = 71 spines from 5 cells, one week of plating). **(C)** Cumulative frequency plot of the data from B, demonstrating the statistically significant difference between the two distributions (Ctrl vs Nic, $p < 0.001$, KS).

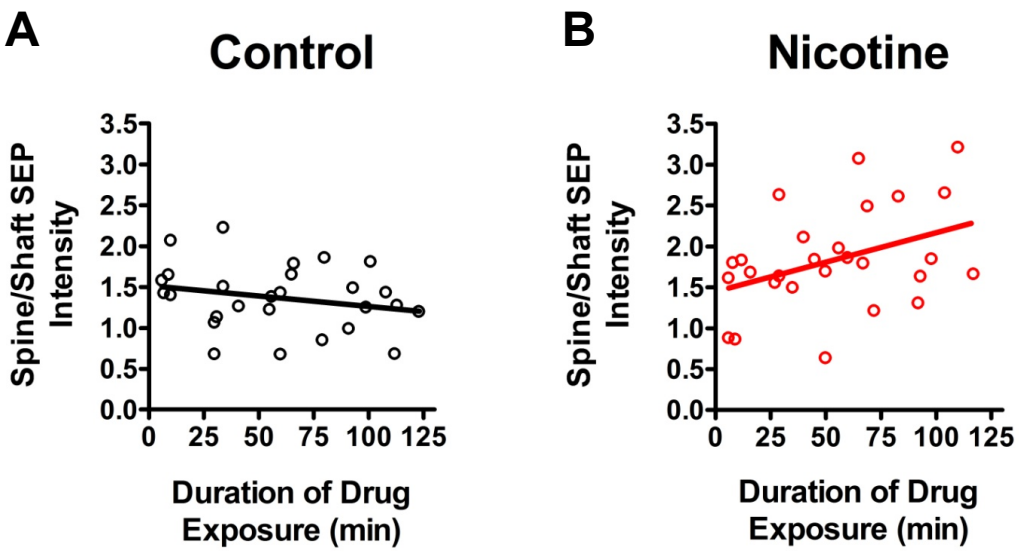

Fig. S3 The nicotine effect on GluR1-SEP spine enrichment occurs gradually over two hours. Cultures were transferred into a perfusion chamber with 1 μM nicotine and imaged for 2 hrs. **(A, B)** Data represent the spine enrichment of GluR1-SEP measured from the baseline images of corresponding FRAP experiments. Values were fit with a linear regression demonstrating a trend in nicotine-treated cultures, but not control cultures (Control: slope -0.003 ±0.002, y-intercept 1.52 ±0.15 n = 27, r^2 = 0.0554, p = 0.2373; Nicotine: slope 0.007 ±0.003, y-intercept 1.446 ±0.21, n = 27, r^2 = 0.1572, p = 0.0406).

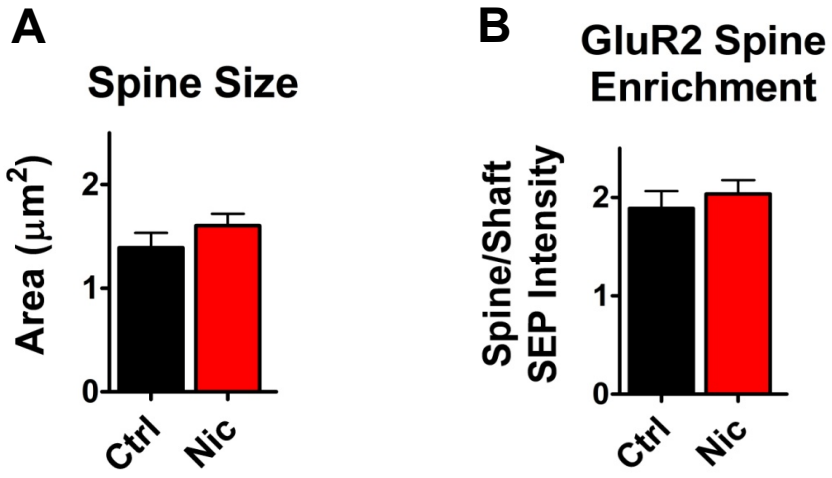

Fig. S4 Nicotine does not affect spine size or SEP enrichment at spines in neurons expressing GluR2-SEP. **(A)** Pre-bleach images from FRAP experiments (see Fig. 2E) were used to quantify spine area by measuring the RFP signal, but yielded no significant difference between control and nicotine-treated cultures (Ctrl vs Nic: 1.39 ± 0.14 vs 1.6 ± 0.11 μm^2; n = 15,18; p = 0.25). **(B)** Pre-bleach images from the same FRAP experiments were used to quantify spine enrichment of GluR2-SEP by measuring spine/shaft optical density, but yielded no significant difference between control and nicotine-treated cultures (Ctrl vs Nic: 1.89 ± 0.18 vs 2.04 ± 0.14; n = 15,18; p = 0.51).

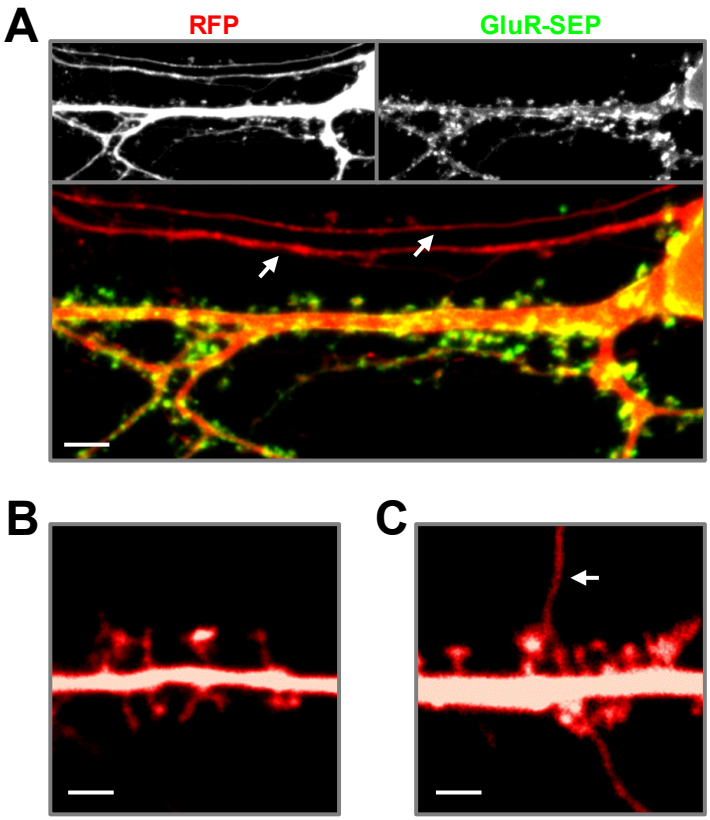

Fig. S5 Identification of transfected axons using cytosolic-RFP signal. **(A)** A representative image of a neuron in dissociated hippocampal culture expressing cytosolic RFP (red) and a SEP-tagged GluR subunit (green). Individual channels are in gray-scale in the images above, and a merged image is below. GluR-SEP fluorescence, representing surface receptors, is found on the cell body, along the dendritic shaft, and on dendritic spines. It is lacking from putative axons which only express RFP, indicated by arrows. Scale bar, 5 μm. **(B)** A representative RFP image of an experimentally acceptable scenario free of transfected axons. Scale bar, 2 μm. **(C)** An RFP image exemplifying an unacceptable experimental scenario in which a transfected axon runs juxtaposed to a dendritic spine, possibly forming a synapse. The arrow indicates the putative axon. Scale bar, 2 μm.

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CHAPTER 2

PROLONGED NICOTINIC MANIPULATION INCREASES AMPA RECEPTOR MOBILITY AT SPINES IN THE HIPPOCAMPUS

By

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SUMMARY

We have previously described the effects acute nicotine exposure has on AMPA receptor trafficking in hippocampal neurons. The consequences of a more prolonged nicotine exposure, however, are unknown. Here we report preliminary results suggesting that chronic nicotine administration for 1-2 days destabilizes GluR2-containing AMPA receptors at spines in cultured neurons from the hippocampus. Surprisingly, chronic treatment with nicotinic antagonists have the same effect, suggesting an atypical mechanism, distinct from that which was reported in Chapter 1.

INTRODUCTION

Nicotinic signaling, through nicotinic acetylcholine receptors (nAChRs), is known to affect higher order cognitive functions as well as play a role in a number of neurological disorders (Peeke and Peeke, 1984; Newhouse et al., 1997; Levin et al., 1998; Zoli et al., 1999; Picciotto and Zoli, 2002; Kenney and Gould, 2008; Tizabi, 2007; Thiel et al., 2008). Chronic nicotine administration leads to changes in synaptic plasticity thought to underlie addiction (Dani et al., 2001; Mansvelder and McGehee, 2002; O'Dell et al., 2007). In the hippocampus, chronic nicotine administration lowers the threshold for induction of long-term potentiation (LTP), a processes believed to contribute to contextual memory formation and its role in addictive behavior (Fuiji et al., 1999; Kenney and Gould, 2008; Placzek et al., 2009). A widely accepted model for LTP involves the activity-dependent recruitment of GluR1-containing AMPA receptors (GluR1s) to spine synapses which act as placeholders for the subsequent replacement with GluR2-containing AMPA receptors (GluR2s) in a constitutive, activity-independent

manner (Malinow and Malenka, 2002; Tanaka and Hirano, 2012). Current models suggest that extrasynaptic surface receptors are the immediate source of new AMPARs added during LTP and that they are recruited to synapses through lateral diffusion (Makino and Malinow, 2009; Petrini et al., 2009; Opazo and Choquet, 2011). Once incorporated at synapses the mobility of these surface receptors is greatly reduced (Makino and Malinow, 2009, 2011; Opazo et al., 2010, 2011; Opazo and Choquet, 2011). In Chapter 1 we described a novel mechanism, reminiscent of LTP, by which acute nicotine exposure, independent of fast excitatory transmission, leads to the synaptic incorporation of GluR1s in hippocampal neurons, with no effect on GluR2 trafficking. Here we report that a prolonged, multi-day exposure to nicotine or nicotinic antagonists results in a destabilization of GluR2s at spines, with no effect on GluR1.

RESULTS

To determine the effect that chronic nicotine exposure has on GluR2 trafficking we exposed days-in-vitro (DIV) 15-16 dissociated hippocampal neurons to nicotine (1 μM) for 1-2 days. The cultures were transfected with cytosolic red fluorescent protein (RFP) and a recombinant GluR2 tagged with the pH sensitive fluorophore super ecliptic pHluorin (GluR2-SEP) which only fluoresces on the cell surface (Fig. 1A; Miesenböck et al., 1998; Kopec et al., 2006). Fluorescence recovery after photobleaching (FRAP) revealed that the mobility of GluR2-SEP at spines was higher in cells treated with nicotine than in corresponding controls (Fig. 1B,C). Control levels of FRAP were comparable to those for GluR2 described in Chapter 1 and agree with published studies (Makino and Malinow, 2009). These findings suggest that chronic nicotine exposure

leads to a destabilization of GluR2 at spines, possibly representing a reduction in synaptic efficacy.

AMPA receptor (AMPAR) mobility is often inversely related to the degree of receptor enrichment at spines compared to the surrounding dendrite (Chapter 1; Heine et al., 2008; Makino and Malinow, 2011). Surprisingly, no corresponding reduction in GluR2-SEP enrichment was observed in the spines displaying increased GluR2-SEP mobility (Fig. 2A,B). Furthermore, no change was seen in spine size, spine density, or in the mobility of GluR1-SEP in cells exposed to nicotine (1 μM) for 1-2 days (Fig. 2C,D,E), indicating that the effect of chronic nicotine is selective in destabilizing GluR2-SEP at spines.

To determine which major subtype of nAChR was necessary for the nicotine-induced increase in mobility of GluR2-SEP at spines, we blocked activity through α7-nAChRs with MLA (100 nM) and α4β2-nAChRs with DHβE (1 μM) during the chronic nicotine exposure. Neither antagonist prevented the increase in GluR2-SEP mobility (Fig. 3A), and most surprisingly chronic treatment with both antagonists in the absence of nicotine had the same effect on GluR2-SEP mobility as did nicotine alone (Fig. 3B). These unlikely results were confirmed in slice culture where 3 days of antagonist exposure (DHβE + MLA) led to an increase in GluR2-SEP mobility at spines in the basal dendrites of CA1 pyramidal neurons (Fig 4). These results suggest that it is not simply the activity through nAChRs that leads to the destabilization of GluR2-SEP at spines, but rather a more unorthodox mechanism.

DISCUSSION

The results described here indicate that whereas 1-3 hrs of nicotine leads to a stabilization of GluR1s at spines with no effect on GluR2s (Chapter 1), extending the nicotine exposure for a few days leads to the opposite effect, namely a destabilization of GluR2s with no effect on GluR1s. The stability of AMPARs at spines is thought to represent their relative degree of synaptic incorporation (Borgdorff and Choquet, 2002; Triller and Choquet, 2005; Ehlers et al., 2007; Makino and Malinow, 2009, 2011). This, along with the increase in synaptic efficacy reported in Chapter 1, suggests that the first few hours of nicotine exposure leads to a strengthening of synapses. The results described here, however, indicate that prolonging the treatment for a few days ultimately results in a net reduction in synaptic efficacy. A likely explanation for this apparent discrepancy lies with the theory of homeostatic synaptic scaling (Turrigiano et al., 1998). In cases of heightened or reduced input, neurons will globally adjust the strength of excitatory synaptic inputs to stabilize firing rate. This process occurs in opposition to Hebbian plasticity, through the postsynaptic regulation of GluR2s, to maintain optimal levels of network activity (Turrigiano, 2008; Gainey et al., 2009). Nicotine is likely to increase glutamate release from presynaptic terminals (McGehee et al., 1995; Gray et al., 1996; Maggi et al., 2003) which, over time, could trigger the homeostatic synaptic scaling response, resulting in increased GluR2 mobility.

A different possibility is that chronic nicotine may lead to repeated postsynaptic potentiation through the addition of GluR1s (Chapter 1), leading to increases in synaptic input which, in turn, trigger homeostatic mechanisms to scale back input via the destabilization of GluR2s. This is not consistent, however, with the finding that no

difference was observed in the mobility of GluR1-SEP between control and chronically nicotine-treated cultures. As a caveat, it should be noted that these GluR1 data are preliminary and uncertain, given that control levels were not as high as usual (compare with Chapter 1). Further investigation is warranted.

If the observed increase in GluR2 mobility indeed correlates with a decrease in synaptic incorporation we would expect to see a reduction in the enrichment of GluR2-SEP at spines (Chapter 1; Heine et al., 2008; Makino and Malinow, 2011), but we do not. A plausible conclusion would be that although receptor mobility is affected, there is no corresponding change in synaptic efficacy. This may not be the case, however, because the spine enrichment value is a ratio of fluorescence on the spine compared to the dendritic shaft and, as such, may mask global reduction in total surface GluR2s. Extreme variation in transfection efficiency prohibited accurate direct measurement of total surface expression of GluR2-SEP. Future investigation of this issue could involve immunoblot analysis of surface receptors and electrophysiological recordings of miniature excitatory postsynaptic currents from untransfected cultures to determine changes in total surface levels and synaptic incorporation of GluR2s.

Changes in AMPAR mobility are often associated with changes in spine size (Kopec et al., 2006; Makino and Malinow, 2009, 2011). We did not, however, observe this with chronic nicotine exposure. Due to experimental limitations, spine size was measured using the GluR2-SEP signal, which may be less accurate than using cytosolic RFP. The same applies to spine density, which may also be inaccurate due to the limited length of dendrite available in the zoomed-in images. Future investigation could overcome these issues by performing experiments as in Chapter 1.

We observe that chronic exposure to both nicotine and nicotinic-antagonists lead to an increase in GluR2-SEP mobility. A possible explanation is that nAChR inactivation, either through blockade or desensitization (Edelstein et al., 1996; Wooltorton et al., 2003), produces these effects. This model assumes that ongoing endogenous nicotinic signaling is necessary for maintenance of GluR2 stability at spines, and that inhibiting this input leads to the observed increase in GluR2 mobility. A similar model has been suggested for an antagonist-driven α7-nAChR-dependent mechanism affecting presynaptic NMDA receptor trafficking in cortical cultures (Lin et al., 2010). Although dissociated hippocampal cultures lack the major cholinergic input provided to the hippocampus in vivo, namely input from the medial septum and diagonal band of broca (Frotscher and Léránth, 1985; Woolf, 1991; Dutar et al., 1995), nicotinic activation in the cultures may still be provided by cholinergic neurons endogenous to the hippocampus (Frotscher et al., 1986, 2000). The culture medium also contains low levels of choline which can serve as an agonist for α7-nAChRs (Neurobasal-E, Invitrogen; Alkondon et al., 1997).

An interesting alternative is that both nicotine and the antagonists may be acting through non-canonical pathways. Although the primary role of ionotropic receptors is to permit ion flux through their internal pores, AMPARs, for example, can participate in signal transduction mechanisms independent of ion flux. These include the ability of postsynaptic AMPARS to recruit presynaptic components, and the participation of presynaptic AMPARS in metabatropic signaling cascades independent (Schenk and Matteoli, 2004; Ripley et al., 2011). Furthermore, the downregulation of surface NMDA receptors can be initiated by agonist binding to the NMDA receptors, independent of ion

flux (Vissel et al., 2001). Nicotinic receptors may also participate in signaling that does not require ion flux. For example, a two-day treatment with nicotine or DHβE accelerates α4β2-nAChR export from the endoplasmic reticulum and, as a result, is thought to alter the "unfolded protein response" and the endoplasmic reticulum "stress response" (Srinivasan et al., 2012). Recently α7-nAChRs have been reported to interact directly with a G protein signaling cascade. It is not known, however, if ion flux through the receptors is required for the initiation of this signaling cascade (Nordman and Kabbani; 2012). A final possibility to consider is that nicotinic agonists and antagonists may exert effects independent of classical nicotinic receptors. A one-day nicotine treatment in vivo has been shown to alter AMPAR protein levels in cortical neurons and the underlying mechanism is thought to reflect a direct interaction between nicotine and the proteasome (Rezvani et al., 2007).

Future directions for this project could make use of pore-dead nicotinic receptors (Criado et al., 2011) and pharmacological inhibition of alternative pathways, such as the proteasome, to narrow down the mechanisms underlying the nicotinic effect on GluR2 mobility. It should be noted, however, that although DHβE + MLA increased the mobility of GluR2 at the basal dendrites of CA1 pyramidal neurons in slice culture, nicotine alone did not do so (data not shown; DIV14-16 and DIV21-24). It may also be relevant that the experiments in dissociated cell culture were not performed under optimal conditions, and thereby prohibited proper compensatory normalization during analysis (see Experimental Procedures). This could lead to high variability and inaccurate results. Future experiments should be carried out as described in Chapter 1 for greater accuracy.

Despite shortcomings, the present results do offer insight into how a more long-term nicotine exposure may affect AMPAR trafficking. A specific hypothesis to test is that destabilization of GluR2s may make synapses more susceptible to subsequent bouts of potentiation, and that could serve as a mechanism by which chronic nicotine treatment lowers the threshold for LTP induction (Fuiji et al., 1999; Kenney and Gould, 2008; Placzek et al., 2009). Mechanisms of this type would be expected to have consequences for hippocampal-dependent behaviors such as learning and memory.

EXPERIMENTAL PROCEDURES

All experiments and analyses were carried out under the conditions described in Chapter 1 except when stated otherwise. Exceptions include the following. Dissociated cultures were used DIV15-16. Only the SEP recovery was used for FRAP analysis in dissociated culture with no background subtraction, RFP normalization, or incidental bleach normalization, and spines that underwent substantial changes in size were discarded. SEP signal, not RFP, was used to measure spine size and density. Analysis of FRAP in slice culture, however, was carried out in the same manner described in Chapter 1.

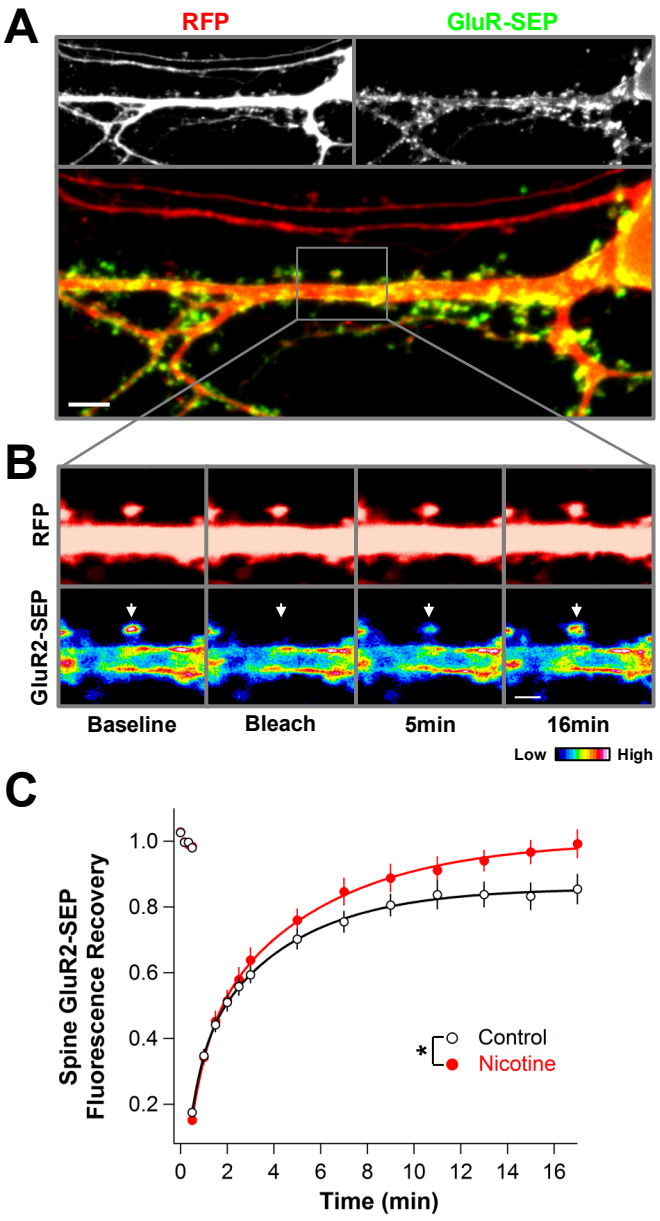

Fig. 1 Chronic nicotine exposure increases the mobility of GluR2-SEP at spines. **(A)** A representative image of a neuron in dissociated hippocampal culture expressing cytosolic RFP (red) and GluR2-SEP (green). Individual channels are in grayscale in the images above, and a merged image is below. GluR2-SEP fluorescence, representing surface receptors, is found both on spines and along the dendrite. Scale bar, 5 µm. **(B)** Images of GluR2-SEP FRAP under control conditions with accompanying cytosolic-RFP images. Arrows indicates bleached spine. Scale bar, 2 µm. **(C)** In cultures treated with 1 µM nicotine for 1-2 days, GluR2-SEP fluorescence recovers to a greater extent in 16 min following photobleaching than do corresponding controls, indicating increased mobility after nicotine treatment (Ctrl vs Nic 85 ± 5 vs 99 ± 5%; n = 18, 22; $p < 0.05$).

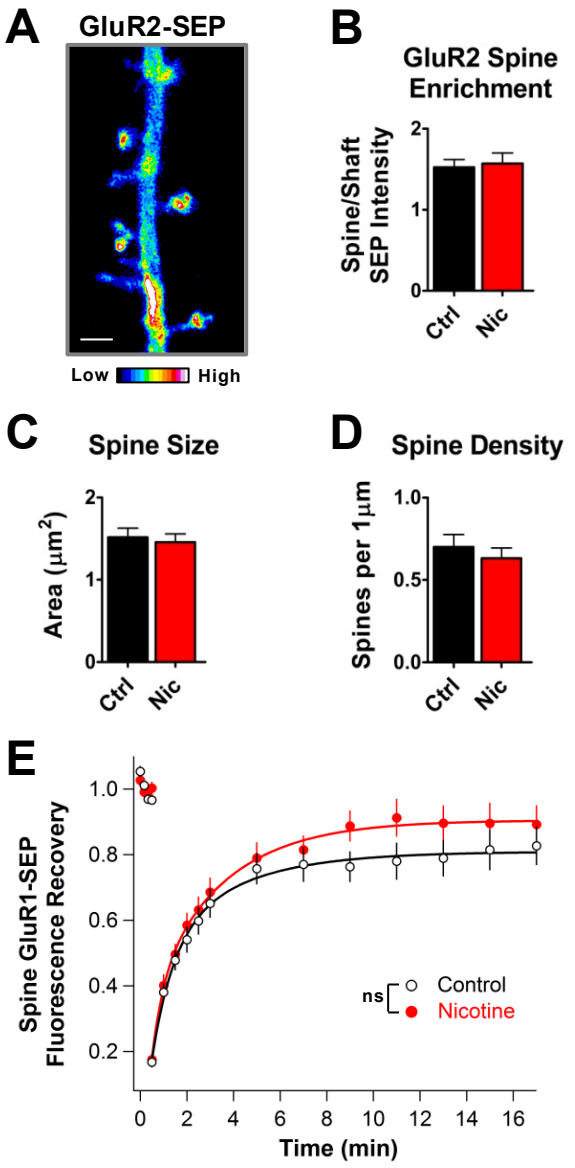

Fig. 2 Chronic nicotine treatment has no effect on GluR2-SEP spine enrichment, spine size, spine density, or on the mobility of GluR1-SEP at spines. **(A)** A typical dendrite from a neuron in dissociated hippocampal culture expressing GluR2-SEP from which measurement were taken. Scale bar, 2 μm. **(B)** Quantification of GluR2-SEP spine/shaft enrichment reveals no change in cultures treated with 1 μM nicotine for 1-2 days (Ctrl vs Nic: 1.52 ± 0.1 vs 1.57 ± 0.13; n = 24,25; p = 0.77). **(C, D)** Measuring the GluR2-SEP signal demonstrated that 1-2 days of 1 μM nicotine did not change the size or number of spines containing GluR2-SEP (Spine size: Ctrl vs Nic; 1.52 ± 0.11 vs 1.46 ± 0.1 μm^2; n = 33,34; p = 0.69; Spine density: Ctrl vs Nic, 0.7 ± 0.08 vs 0.63 ± 0.06 spines/μm; n = 33,34; p = 0.48). **(E)** Unlike GluR2-SEP, 1-2 days of nicotine had no effect on the FRAP of GluR1-SEP. GluR1-SEP signal in both control- and nicotine-treated spines recover to similar levels in 16 min following photobleaching (Ctrl vs Nic: 83 ± 6 vs 93 ± 5%; n = 12,8; p = 0.25).

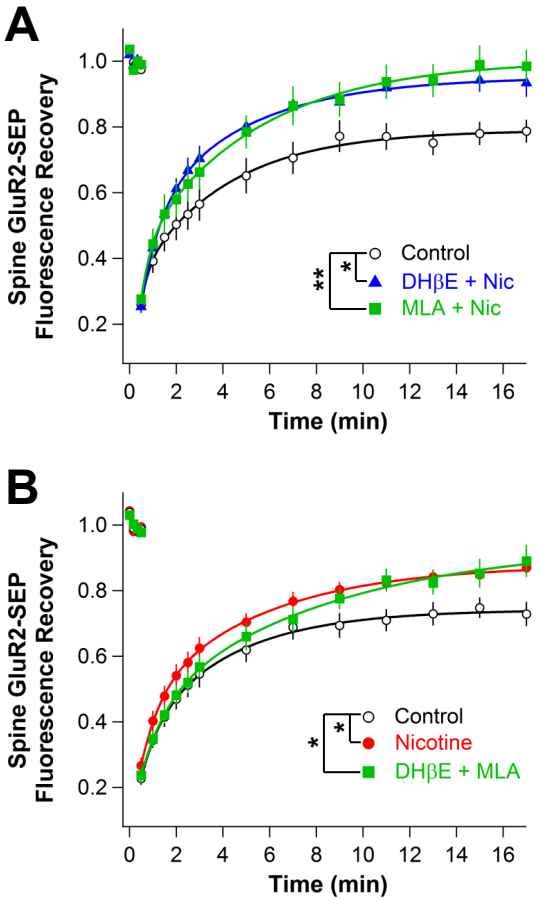

Fig. 3 Nicotinic antagonists have the same effects as nicotine on GluR2-SEP mobility. **(A)** Blocking α4β2-nAChRs with 1 μM DHβE and α7-nAChRs with 100 nM MLA concurrent with the 1-2 day incubation with 1 μM nicotine does not prevent the increase in GluR2-SEP mobility (Ctrl: 79 ± 4%, n = 12; DHβE + Nic: 93 ± 4%, n = 11; MLA + Nic: 99 ± 5%, n = 11; one-way ANOVA: Ctrl vs DHβE + Nic, $p < 0.05$; Ctrl vs MLA + Nic, $p < 0.01$). **(B)** Treating cultures with DHβE and MLA for 1-2 days in the absence of nicotine has the same effect as treating with nicotine alone (Ctrl: 73 ± 4%, n = 12; Nic: 87 ± 2%, n = 14; DHβE + MLA: 85 ± 5%, n = 12; one-way ANOVA: Ctrl vs Nic, $p < 0.05$; Ctrl vs DHβE + MLA, $p < 0.05$).

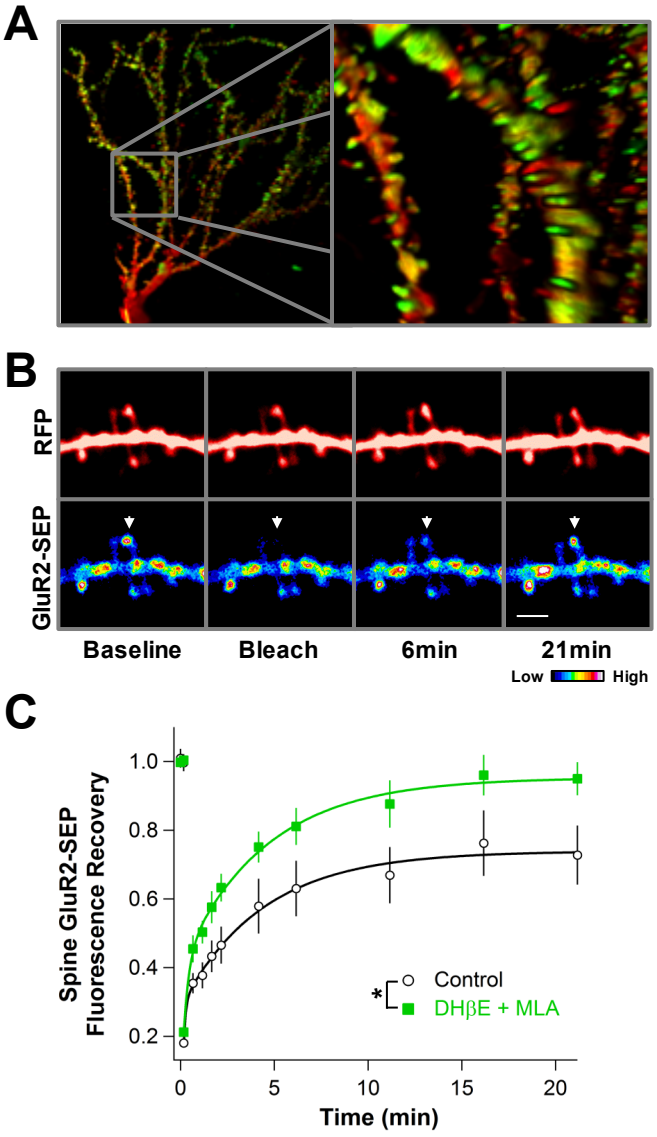

Fig. 4 Chronic administration of nicotinic antagonists increase the mobility of GluR2-SEP at spines in slice culture. **(A)** 3D rendering of the basal dendrites of a CA1 pyramidal neuron in slice culture transfected with cytosolic-RFP (red) and GluR2-SEP (green). GluR2-SEP, indicating surface receptors, is found both on spines and along the dendrite. **(B)** Images of GluR2-SEP FRAP under control conditions with accompanying cytosolic-RFP images. Arrows indicate the bleached spine. Scale bar, 2 μm. **(C)** In cultures treated with 1 μM DHβE + 100 nM MLA for 3 days, GluR2-SEP fluorescence recovers to a greater extent in 16 min following photobleaching than corresponding controls, indicating increased mobility when incubated with the antagonists (Ctrl vs DHβE + MLA 97 ± 6 vs 73 ± 9%; $n = 10,9$; $p < 0.05$).

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DISCUSSION OF THE DISSERTATION

The findings presented in this dissertation demonstrate novel mechanisms by which nicotinic signaling controls glutamate receptor trafficking. Acute stimulation of hippocampal neurons with nicotine leads to a stabilization and accumulation of GluR1-containing AMPA receptors (GluR1s) at spines. This is likely to occur through activation of postsynaptic α7-nicotinic acetylcholine receptors (α7-nAChRs), independent of coincident fast excitatory glutamate transmission. Activation of the α7-nAChRs apparently raises intracellular calcium which leads to the synaptic incorporation of GluR1s through PDZ interactions with PSD-95 scaffold protein family members. The result of this pathway is enhanced synaptic transmission. Prolonging the nicotine treatment for a few days, however, leads to the destabilization of AMPA receptors (AMPARs) at spines. This seemingly contradictory effect is specific to GluR2-containing AMPARs (GluR2s) and is also induced by nicotinic antagonists in the absence of nicotine. These results further our understanding of how nicotinic cholinergic signaling modulates glutamate transmission.

The timing and duration of nicotinic manipulation are critical factors in determining the specific effect on glutamate transmission. Not only do chronic and acute nicotine administrations result in different types of synaptic plasticity, but even millisecond differences in coincident nicotinic cholinergic and glutamatergic stimulation protocols separate long-term potentiation (LTP) from long-term depression (LTD; Fuiji et al. 1999; Ji et al., 2001; Kenney and Gould, 2008; Placzek et al., 2009; Gu et al., 2012; Yakel, 2012). It is therefore not surprising that we observed such different effects on

AMPAR trafficking between a few hours and a few days of nicotine treatment. What is lacking, however, is an understanding of the long-term consequences elicited by the acute nicotine exposure described in Chapter 1. Future experiments should involve inducing these changes with a short nicotine exposure, washing out the drug, and then investigating the effects days later. Furthermore, it would be useful to determine if a shorter nicotine challenge, on the order of minutes, is sufficient to induce GluR1 synaptic incorporation hours later. This would set the stage for experiments involving the focal application of nicotine to determine if local α7-nAChR activation, rather than cell-wide activation, drives the observed increases in synaptic efficacy. In addition, longitudinal studies will help determine if nicotine leads to a shift in the GluR1 enrichment on all spines or the selective enhancement of a few predisposed synapses.

Prior work from our lab has shown that nicotine can affect glutamate synapse formation in developing neurons via similar mechanisms involving postsynaptic nAChRs (Campbell et al., 2010; Lozada et al., 2012b). The experiments described in both chapters of this dissertation were performed on neurons after two weeks in culture, a time when the neurons are, in many respects, considered to be mature, though still in the process of becoming fully innervated (De Simoni et al., 2003; Grabrucker et al., 2009). Future directions could involve performing similar nicotinic manipulations on older and younger cultures to determine the age dependence of these effects.

An attractive continuation of this project will be to extend the studies in vivo. To do so, the GluR-SEP constructs can be expressed in living animals using in utero electroporation or viral injection (Lowery and Majewska, 2010; Makino and Malinow, 2011). Imaging could then be preformed either in acute slice or even in vivo using two-

photon technology to measure receptor mobility and enrichment (Golshani and Portera-Cailliau, 2008; Makino and Malinow, 2011). Utilizing these techniques in slices from animals expressing the photo-activatable channelrhodopsin in cholinergic terminals will permit investigations into how endogenous acetylcholine affects AMPAR trafficking (Gu and Yakel, 2011; Gu at al., 2012). In vivo manipulations have been shown to result in lasting changes in GluR-SEP trafficking that persist in acute slices (Makino and Malinow, 2011). This suggests that nicotine studies need not be restricted to the cortex for in vivo imaging. They could also be extended to the acute slice to study the deeper brain structures involved in addiction such as the ventral tegmental area and nucleus accumbens, as well as the hippocampus (Dani et al., 2001; Mansvelder and McGehee, 2002). These experiments represent an exciting new direction that may help shed light on how nicotine addiction-related behaviors, like self-administration or place-preference (Steiner and Picciotto, 2006), are correlated with changes in AMPAR trafficking in brain regions associated with addiction.

Nicotinic stimulation has been demonstrated to affect a number of hippocampal dependents tasks, yet it is difficult to draw conclusions about how changes in AMPAR trafficking might translate to these differences in behavior. Nicotinic agonists improve learning and memory in humans and non-human primates (Levin et al., 2006). In rodent models nicotinic manipulation alters animal performance on spatial learning tasks such as the Morris Water Maze, working memory in the radial arm maze, and contextual learning in fear conditioning assays (Bernal et al., 1999; Gould and Wehner, 1999; Levin et al., 2002; Kenney and Gould, 2008; Placzek et al., 2009). These behavioral effects are often, at least in part, attributed to the effect nicotinic cholinergic signaling has on synaptic

plasticity in the hippocampus (Martin et al., 2000; Whitlock et al., 2006; Kenney and Gould, 2008; Placzek et al., 2009). Unlike the mechanism described in this dissertation, most known effects of nicotine on synaptic plasticity require coincident glutamate transmission (Fuiji et al., 1999; Ji et al., 2001; Kenney and Gould, 2008; Placzek et al., 2009; Gu et al., 2012; Yakel, 2012). Our finding that a few hours of nicotine administration can lead to a strengthening of glutamate synapses independent of coincident fast, excitatory glutamate transmission may represent an example of non-Hebbian plasticity. Addressing how this translates to the behavioral effects of nicotine poses an intriguing question for further study. One avenue to do so maybe to further elucidate the underlying mechanisms perhaps identifying a unique link in the signal transduction pathway. A likely candidate for this would be a specific kinase leading to the immobilization of GluR1-SEP through the phosphorylation of an auxiliary TARP subunit (Chen et al., 2000; Bats et al., 2007; Opazo et al., 2010, 2011). If found and shown to be specific, this component of the pathway could be manipulated in vivo and in conjunction with behavioral tasks to determine the specific effect nicotine-induced changes on AMPAR mobility has on behavior.

What remains unclear is whether nicotine leads to a global increase in all synapses, or whether it favors the heavy enrichment of only a predisposed few (see Chapter 1, Discussion). A global increase in synaptic strength could indeed correlate with the known ability of nicotine to increase overall attention (Peeke and Peeke, 1984; Levin at al., 1998; Thiel and Fink, 2008). The selective enhancement of predisposed synapses, on the other hand, might represent a delayed form of Hebbian plasticity in which nicotine relies on the prior replenishment of extrasynaptic reserve pools of surface receptors by

local synaptic activity (Chapter 1; Makino and Malinow, 2009). This would be more consistent with the effect nicotine has on enhancing cue-induced conditioning where prior events are associated with forthcoming rewards (Reid et al., 1998; Gould and Wehner, 1999; Steiner and Picciotto, 2006; Kenney and Gould, 2008, Placzek et al., 2009). These conjectures, however, are confounded by the findings presented in Chapter 2, which suggest that a longer nicotine treatment destabilizes AMPARs at spines in the hippocampus. Also problematical is the open question of how long the effects of acute nicotine administration on GluR1 mobility last. The proposed future explorations outlined above aimed at addressing these questions will be critical, therefore, in establishing a link between nicotinic effects on AMPAR trafficking and behavior. The long-term goal of these endeavors will be to further our collective understanding of how the brain functions on a basic level to produce complex behavior and possibly lead to improvements in the health of those suffering from nicotinic-related disease.

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