UNIVERSITY OF CALIFORNIA, SAN DIEGO

Nicotinic Regulation of Hippocampal Development

A Dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in

Neurosciences

by

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2008

The dissertation of Kerri A. Massey is approved, and it is acceptable in quality and form for publication on microfilm:

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(Chair)

University of California, San Diego

2008

Shared pain is lessened, shared joy increased.
--Spider Robinson

To everyone who shared the laughter,
the tears,
and the foot-stomping tantrums,

Thanks for all the stories.

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ACKNOWLEDGEMENTS

First and foremost, I need to acknowledge Darwin, whose direction, support, and unwavering belief have made this work possible. I have always been amazed at Darwin's ability to sift through huge amounts of data and pull out the positive. His enthusiasm for science is contagious and was a driving factor in my success. Darwin believes strongly in each and every person that he brings into the lab. It is his wisdom that assembled the amazing group of people that I have spent the last five years working with. A few deserve mention for uncommon greatness. Bill and Lynn were and are my founts of knowledge. Jingming spent the last few months of his postdoc sharing his rig with me and teaching me how to patch clamp; his limitless patience is something I'll forever aspire to. Rob has always had an uncommon knack for knowing when I needed a cookie, and when I needed a swift smack upside the head. Given enough duct tape and super glue, Adrian can get anything to work; his ingenuity, perseverance, and sheer power of belief have brought the lab to a whole new level. Together with the other Berg Lab members, past and present, these are the people who have made the last five years an adventure.

It has been thrilling to work in such a thriving scientific community. My dissertation committee were always forthcoming with suggestions, and threw open the doors on their expertise and their freezers more than once to help me accomplish my goals. Nick Spitzer and his lab were a vital part of weekly lab meetings. The Gentry group's patience and generosity enabled much of the final work in this dissertation.

My friends have been a wonderful source of support, encouragement, and fun. They are all part of this wonderful story. I can only hope that it continues long past the final page of this dissertation. To my loving family, and especially to my little sister and all my nieces and nephews, let this stand as proof positive that dreams do come true if you put your head down and keep trying. My husband, Shane, has been with me through every step of this journey. He believed in me when I forgot how and his constant strength, support, and love have had an immeasurable impact on my life. I may have gotten here without him, but I wouldn't be nearly as good a person.

This dissertation is organized as free-standing chapters. Chapter 2 is a reprint of the material as it appears in "BDNF up-regulates alpha7 nicotinic acetylcholine receptor levels on subpopulations of hippocampal interneurons" by Massey, Kerri A; Zago, Wagner M; and Berg, Darwin in Molecular and Cellular Neuroscience (2006), volume 33, pages 381-8. Chapter 3 is a reprint of the material as it appears in "Nicotinic activity stabilizes convergence of nicotinic and GABAergic synapses on filopodia of hippocampal interneurons" by Zago, Wagner M; Massey, Kerri A; and Berg Darwin K in Molecular and Cellular Neuroscience (2006), volume 31, pages 549-59. In each case, permission to reprint these articles was obtained from the editors. Chapter 4 represents a manuscript in preparation for publication. Darwin K. Berg, a co-author on these papers, directed and supervised the work which is the basis for this dissertation. The contributions of authors other than Darwin and myself are acknowledged at the end of each chapter. In all cases, manuscripts are reproduced with the permission of all authors.

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Lozada AF, Massey KA, Liu ZP, Berg DK. Nicotinic Promotion of Non-Nicotinic Synapse Formation in the Developing Hippocampus. In preparation.

Abstracts

Massey KA, Zago WM, Berg DK. BDNF up-regulates alpha7 nicotinic acetylcholine receptor levels on subpopulations of hippocampal interneurons. Poster #D20, Society for Neuroscience 2006. Washington, DC.

FIELDS OF STUDY

Major Field: Neurosciences

Studies in Molecular and Cellular Neuroscience
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ABSTRACT OF THE DISSERTATION

Nicotinic Regulation of Hippocampal Development

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Doctor of Philosophy in Neurosciences

University of California, San Diego, 2008

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Nicotinic acetylcholine receptors (nAChRs) are a class of ligand-gated ion channels that are found throughout the central nervous system where they participate in higher order cognitive functions as well as neurological disorders. The nAChRs reach peak levels during early postnatal life. The hippocampus, a forebrain structure essential for memory, expresses high concentrations of nAChRs, which are important in the maturation of GABAergic signaling. This dissertation explores the role of nAChRs in shaping the functional organization and maturation of the developing hippocampus.

In early development nAChRs containing the α7 subunit (α7-nAChRs) are found postsynaptically on interneurons. Brain-derived neurotrophic factor acts through glutamatergic signaling to upregulate α7-nAChRs levels on a subset of interneurons, namely those that innervate pyramidal neurons. Levels of α7-nAChRs are unaffected on interneurons that inhibit other interneurons and pyramidal cells.

To study the role of activity in nicotinic synapse development, septal explants were placed alongside hippocampal slices in organotypic cultures. The septal explants sent out cholinergic and GABAergic processes which invaded the hippocampal slice to form functional synapses, allowing the study of nicotinic signaling in synapse formation in culture. The α7-nAChR and $GABA_A$α1 receptors are found colocalized at the tips of filopodia where they receive convergent cholinergic and GABAergic innervation. When α7-nAChRs are blocked, filopodia become more motile and cholinergic synapses are lost, suggesting that the formation or stabilization of nicotinic synapses is activity-dependent.

Nicotine rapidly induces spines in hippocampal slice culture by acting through β2-containing (β2*) nAChRs on the presumptive postsynaptic cell. Activation of α7-nAChRs is necessary to recruit presynaptic contacts and postsynaptic glutamatergic receptors to dendrites. Similar processes exist in vivo. Direct infusion of nicotine into the hippocampus induces spines, while young animals lacking both α7- and β2*-nAChRs show a reduced number of spines. Susceptibility to nicotine is restricted to early development; nicotine is unable to induce spines in older animals.

Together these results show that nAChRs have diverse functions in the developing nervous system, helping to form both cholinergic and noncholinergic synapses. The location and regulation of nAChRs receptors are, therefore, likely to have a long-lasting impact on hippocampal connectivity.

CHAPTER 1

Introduction

The human brain contains approximately 100 billion neurons (Williams and Herrup, 1988), each laced together with other neurons through synaptic connections. The precise interconnection of neurons underlies everything we think, feel, say, or do. Synaptic modulation can occur throughout life and is thought to be the fundamental basis for learning and memory (Lynch, 2004), addiction (Hyman et al., 2006) and neurodegeneration (Coleman et al., 2004). Early postnatal development is a period of extensive synaptogenesis, during which chemical synapses are rapidly formed, refined, and eliminated. Plasticity is inherent in developing systems (Waites et al., 2005). Active synapses are strengthened while inactive synapses are weakened or lost. Both spontaneous activity as well as sensory input from the environment helps drive refinement (Chiu and Weliky, 2003; McCormick, 1999), making early postnatal development a particularly susceptible time when alterations can have a lifelong impact.

Some plasticity is maintained into adulthood, where coincident activation can still strengthen synapses (LTP), while asynchronous activation can weaken synapses (LTD) (Dan and Poo, 2006). One of the most plastic regions of the brain is the hippocampus. The hippocampus receives major afferent input from the entorhinal cortex, which consolidates cortical information across modalities, consistent with the hippocampus' role in learning and memory. Entorhinal afferents enter the

hippocampus through the perforant pathway and synapse on granule cells within the dentate gyrus. Granule cells extend axons through the mossy fiber pathway to innervate CA3 pyramidal neurons, which in turn project to CA1 neurons through the Schaeffer collaterals (Amaral and Witter, 1989).

Superimposed on top of the excitatory pathways are local circuits containing interneurons, which play diverse roles in the hippocampus (Freund and Buzsaki, 1996). Interneurons that synapse onto the somas or axon initial segments of pyramidal neurons can inhibit and synchronize the firing of large regions of pyramidal neurons as well as sharply defining spike timing (Cobb et al., 1995; Galarreta and Hestrin, 2001). Interneurons can also inhibit other interneurons, leading to disinhibition of the hippocampus (Freund and Gulyas, 1997). Populations of interneurons act together to form the basis of rhythmic oscillations such as the gamma (30-100 Hz) and theta (4-10 Hz) activity observed during active exploration (Klausberger et al., 2003).

During early postnatal hippocampal development, a distinctly different type of network oscillation is observed. Giant depolarizing potentials (GDPs) are slow waves of excitatory network activity that are observed only in young animals when GABA acts as an excitatory neurotransmitter and glutamatergic activity is mainly mediated by NMDA receptors (Ben-Ari et al., 1989). Synchronous activity helps coordinate groups of neurons and drive synaptic development. As synapses mature and GABAergic signaling becomes inhibitory, GDPs are lost and replaced with mature oscillatory patterns (Leinekugel et al., 2002).

Neurons express GABA receptors and respond to ambient GABA even before chemical synapses are formed. Before inhibitory signaling matures, GABA is thought to act as a trophic factor, influencing cell differentiation, dendritic ramifications, and synaptogenesis (Ben-Ari et al., 2007). Though classically an inhibitory neurotransmitter, GABA has been found to be initially depolarizing in all systems studied. Depolarizing effects of GABA arise due to an immature chloride gradient (Cherubini et al., 1991). As chloride transporters shift to mature levels of expression, GABA becomes inhibitory. In the hippocampus, GABAergic neurons both develop synapses and mature earlier than the pyramidal neurons. By P12, GABAergic signaling is predominantly inhibitory, in vivo as well as in culture (Ganguly et al., 2001; Gozlan and Ben-Ari, 2003).

As GABAergic signaling matures, glutamatergic synapses begin to form (Hennou et al., 2002). Initially, glutamatergic synapses contain only NMDA receptors and require excitatory GABA to relieve the magnesium block and achieve efficient activation. Activation helps recruit AMPA receptors to the synapses, which eventually become independent of GABAergic signaling (Ben-Ari et al., 2007). Most excitatory glutamatergic synapses are formed onto dendritic spines, structural compartments that isolate the chemical signals allowing for synapse specific events. More spines are thought to reflect more functional synapses. Especially during early development, spines can be rapidly generated; however, mature synapses can take several days to form (Holtmaat et al., 2005; Nagerl et al., 2007).

The amount of excitatory activity within the hippocampus is thought to drive GABAergic maturation from excitatory signaling to inhibitory signaling, and GABA itself may promote the development of inhibitory signaling (Ganguly et al., 2001). Brain-derived neurotrophic factor (BDNF) can also influence GABAergic maturation. BDNF is a neurotrophin released by glutamatergic cells in an activity-dependent manner (Lu, 2003). BDNF can increase the number and size of GABAergic synapses (Yamada et al., 2002), as well as promote expression of KCC2, a transporter that extrudes chloride to establish a mature chloride gradient (Aguado et al., 2003). Additionally, BDNF promotes dendritic ramifications and affects synaptic plasticity by acting presynaptically to induce neurotransmitter release (Li et al., 1998) and postsynaptically to activate voltage-gated sodium channels (Blum et al., 2002). BDNF exerts the majority of its actions through binding to tyrosine receptor kinase B (TrkB), the high affinity receptor for BDNF. Binding of BDNF to TrkB activates numerous downstream signaling cascades (Patapoutian and Reichardt, 2001). Since TrkB is expressed on the majority of neurons in the hippocampus, BDNF can have broad effects, acting on both pre- and post-synaptic compartments of pyramidal cells and interneurons.

Acetylcholine (ACh) is a modulatory neurotransmitter in the hippocampus provided by septal cholinergic innervation to the hippocampus. Fibers arrive on embryonic day (E) 17 and form discreet synapses onto both glutamatergic and GABAergic neurons (Super and Soriano, 1994). Endogenous cholinergic signaling can

activate two broad classes of receptors; metabotropic muscarinic acetylcholine receptors and ionotropic nicotinic acetylcholine receptors (nAChRs).

Muscarinic receptors are widespread throughout the hippocampus and can influence excitability of cells by postsynaptically activating passive conductances as well as by activating downstream signaling pathways. Presynaptic muscarinic receptors can also act to inhibit release of neurotransmitters. Direct application of muscarine, a specific muscarinic agonist, causes a slow, long-lasting depolarization of pyramidal neurons. The effects on interneurons are more diverse; some interneurons are depolarized, some hyperpolarized, some have a biphasic response, while still others show no response at all (van der Zee and Luiten, 1999).

The nAChRs have a more straight-forward action in the hippocampus. The nAChRs are a class of ligand-gated ion channels that respond to both acetylcholine and nicotine. In vertebrates, nAChRs pass a depolarizing, cationic current in vertebrates. Nine alpha subunits and three beta subunits have been identified, and combine to form functional nicotinic receptors (Sargent, 1993). In spite of the possible diversity, only the α7 homomers and the α4/β2 heteromers exist at high levels within the hippocampus (Zhang et al., 1998).

The α7-nAChR is highly permeable to calcium and both activates and inactivates rapidly (Seguela et al., 1993). In the adult, it is found pre- and postsynaptically at most synapses (Fabian-Fine et al., 2001). However, during peak α7-nAChR expression early in development, the majority of receptors appear postsynaptic, and the highest levels are found on interneurons (Kawai et al., 2002).

The hippocampus contains the highest levels of α7-nAChRs found in the central nervous system. Activation of presynaptic α7-nAChRs can lead to neurotransmitter release (McGehee et al., 1995). Postsynaptically, the receptors can both depolarize the neurons and activate second messenger systems, altering gene expression (Broide and Leslie, 1999, Hu et al., 2002). Mice lacking α7-nAChRs (α7KO) appear similar to wildtype (WT) littermates, showing normal development and brain structure (Orr-Urtreger et al., 1997) though more subtle effects do exist. The α7KO mice have sporadic liters that are small in size and show reduced signs of withdrawal from nicotine (Salas et al., 2007). Additionally, some groups find mild defects in attention, motivation, and working memory (Fernandes et al., 2006; Keller et al., 2005), while others fail to find such effects (Paylor et al., 1998).

The α4/β2-nAChRs are much less permeable to calcium than α7-nAChRs (Tapia et al., 2007), but the has slower kinetics of the receptor allows it to exert a greater depolarizing influence (Buisson et al., 1996). The α4/β2-nAChRs can act presynaptically to influence neurotransmitter release by depolarizing the presynaptic terminal and activating voltage-gated calcium channels (McGehee et al., 1995). Postsynaptically, they can depolarize the cell. The α4/β2-nAChRs are found at moderate levels in the hippocampus and at high levels in areas such as the ventral tegmental area (VTA) (Zoli et al., 1995), where they are thought to mediate the effects of nicotine addiction. Mice lacking the β2-nAChR (β2KO) subunit breed normally but exhibit altered waves of retinal activity in early development (Feller, 2002), fail to

self-administer nicotine (Picciotto et al., 1998), and show altered adult neurogenesis (Harrist et al., 2004; Mechawar et al., 2004).

Mice lacking both α7- and α4/β2-nAChRs (dKOs) have lost essentially all nicotinic signaling in the hippocampus, though other receptors may increase slightly to compensate, at least in the α7KO animals (Yu et al., 2007). Little work has been done with dKO mice, but they seem to exhibit reduced anxiety, reduced passive avoidance, and superior rotor rod skills when compared to WT animals (Marubio and Paylor, 2004). Presumably, the changes in behavior in the dKO mice are in addition to those shown by the parent strains.

The fact that nAChRs reach peak levels of expression during early development of the hippocampus (Zhang et al., 1998) suggests that cholinergic signaling may play a role in synapse formation, refinement, and plasticity. Within the spinal cord, acetylcholine is needed for early rhythmic activity that helps coordinate networks of cells involved in locomotion (Myers et al., 2005). Early retinal waves depend on nAChR activation (Feller, 2002). Without nAChR signaling, retinal ganglion cells take longer to develop fully ramified dendritic arbors (Bansal et al., 2000). In the autonomic ciliary ganglion, spinal cord, and hippocampus, blocking or eliminating α7-nAChRs delays maturation of GABAergic signaling (Liu et al., 2006). Within the hippocampus, exposure to nicotine increases the frequency of GDPs (Le Magueresse et al., 2006), and thus overall hippocampal activity.

Though α7-nAChR expression is known to peak in early hippocampal development, the cellular distribution of the receptors have only been studied later in

development. What cholinergic input means to the hippocampus depends vitally on where α7-nAChRs are located and how they are modulated. BDNF can upregulate α7-nAChR expression directly in the chick ciliary ganglion (Zhou et al., 2004) and through previously unknown pathways in the hippocampus (Kawai et al., 2002). In Chapter 2, we show that different interneuron populations express distinct levels of surface α7-nAChRs. Dissociated cell culture were immunostained to reveal the location and regulation of α7-nAChRs. BDNF acts through glutamatergic signaling to up-regulate postsynaptic α7-nAChRs on subpopulations of hippocampal interneurons, namely those that inhibit pyramidal neurons. This was the first close examination of receptor expression during early postnatal development and the first time differential effects of BDNF were seen within interneuron populations.

When hippocampal and septal tissue are grown together in organotypic slice cultures, the septum sends out cholinergic and GABAergic projections that make specific and functional connections as they would in vivo. Much like in vivo, synapses are thought to form first onto filopodia, which retract towards the dendritic shaft to stabilize and become spines or shaft synapses (Gupton and Gertler, 2007). Little was known about cholinergic synaptogenesis within the hippocampus. Dendritic filopodia express clusters of α7-nAChRs which can allow calcium to enter the cell. Ca^{++} is known to regulate filopodia motility during development (Lohmann et al., 2005), suggesting that α7-nAChRs might play an active role in shaping the activity of filopodia. In Chapter 3, we show that blocking α7-nAChR activity increases filopodial motility. Activity through α7-nAChRs is also needed to stabilize cholinergic contacts,

suggesting that cholinergic synapses form in an activity-dependent manner. The α7-nAChRs found at the tips of filopodia are often coexpressed with $GABA_A$ receptors and receive both cholinergic and GABAergic innervation. The coincident innervation of filopodia by both cholinergic and GABAergic systems suggests that the neurotransmitter systems may interact, especially during early development when α7-nAChRs are at their highest levels. Additionally, α7-nAChRs can activate gene transcription, raising the possibility that cholinergic activation plays a role in GABAergic maturation by influencing transcription of chloride transporters.

Recent work in our lab has shown that α7-nAChRs are important for determining when GABAergic signaling becomes inhibitory: α7KO mice develop mature GABAergic signaling later than WT animals (Liu et al., 2006). Altering the timing of GABAergic inhibition can have a profound impact on the maturation of neurons. Newborn neurons in the adult brain that are forced to mature too soon show a stunted dendritic arbor and fewer GABAergic and glutamatergic synapses (Ge et al., 2006).

Structural changes in spines and the synapses they represent are the basis for hippocampal plasticity, learning, memory, addiction, and neurodegeneration. In early development, altered spines are associated with mental retardation and other developmental defects (Calabrese et al., 2006). Though cholinergic signaling is widespread throughout the nervous system and has potent developmental effects, studies on the effects of nicotine on hippocampal architecture, especially during early development when nAChRs are likely to have the greatest effects, are lacking. In

Chapter 4, we show that nicotinic receptors act cooperatively to induce functional glutamatergic synapses. Nicotine, acting through postsynaptic β2-containing nAChRs, rapidly induces stable spines during early development. Activation of α7-nAChRs increases presynaptic structures and postsynaptic glutamatergic receptor expression. Cholinergic signaling can also modulate spines in vivo. Direct application of nicotine to the hippocampus increases spine number while α7/β2KO mice showed fewer spines than WT mice. The novel and rapid effect of nicotine on hippocampal neurons is akin to plasticity and suggests that endogenous nicotinic signaling helps shape the connections and the maturation of the hippocampus. The results also suggest that exogenous nicotine may co-opt the normal developmental pathways and have lasting effects on the developing fetus.

Together, the work in this dissertation examines how nicotinic signaling affects synaptogenesis in the hippocampus. The findings illustrate how the location, regulation, and activation of nAChRs help promote cholinergic, GABAergic, and glutamatergic maturation. Further implications of this work are discussed in Chapter 5.

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CHAPTER 2

BDNF up-regulates α7 nicotinic acetylcholine receptor levels on subpopulations of hippocampal interneurons

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Mol. Cell. Neurosci. 33 (2006) 381–388

BDNF up-regulates α7 nicotinic acetylcholine receptor levels on subpopulations of hippocampal interneurons

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Received 20 June 2006; revised 4 August 2006; accepted 30 August 2006
Available online 9 October 2006

In the hippocampus, brain-derived neurotrophic factor (BDNF) regulates a number of synaptic components. Among these are nicotinic acetylcholine receptors containing α7 subunits (α7-nAChRs), which are interesting because of their relative abundance in the hippocampus and their high relative calcium permeability. We show here that BDNF elevates surface and intracellular pools of α7-nAChRs on cultured hippocampal neurons and that glutamatergic activity is both necessary and sufficient for the effect. Blocking transmission through NMDA receptors with APV blocked the BDNF effect; increasing spontaneous excitatory activity with the $GABA_A$ receptor antagonist bicuculline replicated the BDNF effect. BDNF antibodies blocked the BDNF-mediated increase but not the bicuculline one, consistent with enhanced glutamatergic activity acting downstream from BDNF. Increased α7-nAChR clusters were most prominent on interneuron subtypes known to directly innervate excitatory neurons. The results suggest that BDNF, acting through glutamatergic transmission, can modulate hippocampal output in part by controlling α7-nAChR levels.

Introduction

Brain-derived neurotrophic factor (BDNF) was first described as a component that regulates development of neuronal structure and function both in the peripheral and central nervous systems (Thoenen et al., 1987; Thoenen, 1995; Lewin and Barde, 1996; Cohen-Cory and Lom, 2004). Subsequently it became clear that BDNF also has acute effects at the synapse, serving as an activity-dependent regulator of synaptic plasticity and participating rapidly in synaptic transmission (Schinder and Poo, 2000; Poo, 2001; Blum et al., 2002; Kovalchuk et al., 2004; Bramham and Messaoudi, 2005). Developmentally, BDNF has numerous synaptic actions including maturation of GABAergic signaling and stabilization of newly formed synapses (Huang et al., 1999; Alsina et al., 2001; Yamada et al., 2002; Bramham and Messaoudi, 2005). Many of these effects have been demonstrated in the hippocampus where BDNF is released from both the dendrites and axons of pyramidal neurons (Haubensak et al., 1998; Hartmann et al., 2001; Balkowiec and Katz, 2002).

BDNF can regulate the level of nicotinic acetylcholine receptors (nAChRs) containing α7 subunits in the hippocampus and other systems (Kawai et al., 2002; Zhou et al., 2004). These α7-nAChRs are expressed at relatively high levels on interneurons in early postnatal hippocampus (Jones and Yakel, 1997; Zhang et al., 1998; Liu et al., 2001; Adams et al., 2002; Kawai et al., 2002). Due to the high calcium permeability of α7-nAChRs (Bertrand et al., 1993; Seguela et al., 1993), they can both depolarize the cell and influence a variety of calcium-dependent events (Broide and Leslie, 1999; Berg and Conroy, 2002). As a result, the distribution and regulation of these receptors can profoundly impact network function.

Here we examine the effects of BDNF on dissociated rat hippocampal neurons in culture and show that BDNF increases both surface and internal pools of α7-nAChRs. The BDNF-mediated increases are dependent on glutamatergic activity and are confined to distinct neuronal subtypes. Glutamatergic neurons show no α7-nAChR increase with BDNF treatment. Within GABAergic neurons, those showing the greatest increases are interneurons that directly innervate pyramidal neurons. The results suggest that BDNF acts through glutamatergic signaling to selectively elevate α7-nAChRs on interneurons positioned to inhibit glutamatergic cells.

Results

BDNF up-regulates both surface and internal α7-nAChRs

Clusters of α7-nAChRs are prominent on the soma and dendrites of hippocampal interneurons in culture. This was found by labeling intact cells with Alexa488-α-bungarotoxin (Alexa-αBgt) to reveal surface clusters of α7-nAChRs (Figs. 1A, D). Cells were then rinsed, fixed, and immunostained with antibodies against glutamic acid decarboxylase 65/67 (GAD) to identify GABAergic interneurons (Figs. 1B, E). About 60% of the GAD-positive cell

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Available online on ScienceDirect (www.sciencedirect.com).

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doi:10.1016/j.mcn.2006.08.011

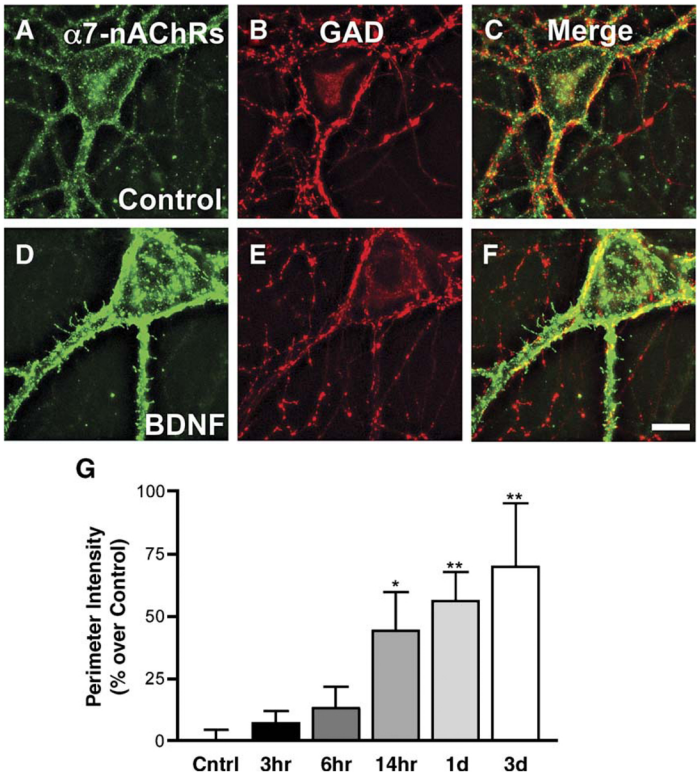

Fig. 1. Time dependence of α7-nAChR increases by BDNF. Dissociated hippocampal cells were treated with control media (A–C) or with 50 ng/ml BDNF (D–F) for 24 h and then stained with Alexa-αBgt for α7-nAChRs (A, D), fixed with 4% PFA, permeabilized, and co-stained for GAD (B, E), and the paired images merged (C, F). Cells were treated with BDNF for the indicated times (G), and the relative staining levels were quantified. Values for individual neurons were normalized to mean control values in each experiment; net increases over control are shown. A 14-h exposure to BDNF produced a significant increase in α7-nAChR staining. Results were compared using ANOVA with the Dunnett post hoc test. Scale bar: 10 μm. Asterisk, $p<0.05$; double asterisk, $p<0.01$.

bodies had significant levels of α7-nAChR staining. Previous studies showed that Alexa-αBgt staining under these conditions co-distributes with surface staining by an antibody against α7-nAChRs (Kawai et al., 2002). Additionally, pre-incubating neurons with an excess of methyllycaconitine (MLA), a specific antagonist of α7-nAChRs, blocked the Alexa-αBgt staining, providing additional evidence for specificity. In comparison to control cells (Figs. 1A–C), 24-h treatment with BDNF (Figs. 1D–F) led to a significant increase in surface expression of α7-nAChRs without significantly changing the number of stained cells. The results were normalized to the mean signal obtained for untreated GABAergic neurons in the same experiments, thereby compensating for the differences in baseline levels among experiments. The increase was first evident after 14 h of BDNF treatment. Longer treatments led to slightly greater effects (Fig. 1F). In all subsequent experiments, cells were treated with BDNF for 16 or 24 h, as indicated.

The time dependence of the BDNF effect was consistent with de novo receptor synthesis and trafficking to the cell surface. Treating the cells with brefeldin-A (BFA) prior to and during a 16-h exposure to BDNF to block transport from the Golgi completely blocked the BDNF-induced increase in surface α7-nAChR staining. No decrement was seen in controls treated with BFA, indicating that little basal turnover of receptor occurs during the test period (Fig. 2A). BFA does block nicotine-induced endocytosis and exocytosis of α7-nAChRs (Liu et al., 2005). The effects of long-term BFA treatment on BDNF-treated cells, however, may be complex: while short-term treatments with BFA do not affect synaptic release at mature synapses (Zakharenko et al., 1999), long-term exposure may interfere. Experiments with blockers of transcription and protein synthesis were also problematical over this time span because of toxic effects. This motivated us to test whether internal pools of α7-nAChRs could be identified and determine how they were affected by the BDNF treatment. Internal α7-nAChRs were visualized by first treating intact cells with Alexa-αBgt to label surface receptors (Figs. 2B, E), and then rinsing, lightly fixing and permeabilizing with methanol, and labeling intracellular receptors with rhodamine (rho)-αBgt (Figs. 2C, F). Prominent clusters of intracellular α7-nAChRs could be identified in this manner, sometimes lying immediately beneath surface clusters of the receptors (Figs. 2D, G). The staining is likely to represent fully assembled receptors because only the pentameric α7-nAChRs are thought to bind significant amounts of αBgt (Anand et al., 1993). Including MLA with the rho-αBgt eliminated the internal clusters. In comparison to

controls (Figs. 2B–D), 24-h treatment with BDNF (Figs. 2E–G) significantly increased the dendritic area occupied by intracellular pools of α7-nAChRs (Fig. 2H). The results show that BDNF increases both surface and internal α7-nAChR pools, consistent with net receptor accumulation occurring either through increased synthesis or stabilization.

BDNF depends on glutamatergic signaling

BDNF, acting through TrkB receptors, can exert effects directly on a target cell or indirectly through changes in synaptic or electrical activity (Li et al., 1998; Kafitz et al., 1999; Schuman, 1999; Blum et al., 2002; Matsumoto et al., 2006). Immunostaining reveals that essentially all hippocampal neurons in culture have substantial amounts of both surface and internal TrkB receptors (data not shown). A known pathway of BDNF action involves TrkB receptor activation of Nav1.9 sodium channels to depolarize the cell (Kafitz et al., 1999; Blum et al., 2002). It is unlikely, however, that this depolarization drives the effects seen on hippocampal α7-nAChR expression. Overnight treatment with saxitoxin (10 nM), which blocks a subset of voltage-gated sodium channels that include Nav1.9, depressed α7-nAChR levels but was unable to prevent the BDNF-induced increase in surface receptors (surface staining in arbitrary units: 19±2 for saxitoxin vs. 26±1 for saxitoxin plus BDNF; mean±SEM, $n=3$).

BDNF can also modulate glutamatergic transmission, potentiating release of glutamate presynaptically and regulating postsynaptic receptor function as well (Levine et al., 1998; Li et al., 1998; Levine and Kolb, 2000). Blocking NMDA receptors with 50 μM 2-amino-5-phosphonopentanoic acid (APV) prevented the BDNF-mediated increase in α7-nAChR staining without affecting basal levels of α7-nAChRs over the same time period (Fig. 3A). The results suggested that BDNF may exert its effects on α7-nAChR levels by acting through glutamatergic transmission. To test this, we treated cultures with 20 μM bicuculline for 24 h to block inhibitory transmission through $GABA_A$ receptors; this protocol increases spontaneous excitatory activity in the cultures (Arnold et al., 2005). The excitation is likely to be confined to glutamatergic pathways since the hippocampus in thought to lack endogenous cholinergic neurons. Bicuculline mimicked BDNF in increasing α7-nAChR staining, and it was not additive with BDNF (Fig. 3B). The results are consistent with a mechanism in which NMDA receptors act downstream of BDNF. Supporting this view, we found that treating cultures with anti-BDNF antibodies prevented BDNF-mediated increases but was unable to prevent the bicuculline-induced increases in α7-nAChR levels (Fig. 3B). The results show that bicuculline can up-regulate α7-nAChR levels without requiring BDNF. The simplest interpretation is that BDNF increases α7-nAChR levels by increasing glutamatergic activity.

BDNF distinguishes subsets of interneurons

The fact that not all GAD-positive hippocampal neurons showed increased α7-nAChR staining in response to BDNF suggested that the effect might be specific for distinct interneuron subtypes. The hippocampus contains multiple classes of interneurons with different functions; immunostaining for neuropeptides provides a means of distinguishing among the classes (Freund

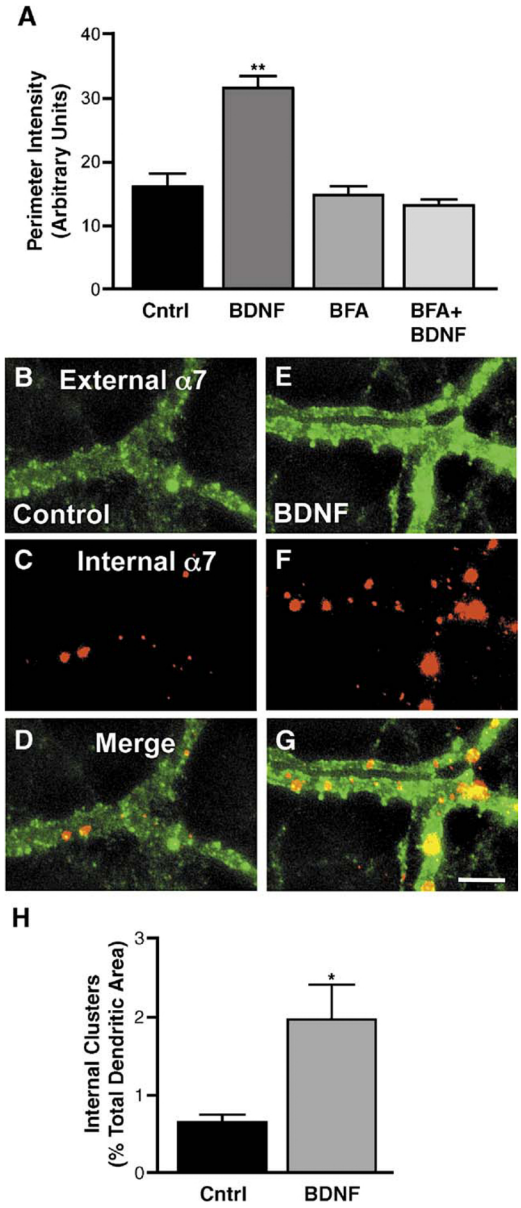

Fig. 2. BDNF effects on internal α7-nAChR pools and dependence on vesicle trafficking. Treating cells with 10 μg/ml BFA, which prevents trafficking of proteins from the Golgi, completely blocked the ability of BDNF (50 ng/ml for 16 h) to increase surface levels of α7-nAChRs (A). Results were compared using ANOVA with the Bonferonni post hoc test for selected pairs of means. Internal pools of α7-nAChRs were visualized by first staining intact cells with Alexa-αBgt to label surface receptors (B, E) and then fixing in methanol and labeling internal receptors with rho-αBgt (C, F). Individual dendrites are shown lined with α7-nAChR clusters on the surface and containing internal clusters as well (D, G). Comparing control cells (B–D) and cells treated with BDNF for 24 h (E–G) showed that the latter had undergone a significant increase in the percentage of dendritic area that was occupied by internal clusters of receptors (H). Results were compared using a Student's two-tailed *t*-test. Scale bar: 10 μm. Asterisk, $p<0.05$; double asterisk, $p<0.01$.

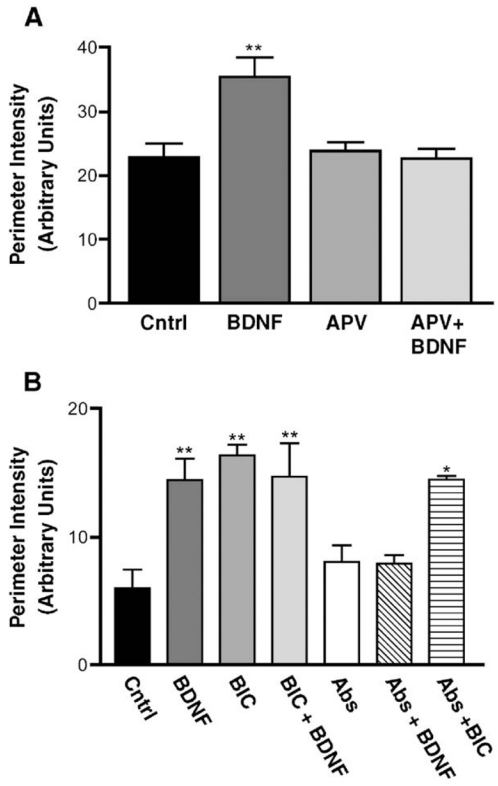

Fig. 3. Dependence of BDNF effect on glutamatergic signaling. Hippocampal cells in culture were treated with BDNF (50 ng/ml), APV (50 μM), or a combination of the two. Surface staining for α7-nAChRs was quantified (A). APV did not change basal levels of α7-nAChR staining, but it completely prevented the increase seen with BDNF. Bicuculline (20 μM), used to block GABAergic inhibition and thereby increase glutamatergic excitation in the cultures, mimicked the BDNF effect but was not additive with it (B). Functional antibodies against BDNF (Abs) prevented the increase seen with BDNF alone but did not prevent the increase seen with bicuculline. The results are consistent with glutamatergic signaling acting downstream of BDNF to elevate α7-nAChR levels. Results were compared using ANOVA with the Bonferonni post hoc test for selected pairs of means. Asterisk, $p<0.05$; double asterisks, $p<0.01$.

and Buzsaki, 1996; Soltesz, 2006). Somatostatin-positive (SS) hippocampal cells include HIPP cells in the dentate gyrus and OL-M cells in the CA regions, both of which directly innervate glutamatergic neurons and mediate feedback inhibition (Katona et al., 1999). Estrogen receptor β (ER-β) is an early marker for parvalbumin-positive fast-spiking basket cells that innervate glutamatergic cells (Freund and Buzsaki, 1996; Blurton-Jones and Tuszynski, 2002). Most vasoactive intestinal peptide (VIP)-positive hippocampal cells represent interneurons that selectively innervate other interneurons (Acsady et al., 1996a,b; Hajos et al., 1996). SS-, parvalbumin-, and VIP-positive cells form three major non-overlapping populations of neurons in the hippocampus. Though it was not possible to stain for parvalbumin in these young cultures, it is likely that ER-β cells form a representative subpopulation of these neurons.

Co-staining hippocampal cultures for surface α7-nAChRs and either SS, ER-β, or VIP demonstrated the heterogeneity in responses to 24 h of BDNF treatment. When normalized to mean values across untreated GABAergic neurons, those cells expressing SS had low levels of surface α7-nAChRs and underwent a substantial increase in response to BDNF (Fig. 4). BDNF treatment also increased the moderate levels of surface α7-nAChRs on neurons defined by ER-β. In contrast, BDNF had no effect on neurons expressing VIP. The distribution of neurons among the GABAergic subpopulations was unchanged by BDNF treatment (43±8% SS-positive, 7±2% were VIP-positive, and 32±9% were ER-β positive). GAD-negative neurons were presumed to be glutamatergic cells and formed a distinct population. They displayed low levels of surface α7-nAChRs and showed no increase in response to BDNF treatment (Fig. 4).

SS and VIP neurons responded to BDNF in different manners and were investigated further. BDNF effects on internal α7-nAChR pools mimicked those seen for surface receptors across neuronal cell type. This was shown by using a modified procedure which preserved immunostaining for neuropeptides while also visualizing intracellular α7-nAChRs. Cells were first incubated with unlabeled αBgt to block surface α7-nAChRs and then were lightly fixed with 2% PFA and permeabilized. A second incubation included primary antibodies for SS and VIP along with Alexa-αBgt for intracellular α7-nAChRs. The fixation reduced the intensity of Alexa-αBgt staining but still enabled internal α7-nAChR clusters to be seen. Few GAD-negative neurons had detectable α7-nAChR clusters, and BDNF treatment did not increase the number of such cells (Fig. 5). In contrast, many GAD-positive neurons displayed intracellular α7-nAChR clusters, and the proportion nearly doubled

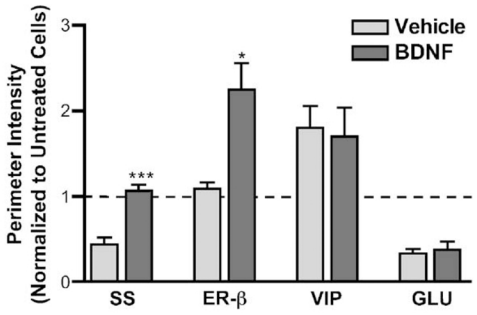

Fig. 4. Identification of interneuronal subtypes that display BDNF-mediated increases in surface α7-nAChRs. Hippocampal cultures were treated with BDNF (50 ng/ml) for 24 h, then stained with Alexa-αBgt, fixed in 4% PFA, permeabilized, and stained for either SS, ER-β as an early marker of parvalbumin positive interneurons, or VIP, and co-stained for GAD. GABAergic and glutamatergic neurons were distinguished by the presence and absence, respectively, of somatic GAD staining. BDNF treatment significantly increased surface staining for Alexa-αBgt on SS-positive and ER-β-positive neurons. BDNF had no effect on VIP-positive cells or on glutamatergic (GLU) cells. BDNF did not alter the percentage of GABAergic cells that were positive for SS, VIP, or ER-β. Surface fluorescence was normalized to the mean fluorescence signal across untreated GABAergic neurons (dashed line). The effect of BDNF on each subpopulation was compared by Student's two-tailed *t*-test. Asterisk, $p<0.05$; triple asterisks, $p<0.001$.

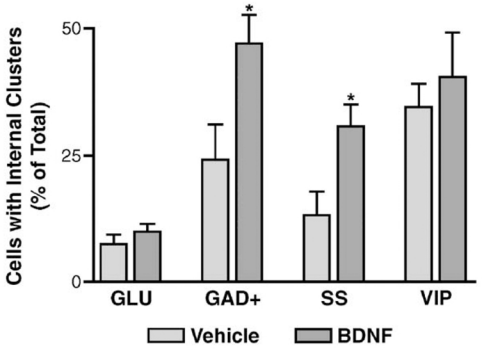

Fig. 5. BDNF effects on internal pools of α7-nAChRs within neuronal cell types. After blocking surface α7-nAChRs with unlabeled αBgt, hippocampal cultures were lightly fixed in 2% PFA and incubated with Alexa-αBgt to label intracellular pools of α7-nAChRs. Cells having one or more internal clusters were scored as positive. The percentage of cells having at least one internal α7-nAChR cluster was quantified for each condition. As seen for surface expression of α7-nAChRs, SS-positive cells showed an increase, while VIP-positive and glutamatergic cells did not. Individual categories of cells were compared to vehicle using Student's two-tailed *t*-test. Asterisk, $p<0.05$.

following BDNF treatment. Only a small fraction of SS-containing neurons displayed the intracellular clusters, but BDNF again increased the proportion significantly. As seen for surface α7-nAChR labeling, a substantial proportion of VIP-expressing neurons contained internal α7-nAChR clusters, but the proportion was not changed by BDNF treatment. The results show that BDNF exerts largely the same effects on internal α7-nAChR pools as it does on surface receptors, and that the same subsets of neurons respond.

Discussion

We show here that BDNF up-regulates α7-nAChR levels on specific subpopulations of hippocampal neurons in culture and that the up-regulation applies both to surface and intracellular receptor pools. The BDNF effect requires glutamatergic transmission and apparently acts through it to achieve the up-regulation. Most interestingly, the up-regulation occurs on interneuron subtypes that directly innervate excitatory neurons in the hippocampus and, at least in part, provide feedback inhibition. Other types of hippocampal neurons do not up-regulate α7-nAChRs in response to BDNF even though they express TrkB receptors. These include excitatory neurons, which express low levels of α7-nAChRs, and interneurons that innervate other interneurons, many of which express high levels of α7-nAChRs. The results suggest that BDNF, acting through glutamatergic regulation of α7-nAChRs, specifically affects hippocampal excitability.

The imaging of α7-nAChRs achieved here with Alexa-αBgt was specific for α7-nAChRs since it was blocked by 5 μM MLA. This antagonist excludes the possibility that the toxin sites include $GABA_A$ receptors with β3 subunit; such GABA receptors have a lower affinity for αBgt and a different pharmacology (McCann et al., 2006). The fact that a long exposure to BDNF was required for detectable increases in α7-nAChR staining suggests that it relied on de novo synthesis of receptors. Blockers of protein synthesis could not be tested because they were toxic over this time period. The inference that de novo synthesis was required, however, was consistent both with the ability of BFA to block the increase and the finding that surface and intracellular pools increased in parallel. BDNF has previously been shown to elevate α7-nAChR levels on chick ciliary ganglion neurons by de novo synthesis (Zhou et al., 2004). Nonetheless, we cannot exclude the possibility that the increased receptor staining reflected, in part, lateral migration of previously dispersed receptors to generate readily visible receptor clusters. Measuring total α7-nAChR levels in the cultures was not informative because BDNF-responsive populations represented a small fraction of the total cells expressing α7-nAChRs.

The BDNF-induced increases in α7-nAChR levels required activation of NMDA receptors. The simplest model accounting for these results is that BDNF increases glutamatergic activity and this, in turn, mediates α7-nAChR expression and accumulation. The effects of glutamate could not be tested directly because extended exposure proved toxic. However, other methods provided strong support of the model. First, blocking NMDA receptors with APV prevented the BDNF effect, indicating that transmission through NMDA receptors was necessary. Second, bicuculline treatment, known to increase excitatory activity in the cultures, mimicked the BDNF effect but was not additive with it. This is consistent with an increase in glutamatergic activity causing an increase in surface receptor expression. These treatments did not alter α7-nAChR basal levels, though longer treatments can do so (Kawai et al., 2002). The bicuculline effect was not seen previously because only a narrow range of α7-nAChR staining was examined and the analysis was less quantitative (Kawai et al., 2002). Lastly, though the BDNF-mediated increase in surface α7-nAChRs could be prevented with function-blocking BDNF antibodies, these antibodies had no effect on the bicuculline-driven increase. Taken together, the results suggest that glutamatergic activity can act independently from BDNF to increase α7-nAChR levels. This places glutamatergic transmission downstream from BDNF activity in the regulatory pathway.

Glutamatergic neurons in the hippocampus release BDNF from both dendritic and axonal locations (Haubensak et al., 1998; Hartmann et al., 2001; Balkowiec and Katz, 2002), and this, in turn, can enhance glutamatergic transmission both pre-and postsynaptically (Levine et al., 1998; Li et al., 1998; Levine and Kolb, 2000). Most hippocampal neurons in culture receive glutamatergic input and have surface TrkB receptors, as is true in vivo (Yan et al., 1997; Drake et al., 1999). Accordingly, BDNF enhancement of glutamatergic transmission, whether presynaptic or postsynaptic in mechanism, could apply broadly across the neuronal population but does not. The fact that only certain interneuronal subtypes display increased α7-nAChR levels in response to BDNF strongly suggests the existence of cell-type-specific regulatory mechanisms engaged by the increased input.

Interestingly, interneurons that increase their α7-nAChR levels in response to BDNF are predominantly those thought to innervate glutamatergic neurons. Anatomical and electrophysiological studies show that both SS-positive neurons and parvalbumin-positive neurons (as identified by ER-β staining) inhibit pyramidal cells and act to decrease activity in the hippocampus. In contrast, VIP neurons do not elevate α7-nAChR levels in response to BDNF, and many VIP neurons selectively inhibit other interneurons. Increased activity in these cells would produce disinhibition of the hippocampus (Katona et al., 1999; reviewed in Freund and Buzsaki, 1996; Soltesz, 2006).

BDNF-induced up-regulation of α7-nAChRs may be designed to support hippocampal homeostasis. Release of BDNF from glutamatergic neurons, perhaps triggered by periods of elevated activity, will increase α7-nAChRs on interneurons inhibiting hippocampal glutamatergic neurons. Since α7-nAChRs are excitatory, increased levels of the receptors should enhance firing of the interneurons, thereby increasing inhibition of the downstream glutamatergic neurons. Activation of the receptors would arise from the diverse cholinergic projections to the hippocampus provided by the septum (Freund and Buzsaki, 1996). In addition to excitatory effects, α7-nAChRs can act in other ways to influence hippocampal function. Since α7-nAChRs have a high relative calcium permeability (Bertrand et al., 1993; Seguela et al., 1993), they can modulate neuronal function through regulation of calcium-dependent processes, including changes in gene expression. Activation of α7-nAChRs has been shown to stabilize cholinergic synapses on hippocampal interneurons in culture (Zago et al., 2006). Thus BDNF may effect specific subpopulations of hippocampal neurons by enhancing excitability, altering gene expression, or increasing the probability of receiving cholinergic innervation. The outcome could alter the balance of excitation and inhibition in the hippocampus, as well as contributing to network properties such as rhythm generation. The overall effects on spike timing and hippocampal network activity will depend on the precise functional connectivity and synaptic properties of the interneuron subpopulations involved.

Experimental methods

Cell cultures

Hippocampal cultures were prepared from 18- to 19-day-old Sprague Dawley rat embryos as previously described (Kawai et al., 2002). Briefly, hippocampi were removed rapidly under stereomicroscopic observation, cut into small pieces, and digested with 20 U/ml of trypsin (Invitrogen; Gaithersburg, MD) in Hanks balanced salt solution (HBSS; Invitrogen) at 37°C for 12 min. The tissue segments were then transferred to Neurobasal medium (Invitrogen) with 10% heat-inactivated horse serum (Invitrogen), triturated with a fire-polished Pasteur pipette and plated at 4×10^4 cells per 12-mm glass coverslip previously coated with poly-D-lysine (>300 kDa; Sigma, St. Louis, MO). Subsequent feeding occurred twice weekly, each time replacing half the volume with fresh Neurobasal media with 2% B-27 (Invitrogen). On day 6, some cultures received 5 μM cytosine-D-arabinofuranoside to inhibit further proliferation of non-neuronal cells. The cultures were maintained in a humidified tissue culture incubator with 5% CO_2 until use. Where indicated, the following compounds were applied to the cells during the last day in culture: 50 ng/ml BDNF (recombinant BDNF; Chemicon, Temecula, CA), 10 μg/ml brefeldin A (Calbiochem, San Diego, CA), 10 nM saxitoxin, 50 μM APV, 20 μM bicuculline, and 10 μg/ml chicken anti-BDNF polyclonal antibodies (Promega, Madison, WI).

Fluorescent labeling of α7-nAChRs

Surface α7-nAChRs were labeled by incubating 20-day-old cultures of dissociated hippocampal cells with 100 nM Alexa-αBgt (Molecular Probes, Eugene, OR) for 1 h at 37°C (Kawai et al., 2002). Cells were then washed in Neurobasal medium and phosphate-buffered saline (PBS) and processed for fluorescent immunocytochemistry. Two methods were used to label internal α7-nAChRs. Some cells were stained for surface α7-nAChRs as above, then fixed and lightly permeabilized in 95% methanol at −20°C for 10 min. Cells were washed in room temperature PBS and then incubated with 100 nM rho-αBgt to reveal internal clusters. A different method was used when needing to preserve intracellular epitopes for identifying neuronal subtypes. In these cases, surface α7-nAChRs were blocked with unlabeled 100 nM αBgt for 1 h at 37°C and then washed and lightly fixed with 2% paraformaldehyde (PFA) for 20 min. After washing again in PBS, cells were incubated with primary antibodies and 100 nM Alexa-αBgt to label intracellular pools of receptors, and then processed for fluorescent immunocytochemistry. Nonspecific binding was routinely assessed by including 5 μM MLA in the incubation with the Alexa-αBgt, but equivalent blockade was also obtained with 50 nM MLA. Co-staining with a goat anti-α7-nAChR antiserum (Santa Cruz Biotechnology, Santa Cruz, CA) yielded co-distribution with Alexa-αBgt, providing additional confirmation of specificity.

Immunocytochemistry

Cells were washed in PBS, fixed in 2% PFA followed by 4% PFA, each for 10 min, and then washed again in PBS and stained. Primary antibodies were diluted in 0.1% Triton X-100 plus 5% normal donkey serum in PBS and incubated with the cells for 1 h at 37°C. These included a mouse anti-GAD monoclonal antibody (1:500–1:1000; MAB351; Chemicon International, Temecula, CA), a rabbit anti-GAD polyclonal antibody (1:1000; Chemicon), a mouse anti-VIP polyclonal antibody (1:500; Immunostar, Hudson, WI), a rabbit anti-SS polyclonal antibody (1:500; Santa Cruz Biotechnology), a rabbit anti-ER-β polyclonal antibody (1:250; Invitrogen, Carlsbad, CA), and a rabbit anti-TrkB polyclonal antibody (1:100; TrkB (H-181); Santa Cruz Biotechnology, Santa Cruz, CA). After labeling, the cells were washed in PBS and incubated for 1 h at room temperature with appropriate secondary antibodies raised in donkey and conjugated to Cy3, FITC, Cy5, or Alexa-647 fluorophores (1:200–1:500 dilution in 0.1% Triton X-100 and 5% normal donkey serum in PBS; Jackson ImmunoResearch, West Grove, PA). The cells were then washed three more times in PBS and mounted using anti-fade mounting solution (Vectashield; Vector Laboratories, Burlingame, CA).

Image acquisition and quantification

Digital images of fluorescently labeled cells were collected using a CCD camera mounted on a Zeiss Axiovert (63× oil immersion objective, 1.4 numerical aperture lens) and equipped with SlideBook deconvolving software (Intelligent Imaging Innovations, Santa Monica, CA). GABAergic cells were identified by eye based on GAD-positive fluorescence present in the cell body but absent from the nucleus. Ten to twenty GABAergic neurons were imaged per coverslip. Neurons in relative isolation were chosen to facilitate quantification of fluorescence along the dendrites. Controls in which one or more primary antibodies were omitted showed no significant cross-contamination among fluorescence channels. Images were collected in z-stacks of 11 focal planes separated by 0.5 μm. SlideBook was used to deconvolve the images and to quantify surface intensity of α7-nAChRs. For each image, one edge of at least two neurites was masked and the mean pixel intensity under the mask was calculated. In some cases, data were normalized to the mean of control GABAergic cells on an independent coverslip. Each separate culture was counted as a single experiment (n) for statistical purposes. To exclude unhealthy cultures, experiments were discarded if control cultures lacked visibly detectable staining for α7-nAChRs or if BDNF-treated cultures showed no visibly detectable increase in α7-nAChR staining.

For measurements of internal α7-nAChRs, a 2D projection image was generated to visualize clusters in all planes. Control cultures in each experiment were used to set the display ranges to be measured. Maximum intensities were calculated for individual cells by averaging the intensity of the brightest clusters (4 per neuron) across four neurons per experiment. Minimum intensities were set to the mean non-cellular background across four control images. These ranges were applied to all experiments for a given culture. Measurements of cluster size, area, and number were performed using ImagePro 3.0 software (Media Cybernetics, Silver Spring, MD). Clusters were defined as having at least nine contiguous pixels (0.9 μm^2) with at least 50% maximal intensity, and were counted along dendritic segments. The total area of dendrites measured per cell was

recorded and used to calculate the percent area occupied by internal α7-nAChR pools. The same procedure was used to analyze the percentage of cells expressing internal clusters.

Statistics

Values represent the mean±SEM of results from 3–14 experiments. Statistical differences between two means were determined using the two-tailed Student's *t* test; comparisons among more than two were determined by ANOVA. The Dunnett post hoc test was used to compare experimental means to a control mean, and the Bonferroni post hoc test was used to compare selected pairs of means.

Materials

Unless otherwise indicated, all drugs and compounds were purchased from Sigma.

Acknowledgments

We thank Xiao-Yun Wang for the expert technical assistance and Honey B. Tijerina for the assistance with preliminary data analysis. Support was provided by NIH grants NS12601 and NS35469, and by a grant from Phillip Morris, USA Inc. and International. K.A.M. and W.M.Z. are Tobacco-Related Disease Research Program Predoctoral and Postdoctoral Fellows, respectively.

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ACKNOWLEDGEMENT

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CHAPTER 3

Nicotinic activity stabilizes convergence of nicotinic

and GABAergic synapses on filopodia of hippocampal interneurons

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Nicotinic activity stabilizes convergence of nicotinic and GABAergic synapses on filopodia of hippocampal interneurons

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Received 6 July 2005; revised 28 October 2005; accepted 17 November 2005
Available online 5 January 2006

Nicotinic acetylcholine receptors containing α7 subunits occupy pre- and postsynaptic sites in the adult hippocampus. We find that embryonic hippocampal slices in culture display the receptors most prominently on interneurons where they form clusters localized in part on filopodia. The receptors often co-distribute specifically with $GABA_A$ receptors. In septal–hippocampal co-cultures, the filopodia become co-innervated by cholinergic and GABAergic terminals abutting the receptor clusters. Nicotinic transmission appears to stabilize the cholinergic contacts: pharmacological blockade of the α7-containing nicotinic receptors increases the rate of filopodia movement and decreases the incidence of the clusters being adjacent to cholinergic terminals. Immunostaining fresh hippocampal slices from neonatal rat pups confirms that cholinergic and GABAergic terminals contact α7-containing nicotinic receptor clusters in vivo, and the clusters appear to include filopodial sites. The results indicate a convergence of nicotinic and GABAergic input at specific sites on developing hippocampal interneurons and suggest that synaptic activity helps stabilize the nicotinic contribution.

Keywords: Nicotinic; Receptor; Hippocampus; Filopodia; Interneuron; Synapse; Postsynaptic; Spine; Nicotinic receptors; Cholinergic; GABAergic

Introduction

Nicotinic cholinergic transmission in vertebrates results from the transmitter acetylcholine (ACh) activating cation-selective ligand-gated ion channels termed nicotinic acetylcholine receptors (nAChRs). Though nAChRs are widespread in the central nervous system, the physiological role of nicotinic cholinergic transmission in the brain is poorly understood. At the cellular level, the best-documented action is that of nAChRs modulating transmitter release. Nicotinic receptors containing the α7 gene product (α7-nAChRs) are well suited for this because they are among the most abundant in the CNS (Broide and Leslie, 1999; Berg and Conroy, 2002) and have a high relative permeability to calcium (Bertrand et al., 1993; Seguela et al., 1993). Indeed, they have been shown to enhance transmitter release from a variety of nerve terminals (McGehee et al., 1995; Gray et al., 1996; Alkondon et al., 1997, 1999; Guo et al., 1998; Li et al., 1998; Radcliffe and Dani, 1998; Jones et al., 1999; Dajas-Bailador and Wonnacott, 2004).

Electrophysiological studies demonstrate that α7-nAChRs also function postsynaptically in the CNS (Alkondon et al., 1998; Frazier et al., 1998; Hefft et al., 1999; Hatton and Yang, 2002; Khiroug et al., 2003). Ultrastructural analyses identify α7-nAChR clusters at postsynaptic sites in adult hippocampus and sensory cortex (Fabian-Fine et al., 2001; Levy and Aoki, 2002). The distribution and abundance of such clusters suggest that many are located at noncholinergic synapses. Hippocampal interneurons express α7-nAChRs (Jones and Yakel, 1997; Sudweeks and Yakel, 2000; Khiroug et al., 2003) and display them in clusters when grown in dissociated cell culture (Liu et al., 2001). Many of the clusters co-localize postsynaptically with $GABA_A$ receptors ($GABA_A$Rs; Kawai et al., 2002) and can be found at the tips of dendritic filopodia.

Dendritic filopodia are likely to serve as postsynaptic precursors, forming a bridge to an axon and subsequently retracting to form either a dendritic spine or shaft synapse (Dailey and Smith, 1996; Ziv and Smith, 1996). Both spiny and aspiny neurons display numerous filopodia during development; the timing and location of such protrusions suggest that they may evolve into shaft synapses at least as frequently as spines. Electron microscopic images confirm that filopodia can participate in synaptogenesis (Saito et al., 1997; Fiala et al., 1998). Whether filopodia also form cholinergic synapses between CNS neurons is unknown.

Here, we examine embryonic hippocampal slices maintained in culture during the period in which α7-nAChRs reach their highest relative levels in vivo (Adams et al., 2002). We find that discrete α7-nAChR clusters are often localized at the tips of filopodia emanating from interneurons in the slices. Moreover, such α7-nAChR clusters usually co-localize with $GABA_A$R clusters, even in the absence of presynaptic specializations contacting the filopodia. The septum is the main source of cholinergic input to the hippocampus in vivo. In co-culture, we find that it provides both cholinergic and GABAergic terminals that converge onto

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doi:10.1016/j.mcn.2005.11.009

hippocampal filopodia tipped with α7-nAChR clusters. Co-localization of cholinergic and GABAergic terminals over α7-nAChR clusters can be identified in vivo as well. Nicotinic transmission increases the incidence of cholinergic contacts in slice culture. The resulting nicotinic input is well positioned to regulate GABAergic signaling during postnatal development.

Results

Clusters of α7-nAChRs and $GABA_A$Rs co-localize at interneuron filopodial tips in slice culture

High levels of α7-nAChRs are found on interneurons identified by GAD staining in hippocampal slice cultures. In 3-week-old cultures, α7-nAChR clusters labeled with AlexaαBgt can be readily visualized on the cell body and along the dendrites. Cells with the highest levels also display the receptor clusters localized at the tips of filopodia extending from the dendrites. This can be seen by co-staining with anti-MAP2 to identify neurons and with rhodamine-phalloidin for filamentous actin in the filopodia (Figs. 1A–D). Because the slices are thin, neurons near the periphery of the slice can sometimes be viewed with relatively little contamination of actin staining from nearby cells, as in the example shown. The localization of α7-nAChR clusters on filopodial tips can also be seen by staining surface membrane with DiI to visualize the contours (Fig. 1E). Typically the filopodia appear long, thin, and devoid of a bulbous ending. By 4–5 weeks in culture, α7-nAChR clusters can often be found at the tips of shorter projections emanating from interneuron dendrites. Such projections have more rounded endings and resemble stubby spines (Figs. 1F, G).

Interestingly, co-staining for α7-nAChRs and $GABA_A$Rs in 3-week-old hippocampal slice cultures showed significant co-localization at filopodial tips extending from interneuron dendrites (Figs. 2A–D). Over half of the filopodia ending in α7-nAChR clusters had coincident $GABA_A$R clusters (56 ± 6%; mean ± SEM for 20 neurons; 3 experiments). They usually lacked glutamate receptor clusters (Figs. 2E–H). Thus, only 11 ± 5% (20 neurons; 2 experiments) and 18 ± 5% (11 neurons; 2 experiments) of the filopodia ending in α7-nAChR clusters showed co-staining for GluR1-containing AMPA receptors and NR1-containing NMDA receptors, respectively.

Co-clustering of α7-nAChRs and $GABA_A$Rs did not require innervation of the filopodia. The majority of filopodia ending in α7-nAChR clusters were not contacted by axon terminals when hippocampal slices were grown alone in culture, as judged by the absence of staining for GAD (Fig. 2C) or synaptophysin (Figs. 2E–H; 69 ± 4%; 109 filopodia analyzed on 15 neurons). The terminals that did innervate filopodia were GABAergic or glutamatergic, as judged by staining for GAD and VGluT, respectively; none were cholinergic as judged by VAChT staining (not shown). Clusters of α7-nAChRs on dendritic shafts co-localized to a limited extent with $GABA_A$R clusters in slice culture (13 ± 5%; 54 neurons; 6 experiments).

Cholinergic and GABAergic septal projections converge on hippocampal filopodia with α7-nAChRs

Since the septum provides the cholinergic innervation of the hippocampus in vivo, we combined septal explants with hippocampal slices in culture to assess the effects of cholinergic input on filopodia having α7-nAChR clusters. Staining either for VAChT or for ChAT (not shown) revealed cholinergic fibers extending from the septal explants and entering the hippocampal slices; no such fibers were apparent in hippocampal slices grown alone. Co-staining for α7-nAChRs and VAChT in the co-cultures revealed cholinergic terminals abutting receptor clusters at the tips of hippocampal filopodia and also on the occasional stubby spine-like

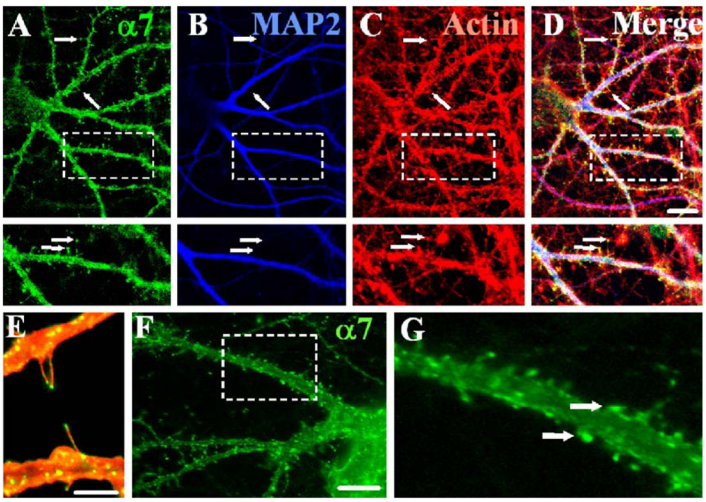

Fig. 1. Clusters of α7-nAChRs on dendritic and somatic filopodia. Hippocampal neurons in slice culture were stained with AlexaαBgt for surface α7-nAChRs (A, E, F) and co-stained either with anti-MAP2 mAb for microtubule-associated protein (B) and rhodamine-phalloidin for F-actin (C) and viewed in overlay (D) or with DiI for membrane contours and viewed in overlay (E). At 3 weeks in culture, the α7-nAChR clusters are usually found on the cell body and dendrites and at the tips of actin-rich filopodia in the slice (arrows). The expanded boxes (A–D) show additional detail for labeled spines. The cell shown was located near the edge of the slice, minimizing the contributions of neighboring cells to the actin stain. At 4 weeks, the receptor clusters can also be found on stubby spine-like structures emanating from the dendrites (F; delineated box enlarged in G). Scale bars, 10 μm.

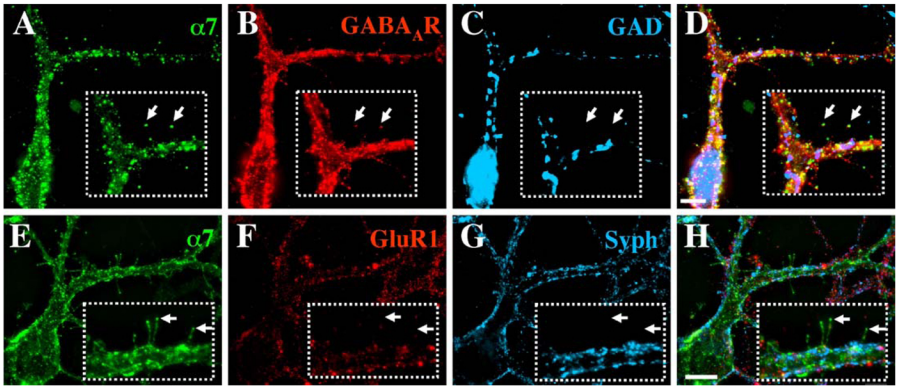

Fig. 2. Co-localization of α7-nAChR and GABA$_A$R clusters on filopodia. Hippocampal neurons at 3 weeks in slice culture were stained with AlexaαBgt for surface α7-nAChRs (A, E) and co-stained either for surface GABA$_A$R α1 subunit (B) and GAD65 (C), or for AMPA receptor GluR1-subunit (F) and synaptophysin (G), and corresponding panels viewed in overlay to the right (D, H, respectively). Boxed regions show expanded dendritic areas. Filopodial tips displaying α7-nAChR clusters often coincide with GABA$_A$R clusters; co-localization of α7-nAChR and glutamate receptor clusters is much less frequent. In the absence of septal explants, filopodia in hippocampal slice culture usually lack contact with presynaptic terminals (GAD, synaptophysin). Scale bars, 10 μm.

structure (Figs. 3A–C). At 3 weeks in culture, 59 ± 5% of the filopodia with α7-nAChR clusters at or near the tip were contacted by cholinergic terminals at the cluster site (112 filopodia from 19 neurons; 3 experiments). In contrast, a much smaller fraction of filopodia with GABA$_A$R clusters, but lacking α7-nAChR clusters (typically on interneurons with lower α7-nAChR levels overall), was contacted by a cholinergic terminal (26 ± 4%; 192 filopodia from 20 cells; 3 experiments). The results suggest that cholinergic terminals preferentially contact filopodia with prominent α7-nAChR clusters and often terminate on the receptor cluster itself.

The septum also provides strong GABAergic innervation to the hippocampus, particularly to interneurons (Freund and Antal, 1988). Triple staining for α7-nAChRs, GABA$_A$Rs, and GAD in septal–hippocampal co-cultures revealed GABAergic synapses on the same filopodia ending in α7-nAChR clusters. Thus, the filopodia co-stained for overlapping clusters of α7-nAChRs and GABA$_A$Rs (Figs. 3D, E) and were contacted by GAD-positive nerve terminals (Fig. 3F). Typically, the GAD-positive terminals ended adjacent to but not co-extensive with the receptor clusters, as expected for a presynaptic terminal innervating a postsynaptic structure (Fig. 3G). Analyzing a number of filopodia ending in α7-nAChR clusters indicated that 41 ± 7% of them received GAD-positive terminals in the co-cultures (72 filopodia from 8 neurons; 3 experiments). Only 14 ± 4% did so in hippocampal slices lacking septal explants (587 filopodia from 34 neurons; 7 experiments). This three-fold difference is consistent with the in vivo situation where septal projections appear to provide much of the GABAergic innervation of hippocampal interneurons (Freund and Antal, 1988). Triple staining for α7-nAChRs, cholinergic terminals with VAChT, and GABAergic terminals with GAD confirmed that both cholinergic and GABAergic terminals do converge on the same filopodium tipped with an α7-nAChR cluster (Fig. 3H). About a

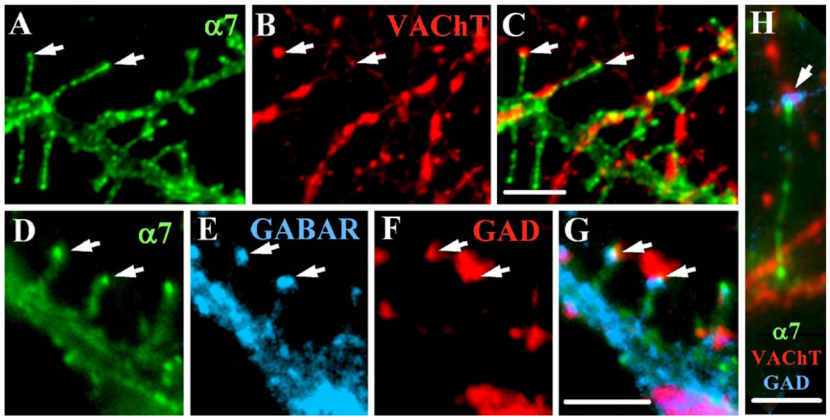

Fig. 3. Innervation of α7-nAChR filopodia by cholinergic and GABAergic terminals from septum. Three-week-old septal–hippocampal co-cultures were stained for surface α7-nAChRs with AlexaαBgt (A, D, H) and co-stained either for VAChT with antibodies (B) and shown in overlay (C), or co-stained for α1-GABA$_A$R subunit (E) and GAD65 (F) with appropriate antibodies and shown in overlay (G), or co-stained for both VAChT and GAD and shown in overlay (H). Arrows indicate examples of receptor clusters on filopodia being innervated by cholinergic (VAChT-positive) and/or GABAergic (GAD-positive) boutons. Scale bars, 10 μm.

third of the filopodia with α7-nAChR clusters were dually innervated (31 ± 2%; 31 filopodia on 5 cells; 3 experiments).

Nicotinic activity stabilizes cholinergic innervation of filopodia having α7-nAChR clusters

The role of synaptic activity in guiding synapse formation on filopodia was examined in septal–hippocampal slice co-cultures. Blocking nicotinic stimulation for 3 days by using either the panspecific nAChR blocker dTC (10 μM) or the α7-nAChR-specific blocker MLA (10 nM) significantly reduced the proportion of α7-nAChR filopodia contacted by VAChT-positive terminals (Fig. 4A). Similarly, depleting vesicular ACh with vesamicol (5 μM) reduced the proportion, while treating either with atropine (10 μM) to block muscarinic signaling or with bicuculline (20 μM) to block activation of $GABA_A$Rs had no effect (Fig. 4A). No obvious differences were noted in either the number of α7-nAChR filopodia or the abundance of VAChT-positive axons in the fields

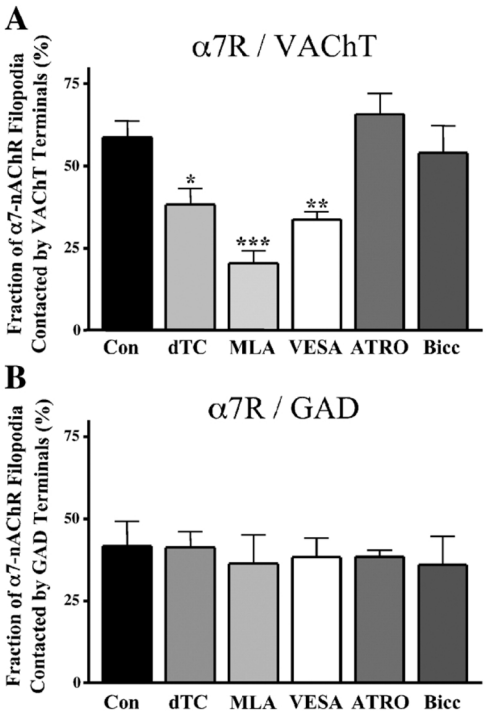

Fig. 4. The effects of synaptic activity on innervation of hippocampal filopodia with α7-nAChRs. The proportions of α7-nAChR filopodia contacted by cholinergic (A) or GABAergic (B) terminals were scored after a 3-day treatment of 3-week-old septal–hippocampal co-cultures with dTC (10 μM), MLA (10 nM), vesamicol (VESA, 5 μM), atropine (atro, 10 μM), or bicuculline (bicc, 20 μM). Blockade of nicotinic cholinergic transmission either in general with dTC or vesamicol or specifically at α7-nAChRs with MLA decreased the incidence of innervation of the filopodia by cholinergic terminals. Blockade of either muscarinic or GABAergic signaling had no effect. GABAergic innervation of the filopodia was not altered by any of the compounds. Values represent the means ± SEMs of results from 3–9 experiments. Statistical differences were determined by ANOVA; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ with respect to control. (The values for dTC, MLA, and VESA were not significantly different from each other).

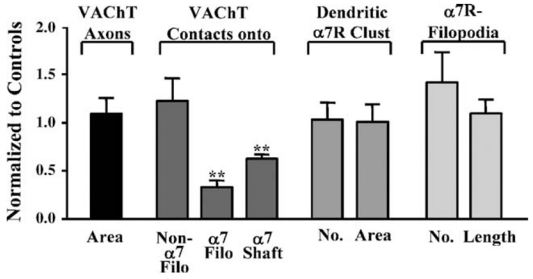

Fig. 5. Consequences of blocking α7-nAChRs for cholinergic structures. Septal–hippocampal co-cultures incubated with MLA for 3 days as in Fig. 4 to block α7-nAChRs were measured for the extent of cholinergic projections as seen by VAChT staining of processes (VAChT axons: area). The incidence of VAChT-positive terminals contacting putative postsynaptic structures (VAChT Contacts onto) was scored separately for filopodia lacking α7-nAChR clusters (Non-α7 Filo), for filopodia containing α7-nAChR clusters (α7 Filo), and for α7-nAChR clusters on dendrite shaft segments (α7 Shaft). Also quantified for α7-nAChR clusters on dendritic shafts (Dendritic α7R Clust) were the number of clusters per unit shaft area (No.) and the fractional shaft area occupied by the clusters (Area). Filopodia with α7-nAChR clusters (α7R-Filopodia) were quantified for the number of such filopodia extending per unit area of dendritic shaft (No.) and for the mean length of such filopodia (Length). All categories were imaged in the same fields of view, and all data were normalized to control values obtained in the absence of MLA treatment. Control values were the following. For VAChT axon area: 11 ± 1% of the surface area in the field of view; for α7 Shaft with VAChT contacts: 43 ± 2%; and under Dendritic α7R Clust, values for No. and area were 20 ± 3 per 10 μm^2 and 8 ± 1%, respectively of the shaft segment area. Under α7R-filopodia, control values were 1.0 ± 0.7 per 10 μm^2 of shaft area for No. and 7 ± 1 μm for filopodia length. Other control values are given in the text. The results represent the mean ± SEM from 3–4 separate experiments with 1–2 coverslips per condition in each experiment and multiple fields of view per coverslip. Statistical differences were determined by Student's two-tailed t test; $**P < 0.01$.

of view following any of the treatments, with the exception of a small increase in VAChT-stained axons following vesamicol treatment. The results indicate that nicotinic signaling is needed either to form or maintain cholinergic innervation of α7-nAChR filopodia.

In contrast to effects on cholinergic innervation, none of the blockers had any impact on the incidence of GAD-positive terminals contacting α7-nAChR filopodia in septal–hippocampal slice cultures (Fig. 4B). Not surprisingly, the cholinergic blockers also failed to affect cholinergic innervation of filopodia lacking α7-nAChRs. Thus, filopodia displaying only $GABA_A$R clusters, as found on a subset on interneurons, were less frequently contacted by cholinergic terminals (26 ± 4% as noted above) in controls, and MLA did not further decrease this level (32 ± 6%; 94 filopodia from 20 cells; 2 experiments). Such neurons generally express lower levels of α7-nAChRs, arranging them in clusters on the soma and dendrites but not prominently on filopodia.

The blockade by MLA did not decrease the abundance of cholinergic projections in the vicinity of interneurons nor, as indicated above, did it change the basal level of cholinergic terminals contacting filopodia lacking α7-nAChR clusters (Fig. 5). These results indicate that the MLA treatment is unlikely to have directly impacted the cholinergic fibers but rather reduced their ability to preferentially innervate filopodia with α7-nAChRs clusters. This reduction extended to α7-nAChR clusters on dendritic shafts, though less dramatically (Fig. 5). Since the

MLA treatment did not change either the number of dendritic α7-nAChR clusters nor the area they occupied, the decrease represents a net reduction in cholinergic innervation of the dendritic receptor clusters, albeit less severe than that seen for filopodial α7-nAChR clusters (Fig. 5).

Taken together, the results show that nicotinic transmission is important for preferential innervation of α7-nAChRs, and that the effect is more pronounced for filopodia receptors than for dendritic shaft receptors. Nicotinic transmission is not required to sustain the availability or abundance of cholinergic projections in the vicinity nor to permit the basal innervation found at non-α7-nAChR sites. The effect is confined to nicotinic contacts: neither cholinergic nor GABAergic transmission is important over the same time frame for GABAergic innervation of filopodia with α7-nAChR clusters.

Nicotinic activity inhibits motility of filopodia with α7-nAChR clusters

The pronounced effect of nicotinic blockade on filopodial innervation focused attention on these structures. Dendritic filopodia are motile structures that may explore the local environment for axons, preparatory to synapse formation (Yuste and Bonhoeffer, 2004). Calcium influx through NMDA receptors inhibits filopodial motility (Fischer et al., 2000; Chang and De Camilli, 2001; Portera-Cailliau et al., 2003). Like NMDA receptors, α7-nAChRs readily promote calcium influx. The finding that MLA decreased the incidence of α7-nAChR filopodia being contacted by cholinergic terminals suggested that nicotinic transmission might act locally on α7-nAChRs to repress motility and thereby stabilize filopodial–axon contacts. To test this, we first identified neurons with α7-nAChR filopodia by labeling with AlexaαBgt. We then marked the neurons by focally applying DiI to the soma, allowing visualization of the entire cell and associated processes. The neurons were incubated overnight to allow resumption of nicotinic signaling as the bound AlexaαBgt either dissociated or was removed from the surface by receptor turnover. Analysis of the DiI-stained filopodia was then carried out on neurons lacking residual AlexaαBgt staining on the surface; some internalized AlexaαBgt was often still present.

Time-lapse imaging indicated that α7-nAChR filopodia identified in this way in septal–hippocampal co-cultures were relatively stable over a period of minutes when in the vicinity of cholinergic terminals (Fig. 6A, left). As indicated above, many such filopodia were directly contacted by cholinergic terminals. If MLA was used to block nicotinic transmission at α7-nAChRs, filopodial motility was much increased (Fig. 6A, right). This was quantified by measuring the overall length of an individual filopodium at regular time intervals (2 min) in the absence of MLA and then calculating the mean length over the whole time period in the absence of MLA (20 min). The value of filopodial length at each time point both in the presence and absence of MLA was then expressed as a percent of that mean. Averaging these normalized values across a population of filopodia for a given time point yielded a population mean ± SD for each individual time. There was no significant decrement in population mean length over time and no specific overall change in length induced by the MLA treatment. Instead, there was a large increase in the variance when MLA was present (Fig. 6B). This increased variability signals an

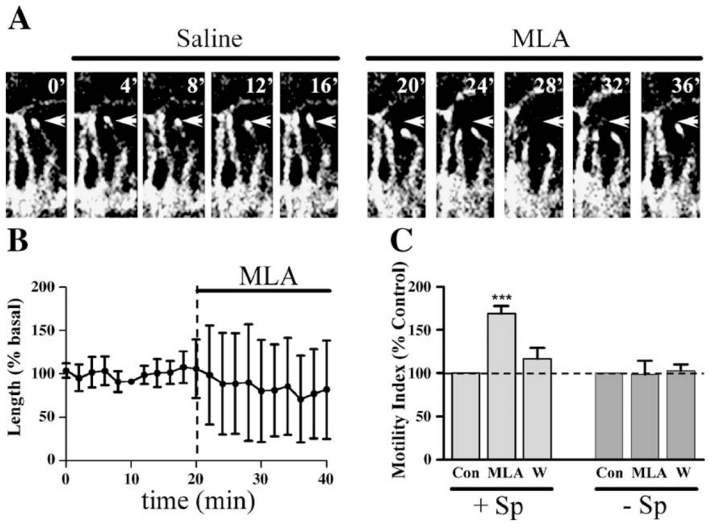

Fig. 6. Regulation of filopodial motility by signaling through α7-nAChRs. Hippocampal filopodia with α7-nAChR clusters were identified by labeling with AlexaαBgt and then staining with focal application of DiI to delineate surface membrane, rinsing and incubating for 24 h to allow toxin removal or dissociation. Time-lapse imaging was then performed at 2- to 4-min intervals for 36–48 min (see Experimental methods). Saline and MLA (10 nM) were bath-applied sequentially on the third and nineteenth minute, respectively, and wash-out of MLA (W) was begun on the thirty-seventh minute. The time-lapse sequences show higher filopodia motility after α7-nAChR blockade by MLA (A). The variability in average filopodial length was also increased by MLA, indicating greater movement within the population (B); values represent means ± SDs for 15 filopodia. Motility indices for hippocampal filopodia were constructed for 16-min intervals before (Con), during (MLA), and after removing (W) MLA in cultures either with (+Sp) or without (−Sp) visible septal input in the vicinity. The results indicate that the α7-nAChR antagonist MLA reversibly increased relative motility when septal input was present (C). The data were normalized to control values (100%) and expressed as means ± SEMs of 72–75 filopodia from 6 neurons combined from 3 experiments. Statistical differences were determined by ANOVA; $***P < 0.001$.

increase in filopodial motility with more contractions and extensions being seen within the population for a given time point when α7-nAChRs were blocked.

An index of motility was constructed by calculating the mean absolute value of changes in filopodial length seen during a 16-min time period before, during, and after an incubation with MLA (Fig. 6C). Normalizing the results to the mean change seen prior to MLA treatment (100%, controls) indicated that MLA nearly doubled the motility of α7-nAChR filopodia in hippocampal regions innervated by septal slices (+Sp). Removing the MLA (washout) returned the values to control levels. In contrast, the motility of filopodia in hippocampal regions devoid of septal input showed no additional increase when incubated in MLA (−Sp). (The variability in filopodial motility did not permit direct comparison of absolute motility rates in +Sp versus −Sp cultures.) At the end of the experiment, the cells were relabeled with AlexaαBgt; the vast majority of DiI-labeled filopodia retained α7-nAChR clusters (not shown). The results demonstrate that nicotinic transmission through α7-nAChRs reduces the motility of filopodia having the receptor clusters. This may explain in part how blockade of α7-nAChRS by MLA leads to fewer filopodia displaying cholinergic innervation. Altered motility is not likely to be the entire explanation, however, because it does not readily extend to α7-nAChR clusters on dendritic shafts which also show an MLA-dependent reduction in cholinergic innervation, though not as severe.

Cholinergic and GABAergic terminals co-innervate α7-nAChR clusters in vivo

To determine whether α7-nAChR clusters in vivo receive both cholinergic and GABAergic innervation, we examined fresh hippocampal slices prepared from P9–13 rats. Co-staining with AlexaαBgt for α7-nAChR clusters and with anti-VAChT antibodies for cholinergic terminals revealed close and specific apposition of the two kinds of structures (Figs. 7A–C). Analysis of adjacent optical sections (z-stack) indicated that the cholinergic terminal was immediately adjacent to, rather than overlapping with, the α7-nAChR cluster (Fig. 7D). This is consistent with a postsynaptic location for the receptor cluster. Similarly, co-staining for α7-nAChRs as above and for GABAergic terminals using anti-GAD antibodies also indicated close and specific apposition of the two (Figs. 7E–G). Again, analysis of z-stack optical sections demonstrated the GAD-positive structure as being adjacent to,

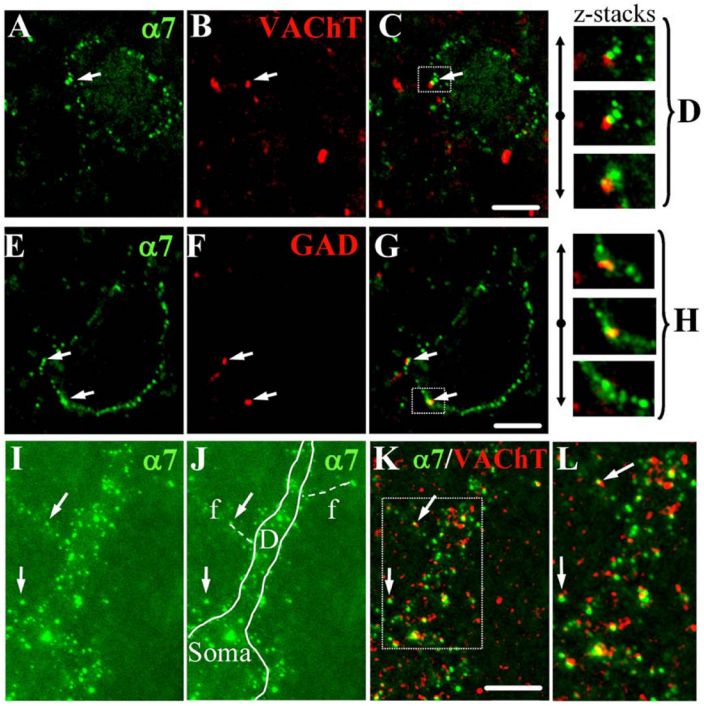

Fig. 7. Innervation of α7-nAChR clusters in vivo by cholinergic and GABAergic terminals. Fresh hippocampal slices from P9–13 rat pups were stained with AlexaαBgt for α7-nAChRs (A) and VAChT for cholinergic terminals (B) and shown in overlay (C). Three optical sections adjacent in the *z*-axis show the cholinergic terminal abuts rather than co-localizes with the receptor cluster (D), as expected for postsynaptic receptors. Similar slices stained with AlexaαBgt (E) and GAD for GABAergic terminals (F) are shown in overlay (G). Again, optical sections adjacent in the *z*-axis indicate the terminal abuts on rather than co-distributes with the receptor cluster (H). Staining for AlexaαBgt and collapsing several adjacent sections in the *z*-axis not only reveal receptor clusters but also indicate the contours of dendrites and cell bodies (I) as indicated by the lines (J) demarcating the soma and attached dendrite (D). Examples of two likely filopodia emanating from the dendrite and containing receptor clusters are indicated by dashed lines (f). The arrows designate receptor clusters followed in subsequent panels. Deconvolving and including the VAChT staining show numerous points at which cholinergic terminals contact α7-nAChR clusters (K). Enlarging the indicated box shows the two previously identified receptor clusters (arrows) as being innervated by VAChT-containing terminals and likely examples of clusters located on filopodia, given their displacement from the dendrite (L). Scale bars, 10 μm.

rather than overlapping with, the receptor clusters (Fig. 7H). The co-stained sites could be identified on cell bodies and on what appeared to be filopodia extending from cell bodies and dendritic shafts. These could best be visualized by viewing stacks of optical sections in the *z*-axis prior to deconvolving. Comparing the background staining in adjacent optical sections revealed the outlines of the cell bodies and dendritic shafts. Then analyzing individual deconvolved sections readily showed α7-nAChR clusters with associated VAChT-staining terminals lying at discrete distances from a soma or dendrite (Figs. 7I–L). Analyzing adjacent sections in the *z*-axis confirmed that the cluster and associated terminal were not part of the dendrite but rather displaced a distance consistent with a filopodial location. Examples were particularly prevalent in the stratum oriens of the hippocampus, reflecting a subpopulation of GABAergic interneurons. The results suggest that cholinergic and GABAergic axons converge on hippocampal α7-nAChR clusters in vivo as they do in slice culture, and that many of these sites may represent filopodia.

Discussion

The principal findings reported here are that α7-nAChRs form clusters that co-distribute with $GABA_A$Rs on interneuron filopodia in hippocampal slice culture, and that the filopodia can be jointly innervated by cholinergic and GABAergic axons from septal slices. Additionally, nicotinic transmission reduces motility of the filopodia having α7-nAChR clusters and stabilizes their innervation by cholinergic terminals. Nicotinic transmission also directly or indirectly stabilizes cholinergic innervation of α7-nAChR clusters on dendritic shafts, though to a lesser extent. Analysis of fresh hippocampal slices confirms that both cholinergic and GABAergic terminals focally innervate sites having α7-nAChR clusters in vivo and the sites appear to include filopodia. The results demonstrate that nicotinic transmission through α7-nAChRs shapes cholinergic connections that are well positioned to influence GABAergic signaling in the developing hippocampus.

Co-clustering of α7-nAChRs and $GABA_A$Rs occurs at filopodial tips even in the absence of innervation. This suggests that postsynaptic molecular interactions can determine receptor location at these early stages. Interneurons were the primary focus here because they expressed high levels of α7-nAChRs amenable for imaging (Jones and Yakel, 1997; Sudweeks and Yakel, 2000; Khiroug et al., 2003). The neurons are likely to represent previously identified subpopulations in vivo with abundant αBgt binding (Freedman et al., 1993). Pyramidal neurons may also express postsynaptic α7-nAChR clusters with the same properties but not at sufficient levels to permit imaging with the present technique. Ultrastructural studies have shown that α7-nAChRs can be found both pre- and postsynaptically at nearly all synapses in the CA1 region of adult hippocampus (Fabian-Fine et al., 2001), a time when overall α7-nAChR levels in the hippocampus are lower than those found during the stages examined here (Adams et al., 2002).

The conclusion that nicotinic transmission acted through α7-nAChRs to stabilize cholinergic innervation of the filopodia was based on the effects of dTC and MLA. The latter, at the concentration used, specifically blocks α7-nAChRs. The antagonists did not alter the number or size of α7-nAChR clusters (Fig. 5 and Kawai et al., 2002) apparently because the extent and duration of blockade were significantly less than that used previously to achieve upregulation of the receptors (Molinari et al., 1998; Ridley et al., 2001). MLA reduced the number of contacts between cholinergic terminals and α7-nAChR filopodia without reducing either the number of such filopodia overall or the number of cholinergic processes in the vicinity. MLA treatment also acutely increased the motility of filopodia in the vicinity of cholinergic processes, whereas it had no effect on filopodia far from cholinergic input. Presumably in the latter case, the filopodia were free of restraint by cholinergic signaling and were fully motile even in the absence of nicotinic antagonists. Variability in motility among filopodia did not permit a direct comparison of absolute mean values for different populations, hence, the requirement for normalization first and the use of a motility index. A key feature of α7-nAChRs likely to be important for their effects on filopodial motility is their high relative permeability to calcium (Bertrand et al., 1993; Seguela et al., 1993). Calcium influx through the receptors may inhibit motility and promote stabilization of filopodia in a manner similar to that of NMDA receptors (Fischer et al., 2000; Chang and De Camilli, 2001; Portera-Cailliau et al., 2003).

Blockade of nicotinic transmission also diminished cholinergic innervation of α7-nAChR clusters on dendritic shafts as noted above. The proportion of such clusters normally innervated, however, was smaller than on α7-nAChR filopodia, and the reduction caused by MLA was also smaller. It is possible that nicotinic transmission not only reduces filopodial motility but also drives filopodial retraction subsequently, delivering innervated α7-nAChR clusters to the dendritic shaft (see below). This could explain how MLA reduces the number of innervated α7-nAChR clusters on dendrites, but it would not by itself explain why no reduction in the total number of such clusters was seen. An alternative possibility is that constraint of filopodial motility may not be the only nicotinic mechanism helping stabilize cholinergic synapses. Nicotinic transmission may also trigger accumulation or secretion of components from postsynaptic compartments that either promote adherence to or induction of presynaptic structures on nearby cholinergic axons.

Interestingly, neither α7-nAChR nor $GABA_A$R activation appeared to have any effect on GABAergic innervation of α7-nAChR filopodia; the number of such contacts was unchanged by treatment with either MLA or bicuculline. GABAergic innervation must depend on other kinds of recognition events and stabilization mechanisms. Moreover, GABAergic contacts must be able to withstand the kinds of filopodial movement that remain in the absence of inhibition by nicotinic transmission. Conceivably GABAergic innervation occurred early on and may already have gone through an activity-dependent phase prior to cholinergic innervation. The role of nicotinic transmission appears not to include stabilization of GABAergic synapses structurally at this early stage.

The staining of VAChT and ChAT performed here indicated that, both in slice culture and in vivo, cholinergic terminals form discrete punctate contacts on postsynaptic structures with α7-nAChR clusters. It is likely that many of the innervated clusters in vivo were on filopodia as they were in culture, in view of their distribution in fresh slices. This could not be confirmed directly, however, because markers that could have distinguished filopodia from dendritic shafts in fresh slices generated massive staining that prevented adequate correlation with α7-nAChRs. Importantly, the finding of discrete cholinergic terminals abutting α7-nAChR clusters suggests a conventional kind of focal transmission for nicotinic signaling in the hippocampus, in agreement with ultrastructural studies (Leranth and Frotscher, 1987; Heimrich

and Frotscher, 1993). This differs from a proposed "volume transmission" mode of signaling for ACh whereby the transmitter is thought to diffuse over some distance to reach receptors at multiple sites (McKinney et al., 1983; Descarries, 1998; Zoli et al., 1999). Volume transmission may be less effective for α7-nAChRs which rapidly desensitize (Zhang et al., 1994) and almost certainly would not discriminate between pre- and postsynaptic receptors for selective activation when both are present at the synapse.

What might be the function of nicotinic transmission through α7-nAChRs on filopodia? One possibility is that the receptors ultimately drive filopodial retraction, perhaps producing the stubby spine-like structures with α7-nAChR clusters seen in older slice cultures. Stimulation of α7-nAChRs can induce neurite retraction in culture (Quik et al., 1990; Pugh and Berg, 1994; Lipton et al., 1988; but see Wong and Wong, 2001). Though transmission through α7-nAChRs reduced filopodial motility here when examined over the short term, it is quite possible that over the long term, a net retraction occurs due to sustained calcium levels or slowly responding cytoskeletal elements. If GABAergic synapses are co-localized on the filopodia, the retraction may serve as a mechanism for converting the contact into a spine or shaft synapse. Most interneurons have few or no spines, but spiny GABAergic interneurons have been described in many regions of the hippocampus (Freund and Buzsaki, 1996), including the stratum lucidum (Gulyas et al., 1992; Spruston et al., 1997) and hilus of the dentate gyrus (Buckmaster et al., 2002).

A complication, however, is the finding that the 3-day blockade of α7-nAChRs did not alter either the number or length of filopodia displaying the receptors. Possibly the filopodia maintain a steady-state equilibrium in the absence of nicotinic stimulation, remaining motile but unable either to retract into the shaft or to form de novo. It is more difficult, however, to explain the implication that blocking filopodial retraction must somehow reduce the fraction α7-nAChR clusters innervated on dendritic shafts without reducing the total number of such clusters.

An alternative possibility for the role of nicotinic signaling through α7-nAChRs, independent of filopodial movement, might be that of providing calcium-dependent modulation of GABAergic responses. GABAergic signaling converts from an excitatory mode to an inhibitory one during this developmental period in vivo (Ganguly et al., 2001). Inhibitory GABAergic transmission also displays plasticity (Lu et al., 2000; Gubellini et al., 2001; Kirischuk et al., 2002; Wang et al., 2003; Bauer and LeDoux, 2004), and $GABA_AR$ function can be regulated (Kawaguchi and Hirano, 2002; Kittler and Moss, 2003) through mechanisms likely to be calcium-dependent. Calcium influx through α7-nAChRs may offer local control.

Experimental methods

Slice cultures

Hippocampal slice cultures were prepared using either the general membrane insert technique (Stoppini et al., 1991) or the placement of slices directly on glass coverslips treated with poly-D-lysine. Embryonic day 19 Sprague–Dawley rats were decapitated, and their brains were quickly removed and placed in chilled HBSS (Invitrogen, San Diego, CA). The hippocampi were dissected and cut transversely into 100- to 150-μm-thick slices using a vibratome. Two or three hippocampal slices were placed on one 12-mm Millicell CM membrane insert (Millipore, Bedford, MA), the runderside of which was bathed in 300 μl of Neurobasal medium with 2% (v/v) B-27 supplement, 0.5 mM L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin (Invitrogen). For septo-hippocampal co-cultures, a dissected piece of septum (approximately 500 μm in diameter) was placed adjacent to the hippocampal slices. For motility studies, the slices were cultivated directly on glass-bottom dishes treated with poly-D-lysine and covered with 250 μl of culture medium. The medium was initially supplemented with 10% (v/v) heat-inactivated horse serum (GIBCO). Subsequent feedings exchanged half the medium at twice weekly intervals with fresh medium lacking the horse serum. Cultures were kept in a humidified tissue culture incubator with 95% air and 5% CO_2. On day 6, the cultures received 5 μM cytosine-β-D-arabinofuranoside to inhibit further proliferation of nonneuronal cells; subsequent feedings diluted out the compound. Experiments were performed on slice cultures after 3–4 weeks in vitro.

Pharmacological treatments

To manipulate neuronal activity in the slice cultures, one or more of the following was present in the culture medium during the last 3 days in vitro: 5 μM of the vesicular ACh transporter (VAChT) inhibitor vesamicol hydrochloride (Tocris Cookson Inc.; Ellisville, MO), 10 μM of the pan-nicotinic receptor antagonist D-tubocurarine chloride (dTC), 10 nM of the selective α7-nAChR antagonist methyllycaconitine citrate (MLA), 10 μM of the muscarinic receptor antagonist atropine sulfate, or 20 μM of the $GABA_AR$ antagonist bicuculline methiodide.

Labeling surface α7-nAChRs and $GABA_ARs$

For imaging studies, slice cultures were washed twice with Neurobasal medium plus 0.1% BSA and then incubated in the same medium containing 100 nM of Alexa Fluor 488α-bungarotoxin (AlexaαBgt; Molecular Probes; Eugene, OR) at 37°C for 45 min. Surface staining of $GABA_ARs$ was achieved by incubating the slices with a rabbit anti-$GABA_AR$ α1-subunit polyclonal antibody (pAb; 06-868; 1:1000; Upstate Biotechnology; Lake Placid, NY) during this same period. Nonspecific binding was assessed by incubating the slices with nicotine (1 mM) 5 min before and during the labeling with AlexaαBgt. The cultures were then washed four times in 2 ml of Neurobasal medium and fixed for 20 min at room temperature in 4% paraformaldehyde in PBS.

Membrane DiI labeling

Slice cultures grown in glass-bottom dishes were first live-stained with AlexaαBgt as above. A saturated stock of the carbanocyanine membrane tracer DiI (Molecular Probes) prepared in fish oil was applied specifically by micropipette under a microscope (Papa et al., 1995) to soma of neurons with filopodia displaying labeled α7-nAChR clusters. The cultures were then washed twice with fresh Neurobasal medium and incubated overnight in conditioned culture medium from sister cultures before analysis.

Fluorescence immunocytochemistry in slice cultures

For immunolabeling, fixed slices were permeabilized with 0.1% Triton X-100 in PBS and then incubated with antibodies. These included a mouse anti-glutamic acid decarboxylase (GAD) monoclonal antibody (mAb; clone GAD-6 for GAD65, 1:1000; Chemicon International, Temecula, CA), a goat anti-vesicular ACh transporter (VAChT) pAb (SC-7717; 1:1000; Santa Cruz Biotechnology Inc.; Santa Cruz, CA), a rabbit anti-glutamate receptor GluR1 subunit pAb (PC246; 1:100; Oncogene Research Products, San Diego, CA), a rabbit anti-choline acetyltransferase (ChAT) pAb (AB5042; 1:2000; Chemicon International), anti-synaptophysin mAb (clone SVP38; 1:1000), a guinea pig anti-vesicular glutamate transporter 1 (VGluT) pAb (AB5905; 1:5000; Chemicon International), and a mouse anti-microtubule-associated protein 2 (MAP2) mAb (1:1000; Sigma; St. Louis, MO). Nonspecific antibody binding was minimized by pretreatment with 5% donkey serum in 0.1% Triton X-100 PBS for 30 min at room temperature. Primary antibodies were diluted in 0.1% Triton X-100 PBS containing

donkey serum and incubated with the cells for 1 h at 37°C. Cells were washed three times (10-min intervals) in 0.1% Triton X-100 PBS and then incubated for 1 h at room temperature with secondary antibodies raised in donkey and conjugated to Cy3, AMCA, or Cy5 fluorophores (1:250–1:500 dilution in 0.1% Triton X-100 PBS; Jackson ImmunoResearch, West Grove, PA). The cells were then washed three times (10-min intervals) in PBS and mounted using anti-fade mounting solution (Vectashield; Vector Laboratories, Burlingame, CA).

Staining of fresh hippocampal slices

Postnatal day 9 (P9)–P13 rats were overdosed with an intraperitoneal injection of pentobarbital (85 mg/kg) and perfused intracardially through the left ventricle with approximately 60 ml of cold PBS. Following exsanguination, brains were quickly dissected and placed into ice-cold oxygenated artificial cerebral spinal fluid (ACSF) containing (in mM): 119 NaCl, 2.5 KCl, 1.3 $MgCl_2$, 2.5 $CaCl_2$, 1 NaH_2PO_4, 26.2 $NaHCO_3$, and 11 glucose. After dissection, hippocampi were immerged in a block of 2.5% agarose and glued to the stage of a vibratome containing cooled oxygenated ACSF for slicing. Free-floating hippocampal sections (70–100 μm) were stained for α7-nAChRs as described above. After fixing for 30 min at room temperature in 4% paraformaldehyde and 0.25% glutaraldehyde in PBS, slices were washed three times with water and frozen (−20°C) and defrosted two times to improve antibody penetration into the tissue. Immunolabeling was performed as described for slice cultures. To decrease background, stained slices were transferred to a tube containing 25 ml of PBS and irradiated in a microwave oven at 1.5 kW for 30 s, washed three times with water, and mounted as described above.

Image acquisition and quantification

Digital images of fluorescently labeled cells were collected using a CCD camera mounted on a Zeiss Axiovert (63× oil-immersion objective, 1.4 numerical aperture lens) equipped with SlideBook deconvolving software (Intelligent Imaging Innovations, Santa Monica, CA). Reconstructed images were generated from *z*-axis stacks of 15–30 0.3-μm optical sections and processed by nearest neighbor or constrained iterative deconvolution. Digital zoom of 2× with filtering (Gaussian) was used for display purposes in Figs. 3, 5, and 6). Controls in which one or more primary antibodies were omitted showed no significant cross-contamination among fluorescence channels. Background values were determined by averaging the fluorescence signal along dendrites from cultures in which primary antibodies were substituted with nonimmune serum and were subtracted from deconvolved images.

Quantification of appositions between filopodia and presynaptic terminals

Neurons showing high levels of α7-nAChR staining at the tips of filopodia were selected from hippocampal areas which received both septal cholinergic and GABAergic innervation as visualized by immunofluorescence. Appositions were manually scored as cases when a single filopodial tip and a given terminal were localized within a maximum distance of one pixel (0.1 μm). All treatments were performed in sister cultures with slices from the same animals, and counts were done blindly. At least three independent experiments (1 animal/experiment) were done for each condition. The values were expressed as a percentage of total filopodia showing contacts with VAChT- and/or GAD-positive boutons. Statistical analyses were performed using ANOVA.

Additional image quantification

Filopodial length and number were measured manually by first masking individual filopodia and then quantifying their length with Slidebook software. Clusters of α7-nAChRs on dendritic shafts were quantified for number and for the proportion of dendritic area that they occupied, as previously described (Kawai et al., 2002). The length and area of the parent dendrite segment were also measured so that the density of filopodia and dendritic α7-nAChR clusters could also be determined. VAChT-labeled boutons contacting α7-nAChR clusters on dendritic shafts were quantified as described (Kawai et al., 2002) except that the threshold was set to analyze only the brightest clusters. The area occupied by VAChT-stained projections (cholinergic) was quantified by ImagePro (Media Cybermetrics, Inc., Silver Spring, MD); threshold was set at 200% above background. All analyses were done blind; control and MLA values were compared using Student's two-tailed *t* tests.

Motility analysis

For time-lapse analysis of DiI-labeled neurons, cultures were transferred to a microscope stage and perfused with HEPES-buffered medium containing (in mM): NaCl 150, KCl 4, $MgCl_2$ 2, $CaCl_2$ 2, glucose 10, Na-pyruvate 0.23, HEPES 10, CNQX 0.02, APV 0.010, bicuculline 0.020, and 2% (v/v) B-27 supplement. The pH was adjusted to 7.4 with NaOH. For acute blocking of α7-nAChRs, 10 nM of MLA was bath applied at a constant flow rate of 1 ml/min for 16 min followed by a wash-out period of 16 min. Images from proximal dendrites were collected every 2–4 min throughout for a total period of 36–48 min. For each time point, 3–5 focal z-stacks (0.3 μm each) were taken and projected into a single image to correct for drift in the z-plane. The lengths of all filopodia were obtained by manually measuring the distance from the dendrite to the filopodial tip using Slidebook software. Analyses were performed by blind or double-blind subjects. A motility index was calculated as the absolute difference in length of protrusions for two consecutive time frames, and the values summed for the period covered by each treatment (total of 4–8 frames each). Filopodia which disappeared at any time-point were excluded from the analysis. Because of variability in basal motility among cells, the data were normalized as percent change in filopodial length compared to the mean value obtained over the entire time period (100%) prior to MLA treatment. All statistical analyses were performed with analysis of variance (ANOVA) using the software Prism (GraphPad, San Diego, CA).

Materials

All reagents were purchased from Sigma unless otherwise indicated.

Acknowledgments

Grant support was provided by the National Institutes of Health Grants NS12601 and NS35469 and by Phillip Morris USA/International. We thank Xiao-Yun Wang and Honey Tijerina for expert technical assistance. W.M.Z. and K.A.M. are Tobacco-Related Disease Research Program Post- and Predoctoral Fellows, respectively.

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ACKNOWLEDGEMENT

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CHAPTER 4

Nicotinic Promotion of Non-Nicotinic Synapse

Formation in the Developing Hippocampus

SUMMARY

Nicotinic cholinergic signaling uses the transmitter acetylcholine to activate ligand-gated ion channels in the nervous system; it contributes to a variety of higher order functions (Picciotto et al., 1995; Bannon et al., 1998; Marubio et al., 1999; Cui et al., 2003; Levin et al., 2006; Bitner et al., 2007), neurological disorders (Newhouse et al., 1997; Picciotto and Zoli, 2002; Raggenbass and Bertrand, 2002; Teper et al., 2007), and addiction (Mansvelder and McGehee, 2002; Maskos et al., 2005). Nicotinic acetylcholine receptors (nAChRs) are widely expressed and reach their highest relative levels during early postnatal life (Zhang et al., 1998; Adams et al., 2002), a time when spontaneous waves of nicotinic excitation occur in the nervous system. The waves have been linked to a variety of phenomena including dendritic development and target selection (Bansal et al., 2000; Myers et al., 2005; Le Magueresse et al., 2006), but the central role and underlying mechanisms remain unclear. We show here that nicotinic stimulation in the early postnatal hippocampus quickly induces dendritic spines. Continued stimulation stabilizes the spines and causes them to become innervated. The two major nAChR subtypes in brain play complementary roles in this: one causes pyramidal neurons in hippocampal slices to extend spines while the other acts recruits presynaptic boutons and increases surfaces expression of postsynaptic AMPA receptors. In vivo exposure to nicotine quickly induces spine extension on hippocampal neurons, and mice lacking the two major nAChR subtypes show retarded acquisition of spines during early postnatal development. The results indicate a fundamental role for endogenous nicotinic

activity in promoting and coordinating glutamate synapse formation in the early postnatal brain and reveal a vulnerability of the process to manipulation by nicotine.

RESULTS

Nicotine quickly induces dendritic spines in early postnatal hippocampal neurons. This was shown by transferring postnatal day 4 (P4) mouse hippocampal slices to organotypic culture, infecting neurons with a Sindbis viral construct encoding GFP so that spines could be visualized, and then incubating with 1 μM nicotine for 1 hour prior to imaging (Fig. 1A). Quantification of spine number on pyramidal neurons in CA1 indicated a 50% increase (Fig. 1B). The induction was not blocked by antagonists of ionotropic and metabotropic glutamate and GABA receptors or by blockade of activity by tetrodotoxin (Fig. 1B). It was blocked, however, by preincubating slices with BAPTA-AM, indicating that calcium was essential. Rapid spine induction by nicotine is a feature of developing neurons; older neurons, e.g. those in P24 slices, did not show additional spines when treated with 1 μM nicotine even for 14 hours (Fig. 2).

Spine induction by nicotine in early postnatal slices was initially reversible. After an extended exposure, however, the spines became stable and remained upon nicotine removal (Fig. 3A). Blockade of AMPA, NMDA, and $GABA_A$ receptors again had no effect (Fig. 3B). The induction depended on β2-containing nAChRs, one of the two major nAChR subtypes found in brain. This was shown by

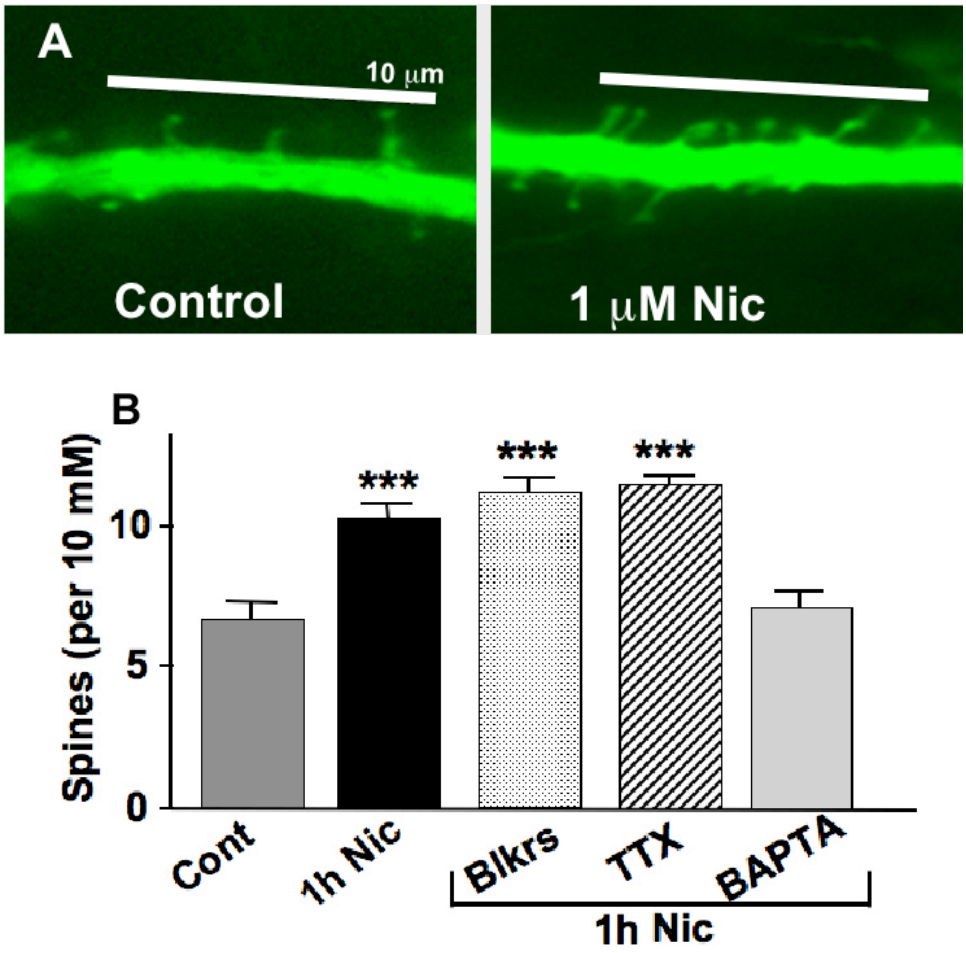

Fig. 4.1. Nicotine induces dendritic spines on hippocampal pyramidal neurons. P4 mouse hippocampal slices were placed in organotypic culture, infected with Sindbis-GFP on the third day, and analyzed on the fourth day. **(A)** Incubating slices for 1 hour with 1 μM nicotine increase the number of spines visible with Sindbis-GFP expression (right) compared to control slices (left). **(B)** Quantifying the number of spines per 10 μm segment of dendrite showed a 50% increased following nicotine treatment, and the increase was not blocked by 50 μM APV, 20 μM NBQX, 20 μM gabazine, 0.5 mM phaclofen, and 250 μM (RS)-MCPG for, respectively, NMDA, AMPA, $GABA_A$, $GABA_B$, and metabotropic glutamate receptors (blkers). Nor was it blocked by 1 μM tetrodotoxin (TTX); it was blocked by 100 μM BAPTA-AM, showing that calcium was required. Scale bars: 10 μm. Values represent means ± SEM of 10-16 neurons in slices from ≥ 3 animals per condition. (***$p < 0.001$ by one-way ANOVA followed by Bonferroni post-hoc test.)

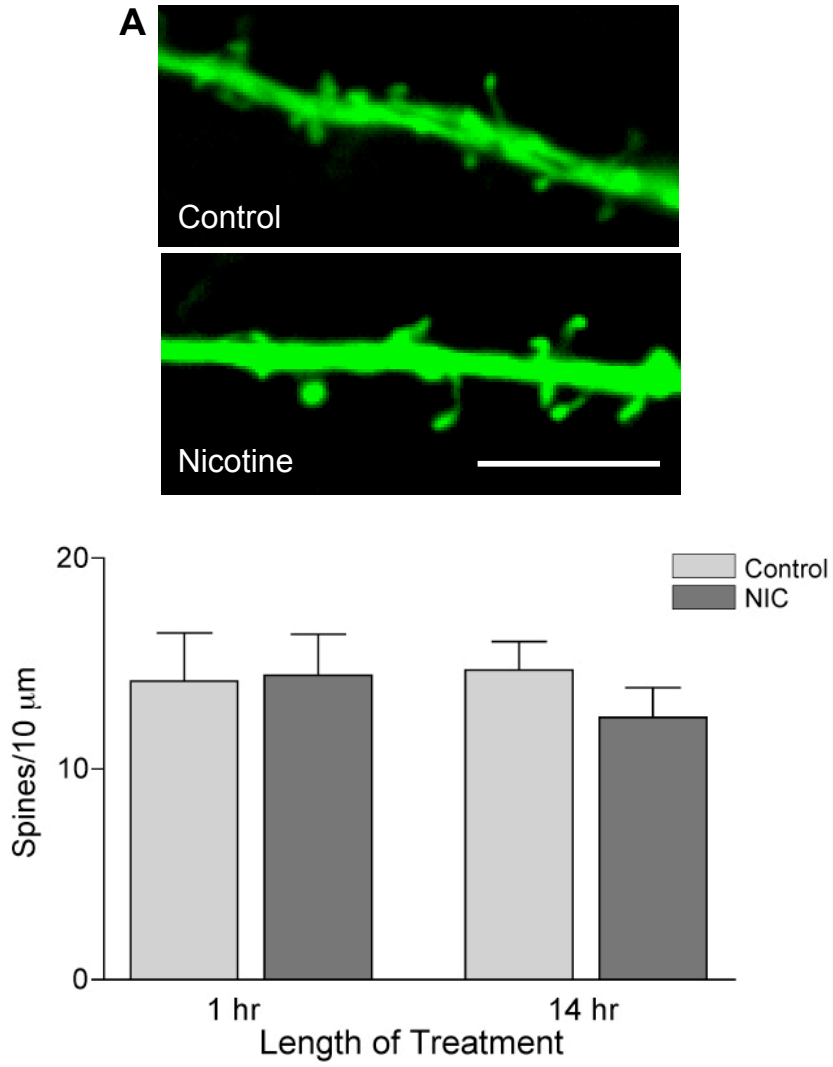

Fig. 4.2. Hippocampal slices from P24 mice show no increase in spine number even after treatment with 1 μM nicotine for 14 hours. Slices from mice expressing GFP under the Thy1 promoter were cultured for 15 hours then imaged for GFP fluorescence to identify spines. **(A)** Control (top) cells were cultured for 15 hours. In Nicotine-treated cultures (bottom), drugs were added one hour after culturing and slices were incubated an additional 14 hours. **(B)** Slices were treated with nicotine for the final hour (1 hr) or 14 hours (14 hr) of culture. Scale bars: 5 μm. Quantification of spines per 10 μm dendritic apical segment from CA1 pyramidal neurons. Values represent the mean ± SEM of ≥ 4 neurons/animal from 5-8 animals per condition. ($p > 0.05$, by Student's t-test)

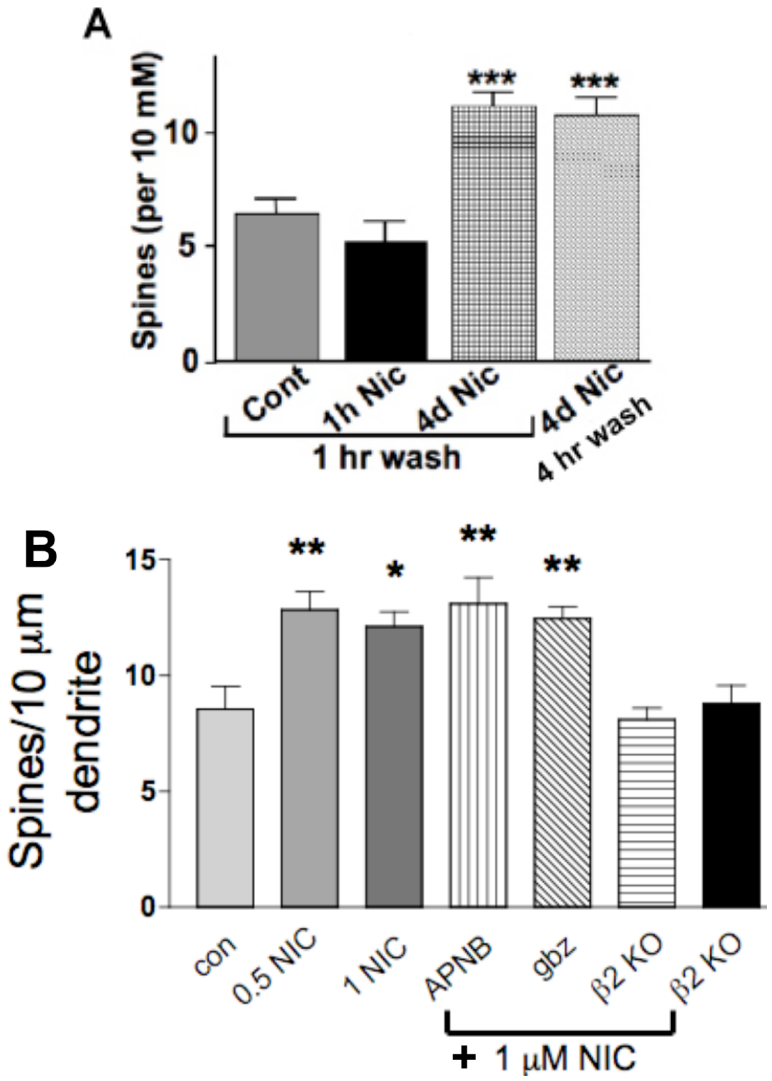

Fig. 4.3. Nicotine-induced spines are stable. P4 mouse hippocampal slices were placed in organotypic culture for 4 days and infected with Sindbis-GFP on the third day to visualize spines. **(A)** Slices were treated with nicotine for 1 hour or 4 day and then washed 1 hour or 4 hours before analysis. **(B)** Quantification (from optical sections comprising z-stacks) showed that both 0.5 and 1 µM nicotine increased spine number by half; the increase was not blocked by APV+NBQX (APNB) or by gabazine (gbz), and could not be elicited in slices from β2KO mice. Values represent means ± SEM of 10-16 neurons in slices from ≥ 3 animals per condition. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ by one-way ANOVA followed by Bonferroni post-hoc test)

comparing slices from wildtype (WT) and β2 knockout (β2KO) mice: even 4 days of nicotine did not increase the number of spines in β2KO slices (Fig. 3B). The other major nAChR subtype in brain, α7-nAChRs, appeared unnecessary for spine induction because blocking them with methyllycaconitine (MLA) did not prevent the effect (Fig. 3B).

Extended treatment of developing rat hippocampal neurons in dissociated cell culture produced an even larger increase. Spines were visualized by transfecting the neurons with a construct encoding green fluorescent protein (GFP). Twice as many spines were present when neurons were treated with 1 μM nicotine for a number of days (Fig. 4A). Immunostaining for PSD-95 indicated that essentially all of the spines in nicotine-treated cultures contained the scaffold component, as did spines in control cultures (Fig. 4B). The nicotine-induced doubling was prevented by dihydro-β-erythroidine (DHβE) which blocks β2-containing nAChRs, but not by α-bungarotoxin (αBgt) which blocks α7-nAChRs. Blockers of AMPA, NMDA, and $GABA_A$ receptors did not prevent the induction (Fig. 4C). The results indicate that chronic stimulation of β2-containing nAChRs induces spines with components expected for glutamatergic synapses on pyramidal neurons.

Immunostaining slices for presynaptic components demonstrated that nicotine-induced spines become innervated. Almost all spines in nicotine-treated slices were in close contact with boutons containing the presynaptic marker synapsin (Fig. 5A,B). In fact the proportion of spines thus innervated was

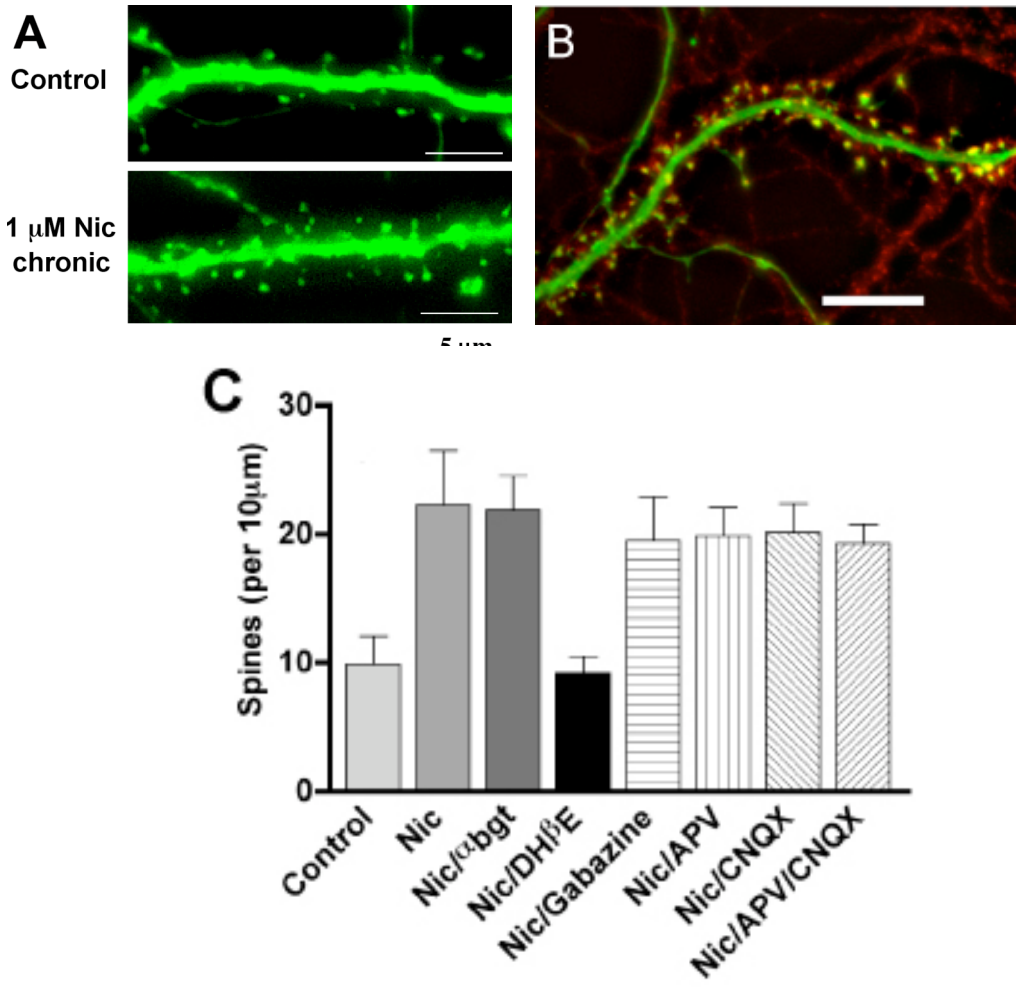

Fig. 4.4. Nicotine induces spines in rat hippocampal cultures. E18 rat hippocampal neurons were grown 20 days in medium containing the indicated compounds and were transfected with GFP on day 3 to reveal individual dendrites and spines. **(A)** Images of dendrites from untreated (Control, upper) and nicotine-treated (1 μM Nic chronic, lower) cultures. Scale bars: 5 μm. **(B)** Immunostaining for PSD-95 (red) overlaid with the GFP-expressing cells (green) revealed the scaffold protein in essentially all spines (yellow). Scale bar: 10 μm. **(C)** Quantification of spines along segments of secondary dendrites (3-5 per neuron) extending from the branch point outward 50 μm. Nicotine treatment doubled the number of spines. This effect was blocked by DHβE, a specific α4β2-nAChRs antagonist, but not by α-bungarotoxin (αbgt) which specifically blocks α7-nAChRs. Ionotropic GABA and glutamate receptors did not appear to be involved since gabazine, APV, and CNQX did not prevent the effect.

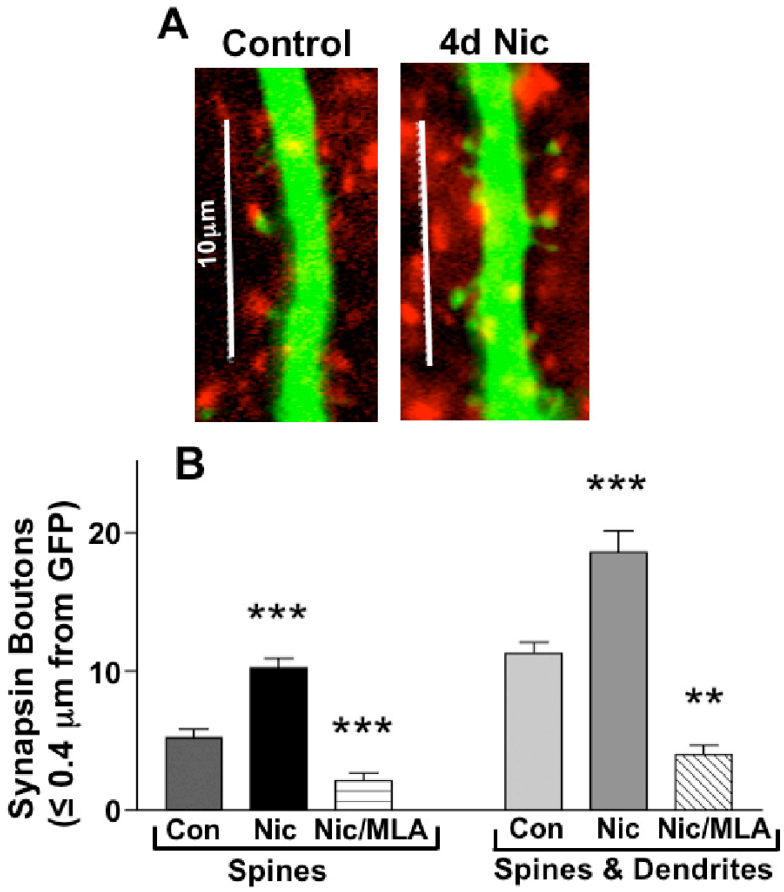

Fig. 4.5. Nicotine induction of presynaptic boutons requires α7-nAChR activation. P4 mouse hippocampal slices were placed in organotypic culture for 4 days and infected with Sindbis-GFP on the third day to visualize spines. **(A)** Immunostaining for synapsin as a presynaptic marker revealed puncta (red) contacting the GFP-filled spines (green). **(B)** Quantification showed that the increased number of synapsin-containing puncta contacting spines matched the increased number of spines, suggesting that spines induced by nicotine acquire presynaptic bouton-like structures. Including 50 nM MLA with nicotine (Nic/MLA) to specifically block α7-nAChRs prevented the increase in synapsin puncta, though earlier experiments indicated it did not prevent the increment in spine number. Notably, MLA reduced the incidence of synapsin-boutons below that seen in controls, suggesting that α7-nAChR activation, perhaps by choline (an α7-nAChR agonist) in the medium, exerts a basal effect. The results suggest that nicotinic activation of α4β2-nAChRs is sufficient to induce spines, but activation of α7-nAChRs may be required for spine innervation. Values represent mean ± SEM; n=7-9 neurons, 4 animals/condition. (**$p < 0.01$, ***$p < 0.001$ by one-way ANOVA followed by Bonferroni post-hoc test)

nominally greater than seen in control conditions. Similarly, the total number of synapsin puncta contacting spines and dendritic surfaces was increased at least as much in nicotine-treated slices as were the number of spines. Blockade of α7-nAChRs with MLA dramatically reduced the number of such boutons, even though it did not reduce spine number (Fig. 5B). The results indicate that α7-nAChRs and β2-containing nAChRs play complementary roles in synapse formation. The α7-nAChR component is profound: MLA treatment depressed overall synapsin puncta levels below those seen in control slices, suggesting that innervation of spines even under control conditions depends in part on α7-nAChR activation (Fig. 5B). This most likely arises from choline in the culture medium serving as a weak agonist for α7-nAChRs (Alkondon et al., 1997). Nicotine induction of synapsin-containing puncta was also blocked by antagonists of AMPA and NMDA receptors, indicating that the effect was mediated by glutamatergic transmission (Fig. 5B).

Functional synapses require postsynaptic receptors. One indication that nicotinic stimulation might also recruit receptors to newly formed synapses came from the observation that nicotine induced quick phosphorylation both calcium, calmodulin-dependent protein kinase II (CamKII) and GluR1-containing AMPA receptors in CA1 pyramidal neurons (Fig. 6). The specific phosphorylation sites were those previously shown to increase CamKII function and AMPA receptor conductance (Barria et al., 1997; Derkach et al., 1999). MLA blocked the phosphorylation events, as did blockers of AMPA and NMDA receptors,

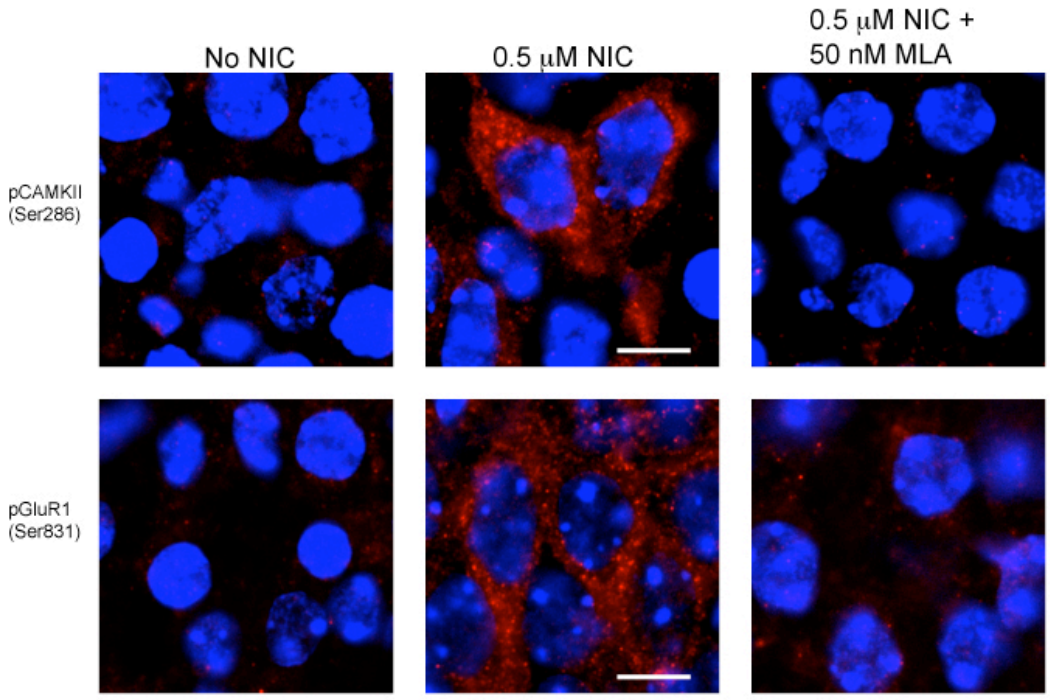

Fig. 4.6. Nicotine induces rapid phosphorylation of CamKII and GluR1-containing AMPA receptors. P4 mouse hippocampal slices were treated with PBS (No Nic,) 0.5 μM nicotine (0.5 μM, middle), or 0.5 μM nicotine with 50 nM MLA (0.5 μM NIC + 50 nM MLA) for 1 hour. Slices were then immunostained phosphorylated on threonine 286 (top) or GluR1 phosphorylated on serine 831 (bottom). Nicotine caused an increase in both phosphorylated CAMKII and phosphorylated GluR1. This increase was blocked by MLA, suggesting that α7-nAChRs are necessary. Images are representative examples from 3 animals for each condition. Scale bars: 10 μm.

demonstrating the involvement of α7-nAChRs and their dependence on glutamatergic transmission.

Since increased CamKII activity can recruit AMPA receptors to the surface (Wang et al., 2005) we tested whether nicotinic stimulation has this effect. P4 mouse hippocampal slices were infected with a Sindbis viral construct encoding the GluR1 subunit coupled to GFP on the N-terminus (Shi et al., 1999). In control cultures the GluR1-GFP could be readily visualized extending throughout the dendritic space; immunostaining for GFP on the external surface indicated that only a small portion of the receptors became inserted into the plasma membrane (Fig. 7A). Treating with 1 μM nicotine for 1 hour, however, induced a doubling of GluR1-GFP staining detectable on the surface, and the increase was blocked by MLA, implicating α7-nAChRs (Fig. 7B-D). Similar experiments substituting a Sindbis viral construct encoding GluR2-GPF (Shi et al., 1999) yielded a similar nicotine-induced increase in surface receptors, again blocked by MLA (data not shown). The results indicate that activation of α7-nAChRs recruits both GluR1- and GluR2-containing AMPA receptors to the surface, potentially making them available for synaptic transmission.

To determine whether nicotine can exert similar effects in vivo, we used stereotaxic intracranial injections to deliver small amounts of 1 μM nicotine directly into the hippocampus of P4-P6 mouse pups expressing GFP under the Thy-1 promoter. An hour after injection the animals were perfusion-fixed, and

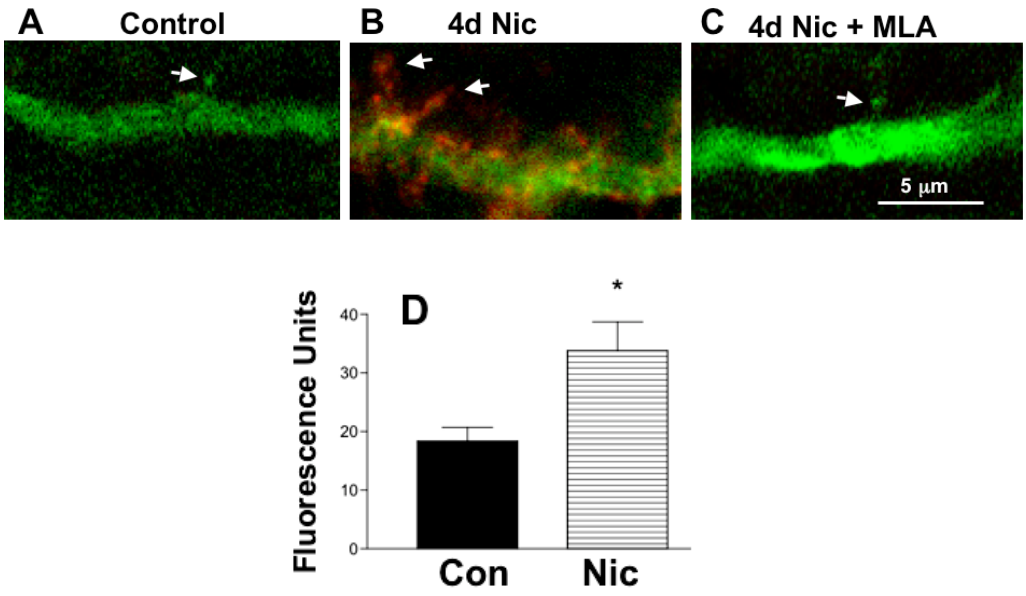

Fig. 4.7. Nicotine recruits GluR1 receptors to the spine surface. P3 mouse hippocampal slices were put in culture with **(A)** no additives (Control), **(B)** 1 μM nicotine (4d Nic), or **(C)** 1 μM nicotine + 50 nM MLA (4d Nic + MLA). On day 3 the cultures were infected with Sindbis-GluR1-GFP, and on day 4 the cultures were rinsed and live-immunostained for GFP (non-permeabilizing conditions) to detect GluR1-GFP on the cell surface (the GFP moiety is on the N-terminal, hence extracellular). Total GluR1-GFP (green); surface GluR1-GFP (red). Arrows indicate spine examples. Nicotine treatment induced appearance of GluR1 on the surface; MLA prevented the effect, implicating α7-nAChRs. **(D)** Quantification shows a doubling (arbitrary fluorescence units). *$p < 0.05$; $n = 3$ animals each condition. Scale bar: 5 μm.

hippocampal slices were analyzed for spine incidence. The nicotine treatment caused a significant increase in the number of dendritic spines visible on GFP-expressing CA1 pyramidal neurons (Fig. 8 A). To determine whether endogenous nicotinic cholinergic activity plays a role in glutamatergic synapse formation in vivo, we compared WT and animals lacking both the β2- and α7-nAChR genes (α7/β2 dKOs). Hippocampal slices were prepared from perfusion-fixed P5 mice and labeled by DiI for imaging. Spines were quantified along apical dendrites of CA1 pyramidal neurons. The α7/β2 dKOs had significantly fewer dendritic spines than did WTs (Fig. 8 B,C). The results indicate that endogenous nicotinic activity helps promote early glutamatergic synapse formation in embryogenesis and early postnatal life.

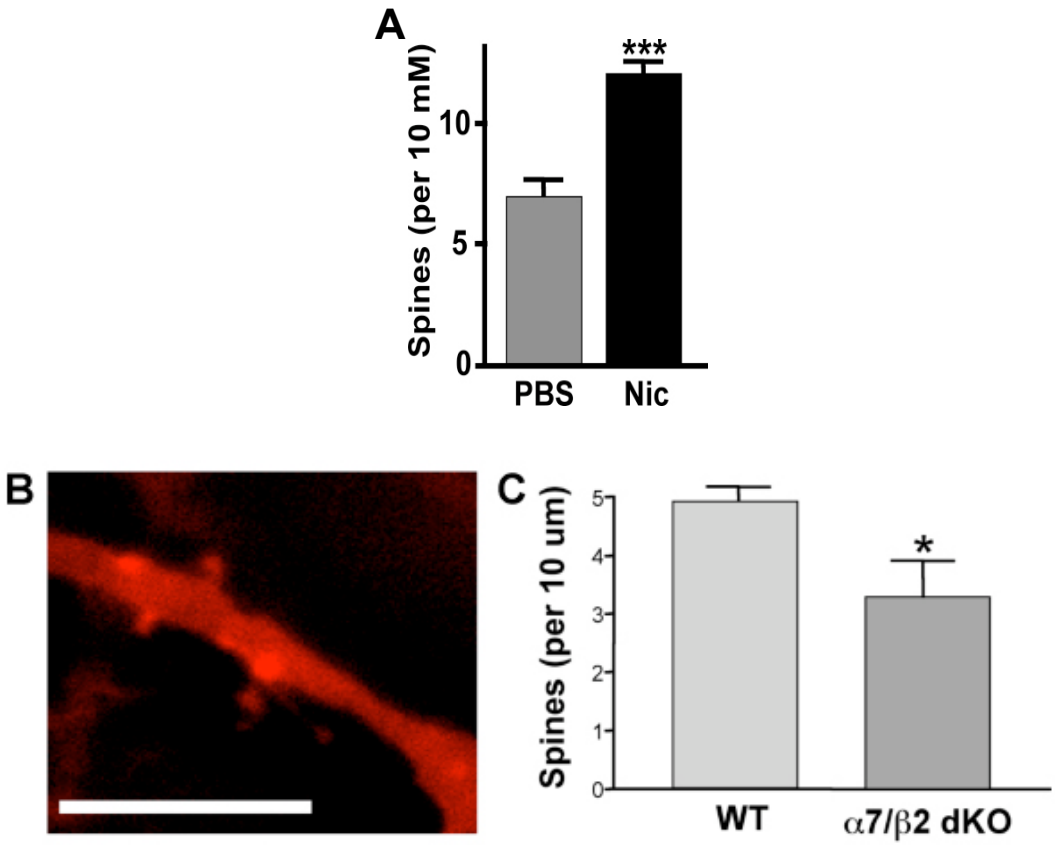

Fig. 4.8. Nicotinic activity in vivo regulates the number of dendritic spines formed in the early postnatal hippocampus. **(A)** P4 Thy-1-GFP mouse pups received a stereotaxic intracranial injection of phosphate-buffered saline (PBS) or 1 μM nicotine (Nic) directly into the hippocampus under anesthesia, were allowed to survive one hour, and then were perfusion-fixed and imaged to reveal spines along primary and secondary apical dendrites of CA1 pyramidal neurons. Quantification showed that nicotine increased the number of spines (***$p < 0.0001$; 4 cells/animal; 3 PBS- and 4 Nic-treated animals). **(B)** P5 WT and α7/β2 dKO mice were anesthetized and perfusion-fixed; their hippocampi were dissected, sliced, incubated 5 days with DiI, and imaged to visualize spines. **(C)** Quantification of spines as in panel B. The β2/α7 dKO neurons have significantly fewer spines. Scale bars: 10 μm. (*$p < 0.01$, two tailed t-test, n = 5 animals per condition)

CONCLUSIONS

It is widely recognized that a period of GABAergic excitation due to an immature chloride gradient is essential for proper innervation of the developing neuron (Ben-Ari, 2002; Gamba, 2005; Represa and Ben-Ari, 2005). Recently it was shown that spontaneous nicotinic cholinergic activity determines when the chloride gradient matures in vivo and terminates the excitatory phase of GABAergic transmission (Liu et al., 2006). The present results showing that nicotinic activity promotes glutamatergic synapse formation during development suggests a mechanism. Reaching a critical level of glutamatergic innervation may be a trigger for altering the chloride gradient and terminating GABAergic excitation.

Nicotinic cholinergic control of glutamatergic synapse formation during development is likely to serve two purposes. First, it will enable the spontaneous waves of nicotinic excitation occurring in the nervous system (Bansal et al., 2000; Myers et al., 2005; Le Magueresse et al., 2006) to coordinate and accelerate innervation across large populations. Neurons unable to participate may be at a disadvantage subsequently when they are less plastic, causing them to be less integrated into major circuits. Second, nicotinic cholinergic control may help position non-nicotinic synapses in the immediate vicinity of postsynaptic nAChRs. Pyramidal neurons express $\alpha 4\beta 2$-nAChRs (Nashmi et al., 2007) and distribute them in clusters along dendritic branches in culture (W. Zago, A. Halff, & D Berg, unpublished observations). If the calcium-dependent events underlying spine induction by $\beta 2$-

containing nAChRs act locally, the synapses formed would be in close proximity to nAChR clusters. This arrangement could provide a platform for nicotinic cholinergic regulation of higher order function subsequently in the nervous system (Picciotto et al., 1995; Bannon et al., 1998; Marubio et al., 1999; Cui et al., 2003; Levin et al., 2006; Bitner et al., 2007). Exogenous nicotine can abrogate the process, driving excessive spine formation early on; imbalance could result with long-term consequences of nicotinic cholinergic control of system function in the adult.

METHODS

Animals: All animal procedures performed were in accordance with the institutional guidelines and approved by the UCSD Institutional Animal Care and Use Committee. C57/BL6 mice of both sexes were used from p3- p24. α7KO mice were initially obtained from Jackson Laboratories (Bar Harbor, ME). β2KO were a kind gift from Dr. Marla B Feller (University of California, San Diego). Thy1M mice were a kind gift from Dr. Anirvan Ghosh (University of California, San Diego). WT mice were purchased from Harlan Sprague-Dawley.

Cell Cultures: Dissociated hippocampal cultures were prepared as described (Kawai et al., 2002). Organotypic slice cultures were prepared by rapidly removing brains into ice-cold ACSF saturated with 95% O2 and 5% CO2, containing in mM, NaCl 119, KCl 2.5, CaCl2 2.5, MgSO4 1.3, NaH2PO4 1.0, NaHCO3 26.2 and glucose 11. Hippocampi were excised from brain slices, plated onto membrane inserts

(Millipore) coated with poly-D-lysine, and incubated at 37C in a humidified chamber with 5% CO2.

Virus: The Sindbis viral construct encoding GFP was obtained from Dr. Gentry Patrick. (University of California, San Diego) Lentiviral constructs expressing GFP, GluR1, or GluR2 were obtained from Dr. Inder Verma (Salk Institute, La Jolla, CA). Viral constructs were added to cell culture media or injected into organotypic cultures with a nanoinjector (Drummond). Cells were incubated for 14hr-9days to allow expression.

Immunofluorescent labeling: 21 DIV hippocampal cultures, cryostat sections, and organotypic cultures were stained as previously described (Massey et al., 2006). Primary antibodies used were mouse anti PSD-95 (1:500, Neuromab), rabbit anti Glur1-phosphoSer845 (1:1000, Upstate), rabbit anti Synapsin (1:100, Chemicon), and CamKII (1:1000, Promega). Fluorescent secondaries (1:500) were acquired from Jackson Immunoresearch.

Fixed tissue preparation: Animals were anesthetized with ketamine/xylazine and transcardially perfused with ice-cold 4% PFA then postfixed overnight. Brains were sliced on a vibratome at 300 um and stored in PBS until use.

Nicotine Treatment of Fresh Slices: 300 um brain slices were allowed to recover in ACSF at room temperature for one hour. Slices were treated with drugs as indicated for 34 minutes, then washed, fixed in 4% PFA. Fresh tissue slices were fixed in 4% PFA, cryoprotected overnight, then sectioned on a cryostat at 10 μm.

DiI: Glass pipettes were dipped into 20% DiI (1,1'-dilinoleyl-3,3,3',3'-tetramethylindocarbocyanine, 4-chlorobenzenesulfonate) in DMSO and dried overnight at 15C. Pipettes were lowered into fixed hippocampal slices for 2 minutes. Slices were incubated in PBS at 4C for 5 days, then mounted and imaged.

Stereotaxic injection: Mice were anesthetized with ketamine/xylazine and placed into a stereotaxic apparatus fitted with an oxygen tube. The skull was revealed and a hole bored through the skull at, from Bregma, A/P: -3.0 mm, M/L +/- 1.5 mm. A syringe was lowered 1.3 mm into brain, relative to the surface of the cortex and 1 μl of 1 μm nicotine over two minutes was injected. 2.5% Fastgreen FCF was included in the needle to verify stereotaxic placement within the hippocampus. The needle was left in place for 2 minutes, and then removed. The mice were allowed to recover for one hour before tissue was processed.

Imaging and Analysis: Digital images of fluorescently labeled cells were collected using a CCD camera mounted on a Zeiss Axiovert (63× oil immersion objective, 1.4 numerical aperture lens) and equipped with SlideBook deconvolving software (Intelligent Imaging Innovations, Santa Monica, CA). Three dimensional z-stacks of images were acquired for analysis. Spines were classified as clear dendritic protrusions and counted along 10 um sections of primary and secondary apical dendrites, avoiding areas within 50 um of the soma. Synapsin boutons were defined as 5 contiguous (0.5 um2) above background. A bouton had to be within 0.4 um of spine to be quantified as a positive contact.

Materials: Unless otherwise state, all materials were obtained from Sigma-Aldrich.

ACKNOWLEDGEMENTS

Grant support was provided by the National Institutes of Health Grants NS12601 and NS35469 and by the Tobacco-Related Disease Research Program 16RT-0167. We thank Xiao-Yun Wan for expert technical assistance and Jeffrey D. Schoelman for maintaining the breeding colony of mutant mice. Lentiviral constructs were a generous gift of Inder Verma (Salk Institute, La Jolla, CA). Sindbis constructs were a generous gift from Dr. Gentry Patrick (University of California, San Diego).

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ACKNOWLEDGEMENT

This chapter represents a portion of a manuscript in preparation for publication. Permission to reproduce the manuscript was obtained from the other authors, Adrian Lozada and Zhaoping Liu. The dissertation author was a secondary researcher and co-author of this manuscript. Adrian Lozada directed research and Zhaoping Liu performed dissociated cell culture work. The dissertation author generated data used in Figures 2 and 8, and assisted with writing and revising the manuscript.

CHAPTER 5

Conclusion

The formation and modulation of synapses occurs rapidly during neonatal development and is necessary for a functioning nervous system (Waites et al., 2005). Cholinergic signaling has been broadly implicated in development (Bansal et al., 2000; Le Magueresse et al., 2006; Liu et al., 2006; Myers et al., 2005); however, little is known about how nicotinic acetylcholine receptors (nAChRs) contribute to the effects. This dissertation has focused on how the location, modulation, and activation of nAChRs play a role in hippocampal development. In culture, α7-nAChRs are modulated by BDNF and needed for stable cholinergic synapses. Additionally, α7- and β2*-nAChRs act together to promote glutamatergic synapses both in culture and in vivo. These results show that cholinergic activity has broad reaching effects throughout development and suggests that early postnatal development may be particularly susceptible to nicotinic influences.

In Chapter 2, brain-derived neurotrophic factor (BDNF) was shown to upregulate α7-nAChRs on subpopulations of interneurons. Though the majority of cultured hippocampal neurons express TrkB receptors and are capable of responding to BDNF, only interneurons that innervate pyramidal cells respond by increasing levels of α7-nAChRs. Pyramidal cells and interneurons that innervate other interneurons show no change. Initial surface or internal levels of α7-nAChRs are not a clear indicator of whether a cell will respond to BDNF, suggesting that the ability to

make α7-nAChRs or to traffic them to the surface is not the source of differential responses to BDNF.

Glutamatergic cells release BDNF in an activity-dependent manner both from dendrites and from axonal terminals (Haubensak et al., 1998; Lu, 2003). The BDNF-induced increase in α7-nAChRs could, in principle, be occurring on a neuron either presynaptic or postsynaptic to the pyramidal neuron. BDNF can affect presynaptic terminals, acting in a retrograde fashion to influence gene expression in the presynaptic neuron (Watson et al., 1999). But the fact that increasing glutamatergic activity can increase α7-nAChR expression even when BDNF activity is blocked, likely excludes a mechanism in which BDNF acts presynaptically to induce α7-nAChR expression. Postsynaptic mechanisms that could shape the response of specific types of interneurons to BDNF include expression of distinct subtypes of glutamatergic receptors or activation of different downstream signaling cascades, either of which could lead to a differential response to BDNF.

Activation of α7-nAChRs will depolarize the neurons, and cells with more nAChRs receptors will be more excitable in the presence of acetylcholine or nicotine. As the increase in α7-nAChRs is confined to interneurons, this would increase the level of GABAergic signaling. If such mechanisms exist in vivo, the effects of α7-nAChRs will depend on the age of the animal. In the early postnatal hippocampus, neurons have an immature chloride gradient and are excited by GABAergic signaling (Cherubini et al., 1991). Correspondingly, increasing α7-nAChRs levels and the excitability of interneurons will drive excitation in the hippocampus. Interneuron

activity may facilitate synapse formation and maturation of GABAergic inhibition. In later development when GABAergic signaling is inhibitory, upregulation of α7-nAChRs could play a different role. As activity increases, more BDNF will be released, which in turn will upregulate α7-nAChRs only on interneurons that inhibit pyramidal neurons. By increasing inhibition onto pyramidal neurons, excitatory activity in the hippocampus will be decreased. By acting to increase inhibition during periods of high excitation, BDNF modulation of α7-nAChRs can act as a slow regulator of hippocampal activity.

In Chapter 3, we show that $GABA_A$α1 receptors (GABARs) are coexpressed with α7-nAChRs at the tips of dendritic filopodia. The filopodia receive coincident GABAergic and cholinergic innervation. Activity through α7-nAChRs is necessary to stabilize both dendritic filopodia and cholinergic synapses onto the filopodia. By upregulating α7-nAChRs on specific populations of interneurons, BDNF may regulate the pattern of cholinergic innervation within the hippocampus.

If α7-nAChRs are necessary for cholinergic synapse formation in the hippocampus, cholinergic synapses should be lost in mice lacking α7-nAChRs (α7KO). To test the in vivo effects of α7-nAChRs, cholinergic synapses onto VIP+ interneurons were compared between P12 hippocampi from wildtype mice (WT) and mice lacking α7-nAChRs (α7KOs). No change was seen (data not shown); however, synapses were measured by immunofluorescence and only contacts onto the cell body could be counted. Electrophysiology would provide a more sensitive measure of

cholinergic contacts throughout the neuron, though isolating subpopulations of interneurons could be difficult.

Calcium can either increase or decrease filopodial motility, depending on the state of the cell (Lohmann et al., 2005). The α7-nAChR has a high relative Ca^{++} permeability, and should be able to influence filopodia motility, and through that, synapse stability. Though most α7-nAChRs are found post-synaptically in early postnatal development, presynaptic receptors almost certainly exist, albeit at lower levels (Fabian-Fine et al., 2001; Gray et al., 1996; McGehee et al., 1995). Either or both receptor pools may be necessary for stabilizing filopodia and cholinergic synapses. Using organotypic cultures from α7KO mice provides a direct way to manipulate pre- and postsynaptic receptors independently. Using α7KO hippocampi will eliminate postsynaptic α7-nAChRs while using α7KO septum will eliminate presynaptic α7-nAChRs. Together, these experiments would indicate whether filopodial and synaptic stability is driven through septal activity or hippocampal activity.

Though GABARs and α7-nAChRs are in close proximity at the tips of dendritic filopodia, α7-nAChRs and GABARs do not colocalize along the dendritic shaft. The absence of uniform colocalization in a single neuron suggests that interactions between GABARs and α7-nAChRs are indirect and that colocalization arises from both receptors being targeted to dendritic filopodia. The α7-nAChRs are frequently found at or near GABAergic synapses along dendritic shafts (Kawai et al., 2002). The proximity between α7-nAChRs, GABARs, and presynaptic terminals may

be maintained when filopodia retract to form shaft synapses (Cohen-Cory, 2002; Fiala et al., 1998), placing α7-nAChRs at GABAergic synapses. The apposition of cholinergic and GABAergic signaling systems suggests that crosstalk may exist between the neurotransmitter systems.

Nicotinic signaling through α7-nAChRs can rapidly influence GABAergic signaling by reducing GABA mediated postsynaptic currents (Zhang and Berg, 2007). Might colocalization between α7-nAChRs and GABAergic synapses form the basis for crosstalk? One way to explore the need for crosstalk between GABAergic and cholinergic systems is to spatially separate the inputs. GABA and nicotine could be applied to neurons through a picospritzer to allow local stimulation of both neurotransmitter systems. If nicotinic stimulation must occur near GABAergic stimulation to decrease GABA currents, this would suggest that crosstalk is necessary for nicotinic effects on GABAergic signaling.

In addition to the effects on GABAergic signaling, in Chapter 4, we show that nicotinic signaling rapidly induces glutamatergic spines, which likely become innervated in the following days. Postsynaptic activation of β2*-nAChRs rapidly induces spines while activation of α7-nAChRs increases presynaptic contacts and induces postsynaptic AMPA receptor expression.

Endogenous cholinergic signaling also supports spine growth; mice lacking both α7- and β2*-nAChRs (dKOs) exhibit fewer spines than WT mice at early developmental points. In organotypic slice cultures, β2*-nAChRs are needed to drive

spine formation; α7-nAChRs are not needed. In vivo, no effect is seen until both α7- and β2*-nAChRs are lost.

All mice used here were constitutive knockouts; they lacked nicotinic signaling from conception till death. Compensation may have occurred in dKO animals and account for the in vivo results. Alternatively, additional mechanisms and brain systems found in the intact animal may be sufficient to mask the loss of a single type of nAChR. Such additional mechanisms cannot, however, overcome the synergistic deficits seen when both nAChRs are lost. The α7KOs showed a delayed GABAergic maturation. Determining what developmental effects the β2KO and dKO mice exhibit would be a first step in understanding the long-term consequences of removing both receptor classes. Short-term effects of nicotinic signaling can be studied separately from developmental effects by using pharmacology in WT animals. For example, DHβE specifically blocks β2*-nAChRs and would be expected to decrease spines on hippocampal neurons in WT animals. Additionally, RNAi could be used to manipulate nAChR levels.

Early in development, glutamatergic synapses can be formed onto both the dendritic shaft and spines of pyramidal neurons (Boyer et al., 1998; Fiala et al., 1998). GABAergic synapses are preferentially formed along dendritic shafts or neuronal cell bodies (Harris and Landis, 1986). As nicotine increases presynaptic contacts onto both spines and dendritic shafts, GABAergic synapses might be induced as well. It should be straightforward to test this hypothesis by staining for presynaptic GABAergic terminals with GAD and for either endogenous or transfected GABARs.

Two types of specificity are likely to regulate the effects of nicotine on spines. First, expression of nAChRs may help define areas that can respond to nicotine. The β2*-nAChR is found colocalized with NMDARs on dendrites while the α7-nAChRs is found postsynaptically at GABAergic synapses. Either or both of the nAChRs might dictate the location of synapses that can be induced by nicotine treatment. The induction of glutamatergic synapses was studied on spiny pyramidal neurons. Can nicotine also lead to more synapses being formed onto GABAergic neurons? Synapses onto interneurons can be readily explored with the analysis techniques used on pyramidal neurons.

During early development, α7- and β2*-nAChRs help to support endogenous spine growth. However, dKO mice do recover from early deficits in spine formation and are indistinguishable from wildtype mice by P14. What effect does the developmental specificity have on hippocampal development?

Septal innervation provides endogenous cholinergic signaling (Kass and Cohen, 2008) and could induce stable glutamatergic synapses. Glutamatergic synapses will form at or near cholinergic synapses and thus be susceptible to cholinergic control. This correlates with the pattern of α4/β2-nAChR expression seen in dissociated cell culture, where the receptors are found colocalized with NMDA receptors (W Zago, A Halff, and D Berg, unpublished observations). Though glutamatergic synapses will certainly form in the absence of nicotinic receptors, the synapses are likely to be independent of nicotinic septal influences, which are known

to modulate learning, memory, and attention within the hippocampus (Levin et al., 2006).

Nicotine can only induce spines in young animals; the plasticity is lost by in older animals. The developmentally specific response to nicotine raises the possibility of kind of "critical period" within the hippocampus during which nicotinic signaling can induce synapse formation. Previously, the effects of critical periods have been largely confined to sensory systems and are perhaps best studied in the visual system.

In young animals, the visual system is extremely sensitive to sensory experience. Occluding one eye causes drastic changes in the ability of the visual cortex to respond to input from that eye. Ocular dominance (OD) plasticity is limited to a critical period in early development (Wiesel and Hubel, 1963). In the rodent, the visual cortex is first responsive to alterations in visual experience approximately five days after eye opening, around p15-p19 (Fagiolini et al., 1994; Gordon and Stryker, 1996). Changes in experience can alter the timing of plasticity. For example, animals reared in the dark have a delayed onset of OD plasticity (Mower et al., 1983). The opening of the critical period is dependent on GABAergic inhibition. In animals that lack GAD65 and have reduced levels of GABAergic inhibition, the visual cortex is unresponsive to monocular deprivation. Plasticity can be restored by treating the mouse with diazepam, which acts postsynaptically to increase GABA receptor conductance in a use-dependent manner (Hensch et al., 1998). A short period of increased GABAergic inhibition is enough to open the critical period at any point in life, both before WT onset of OD plasticity and well into adulthood. Once the window

is opened, subsequent diazepam treatments cannot induce further plasticity (Iwai et al., 2003). Further genetic manipulations reveal that only a subset of GABAergic receptors are needed to induce OD plasticity. $GABA_A\alpha1$ receptors are sufficient to induce OD plasticity (Fagiolini et al., 2004). $GABA_A\alpha1$ receptors are enriched at synapses from PV+ neurons (Klausberger et al., 2002), suggesting that PV+ interneurons may drive OD plasticity.

One major player in the rate of GABAergic maturation is BDNF. Mice that express low levels of BDNF have persistent deficits in GABAergic transmission (Abidin et al., 2008). In mice that express BDNF prematurely, GABAergic inhibition matures more rapidly and a precocious period of OD plasticity is observed (Huang et al., 1999). Furthermore, a misbalance in retinal BDNF is seen with ocular deprivation. During the critical period, re-expressing BDNF in the closed eye prevents OD plasticity (Gianfranceschi et al., 2003). This suggests that BDNF has a driving role in visual system development.

In the hippocampus, BDNF upregulates α7-nAChRs on PV+ interneurons. The α7-nAChRs also colocalize with $GABA_A\alpha1$ receptors. Heightened expression of α7-nAChRs will increase excitability within the GABAergic pathway, likely increasing the rate of maturation. Within the visual system, a threshold level of inhibitory activity through this exact class of interneuron is likely needed to open the period of OD plasticity (Fagiolini et al., 2004). Increased α7-nAChR could thus facilitate threshold levels of inhibition and the onset of OD plasticity. Indeed, α7KO mice show a delayed onset of GABAergic inhibition. Activation of nAChRs could also act to promote

BDNF release. Nicotinic stimulation to the hippocampus promotes theta wave activity (Cobb et al., 1999), a stimulus pattern which drives maximal release of BDNF (Balkowiec and Katz, 2000; Gartner and Staiger, 2002). BDNF could then act to promote GABAergic maturation.

A major new hypothesis arises from the data presented here. Nicotinic receptors are found throughout the sensory cortex. If cortical α7-nAChRs are found in the same locations and modulated in the same way as they are in the hippocampus, the inescapable idea arises that activation of nAChRs plays a significant role to trigger critical period plasticity. The involvement nAChRs in triggering critical period plasticity may be relatively simple to approach by exploring OD plasticity in mice lacking one or more nAChRs. More specific manipulation of nAChR levels could be obtained by driving RNAi expression in a subset of interneurons.

Though critical period plasticity is traditionally studied in the sensory cortex, the work suggests that the hippocampus undergoes a critical period in early development similar to the sensory cortex. Nicotine could be used to test this theory by first defining when the critical period occurs and if it is susceptible to the same manipulations that alter OD plasticity within the visual system. From there, it would be interesting to determine if plasticity can be driven by sensory experience. Might alterations in early visual activity or whisker input influence hippocampal plasticity or alter septal cholinergic input to the hippocampus. If true, the visual system could then be used as a guide to explore the molecular mechanisms behind critical period plasticity within the hippocampus.

In the hippocampus, the critical period would, in part, define the time in which nicotinic signaling can drive spine and synapse formation, inducing long-lasting changes in neuronal connectivity and coupling glutamatergic synapses to cholinergic terminals. Environmental nicotine may easily co-opt glutamatergic synapse formation, dissociating glutamatergic synapses from cholinergic terminals and possibly setting the stage for later addiction. By determining what factors contribute to developmental specificity, we may be able to reintroduce plasticity into the adult system. Adult plasticity could reverse inappropriate connections, allow addicts to "forget" their drugs of abuse. It might even allow nicotine to induce new synapses to replace those lost during neurodegeneration.

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