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Nicotinic excitatory postsynaptic potentials in hippocampal CA1 interneurons are predominantly mediated by nicotinic receptors that contain $\alpha 4$ and $\beta 2$ subunits

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Abstract

In the hippocampus, activation of nicotinic receptors that include $\alpha 4$ and $\beta 2$ subunits ($\alpha 4\beta 2^*$) facilitates memory formation. $\alpha 4\beta 2^*$ receptors may also play a role in nicotine withdrawal, and their loss may contribute to cognitive decline in aging and Alzheimer's disease (AD). However, little is known about their cellular function in the hippocampus. Therefore, using optogenetics, whole cell patch clamping and voltage-sensitive dye (VSD) imaging, we measured nicotinic excitatory postsynaptic potentials (EPSPs) in hippocampal CA1. In a subpopulation of inhibitory interneurons, release of ACh resulted in slow depolarizations (rise time constant 33.2 ± 6.5 ms, decay time constant 138.6 ± 27.2 ms) mediated by the activation of $\alpha 4\beta 2^*$ nicotinic receptors. These interneurons had somata and dendrites located in the stratum oriens (SO) and stratum lacunosum-moleculare (SLM). Furthermore, $\alpha 4\beta 2^*$ nicotinic EPSPs were largest in the SLM. Thus, our data suggest that nicotinic EPSPs in hippocampal CA1 interneurons are predominantly mediated by $\alpha 4\beta 2^*$ nicotinic receptors and their activation may preferentially affect extrahippocampal inputs in SLM of hippocampal CA1.

Keywords

Inhibitory interneuron; nicotinic excitatory postsynaptic potential; hippocampus; voltage-sensitive dye imaging; optogenetics

1. Introduction

Hippocampal cholinergic input from the medial septum/diagonal band of Broca complex (MS/DBB) is crucial for normal hippocampal network function. The cholinergic MS/DBB input influences hippocampal function by activating muscarinic and nicotinic receptors. Indeed, the systemic blockade of muscarinic cholinergic receptors impairs memory formation (Atri et al., 2004; Hasselmo and Giocomo, 2006) and direct injection of

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muscarinic receptor antagonists into the hippocampus compromises encoding of spatial information in rodents (Blokland et al., 1992). Furthermore, nicotinic cholinergic receptor antagonists impair hippocampal-dependent memory tasks in rodents (Levin et al., 2002; Nott and Levin, 2006). Therefore, understanding precisely how acetylcholine (ACh) release affects the cells and synapses in the hippocampus will provide important information regarding how the MS/DBB contributes to hippocampal network function.

MS/DBB cholinergic inputs in part influence hippocampal function through the activation of nicotinic receptors. There are 12 known nicotinic receptor subunits expressed in the central nervous system, 9 of which have been found in the hippocampus (Sudweeks and Yakel, 2000). Functionally, nicotinic receptors that include the $\alpha 7$ subunit ($\alpha 7^*$) (Alkondon et al., 1997; Alkondon et al., 1999; Frazier et al., 1998b; Jones and Yakel, 1997; McQuiston and Madison, 1999), $\alpha 4$ and $\beta 2$ subunits ($\alpha 4\beta 2^*$) (Alkondon et al., 1999; McQuiston and Madison, 1999; Sudweeks and Yakel, 2000), and possibly $\alpha 2$ subunits (Jia et al., 2009; McQuiston and Madison, 1999; Sudweeks and Yakel, 2000) have been identified on inhibitory interneurons. In contrast, there is little evidence for the presence of functional nicotinic receptors on the soma or dendrites of pyramidal neurons; however, functional $\alpha 7^*$ nicotinic receptors have been demonstrated on glutamatergic presynaptic terminals in the hippocampus (Gray et al., 1996). Indeed, synaptically released ACh in the hippocampus has been shown to produce $\alpha 7^*$ nicotinic receptor mediated excitatory postsynaptic responses in hippocampal interneurons (Alkondon et al., 1998; Chang and Fischbach, 2006; Frazier et al., 1998a; Stone, 2007). However, despite the evidence for the presence of functional $\alpha 4\beta 2^*$ nicotinic receptors on hippocampal interneurons, to our knowledge, there has been no demonstration that these receptors are activated by synaptically released ACh.

$\alpha 4\beta 2^*$ nicotinic receptors are pentameric receptors that consist of at least one $\alpha 4$ and one $\beta 2$ subunit. The remaining three subunits of the nicotinic receptor may include additional $\alpha 4$ and $\beta 2$ subunits or other types of nicotinic receptor subunits. In the hippocampus, $\alpha 4\beta 2^*$ receptors are primarily localized to astrocytes and inhibitory interneurons (Gahring et al., 2004a; Gahring et al., 2004b; Gahring and Rogers, 2008). In the hippocampus, the $\beta 2$ nicotinic subunit may be involved in withdrawal from chronic nicotine use (Davis and Gould, 2009). Indeed, high affinity nicotinic receptors in the hippocampus are upregulated in smokers (Perry et al., 1999), and the upregulated receptors are likely to be of the $\alpha 4\beta 2^*$ subtype (Nguyen et al., 2004). In addition to effects on smoking and smoking cessation, $\alpha 4\beta 2^*$ nicotinic receptors in the hippocampus may be involved in cognitive decline and memory loss associated with aging and AD. This is exemplified by a significant loss of $\alpha 4$ subunit expression in aging mice (Gahring et al., 2005a; Gahring et al., 2005b; Rogers et al., 1998) and as much as an 80% decrease in $\alpha 4$ subunit expression in AD patients (Kellar et al., 1987; Marutle et al., 1998; Perry et al., 1999; Perry et al., 2000). Indeed, $\alpha 4\beta 2^*$ receptors may be particularly sensitive to inhibition by the beta amyloid protein associated with the etiology of AD (Wu et al., 2004). Finally, mutations in the $\alpha 4$ and $\beta 2$ subunits have been correlated with autosomal dominant nocturnal frontal lobe epilepsy (De et al., 2000; Hoda et al., 2008; Phillips et al., 1995; Phillips et al., 2001; Steinlein et al., 1997; Steinlein et al., 2000). Therefore, determining which hippocampal interneuron subtypes are activated by the synaptic release of ACh onto $\alpha 4\beta 2^*$ receptors in the hippocampus will provide novel important information regarding how the hippocampus is affected by $\alpha 4\beta 2^*$ nicotinic receptors and how their dysfunction leads to neuropathological problems associated with smoking cessation and cognitive deficits associated with aging and AD.

Therefore, using optogenetics, whole cell patch clamping and VSD imaging, we found that inhibitory interneurons in hippocampal CA1 produced nicotinic EPSPs in response to the release of ACh from MS/DBB cholinergic terminals. Using pharmacological methods, we demonstrated that the nicotinic EPSPs were predominantly mediated by the activation of

$\alpha 4\beta 2^*$ nicotinic receptors. Finally, we used morphological reconstructions and VSD imaging to identify the anatomical location of interneurons that produced nicotinic EPSPs.

2. Methods

2.1 Animal use

A total of 51 animals were used in these studies that were housed in an animal care facility approved by the American Association for the Accreditation of Laboratory Animal Care (AAALAC). Animal experimental procedures followed a protocol approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University (protocol AD20205). This protocol adhered to the ethical guidelines described in the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978). All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.2 Generation of retroviral adeno-associated virus (rAAV) expressing oChIEF in a Cre-dependent manner

We obtained the mammalian codon optimized clone of ChIEF (oChIEF) fused to tdTomato (Lin et al., 2009; Lin, 2010) (pCAGGS-I-oChIEF-tdTomato-I-WPRE) (gift of R. Tsien) and ligated it into rAAV-FLEX-rev-ChR2-tdTomato (Addgene, donated by Scott Sternson, (Atasoy et al., 2008)) at *KpnI* and *XbaI* sites replacing the ChR2-tdTomato sequence. rAAVs (serotype 2/1) were produced by Vector Biolabs (Philadelphia) (1.05×10^{13} VG/ml). We refer to this virus as rAAV-Flex-rev-oChIEF-tdTomato. Because the sequence coding for oChIEF-tdTomato was reversed and floxed by two incompatible LoxP sites, oChIEF-tdTomato's expression only occurred in infected cells that also expressed Cre recombinase (Atasoy et al., 2008).

2.3 Stereotaxic injection of rAAV-Flex-rev-oChIEF-tdTomato into the MS/DBB of Chat-Cre mice

All Chat-cre mice (JAX Stock No. 006410) were initially anesthetized via an intraperitoneal injection of ketamine (100 mg/kg IP) and xylazine (2.5 mg/kg IP). Anesthesia was maintained with O₂ supplemented with 1.0% isoflurane. An incision was made in the skin along the midsagittal suture, and a very small hole was drilled in the skull overlying the septum. A aluminum silicate glass pipette containing undiluted rAAV-Flex-rev-oChIEF-tdTomato was lowered to the level of the MS/DBB, and two midsagittal tracks of viral injections were performed, 1.0 mm and 0.7 mm rostral to Bregma at a rate of 100 nl/min using a motorized nanoinjector (Leica). At each coordinate, 8×100 nl injections were made between 3.5–5.0 mm in depth. Two weeks post viral injection, mice were killed for experimentation.

2.4 Preparation of hippocampal slices

Mice (38 to 49 days old) were used for experimentation. Animals deeply anaesthetized with ketamine (200 mg/kg) and xylazine (20 mg/kg), administered by intraperitoneal injection. Once the animals' heart rate and respiration approached zero and the animals no longer responded to toe pinch, the animals were transcardially perfused with ice cold saline (consisting of (in mM): Sucrose 230, KCl 2.5, CaCl₂ 2, MgCl₂ 6, NaHPO₄ 1, NaHCO₃ 25, glucose 25) and sacrificed by decapitation. The brain was removed, hemi-sectioned, and horizontal slices containing the mid temporal hippocampus were cut at 350 μ m on a vibratome 3000 (Ted Pella Inc, Redding CA). Sections were incubated in a holding chamber kept at 36°C for 30 min and then allowed to return to room temperature. The holding

chamber solution consisted of normal saline (in mM): NaCl 125, KCl 3.0, CaCl₂ 1.2, MgCl₂ 1.2, NaHPO₄ 1.2, NaHCO₃ 25, glucose 25 bubbled with 95% O₂ / 5% CO₂.

2.5 Light-evoked release of acetylcholine from MS/DBB cholinergic axon terminals

Neurons expressing oChIEF-tdTomato were stimulated by blue light transmitted through the epi-illumination light path of an Olympus BX51WI microscope and a 20x water immersion objective (0.95 NA). Blue light flashes (1 ms in duration) were generated from a flash lamp (JML-C2, Rapp Optoelectronic) by passing light through a D455/70x excitation filter focused into a liquid light guide. Blue light exiting the light guide was focused into the epillumination light path of the Olympus BX51WI microscope and back aperture of the 20x water immersion objective (0.95 NA) using a Flashcube 70 (Rapp Optoelectronics) and two dichroic mirrors (515dcxr, Chroma Technology).

2.6 Voltage-sensitive dye imaging

Slices were stained for 30 to 60 min with the voltage-sensitive dye (VSD) NK3630 (0.02 to 0.05 mg/ml) prior to experimentation. Following staining, slices were submerged and continuously perfused in a glass bottom recording chamber with warmed normal saline. The recording chamber was mounted on a fixed stage under an Olympus BX51WI microscope equipped with differential interference contrast (DIC) optics. The image of the slice was collected using transmitted near infrared light (> 775 nm) with a 20x (0.95 NA) water immersion objective. The image was captured (Foresight I-50 frame grabber) with a DAGE-MTI IR1000 CCD camera with contrast enhancement.

For voltage-sensitive dye absorbance measurements, slices were illuminated with a tungsten-halogen 100 W lamp passed through a bandpass filter (705 ± 30 nm, Chroma Technology, Rockingham, VT). The transmitted light was collected with a Wutech H-469IV photodiode array that is part of the Redshirtimaging integrated Neuroplex II imaging system, mounted on the front port of the Olympus BX51WI microscope. The data were acquired, displayed and analyzed with Neuroplex software.

To evoke synaptic ACh release in hippocampal CA1, 1 - 3 flashes of blue light were delivered at 20 ms intervals. Stimulating blue light was directed to the back aperture of the 20x objective by a 515dcxr dichroic mirror (Chroma Technology) for photostimulation. Transilluminated light responses were collected by the same objective and passed back through the dichroic mirror and a 675 nm long pass filter (Chroma Technology) onto surface of an hexagonal array of fiber optics that collect light for the Wutech photodiode array. Adjacent groups of photodiodes of the array, which were perpendicularly oriented to the SP, were selected to measure the VSD signals in SO, stratum pyramidale (SP), stratum radiatum (SR) and SLM of hippocampal CA1. The signal from one photodiode was spatially averaged with its immediate surrounding photodiodes (nine photodiodes in total) to represent the electrical response in each layer of CA1. Additionally, the VSD signals were an average of approximately 10 measurements. The VSD signals were sampled at 1.6 kHz and low pass filtered at 126 Hz. To eliminate or reduce the artifacts created by the stimulation light of the flash lamp, responses measured in the presence of TTX were subtracted from all measurements.

2.7 Electrophysiological Measurements

Whole cell patch clamp recordings on hippocampal CA1 interneurons were performed using patch pipettes (2 to 5 MΩ) pulled from borosilicate glass (8250 1.65/1.0 mm) on a Narishige PP830 pipette puller filled with (in mM): KMeSO₄ 135, NaCl 8, MgATP 2, NaGTP 0.3, HEPES 10, BAPTAK₄ 0.1. Membrane potentials were measured with a Model 2400 patch clamp amplifier (A-M Systems, Port Angeles, WA) and converted into a digital signal by a

PCI-6040E A/D board (National instruments, Austin, TX). WCP Strathclyde Software was used to store and analyze membrane potential responses on a PC computer (courtesy of Dr. J Dempster, Strathclyde University, Glasgow, Scotland). Further analysis was performed with Originpro 8.1 (OriginLab Corp., Northampton, MA, USA) and Instat 3.0 (Graphpad Software, La Jolla, CA, USA).

2.8 Morphological reconstruction of interneurons displaying nicotinic EPSPs

Slices were fixed in 4% paraformaldehyde in 0.1M phosphate buffer overnight, washed 6×10 minutes in phosphate buffered saline (PBS) and incubated for 3 days at 4 degrees in 1:1000 streptavidin Alexa Fluor 633 (Invitrogen) in PBS containing 0.4% Triton X 100 (Fisher). After incubation, the slices were washed 3×60 minutes in PBS and mounted in anti-fade mounting media. Processed slices were then reconstructed using a Zeiss LSM 510 META NLO confocal microscope (Carl Zeiss, Jena, Germany). Alex 633 was excited with the 633 nm line of the red HeNe 5W laser and cells were visualized using a 10 x dry lens (0.3 N.A., voxel dimensions $0.57 \times 0.57 \times 7.8 \mu\text{m}$).

2.9 Statistics and data analysis

Data were analyzed using WCP software for the electrophysiological measurements and Neuroplex II software for the VSD recordings. Statistics were performed using GraphPadInstat (GraphPad Software, La Jolla, CA). Statistical significances were determined using a one way ANOVA or a repeated measures ANOVA and Bonferroni post hoc tests. Differences were determined to be statistically significant for p values less than 0.05. All data was reported as the mean $\pm$ standard error of the mean (SEM).

2.10 Chemicals

All chemicals were purchased from VWR unless otherwise indicated. NK3630 was obtained from Hayashibara Co. (Japan). Dihydro- β -erythroidine (DH β E) and α -Conotoxin PnIA (α -CTX PnIA) were obtained from Tocris Bioscience (Ellisville, Missouri) and 6,7-Dinitroquinoxaline-2,3-dione (DNQX), DL-2-Amino-5-phosphonopentanoic acid (APV), methyllycaconitine (MLA) from Ascent Scientific (Bristol, U.K.).

3. Results

We studied cholinergic synaptic transmission in hippocampal CA1 by injecting a recombinant adenoassociated virus (rAAV, serotype 2/1) containing the genetic code for the excitatory optogenetic protein oChIEF-tdTomato (Lin et al., 2009) into the MS/DBB (Fig. 1). Because the sequence coding for oChIEF-tdTomato was reversed and floxed by two incompatible LoxP sites, oChIEF-tdTomato's expression only occurred in cells that also expressed Cre recombinase (Atasoy et al., 2008). Thus, injecting this rAAV (rAAV-Flex-rev-oChIEF-tdTomato) into mice that selectively express Cre under the control of the choline acetyltransferase promoter (Chat-cre mice, B6;129S6-*Chat^{tm1(cre)Low}/J*) permitted the selective expression of oChIEF-tdTomato in approximately 37% of Chat-immunopositive neurons in the MS/DBB (Fig. 1, estimated from multiple slices from 4 animals). Long range projecting oChIEF-tdTomato fibers were visible in mid-temporal hippocampal slices, which permitted the synaptic release of ACh by blue light flashes. Responses in individual hippocampal CA1 interneurons were measured using whole cell patch clamp methods, and CA1 network responses were measured with VSD imaging.

3.1 Synaptic release of ACh primarily activates $\alpha 4\beta 2^*$ nicotinic receptors

Previous studies have demonstrated that nicotinic receptors in the hippocampus are located on inhibitory interneurons (Gahring et al., 2004a; Seguela et al., 1993; Wada et al., 1989).

Activation of nicotinic receptors on hippocampal interneurons by the exogenous application of cholinergic agonists generated currents largely suppressed by $\alpha 7$ nicotinic receptor antagonists (Alkondon et al., 1997; Alkondon et al., 1999; Frazier et al., 1998b; Jones and Yakel, 1997; McQuiston and Madison, 1999). Indeed, synaptic $\alpha 7$ responses have been reported in hippocampal interneurons (Alkondon et al., 1998; Chang and Fischbach, 2006; Frazier et al., 1998a; Stone, 2007). Nevertheless, the presence of $\alpha 4\beta 2^*$ nicotinic receptors has also been reported to be functionally expressed in hippocampal interneurons (Alkondon et al., 1999; McQuiston and Madison, 1999; Sudweeks and Yakel, 2000).

Using optogenetics, we synaptically released ACh in mouse mid-temporal hippocampal slices and measured EPSPs in hippocampal CA1 interneurons using whole cell patch clamp methods. In a subset of hippocampal CA1 interneurons (28 of 81 interneurons that responded to light flashes – the other 53 interneurons produced muscarinic depolarizations, hyperpolarizations or biphasic responses), we observed slow EPSPs (approximately a few hundred milliseconds in duration) in response to light evoked release of ACh. Occasionally, the EPSPs were large enough to produce action potentials (AP) (Fig. 2A, 2 of 12 interneurons). However, they varied in size (0.7 to 7.0 mV) with mean amplitude of 2.9 ± 0.9 mV. The EPSPs were slow in kinetics with slow rise and decay times (Fig. 2B, average rise time constant 33.2 ± 6.5 ms, decay time constant 138.6 ± 27.2 ms). The EPSPs were not due to the activation of muscarinic receptors because they were either unaffected or potentiated by the muscarinic receptor antagonist atropine (Fig. 2C). The $\alpha 7$ receptor antagonist methyllycaconitine (MLA, 10-100 nM) did not suppress this response (Fig. 2C). However, all EPSPs were completely inhibited by the nicotinic $\alpha 4\beta 2^*$ receptor antagonist dihydro- β -erythroidine (DH β E, Fig. 2C). Because the probability of release from MS/DBB cholinergic terminals onto nicotinic receptors may be low or require that ACh diffuse a considerable distance to bind to their receptors (Umbriaco et al., 1995), we tested whether trains of light flashes (10×20 Hz) could release larger amounts of ACh and thereby activate other subtypes of nicotinic receptors. Trains of light flashes produced summing EPSPs that produced ramp like depolarizations in interneuron membrane potentials (Fig. 2D). These depolarizations were potentiated by atropine and the amount of potentiation increased with subsequent light flashes later in the train (Fig. 2F, repeated measures ANOVA $p < 0.0001$, post hoc test for linear trend $p < 0.0001$). Although, on average atropine potentiated nicotinic receptor responses (Fig. 2G), statistical significance was only achieved for ACh release by the tenth flash and not the first flash of a train (one-way ANOVA, $p < 0.001$ Bonferroni post hoc test). In contrast, the depolarizations produced by trains of light flashes were not affected by MLA (10 – 100 nM, Fig. 2D and G, $n = 9$) or by the selective $\alpha 3\beta 2$ antagonist α -CTX PnIA (10 μ M, Fig. 2E and G, $n = 8$); however, they were completely inhibited by DH β E (100 nM ($n = 6$), 1 μ M ($n = 9$), 10 μ M ($n = 4$), Figs. 2D, E, G, one-way ANOVA, $p < 0.001$, Bonferroni post hoc test, $n = 19$). Therefore, it appears that ACh released from MS/DBB cholinergic inputs in this Chat-cre line preferentially activate $\alpha 4\beta 2^*$ nicotinic receptors on a subset of hippocampal CA1 interneurons.

We next characterized the electrophysiological properties of interneurons displaying nicotinic EPSPs by injecting depolarizing and hyperpolarizing currents. We attempted to categorize them based on their firing patterns in response to suprathreshold depolarizing current injections and by the presence or absence of a depolarizing sag in the membrane potential in response to hyperpolarizing current injections. However, no pattern could be discerned from our data. Some interneurons produced accommodating APs to suprathreshold depolarizing currents (Fig. 3A, $n = 4$), where other interneurons showed little accommodation (Fig. 3B, $n = 2$), fired irregularly with stuttering patterns (Fig. 3C, $n = 2$), or showed a delay to the first AP (Fig. 3D $n = 1$). Interneurons could not be classified by their presence or absence of a depolarizing sag in their membrane potential during hyperpolarizations. Some produced no sag ($n = 3$), some produce fast sags ($n = 3$), and some

produced slow sags ($n = 3$). Thus, CA1 interneurons with nicotinic EPSPs had no specific electrophysiological phenotype.

3.2 Interneurons displaying nicotinic EPSPs have somata and dendrites preferentially located in stratum lacunosum-moleculare and stratum oriens

To identify interneuron subtypes that responded to the synaptic release of ACh via the activation of nicotinic receptors, we included biocytin in the intracellular solution for post hoc anatomical identification of the recorded cell. Streptavidin conjugated to a fluorescent probe permitted the reconstruction of recorded neurons by confocal microscopy. This allowed us to characterize interneurons based on their somatodendritic and axonal locations. From our recordings, we recovered 16 neurons with somatic and dendritic labeling, 12 of which had some axonal fluorescence. The somata of interneurons displaying nicotinic EPSPs were either located in the SO (Fig. 4A-C, $n = 9$), or in SLM or the border of SLM and SR (Fig. 4D-F, $n = 7$). Similarly, these interneurons had dendrites that preferentially arborized in SO (Fig. 4A-C, $n = 9$) or SLM (Fig. 4D, E, $n = 9$). However, some interneurons had dendrites also located in SR (Fig. 4F, $n = 4$). In contrast, axonal arborization showed no layer or interneuron subtype specific patterns (Klausberger, 2009; Klausberger and Somogyi, 2008; Somogyi and Klausberger, 2005). Axons could be observed in all layers of hippocampal CA1: SO (Fig. 4A, B, $n = 2$), SP (Fig. 4E, $n = 3$), SR (Fig. 4B, $n = 4$) and SLM (Fig. 4D, $n = 6$). Based on morphological classifications described by Klausberger and Somogyi (2008), 2 interneurons had morphology similar to cholecystokinin basket cells, 3 were consistent with O-LM interneurons, 2 had morphology consistent with back propagation, oriens-retrohippocampal or double projection interneurons, and 3 were similar to perforant-path associated or neurogliaform interneurons. Two interneurons did not have enough axonal fill to be identified. Therefore, based on our anatomical data, interneurons with somata and dendrites in SO and SLM appear to be the primary target of cholinergic inputs that activate $\alpha 4\beta 2^*$ nicotinic receptors. However, our data do not provide evidence for a specific discrete postsynaptic target of interneurons that display nicotinic EPSPs.

3.3 Synaptic activation of $\alpha 4\beta 2^*$ nicotinic receptors preferentially occurs on postsynaptic elements in stratum lacunosum-moleculare and stratum oriens

Our morphological data suggests that interneurons with somata and/or processes located in SO or SLM are preferentially activated by the synaptic release of ACh onto $\alpha 4\beta 2^*$ nicotinic receptors. To examine this further, we measured the impact of ACh release on hippocampal CA1 network activity using voltage-sensitive dye imaging. Hippocampal slices were stained with the absorption dye NK3630 and changes in light intensity in response to synaptic activation of nicotinic receptors were measured with a photodiode array.

Consistent with our morphological data, brief trains of light flashes (3×50 Hz, 1 ms duration) resulted in increased activity mostly confined to the SLM and SO, as demonstrated by the pseudo color plot (Fig. 5A). Representative averaged traces taken from each layer of CA1 showed that the synaptic release of ACh produced larger amplitude excitatory events in SLM and SO of CA1 (Fig. 5B, black traces). The excitatory responses in SLM were larger than the responses measured in any other layer of hippocampal CA1 (Fig. 5C, one way ANOVA $p < 0.001$, Bonferroni post hoc test $p < 0.001$, $n = 14$), and the excitatory events in SO were significantly larger than the events in SP and SR (Bonferroni post hoc test $p < 0.001$). These responses were not due to the activation of muscarinic receptors as they were not blocked by atropine (Fig. 5B, C, red). On average, there appeared to be a trend toward larger excitatory events in the presence of atropine (Fig. 5C, $n = 14$), but these differences did not reach statistical significance. Furthermore, the excitatory responses were not indirectly due to the release of glutamate (Gray et al., 1996), because they were not affected by ionotropic glutamate receptor antagonists (Fig. 5D and G, $n = 5$). The $\alpha 7$ nicotinic

receptor antagonist MLA (10 – 100 nM) had no effect on excitatory responses (Fig. 5E and G, n = 6). In contrast, the $\alpha 4\beta 2^*$ nicotinic receptor antagonist DH β E (1-10 μ M) completely blocked the excitatory synaptic response in all slices examined (Fig. 5 E-G, one way ANOVAs, Bonferroni post hoc test, $p < 0.001$, n = 13). Although DH β E also has efficacy at $\alpha 3\beta 2$ nicotinic receptors, the $\alpha 3\beta 2$ antagonist α -CTX PnIA (10 μ M) had no effect on the excitatory synaptic events (Fig 5F and G, n = 5). Therefore, our pharmacological data suggests that ACh release from the MS/DBB predominately activates $\alpha 4\beta 2^*$ nicotinic receptors on processes and cell bodies in the SLM and SO of hippocampal CA1.

4. Discussion

Our data have shown that ACh release from MS/DBB terminals activated nicotinic receptors in a subset of inhibitory interneurons in hippocampal CA1. These nicotinic EPSPs had slow kinetics (hundreds of milliseconds in duration) and were predominantly mediated by $\alpha 4\beta 2^*$ receptor activation based on their complete inhibition by DH β E (100 nM – 10 μ M) and lack of effect of the $\alpha 7$ specific antagonist MLA (10-100 nM), the muscarinic receptor antagonist atropine (5 μ M) and the $\alpha 3\beta 2$ receptor antagonist α -CTX PnIA (10 μ M). The interneurons displaying nicotinic EPSPs had somata and dendrites primarily located in the SO and SLM of hippocampal CA1. Moreover, population nicotinic EPSPs in different hippocampal CA1 anatomical layers had varying amplitudes with the following rank order of magnitude: SLM>SO>SR=SP. In contrast, there was no specific laminar distribution for axonal processes of these inhibitory interneurons. To our knowledge, this is the first description of $\alpha 4\beta 2^*$ nicotinic EPSPs in the hippocampus.

The nicotinic EPSPs that we observed differed significantly from synaptic nicotinic responses reported by others in the central nervous system. Our nicotinic synaptic responses had durations in the order of a few hundred milliseconds (rise time constant ~33 ms, decay time constant ~139 ms), which is significantly slower than the $\alpha 7^*$ responses (rise time ~4 ms, decay time constant ~5 ms) reported in hippocampal interneurons (Frazier et al., 1998a) but much faster than nicotinic responses reported in the interpeduncular nucleus (lasting >15s with a decay time constant of ~5 s) (Ren et al., 2011). Furthermore, unlike cholinergic synapses in the interpeduncular nucleus, ionotropic glutamate receptor antagonists did not affect light-evoked nicotinic responses in the hippocampus suggesting that cholinergic inputs from the MS/DBB do not co-release glutamate. Moreover, our nicotinic responses could be elicited by a single 1 ms flash of light whereas 20 s trains of more than 400 light flashes were used to produce nicotinic responses in the interpeduncular nucleus (Ren et al., 2011). Therefore, based on kinetics, the presence and absence of co-transmitters, and the stimuli required to elicit nicotinic postsynaptic responses, the nicotinic EPSPs observed in our studies are significantly different than nicotinic responses reported previously.

Hippocampal interneurons have been reported to express a total of 9 nicotinic receptor subunits (Sudweeks and Yakel, 2000). Studies using the exogenous application of nicotinic receptor agonists to activate hippocampal interneurons have shown that $\alpha 7$ subunits contribute to the largest and most prevalent type of nicotinic response (Alkondon et al., 1997; Frazier et al., 1998b; Jones and Yakel, 1997; McQuiston and Madison, 1999; Sudweeks and Yakel, 2000). Indeed, $\alpha 7$ nicotinic EPSPs have been reported in hippocampal interneurons (Alkondon et al., 1998; Chang and Fischbach, 2006; Frazier et al., 1998a; Stone, 2007). However, there were also subpopulations of interneurons that displayed small $\alpha 4\beta 2^*$ subunit responses (Alkondon et al., 1997; McQuiston and Madison, 1999) and another subpopulation of interneurons that produced slower nicotinic responses postulated to result from the activation of $\alpha 2$ subunit containing nicotinic receptors (Jia et al., 2009; McQuiston and Madison, 1999; Sudweeks and Yakel, 2000). In our studies, we have demonstrated the presence of a small depolarization lasting hundreds of milliseconds that is

completely inhibited by DH β E at concentrations as low as 100 nM. At 100 nM, DH β E preferentially blocks $\alpha 4\beta 2^*$ nicotinic receptors with little effect on $\alpha 2$ containing receptors (Chavez-Noriega et al., 1997; Harvey et al., 1996; Khiroug et al., 2004; Stauderman et al., 1998). Furthermore, the $\alpha 7$ nicotinic receptor antagonist MLA and the $\alpha 3\beta 2$ receptor antagonist α -CTX PnIA had no effect on the nicotinic EPSPs. Therefore, our data suggest that nicotinic EPSPs described in these studies are most likely due to the activation of $\alpha 4\beta 2^*$ nicotinic receptors.

Due to their potential roles in schizophrenia and AD (Guan et al., 2000; Massoud and Gauthier, 2010; Ross et al., 2010; Taly et al., 2009), there has been more attention given to functional studies of $\alpha 7$ nicotinic receptors than any other nicotinic subunit found in the hippocampus (Alkondon et al., 1997; Alkondon et al., 1999; Frazier et al., 1998b; Jones and Yakel, 1997; McQuiston and Madison, 1999). Importantly, rapid $\alpha 7$ nicotinic synaptic responses have been recorded in hippocampal interneurons (Alkondon et al., 1998; Chang and Fischbach, 2006; Frazier et al., 1998a; Stone, 2007). However, we never observed a fast $\alpha 7$ nicotinic EPSP in our studies. We interpret our inability to observe a fast $\alpha 7$ EPSP to be due to the fact that only 7% of cholinergic terminals appear to form conventional synaptic junctions with postsynaptic partners (Umbriaco et al., 1995). Consequently, the majority of cholinergic communication in the hippocampus has been proposed to occur through volume transmission and diffusion of ACh. Thus, the majority of responses to ACh release would be expected to have slow time courses as observed with our nicotinic responses. Furthermore, the effective concentration of ACh at its nicotinic receptors would be expected to be dramatically reduced due to the requirement for diffusion. This would favor the activation of high affinity $\alpha 4\beta 2^*$ nicotinic receptors over lower affinity $\alpha 7^*$ nicotinic receptors (Zhou et al., 2003), as observed in our data. Therefore, our data suggest that nicotinic $\alpha 4\beta 2^*$ receptors are the predominant receptor subtype mediating nicotinic volume transmitted responses in hippocampal interneurons.

Nicotinic EPSPs in hippocampal CA1 interneurons were mostly subthreshold for action potential activation and had durations hundreds of milliseconds long. These data suggest that the nicotinic EPSPs would be favorable for temporal and spatial integration with other excitatory synaptic inputs. In particular, our anatomical and VSD imaging data have shown that nicotinic EPSPs were most prevalent in the SLM of hippocampal CA1. Although our anatomical data has shown that $\alpha 4\beta 2^*$ nicotinic receptors are located on interneurons in the SLM of hippocampal CA1, $\alpha 4\beta 2^*$ receptors have also been described on extrahippocampal excitatory glutamatergic synaptic terminals that innervate SLM (Nashmi et al., 2007). Therefore, the nicotinic VSD signals measured in the SLM may result from the activation of excitatory presynaptic terminals as well as postsynaptic interneuron processes. This suggests that nicotinic EPSPs could boost inputs from the entorhinal cortex (Empson and Heinemann, 1995) and/or reuniens nucleus of the thalamus (Dolleman-van der Weel MJ et al., 1997; Dolleman-van der Weel MJ and Witter, 1996; Dolleman-van der Weel MJ and Witter, 2000; Wouterlood et al., 1990) by presynaptically augmenting the release of glutamate and/or by postsynaptically summing with glutamatergic EPSPs in interneurons. Therefore, although nicotinic EPSPs were observed in other layers of CA1, nicotinic synaptic transmission in hippocampal CA1 may have a larger influence on extrahippocampal inputs vs. intrahippocampal inputs from CA3.

Our data have shown that nicotinic EPSPs were not uniformly distributed among the hippocampal layers. Based on VSD imaging, larger population $\alpha 4\beta 2^*$ nicotinic EPSPs were observed in the SLM relative to other layers of hippocampal CA1. Although cholinergic input from the MS/DBB show no layer selective innervation (Aznavour et al., 2002; Frotscher and Leranth, 1985), $\alpha 4\beta 2^*$ nicotinic receptors in the human hippocampus are found in higher density in the SLM (Perry et al., 1999). Thus a higher density of $\alpha 4\beta 2^*$

receptors on interneuron somata and dendrites in the SLM may contribute to the larger nicotinic EPSPs observed in this layer. In addition, MS/DBB cholinergic varicosities may be closer to $\alpha 4\beta 2^*$ nicotinic receptors residing on somata and processes in the SLM compared to interneurons in other layers of hippocampal CA1. Thus, larger concentrations of ACh may occur at $\alpha 4\beta 2^*$ nicotinic receptors in SLM.

It is also possible that our viral vector may preferentially infect cholinergic neurons in the MS/DBB that project to and target $\alpha 4\beta 2^*$ expressing interneurons in the SLM due to serotype-specific infection (Nathanson et al., 2009). However, this is unlikely because the titer of virus we used in our experiments exceeds AAV titer concentrations reported to confer cell type specificity (Nathanson et al., 2009). Secondly, it is possible that oChIEF-tdTomato expresses better in cholinergic neurons that innervate $\alpha 4\beta 2^*$ expressing interneurons in SLM. However, this is also unlikely because we measured muscarinic responses in a larger portion of interneurons than nicotinic responses (unpublished data) suggesting a lack of preference. Because our injection protocol produced expression of oChIEF-tdTomato in approximately 37% of Chat positive neurons in the MS/DBB, we believe that oChIEF-tdTomato was equally expressed in all types of Chat neurons in the MS/DBB. Therefore, our data suggests that the activation of $\alpha 4\beta 2^*$ nicotinic receptors in hippocampal CA1 will have a more significant effect on the integration of extrahippocampal inputs through its preferential activation of interneuron somata and dendrites in the SLM of hippocampal CA1.

In the hippocampus, $\alpha 4\beta 2^*$ receptors may be involved in withdrawal from nicotine addiction (Davis and Gould, 2009). Furthermore, $\alpha 4\beta 2^*$ receptor loss has been correlated with cognitive decline in aging (Gahring et al., 2005a; Gahring et al., 2005b; Rogers et al., 1998) and AD (Kellar et al., 1987; Marutle et al., 1998; Perry et al., 1999; Perry et al., 2000). Indeed, the role of $\alpha 4\beta 2^*$ receptors in AD may be particularly interesting as beta amyloid protein appears to have a high affinity for $\alpha 4\beta 2^*$ receptors (Wu et al., 2004). However, previous to this study, little was known about the cellular or network properties of synaptically activated $\alpha 4\beta 2^*$ receptors in the hippocampus. Thus, our studies provide a foundation for investigating changes in hippocampal $\alpha 4\beta 2^*$ receptor function associated with nicotine withdrawal, aging, and AD.

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Abbreviations

α-CTX PnIA	α -conotoxin PnIA
$\alpha 4\beta 2^*$	nicotinic receptors containing but not limited to $\alpha 4$ and $\beta 2$ subunits
$\alpha 7^*$	nicotinic receptors containing but not limited to $\alpha 7$ subunits
AD	Alzheimer's disease
ANOVA	analysis of variance
AP	action potential
APV	DL-2-Amino-5-phosphonopentanoic acid
CA1	cornu ammoni 1

CA3	Cornuammon 3
DHβE	dihydro- β -erythroidine
DNQX	6,7-Dinitroquinoxaline-2,3-dione
DIC	differential interference contrast
MLA	methyllycaconitine
MS/DBB	medial septum/diagonal band of Broca complex
rAAV	recombinant adenoassociated virus
SC	Schaffer collateral
SEM	standard error of the mean
SLM	stratum lacunosum-moleculare
SO	stratum oriens
SP	stratum pyramidale
SR	stratum radiatum
VSD	voltage-sensitive dye

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Highlights

1. ACh release activates $\alpha 4\beta 2^*$ nicotinic receptors on hippocampal interneurons.
2. Nicotinic EPSP activation and inactivation kinetics are slow.
3. Nicotinic EPSPs are most prevalent in stratum lacunosum-moleculare.

Figure 1.

Confocal images of the injection site into the MS/DBB of Chat-cre animals. Scale bar represents 50 μm . Neurons were visualized with a 10 x dry lens (0.3 N.A., voxel dimensions $0.48 \times 0.48 \times 6.6 \mu\text{m}$). Sequential scanning and 4 x line averaging was used to eliminate crosstalk and minimize background noise. **A.** Image of presumed cholinergic neurons in the medial septum expressing oChIEF-tdTomato. Images were collected using a 561 DPSS laser. **B.** Image of neurons labeled with anti-Chat antibody (1:200 Millipore AB144P) coupled to Alexa Fluor 633 (Invitrogen), imaged using the red HeNe laser. **C.** Overlay of images A and B.

Figure 2.

Neurotransmitter release from MS/DBB cholinergic terminals activates $\alpha 4 \beta 2^*$ containing nicotinic receptors on hippocampal CA1 interneurons. **A.** A blue light flash (blue bar, 1 ms) transiently depolarized a CA1 interneuron. Responses to 5 consecutive stimuli have been superimposed. Two of the 5 stimuli resulted in the production of action potentials. **B.** Blue light flash produced an excitatory postsynaptic potential (EPSP, black line) in a CA1 interneuron. The EPSP had slow kinetics (green and red dashed lines). **C.** The EPSP (from B, black) was potentiated by atropine (5 μ M, red). Methylyaconitine (MLA, 20 nM, green) did not affect the EPSP. Dihydro- β -erythroidine (DH β E, 10 μ M, dark blue) completely inhibited the EPSP. **D.** A train of blue light flashes (10 $\times$ 20 Hz) produced a summing depolarizing plateau response (black) in the same interneuron as B and C. Atropine (5 μ M, red) potentiated the depolarization to a greater extent toward later stimuli. MLA (20 nM, green) had no effect on the plateau response. DH β E (10 μ M, dark blue) completely inhibited the plateau response. **E.** In the presence of atropine (5 μ M) a train of blue light flashes (10 $\times$ 20 Hz) produced a depolarizing response in another interneuron (black). The depolarizing response was unaffected by α -conotoxin PnIA (α -CTX-PnIA, 10 μ M, light blue). DH β E (1 μ M, dark blue) completely inhibited the depolarizing response. **F.** Histogram of the effect of atropine (1-5 μ M) on the amplitude of the depolarizing nicotinic response produced by a train of blue light flashes (10 $\times$ 20 Hz). **G.** Histogram showing the effect of atropine and nicotinic receptor antagonists on the amplitude of nicotinic EPSPs during a train of blue light flashes (10 $\times$ 20 Hz). The amplitudes following the first and tenth flashes have been plotted.

Figure 3.

Representative electrophysiological properties of interneurons displaying nicotinic synaptic responses. **A.** Interneurons with accommodating action potentials (AP) with (left) and without (right) a depolarizing sag during a hyperpolarizing current injection. **B.** Interneurons with little or no accommodating APs with slow depolarizing sags during hyperpolarizing current pulses. **C.** Interneurons with irregular or stuttering AP firing patterns with (left) or without (right) depolarizing sags in the membrane potential during hyperpolarizing current pulses. **D.** An interneuron that displays a delay to AP firing in response to a depolarizing current injection also produced a depolarizing sag in response to hyperpolarizing currents.

Figure 4.

Morphology of CA1 interneurons displaying nicotinic EPSPs. Biocytin-labeled cells were processed for confocal imaging using streptavidin-633. **A.** An interneuron with its soma and dendrites localized to the stratum oriens (SO) and axon ramifying in SO and the stratum lacunosum-moleculare (SLM). **B.** An interneuron with its soma and dendrites localized to the SO and axon ramifying in SO and the stratum radiatum (SR). **C.** An interneuron with its soma and dendrites primarily localized to the SO and axon projecting toward SLM. **D.** An interneuron with its soma and dendrites localized primarily to the SLM and some axon in the SLM. **E.** An interneuron with its soma near the border of SR and SLM, dendrites projecting into the SLM and axon projecting within the expanded stratum pyramidale (SP) and SO. **F.** An interneuron with its soma in the SLM and dendrites located in all layers of hippocampal CA1.

Figure 5.

Synaptic activation of nicotinic receptors primarily occurs on neuronal cell bodies and processes located in SLM. **A.** Pseudo color images of voltage-sensitive dye (VSD) responses superimposed on the hippocampal slice from which they were measured. Each frame is separated by 25 ms. A burst of blue light flashes (3×50 Hz, second frame, blue result of flash artifact) produced depolarizing responses (green – small depolarizations; red – large depolarizations) primarily localized to the SO and SLM. **B.** Representative VSD responses traces taken from photodiodes overlying each anatomical layer of hippocampal CA1. A burst of blue light flashes produced depolarizing responses in each layer of hippocampal CA1 (black). Atropine ($5 \mu\text{M}$, red) had little effect on the amplitude of the depolarizing responses. **C.** Histogram of VSD depolarizing amplitudes (normalized to SLM amplitudes) in each layer of hippocampal CA1 in response to a burst of blue light flashes (black bars). The amplitude of VSD signals in SLM was significantly larger than all other layers of CA1. The VSD signals in SO were significantly larger than those in SR and SP. Atropine (red) did not significantly affect VSD signals in any layer of CA1. **D.** VSD signals (black) were not inhibited by DNQX ($30 \mu\text{M}$) and APV ($50 \mu\text{M}$) (magenta). **E.** VSD signals in response to a single blue light flashes were unaffected by MLA (50 nM , green) but were inhibited by DH β E ($1 \mu\text{M}$, dark blue). **F.** VSD signals in response to a burst of blue light flashes were unaffected by α -CTX-PnIA ($10 \mu\text{M}$, light blue) but were inhibited by DH β E ($5 \mu\text{M}$, dark blue). **G.** Histogram of VSD depolarizing amplitudes normalized to control amplitudes in each layer of hippocampal CA1. DNQX ($30 \mu\text{M}$) and APV ($50 \mu\text{M}$) had no effect on VSD amplitudes (magenta bars). MLA (10 to 100 nM) had no significant effect on VSD depolarizing amplitudes (green bars). DH β E (1 to $10 \mu\text{M}$) completely inhibited VSD depolarizing amplitudes (dark blue bars). α -CTX PnIA ($10 \mu\text{M}$) had no effect on VSD depolarizing amplitudes (light blue bar).