Published in final edited form as:

Eur J Neurosci. 2011 May ; 33(10): 1786–1798. doi:10.1111/j.1460-9568.2011.07671.x.

A Transgenic Mouse Model Reveals Fast Nicotinic Transmission in Hippocampal Pyramidal Neurons

Michael J. Grybko^{1,2,^}, Eu-teum Hahm^{1,2,^}, Wesley Perrine¹, Jason A. Parnes^{3,4}, Wallace S. Chick⁴, Geeta Sharma^{2,4}, Thomas E. Finger^{2,3,4}, and Sukumar Vijayaraghavan^{1,2,3,*}

¹ Department of Physiology and Biophysics, University of Colorado, School of Medicine, Aurora, CO 80045

² Neuroscience Program, University of Colorado, School of Medicine, Aurora, CO 80045

³ Rocky Mountain Taste & Smell Center, University of Colorado, School of Medicine, Aurora, CO 80045

⁴ Department of Cell and Developmental Biology, University of Colorado, School of Medicine, Aurora, CO 80045

Abstract

The relative contribution, to brain cholinergic signaling, by synaptic- and diffusion-based mechanisms remains to be elucidated. In this study, we examined the prevalence of fast nicotinic signaling in the hippocampus. We describe a mouse model where cholinergic axons are labeled with the *tauGFP* fusion protein driven by the choline acetyltransferase (ChAT) promoter. The model provides for the visualization of individual cholinergic axons at greater resolution than other available models and techniques, even in thick, live, slices. Combining calcium imaging and electrophysiology, we demonstrate that local stimulation of visualized cholinergic fibers results in rapid EPSCs mediated by the activation of $\alpha 7$ -subunit containing nicotinic receptors ($\alpha 7$ -nAChRs) on CA3 pyramidal neurons. These responses were blocked by the $\alpha 7$ -nAChR antagonist methyllycaconitine (MLA) and potentiated by the receptor specific allosteric modulator 1-(5-chloro-2,4-dimethoxy-phenyl)-3-(5-methyl-isoxanol-3-yl)-urea (PNU-120596).

Our results suggest, for the first time, that synaptic nAChRs can modulate pyramidal cell plasticity and development. Fast nicotinic transmission might play a greater role in cholinergic signaling than previously assumed. We provide a model for the examination of synaptic properties of basal forebrain cholinergic innervation in the brain.

Keywords

cholinergic; acetylcholine; *tauGFP*; choline acetyltransferase; synaptic transmission

Introduction

The cholinergic system in the brain has been implicated in a number of cognitive functions including attention, memory, and learning. However, while its importance in regulating behavior remains unchallenged, the mechanics of cholinergic signaling in the brain are

* Address Correspondence to Sukumar Vijayaraghavan, Department of Physiology and Biophysics, University of Colorado, School of Medicine, MS 8307, RC1-N, 12800 E. 19th Avenue, Aurora CO 80045., Phone 303-724-4531, Fax: 303-724-4501, sukumar.v@ucdenver.edu.

[^]These authors contributed equally to the work presented in this manuscript.

largely unknown. Major cholinergic innervation of the cortex arises mainly from relatively few neurons in the basal forebrain region, and is then diffusely spread throughout the region (Mesulam et al, 1983; Mesulam et al, 1986). The lack of distinct fiber tracts, in most areas, makes it technically more challenging to examine stimulated transmission in *in vitro* slice preparations.

A few studies have examined cholinergic transmission in acute slices from the brain (Frazier et al, 1998), but they have been hampered by the necessity to use large intensity bulk stimulations, and even then, with low success rates (e.g. < 10%; Frazier et al, 1998). This study, and a few others (Alkondon et al, 1998; Hefft et al, 1999) have led to the suggestion that fast nicotinic synapses might not be very prevalent in cortical areas, unusual for a transmitter system expected to signal, in part, via the activation of a family of fast, ligand-gated ion channels. Anatomical studies show that while cholinergic axons possess a number of transmitter containing varicosities, few of these putative release sites appear to have postsynaptic specializations apposing them (Contant et al, 1996; Descarries et al, 1997; Turrini et al, 2001; Mechawar et al, 2002). These studies have led to the idea that ACh might signal via diffusion based or 'volume' transmission.

The relative contributions, to cholinergic signaling, by conventional synaptic- versus diffusion-based mechanisms is unknown. At the same time, this is a pivotal issue in our understanding of endogenous cholinergic signaling. In this work we examine fast synaptic component of nAChR signaling.

Having live *in vitro* preparations where incoming cholinergic fibers can be visualized, allows for more defined and local stimulation of these inputs. The development of transgenic models expressing EGFP driven by the choline acetyltransferase (ChAT) has aided in the study of cholinergic neurons (Tallini et al, 2006; von Engelhardt et al, 2007). However, these models do not provide significant advantage in examining transmission *per se*. The reason is that soluble GFP does not label distal portions of axons well, probably due to the low axoplasmic volume. This is definitely a complication when dealing with thick live slices of brain tissue.

To circumvent these problems, we have developed a transgenic mouse model, expressing a *tau*-EGFP fusion protein driven by the ChAT promoter. As *tau* is selectively targeted to axons, the fusion protein labels cholinergic processes in fine detail. Using this model, we show specific CA3 pyramidal neurons are activated upon local cholinergic stimulation. Based on electrophysiological recordings from these neurons, cholinergic activation causes a fast synaptic current mediated by nAChRs that contain the $\alpha 7$ subunit ($\alpha 7$ -nAChRs). This mouse model, therefore, provides a useful tool to study mechanics of cholinergic transmission in the brain under physiological and pathological conditions.

Materials and Methods

Animals

FVB/N mice were used for the experiments described in this manuscript. Generation and breeding of mouse lines was performed at the transgenic facility at the Center for Comparative Medicine, University of Colorado, Denver, School of Medicine. Tails were used for genotyping using a PCR kit and GFP-positive animals were identified. All protocols were approved by the Institutional Animal Care and Use Committee (IACUC).

Generation of ChAT-*tau*GFP transgenic mice

The protocol for the generation of the ChAT-BAC construct is shown in Figure 1. BAC clone RP23-431D9 which harbors the entire *Chat* gene was obtained from Children's

Hospital Oakland Research Institute (CHORI). BAC DNA was purified by NucleoBond DNA purification kit (Clontech) and subsequently transformed into recombinogenic bacterial strain SW102. A targeting vector was constructed to insert the *tauGFP-3XpolyA* cassette at the ATG site of the Chat gene of BAC 431D9 by homologous recombination in SW102 strain of bacteria. To increase the efficiency of recombination, long homologous arms of the vector was generated by PCR and subcloning. Briefly, a 869bp PCR fragment (with *KpnI* site at 5' and *ApaI* site at 3' end) upstream and a 866bp PCR fragment (with *ApaI* site at 5' and *EcoRI* site at 3' end) downstream of Chat ATG were amplified and cloned into pBluescript (digested with *KpnI* and *EcoRI*) by 3-fragment ligation to produce pChatH. Using ptGpA as template, a *tauGFP-3XpolyA* cassette was generated by PCR with *ApaI* site engineered at both the 5' and 3' end. This PCR fragment was cloned into the unique *ApaI* site of pChatH to yield ptGpA-ChatH. Finally, a kanamycin resistant gene (*aphAI*) released from *SacI* digestion of pEP-kanS2 was cloned into the *ClaI* site (immediately downstream of *tauGFP-3XpolyA*) of ptGpA-ChatH by blunt-end ligation to yield ptGpA-ChatH-Kan.

BAC clone RP23-431D9/SW102 was grown at 32°C to log-phase after which the bacteria were heat-shocked at 42°C for 15 minutes to induce Red proteins expression. Cells were then washed twice with ice-cold 10% glycerol. Linear DNA cassette (200 ng) of *tauGFP-3XpolyA*-Kan flanked by Chat homologous arms released from *KpnI* and *SacI* digestion of ptGpA-ChatH-Kan was electroporated into the prepared competent cells. After a 1-hour recovery in LB at 32°C, transformed cells were spread onto LB-Chlor+Kan plates. Surviving bacterial colonies were screened for correct recombination by PCR. BAC DNA of correctly targeted clones was isolated and analyzed by restricted enzyme digestion. The insertion of *tauGFP-3XpolyA* was confirmed by DNA sequencing.

Immunohistochemistry

Animals were anesthetized using chloral hydrate (80mg/kg) or pentobarbital (60mg/kg with supplemental dose at 25mg/kg as necessary) and fixed by intracardiac perfusion with either 4% paraformaldehyde (PFA) in 0.1M phosphate buffered saline (PBS) or Periodate-lysine-paraformaldehyde (PLP) fixative (Lysine 75 mM, Formaldehyde 1.6%, Sodium Periodate 10mM) in 0.1M phosphate buffer, pH 7.4. Brains were removed and fixed for an additional 2-3 h. The tissue was then cryoprotected overnight using a 20 % sucrose solution in PBS.

Cryostat sections (20 μ m 50 – μ m) were collected in 0.1M phosphate buffer and processed as free-floating sections. For 2 cases, every third 40 μ m section was collected to yield an entire series of sections spaced at 120 μ m. Sections were washed in PBS followed by incubation with blocking solution containing 2% normal donkey serum, 0.3% Triton X-100 and 1% bovine serum albumin. All primary antibodies were diluted in the blocking solution. Primary antibodies used were goat anti-ChAT (AB144P from Millipore, Billerica, MA, affinity-purified antiserum raised against human placental choline acetyltransferase, and used at a dilution of 1:200), and chicken anti-GFP (GFP-1020 Aves Labs Inc, generated against purified recombinant green fluorescent protein, and used at 1:1000). Secondary ALEXA and Dylight fluorescent antibodies were obtained from Invitrogen (donkey anti—goat IgG, preadsorbed against rabbit, rat, mouse and human IgG. Carlsbad, CA) and Jackson ImmunoResearch (AffiniPure Donkey Anti-Chicken IgY (IgG) (H+L); preabsorbed against Bov, Gt, GP, Hms, Hrs, Hu, Ms, Rb, Rat, Shp Serum Protein; West Grove, PA,). A fluorescent Nissl counterstain (Neuro Trace 640/660, Invitrogen, N21483) was applied for 25 minutes 1:100 in blocking solution.

Sections were then mounted on slides using Fluormount –G (EM Sciences, Hatfield, PA). Imaging was done on Olympus Spinning Disk Confocal microscope as well as by standard epifluorescence microscopy using an Olympus BX41 Fluorescence Imaging Station with

Retiga-4000RV monochrome camera. Images were composed in Photoshop using the adjust levels controls to manage brightness and contrast of entire figures.

Calcium imaging

Three hundred micron hippocampal slices were prepared from P11-P16 mouse brains using a Leica VT-1000 or a Dosaku DSK-ZeroZ slicer (Ted Pella Inc., Redding CA). Slicing conditions and recording methods were as described in previous studies from our lab (Sharma and Vijayaraghavan, 2003; Sharma et al, 2008). Briefly, all recordings were performed at room-temperature (21°C–23°C), using a Zeiss Axioskop epifluorescence microscope. Slices were continuously perfused with bubbled aCSF containing 2.5 mM CaCl₂, 1 mM MgCl₂, 100 μM DNQX, 20 μM APV, 2 μM atropine, and 10 μM Gabazine. For imaging experiments, 10 μM PPADS and 1 mM MCPG were added to block P2X receptors and type I mGluRs, respectively. Zeiss water immersion objectives (40 × and 20 ×) were used for imaging. Efficacies of antagonists were tested using exogenous agonist application (data not shown). In addition, we have previously demonstrated that all GluR currents can be blocked at these concentrations of antagonists (Sharma and Vijayaraghavan, 2003).

Slices were loaded with 10 μM fura 2-AM as described (Grybko et al, 2010). Images were acquired using a Cooke Sensicam (PCO, Germany) ccd camera and the SlideBook software (Intelligent Imaging Innovations, Denver). Excitation was achieved using a Sutter DG-IV wavelength switcher (Sutter Instruments, Novato, CA). All filters were obtained from Chroma Technologies (Rockingham, VT).

Identified, GFP-expressing, fibers in the stratum lucidum or at the stratum oriens were targeted for stimulation. No amplification or filtering was used. The likelihood of photodamage was reduced by keeping exposure times to a minimum using 4×4 binning of images. FITC excitation was not used during calcium imaging, only for the initial placement of pipettes. Our data with the consistency of the elicited responses speak to this issue. No fibers were detected upon excitation with 340 or 380 nm, not surprising because of the fact that these are thin processes, unlikely to be significantly stained with bulk loading. The pyramidal cells where fura signals are imaged do not express GFP, removing the concern that the calcium signals might be quenched.

A glass stimulation pipette was placed on an identified fiber. Stimulation pipette sizes varied between 2–5 μm. Stimuli were provided using a WPI A 265 stimulus isolator (World Precision Instruments, Sarasotta, FL). Stimulation protocol was varied ranging from a single 100 μs stimulus to a train of 100 stimuli given at 100 Hz. Once a response was elicited to a given stimulus paradigm, conditions were kept constant throughout measurements.

Electrophysiology

Slices were loaded with fura 2-AM as above. Calcium responses to 5, 100 μs pulses, given at a 100 Hz were recorded in aCSF containing 2.5 mM CaCl₂, 1 mM MgCl₂, 2 μM atropine, 50 μM DNQX, 100 μM APV, and 10 μM Gabazine. Once responding cells were identified, they were patched and recorded using the whole cell voltage-clamp configuration. Internal solution (pH 7.2, 290 mOsm) contained 130 mM K-Gluconate, 5 mM KCl, 10 mM EGTA, 10 mM HEPES, 2 mM ATP (magnesium salt), and 0.2 mM GTP (sodium salt). Acquisition rate was 10 KHz. Access resistance was periodically monitored to ensure recording stability as described (Sharma et al, 2008) and cells with changes in access resistance > 15% were discarded. No series compensation was used. All recordings were performed at 32 °C - 34 °C, and signals were recorded with an Axopatch 200 B patch amplifier using pClamp 9 software (Molecular Devices, Sunnyvale, CA). Holding potential was 60 mV.

Recordings were carried out using a 40 X water immersion objective (NA 0.8). In this case, the responding neurons were within 50 μm - 100 μm from the stimulus pipette. Stimulations were done with a glass pipette as described above. Single 100 μs stimuli were used in the recordings given 20 s apart, unless indicated otherwise. In some cases, stimulus artifacts were truncated by subtracting traces showing failures or a trace recorded in the presence of 1 μM TTX. A few neurons responded with fast EPSCs only at high stimulus intensities and were counted as responders. Only neurons that responded consistently to multiple stimuli were used for the experiments described below. Data were analyzed using the Origin 6 software (OriginLabs, Northampton, MA). Data from individual traces were compiled and compared using paired or unpaired Students t-test. Minimum acceptable significance level was $p < 0.05$.

Materials

Except where stated in the text, chemicals were obtained from Tocris (Ellisville, MO) or Sigma (St. Louis, MO).

Statistics

All cumulative data are expressed as Mean $\pm$ SEM. All comparisons were made using Student's t-test. $P < 0.05$ was taken as acceptable level of significance. The Origin 6 software (Microcal, Northampton, MA) or the Minianalysis software (Synaptosoft, Decatur, GA) were used to calculate significance levels.

Results

Expression of *tau*-EGFP

ChAT-driven GFP expression was visible widely throughout the brain corresponding to cells and regions known to express ChAT based on immunohistochemical studies of rats and mice (Armstrong et al, 1983; Woolf et al, 1984; Barmack et al, 1992). Particularly high levels of GFP expression were apparent in the habenulo-interpeduncular system (Fig. 2 A & D). Although ChAT-immunohistochemistry (IHC) also reveals reactivity in this system (as reported by others, e.g. Armstrong et al., 1983), the intensity of the transgene-driven GFP expression in the habenulo-interpeduncular system was disproportionately high compared to the IHC signal. Similarly, examination of the ventral surface of the brain with a fluorescence dissecting microscope reveals strong GFP expression in the interpeduncular nucleus. In addition, GFP fluorescence is visible as elongate patches associated with the islands of Calleja in the olfactory tubercle (Fig. 2 B) and in the parabigeminal-collicular tract running beside the optic tract (Fig. 2 B, PbC) as described by (Motts et al, 2008).

Detailed comparison of transgene-driven GFP and ChAT immunohistochemistry showed nearly complete co-localization of these markers in all areas of the brain and peripheral nervous system. The major ChAT-expressing cell groups of the brain are: motor nuclei, both somatic and parasympathetic, mesopontine tegmentum, striatum, and basal forebrain (Wainer et al, 1984). Numerous other cells within the neuraxis express ChAT, including some cortical interneurons (Levey et al, 1984), and will be considered only briefly in the description below.

Peripheral Nervous System—Obvious label was detected in known cholinergic fibers and terminals including the enteric nervous system and motor endplates (Fig. 2 E, F). Also evident were labeled solitary chemosensory cells in the airways and a few cells in most taste buds (Fig. 2 G), as reported by others (Ogura et al, 2007) .

Hindbrain Motor and Sensory Nuclei—All brainstem motor nuclei exhibited both immunostaining for ChAT and GFP expression, although the levels varied. Since the *tauGFP* was targeted to axons, the motor nerve roots were well-labeled (Fig. 3 A, C). Many motoneurons were not obviously labeled when viewing the GFP fluorescence alone, but were clearly labeled when anti-GFP antisera were utilized. Many cells within the caudal nucleus solitarius also exhibited both GFP fluorescence and GFP immunoreactivity (Fig. 3A). As reported by others for ChAT immunoreactivity rats (Barmack et al, 1992; Jaarsma et al, 1996), vermal areas of the cerebellum showed distinctive ChAT-driven GFP immunoreactive mossy fiber terminals.

Mesopontine Tegmentum—Some scattered, large neurons of the pedunclopontine nucleus and laterodorsal tegmental nucleus were clearly evident in tissue immunoreacted with anti-GFP antisera (Fig. 3 B). However many cells of the pedunclopontine complex were immunoreactive for ChAT protein but exhibited no detectable GFP immunoreactivity (magenta cells in Fig. 3 B). In addition to the conventional pedunclopontine cholinergic neurons, well-labeled ChAT and GFP-immunoreactive neurons were evident in the lateral parabrachial nucleus, likely equivalent to the cholinergic parabrachial neurons described in guinea pig (Motts et al, 2008).

Striatum—Large cholinergic neurons of the striatum were evident both from intrinsic GFP fluorescence and by GFP immunohistochemistry (Fig. 4 A, D). The meshwork of fine processes from these neurons is visible in both the native GFP and GFP-immunoreacted material.

Basal Forebrain and Cortex—The classically described cholinergic cell groups of the basal forebrain are readily visible with both ChAT-driven GFP and with anti-GFP antisera including cells of the medial septum and magnocellular basal forebrain complex (Fig. 4 A). Essentially all GFP-labeled cells exhibit immunoreactivity for the ChAT protein. Particularly striking is the extensive plexus of labeled processes visible within the olfactory tubercle which also contains some scattered cholinergic somata (Fig. 4 B). This ChAT-GFP plexus is even apparent in a ventral view of the brain (Fig. 2 B).

Of particular interest is frontal cortex, which contains small interneurons immunoreactive for ChAT protein. Mostly, these are bipolar neurons in the upper layers of the cortex as described previously (e.g. (Houser et al, 1985; Consonni et al, 2009)). These cells do not express GFP (Fig. 4 C) although substantial GFP can be detected in fibers coursing through the cortical layers and ramifying extensively in layer I of the cortex. The extensive network of GFP-labeled processes visible in this transgenic line is noteworthy in making apparent the heavy cholinergic innervation of this layer. All of these GFP-labeled fibers also are immunoreactive for ChAT protein, but are less obvious than in the GFP preparations.

The slight differences between transgene expression and expression of native ChAT protein suggest that these coding sequences may fall under slightly different regulatory regimes. The ChAT protein is transcribed from a number of distinct promoter regions that arise from alternate splicing of non-coding regions (Misawa et al, 1992; Naciff et al, 1999). In addition, there might be splice variants involving the coding region that result in a different enzyme isoforms (Matsuo et al, 2005). These modifications result in differential localization and regulation of the ChAT protein (Misawa et al, 1993; Matsuo et al, 2005). The qualitative pattern of the *tauGFP* fusion protein distribution would suggest that these regulatory processes do not significantly affect the transgene expression. In addition, our results would suggest that in the line described in this manuscript, the insertion locus does not grossly affect region-specific expression of the transgene and that all GFP expressing cells are cholinergic.

***Tau*-GFP expression in the hippocampus**

In the hippocampus, a diffuse pattern of GFP expressing fibers were observed as has been described from other studies, using immunohistochemical approaches to detect ChAT or acetylcholinesterase (AChE) expression (Frotscher and Leranthe, 1985; Bialowas and Frotscher, 1987; Henderson et al, 1998). Figure 5 shows the expression of *tau*GFP in the hippocampus. While all areas within the hippocampus had fibers expressing the transgene, there were qualitative differences in the extent of innervation. In general, the molecular layer of the dentate gyrus showed the highest intensity of GFP-labeled axons (Figure 5 A & 5 C, right), followed by the CA3 stratum oriens (Figure 5 A & 5 B right), stratum lucidum (Figure 5 B & 5 C, left), and the hilus (Figure 5 A & 5 C right). Often, the fibers were present as a denser band on either side of the CA3 pyramidal cell layer as well as the granule cell layer (Figure 5 A). The fibers also intercalated into the cellular layers (Figure 5 B & C).

The individual axons were highly branched and punctate, showing distinct GFP- and ChAT-positive varicosities, these might represent either release sites or branch points. Though not directly tested here, these varicosities have been shown to be putative release sites from electron microscopic studies (Bialowas and Frotscher, 1987; Deller et al, 1999; Turrini et al, 2001; Casu et al, 2002). The punctate structures were also observed within the pyramidal and granule cell layer, indicating the possibility of somatic synapses on to these neurons (Figure 5B&C).

Scattered cholinergic interneurons were observed within the hippocampus (e.g. Figure 5 B, left panel) as reported earlier (Frotscher et al, 2000). These were often multipolar and more commonly observed in the in the region of stratum oriens closest to the fimbria and in the hilus, though other regions had these interneurons as well.

nAChR-mediated calcium signaling in CA3 pyramidal neurons

Results from our laboratory have shown a robust modulation of CA3 pyramidal neuron firing by presynaptic $\alpha 7$ -nAChRs on mossy fiber terminals (Sharma and Vijayaraghavan, 2003; Sharma et al, 2008). Very small to no postsynaptic nicotinic currents were observed from either these neurons or from CA1 pyramidal cells (Ji et al, 2001; Khiroug et al, 2003; Sharma et al, 2008). At the same time, we reported large calcium signals in response to $\alpha 7$ -nAChR activation by exogenous agonists in CA3 pyramidal neurons (Grybko et al, 2010) in the absence of action potentials, GluR- and GABAR-activation, which were blocked by respective receptor antagonists. In addition, recent EM studies indicate the existence of contacts between cholinergic fibers and pyramidal cell dendrites, indicating the possibility of synaptic signaling in this area (Yamasaki et al, 2010).

These results suggested that the presence of low levels of $\alpha 7$ -nAChRs is sufficient to generate large calcium transients by amplification via voltage-gated calcium channels and/or calcium stores (Brain et al, 2001; Dajas-Bailador et al, 2002; Sharma and Vijayaraghavan, 2003). Secondly, the receptors might be localized e.g. at synapses, to make exogenous activation less effective. We asked whether the use of the *tau*GFP mice, coupled with calcium imaging and electrophysiology, might reveal potential synaptic nAChR signaling by endogenous ACh release on CA3 pyramidal neurons. A few experimental results were confirmed using hilar interneurons (see below).

Slices were loaded with fura 2-AM as described under Methods. Fura 2 was chosen over other calcium dyes because it was empirically determined to load pyramidal neurons better and because it facilitated the identification of putative healthy neurons (based on robust fluorescence from 380 nm excitation and low signals from 340 nm excitation, which facilitates identification of cells with low resting calcium levels).

At the end of the loading period, the slice was visualized under an epifluorescence microscope. Experiments were carried out in the presence of blocker of mAChR, metabotropic and ionotropic GluRs, P2xRs and GABARs (see Methods).

For CA3 pyramidal neurons, cholinergic processes in stratum oriens or stratum lucidum were identified by their GFP fluorescence and a stimulation pipette was placed on a visualized fiber (Figure 6B). Neurons were imaged at 1Hz. At a defined time point, a stimulus (100 pulses, 100 μ s pulse duration delivered at 100 Hz) was administered. Changes in calcium signals were monitored (Figure 6C). Stimulating the nerve in the presence of TTX showed no calcium responses (1.7 ± 1.3 % of responses elicited in the absence of the toxin, $n = 9$, $p = 0.004$), consistent with action potential-dependent signaling.

For a given stimulus, very few neurons in the field responded, with rare exceptions (e.g. Figure 6B). Figure 6D shows data from 2 experiments. From a total of 146 neurons measured in the two fields shown in Figure 6D, only 6 neurons showed rapid, time-locked, calcium transients. While a qualitative correlation could be made between the trajectory of visualized fibers and responding neurons, these cells were interspersed with others that did not show calcium changes (Figure 6B, C, and D). The extensive branching of the fibers and multiple varicosities made an *a priori* prediction of a responding neuron unreliable. Calculating the distance between the stimulating pipette and the pyramidal neurons, no distinct spatial correlation was observed for cells that responded to the stimulus versus those that did not. In Figure 6D, the responding neurons were spread over a distance of 83 μ m - 245 μ m from the stimulating pipette. This is possibly due to more specific stimulation and few synaptic connections in relation to total number of putative release sites (Mechawar et al, 2002; Aznavour et al, 2005). Repeated stimulations did not result in additional neurons responding (data not shown) while multiple responses could be recorded from the neurons that responded with the first stimulus (see below). The spatial distribution of the responding neurons and the consistency of responses provide additional evidence that we were monitoring calcium signals due to specific synaptic stimulation.

A single 100 μ s stimulus induced a calcium transient (Figure 7A). However, these showed significant number of failures and were difficult to analyze as they often overlapped with spontaneous oscillations, which had similar kinetics. As the purpose of imaging in this study was to identify responding neurons, in order to characterize them using electrophysiology, we used the 100 Hz stimulus protocol described above in order to obtain consistent, time-locked signals. The calcium transients were rapid, rising to peak within a single frame (1 s). The averaged transient showed a biphasic decay with a τ_{fast} of 1.7 s and a τ_{slow} of 10 s ($n = 21$; Figure 7B; $R^2 = 0.94$, values obtained using the Non-Linear Least Squares fitter function built-in the Microcal Origin software).

The low percentage of responders was not due to lack of functional nAChRs on most pyramidal cells. To demonstrate this, stimulated fields were then challenged with 5 μ M nicotine for 10 s. Both neurons that responded to electrical stimuli as well as those that didn't showed robust responses to exogenous nicotine (Figure 7C). The mean fractional change in ratio was $15 \pm 3\%$ for cells responding to the stimulus ($n = 4$) and $13.4 \pm 1.5\%$ for those that didn't respond ($n = 23$; $p = 0.6$, unpaired t-test).

The responses were attenuated by 200 μ M mecamylamine, a general nicotinic antagonist (Figure 7D). For the purpose of quantification, we used the integral of the transient. We separately estimated the contributions from the two components of the response identified in Figure 7B. Two consecutive responses, 20–30 min apart gave responses not significantly different from each other (data not shown). In the presence of mecamylamine, responses were significantly reduced to $49 \pm 8\%$ and $11 \pm 13\%$ of the control for the Fast and slow

components, respectively ($n = 19$; $p = 8.61\text{E-}4$, and $p = 5.52\text{E-}6$, for the fast and slow components, paired t-test). The lack of complete block of the fast component is probably due to amplification of calcium signals by nAChR-mediated increased firing rates of the pyramidal neurons, which are spontaneously active, and the possible activation of recurrent networks. Resolution of these signals would require much faster acquisition of images. Such rapid imaging would require single wavelength excitation, increasing the risk of artifacts (Vogt and Regehr, 2001) for the purpose of this study. Incomplete blocks were also evident in responses to exogenous agonists when the cells were allowed to fire action potentials (data not shown).

Synaptic $\alpha 7$ -nAChR currents on pyramidal neurons

Once neurons responding to local stimulation were reliably identified, we then asked whether synaptic nAChR currents could be elicited from them. Slices were loaded with fura 2-AM as described. Responses to single stimuli using a glass pipette, placed at stratum oriens on identified axons, were recorded from CA3 pyramidal neurons. Changes in calcium responses to a 5 pulse stimulation (100 μs /pulse), given at 100 Hz, were recorded as described above. Cells responding with a rapid calcium transient were identified within a 40 $\times$ field. Identified neurons were then held in the whole cell voltage-clamp configuration.

Some experiments were also carried out in hilar interneurons as fibers were more readily identifiable in this region than in the stratum radiatum (see Figure 5), and as these cells have been shown to express functional $\alpha 7$ -nAChRs (Frazier et al, 2003). In response to single electrical pulses (100 μs), both pyramidal cells and hilar interneurons, showed rapid EPSCs (Figure 8 A), consistent with synaptic activation. The pyramidal cell currents had fast rise (10–90 rise time 0.53 ± 0.11 ms) and decay times (with a τ of 3.32 ± 0.17 ms; data from 5 cells, values obtained using the Non-Linear Least Squares fitter function built-in the Microcal Origin software) at 32°C – 34°C . The synaptic delay (stimulus onset to current peak) was 2.1 ± 0.2 ms ($n = 11$ cells).

Not all cells that responded with calcium transients upon electrical stimulation showed EPSCs. The response was position specific (see example in Figure 8 C) and even slight drifts, over time, in the position of the stimulation pipette affected the probability of getting EPSCs (data not shown). Among recordings where the pipette position was carefully monitored, 38 % of cells did not show a fast EPSC (14/37 cells did not respond). In addition, a fraction of neurons gave rapidly attenuating responses. Only neurons that responded to multiple stimuli were used for our experiments. Five to ten responses, elicited 20 s apart, were averaged for each condition. Response amplitudes varied considerably. The mode of the amplitude distribution was 15 pA (Figure 8A). The responses were all or none suggesting that these are unitary EPSCs, however very few sEPSCs were detected, perhaps because of the fact that these axons are cut. Some large currents were observed (Figure 8B) that appeared all or none responses as well. Coupled with results from anatomical studies (Figure 5), these results suggest that a number of boutons from axonal branches impinge upon individual pyramidal neurons, and these are probably distributed across the length of the soma and dendrites.

A 20 s local application of 50 nM MLA, using a puffer pipette, blocked the response (Figure 9 A). In the presence of the $\alpha 7$ -nAChR antagonist, the mean responses were 7 ± 2 % on pyramidal neurons and 5 ± 2 % on hilar interneurons ($n = 4$ and 5 ; $p = 0.017$; $p = 4.42231\text{E-}4$, respectively, paired t-test). Washing the drug out for 1-2 minutes showed recovery of response in 2 cells, in both cases (Figure 9 B).

Incubating of the slice with 10 μM 1-(5-chloro-2,4-dimethoxy-phenyl)-3-(5-methyl-isoxanol-3-yl)-urea (PNU-120596) for 15 minutes, affected the EPSCs (Figure 9 C). In the

presence of this allosteric modulator, the average peak amplitude of the EPSC 2.1 ± 0.4 fold of the control ($n = 3$; PNU-120596 response from each cell was significantly different from its control at $p = 0.009$, $p = 0.004$, and $p = 0.029$, respectively; paired t-test). The decay rate in the presence of PNU-120596 was not significantly altered and was 3.32 ± 0.17 ms (control) vs 3.06 ± 0.28 ms ($n = 5$, $p = 0.2$, unpaired t-test). One interpretation of this result is that significant desensitization occurs at the rising phase of the current, as expected from the fast decay kinetics, which is slowed down by PNU-120596, resulting in increased peak amplitudes. The result might also imply that the rate limiting step in determining the decay of the synaptic current might be transmitter clearance. The clearance might be either due to rapid diffusion of ACh from synapses or due to the action of AChE. In our hands (data not shown) and from other studies (Pereira et al, 1993; Pereira et al, 1994; Clarke et al, 1994), a number of AChE inhibitors appear to have non-specific actions on nAChR currents. Thus, the role of AChE in diffusion of ACh awaits careful characterization of the specificities of various inhibitors.

Discussion

We use a transgenic mouse model to examine the mechanics of cholinergic signaling in the brain. In this model, the expression of a *tauGFP* fusion protein driven by the ChAT promoter, allows for the resolution of single cholinergic axons in order to examine responses to focal electrical stimulation. Using this model we demonstrate rapid synaptic currents mediated by the activation of $\alpha 7$ - nAChRs in both CA3 pyramidal cells and interneurons. The work presented here represents a considerable advancement in the field and makes a number of important contributions. A) The model is of considerable utility for investigators in the general field of cholinergic signaling both from anatomical and functional viewpoint. B) The existence of fast nicotinic synaptic transmission is novel in general. The issue of fast transmission versus diffuse signaling has been central to the field of cholinergic signaling. In this context, the importance of fast nicotinic transmission has been considered minimal for the simple reason that its existence has been controversial. Our study provides evidence, for the first time, for the prevalence of nicotinic transmission in the brain and demonstrates that this form of signaling might be more common than previously assumed. C) The assumption in the field is that pyramidal cell excitation by nicotine is mostly, if not completely, by presynaptic modulation of glutamate and GABA release (Gray et al, 1996; Sharma and Vijayaraghavan, 2003; Sharma and Vijayaraghavan, 2008). The finding that there is rapid synaptic transmission in pyramidal neurons is likely to change our ideas about how glutamatergic output in the hippocampus is modulated by nAChR activation upon endogenous cholinergic stimulation. Further, this fast transmission might have important developmental implications as discussed below.

While the cholinergic system has been thought to play a role in a number of pathological conditions (Coyle et al, 1983; Adler et al, 1998; Mansvelder et al, 2003; Bohnen et al, 2010), mechanistic information of how the system functions is sorely lacking. At the same time, a number of treatment strategies target this system both by regulating transmitter levels (Pepeu and Giovannini, 2010), as well as by modulating nAChR function (Newhouse et al, 2001; Levin and Rezvani, 2002; Haydar and Dunlop, 2010), emphasizing the need for elucidating a functional role for the system.

The prevalence, in cortical regions, of fast excitatory transmission mediated by nAChRs is unknown. The relatively sparse literature demonstrating the existence of fast nicotinic transmission has given credence to the idea of 'volume' transmission. Part of diffusion-based signaling model for nAChRs in the CNS also comes from the findings that they are located on synaptic terminals of other transmitter systems where the predominant effect of the receptors seems to be modulation of transmitter release (McGehee et al, 1995; Gray et al,

1996; Sharma and Vijayaraghavan, 2003). The existence of release sites *sans* postsynaptic specializations (Contant et al, 1996; Descarries et al, 1997; Mechawar et al, 2002) has further strengthened this idea.

Examination of fast nicotinic transmission has been hampered by the lack of adequate animal models to overcome the technical difficulties presented by the anatomy of the brain cholinergic system. While the identification of cholinergic neurons using GFP expressing mice is useful in order to examine their function (von Engelhardt et al, 2007), studying the long distance synaptic signaling requires much clearer labeling of the incoming axons, and their branches in a target area. This is more so in live, acute slices, where the thickness of the slice greatly impairs the ability to visualize processes. Based on our results, the use of the *tau*GFP fusion protein to label axons allows for visualization of single fibers, even in 300 -350 μ m slices. Potential over expression of *tau* is not a concern at the ages used to examine synaptic transmission in this study. We haven't observed any large scale neuronal degeneration (up to 6 -8 months of age) arguing against adverse effects of the transgene, even in the adult. In addition, *tau*GFP has been used in other brain regions with no significant signs of degeneration (Rodriguez et al, 1999; Treloar et al, 2002; Sekirnjak et al, 2003; Walz et al, 2006; Walz et al, 2007), and allows for the resolution of axons and synapses in exquisite detail (Sekirnjak et al, 2003).

The use of calcium signaling for the initial identification of neurons responding to synaptic stimulation allowed for the identification of specific responders. The AP-dependent, and consistent, calcium responses obtained from a very small fraction of pyramidal neurons (and hilar interneurons) speaks to the specificity of the recordings. Possible interpretations for the lack of a broader response might be that large distance, diffusion-based, signaling might require stimulation of a larger number of fibers, or that most diffusion based effects on principal neurons are secondary, via the activation of other presynaptic terminals or astrocytes. The lack of spatial correlation between the stimulus locus and the target reflects the highly complex geometry of the cholinergic axons. At the same time it also predicts that the odds of finding synaptic pairs by local stimulation would be small in wildtype mice, consistent with studies obtained from bulk stimulation experiments (Frazier et al, 1998). Our results also suggest that random placement of stimulus electrodes is unlikely to improve odds of finding pairs. Identification of targets by calcium imaging only provides a rough estimate of target neurons. The reason is that the number of transmitter systems that signal via calcium signals is enormous, and it is not feasible to block all known systems using pharmacology. Further, in practice, random placement of electrodes is likely to be a low yield experiment as has been shown before (Frazier et al, 1998). In the absence of an identifiable field (as shown in Figure 6 and stated in the text), the responses could easily lie outside the field of imaging and randomly activate pyramidal neurons, interneurons, or astrocytes in a field. The identification becomes critical when examining other aspects as well e.g. mossy fiber bouton (Sharma et al, 2008) activation by ACh and for combining evoked glutamatergic signaling with cholinergic stimulation. Under these conditions, the proximity of an identified cholinergic fiber becomes paramount. Indeed, the controversy regarding nAChR contributions to cholinergic signaling can be explained by the simple fact that, in the presence of spontaneous neuronal firing, pharmacological dissection of randomly stimulated inputs is difficult.

The block of the calcium transients by mecamylamine, demonstrated the nicotinic nature of the calcium responses. The arguments raised above justify our decision not to use calcium imaging for detailed pharmacology.

Electrophysiological recordings from CA3 pyramidal neurons, responding with calcium transients to stimulation, revealed the presence of fast EPSCs. The α 7-nAChR EPSCs,

sensitive to MLA, had fast rise and decay times, comparable to fast AMPA synaptic currents. In addition, these currents were modulated by PNU-120596, an allosteric modulator of $\alpha 7$ -nAChRs (Hurst et al, 2005; Papke et al, 2009). Based on the kinetics of the responses, it would be reasonable to conclude that the responses arise from synaptic receptors, or at least receptors within synaptic distances from release sites.

The presence of synaptic $\alpha 7$ -nAChRs on pyramidal neurons suggests an additional role for the receptors in the hippocampus. Based on our data, $\alpha 7$ -nAChRs can act pre- and post-synaptically at the mossy fiber-CA3 synapse. Simultaneous activation of the receptors on mossy fiber terminals as well as the CA3 neurons can provide a timing-dependent modulation of pyramidal cell plasticity (Ji et al, 2001). ACh might also be involved in the formation of functional glutamatergic and GABAergic synapses. In NMDA only synapses, seen early in development (Choi et al, 2000; Montgomery et al, 2001) timed nAChR transmission could substitute for AMPAR-mediated postsynaptic depolarization. These possibilities need to be examined.

The fidelity of expression of the *tauGFP* fusion protein makes this transgenic model ideal for examination of the cholinergic system both in the brain as well as peripheral nervous system, including the neuromuscular junction. In comparison to optogenetic systems, like channelrhodopsin expression, where single fiber stimulation involve significant technical complexity, *if at all possible*, the *tauGFP* mouse can potentially achieve this end using standard protocols. Combined with channelrhodopsin models where bulk activation can be easily triggered without the complications inherent to bulk electrical stimulations, or the use of pharmacological blockers, the two models could go a long way in addressing the issue of prevalence of synaptic versus volume transmission in the cholinergic system.

Key issues remain unresolved regarding cholinergic transmission in the brain. One issue is the relative prevalence of direct synaptic signaling versus transmission via diffusion. Identification of AChE inhibitors that do not interfere with nAChR kinetics will help in addressing this issue.

Also unknown whether cholinergic signaling is tonic or phasic and whether activation of basal forebrain cholinergic neurons results in local or widespread signaling among various target populations. Anatomical studies imply that cholinergic neurons arise from four nuclei in the basal forebrain; termed Ch1-Ch4, which have overlapping domains (Selden et al, 1998). Do these nuclei contain distinct subpopulations of cholinergic neurons under different modulatory controls or does cholinergic activation in response to a specific task lead to a generalized arousal of the entire cortical region?

The cholinergic system plays an essential role in a number of pathological conditions and many therapies currently available target this pathway. While it is not reasonable to expect that therapeutic interventions wait for complete physiological information on a signaling pathway, there is much less known about the latter. It stands to reason that in the long run, mechanistic information on the actions of these systems will be essential for rational and efficacious drug design to combat these illnesses. The use of transgenic mouse models such as the one described in this study will go help in the understanding the nature of cholinergic transmission in the brain.

Acknowledgments

This work was supported by NIDA Grants (RO1DA 10266 and Cutting-Edge Basic research Grant R21 DA 019453) to S.V. and NIDCD grants RO1DC008855 to S.V., and P30 DC04657 to Diego Restrepo & T.E.F.

References

- Adler LE, Olincy A, Waldo M, Harris JG, Griffith J, Stevens K, Flach K, Nagamoto H, Bickford P, Leonard S, Freedman R. Schizophrenia, sensory gating, and nicotinic receptors. *Schizophr Bull.* 1998; 24 :189–202. [PubMed: 9613620]
- Alkondon M, Pereira EF, Albuquerque EX. alpha-bungarotoxin- and methyllycaconitine-sensitive nicotinic receptors mediate fast synaptic transmission in interneurons of rat hippocampal slices. *Brain Res.* 1998; 810:257–263. [PubMed: 9813357]
- Armstrong DM, Saper CB, Levey AI, Wainer BH, Terry RD. Distribution of cholinergic neurons in rat brain: demonstrated by the immunocytochemical localization of choline acetyltransferase. *J Comp Neurol.* 1983; 216:53–68. [PubMed: 6345598]
- Aznavour N, Watkins KC, Descarries L. Postnatal development of the cholinergic innervation in the dorsal hippocampus of rat: Quantitative light and electron microscopic immunocytochemical study. *J Comp Neurol.* 2005; 486:61–75. [PubMed: 15834959]
- Barmack NH, Baughman RW, Eckenstein FP, Shojaku H. Secondary vestibular cholinergic projection to the cerebellum of rabbit and rat as revealed by choline acetyltransferase immunohistochemistry, retrograde and orthograde tracers. *J Comp Neurol.* 1992; 317:250–270. [PubMed: 1577999]
- Bialowas J, Frotscher M. Choline acetyltransferase-immunoreactive neurons and terminals in the rat septal complex: a combined light and electron microscopic study. *J Comp Neurol.* 1987; 259:298–307. [PubMed: 3294933]
- Bohnen NI, Muller ML, Kotagal V, Koeppe RA, Kilbourn MA, Albin RL, Frey KA. Olfactory dysfunction, central cholinergic integrity and cognitive impairment in Parkinson's disease. *Brain.* 2010
- Brain KL, Trout SJ, Jackson VM, Dass N, Cunnane TC. Nicotine induces calcium spikes in single nerve terminal varicosities: a role for intracellular calcium stores. *Neuroscience.* 2001; 106:395–403. [PubMed: 11566509]
- Casu MA, Wong TP, De Koninck Y, Ribeiro-da-Silva A, Cuello AC. Aging causes a preferential loss of cholinergic innervation of characterized neocortical pyramidal neurons. *Cereb Cortex.* 2002; 12 : 329–337. [PubMed: 11839606]
- Choi S, Klingauf J, Tsien RW. Postfusional regulation of cleft glutamate concentration during LTP at 'silent synapses'. *Nat Neurosci.* 2000; 3:330–336. [PubMed: 10725921]
- Clarke PB, Reuben M, el Bizri H. Blockade of nicotinic responses by physostigmine, tacrine and other cholinesterase inhibitors in rat striatum. *Br J Pharmacol.* 1994; 111:695–702. [PubMed: 8019748]
- Consonni S, Leone S, Becchetti A, Amadeo A. Developmental and neurochemical features of cholinergic neurons in the murine cerebral cortex. *BMC Neurosci.* 2009; 10:18. [PubMed: 19272148]
- Contant C, Umbriaco D, Garcia S, Watkins KC, Descarries L. Ultrastructural characterization of the acetylcholine innervation in adult rat neostriatum. *Neuroscience.* 1996; 71:937–947. [PubMed: 8684624]
- Coyle JT, Price DL, DeLong MR. Alzheimer's disease: a disorder of cortical cholinergic innervation. *Science.* 1983; 219:1184–1190. [PubMed: 6338589]
- Dajas-Bailador FA, Mogg AJ, Wonnacott S. Intracellular Ca²⁺ signals evoked by stimulation of nicotinic acetylcholine receptors in SH-SY5Y cells: contribution of voltage-operated Ca²⁺ channels and Ca²⁺ stores. *J Neurochem.* 2002; 81:606–614. [PubMed: 12065669]
- Deller T, Katona I, Cozzari C, Frotscher M, Freund TF. Cholinergic innervation of mossy cells in the rat fascia dentata. *Hippocampus.* 1999; 9:314–320. [PubMed: 10401645]
- Descarries L, Gisiger V, Steriade M. Diffuse transmission by acetylcholine in the CNS. *Prog Neurobiol.* 1997; 53:603–625. [PubMed: 9421837]
- Frazier CJ, Buhler AV, Weiner JL, Dunwiddie TV. Synaptic potentials mediated via alpha-bungarotoxin-sensitive nicotinic acetylcholine receptors in rat hippocampal interneurons. *J Neurosci.* 1998; 18:8228–8235. [PubMed: 9763468]
- Frazier CJ, Strowbridge BW, Papke RL. Nicotinic receptors on local circuit neurons in dentate gyrus: a potential role in regulation of granule cell excitability. *J Neurophysiol.* 2003; 89:3018–3028. [PubMed: 12611982]

- Frotscher M, Leranath C. Cholinergic innervation of the rat hippocampus as revealed by choline acetyltransferase immunocytochemistry: a combined light and electron microscopic study. *J Comp Neurol*. 1985; 239:237–246. [PubMed: 4044938]
- Frotscher M, Vida I, Bender R. Evidence for the existence of non-GABAergic, cholinergic interneurons in the rodent hippocampus. *Neuroscience*. 2000; 96:27–31. [PubMed: 10683406]
- Gray R, Rajan AS, Radcliffe KA, Yakehiro M, Dani JA. Hippocampal synaptic transmission enhanced by low concentrations of nicotine [see comments]. *Nature*. 1996; 383:713–716. [PubMed: 8878480]
- Grybko M, Sharma G, Vijayaraghavan S. Functional distribution of nicotinic receptors in CA3 region of the hippocampus. *J Mol Neurosci*. 2010; 40:114–120. [PubMed: 19693709]
- Haydar SN, Dunlop J. Neuronal nicotinic acetylcholine receptors - targets for the development of drugs to treat cognitive impairment associated with schizophrenia and Alzheimer's disease. *Curr Top Med Chem*. 2010; 10:144–152. [PubMed: 20166959]
- Hefft S, Hulo S, Bertrand D, Muller D. Synaptic transmission at nicotinic acetylcholine receptors in rat hippocampal organotypic cultures and slices. *J Physiol (Lond)*. 1999; 515 (Pt 3):769–776. [PubMed: 10066903]
- Henderson Z, Harrison PS, Jagger E, Beeby JH. Density of choline acetyltransferase-immunoreactive terminals in the rat dentate gyrus after entorhinal cortex lesions: a quantitative light microscope study. *Exp Neurol*. 1998; 152:50–63. [PubMed: 9682012]
- Houser CR, Crawford GD, Salvaterra PM, Vaughn JE. Immunocytochemical localization of choline acetyltransferase in rat cerebral cortex: a study of cholinergic neurons and synapses. *J Comp Neurol*. 1985; 234:17–34. [PubMed: 3980786]
- Hurst RS, Hajos M, Raggenbass M, Wall TM, Higdon NR, Lawson JA, Rutherford-Root KL, Berkenpas MB, Hoffmann WE, Piotrowski DW, Groppi VE, Allaman G, Ogier R, Bertrand S, Bertrand D, Arneric SP. A novel positive allosteric modulator of the $\alpha 7$ neuronal nicotinic acetylcholine receptor: in vitro and in vivo characterization. *J Neurosci*. 2005; 25:4396–4405. [PubMed: 15858066]
- Jaarsma D, Dino MR, Cozzari C, Mugnaini E. Cerebellar choline acetyltransferase positive mossy fibres and their granule and unipolar brush cell targets: a model for central cholinergic nicotinic neurotransmission. *J Neurocytol*. 1996; 25:829–842. [PubMed: 9023728]
- Ji D, Lape R, Dani JA. Timing and location of nicotinic activity enhances or depresses hippocampal synaptic plasticity. *Neuron*. 2001; 31:131–141. [PubMed: 11498056]
- Khiroug L, Giniatullin R, Klein RC, Fayuk D, Yakel JL. Functional mapping and Ca^{2+} regulation of nicotinic acetylcholine receptor channels in rat hippocampal CA1 neurons. *J Neurosci*. 2003; 23:9024–9031. [PubMed: 14534236]
- Levey AI, Wainer BH, Rye DB, Mufson EJ, Mesulam MM. Choline acetyltransferase-immunoreactive neurons intrinsic to rodent cortex and distinction from acetylcholinesterase-positive neurons. *Neuroscience*. 1984; 13:341–353. [PubMed: 6514183]
- Levin ED, Rezvani AH. Nicotinic treatment for cognitive dysfunction. *Curr Drug Target CNS Neurol Disord*. 2002; 1:423–431.
- Mansvelder HD, De Rover M, McGehee DS, Brussaard AB. Cholinergic modulation of dopaminergic reward areas: upstream and downstream targets of nicotine addiction. *Eur J Pharmacol*. 2003; 480:117–123. [PubMed: 14623355]
- Matsuo A, Bellier JP, Hisano T, Aimi Y, Yasuhara O, Tooyama I, Saito N, Kimura H. Rat choline acetyltransferase of the peripheral type differs from that of the common type in intracellular translocation. *Neurochem Int*. 2005; 46:423–433. [PubMed: 15737440]
- McGehee DS, Heath MJ, Gelber S, Devay P, Role LW. Nicotine enhancement of fast excitatory synaptic transmission in CNS by presynaptic receptors [see comments]. *Science*. 1995; 269:1692–1696. [PubMed: 7569895]
- Mechawar N, Watkins KC, Descarries L. Ultrastructural features of the acetylcholine innervation in the developing parietal cortex of rat. *J Comp Neurol*. 2002; 443:250–258. [PubMed: 11807835]
- Mesulam MM, Mufson EJ, Wainer BH, Levey AI. Central cholinergic pathways in the rat: an overview based on an alternative nomenclature (Ch1–Ch6). *Neuroscience*. 1983; 10:1185–1201. [PubMed: 6320048]

- Mesulam MM, Volicer L, Marquis JK, Mufson EJ, Green RC. Systematic regional differences in the cholinergic innervation of the primate cerebral cortex: distribution of enzyme activities and some behavioral implications. *Ann Neurol*. 1986; 19:144–151. [PubMed: 3963756]
- Misawa H, Ishii K, Deguchi T. Gene expression of mouse choline acetyltransferase. Alternative splicing and identification of a highly active promoter region. *J Biol Chem*. 1992; 267:20392–20399. [PubMed: 1400357]
- Misawa H, Takahashi R, Deguchi T. Transcriptional regulation of choline acetyltransferase gene by cyclic AMP. *J Neurochem*. 1993; 60:1383–1387. [PubMed: 8384248]
- Montgomery JM, Pavlidis P, Madison DV. Pair recordings reveal all-silent synaptic connections and the postsynaptic expression of long-term potentiation. *Neuron*. 2001; 29:691–701. [PubMed: 11301028]
- Motts SD, Slusarczyk AS, Sowick CS, Schofield BR. Distribution of cholinergic cells in guinea pig brainstem. *Neuroscience*. 2008; 154:186–195. [PubMed: 18222049]
- Naciff JM, Behbehani MM, Misawa H, Dedman JR. Identification and transgenic analysis of a murine promoter that targets cholinergic neuron expression. *J Neurochem*. 1999; 72:17–28. [PubMed: 9886050]
- Newhouse PA, Potter A, Kelton M, Corwin J. Nicotinic treatment of Alzheimer's disease. *Biol Psychiatry*. 2001; 49:268–278. [PubMed: 11230878]
- Ogura T, Margolskee RF, Tallini YN, Shui B, Kotlikoff MI, Lin W. Immuno-localization of vesicular acetylcholine transporter in mouse taste cells and adjacent nerve fibers: indication of acetylcholine release. *Cell Tissue Res*. 2007; 330:17–28. [PubMed: 17704949]
- Papke RL, Kem WR, Soti F, Lopez-Hernandez GY, Horenstein NA. Activation and desensitization of nicotinic alpha7-type acetylcholine receptors by benzylidene anabaseines and nicotine. *J Pharmacol Exp Ther*. 2009; 329:791–807. [PubMed: 19223664]
- Pepou G, Giovannini MG. Cholinesterase inhibitors and memory. *Chem Biol Interact*. 2010; 187:403–408. [PubMed: 19941841]
- Pereira EF, Alkondon M, Reinhardt S, Maelicke A, Peng X, Lindstrom J, Whiting P, Albuquerque EX. Physostigmine and galanthamine: probes for a novel binding site on the alpha 4 beta 2 subtype of neuronal nicotinic acetylcholine receptors stably expressed in fibroblast cells. *J Pharmacol Exp Ther*. 1994; 270:768–778. [PubMed: 8071869]
- Pereira EF, Alkondon M, Tano T, Castro NG, Froes-Ferrao MM, Rozental R, Aronstam RS, Schratzenholz A, Maelicke A, Albuquerque EX. A novel agonist binding site on nicotinic acetylcholine receptors. *J Recept Res*. 1993; 13:413–436. [PubMed: 8450498]
- Rodriguez I, Feinstein P, Mombaerts P. Variable patterns of axonal projections of sensory neurons in the mouse vomeronasal system. *Cell*. 1999; 97:199–208. [PubMed: 10219241]
- Sekirnjak C, Vissel B, Bollinger J, Faulstich M, du LS. Purkinje cell synapses target physiologically unique brainstem neurons. *J Neurosci*. 2003; 23:6392–6398. [PubMed: 12867525]
- Selden NR, Gitelman DR, Salamon-Murayama N, Parrish TB, Mesulam MM. Trajectories of cholinergic pathways within the cerebral hemispheres of the human brain. *Brain*. 1998; 121 (Pt 12):2249–2257. [PubMed: 9874478]
- Sharma G, Grybko M, Vijayaraghavan S. Action Potential-Independent and Nicotinic Receptor-Mediated Concerted Release of Multiple Quanta at Hippocampal CA3-Mossy Fiber Synapses. *J Neurosci*. 2008; 28:2563–2575. [PubMed: 18322100]
- Sharma G, Vijayaraghavan S. Modulation of presynaptic store calcium induces release of glutamate and postsynaptic firing. *Neuron*. 2003; 38:929–939. [PubMed: 12818178]
- Sharma G, Vijayaraghavan S. Nicotinic receptors containing the alpha7 subunit: a model for rational drug design. *Curr Med Chem*. 2008; 15:2921–2932. [PubMed: 19075644]
- Tallini YN, Shui B, Greene KS, Deng KY, Doran R, Fisher PJ, Zipfel W, Kotlikoff MI. BAC transgenic mice express enhanced green fluorescent protein in central and peripheral cholinergic neurons. *Physiol Genomics*. 2006; 27:391–397. [PubMed: 16940431]
- Treloar HB, Feinstein P, Mombaerts P, Greer CA. Specificity of glomerular targeting by olfactory sensory axons. *J Neurosci*. 2002; 22:2469–2477. [PubMed: 11923411]

- Turrini P, Casu MA, Wong TP, De Koninck Y, Ribeiro-da-Silva A, Cuello AC. Cholinergic nerve terminals establish classical synapses in the rat cerebral cortex: synaptic pattern and age-related atrophy. *Neuroscience*. 2001; 105:277–285. [PubMed: 11672595]
- Vogt KE, Regehr WG. Cholinergic modulation of excitatory synaptic transmission in the CA3 area of the hippocampus. *J Neurosci*. 2001; 21:75–83. [PubMed: 11150322]
- von Engelhardt J, Eliava M, Meyer AH, Rozov A, Monyer H. Functional characterization of intrinsic cholinergic interneurons in the cortex. *J Neurosci*. 2007; 27:5633–5642. [PubMed: 17522308]
- Wainer BH, Bolam JP, Freund TF, Henderson Z, Totterdell S, Smith AD. Cholinergic synapses in the rat brain: a correlated light and electron microscopic immunohistochemical study employing a monoclonal antibody against choline acetyltransferase. *Brain Res*. 1984; 308:69–76. [PubMed: 6478204]
- Walz A, Feinstein P, Khan M, Mombaerts P. Axonal wiring of guanylate cyclase-D-expressing olfactory neurons is dependent on neuropilin 2 and semaphorin 3F. *Development*. 2007; 134:4063–4072. [PubMed: 17942483]
- Walz A, Omura M, Mombaerts P. Development and topography of the lateral olfactory tract in the mouse: imaging by genetically encoded and injected fluorescent markers. *J Neurobiol*. 2006; 66:835–846. [PubMed: 16673392]
- Woolf NJ, Eckenstein F, Butcher LL. Cholinergic systems in the rat brain: I. projections to the limbic telencephalon. *Brain Res Bull*. 1984; 13:751–784. [PubMed: 6532518]
- Yamasaki M, Matsui M, Watanabe M. Preferential localization of muscarinic M1 receptor on dendritic shaft and spine of cortical pyramidal cells and its anatomical evidence for volume transmission. *J Neurosci*. 2010; 30:4408–4418. [PubMed: 20335477]

Figure 1. BAC transgenic vector for generation of the ChAT-*tauGFP* mouse

A *tauGFP*-polyA cassette was introduced precisely at the first codon of the ChAT gene coding exon 3 of the BAC clone RP23-431D9, by Red-mediated homologous recombination in *E. coli*. The modified BAC was injected to make transgenic mice.

Figure 2. Expression of *tauGFP* in the brain and peripheral nervous system

A. Thick (250 µm transverse section through the diencephalon showing the bright fluorescence of the habenulo-interpeduncular tract (HabInttr).

B. Ventral view of the forebrain of a ChAT-GFP mouse. Bright GFP fluorescence is evident in the interpeduncular nucleus (nIp). Endogenous GFP fluorescence also is visible in linear arrays associated with the Islands of Calleja within olfactory tubercle (Olf Tub) (See also Fig. 4 A, B). The cholinergic fibers of the parabrachial-collicular tract (PbC) are visible within the optic tracts.

C. Transverse section through the interpeduncular nucleus showing the bright fluorescence of the terminals of the habenlointerpeduncular tract. Fascicles of the oculomotor nerve (N.III) appear lateral to the interpeduncular nucleus.

D. Transverse section through the medial habenular nucleus (mHab) and the habenlointerpeduncular tract.

E. GFP fluorescence in the enteric nervous system of the gut.

F. Fluorescent motor endplate on striate muscle of the tongue.

G. Section through the vallate papilla showing GFP fluorescence in a subset of taste cells (Type III cells) of the taste buds.

Figure 3. *tauGFP* expression in the hindbrain revealed by GFP immunofluorescence

A. Transverse section through the rostral medulla showing the hindbrain motor nuclei and nerve roots. Scattered cholinergic cells also are present within the nucleus of the solitary tract (nTS). DMNX, dorsal motor nucleus of the vagus, nA, nucleus ambiguus, nTS, nucleus of the solitary tract; N X, vagus nerve root; N XII, hypoglossal nerve root; nXII, hypoglossal nucleus. V = 4th ventricle.

B. Transverse section through the level of the pons showing the trochlear nerve root (N IV) and raphe nuclei (nR). Some of the large neurons of the pedunculopontine nucleus (PPn) exhibit immunoreactivity for GFP (green) but most show only ChAT protein immunoreactivity (magenta).

C. Transverse section through the rostral medulla at the level of entrance of the facula nerve (N VII). The motor nucleus (n VI) and root fibers (N VI) of the abducens nerve also are visible at this level. Large mossy fiber terminals are visible within the cerebellar vermis (CbV). Scale same as in A.

D. Higher magnification of the vermis slightly anterior to the level of panel C showing large cholinergic mossy fiber terminals.

Figure 4. Transgene expression in the basal forebrain

Immunofluorescence as revealed by double label with anti-GFP (green) and anti-ChAT (red). Nissl counterstain is shown in blue.

A. Low magnification section through the anterior telencephalon at the level of the septal nuclei. Numerous cells double labeled for ChAT and GFP are visible in the nuc. diagonal band (nDB) and caudoputamen (CP) (shown at higher magnification in panel D). In addition, a dense cholinergic plexus is associated with the Islands of Calleja (ICj) within the ventral pallidum and olfactory tubercle (Olf Tu). AC, root of the anterior commissure.

B. Higher magnification of the ventromedial portion of the telencephalon showing dense cholinergic innervation of the ventral pallidum and olfactory tubercle.

C. High magnification of a section through frontal cortex, pia is shown at the top. Small cholinergic interneurons of the cortex (magenta arrows) show immunoreactivity for ChAT protein, but lack reactivity for GFP. In contrast the dense fiber plexus of layer I shows robust GFP and ChAT immunoreactivity.

D. High magnification image of the large cholinergic cells of the caudoputamen. The dense plexus of cholinergic fibers within this structure is readily apparent in these tauGFP transgenic animals.

Figure 5. tauGFP positive fiber distribution in the hippocampus

A. Low magnification image of a hippocampus from an adult mouse. Left panel: an image of the entire hippocampus, stained with anti-GFP antibody, showing the distribution of tauGFP. Scale Bar 250 μ m Right panel: Green- Cholinergic fibers stained with anti-GFP antibody. Red- staining with anti-ChAT antibody. Yellow – ChAT- and GFP-positive structures. Blue- DAPI staining to label the cellular layers. There is a widespread distribution of cholinergic fibers throughout the hippocampus. Higher density of staining can be observed adjoining the CA3 pyramidal and granule cell layers. Scale Bar- 200 μ m

B. Higher magnification images of the CA3 region. Green- Anti-GFP Ab staining. Blue- Nissl staining. Left panel shows the fiber distribution in the stratum lucidum (SL). A cholinergic interneuron is visible in this image. Right panel; the pyramidal cell layer showing the highly intercalated, punctate cholinergic fibers suggesting the existence of somatic synapses (SO - stratum oriens). Scale Bar. 20 μ m

C. Left panel: The dentate granule cell layer (GCL) showing a similar diffuse arrangement of cholinergic fibers in the cellular layer. Right panel: The hilus (H) and the molecular layer (ML) showing relatively high expression of fibers. Scale Bar. 20 μ m

Figure 6. Imaging calcium changes in CA3 pyramidal neurons to electrical stimuli

A. Left panel: Pseudocolor image of fura 2 stained CA 3 pyramidal neurons (Red) from an acute hippocampal slice. Middle panel: GFP fluorescence (Green) from the same field. Note the highly branched nature of the processes. Right panel: A merged image showing the fibers and the fura 2 loaded pyramidal neurons.

B. Same merged image as in A. showing the position of the stimulating pipette and cells that responded to the stimulus (yellow) and some of the neurons that did not respond (white).

C. Stimulus evoked calcium transients from the responding neurons (Left traces) and from neurons that did not respond (Right traces). Responding neurons showed rapid calcium transients in response to axonal stimulation.

D. Two panels (under $20\times$ magnification) showing the position of the stimulation pipette placed either in the stratum oriens (Left panel) or in stratum lucidum (Middle panel). Red arrows indicate positions of the responding pyramidal neurons. Right panel: Data showing the distance (in μm) of neurons from the stimulating pipette. Y-axis represents the responders (1) and non-responders (0). Of a total of 146 neurons analyzed from the two fields, only six responded, and these were scattered across the field. Two of the responding neurons had identical distances from the pipette.

Figure 7. Properties of stimulus evoked calcium transients in CA3 pyramidal neurons

A. Calcium transients, from two cells (Left and Right panel), evoked by single 100 μ s pulses given 10 s apart. While a single stimulus gave a rapid time locked (within 1 s) signal, there were distinct failures and the signals were also accompanied by spontaneous calcium oscillations.

B. Left panel: Calcium transients averaged from 21 neurons (bars show SEM), in response to 100 pulses given at 100 Hz. The stimulus paradigm reliably elicited time locked signals. Right panel: The calcium transients were rapid and decayed in a biphasic manner with τ_{fast} of 1.7 s and a τ_{slow} of 10 s.

C. The small numbers of responding neurons were not due to lack of nAChRs in the others. Top pair; response from a pyramidal neuron to an electrical stimulus and to a 5 s application of 5 μ M nicotine. Bottom pair: a different neuron, not responding to an electrical stimulus but nonetheless showing a robust transient in response to nicotine application.

D. Left panel- Response of a single neuron in the absence (Black), or presence (Red) of 200 μ M mecamylamine. There was a significant attenuation of the response in the presence of the general nAChR antagonist. Right panel- Compilation of mecamylamine inhibition. Normalized data from 19 cells-Control (open bars), mecamylamine (hatched bars). The responses were integrated from onset to 5 s (Fast), from 5 s to 40 s (Slow). All components were significantly blocked by the antagonist (***- $p < 0.001$).

Figure 8. Synaptic EPSCs evoked by stimulation of cholinergic fibers

A. Left: Evoked EPSCs on CA3 pyramidal neurons. A series of 10 responses to focal stimulation (100 μ s). Right: Amplitude distribution of 236 individual EPSCs.

B. Left trace: Calcium signals from individual excitation wavelengths (340 nm & 380 nm) showing changes in the opposite direction. Middle trace: Change in Ratio from the same pyramidal neuron. Right trace: the same neuron under voltage clamp reveals a large and fast EPSC in response to a single 100 μ s pulse at 80 μ A intensity.

C. Position specificity of the evoked responses. An evoked response was elicited by placing the stimulation pipette on a cholinergic axon (position 1 in the trace). The stimulation pipette was then moved from its original place to a position approximately equidistant from the recorded neuron (position 2 in the trace). The response was abolished. Returning the pipette to its original position (position 3 in the trace) restored the current.

Figure 9. Pharmacology of the evoked responses

A. Evoked responses from a CA3 pyramidal neuron and a hilar interneuron to a single 100 μ s stimulus. **Stim**- the first stimulated response. **+MLA**- stimulation immediately following a 20 s application of 50 nM MLA using a puffer pipette aimed at the cell soma. **Wash**- responses after 1-2 minutes of washout.

B. Inhibition by MLA in single neurons. In both pyramidal neurons ($n = 4$) and interneurons ($n = 5$) MLA completely attenuated the responses. In two neurons from each cell type, partial to complete recovery was observed upon washout of the antagonist.

C. Left traces: Responses from a pyramidal neuron in the absence (Control) and the presence (+ PNU) of 10 μ M PNU 120596. The neuron was pre-incubated with the drug for 15 minutes. PNU significantly increased the amplitude of the responses. Right traces: Normalized responses from the two conditions showing no significant changes in the decay kinetics.