Published in final edited form as:

J Mol Neurosci. 2010 January ; 40(1-2): 114–120. doi:10.1007/s12031-009-9266-8.

Functional Distribution of Nicotinic Receptors in CA3 Region of the Hippocampus

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Abstract

Nicotinic acetylcholine receptor (nAChR) modulation of a number of parameters of synaptic signaling in the brain has been demonstrated. It is likely that effects of nicotine are due to its ability to modulate network excitability as a whole. A pre-requisite to understanding the effects of nicotine on network properties is the elucidation of functional receptors. We have examined the distribution of functional nAChRs in the dentate gyrus granule cells and the CA3 region of the mammalian hippocampus using calcium imaging from acute slices. Our results demonstrate the presence of functional nAChRs containing the $\alpha 7$ subunit ($\alpha 7$ -nAChRs) on mossy fiber boutons, CA3 pyramidal cells, and on astrocytes. In addition, both CA3 interneurons and granule cells show nicotinic signals. Our study suggests that functional nicotinic receptors are widespread in their distribution and that calcium imaging might be an effective technique to examine locations of these receptors in the mammalian brain.

Keywords

nAChRs; CA3 pyramidal neurons; Granule cells; Nicotinic; Hippocampus

Introduction

Nicotine, an addictive substance and a key component of cigarette smoke, places a tremendous burden on healthcare systems. The drug has also been shown to positively modulate a number of brain functions linked to cognition. It improves attention, arousal, learning, learnt discrimination, and memory functions (Karczmar 1993; Narahashi et al. 2000; Rezvani and Levin 2001). The action of the drug is due to its ability to activate a class of ligand-gated ion channels, the nicotinic acetylcholine receptors (nAChRs). A key subtype of this receptor class, which is widely distributed in the brain, contains the $\alpha 7$ gene product assembled as a pentameric homomer ($\alpha 7$ -nAChRs). The $\alpha 7$ -nAChRs exhibit a high permeability for calcium and effectively raise intracellular free calcium levels ($[Ca^{2+}]_i$), mainly by downstream amplification of signals (Vijayaraghavan et al. 1992; Sharma and Vijayaraghavan 2001; Sharma et al. 2008). Downstream calcium signaling is likely to be an important player in nicotine-mediated synaptic plasticity in the brain.

Here, we demonstrate that the CA3 pyramidal cells, interneurons, and dentate granule cells, exhibit calcium transients due to the activation of nAChRs. In pyramidal neurons, the signal

was mainly due to the activation of $\alpha 7$ -nAChRs. In addition, our previous data also suggest that hippocampal astrocytes and presynaptic mossy fiber boutons (MFBs) possess functional $\alpha 7$ -nAChRs (Sharma and Vijayaraghavan 2001). Our data result in two important findings on the effects of nAChRs on hippocampal functions—(1) functional nAChRs are more widespread than previously thought, necessitating a re-examination of their effects on circuit properties, and (2) in some cases, calcium imaging might be a more sensitive detector than electrophysiology for demonstrating the distribution of functional nAChRs.

Materials and Methods

Materials

All chemicals were obtained from Sigma (St. Louis, MO, USA) or Tocris (Ellisville, MO, USA). Tetrodotoxin was purchased from EMD Biosciences (San Diego, CA, USA), and dyes were purchased from Invitrogen (Carlsbad, CA, USA).

Methods

Hippocampal slices—Parasagittal hippocampal slices, 300 μ m thick, were made as described previously from our lab (Sharma and Vijayaraghavan 2003; Sharma et al. 2008). Slices were prepared from 12- to 16-day-old Sprague–Dawley rats using a Leica VT1000S vibratome, or DTK-zero 1 (Dosaka, Kyoto, Japan), in accordance with institutional guidelines. Slices were then incubated in a bicarbonate based medium (aCSF) containing 120 mM NaCl, 3.5 mM KCl, 26 mM NaHCO₃, 1.25 mM NaH₂PO₄, 10 mM glucose, 2.5 mM MgCl₂, and 1 mM CaCl₂, for at least an hour before the experiment.

Calcium Imaging—Loading of acute slices with the calcium-sensitive dye fura 2-AM and the acquisition of images were done as described (Sharma et al. 2008). Briefly, slices were incubated with 20 μ M fura 2-AM in 0.2% Cremaphor ES for 1 h before taking them for imaging. Imaging was carried out using a Zeiss Axioskop II upright microscope fitted with a CCD camera (Cooke Sensicam) and a Sutter DG IV wavelength switcher. Images were acquired using Slide-Book (Intelligent Imaging Innovations, Denver, CO, USA) and analyzed using Microcal Origin software. Neurons were identified based on location, size, and morphology. Agents were applied for the times stated using a patch pipette as described (Sharma et al. 2008).

Statistics—Statistical significance of all comparisons was evaluated by paired or independent Student *t* tests. Significance was taken at $p < 0.05$.

Results and Discussion

Modulation of presynaptic glutamate release at the principal synapse in the CA3 region, that between the large MFB and the pyramidal cell, is a powerful site for nAChR-mediated alterations in synaptic plasticity in the region. Activation of presynaptic $\alpha 7$ -nAChRs at the MFB, by 20 μ M nicotine or 100 μ M ACh in the presence of 0.5 μ M atropine (ACh/At), causes a dramatic burst of glutamatergic miniature excitatory postsynaptic currents (mEPSCs) recorded from the pyramidal neuron. The onset of this burst of glutamate release is abrupt, and the peak frequency rises to an average of 51-fold above that obtained under control conditions (Sharma and Vijayaraghavan 2003). The burst is initiated by influx of calcium through the $\alpha 7$ -nAChR ion channel, and not through voltage-gated calcium channels (VGCCs). This influx then triggers the release of calcium via the activation of ER ryanodine receptors (RyRs) by calcium-induced calcium release (CICR). This mechanism forms an effective means for the amplification of $\alpha 7$ -nAChR calcium signals.

In addition to the increased release frequencies, the nicotine-induced mEPSC amplitudes were increased as well. The peak amplitudes of mEPSCs ranged from 8 pA (the mode of basal amplitude distribution was 13 pA) to >300 pA, with the average falling at 56 pA, compared to 20 pA under control conditions (Sharma and Vijayaraghavan 2003). The large increase in mEPSC amplitude outlasted the high frequency burst and, like the frequency effect, showed an absolute requirement for ER store calcium release.

Several lines of evidence suggest that the increase in amplitude was not due to postsynaptic alteration in glutamate receptor properties. (1) Chelating postsynaptic calcium with 10 mM BAPTA did not alter the $\alpha 7$ -nAChR effect on the mEPSCs, even though under these conditions other calcium-dependent postsynaptic changes in synaptic currents were reduced or abolished (Yeckel et al. 1999). (2) Rise times of the mEPSCs scaled linearly with amplitude, suggesting that larger amplitude responses might arise due to multiple release events slightly staggered in time (mean stagger of $\sim 100\mu\text{s}$). (3) Slowing release rates by conducting experiments at lowered temperatures (15°C) caused an increase in mEPSC frequencies without any change in amplitudes. This would be expected if concerted release of multiple quanta was required for the amplitude effect because slowing down release rates would dissociate multiple events that would now appear as smaller discernible individual mEPSCs. Among the few large amplitude mEPSCs observed, all had discernible inflections along their rising phase, presumably due to slowed summations of multiple events. (4) The mEPSCs arising from the MFB-CA3 synapse are identifiable because they contain both AMPA and Kainate components. Examining individual contributions of the AMPA and Kainate currents to the mEPSCs, we found that the proportion of the two were identical in both the small and large events (Sharma et al. 2008). This makes a postsynaptic mechanism for the amplitude increase less likely as this would have to arise from an identical modulation of two separate receptor classes.

However, the amplitude increase, in addition to ER store calcium release, also requires the activation of downstream calcium/calmodulin-dependent protein kinase II (CaMKII) activation (Fig. 1).

Based on our data, we conclude that nicotine, via the activation of $\alpha 7$ -nAChRs, causes multiple quanta to be released in a concerted fashion. The MFB is a complex terminal, which forms multiple contacts with a single postsynaptic pyramidal neuron. A single bouton can have as many as 30 active zones, easily allowing for the release of multiple quanta across active zones. Depending on the location of calcium stores in these terminals, calcium release via the activation of RyRs can simultaneously increase calcium around a number of active zones making synchronous release of multiple quanta possible. Alternately, CaMKII-dependent modification of release vesicles might allow for multivesicular release, where multiple vesicles are exocytosed from a single active zone (see Fig. 1).

All the effects described above were mimicked, at physiological temperatures, by a low concentration of nicotine ($0.5\mu\text{M}$), in the range found in the blood of smokers (Henningfield et al. 1993; Sharma and Vijayaraghavan 2008).

A most unusual finding was that the nicotine-mediated mEPSC burst was sufficient to trigger a burst of action potentials (APs) from the postsynaptic neuron (Sharma and Vijayaraghavan 2003; Sharma et al. 2008); this is the first instance of synaptic transmission in the absence of an incoming presynaptic AP. This firing of the postsynaptic neuron occurs only at a time when both the increase in frequency and amplitude are maximal. This implies that the unique short-term plasticity triggered by nicotine at this synapse occurs due to a coincidence of two $\alpha 7$ -nAChR-dependent processes—calcium-dependent increase in release frequency and a calcium- and CaMKII-dependent concerted release of multiple quanta (Fig. 1).

The triggering of synaptic transmission, and consequently, changes in synaptic strength that occurs in the absence of a physiological context, suggests that nicotine effectively “hijacks” this synapse (Fig. 1). Such an effect would imply that stability of these altered synapses now depends on the presence of the drug, and withdrawal of which would lead to destabilization. Whether such an effect is universal among CNS synapses remains untested. If true, such a mechanism would be one of the mechanisms mediating addiction to the drug.

$\alpha 7$ -nAChRs on Astrocytes

Our work also shows that functional $\alpha 7$ -nAChRs are present in purified hippocampal astrocytes (Sharma and Vijayaraghavan 2001). While the current density in these cells is extremely low, application of either ACh/At or nicotine gave a robust calcium response, measured using the calcium-sensitive dye fluo 3. Again, in what appears to be a consistent theme in $\alpha 7$ -nAChR signaling, influx of calcium through the receptor itself did not significantly contribute to the calcium response. Instead, signals observed by us arose from CICR via ER calcium release. In these cells, observed signals were dependent on further calcium release from IP3 receptor activation. Our results suggest a complex interplay of calcium signals triggered by $\alpha 7$ -nAChRs on present astrocytes. This interplay results in nicotine-induced regenerative propagation of calcium transients within and across astrocytes (Sharma and Vijayaraghavan 2002). Initial experiments in acute slices also indicate the presence of $\alpha 7$ -nAChR-mediated calcium signals from astrocytes in the CA3 region of acute slices.

Studies over the last decade have implicated astrocytes as active partners in synaptic signaling (Haydon 2001). Activation of the glial cells can synchronize local networks and regulate neuronal activity (Fellin et al. 2004). The current view is that astrocytes might act as pacemakers, controlling the balance between excitation and inhibition of networks (Fellin et al. 2006). nAChRs on these cells could, therefore, contribute to this control of network excitability. Interestingly, evidence also implicates astrocytes in mechanisms underlying drug addiction (for review, see Haydon et al. 2008). Disruption of astrocytic function alters sensitivity to drugs of abuse, such as cocaine (Bainton et al. 2005). This emerging area of research emphasizes the idea that a complete understanding of the action of drugs of abuse will require taking into account the role of glial cells as well as neurons.

Functional $\alpha 7$ -nAChRs in CA3 Pyramidal Neurons

Responses directly relevant to the CA3-MFB circuit were measured viz. pyramidal neurons, stratum lucidum interneurons, and dentate granule cells. Acute hippocampal slices were loaded with fura 2-AM as described under “Methods”. For these experiments, slices were bathed in 10 μ M DNQX, 100 μ M APV, 10 μ M gabazine, and 1 μ M tetrodotoxin to block ionotropic glutamate receptors, GABA A receptors, and action potentials, respectively. All experiments were carried out at room temperature.

A 10-s application of 5 μ M nicotine elicited calcium transients from all neurons tested (Fig. 2a–c). Neither the actual concentration of the agonist at receptor sites nor the rate of washout are determinable, though evidence exists that the drug might be concentrated in the brain achieving levels that are up to 5-fold that of the serum (Ghosheh et al. 2001). However, we have previously shown that actions of 20 μ M nicotine can be effectively mimicked by biologically relevant concentrations (0.5 μ M) of nicotine at physiological temperatures (Sharma et al. 2008). The current experiments were carried out at room temperature to minimize spontaneous activity, and cells showing calcium oscillations at showing calcium oscillations at room temperature were discarded, making the demonstration of $\alpha 7$ nAChR transients reliable.

Only neurons that responded with a rapid calcium transient to a 10-s application of 75 mM KCl (HiK) at the end of each measurement were taken for analyses to ensure the exclusion of data from unhealthy neurons. The average peak calcium response, measured as the percent change (from resting fluorescence) in the ratio of emission from 340- and 380-nm excitation, was $13 \pm 1.2\%$, $17 \pm 1.28\%$, and 5.5 ± 2.4 (mean $\pm$ SEM from pyramidal cells, interneurons, and granule cells respectively; $p < 0.01$ pyramidal cells vs. granule cells and interneurons vs. granule cells). The interneuron and pyramidal cell responses were not statistically different from each other; $p = 0.6$). To rule out changes in peak due to alterations in the shape and duration of the transients, a number of kinetic parameters were evaluated (Fig. 2d). The integral of the first 90 s of the calcium transient was taken as the total response from a given cell. As expected from the peak response data, only the responses from granule cells was significantly different from those elicited from the other two neuronal types ($p < 0.01$ granule cell vs. pyramidal neurons or interneurons). The differences in nAChR-mediated calcium transients were probably not due to differences in calcium handling capacity of the neurons because both the time to peak and the decay time constant of the transients were not significantly different (Fig. 2d, $p = 0.2-0.6$, unpaired t test). Further, none of the parameters (Peak, Area, Time to peak, Peak, and Decay time constant) measured for the HiK-induced transient were significantly different between granule cells, pyramidal neurons, and interneurons (Fig. 2d). These results suggest that the difference in the peak and total responses to exogenous nicotine must arise from direct differences in nAChR expression/signaling.

We examined the nAChR responses from CA3 pyramidal neurons in detail. A 10-s application of $100 \mu\text{M}$ glutamate, in the presence of DNQX and APV, resulted in attenuation of calcium increase from pyramidal neurons. In the presence of these antagonists, responses to the exogenous glutamate were inhibited by $82 \pm 5\%$. Upon washout of the antagonist, the response recovered to $74 \pm 12\%$ of the first response ($n = 5$ neurons; $p < 0.01$ block vs. recovery; Fig. 3a). These results demonstrate that the glutamate receptors are effectively blocked, and thus, nicotine-induced calcium changes are not due to enhancement of glutamate release and subsequent activation of postsynaptic GluRs. This would also be expected from our previous finding that the mEPSC burst requires a more prolonged application of nicotine and occurs after a delay of many seconds (Sharma and Vijayaraghavan 2003; Sharma et al. 2008).

A second response to $5 \mu\text{M}$ nicotine after a 30-min recovery period resulted in transients that were $87 \pm 14\%$ of the first response ($n = 23$; $p = 0.6$). However, after a 30-min incubation with 100 nM αBTX , nicotine responses were reduced to $14.3 \pm 2.5\%$ of the control ($n = 23$; $p < 0.001$ before vs. after; Fig. 3b). These results suggest that calcium responses from CA3 principal neurons, to nicotine application, arise from the activation of $\alpha 7$ -nAChRs.

Interestingly, responses of the CA3 pyramidal cells to $5 \mu\text{M}$ nicotine were different depending on whether agonist was applied for 1 or 10 s. Cells were considered responders if the change in ratio (ΔR) upon agonist application exceeded 3-fold over baseline RMS noise. While all pyramidal neurons responded to a 10-s application of the agonist, only 65% responded to the shorter application ($n = 25$ and 19 , respectively). Selecting only the responders, the peak response to a 1-s application was 53%, and the total fluorescence change (obtained by taking the integral of the first 90 s of the response) was 27% of the 10-s response ($p < 0.01$ and $p < 0.0001$; respectively). The half time for decay was 9.5 ± 1.8 and 21 ± 2.2 s for the 1- and 10-s responses ($p < 0.005$). Whether these differences arise from better access at 10 s or longer lasting calcium response remains to be seen. The latter possibility would be consistent with the much slower transients seen with 30-s application at MFBs observed by us (Sharma and Vijayaraghavan 2003; Sharma et al. 2008), as well as the prolonged presence of nicotine required for many downstream calcium-dependent effects of $\alpha 7$ -nAChRs (Berger et al. 1998; Dajas-Bailador et al. 2000; Hu et al. 2002).

Taken together, our data suggest that functional nAChRs are present in all the components of the CA3 circuit tested so far. Previous studies have concluded that functional $\alpha 7$ -nAChRs were absent from CA3 pyramidal neurons and dentate granule cells (Sharma and Vijayaraghavan 2003; the CA3 circuit tested so far). Previous studies have concluded that functional $\alpha 7$ -nAChRs were absent from CA3 pyramidal neurons and dentate granule cells (Sharma and Vijayaraghavan 2003; Frazier et al. 2003; Sharma et al. 2008). In the CA1 region of the hippocampus, either no (Khiroug et al. 2003) or small (Ji et al. 2001) responses have been reported from pyramidal neurons consistent with our electrophysiological data obtained with regard to the CA3 pyramidal neurons. At the same time, $\alpha 7$ -nAChR currents elicited from interneurons of the hippocampus have been demonstrated from both the CA1 and the dentate region (Klein and Yakel 2005). As rapid agonist application and washout are difficult to achieve in acute slices, a part of the difficulty in detecting small $\alpha 7$ -nAChR currents in pyramidal neurons and granule cells might be due to the rapid desensitization of $\alpha 7$ -nAChRs (Zhang et al. 1994). A second issue in slices is one of access and location. If the nAChRs are located on processes away from the recorded neuron or non-planar, it might be harder to detect agonist-evoked currents. This remains to be addressed. The ability of calcium signals to be amplified by depolarization, followed by activation of VGCCs (Vijayaraghavan et al. 1992) as well as downstream activation of calcium release from the endoplasmic reticulum (Sharma and Vijayaraghavan 2003; Sharma et al. 2008), makes calcium imaging potentially more reliable. Indeed, our results suggest that this is a valid assumption.

$\alpha 7$ -nAChR currents have been recorded from all hippocampal neurons maintained in dissociated cell culture (Bonfante-Cabarcas et al. 1996; Gray et al. 1996; Alkondon et al. 1996; Radcliffe et al. 1999; Zarei et al. 1999). This discrepancy between experiments in acute slices and cell culture has been interpreted as a consequence of either culture conditions or difference in developmental time courses. Stating that better access to receptors and the ability to rapidly apply agonists in cell cultures might explain part, if not all, of this discrepancy.

On the basis of our results, a complex picture of nAChR function in the hippocampal CA3 area has emerged. The widespread distribution of these receptors (Fig. 4) in the region suggests influences on the entire network. This brings up the issue of relevance and physiological function for these receptors. One interpretation of this ubiquitous distribution across cell types is that by modulating the excitability of an entire network, nAChRs serve to modulate the gain function for a network, achieving an overall increase in arousal and allowing for better discriminability of incoming information. Such a sensitivity control might underlie nAChR effects on attention and learning. The diffuse innervation of all cortical areas by basal forebrain cholinergic innervation is consistent with this idea. Evidence for this hypothesis will require a detailed examination of the mechanics of cholinergic transmission in the CNS. The availability of a transgenic mouse model where cholinergic axons are tagged with GFP to allow visualization in live slices will aid in this endeavor. This is currently being developed in our lab.

Acknowledgments

Funding was provided by the National Institute for Drug Abuse (RO1 DA 10266), the National Institute of Deafness and Communication Disorders (RO1 DC 008855), and a Scientist Development Grant from the American Heart Association (GS).

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Figure 1.

$\alpha 7$ -nAChR-mediated short-term plasticity at the MFB-CA3 pyramidal neuron synapse. Agonist-dependent activation $\alpha 7$ -nAChRs at the terminal of MFB allows calcium influx into the terminal. Incoming calcium transient activates RyRs (cyan cylinders), presumably in the ER, releasing store calcium by CICR (green arrows). Released calcium can trigger exocytosis of single glutamate containing vesicles or, in conjunction with activated CaMKII, results in concerted release of multiple vesicles, resulting in small amplitude and large amplitude mEPSCs, respectively. Coincident increase in mEPSC frequencies and amplitudes drives the postsynaptic neuron above its firing threshold, resulting in a burst of APs. Nicotine induces a

presynaptic AP-independent form of synaptic transmission at the MFB-CA3 pyramidal cell synapse

Figure 2.

Calcium responses to a 10-s application of 5 μ M nicotine. **a** *Left trace* Calcium transient from a CA3 pyramidal neuron. *Right trace* same neuron responding to a 10-s application of 75 mM KCl (HiK). **b** Calcium transient in response to nicotine and HiK from a stratum lucidum interneuron. **c** A dentate granule neuron responds to a 10-s application of nicotine and HiK. **d** *Left graph* Compiled kinetics from pyramidal cells (black bars), interneurons (unfilled bars), and granule neurons (hatched bars), showing the peak response, time to peak, the integral of the response, and the decay time constants. The peak and area were significantly different only for granule neurons ($p < 0.01$, compared to both pyramidal cells and interneurons). The rise times and decay times were not significantly different. *Right graph* Same neurons showing

that the HiK responses were not significantly different between the three cell types for any of the parameters tested

Figure 3.

CA3 pyramidal neurons express functional $\alpha 7$ -nAChRs. **a** *Left* Image of a field of pyramidal neurons loaded with the calcium indicator fura 2. *Middle trace* Response of a pyramidal neuron to 100 μ M glutamate applied for 10 s (*black trace*) after incubation with 10 μ M DNQX and 100 μ M APV (*red trace*) and after a 20-min washout period (*green trace*). A HiK response from the same neuron obtained at the end of the experiment is shown alongside. *Right graph* Compiled responses from six neurons demonstrating block of the glutamate response with the antagonists ($p < 0.01$, block compared to wash). **b** *Left traces* Two CA3 pyramidal neurons (*black and green traces*) responding to 100 mM ACh in the presence of 1 μ M atropine (ACh/At). Both neurons show calcium transients in response to the agonist. The responses are completely blocked after the slice had been incubated with 100 nM α BTX for 30 min. *Right graph* Compiled α BTX effect. Two responses to ACh/At (ACh/At 1 and ACh/At2) demonstrating the consistency of the nicotinic response. Incubation with 100 nM α BTX completely abolishes the responses ($n = 17$; $p < 0.001$, compared with the second response)

Figure 4.

A putative model for nAChR control of the CA3 network. Functional nAChRs are present in granule cells (*G*; *cyan*), CA3 interneurons (*Int*; *green*), pyramidal neurons (*P*; *red*), and astrocytes (*A*; *blue*). In addition, nAChRs are also located on synaptic terminals of the MFB and, possibly, the GABAergic synapses between interneurons and pyramidal cells. Nicotine excites the entire CA3 networks, probably altering the activation threshold of the circuit as a whole, resulting in greater arousal and, possibly, more efficient acquisition of information