Title: The Dominant Functional Nicotinic Receptor in Progenitor Cells in the Rostral Migratory Stream is the α3β4 Subtype.

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Abstract:

Addition of newly generated neurons into mature neural circuits in the adult CNS responds to changes in neurotransmitter levels and is tightly coupled to the activity of specific brain regions. This postnatal neurogenesis contributes to plasticity of the olfactory bulb and hippocampus and is thought to play a role in learning and memory, context and odor discrimination, as well as perceptual learning. While acetylcholine plays an important role in odor discrimination and perceptual learning, its role in adult neurogenesis in the olfactory bulb has not been elucidated. In this study, I have examined the functional expression of nAChRs in progenitor cells of the rostral migratory stream (RMS) in the adult olfactory bulb of mice. I show that most of these cells in the RMS exhibit large nAChR-mediated calcium transients upon application of acetylcholine (ACh). Unlike in the hippocampus, the predominant functional nAChRs on progenitor cells are of α3β4 subtype. Interestingly, functional receptor expression is lost once progenitor cells mature, and are incorporated into the granule cell layer. Instead, nAChRs are now expressed on some presynaptic terminals and modulate glutamate release on to granule cells. My results imply that acetylcholine is a part of the permissive niche and likely plays a role in development of progenitor cells.

Key Words: Cholinergic, Adult Neurogenesis, RMS, Olfactory bulb, nAChRs
Introduction:

New neurons are continuously integrated into existing neuronal circuits in at least two regions of the adult central nervous system: the dentate gyrus and olfactory bulb. While the extent of adult neurogenesis in humans remains controversial (Sanai et al. 2011; Macklis 2012; Bergmann et al. 2012), a number of studies have shown that in rodents, adult neurogenesis contributes to the plasticity of the network in both these areas (Schmidt-Hieber et al. 2004; Lledo et al. 2006; Gao and Strowbridge 2009; Nissant et al. 2009). Functional relevance of this process, however, remains unresolved. Adult neurogenesis has been implicated in physiological processes such as learning and memory (Imayoshi et al. 2008; Deng et al. 2009; Alonso et al. 2012), and odor (Gheusi et al. 2000) and context discrimination (Aimone et al. 2011; Kheirbek et al. 2012) but mechanistic information regarding progenitor regulation of these processes is not understood. Much of the focus has been on adult neurogenesis in the hippocampus and little is known regarding the significance and control of the process in the olfactory bulb (OB). Newborn cells in the sub-ventricular (SVZ) region migrate along the Rostral Migratory Stream (RMS) into the OB, where they form GABAergic granule cells (GCs) and periglomerular (PG) cells surrounding the glomerular neuropil (Merkle et al. 2007; Lledo et al. 2008). Olfactory perceptual learning is diminished in mice where neurogenesis is disrupted (Moreno et al. 2009; Lazarini and Lledo 2011) and mice with impaired neurogenesis show deficits in social recognition, though maternal recognition of pups remains intact (Mak and Weiss 2010; Feierstein et al. 2010). These results suggest an important role for neurogenesis in the adult mammalian OB but the mechanisms that control the proliferation and differentiation of these cells have yet to be understood.

A key modulator of olfactory perceptual learning is acetylcholine (ACh). Studies indicate that blocking either muscarinic AChRs or nicotinic AChRs (nAChRs) can disrupt odor discrimination in mice (Mandairon et al. 2006; Hellier et al. 2012). Whether part of the effects of
this transmitter could be via its modulation of neurogenesis in the OB is yet to be examined.

Cholinergic fibers are present adjacent to newly generated neurons in the olfactory bulb (Whitman and Greer 2007) but unlike receptors for GABA (Liu et al. 2005) and glutamate (Platel et al. 2010a; Platel et al. 2010b), presence of acetylcholine receptors on progenitor cells has not been reported. There is evidence, from the hippocampus, that AChRs might play a role in neurogenesis where α7 and β2 subunit based nAChRs have been shown to modulate progenitor survival (Kaneko et al. 2006; Kotani et al. 2008) and differentiation (Campbell et al. 2010; Lozada et al. 2012). Adult neuronal progenitors isolated from the hippocampus show increased apoptotic cell death when exposed to low levels of nicotine (Berger et al. 1998) by a mechanism mediated by changes in intracellular free calcium ([Ca]i). While cholinergic modulation of neurogenesis in the olfactory bulb has not been examined systematically, it has been reported that increasing acetylcholine levels by blocking acetylcholine esterase can enhance the survival of progenitor cells in OB (Kaneko et al. 2006). Contrary to expectation from this study, a second report (Mechawar et al. 2004) shows that knocking out of β2 subunit of nAChRs, thus decreasing cholinergic influence, results in an increase in the number of granule cells.

The studies summarized above have raised a number of questions. Is the effect of acetylcholine on progenitor cells cell-autonomous? What receptor subtypes mediate these effects? Are these effects mediated, in part, by the ability of nAChRs to alter [Ca]i and activate downstream calcium-dependent signaling cascades? How does functional nAChR expression change as progenitor cells migrate from the RMS into the OB cellular layers? A pre-requisite to answering many of these questions is to know where functional nAChRs are expressed in the migratory pathway and whether there is a change in the receptor expression as incoming progenitor cells migrate into the granule cell (GC) layer of the OB.
Here I address this issue and show that functional nAChRs are expressed in the migrating progenitor cells. These receptors belong to the α3β4 subclass of nAChRs and are efficient at raising [Ca]. I further show that once these cells form mature granule cells, the functional receptor is lost and a large fraction of cholinergic signals arises from presynaptic mechanisms via transmitter release.
Methods:

Olfactory bulb slices and identification of progenitor cells: All experiments were performed under protocols approved by University of Colorado Anschutz Medical Center Institutional Animal Care and Use Committee. C57bl/6 Mice, between ages of 6 to 8 weeks, were housed in ventilated cages in a 10:14 light:dark cycle and given food and water ad libitum. Progenitor cells were identified by either (a) Position of RMS at the center (ependymal layer) of the bulb. We find that most cells in this area are progenitor cells and express doublecortin- a marker for immature neurons. (b) Specific labeling with GFP using replication deficient Lentiviral particles (~10^{10}/ml from Viral Vector Core at Univ. of Pennsylvania). 0.5 to 1 μl virus was injected into the rostral migratory stream using a Kopf Steretoxic instrument at coordinates 3.3A, 0.82L, -2.9 V (Lledo et al. 2008). Mice were euthanized after 8 to 10 days. GFP expressing cells at the elbow, or further along the RMS, were identified and used for recordings.

300 μm sagittal slices of the brain, including the olfactory bulb and RMS, were made using standard protocols (Sharma and Vijayaraghavan 2003; Panzanelli et al. 2009) on a Leica VT1000S vibratome. Cutting solution contained (in mM) 72 Sucrose, 83 NaCl, 1.25 NaH2PO4, 25 NaHCO3, 2.5 KCl, 10 glucose, 3 MgCl2, 0.5 CaCl2, 0.5 ascorbic acid, 2 sodium pyruvate. Slices were incubated in the same solution for an hour before the experiment.

Calcium Imaging Slices were loaded with calcium sensitive dye fura 2-AM as described (Sharma et al. 2008). Briefly, slices were incubated in cutting solution containing 20 μM fura2-AM and 2% Cremaphore ES for 30 min at room temperature. Slices were washed in recording solution and transferred to the recording chamber. Imaging was performed using Zeiss AxioExaminer fitted with a CCD camera (Cooke Sensicam). Sutter DG IV was used for the light source. Images were acquired and analyzed using SlideBook 5 software. Recording solution contained (in mM) 120 Sodium chloride, 1.25 Sodium monophosphate, 25 Sodium Bicarbonate,
10 Glucose, 3.5 potassium chloride, 2.5 magnesium chloride and 1 Calcium Chloride. Images were acquired as a time series at 1 Hz. Data were collected using a 510/80 nm filter and T400LP beam splitter (Chroma Technology). Individual regions of interest (ROI) were specified for each cell in the image by creating masks just large enough to cover an individual cell. Average fluorescence for each ROI / individual cell was measured along a 12 bit scale. Ratio of emission at 340 and 380 nm was normalized to the baseline and plotted as percentage increase over baseline. Experiments were performed on slices from 4 to 7 animals for each condition. Data was pooled from different animals and are presented as mean + SEM across cells. Statistical significance was evaluated by paired or independent Student t-tests.

All experiments were performed with 1mM ACh+ 1μM atropine (ACh/At) to block muscarinic acetylcholine receptors and activate nicotinic receptors only. Drugs were applied using a Picospritzer. Antagonists were present in the bath as well as puffer pipette.

**Immunohistochemistry** Mice were perfused with 4% paraformaldehyde and brains post-fixed for 2 hours and cryoprotected in 20% sucrose. 18 μm sections were cut on a cryostat and free floating sections were stained overnight at 4°C with goat polyclonal doublecortin antibody (SC8066 Santa Cruz, 1:500 dilution). A fluorescent donkey anti-goat secondary antibody (Jackson ImmunoResearch, 1:500 dilution) was used to visualize labeled cells. Control sections were processed using the same protocol but without the primary antibody to determine background staining and antibody specificity. Images were acquired using an Olympus spinning disc microscope.
Results:

Progenitor cells migrate from the SVZ along the RMS to the main olfactory bulb (MOB). After arriving at the bulb they exhibit radial migration and stop either at the granule cell layer integrating into the circuit as granule cells or at the glomerulus developing into periglomerular cells. In this study, I focused on the cells in the subependymal layer of the olfactory bulb of 6 to 10 week old mice. In agreement with previous studies (Darcy and Isaacson 2009), I find that majority of the cells in the subependymal layer express doublecortin – a marker for migrating neural progenitor cells (Figure 1A & B). Dense doublecortin staining was observed in the RMS but not in other layers of the bulb. Based on this result I conclude that most cells in the subependymal layer are migrating progenitor cells.

I then examined for the presence of functional excitatory GABARs on these cells. I used calcium imaging arguing that depolarizing GABAergic signals should result in changes in intracellular calcium concentration ([Ca$_\text{i}$]). Slices were loaded with fura 2-AM (Figure 1C). Local application of 1 mM GABA resulted in rapid calcium transients in cells (n= 7 animals) (Figure 1D). On an average the 340/380 ratio increased by $6.35 \pm 0.47\%$ (mean $\pm$ SEM, n = 88 cells) and decayed with a mean time constant of $58.2 \pm 27.03$ s.

I asked whether migrating cells in the RMS expressed functional nAChRs. A brief application of 1 mM ACh in the presence of 1 µM atropine (ACh/At) resulted in large but variable calcium transients with peak amplitudes ranging from 3 to 22 % above baseline (Figure G & H). Cells in the subependymal zone responded with a mean of $8.03 \pm 0.37\%$ (n=111) increase in intracellular calcium. The concentration of atropine used in these experiments was sufficient to block mAChR-mediated calcium signals (data not shown). My results indicate that migrating progenitor cells in the adult RMS show increase in cytosolic calcium levels via nAChRs.
As progenitor cells exhibit depolarizing responses to both GABA and glutamate from surrounding cells (Young et al. 2010; Platel et al. 2010a), I asked what fraction of nAChR-mediated calcium signals were indirect, due to release of glutamate and/or GABA. When ACh/At-evoked responses were elicited in the presence of 10 µM GABA to block GABAA receptors there was a 20 + 4.5 % decrease in peak amplitude of the response compared to control ACh/At response in absence of the drug (n=147 cells, 6 mice, p<0.004; Fig 2A & B). Blocking ionotropic glutamate receptors with 50 µM DNQX + 100 µM APV resulted in a small but statistically significant reduction in the nAChR-mediated response by 9.6 + 4.5 % (n =78; 5 animals Figure 2C & D). These data suggest that the major component of the calcium signals arise from the activation of nAChRs expressed by migrating progenitor cells.

I then examined the pharmacology of functional nAChRs on progenitor cells. Incubation of slices with 5 µM mecamylamine, a general nAChR blocker, resulted in a large block of the nAChR-induced calcium signals (Figure 3 A 64 + 1.13 % inhibition, n = 110, 4 animals, p < 0.001) confirming that these signals are indeed nicotinic in nature. The α7-nAChR-selective antagonist methyllycaconitine (MLA; 10 nM) failed to affect the nAChR-induced calcium signals ruling out a role for this receptor subtype (n=50, p<0.29). Surprisingly, incubating the slices with 10 nM MLA and 10 µM dihydro-beta-erythroidine (DHβE), a selective blocker of β2 subunit containing nAChRs, also failed to significantly block the calcium transients (p<0.8 compared to control; n=50, 5 animals; Figure 3 C). These results suggest that nAChR signals do not arise from either homomeric or heteromeric α7-nAChRs nor from α4β2 nAChRs, the other common nAChR subtype.

As a role for β2-containing nAChRs can be inferred from a previous study (Mechawar et al. 2004), contrary to my pharmacological results, I examined nAChR signals from progenitors in a β2 knockout (KO) mouse. Progenitor cells from the β2 KOs showed robust calcium transients upon ACh/At application. These responses were significantly smaller (60 + 6.7% of control
response, 7 animals; n= 104) than those in wild type mice. Similar to results from wild-type mice, these responses could be blocked by 5 μM mecamylamine (61.9 + 0.57 % inhibition, n= 77, cells p<0.001). However, as the responses are variable and comparisons were made across animals, it is not clear whether the differences arise due to disparities in calcium responses between the two mice. Combined with my pharmacology results, these data imply that β2-containing nAChRs play a small, if any, part in the nAChR-mediated calcium responses in progenitor cells in the RMS.

The absence of evidence implicating either α7- or β2-containing nAChRs motivated the examination of other possible receptor subtypes. Recent studies (Mineur et al. 2011; D'Souza and Vijayaraghavan 2012) have shown that the α3β4-nAChRs, initially thought to have roles mainly in the autonomic nervous system, might play important roles in CNS functions. I therefore examined the effects of incubating slices with conotoxin AuIB (5 μM), an antagonist specific for α3β4-nAChRs (Luo et al. 1998). The antagonist showed a large inhibition in ACh/At responses from the RMS progenitor cells (Figure 4; 92.6 + 0.76 % inhibition, n = 78 cells, 4 animals, p <0.001). A 15 min washout of the antagonist resulted in a recovery of the responses to 66.5 + 4.6 % of control responses (p<0.001 compared to block). These results suggest that α3β4-nAChRs are the dominant functional nAChR subtype in progenitor cells.

Lastly, I asked whether functional expression of nAChRs was maintained once the cells were incorporated into the granule cell circuit. Responses to local application of ACh/At were examined from fura 2-AM loaded neurons in the granule cell layer. Only a small fraction of GCs (0 to 3 %) responded to ACh/At application in the granule cell layer (Figure 5). Surprisingly, however, the responses in this minor fraction was completely abolished upon application of 50 μM DNQX + 100 μM APV in order to block GluRs, while the ACh/At responses from the RMS cells were unaffected by this treatment (data from 4 animals).
My results suggest that migrating progenitor cells in the RMS express functional nAChRs. Contrary to results from the hippocampus (Liu et al. 2006; Campbell et al. 2010) and expectation from one study in the OB (Mechawar et al. 2004), these cells predominantly express functional $\alpha_3\beta_4$ nAChRs. Once migration stops and cells differentiate in the granule cell layer, the expression of this receptor subtype is suppressed and nAChRs are expressed only on presynaptic terminals forming glutamatergic synapses on granule cells.
Discussion:

In this study, I show that migrating progenitor neurons along the RMS express functional nAChRs in densities sufficient to significantly alter $[\text{Ca}]_i$. Majority, if not all, of the progenitor cells in the subependymal layer express these receptors. While development of hippocampal progenitor cells appears to be modulated by the $\alpha_7$-nAChR subtype (Liu et al. 2006; Campbell et al. 2010), responses in the OB progenitors are mediated by the $\alpha_3\beta_4$ subtype adding to the emerging importance of these receptors in CNS functions. Interestingly, functional nAChR expression is lost in differentiated granule cells and partly replaced by presynaptic regulation of glutamate release by the receptors.

These data contribute to the growing realization that modulation of adult neurogenesis differs greatly between hippocampus and OB. In the hippocampus knocking out $\alpha_7$-nAChR expression specifically in progenitor cells leads to delayed maturation and integration of dentate granule cells (Liu et al. 2006) and $\beta_2$ subunit containing nAChRs modulate dendritic arborization of newly generated dentate granule cells. Furthermore, presence of $\alpha_3\beta_4$ nAChRs has not been reported on progenitor cells generated in the sub granular zone of dentate gyrus.

Acetylcholine has been shown to be neuroprotective as blockers of acetylcholine esterase enhances progenitor cell survival in the RMS (Kaneko et al. 2006). However, a conflicting study (Mechawar et al. 2004) shows that endogenous stimulation of nAChRs can also lead to apoptosis. In this study knock out of $\beta_2$ nAChR subunits lead to a 46% increase in the number of new granule cells generated in the olfactory bulb while the number of PG cells remains unchanged.

Contrary to expectation from the studies mentioned above, my studies clearly demonstrate that the predominant functional subtype expressed in migrating progenitor cells in RMS contains $\alpha_3\beta_4$ nAChR subunits. This receptor subclass modulates diverse processes. For
example, α3β4 nAChRs in neurons expressing pro-opiomelanocortin in the hypothalamus
participate in nicotine-dependent suppression of appetite (Mineur et al. 2011). In addition α3β4
nAChRs modulate release of GABA in the hippocampus (Tang et al. 2011) and play a role in
cholinergic modulation of OB output (D’Souza and Vijayaraghavan 2012). Their expression on
progenitor cells in the RMS but not on mature granule cells would imply that they likely modulate
development of immature granule cells.

My data show that functional α3β4 nAChRs are absent in the soma of mature granule
cells. This is in agreement with an earlier report that found that granule cells do not exhibit
detectable currents in response to application of nicotine (Castillo et al. 1999). It is, however,
possible that in mature granule cells nAChRs (either α3β4 or other subtypes) are specifically
localized at dendritic locations removed from the soma such that their contribution to calcium
changes might have gone undetected.

I did see an increase in [Ca]i in a small fraction of mature granule cells but this was
completely inhibited by ionotropic glutamate receptor blockers. This suggests that once neurons
mature and are incorporated into the granule cell circuit, cholinergic control is ceded to
presynaptic mechanisms. This would be consistent with Hebbian and other activity-dependent
control mechanisms for synapse formation. Similar observation has been made in the
hippocampus where nAChRs modulate hippocampal progenitor cell development by cell
autonomous calcium signaling but this effect is lost once cells differentiate and acquire
adequate calcium buffering capacities (Berger et al. 1998).

Role of the β2 subunit remains unclear. I have observed robust nicotinic responses from
progenitor cells in slices from β2 knockout mice. On average the responses are smaller than
those from control slices. My studies appear contradictory to results from Mechawar et.al.
(Mechawar et al. 2004) where the number of newly generated granule cells is higher in β2
knockouts. This study does not address the step at which β2 subunit might act or whether there
might be compensatory or reactive mechanisms. One plausible explanation is that β2 containing
nAChRs are presynaptic and enhance glutamate release on to newly generate granule cells
leading to excitotoxicity in some cells. If this is the case, knocking out β2 subunit will be
neuroprotective.

Progenitor cells on the RMS have prominent cell bodies with relatively short processes.
While it is clear that majority of the response to acetylcholine is mediated by α3β4-nAChRs, I
cannot completely rule out contributions by nAChRs containing α7, β2 or other subunits that
may be localized to the tips of the processes. Their response may have gone undetected if it is
small, fast or localized at the tip alone. In addition, non-cell autonomous roles of nAChRs and
the exact composition of these receptors on progenitor cells (whether they contain the β2
subunit as well) are yet to be determined.
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Figure Legends:

**Fig.1:** Progenitor Cells in RMS Express Doublecortin and are Depolarized by GABA and ACh.  

A. Fixed frozen sections labeled with doublecortin antibody (red) showing dense labeling in RMS but not in other layers of the olfactory bulb. Nuclei are stained with DAPI (blue). Scale Bar: 100 μm.  

B. Higher magnification of doublecortin labeling in RMS Scale Bar: 10 μm  

C. Image of cells in the RMS loaded with fura 2 AM. Scale Bar: 15 μm  

D. Calcium transients generated in a progenitor cell in the RMS upon a 10s application of 1mM GABA.  

E. Cartoon of sagittal section of brain showing the site of injection.  

F. Two cells in the RMS expressing GFP with processes pointing in the direction of migration.  

G. Calcium transients in response to a 5s puff application of 1 mM ACh and 1 μM Atropine (ACh/AT). All cells in the field respond but the response is variable among cells.  

H. Average response from 36 cells shown in G.  

**Fig2:** Response to ACh/AT is Mainly Due to Nicotinic Receptors Expressed by Progenitor Cells.  

A. Response from one field of cells. Black trace is control response to a 5s puff of ACh/AT from one field. Gray trace is response from same cells in presence of 10 μM GABAzine.  

B. Blocking GABA receptors with 10 μM GABAzine blocks only 20 ± 4.5% of the response. n = 147, 6 animals.  

C. Response to a 10s puff of ACh/AT from one field of cells before (black trace) and after (gray trace) treatment with 50 μM DNQX and 100 μM APV.  

D. Ionotropic glutamate receptor blockers block 9.6 ± 4.5% of response. n = 78 cells, 5 animals.  

**Fig3:** Nicotinic Response is Not Due to Activation of α7 or β2 Subunit Containing nAChRs.  

Responses from single cells. Black traces are control response and Gray traces are after application of specific antagonists.  

A. Response of a single progenitor cell is completely blocked by 5 μM mecamylamine (MEC).  

B. 10 nM MLA, an antagonist specific for α7nAChR has no effect.  

C. Further block by 10 μM DHBE (MLA+DHBE) also fails to block the calcium transient.
D. Response is present in progenitor cells from β2nAChR Knockout mice. Average response from 104 progenitor cells from seven β2nAChR Knockout mice. E. Bar graph comparing average responses under various conditions.

Fig4: Progenitor cells in the RMS Express Functional α3β4nAChRs. A. Average response from 116 cells from 4 animals. Response is inhibited by 5 μM conotoxin AuIB specific for α3β4nAChR receptors (light gray). Response recovers after a 15 min washout of AuIB. B. On an average response was reduced by 92 ± 0.76 % and recovered to 66.5 ± 6.8% of control.

Fig5: nAChRs are Absent in Mature Granule Cells. A. A field of granule cells loaded with fura2 AM. B. Response from these cells to a 5s puff of ACh/AT. Only 3 cells responded in this field. The cell with the largest response is circled in A. Two cells, marked with arrows in A, showed smaller responses. Traces on the right are 15 min after application of DNQX/APV. Responses from the small fraction of granule cells that respond to ACh/AT can be completely blocked by DNQX/APV, unlike that seen in NPCs (inset C).