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Olfactory discrimination varies in mice with different levels of $\alpha 7$ -nicotinic acetylcholine receptor expression

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Abstract

Previous studies have shown that schizophrenics have decreased expression of $\alpha 7$ -nicotinic acetylcholine ($\alpha 7$) receptors in the hippocampus and other brain regions, paranoid delusions, disorganized speech, deficits in auditory gating (i.e., inability to inhibit neuronal responses to repetitive auditory stimuli), and difficulties in odor discrimination and detection. Here we use mice with decreased $\alpha 7$ expression that also show a deficit in auditory gating to determine if these mice have similar deficits in olfaction.

In the adult mouse olfactory bulb (OB), $\alpha 7$ expression localizes in the glomerular layer; however, the functional role of $\alpha 7$ is unknown. We show that inbred mouse strains (i.e., C3H and C57) with varying $\alpha 7$ expression (e.g., $\alpha 7$ wild-type [$\alpha 7^{+/+}$], $\alpha 7$ heterozygous knock-out [$\alpha 7^{+/-}$] and $\alpha 7$ homozygous knockout mice [$\alpha 7^{-/-}$]) significantly differ in odor discrimination and detection of chemically related odorant pairs.

Using [¹²⁵I] α -bungarotoxin (α -BGT) autoradiography, $\alpha 7$ expression was measured in the OB. As previously demonstrated, α -BGT binding was localized to the glomerular layer. Significantly more expression of $\alpha 7$ was observed in C57 $\alpha 7^{+/+}$ mice compared to C3H $\alpha 7^{+/+}$ mice. Furthermore, C57 $\alpha 7^{+/+}$ mice were able to detect a significantly lower concentration of an odor in a mixture compared to C3H $\alpha 7^{+/+}$ mice. Both C57 and C3H $\alpha 7^{+/+}$ mice discriminated between chemically related odorants sooner than $\alpha 7^{+/-}$ or $\alpha 7^{-/-}$ mice. These data suggest that $\alpha 7$ -nicotinic-receptors contribute strongly to olfactory discrimination and detection in mice and may be one of the mechanisms producing olfactory dysfunction in schizophrenics.

Keywords

$\alpha 7$ -nACh; odor discrimination; olfaction; behavior; α -bungarotoxin; MLPEST

1. Introduction

Approximately 0.4–0.6% of the population (Goldner et al., 2002; Bhugra 2005) is diagnosed with schizophrenia, a psychiatric disease characterized by inaccurate perceptions of reality, commonly manifested by auditory and olfactory hallucinations, paranoid delusions, disorganized speech, and deficits in odor detection, odor discrimination, and learning (Isseroff et al., 1987; Dunn and Weller, 1989; Serby et al., 1990; Kopala et al., 1993; Wu et al., 1993; Houlihan et al., 1994; Malaspina et al., 1994; Kopala et al., 1995; Turetsky et al., 2000; Malaspina et al., 2002; Moberg et al., 2003; Doop et al., 2006). Onset typically occurs in early adulthood and symptoms persist for the remainder of the patient's lifespan (Castle et al., 1991; Dickerson 2007). However, the causes of these sensory and behavioral deficits in schizophrenics remain unclear.

Previous studies have linked functional polymorphisms in the promoter region of the human $\alpha 7$ -nicotinic acetylcholine receptor ($\alpha 7$, nAChR) gene (*CHRNA7*) with both schizophrenia and with an auditory gating deficit wherein individuals cannot inhibit neuronal responses to repetitive auditory stimuli (Freedman et al., 1997; Leonard et al., 2002). Postmortem samples from patients with schizophrenia reveal a decreased expression of $\alpha 7$ in hippocampus (Freedman et al., 1995), reticular thalamic nucleus (Court et al., 1999), cingulate cortex (Marutle et al., 2001), and frontal cortex (Guan et al., 1999). Similarly, mice with a polymorphism in the $\alpha 7$ promoter have decreased hippocampal $\alpha 7$ expression and these changes in $\alpha 7$ levels correlate with changes in auditory gating deficits (Stevens et al., 1996; Simosky et al., 2001). Unraveling the neural basis of schizophrenia is challenging because we cannot easily characterize corresponding symptoms in animal models. One successful approach is to study endophenotypes – behavioral or physiological phenotypes present in the disease (Gottesman and Gould, 2003; Gould et al., 2006; Akil et al., 2010) – that can be measured objectively in animal model systems. The auditory gating deficit has been a successful endophenotype to examine nicotinic deficits in schizophrenia. Here we examined the role of $\alpha 7$ in *another* endophenotype – odor discrimination/detection (Rupp et al., 2005a, 2005b; Turetsky et al., 2009b; Moberg et al., 2006; review: Atanasova et al., 2008).

nAChR are ligand-gated ion channel receptors widely distributed in the brain. To date, at least nine α -($\alpha 2$ – $\alpha 10$) and three β -($\beta 2$ – $\beta 4$) subunits have been cloned in chick, rat, and human neuronal tissue (Albuquerque et al., 1995, 2009; Elgoyhen et al., 2001; Graham et al., 2002). Homomeric $\alpha 7$ are characterized by fast kinetics, where they rapidly activate and inactivate (desensitize) and have a high permeability to calcium. These nicotinic-receptors are also efficient in raising intracellular calcium levels in neurons (Vijayaraghavan et al., 1992; Seguela et al., 1993). By modulating intracellular calcium levels, the $\alpha 7$ -nicotinic-receptor may regulate the function of the target cell via calcium-dependent processes, such as activation of second-messenger systems and induction of immediate early gene expression (Albuquerque et al., 1995; Buccafusco et al., 2005; Hansen et al., 2007). Autoradiography using [125 I] α -bungarotoxin (α -BTX, selective $\alpha 7$ antagonist; Drisdell and Green, 2000) has shown that $\alpha 7$ expression is found throughout the mammalian brain (Whiteaker et al., 1999; Leonard and Bertrand, 2001) with localization in the glomerular layer of the OB (Le Jeune et al., 1995, 1996; Alkondon et al., 1996). Because $\alpha 7$ -nicotinic-receptors are expressed in a characteristic manner in the OB, the olfactory system provides a unique opportunity to link $\alpha 7$ -nicotinic-receptor function with one of the sensory deficits characteristic of schizophrenia.

Cholinergic cell bodies in the horizontal limb of the diagonal band of Broca (HDB) project to the OB glomerular layer as well as to the anterior olfactory nucleus and the olfactory tubercle (Le Jeune and Jourdan, 1991; Lauterborn et al., 1993). Previous studies have shown

that lesions of the HDB cholinergic fibers reduce the ability of rats to distinguish chemically-related odorants, but not the ability to discriminate unrelated odorants (Linster et al., 2001). Similarly, intraperitoneal injection of an acetylcholinesterase inhibitor increases the ability of rats to detect low concentrations of butanol in a mixture (Doty et al., 1999). These data suggest that acetylcholine may assist the olfactory system in discriminating small differences in composition of natural odorants (>100 molecules), closely related odorants from various chemical families, and enantiomers (odorant pairs that differ in stereoconfiguration).

In this study, we tested the hypothesis that mice with decreased $\alpha 7$ expression have deficits in odor discrimination as observed in humans with schizophrenia. We show that: 1) expression of $\alpha 7$ -nicotinic-receptors differs by mouse strain and genotype in the OB, and 2) odor discrimination abilities differ by mouse strain and genotype in a manner related to $\alpha 7$ expression levels.

2. Results

Previous studies have shown that $\alpha 7$ -nicotinic-receptor expression varies between mouse strains in the hippocampus (Marks et al., 1996; Stevens et al., 1996; Simosky et al., 2001; Brooks et al., 2009); however, it is unknown if $\alpha 7$ expression varies between strains of mice in the OB. Using autoradiography, we measured α -BGT binding in the OB of mice with different backgrounds and genotypes (i.e., C57 and C3H; $\alpha 7^{+/+}$, $\alpha 7^{+/-}$, $\alpha 7^{-/-}$; Figure 1). α -BGT binding was localized to the glomerular layer of the OB. C57 $\alpha 7^{+/+}$ mice ($n = 8$) had a mean tissue equivalent of 745.2 ± 76.34 nCi/g (mean $\pm$ sem) compared to 465.7 ± 52.9 nCi/g in C3H $\alpha 7^{+/+}$ mice ($n = 7$; Figure 2). Mice with decreased $\alpha 7$ -nicotinic-receptor expression (i.e., $\alpha 7^{+/-}$ and $\alpha 7^{-/-}$; range: 5.0 – 228.2 ± 1.6 – 72.6 nCi/g) had significantly less α -BGT binding compared to $\alpha 7^{+/+}$ mice (Figures 1–2). Finally $\alpha 7^{-/-}$ mice had no α -BGT binding showing that these nicotinic-receptors were truly knocked-out of the genome (Figures 1–2). These data show that $\alpha 7$ expression varies significantly between mouse strains as well as between $\alpha 7^{+/+}$, $\alpha 7^{+/-}$, and $\alpha 7^{-/-}$ mice (See Supplementary Results and Figures S1–S4).

To determine if this variable $\alpha 7$ expression correlates with deficits in odor discrimination, we conducted behavioral experiments using Maximum Likelihood Parameter ESTimation (MLPEST; Linschoten et al., 2001; Clevenger and Restrepo, 2006). This method is reliable and accurately assesses olfactory detection threshold within a single day. As MLPEST is a difficult behavioral task, mice were first trained on a simple go-no-go behavioral task (S+/S–) before testing mice in MLPEST.

The S+/S– behavioral task requires mice to learn to lick on a water-rewarded stimulus (1% isoamyl acetate; S+) and not to lick on an unrewarded stimulus (mineral oil [MO]; S–). After completion of three consecutive blocks with $\geq 85\%$ accuracy, it was determined that the mouse learned the S+/S– task (Figure 3A). Both C57 and C3H mice were able to learn the S+/S– program within 2 weeks.

To establish that mice were cuing on odors and not changes in airflow or valve noise, we removed the odor during the S+/S– session after the mouse was routinely performing at or above criterion (Figure 3B). Finally, in order to ensure that behavioral differences observed in the olfactometer would not be due to strain-or genotype-specific differences in learning and memory, the number of days required training mice from each strain and genotype was determined. Each group of mice learned S+/S– at the same rate (Figure 3C).

We used four pairs of odor sets, three of which have been previously used in behavioral experiments (Doucette et al., 2007). All genotypes of both the C57 and C3H mouse strains

reached criterion the first time they were exposed to either the aldehyde or acetate pairs (data not shown). For the alcohol and dissimilar odorant pairs, however, only $\alpha 7^{+/+}$ mice reached criterion the first time they were presented the stimuli (Figure 4, alcohol odor pair shown). Both $\alpha 7^{+/-}$ and $\alpha 7^{-/-}$ mice needed 3–4 odorant presentations to reach criterion. Nonetheless, all groups of mice were able to reach criterion of each new odor pair before being tested in MLPEST.

Tests in MLPEST resulted in significant differences in mean threshold for the aldehyde and acetate odor pairs (Figure 5). For the aldehyde pair, C57 $\alpha 7^{+/+}$ mice had an average threshold of -2.62 ± 0.26 (mean $\pm$ sem; $n = 5$, concentration measured in log scale, see Experimental Procedures) while C57 $\alpha 7^{+/-}$ ($n = 6$) and $\alpha 7^{-/-}$ ($n = 5$) mice had an average threshold of -1.80 ± 0.11 and -1.88 ± 0.11 , respectively. Similarly, C3H $\alpha 7^{+/+}$ mice had a significantly lower mean threshold of -2.83 ± 0.21 ($n = 7$) compared to $\alpha 7^{+/-}$ (-2.21 ± 0.15 ; $n = 8$) and $\alpha 7^{-/-}$ (-2.14 ± 0.21 ; $n = 5$) mice (* $p < 0.05$, ANOVA with multiple comparisons, Newman-Keuls). However, $\alpha 7^{+/+}$ mice were not significantly different between background strains (Figure 5A). For the acetate odor pair, only C57 $\alpha 7^{+/+}$ mice ($n = 5$) had a significantly lower threshold (-2.66 ± 0.18 ; $p < 0.05$, ANOVA with multiple comparisons, Newman-Keuls; Figure 5B) compared to all other groups of mice (range: -1.92 through -2.25 ± 0.07 through 0.13 ; C57 $\alpha 7^{+/-}$: $n = 8$; C57 $\alpha 7^{-/-}$: $n = 5$; C3H $\alpha 7^{+/+}$: $n = 11$; C3H $\alpha 7^{+/-}$: $n = 9$; C3H $\alpha 7^{-/-}$: $n = 7$). There were no significant differences between all mouse groups for the alcohol or dissimilar odorant pairs (data not shown). These data suggest that for certain odors, mice with decreased $\alpha 7$ -nicotinic-receptor expression have discrimination or detection deficits in olfaction (MLPEST does not provide information on whether the differences are in detection vs. discrimination).

3. Discussion

The principal findings of this study are that: 1) mice bred on dissimilar backgrounds have significantly different levels of $\alpha 7$ expression in the OB, and 2) mice with decreased $\alpha 7$ expression have deficits in detecting or discriminating chemically related odors, which is similarly observed as one of the sensory deficits in schizophrenia. Although the complex mechanisms mediating olfaction are not well understood, our results suggest that the $\alpha 7$ -nicotinic-receptor may contribute to olfactory detection and/or discrimination in adult mice.

α -BGT Binding

To date, no reports have shown if there is a difference in α -BGT binding in the OB of $\alpha 7^{+/+}$ inbred mouse strains. We observed that C57 $\alpha 7^{+/+}$ mice had significantly more α -BGT binding in the glomerular layer of the OB compared to C3H $\alpha 7^{+/+}$ mice (Figures 1–2). This is *different* than what has been previously measured in the hippocampus where C3H $\alpha 7^{+/+}$ mice have more binding (see Supplementary Results for control data). Previous reports show regional differences in α -BGT binding throughout the brain and *in general*, strains of mice with high binding in one region have high binding in other regions (Brooks et al., 2009; Marks et al., 1989). Although we show that C57 mice ($n = 8$) have more α -BGT binding in the OB compared to C3H mice ($n = 7$), we do not think that we under-reported the amount of binding as we consistently measured all sections from the anterior through the posterior OB in all strains of mice and had similar group sizes for this study. Furthermore, Brooks et al. (2009) observed that the $\alpha 7$ -genotype significantly correlates with α -BGT binding across brain regions as well as within specific nuclei in a single brain region, suggesting that different combinations of genes may be involved in regulating α -BGT binding.

As expected, $\alpha 7^{+/-}$ and $\alpha 7^{-/-}$ mice in all background strains had significantly less α -BGT binding in the OB compared to $\alpha 7^{+/+}$ mice (Figure 2). In general, α -BGT binding in $\alpha 7^{+/-}$ mice was $< 50\%$ of binding measured in $\alpha 7^{+/+}$ mice, suggesting that $\alpha 7$ -nicotinic-receptor

expression is also reduced by > 50% in $\alpha 7^{+/-}$ mice. These data are similar to what Adams et al. (2008) measured in the hippocampi of C3H $\alpha 7^{+/+}$ and $\alpha 7^{+/-}$ mice (i.e., 54–64% decreased binding in the dentate gyrus, CA3, and CA1 hippocampal regions of $\alpha 7^{+/-}$ mice compared to $\alpha 7^{+/+}$ mice). This reduction in $\alpha 7$ -nicotinic-receptor expression may cause compensatory changes in the cholinergic system, such as increasing expression of other nicotinic receptors (e.g., $\alpha 4\beta 2$ receptors) or nicotinic receptor subunits (e.g., $\alpha 4$, $\alpha 5$, $\beta 2$, and/or $\beta 4$ subunits) throughout the brain. A previous study, however, showed that the relative amount of cortical, hippocampal, or thalamic mRNA for the $\alpha 4$, $\alpha 5$, $\beta 2$, and $\beta 4$ subunits in adult C57 $\alpha 7^{+/+}$, $\alpha 7^{+/-}$ and $\alpha 7^{-/-}$ mice was the same (Franceschini et al., 2002). In another study, $\alpha 7$ RNA levels were not different between C3H and DBA $\alpha 7^{+/+}$ mice in several brain regions although $\alpha 7$ -protein expression between these two mouse strains was significantly different in the hippocampus and basal ganglia (Brooks et al., 2009). These data suggest that the cholinergic system is relatively stable throughout the brain even in mice with a decrease or lack of $\alpha 7$ -protein expression. However, using autoradiography is not sufficient to conclude absolute differences in $\alpha 7$ -nicotinic-receptor expression levels, as there could be differences in tissue penetration. To address this issue, a more sensitive and quantitative assay for $\alpha 7$ expression would need to be performed (e.g., membrane-binding experiments).

Learning Behavioral Tasks

In this study, we did not see a difference in learning the go-no-go behavioral task between C57 and C3H mice (Figure 3C). Therefore to test odor discrimination thresholds of C57 and C3H mice, we used MLPEST to reduce odorant sensitization that can equalize behavioral responses between strains as observed in other multi-day testing olfactory discrimination paradigms (Wetherhill and Levitt, 1965; Cain et al., 1983; Apter et al., 1999). MLPEST includes both positive and negative controls such that the animals are presented with rewarded and unrewarded stimuli after each test stimulus. This provides maintenance of stimulus control, such that if a mouse fails to lick on the test stimulus and correctly licks on the following S+ and not on S−, then we can determine that the mouse did not detect a difference between the test stimulus and the S− odorant, thus validating the animal's threshold.

We observed that $\alpha 7^{+/+}$ mice had a significantly lower threshold compared to $\alpha 7^{+/-}$ and $\alpha 7^{-/-}$ mice when tested with a closely related aldehyde pair and that only the C57 $\alpha 7^{+/+}$ mice had a significantly lower threshold compared to all other mouse groups when tested with a chemically similar acetate pair (Figure 5). These data suggest that a decrease in $\alpha 7$ -expression reduces olfactory discrimination and/or detection for a subset of chemically-related odor pairs. However, when mice were tested in a related alcohol or dissimilar odor pair we did not see a difference in MLPEST threshold. For these odorants, only $\alpha 7^{+/+}$ mice reached criterion the first time they were tested on either the alcohol or dissimilar odor pairs (Figure 4), whereas $\alpha 7^{+/-}$ and $\alpha 7^{-/-}$ mice reached criterion after 3–4 presentations (one presentation = 200 trials). The $\alpha 7^{+/+}$ mice had less exposure of these odor pairs (1–2 presentations) because they reached criterion so quickly compared to $\alpha 7^{+/-}$ and $\alpha 7^{-/-}$ mice, which may be a reason why we could not identify a significant difference. Another explanation for not observing a significant difference is that $\alpha 7$ -nicotinic-receptors may not be necessary for discriminating similar alcohol odorants.

There are limitations to this study, as we did not manipulate the $\alpha 7$ -nicotinic-receptor with pharmacological agents *in vivo*. These studies are important in identifying how $\alpha 7$ -nicotinic-receptors influence odor discrimination. Specifically, by blocking $\alpha 7$ -nicotinic-receptors with either α -BTX or mecamylamine (selective $\alpha 7$ antagonists) in $\alpha 7^{+/+}$ mice we would expect a decreased odor detection/discrimination in MLPEST when tested with either an aldehyde or acetate odor pair as we observed in $\alpha 7^{-/-}$ mice (Figure 5).

Correlating α -BGT Binding with Discrimination Behavior

Previous studies have demonstrated that damaging cholinergic fibers emerging from the HDB produced deficits in discriminating chemically-related odorants in adult rats (Linster et al., 2001). These results suggest that the cholinergic system may be critical for odor discrimination. Chemically-related odorants often have overlapping glomerular activation patterns (e.g., Johnson et al., 2005), and thus contrast enhancement may be essential for these types of olfactory discrimination tasks (S+/S- and MLPEST). Since α 7-nicotinic-receptors can modulate inhibition (Wanaverbecq et al., 2007), reducing the amount of α 7 may result in decreasing inhibition and reducing contrast enhancement. Furthermore, it has been hypothesized that lateral inhibition begins at the glomerular level in a classic on-center-off-surround pattern (Aungst et al., 2003). Previous reports as well as this study show that α 7-nicotinic-receptors are localized to the glomerular layer and thus placing these receptors in the appropriate location to mediate this process (Le Jeune et al., 1995).

Interglomerular lateral inhibition alone, however, may not be sufficient to mediate olfactory discrimination. Lateral inhibition by granule cells or temporal firing patterns in the piriform cortex may also play a role (Christensen and White, 2000). It has been shown that blocking the synchronization of projection neurons in the honeybee reduces the ability to discriminate chemically similar but not dissimilar odorants (Stopfer et al., 1997). Temporal firing patterns coming into and leaving from the piriform cortex could help mediate olfactory discrimination in rodents (Christensen and White, 2000; Wilson, 2001). Furthermore, Wilson's data (2001) show that cholinergic activation is critical for piriform cortical-mediated olfactory discrimination (Weinberger et al., 1992).

Finally, it is clear that α 7 expression varies in postnatal development suggesting a potential role for these receptors in olfactory system development (Le Jeune et al., 1995). Thus, the changes in olfactory discrimination/detection found in this study could be a result of differences in olfactory system development. Whether this is the case can be tested by future studies where the effects of acute changes in α 7-nicotinic-receptor expression or function are examined.

Correlating α 7-Nicotinic-Receptor Function with Olfactory Dysfunction

Previous studies have shown that auditory gating deficits in humans with schizophrenia can be improved within minutes of smoking cigarettes (Adler et al., 1993). However, no such correlation (i.e., smoking cigarettes) has been shown to improve olfactory dysfunction in schizophrenic patients. Smoking has been hypothesized to exacerbate patients' olfactory impairments, and in fact, smoking has been shown to produce long-term odor identification deficits that are reversible in non-schizophrenics (Frye et al., 1990). In schizophrenia, non-smoking patients had a significantly lower score on the University of Pennsylvania Smell Identification Test (UPSIT) compared to non-smoking controls; however, this was not significant between smokers (Houlihan et al., 1994). Furthermore, significant differences were observed between smoking and non-smoking controls, but not between smoking and non-smoking schizophrenics. There is a lack of data showing that smoking can improve olfactory deficits in patients. This would be a difficult study to carry out, as there are several chemicals in cigarette smoke (e.g., ammonia, formaldehyde, toluene, etc.) that have been reported to damage olfactory sensory neurons (OSN) and mucosa in rodents (Vent et al., 2004). Thus, identifying a correlation that links decreased α 7-nicotinic-receptor function with olfactory impairments in schizophrenia remains elusive.

We have not resolved if decreased α 7 expression and therefore function, is due to physiological or intracellular signaling changes in OSNs. Recent studies have reported that patients with schizophrenia have significantly increased electroolfactogram responses (i.e.,

electrical depolarization of OSNs) compared to controls and this was irrespective of acute symptomatology, antipsychotic medication dosage, or smoking history (Turetsky et al., 2009a). OSNs continually regenerate approximately every three months in humans and re-innervate the OB glomeruli (Takagi, 1969). However, postmortem olfactory epithelial tissue from schizophrenics report an increase in the number of immature OSNs compared to controls suggesting disrupted development and regeneration (Arnold et al., 2001). Furthermore, OSNs in patients with schizophrenia show an odor-specific hyposmia that is consistent with a disruption of cAMP-mediated signal transduction (Turetsky and Moberg, 2009). We do not know if alternations in $\alpha 7$ -nicotinic-receptor function relate to these other hypothesized mechanisms (i.e., changes in OSN function and intracellular signaling).

Although we have shown that α -BTX binding is localized to the glomerular layer (Figure 1), we do not have direct evidence showing that cells in the glomerular layer of the OB could be the site responsible for the differences in behavior that we detected. Other sites in the cholinergic or olfactory pathways may be important. Previous studies have shown that cholinergic input from the HDB to the OB glomerular layer could be under $\alpha 7$ -nicotinic-receptors from other brain regions that project to these areas (e.g., Thomsen et al., 2008) as this is important in medial prefrontal cortical function (Thomsen et al., 2010). To understand the role of $\alpha 7$ -nicotinic-receptor-dependent regulation of acetylcholine neuronal function in the OB and in schizophrenia, further studies need to be performed.

Finally, alternative mechanisms that might contribute to deficits in olfaction for schizophrenic patients could be the primary locus for the therapeutic action of antipsychotic medications in the dopamine system, and there is a rich dopaminergic innervation in the OB (review: Cave and Baker, 2009). The glomerular layer of the OB contains the largest expression of dopaminergic neurons as shown by tyrosine hydroxylase (TH) staining, the first enzyme in the dopamine biosynthetic pathway (Baker and Farbman, 1993; Nagatsu et al., 1964). $\alpha 7$ expression is also localized to the glomerular layer and its activation by nicotine can modulate dopamine biosynthesis via altering tyrosine hydroxylase gene expression (Serova and Sabban, 2002). Data regarding the effects of antipsychotics on olfactory function are mixed (e.g., Turetsky et al., 2009a; Hegg and Lucero, 2004), but for our experiments we did observe a deficit in olfactory discrimination in mice with decreased $\alpha 7$ expression without the presence of antipsychotic medication. However we cannot conclude that the decrease in $\alpha 7$ -nicotinic-receptor expression causes or adds to a primary or secondary dopaminergic dysregulation in the OB and possibly olfactory function.

$\alpha 7$ -nAChR Null Mice

Our data show that both C57 and C3H $\alpha 7^{+/-}$ and $\alpha 7^{-/-}$ mice have deficits in discriminating chemically-related odorants. Correlations between decreased $\alpha 7$ -expression and auditory gating deficits have been established in both humans and mice (Freedman et al., 1997; Leonard et al., 2002; Stevens et al., 1996; Simosky et al., 2001). In another behavioral test, Paylor et al. (1998) showed that compared to C57 $\alpha 7^{+/+}$ mice, $\alpha 7^{-/-}$ mice do not exhibit a deficit in an acoustic startle response when measured by pre-pulse inhibition, suggesting that compensatory mechanisms could be acting in $\alpha 7^{-/-}$ mice to maintain neuronal function (Paylor et al., 1998). Recently, C3H $\alpha 7^{+/-}$ mice showed auditory gating deficits compared to $\alpha 7^{+/+}$ mice (Adams et al., 2008), and our results show deficits in olfactory discrimination in C3H $\alpha 7^{+/-}$ mice compared to $\alpha 7^{+/+}$ mice. Together these data suggest that $\alpha 7^{+/-}$ (heterozygous knock-out) mice are not able to compensate as effectively as $\alpha 7^{-/-}$ (homozygous knock-out) mice, and that the $\alpha 7^{+/-}$ (heterozygous knock-out) mouse may be an animal model for the olfactory endophenotype observed in schizophrenia.

In conclusion, C3H and C57 $\alpha 7^{+/-}$ and $\alpha 7^{-/-}$ mice exhibit a significant reduction in the density of OB $\alpha 7$ -nicotinic-receptors. This reduction in receptor density is associated with a decreased ability to detect chemically similar odorant pairs. These data emphasize the importance of the $\alpha 7$ -nicotinic-receptor's role in modulating olfaction and may provide a useful model for investigating olfactory dysfunction observed in humans with schizophrenia. These results open the possibility but do not prove that this may be a model for the olfactory endophenotype in schizophrenia. Further experiments are needed to explore the validity of this mouse ($\alpha 7^{+/-}$) as a model for this olfactory endophenotype. Finally, there are many other special sense endophenotypes in schizophrenia that our data do not address; thus, the $\alpha 7^{+/-}$ mouse is not a comprehensive model for schizophrenia.

4. Experimental Procedure

Animals and water restriction

C57BL/6J $\alpha 7$ -nAChR null mutant mice ($\alpha 7^{-/-}$) were obtained from Jackson Laboratories while C3H-HeN $\alpha 7^{-/-}$ mice were obtained from the Institute of Behavioral Genetics (Dr. Allan Collins, University of Colorado Boulder). Mice were bred and housed in static micro-isolation cages. A previous study in our laboratory examined two types of caging/housing environments to determine the effects of environment on olfactory function (Oliva et al., 2010). High ventilated caging mechanically exchanges fresh air at a rate of one volume per minute. Low ventilation or static micro-isolation cages passively exchange air through a filter cover. Oliva et al. (2010) noted differences in mouse behavior (e.g., an increase in intermale aggression when housed in "standard", high ventilated caging) as well as neuroanatomical changes that occurred in the OB as a result of the differences in housing conditions. Therefore, this study housed all mice in static micro-isolation cages.

All experimental procedures were performed under approved University of Colorado IACUC protocols. Mice were housed in groups of ≤ 5 mice/cage (but most often cages only had 1–2 mice each), given food and water *ad libitum*, and maintained in a 12:12 light:dark cycle. Male mice were only used for behavioral and anatomical experiments. Behavioral training began at 2.5 months and concluded up to 8 months of age. However, most mice completed training by 6 months of age. Anatomical experiments were performed at the conclusion of behavioral training (i.e., between 6–8 months). Mice were weighed prior to training and daily to ensure they maintained 80–85% of their original unrestricted weight. Mice store water well and are not motivated to learn a task without being water deprived for ~22 hours prior to training/testing (Tucci et al., 2006). During training/testing, mice received at least 1 ml of water daily to maintain their restricted weight.

Odorant preparation and delivery

Odors were delivered using a computer-controlled olfactometer that delivered a known concentration of an odorant. Odors were made weekly with high purity odorants (Sigma-Aldrich; vehicle = mineral oil [MO]; v/v) to a final volume of 10 ml. Volatilized odors (1/40 dilution with air) were presented to mice through an odorant port. To increase task difficulty, mice needed to discriminate between odor mixtures (between odor A and a 1:1 mixture of odor A:odor B; Stevenson, 2001). For these experiments, odor A was unrewarded (S–) while the mixture of odors A and B was rewarded (S+). A previous study showed, that when tested in odor discrimination of odor pairs, the odors in Table 1 differ in perceptual similarity for C57 $\alpha 7^{+/+}$ mice (Doucette et al., 2007). We chose to use three of the same odor pairs for these experiments and added an aldehyde pair. The concentrations of odors were 0.1% in MO except for the alcohol pair, which was a concentration of 1% in MO.

Behavioral training

S+/S——Mice were trained/tested in an olfactometer chamber with a go-no-go protocol (S +/S—; Slotnick and Restrepo, 2005) where a correct response consisted of licking on an S+ trial (reward = 10 μ l of water) or not licking on an S— trial (unrewarded). Mice were presented with 200 trials (block = 20 trials with 10 S+ and 10 S— pseudo-randomly selected presentations) and the percent correct for each block was determined. Following completion of criterion (three consecutive blocks $\geq$ 85%) for two consecutive presentations, training was complete. Mice were then tested on a new odor pair before moving to the detection test (see below).

MLPEST—Following training, mice were tested on their detection threshold using a modified MLPEST program for use with odorant mixtures (Linschoten et al., 2001; Clevenger and Restrepo, 2006). Odor pairs were prepared with decreasing concentrations of odor B (Table 1). All test concentrations contained 0.1% of odor A (v/v in MO), and the concentration of odor B (v/v in MO) was decreased by 0.5 log units for each new concentration (0.1% to 0.0001%, test stimulus). Briefly, mice were tested with a block of S +/S— trials to ensure discrimination of the odor mixture from odor A alone. Once achieving $\geq$ 85% accuracy, mice were presented with MLPEST blocks (30 trials: S+, S—, and a test stimulus presented randomly for each trial). To maintain stimulus control, MLPEST was modified such that licking on an S— stimulus resulted in S+/S— trial presentations until the animal did not respond to two consecutive S— stimuli. The program changed the concentration of odor B in the test stimulus according to the MLPEST algorithm (see Clevenger and Restrepo, 2006). We performed a power analysis (see Statistics) to ensure we would not miss significant differences in threshold between genotypes in the C3H mice (α 7+/+: n = 11; α 7+/-: n = 9; α 7-/-: n = 7).

Tissue Preparation

Mice were euthanized by CO₂ inhalation; brains removed quickly, placed in molds filled with OCT (Optimal Cutting Temperature compound) and then frozen (-80°C). The frozen brains were stored at least overnight and not more than two months before sectioning. On the day of sectioning, the brains were placed in the cryostat (-18°C) for at least 45 min before cutting. Coronal sections (12 μ m) were thaw-mounted on gelatin-subbed microscope slides and stored at -80°C for 2–6 weeks until [¹²⁵I] α -BGT binding was performed.

α -BGT Autoradiography

As previously published (Adams et al., 2008), slides were preincubated with room temperature TBS (50 mM, pH 7.4, 30 min) containing 2 mg/ml BSA and then incubated at 37°C for 3 hours in buffer containing 5 nM [¹²⁵I] α -BGT (specific activity: 2000–2200 Ci/mmol, Amersham Pharmacia Biotech or PerkinElmer). For two experiments, the high-specific activity α -BGT was not available thus we used the low-specific activity (104–107 Ci/mmol, PerkinElmer) along with a conversion factor to include the data in the analysis (see below). Non-specific binding slides were processed identically, except that 5 mM unlabeled nicotine was added to both the prewash and the α -BGT solution. Following incubation, slides were washed with TBS/BSA (5 min), TBS (15 min), and PBS (5 min). Slides were dipped briefly in 4°C dH₂O then quickly dried under cool air. Slides dried overnight at room temperature then exposed to high performance autoradiography film along with ¹⁴C-standards for 24 hr, after which the film was developed.

Quantifying α 7-nAChR Density

Each section was digitally captured under controlled light and exposure conditions. Binding of α -BGT was quantified using software initially developed to map glomeruli within the OB

(Schaefer et al., 2001a, 2001b). For all sections, the glomerular layer of the OB (48–60 μm apart) were outlined using a computer mouse with effort made to maintain the outline as close as possible to the edges of the desired region. The program calculated the darkness of α -BGT binding subtracted from mean non-specific binding based on a density gradient of 0 (black) to 255 (white). The grey-scale values were converted to nCi/g of ^{125}I by using radioactivity standards on the films and a 2nd order polynomial regression to generate a standard curve. All regression analyses were conducted within the linear portion of the standard curves. For the two experiments with low-specific activity, we converted the grey-scale values to nCi/g of ^{125}I by the same method used above and then multiplied the number by the mean counts-per-minute (CPM) of high-specific activity divided by the CPM of the experiment's low-specific activity.

Statistics

Mice were tested in MLPEST five times for each odor pair and responses from all mice were averaged. Power analysis was performed to ensure significant differences in acetate thresholds from C3H mice were not missed (80% power: $n = 7$ mice). Analysis of variance (ANOVA) with a multiple comparisons test (Newman-Kuels) was used to determine significant differences in: 1) learning the S+/S− task, 2) discrimination and detection thresholds between mouse strains and genotypes ($\alpha 7^{+/+}$ vs. $\alpha 7^{+/-}$ vs. $\alpha 7^{-/-}$), and 3) amount of α -BGT binding between mouse strains and genotypes. Significance was accepted when $p < 0.05$, calculated *post hoc*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

$\alpha 7$	$\alpha 7$ -nicotinic acetylcholine receptor
nAChR	nicotinic acetylcholine receptor
+/+	wild type
+/-	heterozygous knock out
-/-	homozygous knock out
α-BTX	[^{125}I] α -bungarotoxin
OB	olfactory bulb
HDB	horizontal limb of the diagonal band of Broca
PC	piriform cortex
MLPEST	Maximum Likelihood Parameter ESTimation
MO	mineral oil
OSN	olfactory sensory neurons

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Figure 1. [^{125}I] α -BGT binding is localized to the glomerular layer of the OB (arrows). **A)** Nonspecific binding. Representative binding in **(B–D)** C57 and **(E–G)** C3H mice. Scale bar = 1 mm.

Figure 2.

Mean specific tissue equivalent α -BGT binding in mice. Unlike the hippocampus (see Supplementary Figures/Results), C57 $\alpha 7^{+/+}$ mice have significantly more binding in the OB compared to C3H $\alpha 7^{+/+}$ mice ($n = 5-8$ mice/strain). Significantly less α -BGT binding was observed in $\alpha 7^{+/-}$ and $\alpha 7^{-/-}$ compared to $\alpha 7^{+/+}$ mice. No significant differences were observed between $\alpha 7^{+/-}$ or $\alpha 7^{-/-}$ mice ($\alpha 7^{+/-}$: $n = 4-5$ mice/strain; $\alpha 7^{-/-}$: $n = 3$ mice/strain, ** = $p < 0.01$, ANOVA with multiple comparisons, Newman-Keuls).

Figure 3. Learning a “go-no-go” odor task. **A)** Sample data from a C57 $\alpha 7^{+/+}$ mouse, where 1% isoamyl acetate was the rewarded response and mineral oil was the unrewarded response. Mice had a 50% chance of getting the correct response, but performance of $\geq 85\%$ correct for three consecutive blocks was defined as reaching criterion or learning the task. Blocks consisted of 20 pseudo-randomly presented rewarded (10) and unrewarded (10) odor trials. **B)** Control data showing that mice cued on the odor for achieving criterion. As soon as the odor was removed, mice achieved the rewarded response by chance. When the odor was returned, mice reached criterion again. **C)** Despite the background of the mouse (C57 or C3H), all genotypes learned the go-no-go odor task at the same rate (ANOVA with multiple comparisons, Newman-Keuls, $p = 0.9$, $n = 8-13$ for each group).

Figure 4.

Before mice were tested with the MLPEST program, the animals were required to complete S+/S- to ensure they were able to detect the differences between the alcohol pair. **A)** C3H $\alpha 7^{+/+}$ mice reached criterion by the 6th block the first time they were exposed to the odor pair (n = 8). **B)** In the last presentation, by the 3rd block, all C3H mice achieved criterion ($\alpha 7^{+/-}$: n = 7; $\alpha 7^{-/-}$: n = 6). **C)** Similar to C3H $\alpha 7^{+/+}$ mice, C57 $\alpha 7^{+/+}$ mice reached criterion by the 7th block in the first presentation. **D)** All C57 genotypes completed S+/S- paradigm by the 3rd block in the last presentation of the alcohol pair (n = 7 for all groups).

Figure 5. Mean discrimination thresholds for $\alpha 7^{+/+}$, $\alpha 7^{+/-}$, and $\alpha 7^{-/-}$ littermates. Odors were mixed with 0.1% octaldehyde (**A**) or 0.1% propyl acetate (**B**). Odorant concentrations decreased by 0.5 log units for each new concentration, ranging from 0.1% to 0.0001%. **A**) For the discrimination task using an aldehyde odor pair, WT mice discriminated an entire log unit lower compared to HET and KO in both strains of mice ($n = 5-11$ mice; * $p < 0.05$, ANOVA with multiple comparisons, Newman-Keuls). **B**) However for an acetate odor pair, only the C57 WT mice had a threshold half a log unit lower compared to all other groups of mice ($n = 5-8$ mice for aldehydes; * $p < 0.05$, ANOVA with multiple comparisons, Newman-Keuls).

Table 1

Odors used as conditioned stimulus pairs.

Odor pair	Odor A (S-)	Odor B (S+)
Aldehydes	Octaldehyde	Heptaldehyde
Acetates	Propyl acetate	Ethyl acetate
Alcohols	1-Heptanol	1-Octanol
Dissimilar odor pair	Propionic Acid	Benzaldehyde