Published in final edited form as:

Hippocampus. 2008 ; 18(8): 737–749. doi:10.1002/hipo.20430.

Nicotinic Acetylcholine Receptor Expression in the Hippocampus of 27 Mouse Strains Reveals Novel Inhibitory Circuitry

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Abstract

Mouse strains are well-characterized to exhibit differences in their physiological and behavioral responses to nicotine. This report examines the expression of the high-affinity nicotine binding receptor subunit, neuronal nicotinic receptor subunit $\alpha 4$ (nAChR $\alpha 4$), in the dorsal hippocampus of 27 inbred mouse strains. Multiple differences among mouse strains in the cellular expression of nAChR $\alpha 4$ between subregions of the hippocampal field are evident. Differences that we describe in the expression of nAChR $\alpha 4$ suggest mouse strains of diverse genetic origin could exhibit significant variation in how this receptor contributes to modulating intra-hippocampal circuitry. These findings define a genetic frame-work in which the strain-specific responses to nicotine include underlying contributions by the varied anatomical context in which nAChRs are expressed.

Keywords

Nicotinic Receptor; mouse strains; hippocampus; genetics; cytoarchitecture

INTRODUCTION

Mouse models have provided many insights into the genetic and physiological mechanisms of disease susceptibility and behavioral variation ranging from normal immune processes to mechanisms of cancer and drug dependency. This includes the demonstration that mice of different strains exhibit often dramatic, yet characteristic, differences in their physiological and behavioral responses to nicotine (Bovet et al., 1966; Marks et al., 1991; Crawley et al., 1997). Therefore, characterization of the differences in the expression of nicotine's target in the central nervous system, collectively termed nicotinic acetylcholine receptors (nAChR), is a starting place to identify how genetics contribute to the individual response to nicotine.

While all mammals appear to share a similar nAChR repertoire of 16 genetically related subunits (Hogg et al., 2003), it is the coincident expression of nAChR $\alpha 4$ and nAChR $\beta 2$ subunits that compose the majority of high affinity nicotine binding sites in the mammalian brain (Flores et al., 1992; Gahring et al., 2004a; Hogg et al., 2003; Whiteaker et al., 2006). Recent studies have demonstrated the central role for this nAChR subtype in the progression of many key physiological responses related to nicotine addiction such as up-regulation in response to chronic nicotine administration, reinforcement, self-administration and cognition (Picciotto et

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al., 1997; Levin, 2002; Lester et al., 2003; McCallum et al., 2006) as well as neuroprotection (Gahring et al., 2003). Studies of mice have proven particularly informative in terms of revealing evidence for a strong genetic pre-deposition that control these strain-distinct characteristics. In terms of nAChR α 4 expression, detailed studies have revealed that approximately 40 percent of the variation among mouse strains in their respective responses to nicotine (Crawley et al., 1997) is likely related to differences in the expression or the response by receptors harboring this subunit (Marks et al., 1989; Crawley et al., 1997). However, despite the identification of polymorphisms in this and other nAChR subunits that can influence their function (Dobelis et al., 2002), the distinct relationship among strains to nicotine sensitivity remains to be elucidated and in fact it is not likely to be straight-forward (Butt et al., 2003; Gahring et al., 2004a; Li et al., 2005).

Another explanation for a genetic link between nAChRs and strain-dependent nicotine responses could be reflected in genetically-determined differences in the anatomical context in which these receptors are expressed (Adams et al., 2001; Gahring et al., 2004a; Gahring et al., 2004b). For example, evidence that mouse strains exhibit significant qualitative and quantitative differences in nAChR expression by inhibitory interneurons of the hippocampus that correlate with differences in the behavioral responses to nicotine has been presented previously for at least 8 eight different mouse strains or lines (Gahring et al., 2004a) and by other groups exploring relationships between hippocampal morphology and related nAChRs (Adams et al., 2001). This leads to the hypothesis that the genetic control of the morphological context in which nAChRs are expressed may be a central contributor to how this modulatory receptor system governs the balance between excitatory and inhibitory circuitry. To test this hypothesis, we have examined the expression of nAChR α 4 within the dorsal hippocampus of 27 inbred mouse strains. This study reveals that the anatomical context of nAChR expression differs between mouse strains. Consequently, it is implied that how nAChRs contribute to modulating local and extended control of inhibitory tone in the hippocampus will vary substantially among strains of differing genetic relatedness. In a parallel study (Rogers et al., submitted), at least part of the difference in the cellular context of nAChR α 4 expression is shown to reside on chromosome 11 of the mouse.

METHODS

Choice of Mouse Strains

All strains studied were obtained from Jackson Laboratories (BarHarbor, ME). Mice were between 5 and 8 months of age for inclusion in this study. Three criteria were used for strain selection. First, to evaluate variation in nAChR α 4 hippocampal cytoarchitecture, strains were selected from 5 major genetically defined subgroups among the 102 strains defined recently (Petkov et al., 2004). In their analysis a phylogenetic tree anchored by 7 defined subgroups was defined. For the 27 strains examined in this study (Table 1), 9 were from group 1 (anchored by BALB/c and C3H/HeN), 5 strains from group 2 anchored by Swiss mice (anchored by BUB/Bn), 6 strains from group 4 (anchored by C57BL/6), 2 strains from group 5 (anchored by 129-related strains), and 5 strains from group 6 (anchored by DBA/2). Mice from groups 3 (Japan and the New Zealand strains) and 7 (wild-derived inbred strains) were not examined. The second criterion was to include mouse strains that have been characterized for their differential response to nicotine (Crawley et al., 1997; Marks et al., 1991). All but 2 (ST/b and C57L) of these 19 strains were included. Data for the strains BALB/c, C3H/HeN, C57BL/6, CBA and DBA/2 have been reported previously (Gahring et al., 2004a; Gahring et al., 2004b) and these values were used in this study. Finally, as indicated above, strain availability contributed to the inclusion or exclusion of some strains from this analysis. All animals were maintained in IACUC approved animal housing with appropriate light/dark cycling.

Genomic Sequencing—Genomic DNA from mouse tails was amplified using long PCR conditions as described previously (Gahring et al., 2004a). For each reaction 100 ng of genomic DNA was used for preparing each sequence-ready template for automated sequencing. Sequence trace files were evaluated to identify consensus genomic sequence at polymorphic positions identified in the forward and the reverse sequences. All sites were homozygous at the polymorphic position in the strains examined.

Immunoreagents and Data Analysis—The antibodies and methods used here to prepare and stain free-floating sections prepared from mice perfused with 2% paraformaldehyde have been described previously (Flores et al., 1992; Gahring et al., 2004a; Rogers et al., 1998; Turne and Kellar, 2005; Gahring et al., 2005b). Primary antibodies to nAChR α 4 included rabbit polyclonal 5009, 6964 or mouse monoclonal 4G10. As reported previously (Gahring et al., 2004a), these antibodies gave equivalent results and the pattern of staining closely parallels findings from in situ hybridization and ^3H -nicotine binding (Rogers et al., 1998). Of note is that the 5009 polyclonal antiserum was one of the immunoreagents used in very early studies (Flores et al., 1992) that originally defined the nAChR α 4 subunit as the principal component of the high-affinity nicotine binding site and the nAChR up-regulated by nicotine administration in the rodent. Similarly, the specificity has been characterized further in studies using cultured cells and rat brain preparations (Turner and Kellar, 2005).

Analysis of coronal brain sections (25 microns thick collected at repeating intervals of 100 microns) included scoring of immunopositive cells from the dorsal hippocampus of at least five animals per mouse strain as before (Rogers et al., 1998; Gahring et al., 2005a). At least 6 sections per animal (left and right side) were captured by the meander-scan method of the NeuroLucida/Virtual Slice software package (MicroBrightField) using a DAGE 300 CCD camera attached to a Zeiss Axiovert 200 microscope and Plan-Neofluar 20X objective. All montages were matched to the same relative anatomical plane (between Bregma -1.7 to -2.2 ; Franklin and Paxinos, 2008), and scored for immunostained cells per subregion (dentate gyrus, CA3 and CA1 as defined in Figure 1a). The CA1 region was subdivided further into the pyramidal layer and the stratum radiatum or stratum oriens which for scoring purposes were defined as beginning 2 cell diameters (~ 25 microns) either side of the pyramidal cell layer. In some cases, especially for scoring the much smaller astrocytes, image montages were generated using a 40X Plan-Neofluar objective.

Sampling and statistical approaches were published previously (Rogers et al., 1998; Gahring et al., 2005a). For quantitative analysis, an immunopositive cell was defined based upon inclusion of the nucleus (majority of the cell body volume within the section) and staining intensity as defined by an objective distinction from background using algorithms in ImagePro-Plus software package (Media Cybernetics). The average value for corresponding anatomically-defined regions of each animal was calculated and these values summed as in Table 1. Standard statistical tests used the GB-Stat v9.0 software package. Image assembly for presentation in the figures was done using Photoshop C2 software.

RESULTS

The expression of nAChR α 4 in the hippocampus, and especially interneurons (defined previously as GAD+; (Gahring et al., 2004a)) can vary considerably between mouse strains (Figures 1). Of note is the striking difference among the density of pyramidal layer-associated interneurons (especially CA1) and the disparity in the prevalence of nAChR α 4+ astrocytes (GFAP+ as determined previously (Gahring et al., 2004a); see Figure 1b and below). Also evident are notable, and in some strains equally dramatic, differences in the expression of nAChR α 4 in the dentate gyrus (Figure 1). This is especially true of neurons in the hilar region and outer molecular layers. In CA3, immunostaining for nAChR α 4 of neurons or astrocytes is

more sporadic and generally restricted to large pyramidal neurons. This region exhibits the least heterogeneity among strains in nAChR α 4 expression of any hippocampal region and is not discussed further. The subiculum also exhibits many nAChR α 4+ interneurons whose density overall appears to be far greater than observed in the CA1 or CA3 regions (not shown). This region was not analyzed in detail, however, since coronal sections are not optimal for quantitation of this region. Finally, members of two strains, 129 and I/Ln, exhibited corpus callosum agenesis (Wahlsten et al., 2006; Wahlsten et al., 1992). While this afflicted only 2 of 5 129 $\times$ 1/Sv mice, all I/Ln mice were altered. The shape of the I/Ln hippocampus also appears distorted, possibly reflecting what appears as a shortened CA3 pyramidal region (not shown). The values scored for this strain are included within the analyses since the CA1 and dentate gyrus regions could not be dismissed based upon obvious visual or statistical differences in nAChR α 4 expression relative to others despite substantially distorted hippocampal morphology.

Quantitation of nAChR α 4 Expression in CA1 defines unique strain-related relationships

Differences in expression of nAChR α 4 were subjected to quantitative analysis. The results of this analysis are presented in Table 1 and Figure 2 where the region-defined expression of nAChR α 4 in the dorsal hippocampus is reported as the average number of cells per region and standard error of the mean. Also shown is the relative genetic-relatedness of strains as reflected by association in 5 groups as defined by the SNP analysis of Petkov and colleagues (Petkov et al., 2004; see Methods). Using the CA1 subregion as a point of reference (Figure 2), there is a considerable range in density of neurons expressing nAChR α 4 among and between genetically-related strains. The number of nAChR α 4+ neurons tends to be greatest in groups 1 and 2 and least in groups 4, 5 and 6. Also some strains differ substantially in the relative ratio of CA1 immunostained astrocytes (compare groups 1+2 versus 4+6; see below and Table 1). Only rarely are nAChR immunostained astrocytes observed outside of CA1 (i.e., dentate gyrus or CA3; Figure 1 and below). Intra-group variation is also evident. Members of group 1 (C3H) can vary in expression by 6-fold, although this variability cannot be generalized since other groups exhibit relatively little variance among related mouse strains (e.g., groups 4 (C57BL/6) and 6 (DBA1)). To examine the relationship between strain relatedness and hippocampal nAChR α 4 expression density, the neuron counts from the dentate gyrus, CA1 and CA3 values in Table 1 were compared using Pearson correlation coefficient analysis and these values were used to generate a tree diagram of mouse strain relatedness (Figure 3) using the Fitch and Margoliash algorithm of PhyloDraw (Choi et al., 2000). Also shown in Figure 3 is representative section from CA1 for each strain examined. Because only two mouse strains from Group 5 were available for analysis, the assignment of these strains was based solely upon their SNP-based group designation. Overall there is excellent agreement with the findings from this study and the genetic-based analyses of Petkov et al., (Petkov et al., 2004). Even certain subtle strain relationships were retained in this measurement. For example, the close relatedness of C57BL/6 and C57BLKS (also referred to as C57BL/Ks) is consistent with the relatively recent origin of the C57BLKS strain from a C57BL/6 $\times$ DBA2 cross where approximately 70 percent of the C57BLKS genome is derived from C57BL/6 (Naggert et al., 1995). The early separation in the CBA and C3H/HeN lineages by these criteria is also noted.

Mouse Strains differ in nAChR α 4 Expression: Hippocampal CA1

The cells most prominently stained for nAChR α 4 expression are pyramidal-associated interneurons of CA1. Also present are interneurons associated with the stratum oriens and stratum radiatum, and their numbers vary considerably among strains (Figure 2 and Figure 4). Sections from three strains (LG, SWR and P) are shown in Figure 4a as examples of the diversity of CA1 nAChR α 4 expression in this region. For the LG strain, the nAChR α 4+ interneurons in association with the pyramidal layer exhibit a well-organized pattern or regular lateral spacing (approximately one nAChR α 4+ interneuron per 100 to 125 microns). This basic

pattern of nAChR α 4+ interneurons is retained in most strains, albeit the interval of separation can vary substantially. This is also seen in Figure 4a where occurrence of interneurons in the SWR strain is almost one-half that of the LG strain and greatly diminished in the P strain mouse. Also in Figure 4 is shown the CA1 region for additional strains where the regularity of spacing in these interneurons is evident. Additional study will be required to better quantitate this presumed pattern, although preliminary 3D-reconstruction is suggestive of regularity suggestive of a possibly more complicated geometric pattern (not shown).

Often in association with the well-spaced pyramidal-associated interneurons are interneurons of distinct morphology in the stratum radiatum that, while infrequent in single sections (see Table 1), tend to exhibit regular lateral spacing (approximately 1 every 350 microns; Figure 4a). The distinct cytoarchitecture includes one process that extends towards the CA1 pyramidal layer while another projects from the cell body to transverse the stratum radiatum roughly in parallel with axons of the Schaffer collaterals. A cell type in the C3H/HeN strain with a similar morphology has been reported previously ((Gahring et al., 2004a); and see Figure 3). Occasionally there is observed a very thin and difficult to distinguish process (presumably an axon) that extends towards the stratum lacunosum moleculare (not shown). While these interneurons are very rarely observed in the presence of astrocytes (see Table 1), they are occasionally present in some strains that also have nAChR α 4+ astrocytes (e.g., BALB/c; Figure 3).

Another highly variable feature among mouse strains is the staining of dendritic arbors of the CA1 nAChR α 4+ interneurons. This difference is readily apparent in photos of strains depicted in Figure 4b where prominent dendritic arbors extend from the cell bodies near the pyramidal layer through the stratum radiatum where they are observed to terminate in prominent bifurcations near the lateral projections of the perforant path in or near the border of the stratum lacunosum moleculare (e.g., LP; Figure 4b). Although a clear pattern of dendritic staining has not been established, there are similarities within in groups. For example, in strains collectively placed in groups 1 or 2, with the notable exception of RIIS, there are extensive dendritic projections that are often rich in varicosities (e.g., group 2 and members of group 1; see Figure 1 and 3 and Rogers et al., 1998). Also, there is an influence by nAChR α 4+ astrocytes. Other strains, however, exhibit prominent projections, but few varicosities (e.g., group 4) while other strains (such as group 6) produce difficult to image processes. In general, when astrocytes are present, the strength of staining in the dendrites is much reduced (Figure 4). Nevertheless, when prominent staining of dendrites is observed in the presence of stained astrocytes, the nAChR α 4+ processes tend to be observed to terminate in the vicinity of astrocyte groupings (see C58 and SF/CamEi in Figure 3 and for C57BL/6 (see Gahring et al., 2004a)) where these processes and astrocytes appear to intermingle (Gahring et al., 2004a; Gahring et al., 2004b). Finally, the other extreme case are exemplified by the P strain (Figure 3a) which exhibits very few nAChR α 4+ stained interneurons and a high density of astrocyte staining (Figure 1b and Figure 4a). The P mouse belongs to the group 6 (DBA2) lineage. All mice of this group (including DBA1; see Figure 1b,) exhibit prominent nAChR α 4 expression by astrocytes and fewer, but strongly stained, interneurons (Table 1, Figure 2 and below) that lack prominent dendritic projections.

Prominent staining of interneurons in the stratum oriens is present and varies according to strain (Table 1, Figure 2, Figure 3 and Figure 4). The heterogeneity of cell morphology in this region is striking. Observed are cells with a triangular-oval soma with processes that extend from two of the triangle apexes laterally and one that usually extends towards the stratum radiatum and transverses to the pyramidal layer (Figure 4c and not shown). Presumptive axons from these cells are also occasionally observed to extend towards the alveus away from the subiculum in the direction CA3. Similar to the sr, the absence of nAChR α 4+ interneurons in the stratum oriens is most often accompanied by the presence of stained astrocytes (e.g., Figure 2 and 3b).

Astrocytes appear near the distal region bordering on the alveus, which is particularly true of mice from groups 4 (C57BL/6) and 6 (DBA2). These mice also exhibit very few interneurons stained with nAChR α 4 in this layer. At least two other strains (A and RIIS) exhibit this substitution-like phenotype which seems unusual since both of these strains are otherwise in groups whose other members generally do not match with these descriptions. In fact, the interneuron:astrocyte relationship does not strictly segregate as a simple genotype:phenotype relationship (Rogers et al., submitted).

The typical hippocampal nAChR α 4 staining pattern for two strains (129 $\times$ 1/Sv and FVB/N) that are often used as the parental background strain for construction of knock-out or knock-in mice is shown in Figure 3c. In these strains there is very rarely observed astrocyte nAChR α 4 and the neuronal pattern of staining is frequently best described as irregular (as for FVB/N). This suggests that strains harboring the 129 $\times$ 1/Sv or FVB/N background would have nAChR α 4 expected from the respective background, which in these cases exhibit only infrequently nAChR α 4+ astrocytes.

Previously it was reported that there is a quantitative inverse relationship between the proportion of CA1 nAChR α 4+ interneurons and astrocytes (Gahring et al., 2004a). This was extended to the 27 strains examined in this study and the relative ratio of nAChR immunostained CA1 neurons versus astrocytes as a percentage of total hippocampal nAChR α 4 expression is shown in Figure 5. The highly significant inverse relationship ($r = -0.87$; $p < 0.001$), confirms that a very strong inverse relationship in the CA1 between the percent of nAChR immunostained astrocytes versus neurons.

Dentate gyrus

Interneurons of the dentate gyrus (DG) have been classified according to several criteria including morphology, neurotransmitter markers and physiological properties (e.g., (Alkondon and Albuquerque, 2004; Freund and Buzsaki, 1996; Somogyi and Klausberger, 2005)). In our measurements of nAChR α 4 expression in the DG (Figure 6), nAChR α 4+ cells ranged in density from an average of fewer than two per section (SM) to over 13 (BALB/c) (see Table 1). Although statistically DG nAChR α 4+ numbers between each group are similar, except for groups 1 and 6 which differ sufficiently to reach statistical significance ($p < 0.04$), other notable differences are apparent. As seen in Figure 6, despite quantitative similarity, there is considerable complexity in nAChR α 4+ cell distribution among or between strains. This is particularly evident in the interneurons of the DG hilar and molecular layer (Figure 6) where the relative distribution of nAChR α 4+ cells, and the apparent complexity in the processes extending from these cells can differ substantially, especially of the hilar region where considerable anatomical diversity occurs between strains. Also evident, as in the pyramidal-associated interneurons of CA1, is the regular spacing of interneurons (~150 microns; Figure 6a–b) that in some strains is best observed in the sub-granular layer (e.g., YBR/Ei; Figure 6b) but in the molecular layer of other strains (e.g., AKR and LG as in Figure 6b; also compare with AKR in Figure 1b). The distinct spacing and location of these interneurons and their importance to nicotinic receptor-related circuitry in the hippocampal structure is returned to below.

The nAChR α 4+ cells in the DG fall in general fall into two regions; those in the molecular layer those in the hilus (Figure 6). Interneurons of the DG molecular layer (ML) can be subdivided further into those in the outer ML (OML) or inner ML (IML). This distinction is relevant because granule cell dendrites and cells of the OML receive excitatory afferents principally from the perforant path (Steward, 1976) whereas the IML receives mostly cholinergic commissural/associational pathway terminals. Overall nAChR α 4+ cells in the molecular layer (IML+ OML; Figure 1) represented less than 15% of the total DG cells. Of these the ratio between IML and OML was approximately 2:1, respectively, in 15 strains, but

two additional subgroups were identified. The first exhibits a reversed ratio where OML cells outnumbered IML cells (BUB/Bn, FVB/N, SJL) by almost 4:1 where they are dispersed throughout the OML (Figure 6). These strains are also exclusive to genetic group 2 mice, and they are among those that exhibit the fewest CA1 astrocytes. The second subgroup consists of 9 strains (129, A, C57BR, DBA1, DBA2, LP, RIIS, SM, YBR/Ei), where nAChR α 4+ cells in the molecular layer (both IML and OML) are very rarely observed. Also of note is the variety of morphologies detected among these stained cells (Figure 1 and 6b and 6c). Neurons matching the description of a “pyramidal-like interneuron” whose cell body is located within the inner third of the OML and whose dendrites extend into the hilar region towards CA3 have been described (Ceranik et al., 1997). While no axonal staining was reliably detected using this method, axons of these OML interneurons are reported to cross the hippocampal fissure (HF) to interact with hippocampal CA1 neurons and those of the subiculum. No OML nAChR α 4+ cells immediately adjacent to the HF were observed. Cells with considerable morphological diversity are revealed in the IML (Figure 6 b–c). These cells range from those with relatively simple oval cell bodies and often a single process projecting towards the granule cell layer or occasionally as for the P strain a bi-processed cell body as in Figure 6c. In some strains the IML neurons resemble displaced granule cells that possess prominent arbors projecting into the OML. Also evident are triangular cells with two projections extending bi-directionally along the granule cell perimeter and one projection through this cell layer (Figure 6c). The soma of these cell is within or adjacent to the IML and they project a single stained process proximal to the cell body directly towards the granule cell layer. Also of interest is the distribution of nAChR α 4+ cells in the DG of the P strain. In contrast to the poor staining of interneurons in the hippocampal CA1 region of the P strain, the staining in both OML cells as well as those of the hilus is robust.

Cells in the hilar region harbor morphology suggestive of spiny cells (Lubke et al., 1998) and basket cells (Figure 6b and 6c). These cells are common and their arbors are consistent with reports that in addition to interacting with commissural/associational fibers, projections from these hilar interneurons may terminate in the IML (Lubke et al., 1998; Sik et al., 1994) as well in other region of the granule cell and CA3 pyramidal cell layers. Collectively, the strategic localization of these diverse interneurons subtypes indicates that the differential nAChR α 4 expression by these hilar neurons would likely impact on the information flow from the dentate gyrus to CA3, as well as the distribution of signaling that enters the dentate gyrus from perforant path fibers. Also, presence of limited astrocyte expression of nAChR α 4 and the broad quantitative differences among strains between nAChR α 4+ interneurons indicates that such inhibitory control may in fact vary considerably among different mice that use the nAChR system.

Modeling strain-dependent inhibitory circuitry revealed by nAChR α 4 expression

Because local and cross-regional inhibitory circuitry controls the establishment, magnitude, and timing of spatially consistent oscillations (Traub et al., 1996), how strain differences in nAChR α 4+ expression by interneurons could be interrelated between the dentate gyrus, CA3 and CA1 was examined. For this analysis, strains were combined according to similarity in relative nAChR α 4 expression by astrocytes (groups 1+2 versus groups 4+6; Table 1) and compared using Pearson correlation coefficient analyses (not shown). The findings are presented graphically in Figure 7. For all strains examined there appears a set relationship that for every nAChR α 4+ interneuron in the dentate gyrus there is a corresponding numerical match with 2 similarly distinguished CA3 interneurons. The relationships between CA3 and CA1 and the cross-regional correspondence between the DG and CA1 differed according to genetic group designations. Groups with few nAChR α 4+ astrocytes displayed significant agreement in the relationship between the nAChR α 4+ interneuron numbers in CA3 and CA1 (1:1). This is in contrast with strains in groups 4+6 where this correspondence is not significant (Figure

8). Since these strains exhibit greater numbers of nAChR α 4+ astrocytes, the result strongly suggests that the inhibitory circuit relationship among these hippocampal subdivisions differs relative to each other in different strains. The inclusion of CA1 astrocyte numbers reveals that in astrocyte-poor strains of groups 1+2, there is no apparent relationship between CA3 and astrocyte numbers, which is consistent with interneuron dominated nAChR relationships in these strains. However for groups 4+6 the absence of a nAChR interneuron relationship between CA3 and CA1 is apparently compensated for by the appearance of a significant, but inverse relationship, between CA3 interneurons and CA1 astrocytes (1:3; respectively, and see Figure 7 and Figure 2). An intriguing possibility for inter-regional inhibitory dynamics between the DG and CA1 is also revealed. In this analysis, there is a statistically significant correspondence between DG and CA1 nAChR interneurons in astrocyte poor, but not astrocyte rich strains where no correlation is found. Interestingly, this relationship corresponds with those strains in which DG-OML nAChR α 4 interneurons are observed versus no correspondence when these cells are absent. Also, since interneurons in the alveus (and stratum oriens) of the CA1 can back-project into CA1, CA3, and dentate gyrus hilar region (Sik et al., 1994) and possibly more distant structure including the septum (Jinno et al., 2007), these data suggest the possibility that nAChR-mediated modulation is long-range and subject to cross-regional feedback inhibition in the hippocampus, particularly in strains belonging to Groups 1 and 2. Hence, the different relationships seen in Groups 1 and 2 versus 4 and 6 suggest basic difference could exist in how these strains configure the relationship between OML interneurons of the dentate gyrus and CA1 interneurons and astrocytes.

DISCUSSION

Specialization of neuro-circuitry through customizing cellular function and manipulation of neurotransmitter diversity are well-recognized mechanisms to impart functional specificity. The hippocampus offers several advantages as a platform for experimentally dissecting these relationships in terms of genetics, brain structure and behavior. As noted above, there is considerable genetic control of variation among CNS regional size and the cell populations between inbred mouse strains (Jucker et al., 2000; Lu et al., 2001; Peirce et al., 2003; Wimer et al., 1976). Similarly, volume differences of almost 2-fold in the human hippocampus (Hewett et al., 1994; Schumann et al., 2007) suggest such differences may not be limited to rodents. Given the wide variety of hippocampus-dependent behavioral traits including memory formation and navigational skills and the fact that nicotine can impact upon many of these behaviors, the possibility of genetic contribution to differences in hippocampal morphology are likely a covariant of the strain-specific nicotine response.

The well-defined architecture of the hippocampus also provides the advantage of measuring a layered network with well defined organization. As noted above, this includes the exceptional definition of neuronal subtypes, to the established interaction between excitatory and inhibitory local circuitry. This has been exploited by numerous groups to demonstrate that multiple nAChR subtypes expressed in the hippocampus function to coordinate and modulate local excitatory:inhibitory tone (Alkondon and Albuquerque, 2002; Alkondon and Albuquerque, 2004; Ji et al., 2001; McGehee, 2002). Our findings are in agreement with these studies and combined with earlier reports (Gahring et al., 2004a) indicate that considerable heterogeneity in nAChR expression by interneurons is possible. This would impact directly on the establishment of excitatory-inhibitory tone through adjusting thresholds for the initiation of action potentials by principal neurons. Therefore, overall differences in nAChR distribution within interneurons will impose considerably different constraints on the temporal and spatial impact of this system on local neurotransmission. Consequently, individual variation in this morphology is likely to be a significant component of the contributor to how different mouse strains respond to nicotine.

In the above context, the quantitative divergence between different mice in terms of the nAChR expression that ranges from mostly interneurons (e.g., mice of group 1 such as BUB/Bn) to other strains with relatively few nAChR α 4+ interneurons (including groups 4 and 6) is exceptional. This would be relevant to how the control of local circuitry may differ between mice of different genetic background. For example, the recent re-evaluations of the role astrocytes play in neurotransmission has considerably increased our understanding of how they participate in directly regulating both inhibitory and excitatory neurotransmission (e.g., (Fellin et al., 2006; Hosli et al., 1994; Sharma and Vijayaraghavan, 2001)). Specific examples include the modulation of excitatory neurotransmission by CA1 astrocyte release of a variety of glutamate receptor modulators including D-serine (Panatier et al., 2006) and kynurenic acid (Schwarcz and Pellicciari, 2002). Consequently, in light of the inverse relationship between nAChR α 4+ inhibitory interneurons and astrocytes (Figure 5), it may be hypothesized that modulation of excitatory circuitry by this nicotinic receptor subtype would impact upon pathways that influence both timing and spatial resolution. For example, interneuron modulation is fast and predominantly regulates local excitatory firing; whereas astrocytes effects would be slower and possible extend through interlaced interactions throughout the region. Such interactions have also been described to relate to timing of oscillatory activity (especially important in the development of the hippocampus; e.g., (Garaschuk et al., 1998)) and nicotine-initiated oscillations in astrocyte precursor cells are known (Rogers et al., 2001). Because thresholds of timing and oscillatory magnitude are relevant to many hippocampal functions, the possibility of these behavioral responses being altered in a strain dependent way provides a direct way for future testing of these relationships. The nAChR+ astrocytes can also make direct contact with blood vessels ((Gahring et al., 2004b), not shown), indicating that animals in which this nAChR expression is dominant may also be more responsive to blood-borne agents that modulate these receptors (e.g. nicotine). There are also reports that nAChR expression differs among mouse strains in other brain regions which includes an age-dependent component. For example, a substantial (2-fold) strain-specific variability in nAChR expression is observed in the striatum (Altavista et al., 1987) as well as the retina (Kang et al., 2004) and cerebellum (Turner and Kellar, 2005). Further, a dynamic relationship between nAChR expression by CA1 neurons and astrocytes has also been suggested to be found throughout the life span of both rodents and humans (Hosli et al., 2000; Graham et al., 2002; Graham et al., 2003; Teaktong et al., 2004; Gahring et al., 2005a). The possibility that DG OML or IML cells are Cajal-Renji cells (von Haebler et al., 1993; Del Rio et al., 1996) can be considered. However, this seems unlikely since this cell type is not positioned tangentially to the hippocampal fissure and the age of the animals studied here is well past the time (P31) these cells are expected to degenerate (Del Rio et al., 1996). Collectively, mouse strain specific behavioral and physiological responses to nicotine would likely be a reflection of a complex interplay between genetic factors that shape differences in expression and cellular architecture of this modulatory neurotransmitter system in the mammalian nervous system.

The developmental changes in nAChR expression by CA1 astrocytes may provide some insight into how this system changes with age. As noted, the general direction of change in nAChR expression begins with high interneuron numbers that diminish in favor of increased astrocyte expression. In this study, the P strain is most notable in this regard. This strain exhibits early neurodegenerative problems that begin around 12 months of age, similar to mice whose nAChR β 2 subunit has been genetically deleted (Zoli et al., 1999). However, even in strains with a high interneuron to astrocyte nAChR expression ratio, (e.g., CBA and C3H/HeN), there is an eventual shift in this ratio to favor astrocytes (Gahring et al., 2005a). Such findings also hint at the nature of long-range and possibly bidirectional control of nAChR neuronal activity in the hippocampus. While there is no evidence in this study for a direct inter-region interaction among nAChR α 4 interneurons or astrocytes among or between the DG or hippocampal subregions, these data do infer that rules regarding the establishment of these ratios are operative in these well defined inbred strains. Such a relationship may be difficult to study

directly, however, because there are too few DG-OML cells to impact upon the statistical correlations generated in this study. Nevertheless, the possibility that additional anatomic or other influences on the cross-regional interaction between the DG and CA1 inhibitory circuitry seems likely and is the subject of future investigations.

Acknowledgments

Thanks to Karina Periyannan and Steele McIntyre for excellent assistance in immunohistochemistry and Janelle Lee for genomic sequencing. Thanks also to Dr. Robert Weiss, Department of Human Genetics, for valuable assistance with performing PhyloDraw-based analyses. This study was funded by NIH grant NIDA/NHBLI PO1 HL72903 and AG17517.

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Figure 1. The expression of nAChR α 4 in the dorsal hippocampus varies between mouse strains
 Typical immunostaining for nAChR α 4 in the dorsal hippocampus of indicated strains. **Panel A.** A diagram of the dorsal aspect of the hippocampus taken from (Franklin and Paxinose, 2008) with modification. Coronal sections were collected from the location approximated on the sagittal drawing by a black line (inset). The coronal section shows boundaries of the respective subregions (arrow heads; see text). For the dentate gyrus, the area engulfed by the fascia dentate (hilar region) is included minus the cells counted within the interceding cells of the CA3 pyramidal layer. Anatomical definitions are: fbr, fimbria; ec, external capsule; DG, dentate gyrus, and hippocampal subfields, CA1, CA2 and CA3, respectively. Subregions of CA1 are defined as; py, pyramidal layer; so, stratum oriens; sr, stratum radiatum; and slm, stratum lucunosum moleculare. For the dentate gyrus (DG) the granule cell, gc; hilar, hi and molecular layer (ml) is indicated. Finally, the CA3 pyramidal layer is marked. **Panel B.** A section from the strains indicated (A) is stained for nAChR α 4 and matched approximately to the diagram in Panel A. Strain-expression is seen in all major subdivisions of the hippocampus (arrows and arrowheads), although the most prominently stained neuronal-like cells are associated with the dentate gyrus (hr and granule cell layers) and the pyramidal layer (Py) of CA1. **Panel C.** Images comparing similar coronal sections taken of a portion of the hippocampal field from the SJL, AKR and P strains, respectively. The quantitative and qualitative differences in expression for this nAChR subunit were measured from similar sections prepared from 27 strains. Labels indicating anatomic definitions are to the left. All bars = 100 microns.

Figure 2.

Graphic presentation of data from Table 1 showing the relative quantitative distribution of nAChRα4+ interneurons and astrocytes in different mouse strains. The data are organized according to major subdivisions of the hippocampal field, genetic group relatedness as indicated (see (Petkov et al., 2004)) and in order of increasing numbers of stained neurons in the CA1 region. All values are the mean $\pm$ the standard error of the mean.

Figure 3.

Mouse family tree generated from combining the strains from genetic groups (Table 1 and (Petkov et al., 2004)) and Pearson correlation values for nAChR α 4 expression the hippocampus (see text). Examples of the staining distribution for nAChR α 4 in CA1 for each strain are also shown for reference. The program PhyloDraw was used to generate the tree. The length and distances of branches between strains are arbitrary. Group anchor strains (Petkov et al., 2004) are boxed. Abbreviations are as in Figure 1. Bar = 100 microns.

Figure 4.

Examples of strain-related differences in nAChR α 4 expression in CA1. **Panel A.** Examples of nAChR α 4 expression in CA1 are shown for three strains as indicated. Notable staining of pyramidal-associated interneurons (py-int) in the LG strain is observed that in some sections exhibits remarkable regularity (asterisks). There are few stained astrocytes and regularly spaced neurons in the stratum radiatum (sr; approximately 100–150 micron intervals) are identified (arrows). Occasional cells in the stratum oriens (so) are also seen (arrowhead). Some strains, such as SWR exhibit fewer py-int, although these may also be distributed at regular intervals across the pyramidal layer (asterisks). Stained cells in the sr and so are infrequent in this section. The P strain shows occasional py-int neurons with occasional spacing reminiscent to that of SWR, although far less frequent. This strain (also shown in Figure 1) exhibits the greatest density of astrocytes in the sr and so and the fewest nAChR α 4+ interneurons. **Panel B.** Examples of additional strains (as indicated) show the diversity in cell morphology and patterns of nAChR α 4 expression encountered. The strains in the upper part of this section exhibit well-spaced py-int cells, many astrocytes and relatively limited arborization of neuronal processes. The A strain also exhibits predominate accumulation of stained astrocytes in the CA1 sr near the border with the stratum lacunosum moleculare. In contrast, LP and SJL each

reflect strains rich in py-int staining and few nAChR α 4+ astrocytes. Again, these neurons show regular spacing and are characterized by extensive arbors that are well defined. **Panel C.** The expression nAChR α 4 in the CA1 region of two strains frequently used for generation of genetically modified mice, 129 and FVB/N. The 129 exhibits a limited nAChR α 4 pattern of py-int expression that usually lacks the regularity suggested by this image. FVB/N is rich in stained interneurons, but the pattern of expression might be described as chaotic. Also, astrocytes that are nAChR α 4+ are rare in these backgrounds. Bar in Panel C = 100 microns for all photos except Panel B; strain A where it is 150 microns.

Figure 5.

Strain-specific CA1 nAChR α 4+ interneuron:astrocyte ratios are inversely related. The percent of the total nAChR α 4+ hippocampal CA1 interneurons plotted against the stained astrocyte ratio as derived from values in Table 1 is shown. The highly significant inverse relationship ($p < 0.001$) between these parameters is similar to that reported previously (Gahring et al., 2004a; Gahring et al., 2004b; Gahring et al., 2005). Some strains are identified for internal reference. Error bars reflect the mean $\pm$ the standard error of the mean for each strain measurement.

Figure 6.

Examples of strain-related differences in nAChR α 4 expression in the dentate gyrus. **Panel A.** The expression of nAChR α 4 in the dentate gyrus of three strains (as indicated) shows the diversity of staining, particularly in interneurons of the hilar region. Bar = 200 microns; gc=granule cell layer; ml = molecular layer. **Panel B.** Increased magnification of the dentate gyrus of YBR/Ei, AKR and LG mice illustrates the often dramatic difference in nAChR α 4 expression by interneurons of the molecular layer. Similar to CA1, the nAChR α 4+ dentate gyrus interneurons occur at regular intervals (asterisks) although their location in the granule cell layer or molecular layer is dependent upon the strain. Also shown are strains that exhibit nAChR α 4 expression in the inner molecular layer (IML) as for AKR or the outer molecular

layer (OML) as for LG. Bar = 100 microns. **Panel C.** Shown are various examples of the morphology of molecular layer cells positive for nAChR α 4 expression. As seen in these examples, the stained interneurons appear morphologically and anatomically heterogeneous (see text). Bars = 35 microns for SWR and AKR and 60 microns for BUB/Bn and P.

Figure 7.

Inter-regional definition of nAChR α 4 differences in the hippocampal formation varies according to genetic relatedness. In these diagrams the data from Table 1 are combined based upon genetic and nAChR α 4+ expression similarities such as cells in dentate gyrus (DG) outer molecular cells (+or -OML) and CA1 astrocytes (+ or - Ast). The relative ratio of nAChR α 4+ neurons (N) or astrocytes (Ast) among hippocampal subregions (arrows) is shown above the arrow and the Pearson correlation coefficient for this relationship below the arrow. Solid arrows reflect statistically significant relationships ($p < 0.05$; P-values boxed). All strains display a significant correspondence between the number of nAChR α 4+ interneurons of (DG) and CA3. However, between CA3 and CA1 there is a notable difference between these groups in the relative organization of nAChR α 4+ expression between these respective hippocampal subregions. These differences suggest that the contribution of this receptor to modulating neurotransmission in the hippocampus will not necessarily be the same among these respective mouse strain groups (see text).

Table 1

Quantitation of nAChR $\alpha 4$ + interneurons and astrocytes in the adult dorsal hippocampus of 27 inbred mouse strains.

Cell Count Mean (S.E.M.)	DG	CA3	CA1 (Total)	CA1: py	CA: so	CA1: sr	All Neurons	CA1 Astros	Group (Petkov et al.)	$\alpha 4/\beta 4$ haplo
129 $\times$ 1/Sv	2.25 (0.31)	2.54 (0.31)	8.50 (1.01)	5.63 (0.71)	2.42 (0.41)	0.46 (0.21)	13.29 (1.17)	6.70 (1.95)	5	T/P
A	5.00 (0.47)	3.46 (0.36)	11.67 (0.96)	9.17 (0.74)	1.79 (0.38)	0.71 (0.38)	20.13 (0.96)	44.50 (8.41)	1	A/L
AKR	3.96 (0.35)	7.42 (0.93)	15.58 (0.90)	10.79 (0.68)	4.00 (0.40)	0.79 (0.19)	26.96 (1.55)	5.60 (8.10)	1	A/L
BALB/c	13.40 (2.10)	23.10 (8.70)	36.90 (6.11)	22.20 (6.70)	10.50 (2.80)	4.20 (0.20)	73.40 (3.70)	32.40 (2.70)	1	T/L
BUB/Bn	5.92 (0.57)	11.17 (1.14)	21.04 (1.57)	15.08 (0.98)	4.46 (0.52)	1.57 (0.90)	38.13 (2.32)	2.75 (1.45)	2	T/L
C3H/HeN	9.80 (2.40)	11.80 (2.50)	24.80 (6.70)	17.70 (5.10)	5.00 (2.90)	2.10 (0.20)	46.40 (2.70)	15.30 (4.70)	1	A/L
C57BL/6	4.80 (0.70)	5.00 (3.10)	10.10 (2.70)	7.10 (1.60)	2.30 (0.90)	0.70 (0.20)	19.90 (1.06)	50.30 (7.50)	4	T/L
C57BL/10	2.79 (0.58)	6.33 (1.22)	10.75 (1.24)	8.71 (0.97)	1.42 (0.31)	0.63 (0.16)	19.88 (2.38)	29.20 (4.36)	4	T/L
C57BLKS	5.59 (0.59)	15.35 (1.06)	13.35 (1.29)	8.24 (0.88)	4.76 (0.54)	0.35 (0.19)	34.29 (2.17)	23.20 (6.50)	4	T/L
C57BR	2.68 (0.68)	6.42 (0.87)	21.37 (1.50)	14.79 (1.26)	5.47 (0.70)	1.31 (0.41)	30.47 (3.11)	18.00 (4.50)	4	T/L
C58	1.88 (0.27)	2.92 (0.48)	7.46 (0.75)	6.04 (0.61)	1.17 (0.21)	0.25 (0.11)	12.25 (1.01)	27.92 (3.72)	4	T/L
CBA	8.30 (1.00)	13.30 (1.20)	43.20 (3.40)	27.30 (1.60)	13.00 (1.40)	2.90 (0.40)	64.80 (2.40)	22.60 (2.20)	1	T/L
CE	7.67 (0.83)	21.75 (1.50)	19.33 (0.93)	13.13 (0.86)	5.42 (0.41)	0.79 (0.20)	48.75 (2.32)	30.50 (2.60)	1	T/P
DBA1	8.54 (0.61)	6.96 (0.71)	12.21 (0.96)	10.17 (0.79)	1.88 (0.30)	0.17 (0.08)	27.71 (1.50)	35.50 (8.53)	6	A/P
DBA2	3.60 (0.90)	5.00 (0.90)	9.90 (1.50)	7.00 (0.80)	2.80 (0.40)	0.10 (0.30)	18.50 (1.09)	44.00 (3.70)	6	A/P
FVB/N	2.92 (0.33)	16.17 (1.72)	32.79 (2.86)	21.50 (1.83)	7.75 (1.00)	3.54 (0.52)	51.88 (4.51)	5.50 (0.75)	2	T/L
I/Ln	3.90 (1.91)	8.45 (0.97)	13.05 (1.27)	11.00 (1.53)	7.20 (1.68)	2.25 (1.06)	25.40 (1.78)	16.80 (2.48)	6	T/L
LG	12.13 (1.20)	27.92 (2.13)	32.75 (1.36)	20.25 (0.94)	10.88 (0.72)	1.63 (0.31)	72.79 (3.82)	10.50 (2.30)	1	T/L
LP	0.79 (0.16)	3.84 (0.51)	7.16 (0.64)	4.47 (0.49)	2.21 (0.40)	0.47 (0.16)	11.79 (0.64)	9.50 (2.76)	5	T/P
P	1.88 (0.40)	1.25 (0.41)	4.83 (0.77)	4.25 (0.71)	0.58 (0.21)	0.00 (0.00)	7.96 (1.28)	81.11 (11.6)	6	T/L
PL	3.25 (0.39)	3.67 (0.57)	10.33 (1.18)	7.42 (0.84)	2.67 (0.62)	0.25 (0.13)	17.25 (1.33)	38.80 (2.93)	1	T/L
RIIS	3.00 (0.86)	6.27 (1.31)	16.50 (2.48)	11.36 (1.85)	4.41 (0.61)	0.73 (0.21)	25.77 (4.29)	6.00 (6.50)	2	A/L
SF-CamEi	11.04 (1.20)	15.58 (1.06)	17.17 (1.12)	13.38 (1.00)	3.75 (0.42)	0.04 (0.04)	43.79 (2.62)	28.50 (2.87)	1	A/L
SJL	4.25 (0.55)	7.38 (0.77)	15.79 (1.06)	13.04 (0.77)	2.33 (0.36)	0.42 (0.17)	27.42 (1.83)	6.50 (4.64)	2	T/L
SM	1.82 (0.40)	10.24 (1.32)	16.29 (1.00)	10.24 (0.76)	3.88 (0.45)	2.18 (0.25)	28.35 (3.16)	22.00 (3.63)	6	T/P
SWR	7.06	12.61	20.83	15.72	4.17	0.94	40.50	2.25	2	T/L

Cell Count Mean (S.E.M.)	DG	CA3	CA1 (Total)	CA1: py	CA: so	CA1: sr	All Neurons	CA1 Astros	Group (Petkov et al.)	$\alpha 4/\beta 4$ haplo
YBR/Ei	(0.83) 11.13 (1.31)	(0.94) 20.33 (2.52)	(1.18) 12.17 (1.35)	(0.77) 8.29 (0.80)	(0.54) 3.67 (0.56)	(0.26) 0.21 (0.10)	(1.71) 43.63 (4.86)	(0.97) 7.46 (2.06)	4	T/L

Each strain (all from Jackson Laboratories) and hippocampal subregion quantitated (Methods) is shown. The mean and standard error of the mean (in brackets) are shown for each strain. Strain relatedness (Group) according to Petkov et al. (Petkov et al., 2004) is listed. The haplotype structure from two known non-synonymous amino acid changes in either nAChR $\alpha 4$ or nAChR $\beta 4$ as confirmed by genomic sequencing is shown (see also Dobelis et al., 2002; Gahring et al., 2004a).