

Published in final edited form as:

*J Comp Neurol.* 2008 November 10; 511(2): 286–299. doi:10.1002/cne.21828.

## Expression of neuronal nicotinic acetylcholine receptor subunit mRNAs in rat hippocampal GABAergic interneurons

Jong-Hyun Son and Ursula H. Winzer-Serhan\*

Department of Neuroscience & Experimental Therapeutics, Texas A&M University System, Health Science Center, College Station, Texas 77843-1114, USA.

Oswald Steward

### Abstract

Hippocampal inhibitory interneurons are a diverse population of cells widely scattered in hippocampus, where they regulate hippocampal circuit activity. The hippocampus receives cholinergic projections from the basal forebrain, and functional studies have suggested the presence of different subtypes of nicotinic acetylcholine receptors (AChR) on GABAergic interneurons. Single cell PCR-analysis had confirmed that several nAChR subunit mRNAs are coexpressed with glutamate decarboxylase 67 (GAD67), the marker for GABAergic interneurons. In this anatomical study, we systematically investigated the coexpression of GAD67 with different nAChR subunits using double *in situ* hybridization with a digoxigenin-labeled GAD67 probe and <sup>35</sup>S-labeled probes for nAChR subunits ( $\alpha 2$ ,  $\alpha 3$ ,  $\alpha 4$ ,  $\alpha 5$ ,  $\alpha 6$ ,  $\alpha 7$ ,  $\beta 2$ ,  $\beta 3$ , and  $\beta 4$ ). The results revealed that most GAD67-positive interneurons expressed  $\beta 2$ , and 67 % also expressed  $\alpha 7$  mRNA. In contrast, mRNA expression of other subunits was limited; only 13 % of GAD67-positive neurons coexpressed  $\alpha 4$ , and less than 10% expressed transcripts for  $\alpha 2$ ,  $\alpha 3$ ,  $\alpha 5$  or  $\beta 4$ . Most GAD67/ $\alpha 2$  coexpression was located in CA1/CA3 stratum oriens, and GAD67/ $\alpha 5$  coexpression was predominantly detected in CA1/CA3 stratum radiatum/lacunosum moleculare and the dentate gyrus. Expression of  $\alpha 6$  and  $\beta 3$  mRNAs was rarely detected in the hippocampus, and mRNAs were not coexpressed with GAD67. These findings suggest that the majority of nicotinic responses in GABAergic interneurons should be mediated by a homomeric  $\alpha 7$  or heteromeric  $\alpha 7^*$ -containing nAChRs. Other possible combinations such as  $\alpha 2\beta 2^*$ ,  $\alpha 4\beta 2^*$ , or  $\alpha 5\beta 2^*$  heteromeric nAChRs could contribute to functional nicotinic response in subsets of GABAergic interneurons but overall would have a minor role.

### Keywords

*in situ* hybridization; hippocampus; nAChR; cholinergic; GAD67

### INTRODUCTION

Neurons in the hippocampus are divided into two subpopulations, principal excitatory neurons and local inhibitory interneurons (Freund and Buzsaki, 1996). Although local interneurons are a heterogeneous population, characterized by various chemical markers such as calcium binding proteins or neuropeptides, most of them contain the inhibitory neurotransmitter  $\gamma$ -amino butyric acid (GABA). In the hippocampus, GABAergic interneurons are widely distributed in CA1 and CA3 fields of ammon's horn (CA1, CA3)

\* Author of correspondence: Dr. Ursula Winzer-Serhan Dept. Neuroscience and Experimental Therapeutics 262 Reynolds Medical Bldg. TAMUS, Health Science Center College Station, TX 77843-1114 Tel: (979) 845-2860 Fax: (979) 845-0790 uwserhan@medicine.tamhsc.edu.

and in the dentate gyrus (DG), and play an important role in regulating the activity of both excitatory principal neurons, and inhibitory interneurons (Alkondon and Albuquerque, 2001; Ji and Dani, 2000; Jinno et al., 1998, Jones et al., 1999).

Neuronal nicotinic acetylcholine receptors (nAChRs) belong to the superfamily of ligand-gated ion channels, and are found in the central and peripheral nervous system (Sargent, 1993 review). Neuronal nAChR are composed of 5 subunits which can be classified into ligand binding  $\alpha$  and structural  $\beta$  subunits. Through molecular cloning at least six mammalian  $\alpha$  ( $\alpha 2$ ,  $\alpha 3$ ,  $\alpha 4$ ,  $\alpha 5$ ,  $\alpha 6$  and  $\alpha 7$ ) and three  $\beta$  ( $\beta 2$ ,  $\beta 3$  and  $\beta 4$ ) subunits have been identified in the brain (Sargent, 1993). Different combinations of  $\alpha$  and  $\beta$  subunits form functional heteromeric nAChRs with distinct physiological and pharmacological properties. The exception is the widely distributed  $\alpha 7$  subunit, which forms homomeric nAChRs (McGehee and Role, 1995, Chen and Patrick, 1997).

The rat hippocampus receives cholinergic input from the medial septum-diagonal band (MSDB) complex of the basal forebrain, with projections terminating at local inhibitory interneurons and principal excitatory neurons (Dougherty and Milner, 1999, Frotscher and L  r  n  th, 1985, Woolf, 1991). Several studies have shown that rat hippocampal interneurons express functional nAChRs (Alkondon et al., 1997; Frazier et al., 1998; McQuiston and Madison, 1999, and Ji and Dani, 2000), and that nicotinic agonists modulate GABAergic input to other hippocampal interneurons and principal neurons via activation of nAChRs (Alkondon et al., 2001, Jones and Yakel, 1997). Based on their electrophysiological and pharmacological properties, at least three distinct functional nAChR subtypes have been described:  $\alpha 7$ ,  $\alpha 4/\beta 2$  and  $\alpha 3/\beta 4$  (Alkondon and Albuquerque, 2004), suggesting the expression of several  $\alpha$  and  $\beta$  nAChR subunits in GABAergic interneurons. This has been supported by a single cell reverse-transcription polymerase chain reaction (RT-PCR) study that demonstrated the expression of several nAChR subunit mRNAs in the same interneuron, and different expression profiles could be correlated to distinct electrophysiological properties of nAChRs (Yakel and Shao, 2004). In addition, recordings from different types of interneurons in different layers of the hippocampal CA1 region found heterogeneity in the functional responses suggesting different subtypes of nAChR (McQuiston and Madison, 1999). This is supported by results from anatomical *in situ* hybridization studies and PCR analysis suggesting that interneurons express  $\alpha 5$  nAChR subunit mRNA in stratum (s.) radiatum, and  $\alpha 2$  mRNA in s. oriens (Winzer-Serhan and Leslie, 2005, Son and Winzer-Serhan, 2006, Sudweeks and Yakel, 2000).

However, a complete overview of the expression of nAChR subunits in GABAergic interneurons is still missing. Therefore, for a comprehensive anatomical analysis we used double-labeling *in situ* hybridization to identify GABAergic interneurons expressing nAChR subunit mRNAs in the adult rat hippocampus, by using a digoxigenin-labeled riboprobe for glutamic acid decarboxylase 67 (GAD67) as a marker for GABAergic neurons and  $^{35}\text{S}$ -labeled cRNA probes for the detection of nAChR subunit mRNAs. In the present study, distinct patterns of expression for the various nAChR subunits in GABAergic interneurons were observed with the majority expressing  $\alpha 7$  and  $\beta 2$ , and limited and restricted expression of  $\alpha 2$ ,  $\alpha 3$ ,  $\alpha 4$ ,  $\alpha 5$  and  $\beta 4$ , and no detectable expression of  $\alpha 6$  and  $\beta 3$  nAChR subunit mRNAs.

## MATERIAL AND METHODS

### Tissue preparation

Adult male Sprague-Dawley rats (~ 300 g) (Harlan Inc., Houston, TX) were kept on a 12 h light/dark cycle with free access to food and water according to the protocol approved by the Institutional Animal Care and Use Committee at Texas A&M, and consistent with National

Institute of Health guidelines. The animals were anesthetized with isofluorane, decapitated, and brains were immediately removed, frozen in  $-20^{\circ}\text{C}$  isopentane, and stored at  $-80^{\circ}\text{C}$  until use. From each animal, 20  $\mu\text{m}$  coronal sections were cut on a cryostat for dorsal (Bregma  $-2.30$  to  $-4.16$  mm) and ventral hippocampus ( $-4.80$  to  $-5.20$  mm) according to the "Rat Brain Atlas" by Paxinos and Watson (1998). For each nAChR subunit probe four dorsal and four ventral sections from three different animals were used. Sections were 200  $\mu\text{m}$  apart from each other for a representative analysis of dorsal and ventral hippocampus. Sections were postfixed with 4% paraformaldehyde in 0.1 M phosphate buffer (PB) (pH 7.4) for 1 h at room temperature (RT), washed in PB, air dried and stored desiccated at  $-20^{\circ}\text{C}$  until use.

### crRNA probe preparation

Plasmids containing full-length sequences for nAChR subunits were kindly provided by Dr. J. Boulter (UCLA, CA), and transcripts were subcloned into pBluescript II SK between T3 and T7 promoter sites.  $^{35}\text{S}$ -labeled crRNA probes for  $\alpha 2$  (1931 bp),  $\alpha 3$  (1858 bp),  $\alpha 4$  (2110 bp),  $\alpha 5$  (1607 bp),  $\alpha 6$  (1760 bp),  $\alpha 7$  (2100 bp),  $\beta 2$  (2196 bp),  $\beta 3$  (1780 bp), and  $\beta 4$  (2522 bp) were synthesized in antisense and sense orientation in the presence of  $^{35}\text{S}$ -UTP (PerkinElmer, Boston, MA).  $^{35}\text{S}$ -labeled full-length probes were further subjected to alkaline hydrolysis to yield products with an average size of 600 bp, except for the  $\alpha 2$  riboprobe, which was not hydrolyzed. A template for GAD67 (185-650; 466 bp) was generated by RT-PCR (forward primer: 5'-ATGGCATCTTCCACGCCTTCG-3', reverse primer: 5'- CCAAATTAACCTTCCATGCC-3'), subcloned into pPCR-Script Amp SK (+) (Stratagene, La Jolla, CA), and sequenced to verify the sequence for rat GAD67 (M76177). Digoxigenin-labeled GAD67 crRNA probes were synthesized using Dig-UTP (Roche Applied Science, Indianapolis, IN).

In addition, a PCR product for GAD67 (1198- 1474; 277 bp) was used as a template to generate  $^{35}\text{S}$ -labeled GAD67 crRNA probe by *in vitro* transcription after adding the T3 promoter sequence to the reverse primer (forward: 5'- TTATGTCAATGCAACCGCAGGC-3', reverse: 5'- AATTAACCCTCAAAGGNNNNNNNNNNNNNACACATCTGGTTGCATCCTTGG-3').

### Double *in situ* hybridization

Sections were pretreated with proteinase K (0.1  $\mu\text{g}/\text{ml}$ ) for 30 min at RT, acetylated, dehydrated through graded ethanols (50, 70, 95 and 100%) and air-dried. Sections were then incubated for 18 h at  $60^{\circ}\text{C}$  with a 1:1 dilution of Dig-labeled antisense GAD crRNA probe (0.1  $\mu\text{g}/\text{ml}$ ) and  $^{35}\text{S}$ -labeled sense or antisense probes ( $2 \times 10^7$  cpm/ml) in hybridization solution (50% formamide, 10% dextran sulfate, 500  $\mu\text{g}/\text{ml}$  tRNA, 10 mM dithiothreitol, 0.3 M NaCl, 10 mM Tris, pH8.0, and 1 mM EDTA, pH 8.0). After hybridization, sections were incubated with RNase A (20  $\mu\text{g}/\text{ml}$ ) for 30 min at  $37^{\circ}\text{C}$ , followed by two 5 min washes (2X standard saline citrate buffer [SSC]), two 10 min washes with 1X and 0.5X SSC at RT, and a 30 min wash in 0.1X SSC at  $65^{\circ}\text{C}$ . After the hot wash, the slides were incubated in Genius buffer 1 (GB1) (100 mM Tris-HCl, 150 mM NaCl, pH 7.5) for 5 min, followed by a 30 min incubation in 5% nonfat dry milk in GB1 plus 0.25% Triton-X (GB2) at RT. The alkaline-phosphatase conjugated anti-Dig Fab antibody (sheep) (Roche Applied Science, Indianapolis, IN), prepared as 1:1,000 dilution in GB1, was applied to the sections and slides were incubated for 3 h at RT. The slides were washed three times for 1, 5, and 10 min in GB1. Slides were incubated with color reagent [200  $\mu\text{l}$  of NBT/BCIP stock solution (18.75 mg/ml NBT, 9.4 mg/ml BCIP) in 10 ml of GB3 (100 mM Tris-HCl, 100 mM NaCl, 50 mM  $\text{MgCl}_2$ , pH 9.5)] (Roche Applied Science, Indianapolis, IN) at RT overnight. The slides were washed twice in GB4 (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and once in double deionized water, dehydrated with brief dips in graded ethanols (50, 70, 95, and 100%), air-dried and apposed to Kodak Biomax MR film for an appropriate period of time. After film

development, slides were coated with 2% parlodion (SPI-Chem, West Chester, PA) in isoamylacetate and dipped in liquid NTB emulsion (VWR, West Chester, PA). After an appropriate period of incubation time, slides were developed in Kodak developer D-19, fixed, cover-slipped, and analyzed.

## Analysis

The hippocampus was divided into three subdivisions: CA1, CA3 and DG. The border between the CA1 region and subiculum was defined with a straight line through the point at which the densely packed pyramidal cell layer of the s. pyramidale disappeared. The border between CA1 and CA3 regions was defined with a straight line that linked the tip of the supra-pyramidale blade of the DG with the onset of more loosely packed pyramidal cell of CA3. The border between the CA3 region and the DG was defined with lines drawn from the edges of the two blades of the granule cell layer to the inner tip of the pyramidal cell layer of the CA3 region. The three subdivisions of the hippocampus were further divided into layers: CA1 and CA3 strata oriens, pyramidale, radiatum/lacunosum moleculare, and DG molecular layer, granule cell layer and hilus, identified in relation to the principal cell layers.

The hybridization signal derived from the non-radioactive Dig-labeled GAD67 probe (dark color reaction product) was analyzed by lightfield, the radioactive hybridization signal (silver grains) by darkfield microscopy using an Olympus BX51 microscope. The criterion for the identification of positive GAD67 mRNA expressing interneurons were determined by the area ( $\geq 30$  and  $\leq 300 \mu\text{m}^2$ ) labeled by non-radioactive in situ hybridization, and its integrated density (gray values) using Image J software ([www.nih.gov](http://www.nih.gov)) ( $\geq 5000$  arbitrary units). To determine positive nAChR subunit mRNA expression the number of silver grains expressed on GAD-positive neurons derived with the sense probes (background) was determined ( $2.5 \pm 1.56/\text{GAD-positive neuron}$ ). A positive signal for radioactive in situ hybridization was set as  $\geq 12$  silver grains overlying a positive Dig-labeled neuron, which is at more than 4 times higher than background. Thus, the criteria for the identification of double labeled neurons were established by the number of silver grains ( $\geq 12$ ) on GAD67 neurons identified as a Dig-labeled area of  $\geq 30$  and  $\leq 300 \mu\text{m}^2$  having an integrated density value of  $\geq 5000$ .

For counting, images were captured by a DP7-1 digital camera. For each nAChR subunit double-labeled cells were counted in four sections from dorsal and four from ventral hippocampus from three adult males ( $n = 3$ ) under an objective lens (X40, NA 1.0) and the percentage of double-labeled cells was compared to those of GAD67 and  $\beta 2$  mRNA coexpression. Since most GAD67 positive cells also expressed transcripts for  $\beta 2$ , this seemed to provide a reasonable estimate of the relative proportion of cells exhibiting double-labeling. For the statistical analysis results from dorsal and ventral sections were combined and analyzed as a percent of total Dig-GAD/ $\beta 2$  positive cells (defined as 100%) using the student t-test.

Autoradiographic images of sections were obtained using computer-based image analysis system (MCID, Imaging Research Inc. St. Catherine, Canada; now InterFocus Imaging Ltd, UK). Darkfield photomicrographic images were captured by a DP7-1 digital camera (software DP manager, Leeds Instruments, Irving, TX) and adjusted for brightness and contrast using the Adobe Photoshop 9.0 image-editing software package (Adobe System, Mountain View, CA).

## RESULTS

In the adult brain the expression of nAChR subunit mRNAs was studied using radioactive *in situ* hybridization with subunit specific cRNA probes. The analysis of the autoradiograms showed expression patterns in coronal brain section for the nAChR subunits identical to those previously reported, with  $\alpha 4$ ,  $\alpha 7$  and  $\beta 2$  subunit mRNAs being the most widely expressed (Fig. 1). In the hippocampus, strong and widespread mRNA expression was only detected for  $\alpha 7$  and  $\beta 2$  (Fig. 1F, G). Other subunits like  $\alpha 4$  and  $\beta 4$  exhibited very low (Fig. 1C, I), scattered ( $\alpha 2$ ,  $\alpha 5$ ) (Fig. 1A, D) or barely detectable mRNA expression ( $\alpha 3$ ,  $\alpha 6$ ,  $\beta 3$ ) (Fig. 1B, E, H) in the hippocampal formation despite intense hybridization signal in other structures such as the medial habenula. The sense probes used to detect non-specific hybridization exhibited only background levels of hybridization signal (Fig. 1J) as shown for the  $\beta 2$  sense probe.

### Expression of nAChR subunit mRNAs in rat hippocampus

Adult coronal brain sections were analyzed using darkfield microscopy to detect the expression of nAChR subunit mRNAs at a cellular level in dorsal (Fig. 2) and ventral hippocampus (Fig. 3). Spatially restricted expression of  $\alpha 2$  mRNA was detected in CA1 s. oriens in dorsal and ventral hippocampus with more cells exhibiting  $\alpha 2$  expression in ventral compared to dorsal hippocampus (Fig. 2A and Fig. 3A). In dorsal hippocampus, sporadic expression of  $\alpha 3$  subunit mRNA was found on a few scattered cells mostly in CA1 and CA3 s. radiatum. In the ventral hippocampus  $\alpha 3$  mRNA expression was restricted to a small area in CA1 s. pyramidale which also exhibited increased expression of  $\alpha 4$ ,  $\alpha 5$  and  $\beta 4$  mRNAs (Fig. 2B and Fig. 3B). The expression of  $\alpha 4$  mRNA was weak throughout the hippocampus, especially when compared to the robust signal detected in cortex and thalamus (Fig. 2C and Fig. 3C). However, numerous scattered cells exhibiting weak  $\alpha 4$  mRNA expression were found in all CA1 and CA3 strata and the DG in dorsal and ventral hippocampus. Strong expression of  $\alpha 5$  mRNA was detected in cell in scattered CA1 s. radiatum and DG in dorsal and to a lesser extent in ventral hippocampus (Fig. 2D and Fig. 3D). The expression of  $\alpha 6$  mRNA was restricted to a few cells (1 to 3 cells per section) randomly distributed in different regions and layers in dorsal and ventral hippocampus (Fig. 2E and Fig. 3E). Strong expression of  $\alpha 7$  was found in all areas of dorsal and ventral hippocampus including strong expression in scattered cells in all strata of CA1 and CA3 and the DG (Fig. 2F and Fig. 3F). Strong expression of  $\beta 2$  mRNAs was found in all areas of dorsal and ventral hippocampus including strong expression in scattered cells in all strata of CA1 and CA3 and the DG (Fig. 2G and Fig. 3G), however, the hybridization signal in interneurons was less intense than for  $\alpha 7$ . Hybridization signal for  $\beta 3$  was detected in randomly distributed single cells, but was limited to 1 to 3 cells per section in dorsal and ventral hippocampus (Fig. 2H and Fig. 3H). Weak expression of  $\beta 4$  mRNA was detected in CA3 s. pyramidal in dorsal hippocampus and CA1 s. pyramidal in ventral hippocampus (Fig. 2I and Fig. 3I) and in a few scattered cells in CA3 s. radiatum. The sense probes as shown for  $\beta 2$  as an example exhibited only background hybridization signal (Fig. 2J).

### Co-expression of nAChR subunit mRNAs with GAD67 mRNAs

To evaluate the expression of nAChR subunit mRNA in GAD67-positive interneurons, combined radioactive and non-radioactive *in situ* hybridization was used. The sensitivity of the method was verified by using two probes for GAD67 mRNA, directed against different sequences to avoid competition for the same complementary sequence. The interneurons were labeled with a 277 bp  $^{35}\text{S}$ -labeled (1198–1474 bp) and a 466 bp Dig-labeled (185–650 bp) GAD67 cRNA probe (Fig. 4A, A'). Hybridization pattern for GAD67 mRNA derived with the Dig- or the  $^{35}\text{S}$ -labeled probes were identical as demonstrated by the pictures taken with lightfield (Dig-signal) (Fig. 4A) or darkfield ( $^{35}\text{S}$ -signal) (Fig. 4A') from the same area



of the same section. The relative signal intensity for GAD67 mRNA hybridization appeared similar for both probes. Cells strongly labeled by Dig-GAD67 also exhibited high numbers of silver grains, and those exhibiting weak Dig-signal showed lower numbers of silver grains (Fig. 4B, B' and B''). This suggests that a strong Dig-signal did not reduce or prevent radiation from the  $^{35}\text{S}$ -hybridization signal from reaching the emulsion which is necessary for the formation of the silver grains. Thus, most if not all neurons labeled by one probe were also positive for the other probe. Therefore, the Dig-labeled GAD67 probe used for double *in situ* hybridization in this study was a highly sensitive and reliable marker for the detection of GABAergic interneurons, and suitable for double *in situ* hybridization.

The double *in situ* hybridization showed co-expression of GAD67 mRNA with  $\alpha 2$ ,  $\alpha 3$ ,  $\alpha 4$ ,  $\alpha 5$ ,  $\alpha 7$ ,  $\beta 2$  and  $\beta 4$  nAChR subunit mRNAs in adult rat hippocampus, but not with  $\alpha 6$  and  $\beta 3$  (Fig. 5, Fig. 6). Quantitative analysis revealed that most GAD67 positive interneurons also expressed transcripts for  $\beta 2$ , and the majority exhibited co-expression with  $\alpha 7$  mRNA, but less than 20% exhibited co-expression with the other subunits (Fig. 7A). Sense probes were used as negative controls for double *in situ* hybridization and exhibited only background signal on Dig-labeled neurons as shown for the  $\alpha 7$  sense probe (Fig. 5J and Fig. 6J).

Co-expression of GAD67 with  $\alpha 2$  mRNA was mostly detected in CA1 s. oriens ( $30.1 \pm 5.3\%$ ) (Fig. 5A, Fig 6A and Fig. 7B), and a few double-labeled neurons were found in CA3 s. oriens ( $14.1 \pm 1.9\%$ ) and radiatum ( $8.1 \pm 2.4\%$ ) (Fig. 7C) but not in the DG. A comparison between dorsal and ventral CA1 s. oriens showed significantly higher coexpression ( $p \leq 0.05$ ) in ventral ( $37.4 \pm 3.5\%$ ) than dorsal hippocampus ( $22.8 \pm 2.0\%$ ). Coexpression of GAD67 and  $\alpha 3$  nAChR mRNAs was restricted to a few neurons located in the CA3 s. radiatum where  $1.8 \pm 1.5\%$  of GAD67 positive neurons also expressed  $\alpha 3$  mRNA (Fig. 5B, Fig. 6B and Fig. 7C).

Expression intensity for  $\alpha 4$  mRNA was very low throughout the hippocampus including expression of  $\alpha 4$  in interneurons (Fig. 5C and Fig. 6C). However,  $12.9 \pm 3.9\%$  of GAD67 positive neurons also expressed  $\alpha 4$  mRNA (Fig. 7A). Coexpression was found in CA1 ( $17.4 \pm 2.4\%$ ) and CA3 s. oriens ( $15.2 \pm 3.3\%$ ) and radiatum ( $17.1 \pm 2.5\%$ ) and in the molecular layer ( $18.0 \pm 8.0\%$ ), granule cell layer ( $12.1 \pm 6.3\%$ ) and hilar region ( $10.8 \pm 2.9\%$ ) of the DG (Fig. 7B, 7C and 7D). Coexpression of GAD67 and  $\alpha 5$  mRNA was detected in  $5.9 \pm 3.6\%$  of GABAergic neurons in the hippocampus (Fig. 5D, Fig. 6D and Fig. 7A), but was restricted to CA1 s. radiatum/ lacunosum moleculare ( $13.7 \pm 6.3\%$ ), CA3 s. radiatum ( $9.4 \pm 7.1\%$ ), and the granule cell layer ( $12.1 \pm 5.9\%$ ) and hilar region ( $13.6 \pm 7.1\%$ ) of the DG (Fig. 7B, 7C and 7D). The coexpression of GAD67 and  $\alpha 5$  mRNA was mostly restricted to dorsal hippocampus.

Expression of  $\alpha 6$  was detected in very few cells which exhibited very strong mRNA expression. However, there was no coexpression with GAD67 mRNA detected in any of the  $\alpha 6$  expressing cells (Fig. 5E and Fig. 6E). Strong coexpression of GAD67 and  $\alpha 7$  mRNAs was also found throughout the hippocampus in  $66.7\% (\pm 9.8\%)$  of GABAergic interneurons (Fig. 5F, Fig. 6F and Fig. 7A). Double-labeling was high in CA1 ( $90.5 \pm 4.99\%$ ) and CA3 s. pyramidal layers ( $86.0 \pm 8.02\%$ ) and DG granule cell layer ( $71.6 \pm 4.97\%$ ) and hilar region ( $74.1 \pm 13.14\%$ ) (Fig. 7B, C and D). However, due to the high expression of  $\alpha 7$  mRNA in pyramidal and granule cells it was not possible to distinguish expression of  $\alpha 7$  mRNA in principal cells from those in interneurons. In other regions such as CA1 s. oriens ( $52.7 \pm 8.5\%$ ) and radiatum/LM ( $59.4 \pm 5.5\%$ ), CA3 s. oriens ( $41.2 \pm 12.9\%$ ) and radiatum ( $49.2 \pm 10.02\%$ ), coexpression was significantly lower.

Of the beta subunits expressed in the hippocampus, only  $\beta 2$  and  $\beta 4$  were coexpressed with GAD67 mRNA (Fig. 5G, I and Fig. 6G, I);  $\beta 3$  mRNA, although expressed in very few cells,

was never detected in GAD67 positive interneurons (Fig. 5H and Fig. 6H).  $\beta 2$  exhibited strong and widespread mRNA expression, whereas hybridization signal intensity for  $\beta 4$  mRNA was low and restricted to a few cells. The microscopic analysis revealed that most GABAergic interneurons also expressed transcripts for  $\beta 2$  mRNA in all areas of the hippocampus (Fig. 5G, Fig. 6G and Fig. 7A). In contrast,  $\beta 4$  mRNAs was restricted to less than  $2.5 \pm 1.36\%$  of interneurons (Fig. 5I, Fig. 6I and Fig. 7A), mostly located in CA1 and CA3 s. radiatum/moleculare.

## DISCUSSION

This study is the first to systematically analyze the expression of nAChR subunit mRNAs in GABAergic interneurons in the rat hippocampus using double *in situ* hybridization. The results show that most GAD67 positive GABAergic interneurons expressed  $\beta 2$  subunit mRNA and about 66% also expressed  $\alpha 7$  mRNA. In contrast, the expression of other subunits was very limited; only 13 % of GABAergic neurons expressed  $\alpha 4$  mRNA followed by 6% for  $\alpha 5$  and  $\alpha 2$  and less than 2% for  $\alpha 3$  and  $\beta 4$ . In addition, the relative expression intensities for  $\alpha 4$ ,  $\alpha 3$  and  $\beta 4$  in GABAergic interneurons were low compared to their expression in other brain areas. No expression of  $\alpha 6$  and  $\beta 3$  mRNAs was detected in GABAergic interneurons, although, a limited number of randomly scattered cells which exhibited very intense mRNA expression were found throughout the hippocampus but their identity remains to be determined.

### Technical considerations

Radioactive-labeled cRNA probes for the detection of nAChR subunit mRNAs together with a dig-labeled probe to visualize GAD67 mRNA were used in this double *in situ* hybridization study. In general double *in situ* hybridization is a powerful tool for the localization of two mRNA transcripts in a single neuron. This method is a well-established technique to measure colocalization of nAChR subunit mRNAs with marker genes to identify specific neuronal populations (Winzer-Serhan and Leslie, 1997, Azam et al., 2002, 2003). In fact, the results for the expression of nAChR subunit mRNAs in tyrosine hydroxylase positive dopaminergic neurons in the substantia nigra and ventral tegmental area were similar to those obtained with single-cell PCR (Azam et al., 2002, Klink et al., 2001). However, there are limitations with this method. In general non-radioactive *in situ* hybridization is considered to be less sensitive, and quenching of the radioactive signal by the nonradioactive one could lower the number of detectable double-labeled cells. We compared the hybridization signal for GAD67 double *in situ* hybridization using two GAD67 probes. The results showed that although the  $S^{35}$ -labeled GAD67 probe was much shorter than the Dig-labeled GAD67 probe (277 vs. 466 bp), the hybridization pattern for both probes were identical. Furthermore, there was no sign of quenching by the color precipitate, because neurons which were strongly labeled by the non-radioactive probe were also strongly labeled by the radioactive probe. For the double *in situ* hybridization with nAChR subunits,  $S^{35}$ -labeled probes were transcribed from full-length transcripts and had probe length between 1607 and 2522 bp to increase the sensitivity of the radioactive signal, and to assure that most if not all cells coexpressing transcripts for nAChR subunits were accounted for. Although, it does not seem that digoxigenin labeling reduced detection of radioactive labeling, we cannot rule out that interneurons, expressing low levels of a nAChR subunit (such as  $\alpha 3$ ,  $\alpha 4$ , or  $\beta 4$ ), did not reach the criteria of 12 silver grains which was set as 4 times above background to avoid false positive results. This could result in an underestimation of coexpressing interneurons, especially. To control for the limitations of the method, the data are calculated as the percentage of GAD67/ $\beta 2$  double labeled cells in the hippocampus, which offers a reasonable estimate of the relative proportion of cells expressing both markers.

## Anatomical evaluation

Our data are in complete agreement with previous anatomic studies. In here, the hybridization patterns derived with the different cRNA probes for the nAChR subunits are identical to those reported previously showing robust and widespread expression for  $\alpha 7$  and  $\beta 2$  mRNA, but limited expression for other subunits (Patrick et al., 1989, Duvoisin et al., 1989, Wada et al., 1990, Wada et al., 1989, Séqu  la et al., 1993). These findings suggest the presence of mainly homomeric nAChRs but very limited expression of the major heteromeric nAChR subtypes in the hippocampus. This is supported by receptor autoradiographic studies demonstrating that in rodent hippocampus, the major nAChR subtype is the  $\alpha 7$  homomeric receptor, exhibiting strong binding throughout the hippocampal formation including interneurons (Clarke et al., 1985, Fuchs and Schwark, 1993, Freedman et al., 1993, Tribollet et al., 2004). In contrast, the number of binding sites for heteromeric receptors, detected with radiolabeled ligands such as nicotine, epibatidine, or cytosine are very low (Clarke et al., 1985, Pauly et al., 1989, Perry and Kellar, 1995, Happe et al., 1995, Tribollet et al., 2004). Although,  $\beta 2$  mRNA is strongly expressed in the hippocampal formation, heteromeric binding sites are not present because of the lack of a corresponding  $\alpha$  subunit required for  $\beta 2$  to form heteromeric receptors.

A similar expression profile of nAChR subunit mRNAs in GABAergic neurons has been described before (Azam et al., 2003). The majority of GABAergic neurons in the basal forebrain express mRNAs for  $\alpha 7$  and  $\beta 2$ , with only a small subpopulation expressing other subunit mRNAs, mainly for  $\alpha 2$ ,  $\alpha 4$ , and  $\beta 4$ . In addition, GABAergic neurons located in the substantia nigra/ventral tegmental area also show mRNA expression of only a limited number of subunits:  $\alpha 4$ ,  $\alpha 7$  and  $\beta 2$  (Azam et al., 2002). This is in stark contrast to dopaminergic and noradrenergic neurons which express a wide array of different nAChR subunit mRNAs (Azam et al., 2002, Klink et al., 2001, Gallardo et al., 1997).

However, a single-cell RT-PCR study suggests that in addition to  $\alpha 7$  and  $\beta 2$  other nAChR subunits such as  $\alpha 2$ ,  $\alpha 3$ ,  $\alpha 4$ ,  $\alpha 5$ ,  $\beta 3$  and  $\beta 4$  are expressed within GAD-positive neurons (Sudweeks and Yakel, 2001). Given the higher sensitivity of RT-PCR over *in situ* hybridization it is possible that very low levels of mRNA expression did not result in a positive hybridization signal and remained undetected in this study.

## Correlation with functional studies

Our anatomical data for  $\alpha 7$  mRNA expression are widely supported by functional studies reporting that the majority of nicotinic responses are mediated by an  $\alpha 7$ -type nAChR (Alkondon and Albuquerque, 1993). However, despite the strikingly low number of heteromeric nAChR binding sites, and low and restricted expression of  $\alpha$  subunits in the hippocampus, functional studies have repeatedly suggested that heteromeric nAChRs located on GABAergic interneurons play a major role in the regulation of hippocampal activity (for review see Alkondon and Albuquerque 2004, Yakel and Shao, 2004). At least three distinct physiological responses to nicotinic agonists have been described in GABAergic interneurons and attributed to different nAChRs subtypes such as the homomeric  $\alpha 7$  (type IA), and the heteromeric  $\alpha 4\beta 2$  (type II) and  $\alpha 3\beta 4$  (type III) nAChRs (Alkondon and Albuquerque, 1993, Alkondon et al., 1997, Jones and Yakel, 1997, Frazier et al., 1998, McQuiston and Madison, 1999, Ji and Dani, 2000). One explanation for this discrepancy is that the characterization of functional nAChRs has been done mostly in hippocampal slices from postnatal rat pups. Several anatomical studies have documented that the expression of nAChR subunit mRNAs is developmentally regulated with increased expression of  $\alpha 2$ ,  $\alpha 3$ ,  $\alpha 4$ ,  $\alpha 5$ ,  $\alpha 7$  and  $\beta 4$  in postnatal hippocampus (Winzer-Serhan and Leslie, 1997, 2005, Adams et al., 2002, Son and Winzer-Serhan, 2006, Huang and Winzer-Serhan, 2006). However, a recent study by Alkondon and co-worker (2007) using



hippocampal slices from adult and postnatal animals came to the conclusion that, although distinct age-dependent differences in the functional response to nicotinic activation exist, the type III response (presumably an  $\alpha 3\beta 4$ -type nAChR) is still present in slices from adult animals.

An alternative explanation is that even very low numbers of mRNA transcripts, not detected by *in situ* hybridization, could be translated into subunit proteins, and subsequently be incorporated into functional nAChRs. It remains to be seen however, if sufficient amounts of proteins are synthesized to significantly contribute to nicotinic hippocampal activity.

On the other hand, the pharmacological tools used to characterize nicotinic responses in hippocampal slices are not selective enough to clearly distinguish between nAChR subtypes. Although, 90% of nicotinic responses are believed to be mediated via activation of  $\alpha 7$  (type IA) nAChRs, discrepancies between the properties of hippocampal type IA and expected properties of homomeric  $\alpha 7$  nAChRs have led to speculations that not all  $\alpha 7$  nAChRs are homomeric (Yakel and Shao, 2004, Khirough et al., 2002). If in fact  $\alpha 7$  subunits also form functional heteromeric nAChRs in hippocampal interneurons, then those could contribute to the functional diversity seen in the nicotinic responses despite an apparent lack of  $\alpha 4$  and in particular  $\alpha 3$  mRNA expression in most interneurons. Support for this idea comes from a recent study presented by Liu et al. (2007) describing a novel  $\alpha 7\beta 2$  heteromeric nAChR which is highly sensitive to inhibition by  $\beta$ -amyloid peptides ( $A\beta$ ), and is expressed in forebrain cholinergic neurons. Basal forebrain cholinergic neurons exhibit a similarly restricted expression pattern of mostly  $\alpha 7$  and  $\beta 2$  nAChR subunits (Azam et al., 2003). Interestingly,  $A\beta$  also inhibits whole-cell and single-channel nicotinic currents from rat hippocampal interneurons at low concentrations (Pettit et al., 2001). Therefore, in addition to forming homomeric receptors,  $\alpha 7$  may also form heteromeric nAChRs and thus, could contribute to the diverse nicotinic responses typically seen in hippocampal interneurons.

In this study we found that  $\alpha 2$  mRNA was clearly expressed in 30% of CA1 s. oriens/alveus GABAergic interneurons, presumably in oriens-lacunosum moleculare (OLM) cells (Son and Winzer-Serhan, 2006, Wada et al., 1989). This finding is supported by the RT-PCR study also describing co-expression of  $\alpha 2$  transcripts with GAD67 in s. oriens (Sudweeks and Yakel, 2001). OLM cells potently inhibit excitatory input to pyramidal cells (Yanovsky et al., 1997), and exhibit two different nicotinic responses, a fast  $\alpha 7$ , and a slow non- $\alpha 7$  mediated response (McQuiston and Madison, 1999). The slow response is insensitive to the  $\alpha 3\beta 2$  nAChR antagonist  $\alpha$ -CTX MII, and relatively insensitive to the  $\alpha 4\beta 2$  nAChR antagonist DH $\beta$ E, therefore, suggesting other types of nAChRs, perhaps with participation of  $\alpha 2$ . A functional study has recently underscored the importance of  $\alpha 2$ -containing nAChRs in regulating long-term potentiation in the hippocampal CA1 region (Nakauchi et al., 2007). Thus,  $\alpha 2$ -containing nAChRs appear to be a major functional contributor to the slow nicotinic response in these neurons and could either be a new type of nicotinic response mediated by  $\alpha 2\beta 2$  nAChRs or represent one of the previously described (type II or III) ones.

The  $\alpha 5$  nAChR subunit mRNA exhibited spatially restricted but robust expression in GABAergic neurons in CA1 and CA3 s. radiatum and in the DG. The  $\alpha 5$  stands out among  $\alpha$  nAChR subunits because  $\alpha 5$  does not form functional nAChRs when expressed alone or in combination with another  $\beta$  subunit (Ramirez-Latorre et al., 1996). However, when combined with another  $\alpha$  and  $\beta$  subunit,  $\alpha 5$  does contribute to the formation of heteromeric channels and significantly alters functional properties such as increasing single channel conductance. In a previous anatomical study co-expression of  $\alpha 5$  and  $\alpha 7$  mRNA in a subpopulation of hippocampal interneurons was detected (Winzer-Serhan and Leslie, 2005), and since most GABAergic interneurons express  $\beta 2$  mRNA (this study), a heteromeric

$\alpha 5\alpha 7\beta 2$  nAChRs may exist. However, since  $\alpha 4$  mRNA is also found in the same areas as  $\alpha 5$  mRNA, an  $\alpha 4\alpha 5\beta 2$  heteromeric receptor type seems also a possibility.

Hippocampal inhibitory interneurons are very diverse, and differ in their projection and innervation patterns, physiology, expression of neurotransmitter receptors and calcium binding proteins. This diversity is thought to reflect functional and structural specializations which evolved to control distinct network operations such as synchronization of neuronal firing, pyramidal cell generated theta rhythm, or basket cell generated gamma oscillations to name a few (Freund and Buzsaki, 1996). The complex expression pattern of nAChR subunits, most likely tailored to the specific functional needs of different interneuron populations contributes to the interneuronal diversity. Depending on the physiological properties of distinct nAChR subtypes nicotine or nicotinic activation could differentially influence subpopulations of interneurons and contribute to the differential control of the hippocampal network. Thus, nAChRs are in a position where they can play a critical regulatory role in hippocampal circuit activity in line with their role in enhancing cognitive functions.

## CONCLUSION

In summary, based on the anatomical analysis presented in this study, the majority of nicotinic responses should be mediated by an  $\alpha 7$ -containing nAChR and some by non- $\alpha 7$  heteromeric receptors such as an  $\alpha 2\beta 2^*$  subtype in the s. oriens and an  $\alpha 5$  containing nAChRs in the s. radiatum and DG. Heteromeric  $\alpha 4\beta 2^*$  nAChRs should represent only a small fraction of the nicotinic response since less than 20% of GAD67 positive neurons were identified as co-expressing  $\alpha 4$  mRNA. Nicotinic responses mediated by heteromeric  $\alpha 3\beta 4^*$  nAChRs should be at best very rare, because the number of GAD67 positive neurons co-expressing  $\alpha 3$  was almost negligible and very limited for  $\beta 4$ .

## Acknowledgments

Grant sponsor: NIH grants DA 106487.

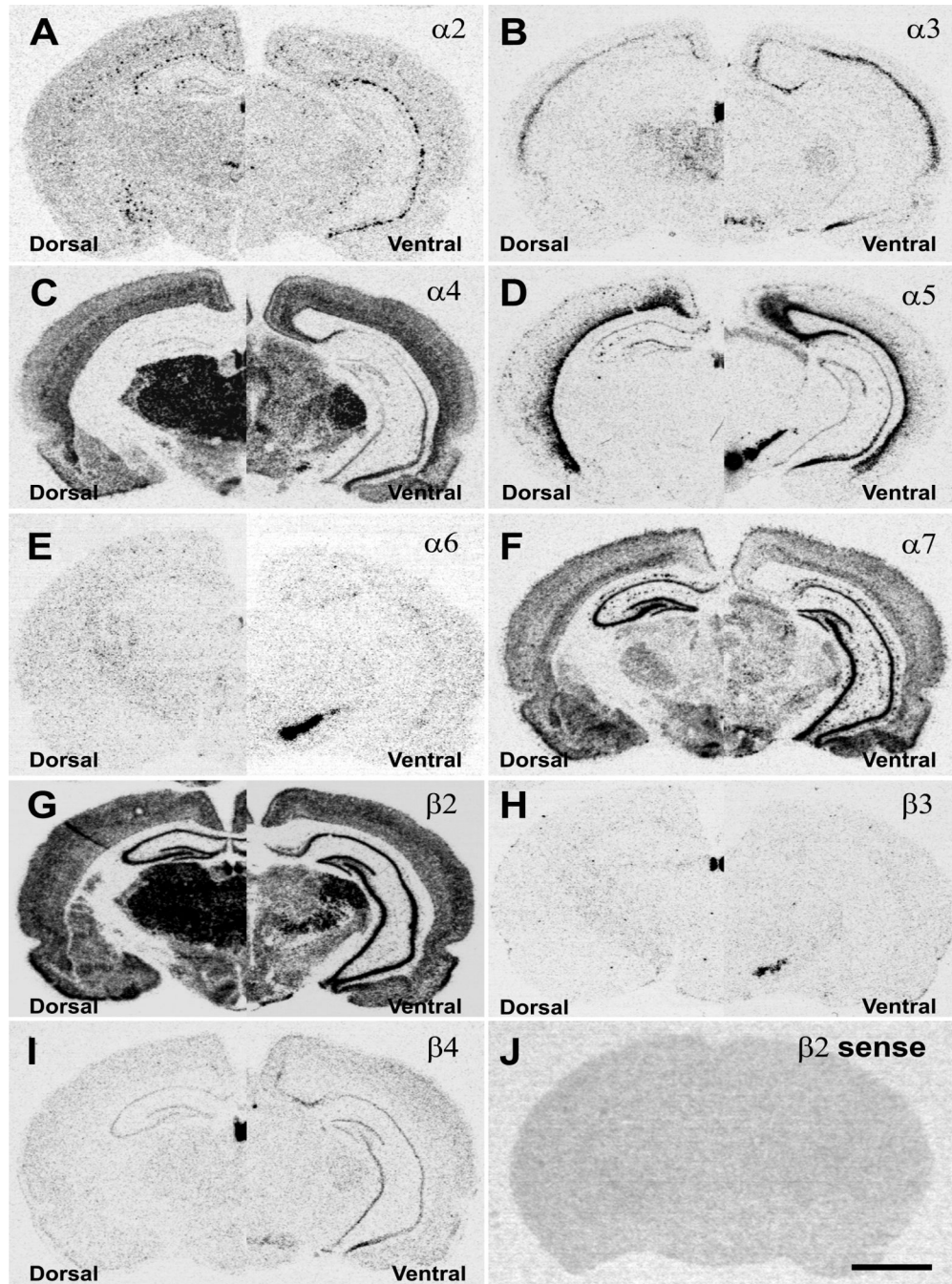
## REFERENCES

- Alkondon M, Albuquerque EX. Diversity of nicotinic acetylcholine receptors in rat hippocampal neurons. I. Pharmacological and functional evidence for distinct structural subtypes. *J Pharmacol Exp Ther.* 1993; 265:1455–1473. [PubMed: 8510022]
- Alkondon M, Albuquerque EX. Nicotinic acetylcholine receptor  $\alpha 7$  and  $\alpha 4\beta 2$  subtypes differentially control GABAergic input to CA1 neurons in rat hippocampus. *J Neurophysiol.* 2001; 86:3043–3055. [PubMed: 11731559]
- Alkondon M, Albuquerque EX. The nicotinic acetylcholine receptor subtypes and their function in the hippocampus and cerebral cortex. *Prog Brain Res.* 2004; 145:109–120. [PubMed: 14650910]
- Alkondon M, Pereira EF, Albuquerque EX. Age-dependent changes in the functional expression of two nicotinic receptor subtypes in CA1 stratum radiatum interneurons in the rat hippocampus. *Biochem Pharmacol.* 2007; 74:1134–1144. [PubMed: 17645875]
- Alkondon M, Pereira EF, Barbosa CT, Albuquerque EX. Neuronal nicotinic acetylcholine receptor activation modulates gamma-aminobutyric acid release from CA1 neurons of rat hippocampal slices. *J Pharmacol Exp Ther.* 1997; 283:1396–1411. [PubMed: 9400016]
- Adams CE, Broide RS, Chen Y, Winzer-Serhan UH, Henderson TA, Leslie FM, Freedman R. Development of the  $\alpha 7$  nicotinic cholinergic receptor in rat hippocampal formation. *Brain Res Dev Brain Res.* 2002; 139:175–187.
- Azam L, Winzer-Serhan UH, Chen Y, Leslie FM. Expression of neuronal nicotinic acetylcholine receptor subunit mRNAs within midbrain dopamine neurons. *J Comp Neurol.* 2002; 444:260–274. [PubMed: 11840479]

- Azam L, Winzer-Serhan UH, Leslie FM. Co-expression of alpha7 and beta2 nicotinic acetylcholine receptor subunit mRNAs within rat brain cholinergic neurons. *Neuroscience*. 2003; 119:965–977. [PubMed: 12831856]
- Chen D, Patrick JW. The alpha-bungarotoxin-binding nicotinic acetylcholine receptor from rat brain contains only the alpha7 subunit. *J Biol Chem*. 1997; 272:24024–24029. [PubMed: 9295355]
- Clarke PB, Schwartz RD, Paul SM, Pert CB, Pert A. Nicotinic binding in rat brain: autoradiographic comparison of [<sup>3</sup>H]acetylcholine, [<sup>3</sup>H]nicotine, and [<sup>125</sup>I]-alpha-bungarotoxin. *J Neurosci*. 1985; 5:1307–1315. [PubMed: 3998824]
- Dougherty KD, Milner TA. Cholinergic septal afferent terminals preferentially contact neuropeptide Y-containing interneurons compared to parvalbumin-containing interneurons in the rat dentate gyrus. *J Neurosci*. 1999; 19:10140–10152. [PubMed: 10559422]
- Duvoisin RM, Deneris ES, Patrick J, Heinemann S. The functional diversity of the neuronal nicotinic acetylcholine receptors is increased by a novel subunit: beta4. *Neuron*. 1989; 4:487–496. [PubMed: 2642007]
- Frazier CJ, Rollins YD, Breese CR, Leonard S, Freedman R, Dunwiddie TV. Acetylcholine activates an alpha-bungarotoxin-sensitive nicotinic current in rat hippocampal interneurons, but not pyramidal cells. *J Neurosci*. 1998; 18:1187–1195. [PubMed: 9454829]
- Freedman R, Wetmore C, Strömberg I, Leonard S, Olson L.  $\alpha$ -Bungarotoxin binding to hippocampal interneurons: immunocytochemical characterization and effects on growth factor expression. *J Neurosci*. 1993; 13:1965–1975. [PubMed: 8478687]
- Freund TF, Buzsaki G. Interneurons of the hippocampus. *Hippocampus*. 1996; 6:347–470. [PubMed: 8915675]
- Frotscher M, Léránth C. Cholinergic innervation of the rat hippocampus as revealed by choline acetyltransferase immunocytochemistry: a combined light and electron microscopic study. *J Comp Neurol*. 1985; 239:237–246. [PubMed: 4044938]
- Fuchs JL, Schwark HD. Distribution of [<sup>3</sup>H]QNB and [<sup>125</sup>I]alpha-bungarotoxin binding and acetylcholinesterase activity in visual system and hippocampal structures of eleven mammalian species. *J Comp Neurol*. 1993; 329:427–437. [PubMed: 8454734]
- Gallardo KA, Winzer-Serhan UH, Chen YC, Leslie FM. Ontogenetic expression of neuronal nicotinic acetylcholine receptor subunits in rat locus coeruleus. *Soc Neurosci Abstr*. 1997; 23:383.
- Happe HK, Peters JL, Bergman DA, Murrin LC. Localization of nicotinic cholinergic receptors in rat brain: autoradiographic studies with [<sup>3</sup>H] cytisine. *Neuroscience*. 1994; 62:929–944. [PubMed: 7870314]
- Ji D, Dani JA. Inhibition and disinhibition of pyramidal neurons by activation of nicotinic receptors on hippocampal interneurons. *J Neurophysiol*. 2000; 83:2682–2690. [PubMed: 10805668]
- Jinno S, Aika Y, Fukuda T, Kosaka T. Quantitative analysis of GABAergic neurons in the mouse hippocampus, with optical dissector using confocal laser scanning microscope. *Brain Res*. 1998; 814:55–70. [PubMed: 9838044]
- Jones S, Sudweeks S, Yakel JL. Nicotinic receptors in the brain: correlating physiology with function. *Trends Neurosci*. 1999; 22:555–561. [PubMed: 10542436]
- Jones S, Yakel JL. Functional nicotinic ACh receptors on interneurons in the rat hippocampus. *J Physiol*. 1997; 504:603–610. [PubMed: 9401968]
- Khiroug SS, Harkness PC, Lamb PW, Sudweeks SN, Khiroug L, Millar NS, Yakel JL. Rat nicotinic ACh receptor alpha7 and beta2 subunits co-assemble to form functional heteromeric nicotinic receptor channels. *J Physiol*. 2002; 540:425–434. [PubMed: 11956333]
- Klink R, de Kerchove d'Exaerde A, Zoli M, Changeux JP. Molecular and physiological diversity of nicotinic acetylcholine receptors in the midbrain dopaminergic nuclei. *J Neurosci*. 2001; 21:1452–1463. [PubMed: 11222635]
- Liu Q, Chang Y, Zhang J, Xue F, Dechon J, Lukas RJ, WU J. A novel  $\alpha$ 7 $\beta$ 2-nicotinic acetylcholine receptor in forebrain cholinergic neurons is highly sensitive to amyloid beta peptides. *Neuroscience Abstract*. 2007; 2007:39.1.
- McGehee DS, Role LW. Physiological diversity of nicotinic acetylcholine receptors expressed by vertebrate neurons. *Annu Rev Physiol*. 1995; 57:521–546. [PubMed: 7778876]

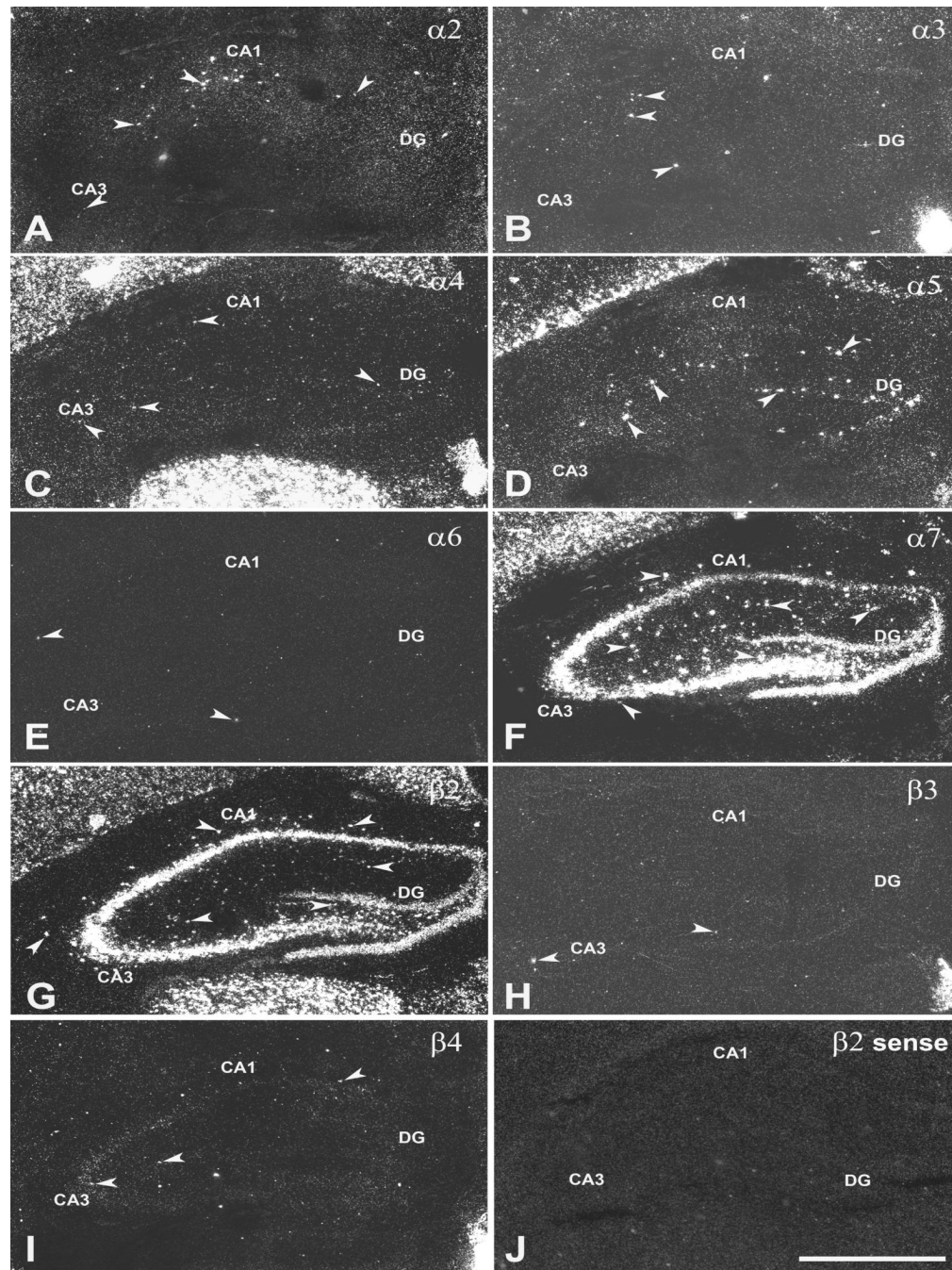
- McQuiston AR, Madison DV. Nicotinic receptor activation excites distinct subtypes of interneurons in the rat hippocampus. *J Neurosci*. 1999; 19:2887–2896. [PubMed: 10191306]
- Nakauchi S, Brennan RJ, Boulter J, Sumikawa K. Nicotine gates long-term potentiation in the hippocampal CA1 region via activation of  $\alpha 2^*$  nicotinic ACh receptors. *Eur J Neurosci*. 2007; 25:2666–2681. [PubMed: 17466021]
- Patrick J, Boulter J, Deneris E, Wada K, Wada E, Connolly J, Swanson L, Heinemann S. Structure and function of neuronal nicotinic acetylcholine receptors deduced from cDNA clones. *Prog Brain Res*. 1989; 79:27–33. [PubMed: 2587747]
- Pauly JR, Stitzel JA, Marks MJ, Collins AC. An autoradiographic analysis of cholinergic receptors in mouse brain. *Brain Res Bull*. 1989; 22:453–459. [PubMed: 2706548]
- Paxinos, G.; Watson, C. The rat brain in stereotaxic coordinates. 4th edition. Academic Press; San Diego, CA: 1998.
- Perry DC, Kellar KJ. [ $^3\text{H}$ ] epibatidine labels nicotinic receptors in rat brain: an autoradiographic study. *J Pharmacol Exp Ther*. 1995; 275:1030–1034. [PubMed: 7473129]
- Pettit DL, Shao Z, Yakel JL.  $\beta$ -Amyloid (1–42) peptide directly modulates nicotinic receptors in the rat hippocampal slice. *J Neurosci*. 2001; 21(RC120(1–5))
- Ramirez-Latorre J, Yu CR, Qu X, Perin F, Karlin A, Role L. Functional contributions of  $\alpha 5$  subunit to neuronal acetylcholine receptor channels. *Nature*. 380:347–351. [PubMed: 8598930]
- Sargent PB. The diversity of neuronal nicotinic acetylcholine receptors. *Annu Rev Neurosci*. 1993; 16:403–443. [PubMed: 7681637]
- Séguéla P, Wadiche J, Dineley-Miller K, Dani JA, Patrick JW. Molecular cloning functional properties, and distribution of rat brain alpha7: a nicotinic cation channel highly permeable to calcium. *J Neurosci*. 1993; 13:596–604. [PubMed: 7678857]
- Son JH, Winzer-Serhan UH. Postnatal expression of alpha2 nicotinic acetylcholine receptor subunit mRNA in developing cortex and hippocampus. *J Chem Neuroanat*. 2006; 32:179–190. [PubMed: 17046198]
- Sudweeks SN, Yakel JL. Functional and molecular characterization of neuronal nicotinic ACh receptors in rat CA1 hippocampal neurons. *J Physiol*. 2000; 527:515–528. [PubMed: 10990538]
- Tribollet E, Bertrand D, Marguerat A, Raggenbass M. Comparative distribution of nicotinic receptor subtypes during development, adulthood and aging: an autoradiographic study in the rat brain. *Neuroscience*. 2004; 124:405–420. [PubMed: 14980390]
- Wada E, Wada K, Boulter J, Deneris E, Heinemann S, Patrick J, Swanson LW. Distribution of alpha2, alpha3, alpha4, and beta2 neuronal nicotinic acetylcholine receptor subunit mRNAs in the central nervous system: a hybridization histochemical study in the rat. *J Comp Neurol*. 1989; 284:314–335. [PubMed: 2754038]
- Wada E, McKinnon D, Heinemann S, Patrick J, Swanson LW. The distribution of mRNA encoded by a new member of the neuronal nicotinic acetylcholine receptor gene family (alpha5) in the rat central nervous system. *Brain Res*. 1990; 526:45–53. [PubMed: 2078817]
- Winzer-Serhan UH, Leslie FM. Codistribution of nicotinic acetylcholine receptor subunit alpha3 and beta4 mRNAs during rat brain development. *J Comp Neurol*. 1997; 386:540–554. [PubMed: 9378850]
- Winzer-Serhan UH, Leslie FM. Expression of alpha5 nicotinic acetylcholine receptor subunit mRNA during hippocampal and cortical development. *J Comp Neurol*. 2005; 481:19–30. [PubMed: 15558717]
- Wolf NJ. Cholinergic systems in mammalian brain and spinal cord. *Prog Neurobiol*. 1991; 37:475–524. [PubMed: 1763188]
- Yakel JL, Shao Z. Functional and molecular characterization of neuronal nicotinic ACh receptors in rat hippocampal interneurons. *Prog Brain Res*. 2004; 145:95–107. [PubMed: 14650909]
- Yanovsky Y, Sergeeva OA, Freund TF, Haas HL. Activation of interneurons at the stratum oriens/alveus suppresses excitatory transmission to apical dendrites in the CA1 area of the mouse hippocampus. *Neuroscience*. 1997; 77:87–96. [PubMed: 9044377]





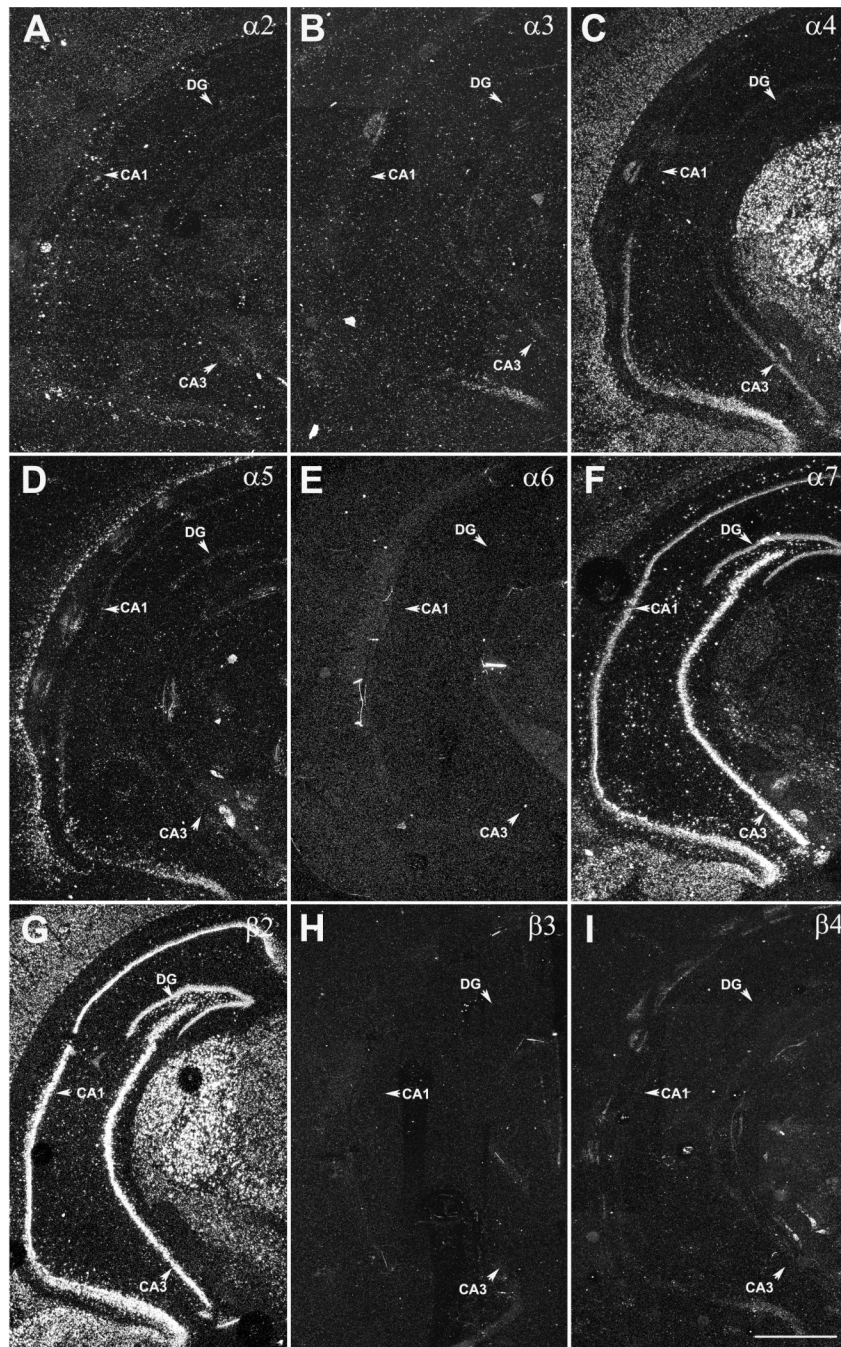
**Figure 1.** Autoradiographic images of nAChR subunit mRNA expression pattern in coronal sections of dorsal (left side) and ventral hippocampus (right side). Specific hybridization signals were derived with  $^{35}\text{S}$ -labeled antisense probes for A)  $\alpha 2$ , B)  $\alpha 3$ , C)  $\alpha 4$ , D)  $\alpha 5$ , E)  $\alpha 6$ , F)  $\alpha 7$ , G)  $\beta 2$ , H)  $\beta 3$ , and I)  $\beta 4$ ; and J) nonspecific hybridization signal detected with a  $\beta 2$  sense probe. Scale bar = 1mm.





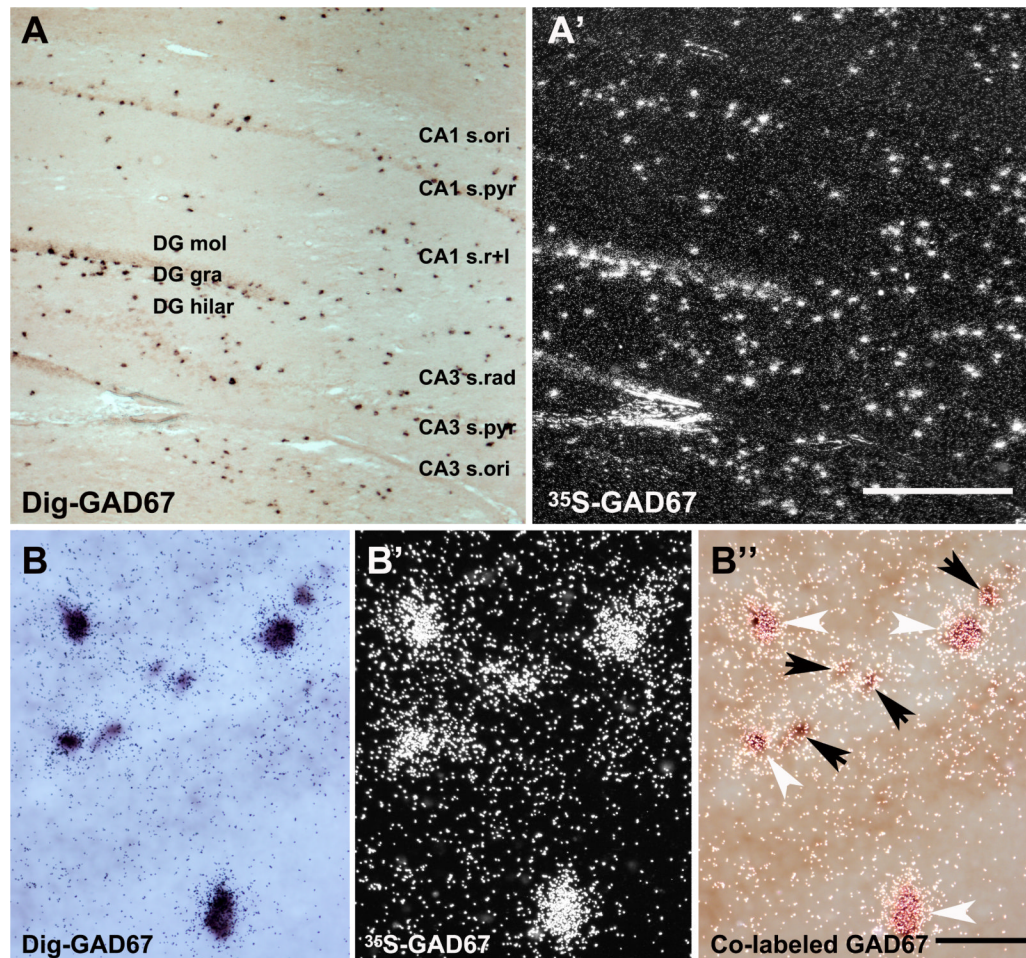
**Figure 2.** Darkfield images of nAChR subunit mRNA expression in coronal sections of dorsal hippocampus. The expression of A)  $\alpha 2$ , B)  $\alpha 3$ , C)  $\alpha 4$ , D)  $\alpha 5$ , E)  $\alpha 6$ , F)  $\alpha 7$ , G)  $\beta 2$ , H)  $\beta 3$ , I)  $\beta 4$ , and J)  $\beta 2$  sense was detected with  $^{35}\text{S}$ -labeled probes. Arrowheads point to specific hybridization in cells in the hippocampus. Abbreviations: CA1 and CA3, CA1 and CA3 hippocampal subfields; DG, dentate gyrus. Scale bar = 1mm.





**Figure 3.** Darkfield images of nAChR subunit mRNA expression in coronal sections of ventral hippocampus. The expression of A)  $\alpha 2$ , B)  $\alpha 3$ , C)  $\alpha 4$ , D)  $\alpha 5$ , E)  $\alpha 6$ , F)  $\alpha 7$ , G)  $\beta 2$ , H)  $\alpha 3$ , and I)  $\beta 4$  was detected with  $^{35}\text{S}$ -labeled probes. Arrows point to principal cell layers of CA1 and CA3 and DG. Abbreviations: CA1 and CA3, CA1 and CA3 hippocampal field; DG, dentate gyrus. Scale bar = 1 mm.

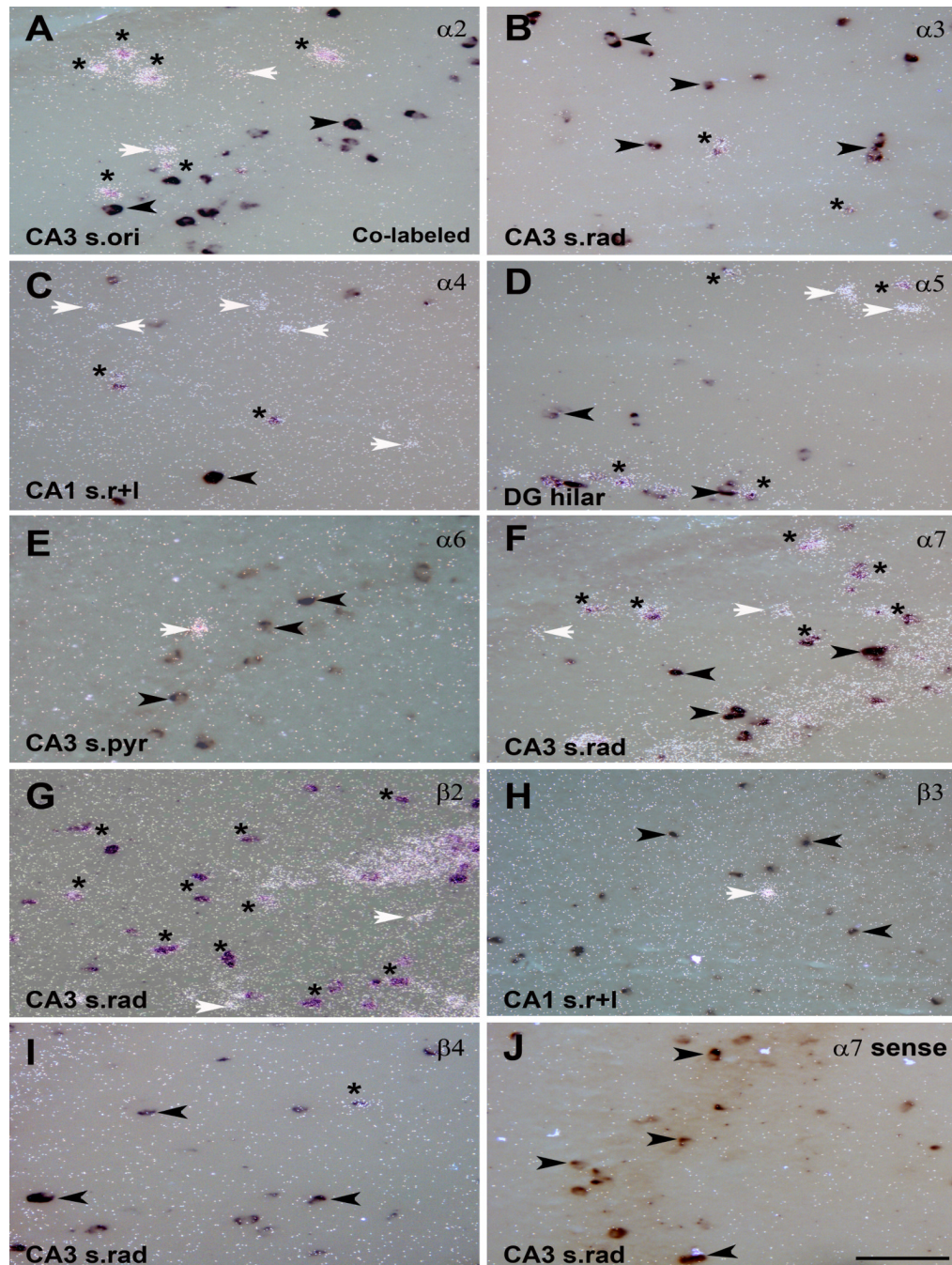




**Figure 4.**

GAD67 mRNA expression detected with double *in situ* hybridization in the hippocampus. Hybridization signals for GAD67 viewed in bright-field detecting non-radioactive signal generated with the Dig-labeled probe (A) and in darkfield detecting radioactive hybridization signal generated with the  $^{35}\text{S}$ -labeled probe (A'). Higher magnification lightfield (B), darkfield (B') and dual exposure (B'') to light- and darkfield simultaneously detecting GAD67 expression in interneurons of the CA3. White arrowheads point to strong and black arrows to moderate expression of GAD67. Abbreviations: CA1 s.ori, CA1 stratum oriens; CA3 s.pyr, CA3 stratum pyramidale; CA1 s.r+l, CA1 stratum radiatum/lacunosum moleculare; DG mol, dentate gyrus molecular layer; DG gra, dentate gyrus granule cell layer; DG hilar, dentate gyrus hilar region. Scale bar = 200  $\mu\text{m}$  (A') and 50  $\mu\text{m}$  (B'').

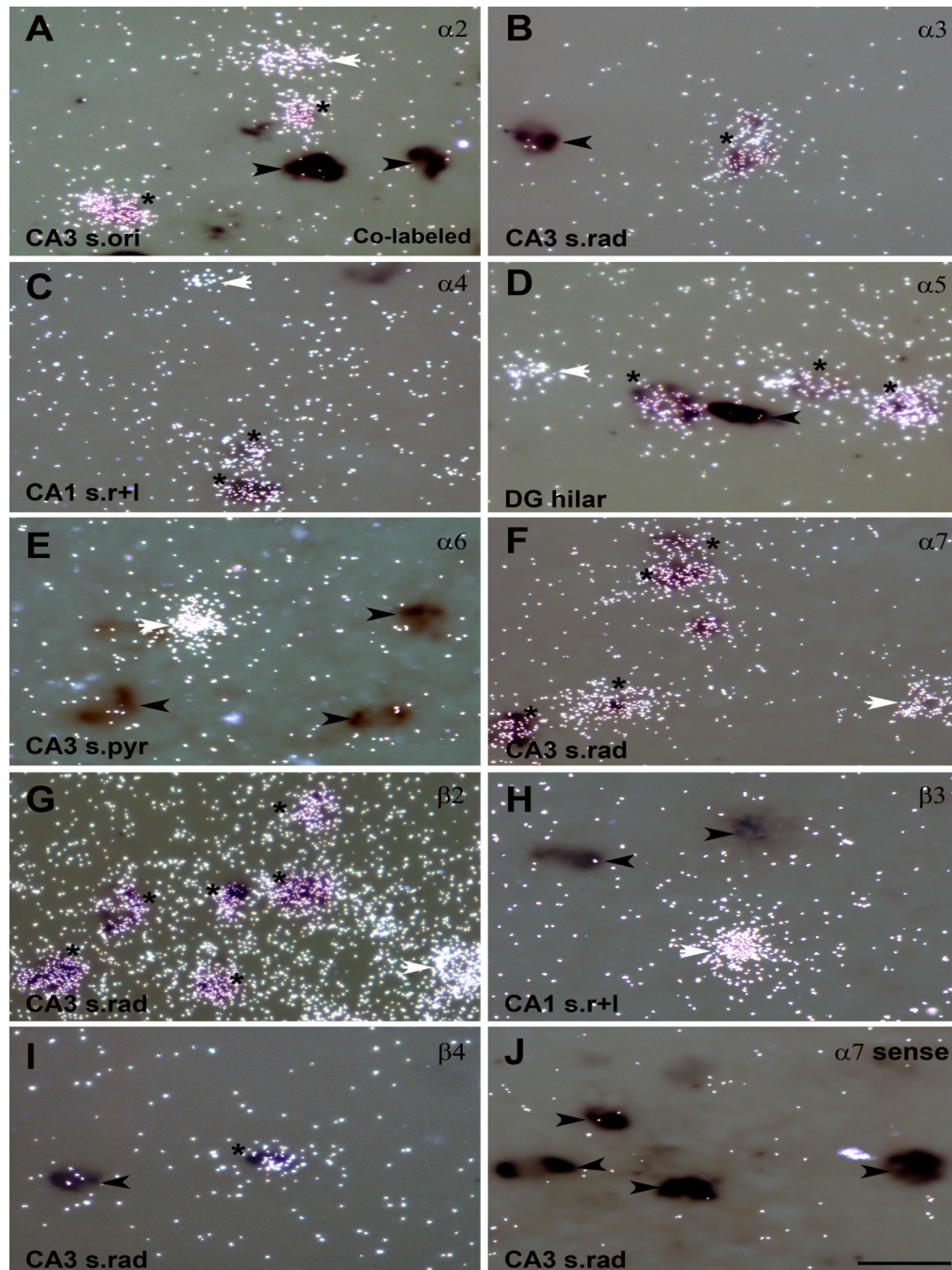




**Figure 5.** Co-expression of GAD67 and nAChR subunit mRNAs in hippocampal interneurons detected with double *in situ* hybridization. Expression of GAD67 and nAChR subunit mRNA for A)  $\alpha 2$ , B)  $\alpha 3$ , C)  $\alpha 4$ , D)  $\alpha 5$ , E)  $\alpha 6$ , F)  $\alpha 7$ , G)  $\beta 2$ , H)  $\beta 3$ , and I)  $\beta 4$  detected by dual exposure to light- and darkfield. J) Double *in situ* hybridization with the Dig-GAD67 probe and a  $^{35}\text{S}$ -nAChR sense probe for  $\alpha 7$  used as a negative control. Asterisks indicate neurons with co-expression of mRNAs for GAD67 and a nAChR subunit; white arrows point to single-labeled neurons expressing nAChR subunit mRNA only; black arrows point to single-labeled GAD67 expressing neurons. Abbreviations: CA1 s.ori, CA1 stratum oriens; CA3

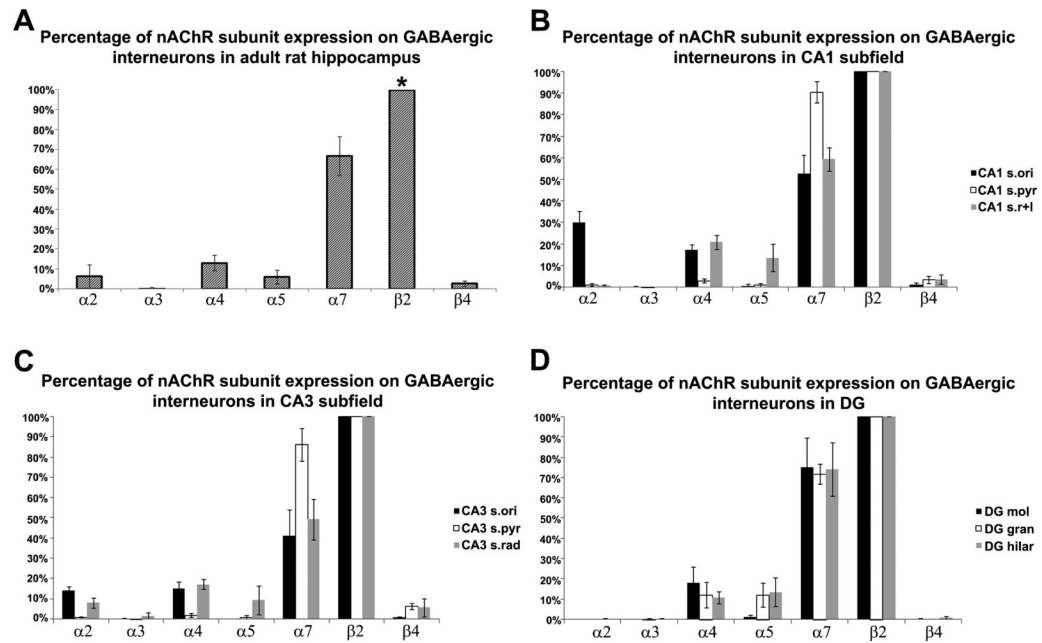
s.pyr, CA3 stratum pyramidale; CA1 s.rad/LM, CA1 stratum radiatum/lacunosum moleculare; DG mol, dentate gyrus molecular layer. Scale bar = 200  $\mu$ m.





**Figure 6.** Higher power images of co-expression of GAD67 and nAChR subunit mRNAs in hippocampal interneurons detected with double in situ hybridization. Expression of GAD67 and nAChR subunit mRNA for A)  $\alpha 2$ , B)  $\alpha 3$ , C)  $\alpha 4$ , D)  $\alpha 5$ , E)  $\alpha 6$ , F)  $\alpha 7$ , G)  $\beta 2$ , H)  $\beta 3$ , and I)  $\beta 4$  detected by dual exposure to light- and darkfield. J) Double *in situ* hybridization with the Dig-GAD67 probe and a  $^{35}\text{S}$ -nAChR sense probe for  $\alpha 7$  used as a negative control. Asterisks indicate neurons with co-expression of mRNA s for GAD67 and a nAChR subunit; white arrows point to single-labeled neurons expressing nAChR subunit mRNA only; black arrows point to single-labeled GAD67 expressing neurons. Abbreviations: CA1

s.ori, CA1 stratum oriens; CA3 s.pyr, CA3 stratum pyramidale; CA1 s.rad/LM, CA1 stratum radiatum/lacunosum moleculare; DG mol, dentate gyrus molecular layer. Scale bar = 50  $\mu$ m.



**Figure 7.** Percent of GAD67 interneurons coexpressing nAChR subunit mRNAs for  $\alpha 2$ ,  $\alpha 3$ ,  $\alpha 4$ ,  $\alpha 5$ ,  $\alpha 7$ , and  $\beta 4$  in (A) entire hippocampus, (B) CA1 subfield, (C) CA3 subfield, and (D) in the dentate gyrus. The percent coexpression for each nAChR subunit was compared to that of  $\beta 2$  (representing 100%). \* indicates that the percentage of GAD67/ $\beta 2$  neurons was significantly different from those of all other subunits. (\* $p < 0.001$ , Student t-test analysis,  $n=3$ ).