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HIPPOCAMPAL INTERNEURONS CO-EXPRESS TRANSCRIPTS ENCODING THE *α7* NICOTINIC RECEPTOR SUBUNIT AND THE CANNABINOID RECEPTOR 1

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Abstract

The notion of functional interactions between the α7 nicotinic acetylcholine (α7 nACh) and the cannabinoid systems is emerging from recent *in vitro* and *in vivo* studies. Both the α7 nACh receptor and the cannabinoid receptor 1 (CB1) are highly expressed in the hippocampus. To begin addressing possible anatomical interactions between the α7 nACh and the cannabinoid systems in the rat hippocampus, we investigated the distribution of neurons expressing α7 nACh mRNA in relation to those containing CB1 mRNA. By *in situ* hybridization we found that the α7 nACh mRNA is diffusely expressed in principal neurons and is highly expressed in a subset of interneurons. We observed that the pattern of distribution of hippocampal interneurons co-expressing transcripts encoding α7 nACh and glutamate decarboxylase (GAD; synthesizing enzyme of GABA) closely resembles the one displayed by interneurons expressing CB1 mRNA. By double *in situ* hybridization we established that the majority of hippocampal interneurons expressing α7 nACh mRNA have high levels of CB1 mRNA. As CB1 interneurons contain cholecystokinin (CCK), we investigated the degree of cellular co-expression of α7 nACh mRNA and CCK, and found that the cellular co-existence of α7 nACh and CCK varies within the different layers of the hippocampus.

In summary, we established that most of the hippocampal α7 nACh expressing interneurons are endowed with CB1 mRNA. We found that these α7 nACh/CB1 interneurons are the major subpopulation of hippocampal interneurons expressing CB1 mRNA. The α7 nACh expressing interneurons represent half of the detected population of CCK containing neurons in the hippocampus. Since it is well established that the vast majority of hippocampal interneurons expressing CB1 mRNA have 5-HT type 3 (5-HT_3) receptors, we conclude that these hippocampal α7 nACh/5HT_3/CB1/CCK interneurons correspond to those previously postulated to relay inputs from diverse cortical and subcortical regions about emotional, motivational, and physiological states.

Keywords

CB1; 5-HT3 receptors; 5-HT; CCK; Alzheimer's disease; schizophrenia

Nicotinic acetylcholine (nACh) receptors are members of the superfamily of ligand-gated ion channels that the includes the GABA receptors ($GABA_A$ and $GABA_C$), the glycine receptor and the 5-HT type 3 receptor (5-HT_3 R). In the nervous system, several subunits of the nACh receptor have been identified, including nine α subunits (α2-α10) and three β subunits (β2-β4) (Sargent, 1993; McGehee and Role, 1995). These subunits are assembled into different

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combinations to form functional pentameric receptors (Sargent, 1993; McGehee and Role, 1995). Electrophysiological, pharmacological and genetic approaches demonstrated that the homomeric α7-nACh receptor has a high affinity for α-bungarotoxin (Couturier et al., 1990; Séguéla et al., 1993; Chen and Patrick, 1997; Orr-Urtreger et al., 1997). The high affinity of the α7-nACh receptor for α-bungarotoxin has been used to map the distribution of this receptor in several brain areas, including the hippocampus (Hunt and Schmidt, 1978; Freedman et al., 1993; Fabian-Fine et al., 2001). Although mRNA encoding the α7 nACh subunit is expressed in both principal neurons and interneurons in the hippocampus and the dentate gyrus (DG) (Séguéla et al., 1993), the presence of functional α7 nACh receptors has been detected in interneurons (Jones and Yakel, 1997; Alkondon et al., 1998; Frazier et al., 1998a; Frazier et al., 1998b; McQuiston and Madison, 1999; Shao and Yakel, 2000; Sudweeks and Yakel, 2000; Buhler and Dunwiddie, 2002; Khiroug et al., 2003). In contrast, the presence of functional α7 nACh receptors in principal neurons of either the hippocampus or the dentate gyrus is controversial, as observations suggesting functional α7 nACh receptors in hippocampal gultamatergic neurons have not been replicated by others (Frazier et al., 1998b; Khiroug et al., 2003; Sudweeks and Yakel, 2000). Activation of α7 nACh receptors located on interneurons within the stratum (s.) oriens mediates strong excitatory effects that result in an increase of GABAergic neurotransmission (Jones and Yakel, 1997; Alkondon et al., 1998; Frazier et al., 1998a,b; McQuiston and Madison, 1999; Shao and Yakel, 2000; Sudweeks and Yakel, 2000; Buhler and Dunwiddie, 2002; Khiroug et al., 2003).

Hippocampal GABAergic neurotransmission is also regulated by the cannabinoid receptor type 1 (CB1) receptors, which are highly expressed in hippocampal interneurons (Mailleux and Vanderhaeghen, 1992; Matsuda et al., 1993; Gatley et al., 1998; Tsou et al., 1998). These CB1 interneurons contain cholecystokinin (CCK) (Katona et al., 1999; Marsicano and Lutz, 1999; Tsou et al., 1999) and co-express the subunit A of the 5-HT_3 R (Hermann et al., 2002; Morales and Backman, 2002; Morales et al., 2004). The CB1 receptor is a G-protein-coupled receptor that mediates the effects of the major psychoactive constituent of marijuana, delta 9-tetrahydrocannabinol (Δ^{9}-THC; Howlett, 1995). Electrophysiological studies, using hippocampal cultures (Ohno-Shosaku et al., 2001) and hippocampal slices (Wilson and Nicoll, 2001), demonstrated that endogenous cannabinoids produced in postsynaptic neurons act on presynaptic CB1 receptors on terminals of interneurons to suppress GABA release. In addition, it has been shown that CB1 receptor activation by synthetic CB1 agonists (Katona et al., 1999; Hajos et al., 2000; Hoffman and Lupica, 2000) or endogenous cannabinoids (Ohno-Shosaku et al., 2001; Wilson and Nicoll, 2001) inhibits hippocampal GABA neurotransmission, and that hippocampal neurons that express high levels of the CB1 mRNA are mostly GABAergic (Morales et al., 2004).

Functional interactions between the nicotinic and cannabinoid systems have been documented (for reviews see, Castañe et al., 2005; Viveros et al., 2006), and results from recent *in vitro* (Oz et al., 2003, 2004) and *in vivo* (Solinas et al., 2007) studies provide evidence for possible functional interactions between the α7 nACh and the cannabinoid systems. These interactions may occur, in part, by direct actions of endogenous cannabinoids on α7 nACh receptor-mediated neurotransmission. Previously, we showed that endogenous cannabinoids inhibit α7 nACh receptor-mediated responses in *Xenopus* oocytes (Oz et al., 2003, 2004). Recent studies with rats demonstrated that systemic administration of a selective α7 nACh receptor antagonist diminishes the discriminative and reinforcing effects of cannabinoids and their ability to increase dopamine levels in the nucleus accumbens (Solinas et al., 2007).

It is not clear which brain regions and neuronal components of the α7 nACh and the CB1 systems may establish functional interactions. Because the hippocampus is particularly rich in both α7 nACh and CB1 receptors, we begin addressing possible anatomical interaction between the α7 nACh and the CB1 systems in this brain region. In this study we investigated the

distribution of neurons expressing α7 nACh mRNA in relation to those containing CB1 mRNA. We confirmed that in the hippocampus both pyramidal neurons and interneurons express α7 nACh mRNA. We established that the α7 nACh expressing interneurons constitute a subset of hippocampal GABAergic neurons co-expressing CB1 mRNA. Thus, the well-documented hippocampal GABAergic neurotransmission regulated by CB1 receptors is also likely to be influenced by acetylcholine acting on α7 nACh receptors within the same interneuron.

EXPERIMENTAL PROCEDURES

Tissue preparation

Adult Sprague-Dawley male rats (200-250 g body weight) were anesthetized with chloral hydrate (35 mg/100 g) and perfused transcardially with a solution of 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.3. Brains were then removed from the skull, postfixed with paraformaldehyde for 2 h at 4 °C, rinsed with PB and sequentially transferred to 12%, 14% and 18% sucrose solutions. Brains were frozen on dry ice and cut in a cryostat to obtain coronal sections of 20 μm in thickness. All animal procedures were approved by the NIDA Animal Care and Use Committee. All experiments conformed to guidelines on the ethical use of animals as regulated by the NIH Guidelines for the Care and Use of Laboratory Animals NIH, and experiments were carried out in careful methods to minimize the amount of suffering and number of animals used.

Synthesis of riboprobes

The pBSmα7 plasmid containing the cDNA template for the M3/M4 intracellular loop of the mouse α7 nACh receptor (positions 1089-1373 according to GenBank sequence L37663) was kindly provided by Dr. J. W. Patrick (Baylor College of Medicine, Houston, TX, USA). The plasmid was cut using the restriction enzyme *Sma*I (for the antisense orientation) or *Eco*RI (for the sense orientation). The cDNAs were transcribed *in vitro* in the presence of [^{35}S]- and [^{33}P]-labeled UTP with either T7 RNA polymerase (antisense) or T3 RNA polymerase (sense). The radioactive antisense and sense probes for the human CB1 receptor (nucleotides 494-1622, accession # M77952) were prepared by cutting the plasmid and transcribing it with either T7 (antisense) or SP6 (sense) RNA polymerases in the presence of [^{35}S]- and [^{33}P]-labeled UTP. Digoxigenin-labeled antisense CB1 riboprobe was prepared using digoxigenin-labeled UTP (Boehringer Mannheim; Indianapolis, IN, USA) as described earlier (Morales et al., 2004). The antisense digoxigenin-labeled probes for the detection of GAD 65 and GAD 67 mRNAs were individually prepared and mixed together during hybridization (Yamaguchi et al., 2007).

In situ hybridization

In situ hybridization was performed as previously described (Morales and Backman, 2002). Cryosections were rinsed 3×10 min in diethyl pyrocarbonate (DEPC) -treated PB, incubated for 10 min in PB containing 0.5% Triton X-100, rinsed 3×10 min with DEPC-PB, treated with 0.2 M HCl for 15 min, rinsed 3×10 min with DEPC-PB, and then acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8.0) for 10 min. Sections were rinsed 3×10 min with DEPC-PB, post-fixed with 4% paraformaldehyde for 10 min, and after three rinses with DEPC-PB were incubated for 2 h in hybridization buffer (50% formamide; 10% dextran sulfate; 5× Denhardt's solution; 0.62 M NaCl; 50 mM DTT; 10 mM EDTA; 20 mM PIPES, pH 6.8; 0.2% SDS; 250 μg/ml salmon sperm DNA; 250 μg/ml tRNA). Sections were then hybridized at 55 °C for 16 h in the same hybridization buffer containing [^{35}S]- and [^{33}P]-labeled single-stranded mouse α7 nACh subunit RNA probes (at 10^7 cpm/ml). Sections were then rinsed with 2× SSC buffer containing 10 mM β-mercaptoethanol for 30 min at room temperature and treated with DNase-free RNAse A at 8 μg/ml at 37 °C for 1 h, washed with 0.5× SSC containing 50% formamide with 10 mM β-mercaptoethanol and 0.5% Sarkosyl for 2 h at 55 °C, and with 0.1×

SSC containing 10 mM β-mercaptoethanol and 0.5% Sarkosyl at 60 °C for 1 h. The sections were then washed 4×10 min in PB, mounted on chrome-alum-coated glass slides and air dried. The air-dried slides were dehydrated in a series of increasing ethanol concentrations (2 min each in 30, 60, 90, 95, 100, and 100%) followed by 2×10 min in Citrisol clearing agent (Fisher Scientific, Fair Lawn, NJ, USA) and two additional rinses in 100% ethanol. To determine the quality of the *in situ* hybridization prior to dipping slide in photographic emulsion, slides were placed in a light tight metal cassette (BAS Cassette 2025, Fuji Photo Film Co., Japan) containing a Fujifilm Imaging Plate (BAS-i.p. MS 2025, Fuji Photo Film Co.). After overnight exposure at room temperature, the plate was read in a Fujifilm Bio-Imaging Analyzer BAS-5000 system (Fuji Photo Film Co.), and digital images of the tissue were obtained. The slides were dipped in Illford K5 nuclear track emulsion and exposed for 2-5 weeks prior to photographic development. We found that material hybridized with [^{35}S]- and [^{33}P]-labeled riboprobes requires exposure times shorter than material hybridized with single [^{35}S]- or [^{33}P]-labeled riboprobes. After development of silver grains the slides were dehydrated again, coverslipped and viewed by microscopy.

Double *In situ* hybridization

The hybridization process was carried out as above except that the [^{35}S]- and [^{33}P]-labeled antisense α7 nACh probe was mixed with digoxigenin-labeled antisense probes for either the human CB1 mRNA or rat GAD mRNA. In addition, following the last SSC wash, sections were rinsed 3×10 min with PB and for 60 min with blocking solution containing 2% bovine serum albumin fraction V (BSA; Sigma-Aldrich, St. Louis, MO, USA), 0.3% Triton X-100, 20 mM Tris buffer, 0.5 M NaCl and 20 mM NaN_3, at pH 8.2. Sections were incubated with alkaline phosphatase-conjugated antibody against digoxigenin (Roche Diagnostics, Indianapolis, IN, USA) overnight at 4 °C at 1:3000 dilution. Sections were rinsed 3×10 min in 0.1 M Tris buffer containing 0.5 mM $MgCl_2$ and 150 mM NaCl, pH 9.5, and the alkaline phosphatase activity was developed using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Roche Diagnostic). After rinsing 4×10 min in PB, the sections were mounted on glass slides and dehydrated and dipped in Illford K5 emulsion as described above.

In situ hybridization and CCK immunocytochemistry

Brain sections were prepared as described above. Following *in situ* hybridization for α7 nACh mRNA, the sections were rinsed with PB, incubated overnight with mouse anti-CCK (Morales and Bloom, 1997) in PB supplemented with 4% BSA and 0.3% Triton X-100. After rinsing (3×10 min) in PB, the sections were incubated with biotinylated anti-mouse antibody and the Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA) followed by detection of peroxidase activity using diaminobenzidine and H_2O_2. Sections were mounted, dehydrated and autoradiographed (for the detection of α7 nACh mRNA) as described above.

Data analysis

We used the computer software Stereo Investigator (MicroBright-Field, Inc., Williston, VT, USA) to outline in each hippocampal section the following areas: the stratum (s.) oriens, s. pyramidale, striatum radiatum, s. lacunosum moleculare and s. lucidum of CA1 and CA3, and the polymorph layer. Within each traced region (4× objective lens), single- and double-labeled neurons were observed at higher power (20× objective lens) and marked electronically. To obtain the number of cells showing label for α7 nACh mRNA, CB1 mRNA, GAD65/67 mRNA or CCK immunostaining, specific markers within the software were ascribed for each of the independent types of cell countings. These results were used to calculate the percentage of cell profiles containing α7 nACh mRNA and either CB1 mRNA, GAD mRNA, or CCK immunostaining within the detected population of either CB1 or α7 nACh mRNA expressing neurons within each region. A cell was considered to express CB1 mRNA, GAD mRNA, or

CCK immunoreactivity when its soma was clearly stained purple (due to alkaline phosphatase detection of the digoxigenin probe) or brown (due to peroxidase detection). A neuron was considered to express α7 nACh mRNA when, following the hybridization with the antisense α7 nACh riboprobe, it was covered with silver grains with a density of at least threefold higher than the surrounding background. A neuron was considered double-labeled when its soma was purple (or brown) and was covered with an aggregate of silver particles clearly on top of it. Control experiments using sense probes showed only very low background level of radioactive or digoxigenin labeling, demonstrating that the labeling with α7 nACh, CB1, or GAD antisense probes was specific. Material was analyzed and photographed under bright field or epiluminescence microscopy using a Nikon Eclipse E 800 microscope equipped with 4×, 10× and 20× objective lenses. Double labeled material was analyzed using epiluminescence to increase the contrast of silver grains, as either dark-field or bright field optics does not allow a clear visualization of silver grains when co-localized with immunoproducts. Pictures were adjusted to match contrast and brightness by using the program Adobe Photoshop (Adobe Systems Incorporated, Seattle, WA, USA).

RESULTS

Both pyramidal neurons and interneurons express α7 nACh mRNA in the hippocampus

By using α7 nACh radioactive antisense riboprobes, we detected expression of α7 nACh mRNA in the CA1, CA2 and CA3 subfields of the hippocampus as well as in the DG (Fig. 1A). Signal for α7 nACh mRNA was not detected when brain sections were hybridized with the corresponding α7 nACh sense riboprobe (Fig. 1B). Within the pyramidal layers the α7 nACh mRNA signal appears uniformly diffused, making the visualization of positive individual principal neurons difficult (Fig. 1A). Interneurons expressing high levels of α7 nACh mRNA were found in the CA1 and CA3 subfields of the hippocampus (with fewer cells in the CA2) as well as in the DG. Interneurons expressing α7 nACh mRNA were clearly seen in the s. oriens, s. radiatum, s. lacunosum moleculare and s. lucidum (Fig. 1A). Within the DG, α7 nACh mRNA was uniformly diffused in the principal neurons of the granular layer, but highly expressed in interneurons within the polymorph layer (Fig. 1A). Interneurons containing α7 nACh mRNA were also found in the s. pyramidale of the hippocampus and in the granular layer of the DG, their distribution within these principal layers was clearly seen when short exposure times, 2 weeks instead of 4-5 weeks, were used (Fig. 1C).

GABAergic nature of cells expressing high levels of α7 nACh mRNA

To validate the GABAergic nature of neurons expressing high levels of α7 nACh mRNA, we determined the degree of cellular co-localization of α7 nACh mRNA and GAD mRNA. This was done by performing double *in situ* hybridization utilizing a mixture of two antisense riboprobes; (i) radioactive antisense probe for detection of α7 nACh mRNA and (ii) antisense digoxigenin-labeled probe for detection of mRNAs of the two isoforms of the enzyme GAD (GAD65/67). Using this approach it became evident that almost all neurons expressing high levels of α7 nACh mRNA contain GAD mRNA (Fig. 2) and are therefore GABAergic. However, it should be noted that many hippocampal interneurons expressing GAD mRNA do not contain α7 nACh mRNA (or contain it in a much lower levels). Thus, α7-nACh/GAD cells constitute a subpopulation of hippocampal interneurons.

Analysis of the percentage of cellular co-expression of α7 nACh mRNA and GAD mRNA in several layers of the hippocampus and the DG indicated that 87% to 99% of interneurons expressing α7 nACh mRNA contain GAD mRNA (Table 1). The degree of co-expression of α7 nACh and GAD may have been underestimated due to the fact that while silver grains (corresponding to α7 nACh signal) are confined to the surface of the section, digoxigenin-labeled neurons are seen throughout the depth of the section. It was not possible to obtain

accurate numbers for α7 nACh mRNA-labeled cells in the s. pyramidale of the hippocampus and in the granular layer of the DG due to the presence of diffuse α7 nACh labeling within the principal neurons. Consequently, layers analyzed in this experiment within the CA1 and CA3 regions were the s. oriens, s. radiatum, and s. lacunosum moleculare; the polymorph layer of the DG was also analyzed.

Co-expression of α7 nACh mRNA and CB1 mRNA in hippocampal interneurons

By using CB1 radioactive antisense riboprobes, we detected cellular expression of CB1 mRNA in the hippocampus and the DG (Fig. 3A). Signal for CB1 mRNA was not detected when brain sections were hybridized with the corresponding CB1 sense riboprobe (Fig. 3B). Interestingly, the distribution of hippocampal interneurons expressing CB1 mRNA (Fig. 3A) closely resembles that of interneurons expressing α7 nACh mRNA (Fig. 1C). To determine the degree of overlapping of the cell populations expressing α7 nACh mRNA or CB1 mRNA, we simultaneously labeled these two cellular populations by double *in situ* hybridization. The cellular distribution of α7 nACh mRNA was visualized using the α7 nACh antisense radioactive-labeled probe, and that of the CB1 mRNA was visualized using digoxigenin-labeled antisense CB1 probe. We found that most of the interneurons expressing α7 nACh mRNA co-expressed CB1 mRNA in the CA1, CA2 and CA3 subfields of the hippocampus, and in the DG (Fig. 4).

Quantitative analysis demonstrated a very high degree of cellular co-expression of α7 nACh mRNA and CB1 mRNA in both the hippocampus and the DG (Table 2). Within the detected population of interneurons expressing α7 nACh mRNA in the CA1, 89%±0.5 (mean±S.E.M.) contained CB1 mRNA in the s. pyramidale, 96%±1.4 in the s. radiatum and 88%±0.6 in the s. lacunosum moleculare. Similar results were determined for the CA3 subfield, where 75%±0.5 of the detected population of the α7 nACh expressing neurons co-expressed CB1 in the s. pyramidale, 96%±0.9 in the s. radiatum, and 80%±0.3 in the s. lucidum. In the polymorph layer of the DG the vast majority of α7 nACh interneurons (91%±1.6) contained CB1 mRNA. Within the hippocampus the lowest levels of α7 nACh mRNA and CB1 mRNA co-expression were constantly found in the s. oriens. In the s. oriens, 37%±1.3 of the detected population of α7 nACh expressing neurons contained CB1 mRNA in the CA1 and 50%±0.8 in the CA2.

We performed further quantitative analysis to determine the extent of representation of the α7 nACh/CB1 containing interneurons in the detected population of CB1 interneurons (Table 2). Within the detected population of interneurons expressing CB1 mRNA in the CA1, 75% ±0.3 co-expressed α7 nACh mRNA in the s. oriens, 85%±0.6 in the s. pyramidale, 81%±1.4 in the s. radiatum, and 83%±0.7 in the s. lacunosum moleculare. The CA3 also contained a high representation of interneurons co-expressing α7 nACh mRNA and CB1 mRNA in the detected population of CB1 interneurons. Of the detected population of the CB1 expressing neurons 84%±0.5 co-expressed α7 nACh mRNA in the s. pyramidale, 78%±1.1 in the s. radiatum and 100%±0.3 in the s lucidum. The lowest concentration of interneurons containing CB1 and co-expressing α7 nACh mRNA was detected in the s. oriens of CA3 where only 51% ±0.8 of the detected population of CB1 expressing interneurons contained α7 nACh mRNA. Most of the CB1 interneurons (87%±1.9) contained α7 nACh mRNA in the polymorph layer of the DG (Table 2).

In summary, within the hippocampus and the DG, with exception of the s. oriens (where the co-expression was less pronounced), the vast majority of α7 nACh expressing interneurons contained CB1 mRNA. Moreover, these α7 nACh/CB1 expressing neurons constitute the main subpopulation of GABAergic neurons within the detected population of CB1 containing interneurons in the hippocampus and DG.

A subpopulation of hippocampal interneurons expressing α7 nACh mRNA contains CCK

It is well established that many hippocampal interneurons expressing CB1 mRNA contain the neuropeptide CCK (Katona et al., 1999; Marsicano and Lutz, 1999; Morales and Backman, 2002; Morales et al., 2004). Thus, we used a combination of *in situ* hybridization (for detection of α7 nACh mRNA) and immunohistochemistry (for detection of CCK) to investigate the degree of overlapping of the cell populations expressing α7 nACh mRNA or CCK immunoreactivity (Fig. 5). Quantitative analysis showed that the number of neurons expressing α7 nACh mRNA that contain CCK is variable in the different layers of the hippocampus (Table 3). Within the detected population of interneurons expressing α7 nACh mRNA in the CA1, 21%±0.9 contained CCK immunoreactivity in the s. oriens, 76%±0.4 in the s. pyramidale, 76% ±0.6 in the s. radiatum, and 48%±0.3 in the s. lacunosum moleculare. In the CA3 subfield, 21% ±0.9 of the detected population of interneurons expressing α7 nACh mRNA neurons contain CCK immunoreactivity in the s. oriens, 48%±0.5 in the s. pyramidale, 79%±0.7 in the s. radiatum and 41%±0.3 in the s lucidum. In the polymorph layer of the DG, as many as 78% ±1.1 of all interneurons expressing α7 nACh mRNA contained CCK.

Further quantitative analysis was performed to determine the extent of representation of the α7 nACh/CCK containing interneurons in the detected population of CCK interneurons (Table 3). Analysis of the CA1 and CA3 subfields of the hippocampus and DG showed that about half of all the CCK immunoreactive neurons expressed α7 nACh mRNA in the s. pyramidale, s. radiatum, s. lacunosum moleculare, s. lucidum and polymorph layer. Within the s. oriens 70% of all CCK neurons had α7 nACh mRNA.

In summary, within the hippocampus and the DG, with exception of the s. oriens, more than half of the α7 nACh mRNA expressing interneurons contained CCK. Moreover, these α7 nACh/CCK expressing neurons account for more than half of the detected population of CCK immunoreactive neurons in the hippocampus and DG.

DISCUSSION

Differential levels of α7 nACh mRNA expression between the hippocampal GABAergic and glutamatergic neurons

We found low levels of α7 nACh mRNA in pyramidal and granular neurons, but high levels of expression in a subset of interneurons distributed in both the hippocampus and the DG. In these interneurons, the protein product from the α7 nACh mRNA is likely to be assembled mostly, if not exclusively, into homomeric α7 nACh receptors. In line with this suggestion, the existence of functional α7 nACh receptors in hippocampal interneurons has been demonstrated by electrophysiological analysis of hippocampal slide preparations (Jones and Yakel, 1997; Alkondon et al., 1998; Frazier et al., 1998a,b; McQuiston and Madison, 1999; Shao and Yakel, 2000; Sudweeks and Yakel, 2000). In contrast, α7 nACh receptor-mediated excitation of pyramidal neurons has not been detected in these preparations (Frazier et al., 1998b; Khiroug et al., 2003), despite the presence of α7 nACh mRNA in glutamatergic neurons (hippocampal pyramidal neurons and granular cells, see Fig. 1A). This apparent contradiction could be due to the presence of low levels of α7 nACh mRNA in principal neurons, leading to a very limited expression of α7 nACh receptors. Alternatively, it would be of interest to find out whether the α7 nACh subunits in principal neurons could be assembled into α7 nACh heteromeric receptors differing in their electrophysiological properties from the regular homo-pentameric receptors. However, α7 nACh-containing heteromeric nACh receptors have not yet been identified.

The vast majority of α7 nACh mRNA expressing interneurons contains CB1 receptors

We established that, with exception of the s. oriens, in both the hippocampus and the DG the vast majority of interneurons containing α7 nACh mRNA co-expresses CB1 mRNA. In

contrast, within the s. oriens only between 37% and 50% of all interneurons expressing α7 nACh mRNA contain CB1 mRNA. In addition, we determined that, with exception of the s. oriens, in both the hippocampus and the DG the interneurons co-expressing α7 nACh mRNA and CB1 mRNA constitute the major population of CB1 containing interneurons. Based on the high degree of co-localization of α7 nACh mRNA and CB1 in these interneurons, it is likely that their GABAergic neurotransmission will be regulated by the synthesis and release of endocannabinoids from the postsynaptic pyramidal neurons (Kim et al., 2002; Neu et al., 2007) and by their cholinergic innervations from the medial septum and diagonal band (Woolf, 1991).

Interneurons expressing α7 nACh mRNA constitute a subpopulation of CCK containing neurons in the hippocampus and the DG

We found that, with the exception of the s. oriens, in both the hippocampus and the DG 40-79% of all interneurons expressing α7 nACh mRNA contain CCK. In contrast, within the s. oriens only 20% of all interneurons expressing α7 nACh mRNA have CCK. The low proportion of interneurons co-expressing α7 nACh mRNA and CCK in the s. oriens is consistent with the detection of the lowest proportion of α7 nACh/CB1 interneurons in this layer, and the fact that CB1 containing neurons in their vast majority have CCK. We conclude that in the s. oriens, as opposed to the other layers of the hippocampus and the DG, there are two major subpopulations of interneurons expressing α7 nACh mRNA; interneurons expressing α7 nACh mRNA together with CB1 and CCK, and interneurons expressing α7 nACh mRNA but lacking CB1 and CCK. The latter subpopulation is likely to contain other neuropeptides such as somatostatin (Freedman et al., 1993).

The α7 nACh receptor-mediated activation of hippocampal interneurons produces either inhibition or disinhibition of pyramidal neurons (Ji and Dani, 2000; Buhler and Dunwiddie, 2002). By combining electrophysiological and anatomical analysis, three kinds of synapses (somatic-pyramidal, dendritic-pyramidal and interneuronal) were identified to participate in the hippocampal inhibitory responses mediated by α7 nACh receptors (Buhler and Dunwiddie, 2002). We propose that axon terminals in these three types of synapses originate in part from interneurons that contain both α7 nACh receptors and CCK. This suggestion is based on our finding that half of the detected population of CCK containing cells co-express α7 nACh mRNA (seeTable 3), and on the fact that CCK interneurons make inhibitory synapses on interneurons as well as on the soma and dendrites of pyramidal neurons (Nunzi et al., 1985; Cope et al., 2002).

Hippocampal α7 nACh/CB1/5HT3$_A$/CCK interneurons

The subunit A (5-HT$_{3A}$), but not the subunit B (5-HT$_{3B}$), of the 5-HT$_3$ receptor is encoded in GABAergic/CCK neurons of the hippocampus and the DG (Morales and Wang, 2002; Morales and Bloom, 1997). These neurons in their vast majority co-express CB1 mRNA (Hermann et al., 2002; Morales and Backman, 2002; Morales et al., 2004). Considering that most of the interneurons expressing α7 nACh mRNA co-express CB1 mRNA, we conclude that the majority of these neurons also contain transcripts encoding the 5-HT$_{3A}$ subunit and the neuropeptide CCK. Based on our previous observations and current results we conclude that within the hippocampus and the DG there is a subpopulation of GABAergic neurons containing CCK, the two ion channel receptors α7 nAChR and 5HT$_3$R, and the G-protein-coupled receptor CB1. Considering the composition of these α7 nACh/5HT3$_A$/CB1/CCK interneurons, it is expected that their activity will be regulated by the integration of the action from several brain regions. Tracing studies had established that CCK hippocampal interneurons receive cholinergic inputs from the septum (Woolf, 1991) and serotonergic afferents from the median raphe nucleus (Freund et al., 1990). In addition, electrophysiological analysis determined that the tonic control of GABA release from hippocampal CCK cells is regulated through the

modulation of the synthesis and release of endocannabinoids from the postsynaptic pyramidal neurons (Kim et al., 2002; Neu et al., 2007). The complex composition of these α7 nACh/$5HT3_A$/CB1/CCK interneurons is in line with the suggestion that hippocampal CCK neurons relay inputs from diverse cortical and subcortical regions about emotional, motivational, and physiological states (Freund, 2003). These neurons are proposed to act as modulators that adapt hippocampal network activity to behavioral states (Freund, 2003).

GABA release from the α7 nACh/$5HT3_A$/CB1/CCK interneurons will depend on balanced functional participation of their receptors. Activation of α7 nACh receptors mediates strong excitatory effects that result in an increase of GABAergic neurotransmission in the hippocampus (Jones and Yakel, 1997; Alkondon et al., 1998; Frazier et al., 1998a,b; McQuiston and Madison, 1999; Shao and Yakel, 2000; Sudweeks and Yakel, 2000). Similarly, activation of the $5HT_3$ receptor results in enhanced GABAergic neurotransmission in cultured hippocampal neurons (Yakel and Jackson, 1988) and in hippocampal slices (Ropert and Guy, 1991; Corradetti et al., 1992; Kawa, 1994; Maeda et al., 1994; Passani et al., 1994). Moreover, 5-HT was shown to directly excite GABAergic interneurons via the $5HT_3$ receptor and consequently to increase the frequency of inhibitory synaptic events recorded in CA1 pyramidal cells of rat hippocampal slices (Ropert and Guy, 1991). In contrast, endogenous cannabinoids produced in postsynaptic neurons act on presynaptic CB1 receptors on terminals of interneurons to suppress GABA release in hippocampal cultures (Ohno-Shosaku et al., 2001) and hippocampal slices (Wilson and Nicoll, 2001). In addition, CB1 receptor activation by synthetic CB1 agonists (Katona et al., 1999; Hajos et al., 2000; Hoffman and Lupica, 2000) or endogenous cannabinoids (Ohno-Shosaku et al., 2001; Wilson and Nicoll, 2001) inhibits hippocampal GABA neurotransmission.

CONCLUSION

In summary, we established that the α7 nACh expressing interneurons constitute a subset of hippocampal GABAergic neurons endowed with the CB1 receptor. These neurons constitute the main subpopulation of CB1 containing interneurons, and represent half of the population of CCK positive neurons. As previous studies demonstrated that CB1 interneurons contain the functional subunit $5\text{-}HT_{3A}$ of the $5\text{-}HT_3$ receptor, these α7 nACh/$5HT3_A$/CB1/CCK interneurons constitute the major population of CCK interneurons postulated to carry information from subcortical pathways about the emotional, motivational and general physiological state of the animal.

Acknowledgments

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Abbreviations:

BSA, bovine serum albumin
CB1, cannabinoid receptor type 1
CCK, cholecystokinin
DEPC, diethyl pyrocarbonate
DG, dentate gyrus
GAD, glutamate decarboxylase
nACh, nicotinic acetylcholine
PB, phosphate buffer
s, stratum
α7 nACh, α7 nicotinic acetylcholine
$5\text{-}HT_3$ R, 5-HT type 3 receptor

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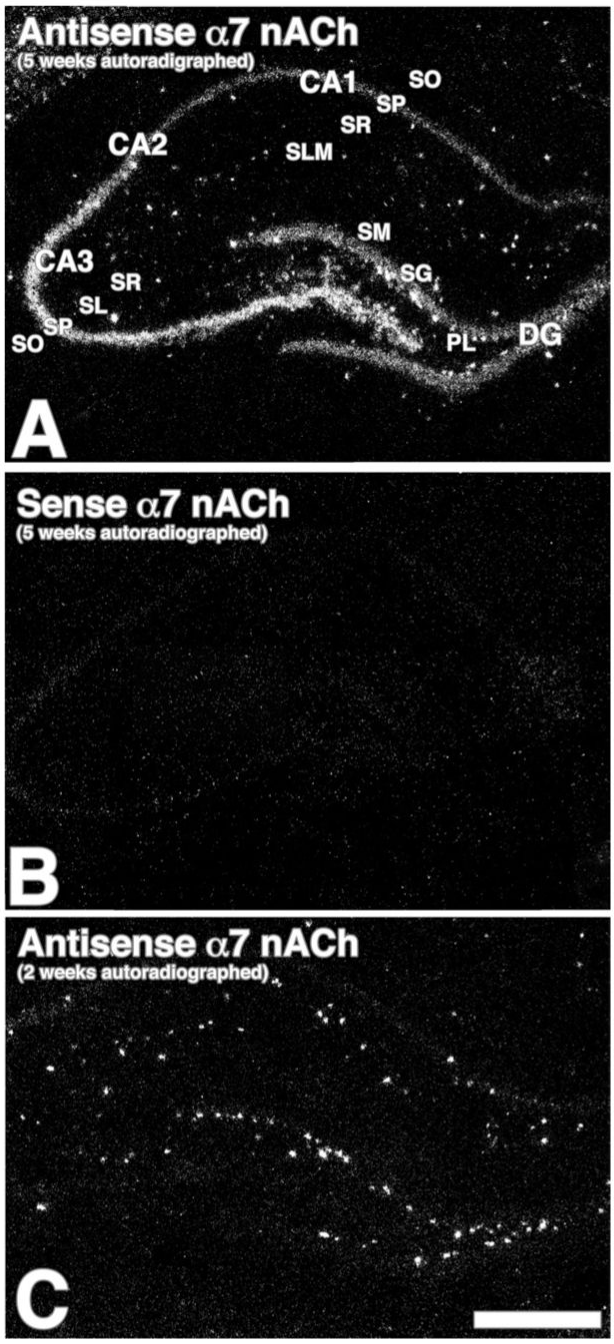

Fig. 1.

Expression of *α*7 nACh mRNA in rat hippocampus and DG. (A) Epiluminescence view of a coronal section hybridized with radioactively-labeled antisense *α*7 nACh probe. Note scattered *α*7 nACh mRNA signal in the pyramidal layers (SP) of CA1, CA2 and CA3 subfields of the hippocampus and granular layer (SG) of the DG. High levels of expression are seen in interneurons in the s. oriens (SO), s. pyramidale (SP), s. radiatum (SR), s. lucidum (SL) and s. lacunosum moleculare (SLM) of the hippocampus. Highly expressing interneurons are also present in the polymorph layer (PL) and stratum moleculare (SM) of the DG. (B) Epiluminescence view of a brain section hybridized with the sense *α*7 nACh probe. Note lack of labeled cells. Sections in A and B were exposed for 5 weeks. (C) Epiluminescence view of

a coronal section hybridized with radioactively labeled antisense α7 nACh probe exposed for 2 weeks, instead of 5 weeks. Note that α7 nACh mRNA signal in material with short exposure is not visualized in the principal layer of CA3. Scale bar=180 μm, shown in C for A, B and C.

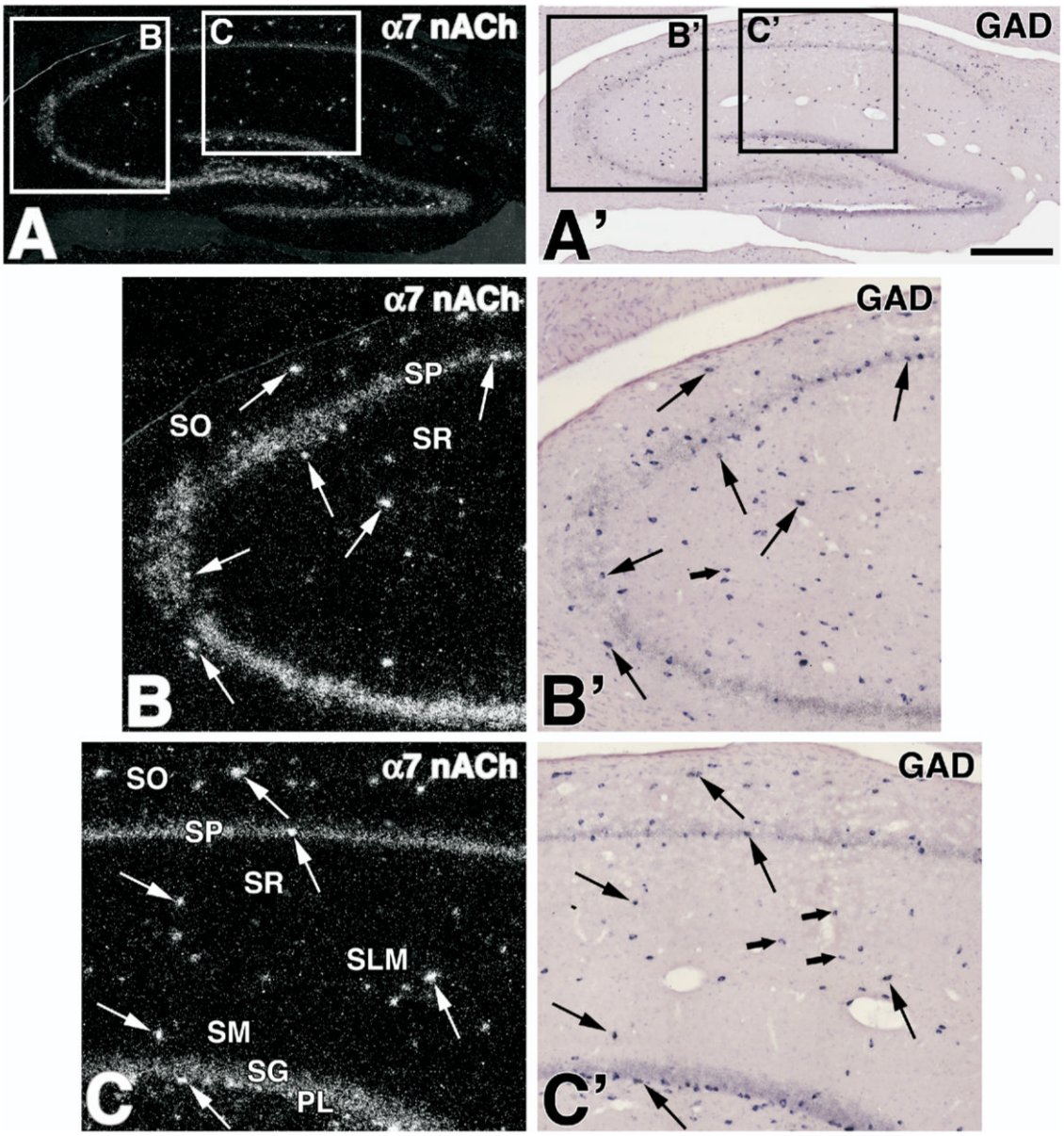

Fig. 2.

Hippocampal interneurons expressing α7 nACh mRNA co-express GAD mRNA. (A, A') Low magnification of epi-fluorescence (A) and bright field (A') views of cellular expression of α7 nACh mRNA (A) and GAD mRNA (A'). Frames B, B' C and C' correspond to higher magnification of areas delineated in panel A and A'. In frames B, B', C and C' the large arrows indicate examples of neurons co-expressing α7 nACh and GAD mRNA. The small arrows in B' and C' indicate examples of neurons expressing GAD mRNA but lacking α7 nACh mRNA. Scale bar=500 μm, shown in A' for A and A'; 180 μm for B, B', C and C'.

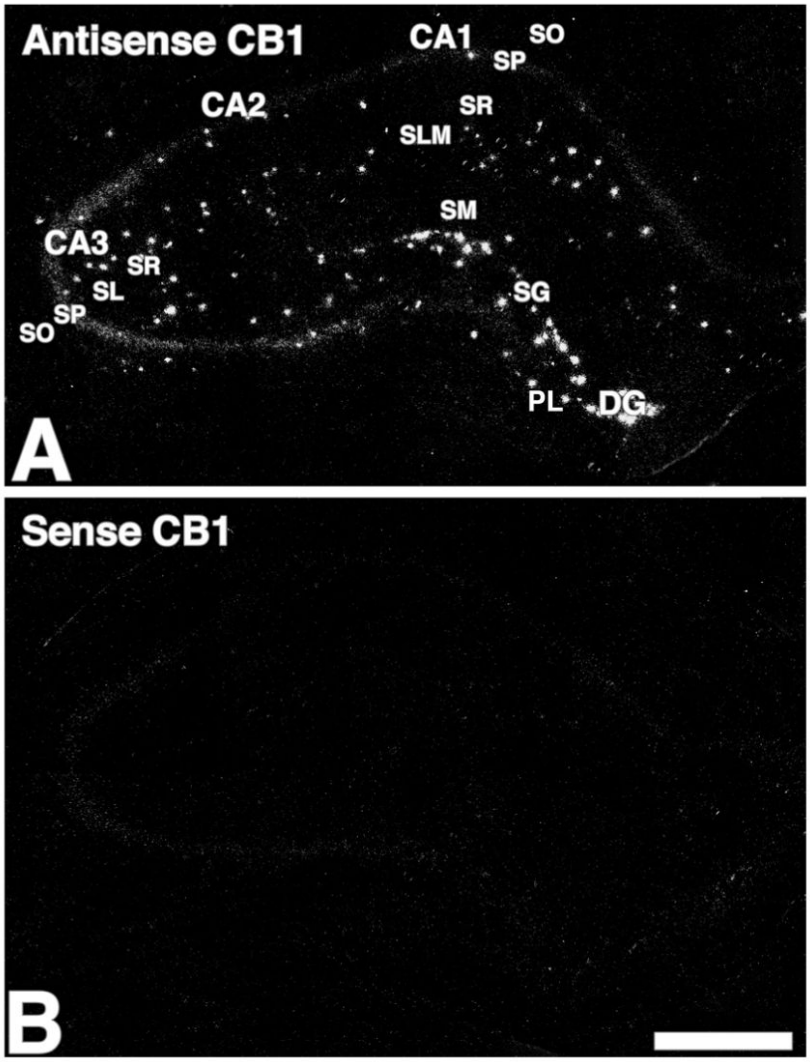

Fig. 3.

Cellular expression of CB1 mRNA in rat hippocampus and DG. (A) Epiluminescence view of a coronal section hybridized with radioactively-labeled antisense CB1 probe. Note high levels of CB1 mRNA expression in interneurons within the stratum s. oriens (SO), s. pyramidale (SP), s. radiatum (SR), s. lucidum (SL) and s. lacunosum moleculare (SLM) of the hippocampus. Highly expressing interneurons are also present in the polymorph layer (PL) and s. moleculare (SM) of the DG. (B) Epiluminescence view of a brain section hybridized with the sense CB1 probe. Note lack of labeled cells. Scale bar=450 μm, shown in B for A and B.

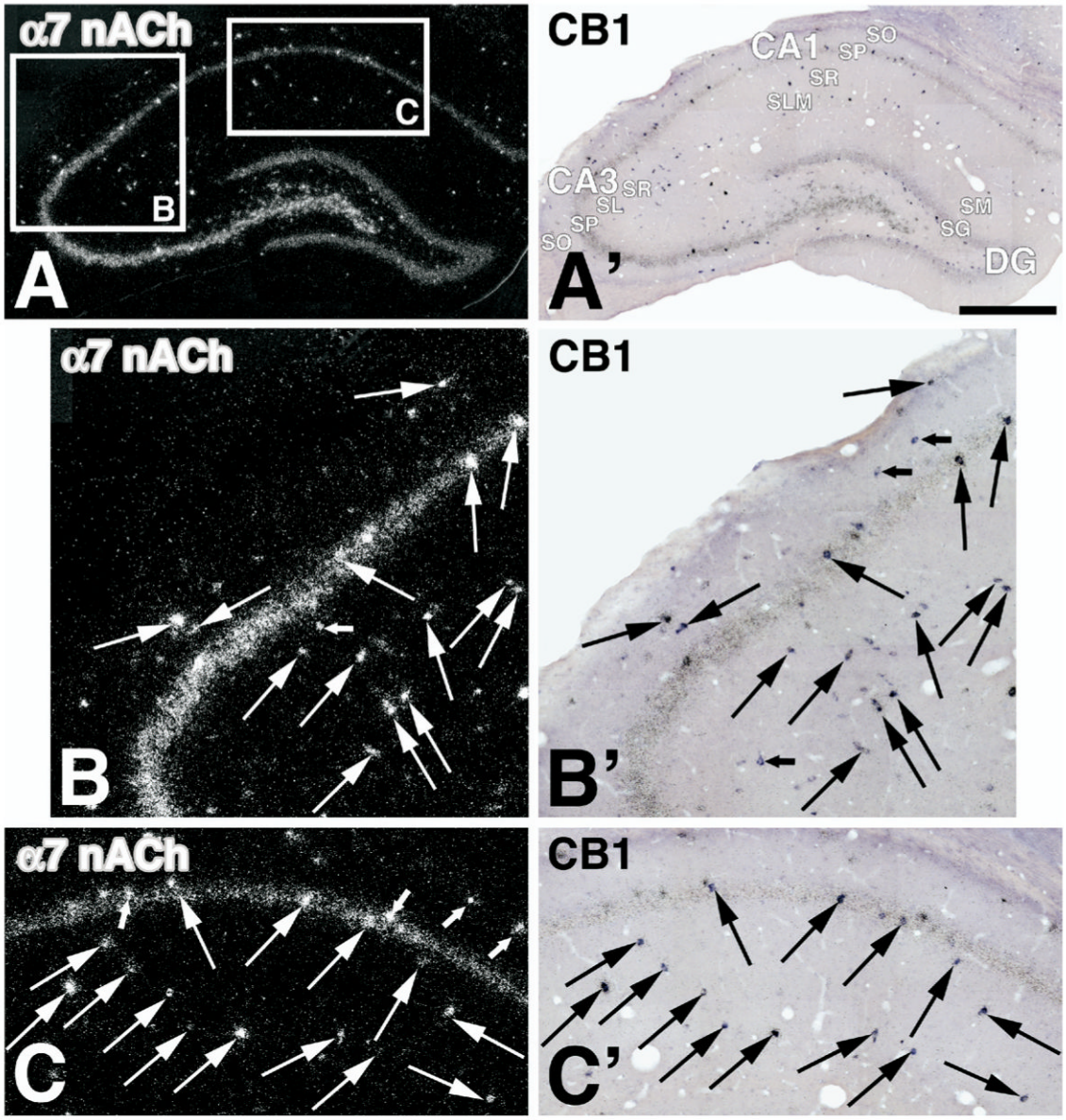

Fig. 4.

High degree of cellular co-expression of α7 nACh and CB1 transcripts in hippocampal interneurons. (A, A') Low magnification of epi-fluorescence (A) and bright field (A') views of cellular expression of α7 nACh mRNA (A) and CB1 mRNA (A'). Frames B, B', C and C' correspond to higher magnification of areas delineated in panel A. Large arrows indicate examples of neurons co-expressing α7 nACh and CB1 mRNAs. Small arrows in B, B', C and C' indicate examples of neurons expressing either α7 nACh or CB1 mRNAs. Scale bar shown in A' represents 470 μm in A and A'; 170 μm for B, B', C and C'.

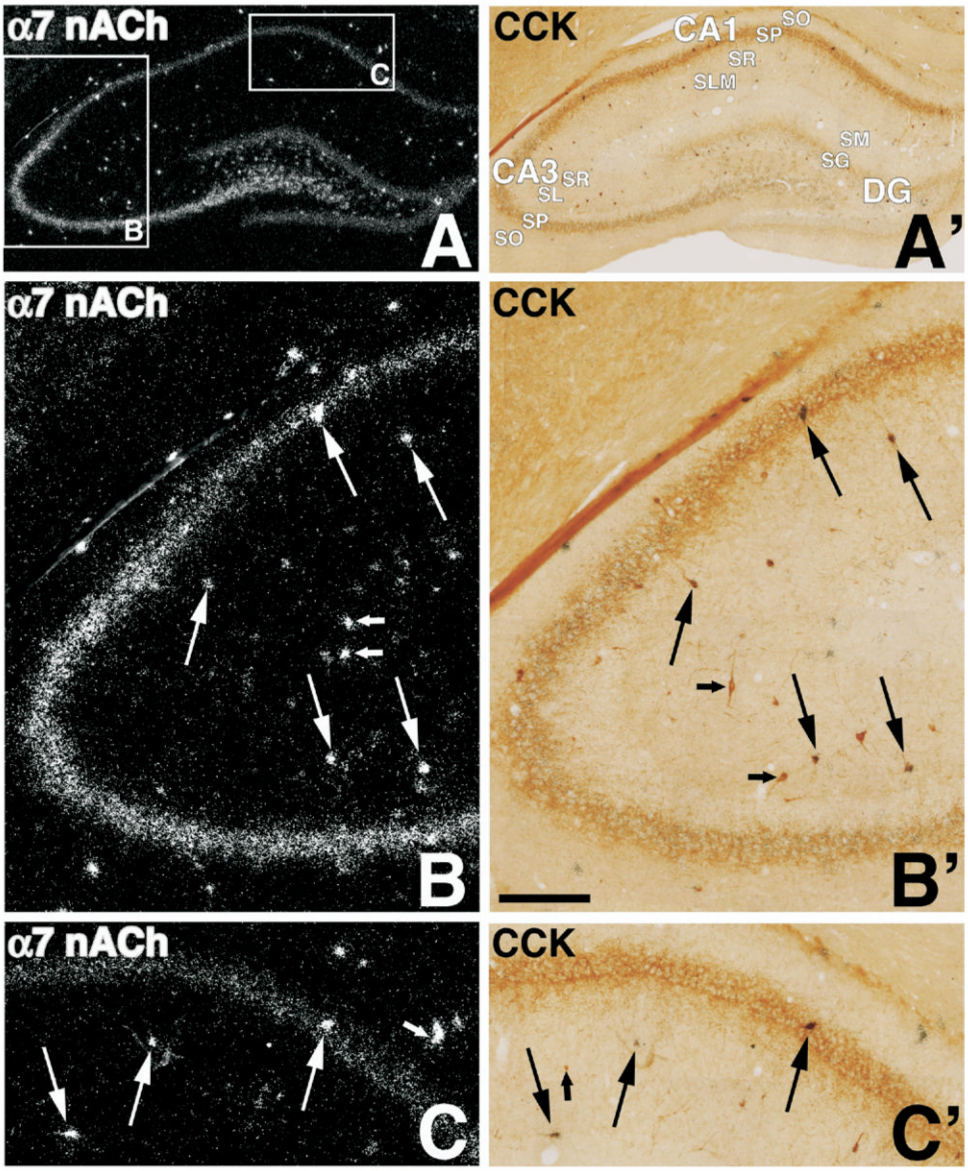

Fig. 5.

Many hippocampal interneurons expressing α7 nACh mRNA contain CCK immunoreactivity. (A, A') Low magnification of epi-fluorescence (A) and bright field (A') views of cellular expression of α7 nACh mRNA (A) and CCK immunoreactivity (A'). Frames B, B', C and C' correspond to higher magnification views of areas delineated in panel A. Large arrows indicate examples of neurons co-expressing α7 nACh mRNA and CCK immunoreactivity. Small arrows in B, B', C and C' indicate examples of neurons expressing either α7 nACh mRNA or CCK. Scale bar shown in B' represents 180 μm for A and A'; 590 μm for B, B', C and C'.

Table 1

Relative percentage of interneurons co-expressing α7 nACh and GAD mRNAs in the hippocampus and the DG in the population of α7 nACh mRNA expressing interneurons

Region	Percentage of interneurons co-expressing α7 nACh and GAD mRNAs in the population of α7 nACh mRNA expressing interneurons,[a] mean±S.E.M.
CA1	
s. oriens	94±0.8 (*n*=85)
s. radiatum	99±1.2 (*n*=38)
s. lacunosum moleculare	97±1.1 (*n*=42)
CA3	
s. oriens	87±0.7 (*n*=72)
s. radiatum	88±1.6 (*n*=61)
s. lucidum	89±0.4 (*n*=25)
DG	
Polymorph layer	98±2.4 (*n*=201)

[a] Interneurons expressing α7 nACh mRNA or GAD mRNA were counted in 13 sections from three different rats. For each layer, the percentage of interneurons co-expressing α7 nAch mRNA and GAD mRNA was calculated from the population of α7 nACh mRNA expressing neurons.

Table 2

Relative percentage of interneurons co-expressing *α7* nACh and CB1 mRNAs in the hippocampus and the DG

Region	Relative percentage of interneurons co-expressing *α7* nACh and CB1 mRNAs in the population of *α7* nACh mRNA expressing interneurons,[a] mean±S.E.M.	Relative percentage of interneurons co-expressing α7 nACh and CB1 mRNAs in the population of CB1 mRNA expressing interneurons,[b] mean±S.E.M.
CA1		
s. oriens	37±1.3 (*n*=64)	75±0.3 (*n*=32)
s. pyramidale	89±0.5 (*n*=75)	85±0.6 (*n*=78)
s. radiatum	96±1.4 (*n*=108)	81±1.4 (*n*=128)
s. lacunosum moleculare	88±0.6 (*n*=52)	83±0.7 (*n*=55)
CA3		
s. oriens	50±0.8 (*n*=61)	51±0.8 (*n*=60)
s. pyramidale	75±0.5 (*n*=70)	84±0.5 (*n*=63)
s. radiatum	96±0.9 (*n*=93)	78±1.1 (*n*=115)
s. lucidum	80±0.3 (*n*=20)	100±0.3 (*n*=16)
DG		
Polymorph layer	91±1.6 (*n*=261)	87±1.9 (*n*=273)

Interneurons expressing *α7* nACh mRNA or CB1 mRNA were counted in 17 sections from three different rats.

[a] For each layer, the percentage of interneurons co-expressing *α7* nACh mRNA and CB1 mRNA was calculated from the population of *α7* nACh mRNA expressing interneurons.

[b] For each layer, the percentage of interneurons co-expressing *α7* nACh mRNA and CB1 mRNA was calculated from the population of CB1 mRNA expressing neurons.

Table 3

Relative percentage of interneurons co-expressing α7 nACh mRNA and CCK immunoreactivity in the hippocampus and the DG

Region	Percentage of interneurons co-expressing *α7* nACh mRNA and CCK immunoreactivity in the population of *α7* nACh mRNA expressing interneurons,[a] mean±S.E.M.	Percentage of interneurons co-expressing *α7* nACh mRNA and CCK immunoreactivity in the population of CCK immunoreactive interneurons,[b] mean±S.E.M.
CA1		
s. oriens	21±0.9 (*n*=128)	70±0.2 (*n*=40)
s. pyramidale	76±0.4 (*n*=55)	51±0.5 (*n*=82)
s. radiatum	76±0.6 (*n*=121)	55±0.6 (*n*=167)
s. lacunosum moleculare	48±0.3 (*n*=52)	56±0.4 (*n*=44)
CA3		
s. oriens	21±0.9 (*n*=120)	72±0.3 (*n*=36)
s. pyramidale	48±0.5 (*n*=87)	58±0.4 (*n*=72)
s. radiatum	79±0.7 (*n*=144)	65±0.7 (*n*=175)
s. lucidum	41±0.3 (*n*=43)	50±0.3 (*n*=36)
DG		
Polymorph layer	78±1.1 (*n*=273)	59±1.8 (*n*=359)

Interneurons expressing *α7* nACh mRNA or CCK immunoreactivity were counted in 21 sections from three different rats.

[a] For each layer, the percentage of interneurons co-expressing *α7* nACh mRNA and CCK immunoreactivity was calculated from the population of *α7* nACh mRNA expressing interneurons.

[b] For each layer, the percentage of interneurons co-expressing *α7* nACh mRNA and CCK immunoreactivity was calculated from the population of CCK interneurons.