The α2-Subunit of the Nicotinic Cholinergic Receptor Is Specifically Expressed in Medial Subpallium-Derived Cells of Mammalian Amygdala

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ABSTRACT

Nicotinic acetylcholine receptor (nAChR) subtypes are expressed in specific neuronal populations, which are involved in numerous neural functions such as sleep, fatigue, anxiety, and cognition, as well as the central processing of pain and food intake. Moreover, mutations in nAChRs subunits have been related to frontal lobe epilepsy, neurodegenerative diseases, and other neurological disorders, including schizophrenia and attention deficit and hyperactivity disorder (ADHD). Previous studies have shown that the α2-subunit of the AChR (Chrna2) is expressed in the basal forebrain, in the septum, and in some amygdalar nuclei in the adult rodent brain. However, although the importance of this amygdalar expression in emotion-related behavior and the physiopathology of neuropsychiatric disorders has been accepted, a detailed study of the Chrna2 expression pattern during development has been lacking. In this study we found that Chrna2 is specifically expressed in medial subpallium-derived amygdalar nuclei from early developmental stages to adult. This finding could help us to better understand the role of Chrna2 in the differentiation and functional maturation of amygdalar neurons involved in cholinergic-regulated emotional behavior. J. Comp. Neurol. 523:1608–1621, 2015.

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Acetylcholine (ACh) is a neurotransmitter released by cholinergic neurons. Neural cells respond to ACh signal through acetylcholine receptors, both muscarinic and nicotinic. Nicotinic ACh receptor (nAChR) subtypes are widely expressed in the peripheral and central nervous system, where they regulate multiple processes related to cell excitability and activity integration in neuronal circuits. These neural processes are crucial for cognition, sleep, anxiety, food intake, and the central processing of pain (McGehee and Role, 1995; Role and Berg, 1996; Gotti et al., 1997; Lindstrom, 1997; Changeux and Edelstein, 2001; Hogg and Bertrand, 2003). nAChRs are preferentially located at the preterminal and presynaptic sites of neuropils regulating the release of ACh, noradrenaline, dopamine, and γ-aminobutyric acid (GABA) neurotransmitters (Gotti et al., 2006). Moreover, alterations in nAChRs seem to constitute risk factors for neurodegenerative diseases as Alzheimer's and Parkinson and neurological disorders including epilepsy, schizophrenia, and attention deficit and hyperactivity disorder (ADHD) (Freedman et al., 1997; Steinlein et al., 1999; Jones et al., 1999; Paterson and Nordberg, 2000; Dani, 2001; Grothe et al., 2009; Posadas et al., 2013). Finally, in the amygdala, nAChR signaling plays a significant role in working-,

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short-, and long-term memory and attention mechanisms (Dumery and Blozovski, 1987; Blozovski and Dumery, 1987; Gallagher and Holland, 1992; Voytko, 1996; Barros et al., 2005).

nAChR subtypes consist of five subunits assembled in the plasma membrane to form a channel permeable to cations. Receptor variety is mainly due to the diversity of the genes encoding these subunits (Fucile, 2004), which are classified into two families, α2–α10 and β2–β4 subunits (Le Novere and Changeux, 1995; Lindstrom, 2000). Homomeric nAChRs made up only of α-subunits (α7–α10) have been identified that are characterized by sensitivity to α-bungatoxin, as well as heteromeric AChRs not sensitive to α-bungatoxin, which consist of α (α2–α6) and β (β2–β4) subunits (Gotti and Clementi, 2004).

Previous studies have described the expression of major nAChR subtypes in mouse nervous system, along with their subunit composition, pharmacology, and localization (Gotti et al., 2007; Posadas et al., 2013). Studies based on the use of oligoprobes and riboprobes have shown that the α2-subunit (Chrna2) is expressed in neurons of the olfactory bulb, amygdala, medial septum, basal forebrain, and hippocampus of the adult prosencephalon. Moreover, the α2 signal was also detected in the midbrain alar plate, regions of the hindbrain, such as dorsal and ventral tegmental nuclei of the pons, median raphe nuclei, and bulbar reticular formation, as well as in the ventral horn of the spinal cord (Marks et al., 1992; Zoli et al., 1998; Lena et al., 1999; Azam et al., 2002; Ishii et al., 2005; revised by Gotti et al., 2007). This α2-subunit is involved in nicotine-regulated emotional memories, nicotine addiction, and other neuropsychiatric diseases related to alterations of normal amygdala function (revised by Gotti and Clementi, 2004; Lotfipour et al., 2013). However, although the importance is well known of spatiotemporal patterns of gene expression during development for the establishment of normal function and in the physiopathology of neuropsychiatric disorders, no detailed study has reported on the distribution of the α2-subunit in the amygdalar complex of the developing brain.

The amygdala is a complex structure of heterogeneous neuronal populations forming a key center of the limbic system in the telencephalon, and involved in emotional regulation, social behavior, and hypothalamic-related vegetative and endocrine functions (LeDoux, 1992, 2000; Aggleton, 2000; Davis, 2000; Gallagher, 2000; Zald, 2003). Data based on expression pattern of genes and fate-map studies have demonstrated that the amygdala consists of pallial and subpallial components (Puelles et al., 2000; Medina et al., 2004; Pombero and Martinez, 2009; Bupesh et al., 2011a,b). The lateral and ventral pallium contains the claustral nuclear complex and the pallial amygdalar nuclei. The ventral pallium forms the lateral and basomedial nuclei, and the anterior posteromedial, cortical, and amygdalohippocampal areas of the amygdala, whereas the lateral pallium forms the amygdalar basolateral nucleus and the posterolateral cortical amygdalar area (Medina et al., 2004; Bupesh et al., 2011a); in addition to the claustrum proper, the lateral pallium also generates the posterior endopiriform nucleus and the dorsal part of the piriform cortex. Regarding the subpallial amygdala, the interstitial nucleus of the posterior limb of the anterior commissure (IPAC), and the central amygdala derive from the striatal subdivision of the subpallium (the lateral ganglionic eminence [LGE]); the lateral part of the bed nucleus of the stria terminalis (BSTL) originates from the pallidal area of the subpallium (the medial ganglionic eminence [MGE]). The anterior, medial intra-amygdaloid, and posteromedial areas of the bed nucleus of the stria terminalis (BSTa, BSTia, and BSTpm), ventromedial and sublenticular extended amygdala (EA and SLEA, respectively), and the anterior and posterodorsal medial amygdala (MeA and MePD) derive from the ventrocaudal part of the MGE (termed the diagonal area [Dg]). Finally, the central part of the posteroventral part of the medial amygdala (MePVc), together with the basal magnocellular complex (BMC), is derived from the preoptic area (POA) (Puelles et al., 2000; Garcia-Lopez et al., 2008).

In relation to the cholinergic system, some cholinergic neurons in the sublenticular extended amygdala project to the cortex (Gritti, 2006). Expression of the α2 mRNA subunit of nAChR in adult rodent brain has been studied by using radioactive and nonradiactive in situ hybridization (Picciotto et al., 2001; Ishii et al., 2005).

Increasing evidence has shown a direct relation between neurodevelopmental alterations and neurodegenerative and psychiatric disorders; thus studies of gene expression during development are necessary to better understand the physiopathological mechanisms underlying these diseases. The present study attempted to increase our knowledge of α2-subunit of the nicotinic cholinergic receptor (Chrna2) expression in the developing amygdalar complex, as a preliminary analysis to establish its involvement in the regulation of nicotine-modulated behaviors, as well as in its possible implication in mechanisms that predispose to the development of neurodegenerative and neuropsychiatric diseases.

MATERIALS AND METHODS

Mouse embryos and in situ hybridization of tissue sections

Wild-type mice (ICR) obtained from the University Animal House, were used for Chrna2 expression analysis. The day of vaginal plug was considered embryonic

day 0.5 (E0.5). The study was carried out at embryonic stages E14.5, E16.5, and E17.5 and at neonatal stage (P0). All the animal experiments were performed in compliance with the Spanish and European Union laws on animal care in experimentation (Council Directive 86/609/EEC), and have been analyzed and approved by the Animal Experimentation Committee of the University Miguel Hernandez and Neuroscience Institute, Alicante, Spain (Reference IN-SM-001-11). All efforts were made to minimize suffering.

Mice were bred and maintained in our animal facilities. Embryos were removed by Caesarean section and decapitated. The head of the mouse embryos was fixed overnight by immersion in 4% paraformaldehyde in phosphate-buffered saline solution (PBS; 0.1 M, pH 7.4). Postnatal mice were transcardially perfused with the same fixative solution, and brains were postfixed in fresh fixative overnight at 4°C, and then embedded in 4% agarose; 70-μm-thick sagittal, transversal, or horizontal sections were cut on a vibratome (Leica).

Floating sections were processed by in situ hybridization using mRNA probes to detect *Chrna 2, Shh* (Shimamura and Rubenstein, 1997), and *Nkx 2.1* (Sussel et al., 1999) transcripts, as indicated by Shimamura et al. (1994). The Nkx 2.1 mRNA probe was kindly provided by J.L. Rubenstein, and the Shh riboprobe by A. McMahon. For in situ hybridization, antisense digoxigenin-labeled riboprobes for these genes were synthesized. First, floating sections were washed once with PBS, pH 7.4, containing 0.1% Tween-20 (Sigma Aldrich, Steinhem, Germany). Then they were incubated in hybridization buffer containing deionized formamide 50%, standard saline citrate pH 7, heparin 50 μg/ml (Sigma-Aldrich), yeast tRNA 50 μg/ml (Sigma-Aldrich), and water free of RNAase and DNAase (Sigma-Aldrich), for 1 hour at 65°C. Thereafter, sections were hybridized overnight at 65°C in hybridization solution containing 2 μl/ml of riboprobe. After hybridization, the sections were washed and incubated overnight at 4°C with alkaline phosphatase-coupled anti-digoxigenin antiserum (1:3,500; Roche Diagnostics, Mannheim, Germany), and then NBT/BCIP or INT/BCIP were used as a chromogenic substrates for the alkaline phosphatase to obtain blue or red labeling, respectively (Boehringer, Mannheim, Germany). Finally, sections were mounted on slides, dehydrated, and covered with Eukitt. Sections were then photographed under a microscope (Leica).

Chrna 2 mRNA probe

For the synthesis of digixigenin-11-UTP–labeled RNA probe, the *Xba*I/*Xba*I fragment (569 bp), from the Chrna 2 cDNA full-length clone (clone name: IRAVp968F1227D; imaGenes, Source BioScience LifeSciences, Berlin, Germany), was subcloned into the pSTBlue-1 Blunt Vector (Novagen, Darmstadt, Germany) between the SP6 and T7 promoter sites. Based on data from the GeneBank database, the sequence of this fragment contains no significant homology to other DNA sequences.

Immunohistochemistry

In other cases vibratome sections were processed by immunohistochemistry by using the following antibodies: goat anti-choline acetyl transferase polyclonal antibody (ChAT; Merck, Darmstadt, Germany, Cat# AB144P, RRID:AB_11214092) and goat anti-somatostatin (SOM) polyclonal antibody (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, Cat# sc-7819, RRID:AB_2302603).

Immunostaining was performed by using the standard biotin-avidin/horseradish peroxidase (HRP) procedure. All floating sections were incubated with 0.9% oxygen peroxide in PBS with 0.1% Triton X-100 (PBS-T) for 30 minutes and then blocked in 1% bovine serum albumin (Sigma) in PBS-T for 1 hour. Primary antibodies were incubated overnight at 4°C. The following day sections were incubated with biotinylated rabbit anti-goat IgG antibody for 1 hour at room temperature, followed by Vectastain ABC reagent for 1 hour (both from Vector, Burlingame, CA), and immunolabeling was revealed with diaminobenzidine tetrahydrochloride. Sections were then mounted on slides, dehydrated, and covered with

Abbreviations

AAv	anterior amygdala, ventral part
ac	anterior commissure
ACx	cortical amygdala
Bas	nucleus basalis
BC	basal amygdalar complex
BL	basolateral amygdalar nucleus
BM	basomedial amygdalar nucleus
BMC	basal magnocellular complex
BST	bed nucleus of stria terminalis
BSTL	bed nucleus of stria terminalis, lateral part
BSTM	bed nucleus of stria terminals, medial part
Ce	central amygdala
Cevm	ventromedial part of the central amygdala
CPu	caudate-putamen
Dg	diagonal area
DP	dorsal pallium
EA	ventromedial extended amygdala
GP	globus pallidus
ic	internal capsule
IPAC	interstitial nucleus of posterior limb of anterior commissure
L	lateral amygdalar complex
MeA	medial amygdala, anterior part
MeP	medial amygdala, posterior part
MePD	medial amygdala, posterodorsal part
MePV	medial amygdala, posteroventral part
MGE	medial ganglionic eminence
MP	medial pallium
OB	olfactory bulb
pac	posterior limb of anterior commissure
Pir	piriform cortex
POA	anterior preoptic area
Se	septum
SLEA	extended amygdala, sublenticular part
VP	ventral pallidum
VPac	ventral pallidum, commissural part

TABLE 1.
Primary Antibodies Used

Antigen	Immunogen	Details	Concentration (immunohistochemistry)
Choline acetyl transferase (ChAT)	Human placental ChAT	Chemicon, goat polyclonal, Cat# ab144P, RRID: AB_11214092	0.01 μg/μl
Somatostatin (SOM)	Neuropeptide mapping near the C-terminus of somatostatin of human origin	San Cruz Biotechnology, goat polyclonal, Cat# sc-7819, RRID: AB_2302603	4 ng/μl

Eukitt. After immunostaining, brain sections were photographed under a microscope (Leica).

Finally, digital images were adjusted for brightness and contrast by using Adobe Photoshop CS2 (San Jose, CA; RRID: SciRes_000161), and the figures were created by using Canvas X (ACD Systems International, Victoria, BC, Canada).

Antibody characterization

See Table 1 for a list of the antibodies used.

The anti-ChAT antibody (Merck, Cat# AB144P, RRID: AB_11214092) is a polyclonal antibody generated in goat against the human placental enzyme and is specific for ChAT found in the central nervous system. The antibody specificity has been checked by Western Blot (Brunelli et al., 2005), and studies based on mRNA expression analysis have shown that the anti-ChAT antibody labeled the same cells as the mRNA (Oh et al., 1992). The anti-SOM antibody (Santa Cruz Biotechnology; goat polyclonal, Cat# sc-7819, RRID: AB_2302603) is a polyclonal antibody, also generated in goat, which was raised against a neuropeptide mapping near the C-terminus of SOM of human origin. This antibody showed an appropriate pattern of labeled cells in the telencephalon according to previous reports (Garcia-Lopez et al., 2008) and according to SOM mRNA expression analysis (Fitzpatrick-McElligott et al., 1988, 1991).

RESULTS

The present study explores the expression pattern of the nicotinic cholinergic receptor α-subunit (Chrna2) jointly with the localization of other amydalar genetic markers, Nkx2.1, Shh, SOM, and ChAT. This combined analysis enabled us to accurately identify the different domains of the subpallial amygdala: the striatal, pallidal, diagonal, and preoptic amygdalar areas. Subdivisions of the amygdala were identified according to the areas described by Garcia-Lopez et al. (2008).

Early Chrna2 expression pattern

During early mouse development (E0.5–E13.5), Chrna2 expression was not detected by in situ hybridization in any brain region. Conversely, α2 mRNA-expressing cells were detected for the first time at stage E14.5. Most of these cells were located in the medial amydalar areas (the BST, EA, and SLEA), whereas some weakly labeled and scattered cells were detected in the basal magnocellular amygdalar complex (BMC; Fig. 1A–I).

Pallidal and striatal amygdala

The subpallial subdivisions MGE and LGE have been shown to give rise to different parts of the centromedial amygdala. These regions are characterized by the combined expression of several genes. Thus, the MGE and its derivatives in the amygdala express Dlx-1, -2, and -5 and Nkx-2.1 (Marin et al., 2000; Puelles et al., 2000; Sussel et al., 1999), and LGE territories are characterized by the expression of Dlx-1, -2, and -5 (Puelles et al., 2000; Medina et al., 2004). In addition, molecular regionalization and structural analysis showed that the lateral part of the BSTL is mainly derived from the MGE, whereas the LGE gives rise to the intercalated amygdalar masses (IPAC) and most of the central amygdala (Ce; Holmgren, 1925; Bayer, 1987; Puelles et al., 2000; Swanson, 2000; Medina et al., 2004; Tole et al., 2005; Garcia-Lopez et al., 2008). The LGE and MGE also contribute to the BSTL and Ce, respectively (Bupesh et al., 2011b). Chrna2-expressing cells were not detected in the globus pallidus cells (GP) at E14.5 (Fig. 1A–L). The GP and ventral pallidum (VP) were identified by Nkx 2.1 expression (Fig. 1J–L), showing that only a few scattered cells were labeled with the Chrna2-probe in the VP (Fig. 1B,D,J). In the case of the LGE-derived regions, the areas corresponding to the striatal amygdala (as is the case for Ce), which has been identified by the expression of the neuropeptide SOM, did not exhibit cells positive for α2 mRNA (Fig. 1M–O). Nevertheless, some cells were detected in the ventromedial part of the central amygdala (Cevm). Therefore, the Chrna2 gene was detected in very few striatal-derived cells of the amygdala (Fig. 1J–O). SOM+ cells were also observed in some pallial-derived areas, such as the piriform cortex (Pir; Fig. 1M–O), the basal amygdalar complex (BM and BL; Fig. 1O), and the

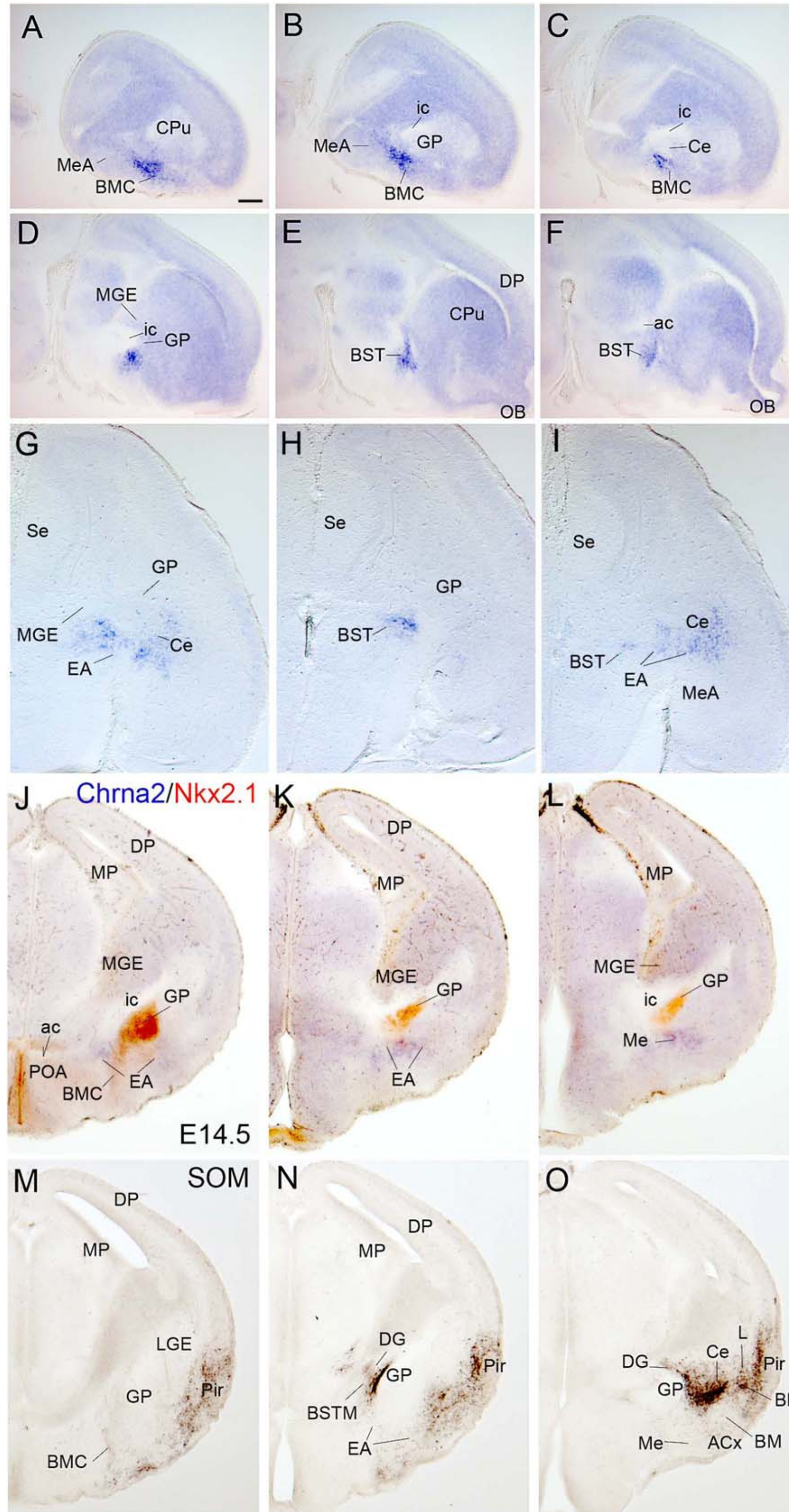

Figure 1. Analysis of Chrna2 expression at stage E14.5. **A–O:** Sagittal (A–F) and horizontal (G–I) sections of embryonic mouse telencephalon were hybridized for Chrna2. Chrna2 expression was found in the BMC, BST, and EA. Combined analysis in coronal sections of Chrna2 and Nkx2.1 showed that Chrna2 does not label pallidal-derived structures (J–L); SOM immunostaining was used to localize striatal amygdalar derivatives as Ce (M–O) that also appeared to be negative for Chrna2 expression. For abbreviations, see list. Scale bar = 200 μm.

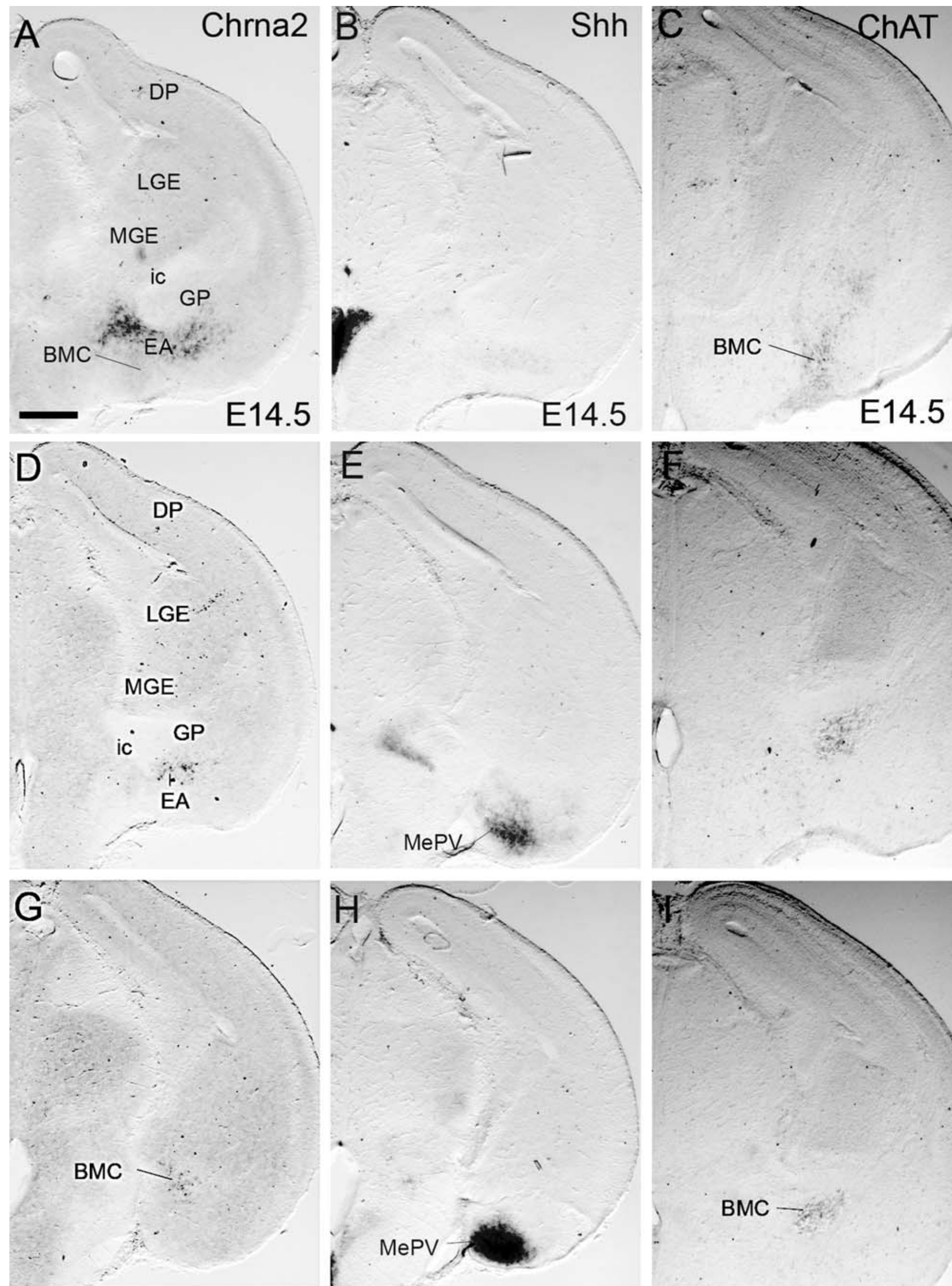

Figure 2. Combined analysis of Chrna2 with Shh and ChAT at stage E14.5. **A–I:** Parallel transversal sections of mouse brain telencephalon were processed by Chrna2 in situ hybridization (A,D,G) and Shh (B,E,H) and anti-ChAT by immunohistochemistry (C,F,I). Scattered Chrna2-expressing cells were found in the BMC (A), also labeled by anti-ChAT (B), whereas the amygdalar areas that express Shh such as the MePV (E) did not show Chrna2-expressing cells (D–I). For abbreviations, see list. Scale bar = 200 μm in A (applies to A–I).

cortical amygdala (ACx; Fig. 1O). All these areas were also negative for Chrna2.

Diagonal-preoptic derived amygdala

Dg and POA nuclear derivatives show strong expression of Nkx-2.1, Dlx-1, -2, and -5, Shh, Lhx-6, and Lhx-7/8 during early development (Shimamura and Rubenstein, 1997; Garcia-Lopez et al., 2008); however, only the POA exhibits Shh expression in its ventricular region. The Dg gives rise to the posterodorsal part of the medial amygdala (MePD), the extended amygdala, which is formed by the ventromedial (EA) and sublenticular amygdalar region (SLEA), and the ventral part of the anterior amygdala (AAv), as well as the medial subdomains of the BST (Remedios et al., 2004; Garcia-Lopez et al., 2008; Bupesh et al., 2011a). Moreover,

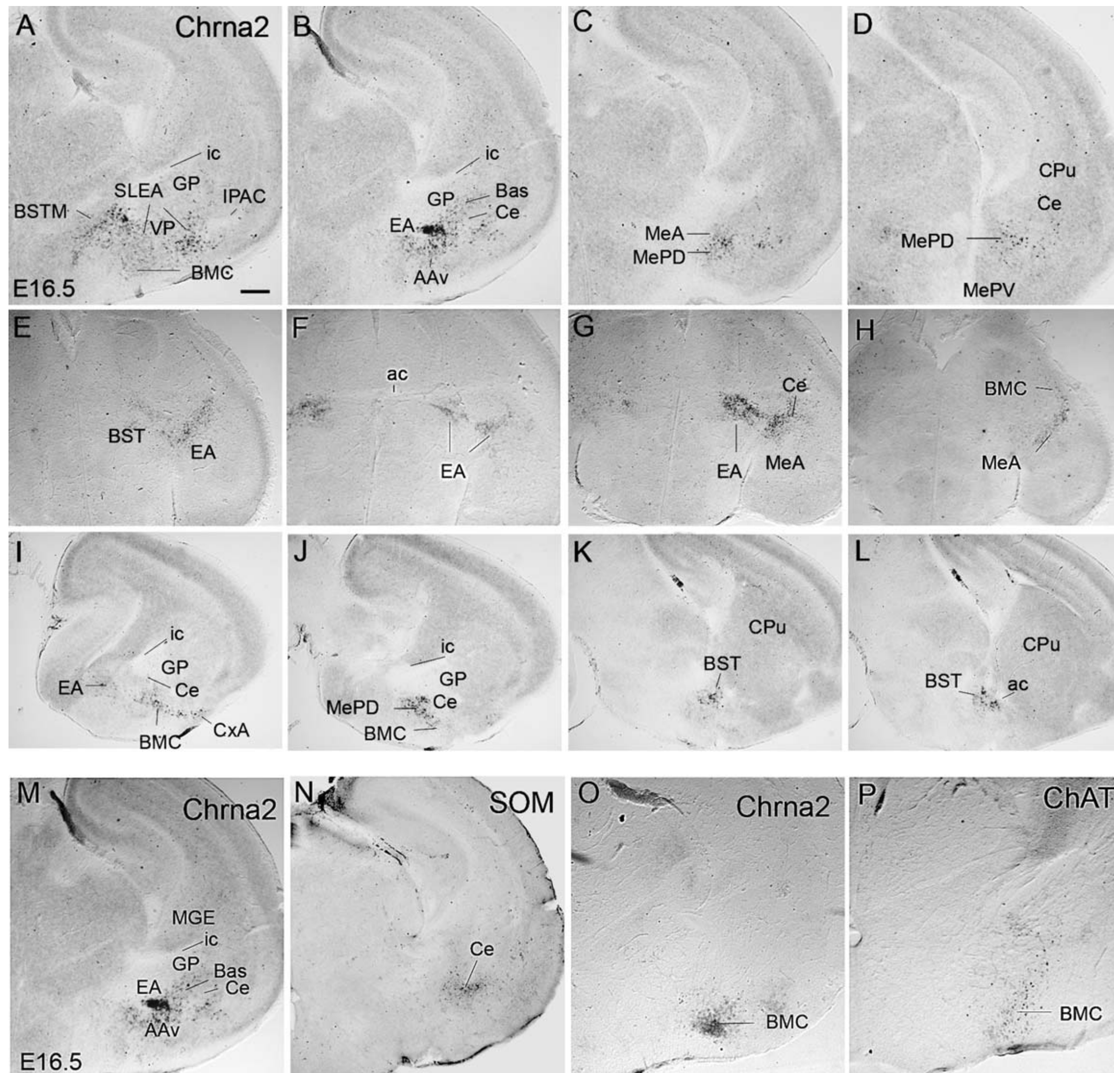

Figure 3. Chrna2 expression studied at stage E16.5. **A–P:** Transversal sections, arranged from rostral to caudal (A–D), horizontal sections from dorsal to ventral (E–H), and sagittal sections (rostral to right) from lateral to medial (I–L), were processed by RNA in situ hybridization to detect Chrna2 expression. These sections showed Chrna2 expression throughout the entire Dg-derived amygdalar nuclei: AAv, SLEA, BSTM, MeA, and MePD. Very few cells were observed in the Ce, identified by SOM immunoreactivity (M–N). The POA-derived region BMC, identified by anti-ChAT immunoreactivity, showed abundant Chrna2-expressing cells (O–P). For abbreviations, see list. Scale bar = 200 μm in A (applies to A–P).

the POA may generate a subset of cells of the postero-ventral and anterior parts of the medial amygdala (MePV and MeA) and of the BSTL (Garcia-Lopez et al., 2008).

Between the GP and the BMC, $\alpha 2$ mRNA was intensely expressed in cells distributed in the area that corresponds to the EA (Figs. 1J,K, 2A). This region is located between the MGE and the POA derivatives and expresses Nkx 2.1 (Fig. 1J,K). Moreover, the ventroposterior part of the medial amygdala (MeVP) was characterized by the expression of Shh (Fig. 2H) and was negative for ChAT expression (Fig. 2F). This ventral area that originates in the POA (Garcia-Lopez et al., 2008; Bupesh et al., 2011a), did not exhibit positive cells for Chrna2 (Fig. 2D,G). Nevertheless, we observed that the BMC, which expresses ChAT and originates from the POA, contained some scattered cells labeled with the Chrna2 riboprobe (Fig. 2A,C).

Chrna2 expression in late stages of amygdala development

During the following embryonic stages (E16.5–17.5), the telencephalon becomes more mature and thus nuclear organization appears more evident. We observed that the expression of Chrna2 was similar to earlier stages.

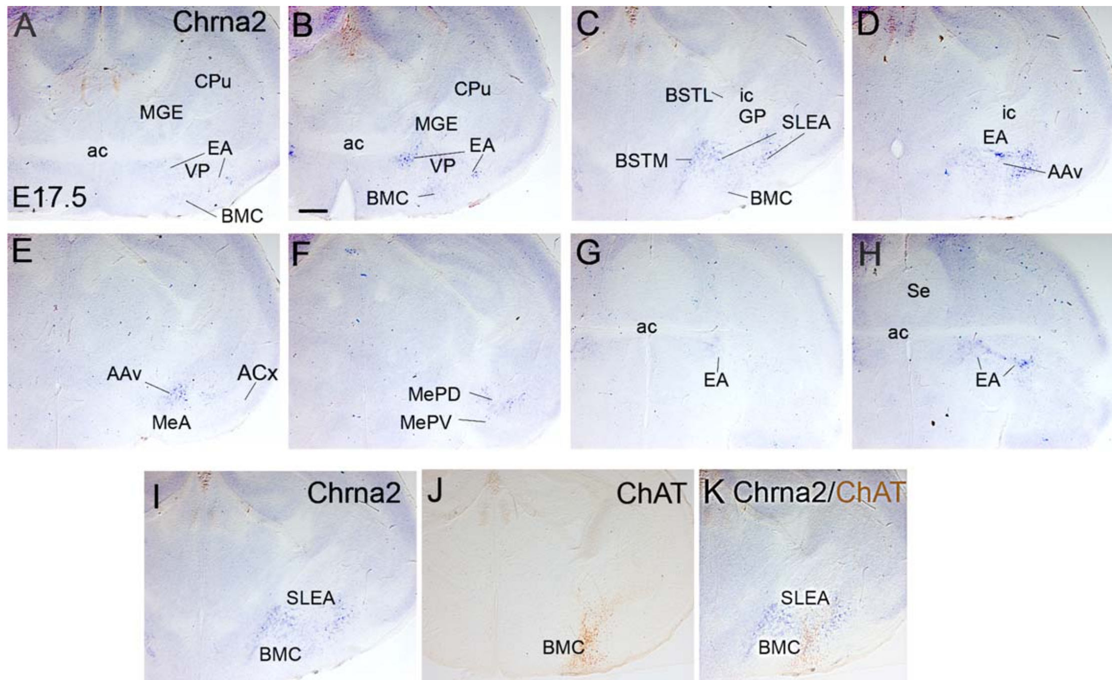

Figure 4. Chrna2 expression in the amygdala at stage E17.5. **A–H:** Transversal (A–F) and horizontal (G–H) brain sections, arranged from rostral to caudal and from dorsal to ventral, respectively, were processed by in situ hybridization, to detect Chrna2 expression. At stage E17.5 the Chrna2 expression pattern was similar to that of earlier stages. It was detected in the SLEA, BSTM, AAv, and MeA nuclei, derived from the Dg region. Moreover, the expression in BMC was also maintained (A–C). **I–K:** Merged picture composed of two consecutive section of the same embryo processed to detect Chrna2 (blue) and ChAT (brown) showing Chrna2/ChAT colocalization in the BMC (K). For abbreviations, see list. Scale bar = 200 μm in B (applies to A–K).

Striatal and pallidal amygdala

At stages E16.5 and E17.5, very few cells positive for α2 mRNA were observed in the pallidal region. The GP was negative for Chrna2, whereas some cells were detected in the VP (Figs. 3A,B, 4A,B). In contrast, the BSTL, which also originates in the pallidal region of the subpallium, did not exhibit cells expressing the α2-subunit. Moreover, the LGE remained negative for Chrna2. Thus, we did not detect α2 mRNA-expressing cells in the caudate-putamen (CPu; Figs. 3A–L, 4A–H) or in the IPAC (Figs. 3A–L, 4A–H). Some positive cells were detected in the Ce (Figs. 3G,M,N, 4A–H). The Ce was identified by the expression of SOM (Fig. 3M,N).

Dg and POA amygdalar derivatives

All areas of the amygdala originated in the Dg showed cells expressing Chrna2 (Figs. 3A–L, A–H). In addition, most of cells expressing Chrna2 were detected in Dg derivatives. We observed that most cells in the extended amygdala (EA and SLEA) were labeled with Chrna2 mRNA (Figs. 3A,B,E–G,I, 4A–D,G,H). Another amygdalar region that seems to be derived from the Dg is the ventral part of the anterior amygdala (AAv; Garcia-Lopez et al., 2008; Bupesh et al., 2011a). This region extends ventrally to the EA and surrounds the nucleus of the lateral olfactory tract (LOT). We observed cells expressing Chrna2 in the AAv at stages E16.5 and E17.5 (Figs. 3B,M, 4D,E). Moreover, the MeA also showed some scattered cells expressing Chrna2 (Figs. 3C,D,G,H, 4E,F). In the case of the medial amygdala nuclei, different histogenetic origins have been described: the VP, POA, and Dg (Garcia-Lopez et al., 2008; Bupesh et al., 2011a). We found that only the region derived from the Dg, the anterior and the posterodorsal parts (MeA and MePD), exhibited cells expressing Chrna2 (Figs. 3C,D,G,H, J, 4E,F).

The BST is a complex area that has been subdivided into lateral, medial, anterior, and posterior components (Alheid et al., 1998; Dong and Swanson, 2001; Paxinos and Franklin, 2004). In our analysis, we observed that the Chrna2 gene is only expressed in the medial region of the BST (BSTM; Figs. 3A,K,L, 4C), which arises from the Dg germinal zone (Garcia-Lopez et al., 2008; Bupesh et al., 2011a).

In contrast, POA derivatives such as the BMC and BAS (Figs. 3A,B,I,J, 4A–C) showed scattered distribution of Chrna2-expressing cells. The BMC was distinguished

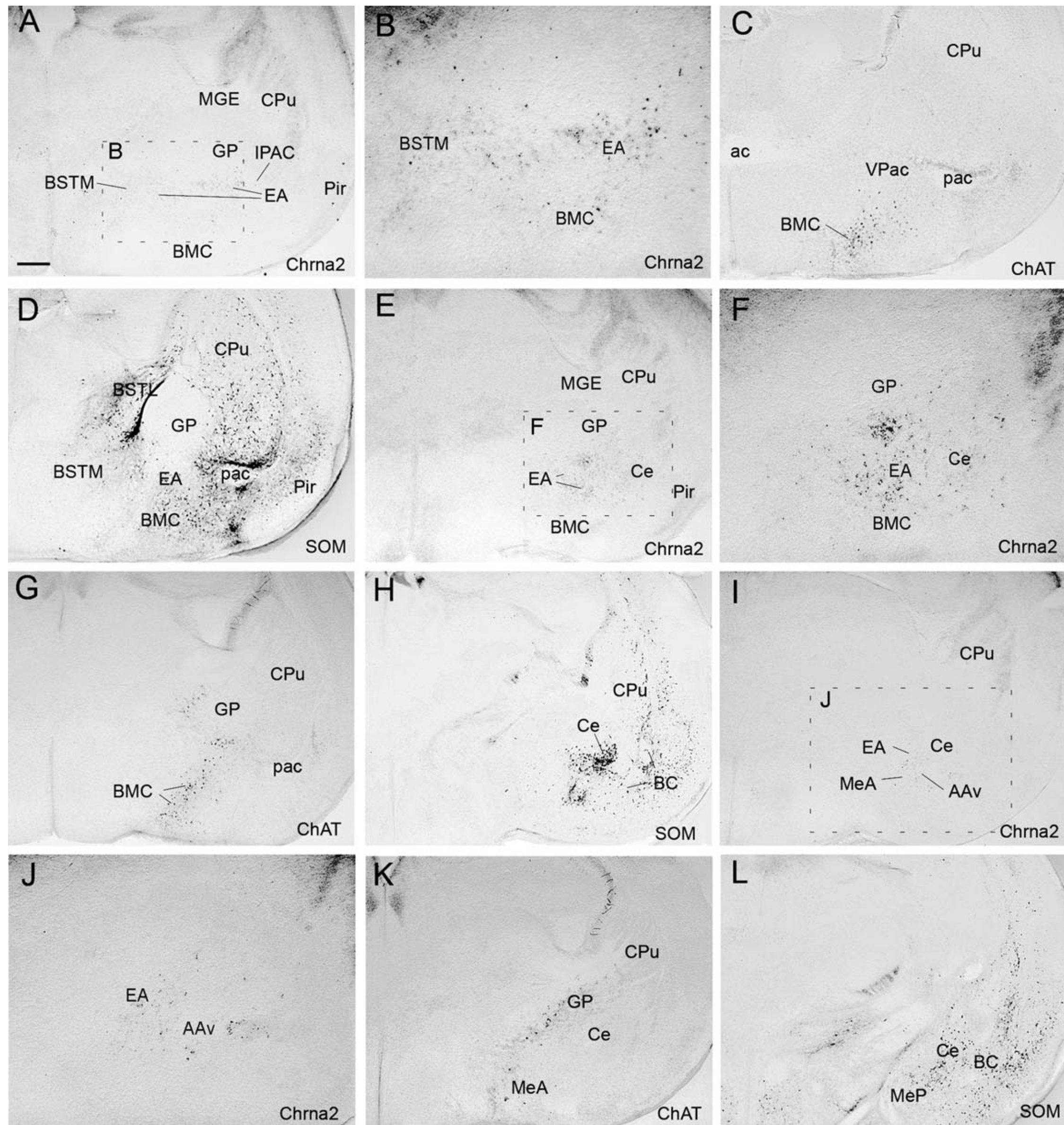

Figure 5. Chrna2 expression at neonatal stage. **A–L:** Parallel transversal sections, arranged from rostral to caudal, were processed to localize Chrna2 by in situ hybridization, and ChAT and SOM expression by immunohistochemostry. The boxed areas in A, E, and I are enlarged in B, F, and J, respectively, to better illustrate the cells expressing Chrna2. Parallel analysis with ChAT (C,G,K) and SOM (D,H,L) was used to identify the different areas of the subpallial amygdala. In the amygdala derived from the Dg, the α2-subunit of the ChR was detected in the SLEA, BSTM, MeA, and AAv. In the Ce, very few α2-subunit–expressing cells were found, whereas the BMC exhibited many cells with Chrna2 labeling. No Chrna2-positive cells were observed in pallidal derivatives such as the GP. For abbreviations, see list. Scale bar = 200 μm (applies to A,C–E,G–I,K,L); 85 μm in B,F,J.

from other regions by the expression of ChAT (Figs. 3O,P, 4I–K).

Chrna2 expression in the amygdala of neonatal mice

The distribution of α2 mRNA-expressing cells in the central nervous system of adult rodents has been previously reported (Wada et al., 1989; Ishii et al., 2005). These authors studied the distribution of Chrna2 by using in situ hybridization in rat and mouse brains and observed mRNA similar patterns in both species (Ishii et al., 2005). Actually, Ishii et al. (2005) mapped Chrna2 expression in medial, central, and basomedial nuclei of the amygdala. Thus, in our results we have observed that the Chrna2 expression pattern in the amygdala during embryonic development appeared to be more restrictive than in the adult. Thus we decided

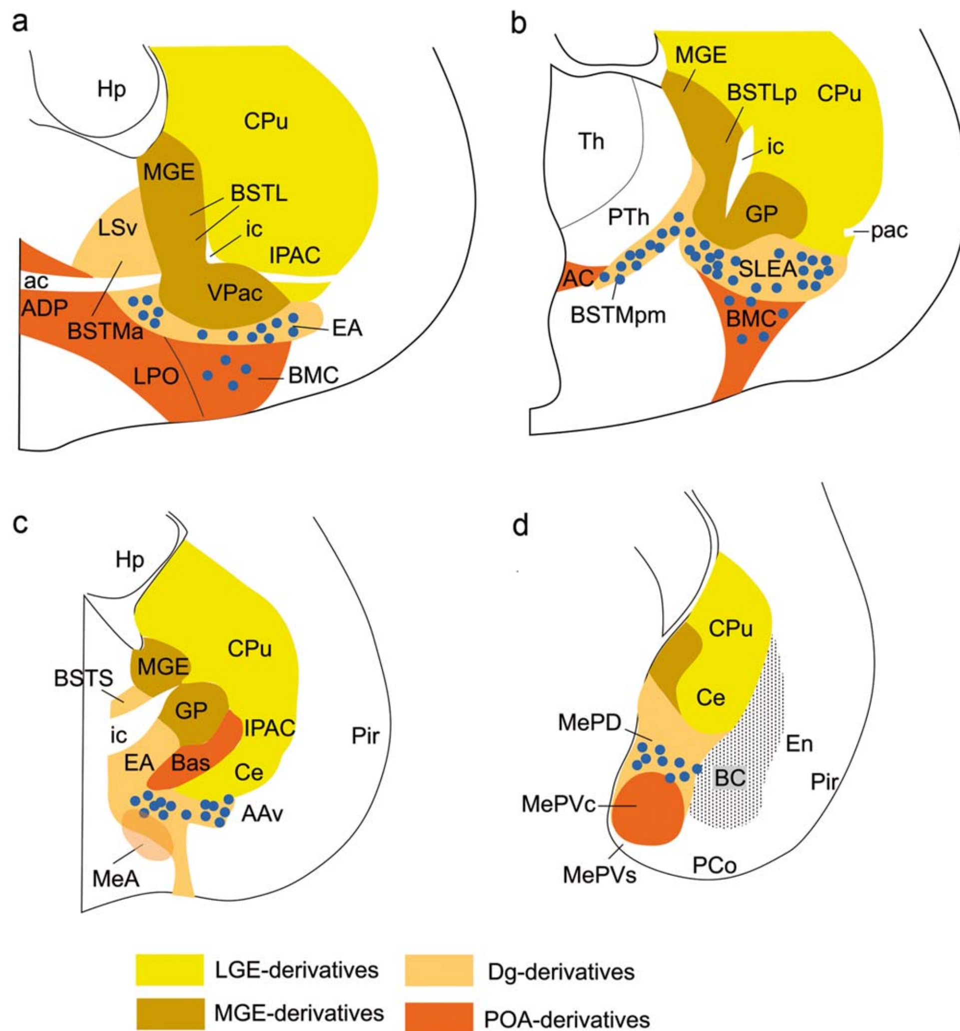

Figure 6. **a–d**: Schematic drawings of telencephalic frontal sections (arranged from rostral to caudal) at stage E16.5 (adapted from Garcia-Lopez et al., 2008. Blue dots represent cells expressing Chrna2 in the amygdala. Different derivatives are identified by a color code. Chrna2 is expressed in almost the entire amygdala developed from the Dg (light orange) and in the BMC that is derived from the POA (dark orange).

to study the location of Chrna2 at P0 with the aim of confirming this mature extended pattern or identifying a conserved restrictive expression pattern between the immature embryonic brain and the adult amygdala.

Pallidal and striatal amygdala

Pallidal and striatal amygdalar derivatives lacked Chrna2 expression at P0. BSTL, Ce, and IPAC amydalar nuclei are derived from the medial and lateral ganglionic eminences and were not populated by complementary-Chrna2 riboprobe-positive cells (Fig. 5A–L). Actually, the BSTL was detected by the expression of SOM (Fig. 5D) and was negative for Chrna2 mRNA (Fig. 5E,F). Only a few cells were observed at these stages in the striatal-derived amygdalar nuclei IPAC and Ce (Fig. 5A,B and E,F, respectively). The study of SOM expression allowed us to identify the location of the Ce (Fig. 5H,L). Chrna2 mRNA expression in scattered neurons in central amygdala nuclei in adult mice brain has been previously described (Ishii et al., 2005).

Dg and POA amygdala

Most of the Dg-derived amygdala and POA basal forebrain derivatives exhibited Chrna2-expressing cells. Although the expression was more abundant in these areas than in others of subpallial amygdala derivatives, the density of the labeled cells was lower at these perinatal stages than at earlier stages. Chrna2 expression was detected in cells located in the EA (Fig. 5A,B,E,F), the AAv (Fig. 5I, J), and the MeA/MePD (Fig. 5I,J). The analysis of Chrna2 and SOM expression in parallel series showed that the medial part of the BST (BSTM)

expressed Chrna2 (Fig. 5A,B), whereas its lateral part (BSTL), also identified by SOM expression (Fig. 5D), did not exhibit any labeled cells (Fig. 5A,B). The expression in the MeA and in the BSTL was preserved in the adult brain (Wada et al., 1989; Ishii et al., 2005).

In relation to POA derivatives, some cells were detected in the BMC (Fig. 5A,B,E, F), which were identified by ChAT expression (Fig. 5C). The density of Chrna2-expressing cells in this POA-derived region was reduced compared with earlier stages; however, it is conserved until adult stages (Ishii et al., 2005).

DISCUSSION

In the present study we demonstrate that Chrna2 is specifically expressed in cells derived from a narrow germinative domain in the subpallial telencephalon, the Dg amygdalar region at the ventrocaudal pole of the medial ganglionic eminence (Fig. 6). To perform this study, we made an mRNA probe using a mouse α2 cDNA fragment (569 bp). We first confirmed that this fragment contained no significant homology to other mouse sequences by searching in the GenBank database. In addition, we used the Eurexpress database to verify the Chrna2 expression pattern at stage E14.5 and to discard possible cross-reactions with other nAChR subunits.

nAChR α2 subunit (Chrna2) is expressed in Dg-derived amygdala and BMC in the basal forebrain

The amygdala is a complex structure composed of multiple regions derived from different histogenetic domains in the telencephalic germinal epithelium. Thus we can identify the pallial amygdala, which is composed of derivatives of the ventral and lateral pallium, and the subpallial amygdala, which consists of derivatives of the subpallium (Puelles et al., 2000; Stenman et al., 2003; Medina et al., 2004; Bupesh et al., 2011a,b). We have observed that Chrna2 is expressed particularly in the subpallial-derived amygdala, which is subdivided into four histogenetic domains: striatal, derived from the LGE; pallidal, derived from the MGE; entopeduncular, derived from the Dg or MGEvc; and preoptic, derived from the POA (Puelles et al., 2000; Garcia-Lopez et al., 2008; Bupesh et al., 2011a,b). These domains give rise to most of the cells that form the centromedial and extended amygdala (Garcia-Lopez et al., 2008; Bupesh et al., 2011b). This is in agreement with the results of Soma et al. (2009) on the nuclear origin of the amygdala using experimental lineage tracing with in utero electroporation, taking into account that they identify as diencephalic derivatives the medial and central amygdala, which are actually structures corresponding to our Dg (or MGEvc in Bupesh et al., 2011a) and POA epithelial regions. Previous studies, based on combined analysis of developmental regulatory genes and phenotypic neural markers, have described the nuclei that derive from the Dg and POA. The BSTM, SLEA, and part of the medial amygdala (anterior and posterodorsal parts) are derived from the Dg. In all these nuclei Chrna2 was expressed from E14.5 to neonatal stages. Moreover, cells expressing Chrna2 were also detected in the BMC, a POA derivative (Fig. 6). In contrast, no positive cells for Chrna2 were observed in other POA-derived amygdalar derivatives such as the medial posteroventral nucleus (MePV). The amygdalar areas derived from striatal and pallidal domains did not show Chrna2 expression at early developmental stages, indicating that this subunit of the cholinergic receptor is specifically expressed in cells originating in the Dg amygdalar domains. In the adult brain, scattered cells expressing the α2 receptor subunit have been detected in almost the entire medial and central amygdalar structures, with the exception of the lateral and basolateral nuclei (pallial amygdala; Ishii et al., 2005; present results). Because we have noted that in the embryo Chrna2 expression is restricted to the Dg-derivative nuclei, Chrna2-expressing cells may undergo migratory movement from localized germinative loci, to populate all of the subpallial-derived amygdala during later developmental and postnatally.

Possible functional implication of Chrna2 expression in the subpallial amygdala

Our results show that cells expressing the α2-subunit of the cholinergic receptor are located in the anterior and posterodorsal areas of the medial amygdalar nuclei. These areas are specifically involved in reproductive behavior, and send inhibitory projections to the medial hypothalamus (Petrovich et al., 2001; Garcia-Lopez et al., 2008), whereas the Chrna2-negative posteroventral part of the medial amygdala is related to defensive activities. Consequently, in mouse developing and adult telencephalon, Chrna2 is expressed in the amygdalar circuits involved in sexual behavior, which correspond with the part of the amygdala derived from the Dg division.

In addition, AAv and SLEA are populated by small stellate cholinergic neurons that are smaller and less anti-ChAT–immunoreactive than other basal forebrain ChAT-positive neurons (Gastard et al., 2002). These cholinergic neurons are contacted by symmetric synaptic specialization of axons coming from the Ce, which have been proposed to be GABAergic (Loopuijt and

Zahm, 2006; Bienkowski et al., 2013). In this way, cholinergic nicotinic receptors containing the α2-subunit could play a role in the regulation of ventral amygdala cholinergic response interacting with the GABAergic innervation coming from the Ce. Therefore, it could be involved in the mechanisms associated with attention tasks and fear conditioning.

It has recently been shown that the behavioral phenotype of Chrna2 mutant mice has as the most relevant alteration a deficit in nicotine-enhanced emotional memories via a sex-dependent mechanism (Lotfipour et al., 2013). Amygdalar expression of Chrna2 in sexual dimorphic nuclei (in the EA and POA) may underlie this functional phenotype, and strongly increases our interest in understanding the developmental pattern of Chrna2 expression in amygdalar development in relation to sexual structural dimorphism. Therefore, although we have exclusively analyzed male embryos, a comparison of these results with temporal–spatial expression patterns in females seems to be an interesting question for the future.

In conclusion, we have described Chrna2 expression in the amygdala and propose that this expression is a marker of the Dg-derived amygdala. This nAChR subunit is related to emotional memories, attention tasks, and fear conditioning. In addition, in humans, CHRNA2 mutations are related to epilepsy, schizophrenia, autism, bipolar disorder, and depression (De Marco et al., 2007; Tabares-Seisdedos and Rubenstein, 2009; Posadas et al., 2013). Future studies are necessary to clarify direct causal correlations between α2-subunit expression in the amygdala and its functional implications for neuropsychiatric disorders.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflicts of interest.

ROLE OF AUTHORS

All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: AP and SM. Acquisition of data: AP. Analysis and interpretation of data: AP and SM. Drafting of the manuscript: AP. Critical revision of the manuscript for important intellectual content: SM. Obtained funding: SM. Technical and material support: AP. Study supervision: SM.

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