A Novel Subpopulation of 5-HT$_{3A}$ Receptor Immunoreactive Interneurons in the Rat Basolateral Amygdala

Franco Mascagni and **Alexander Joseph McDonald**[*]
Department of Pharmacology, Physiology and Neuroscience University of South Carolina School of Medicine Columbia, SC 29208

Abstract

The amygdalar basolateral nuclear complex (BLC) has very high levels of the type 3 serotonin receptor (5-HT$_3$R). Previous studies have reported that 5-HT$_3$R protein in the BLC is expressed in interneurons and that 5-HT$_3$R mRNA is coexpressed with GABA and certain neuropeptides or calcium-binding proteins in these cells. However, there have been no detailed descriptions of the distribution of 5-HT$_3$R+ neurons in the amygdala, and no quantitative studies of overlap of neurons expressing 5-HT$_3$R protein with distinct interneuronal subpopulations in the BLC. The present investigation employed dual-labeling immunohistochemistry using antibodies to the 5-HT-3A receptor subunit (5-HT$_{3A}$R) and specific interneuronal markers to address these questions. These studies revealed that there was a moderate density of nonpyramidal 5-HT$_{3A}$R+ neurons in the BLC at all levels of the amygdala. In addition, immunostained cells were also seen in anterior portions of the cortical and medial nuclei. Although virtually all 5-HT$_{3A}$R+ neurons in the BLC were GABA+, very few expressed neuropeptide or calcium-binding protein markers for individual subpopulations. The main interneuronal marker expressed by 5-HT$_{3A}$R+ neurons was cholecystokinin (CCK), but only 8–16% of 5-HT$_3$R+ neurons in the BLC, depending on the nucleus, were CCK+. Most of these CCK+/5-HT$_{3A}$R+ double-labeled neurons appeared to belong to the subpopulation of large type L CCK+ interneurons. Very few 5-HT$_{3A}$R+ neurons expressed calretinin, vasoactive intestinal peptide, or parvalbumin, and none expressed somatostatin or calbindin. Thus, the great majority of neurons expressing 5-HT$_{3A}$R protein appear to constitute a previously unrecognized subpopulation of GABAergic interneurons in the BLC.

Keywords

serotonin; GABA; peptides; calcium-binding proteins

The amygdala receives a robust serotonergic innervation from the dorsal raphe nucleus that targets all amygdalar nuclei, especially the basolateral nuclear complex (Moore et al., 1978; Steinbusch, 1981; Fallon and Ciofi, 1992; Sadikot and Parent, 1990; Abrams et al., 2005). Microdialysis studies indicate that there is increased serotonin (5-HT) release in the amygdala during behavioral arousal and stress (Kawahara et al., 1993; Rueter and Jacobs, 1996). In addition, certain genetic variations in human serotonin transporter and tryptophan hydroxylase genes, which presumably result in altered extracellular serotonin levels, are associated with

*Correspondence to: Alexander Joseph McDonald, Telephone: 803-733-3378, Fax: 803-733-1523, E-mail: mcdonald@med.sc.edu.
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increased activation of the amygdala by emotional stimuli, as well as anxiety and depression in these individuals (Canli et al., 2005; Hariri and Holmes, 2006).

Animal studies also suggest that serotonergic neurotransmission in the basolateral nuclear complex of the amygdala (BLC) plays a critical role in stress, anxiety, and depression, but the exact effects depend on the type of serotonin receptor activated (Zangrossi and Graeff, 1994; Graeff et al., 1996; Zangrossi et al., 1999; De Mello Cruz et al., 2005; Abrams et al., 2005; Inoue et al., 2004). The type 3 serotonin receptor (5-HT$_3$R) is unique in that it is the only serotonin receptor that is a ligand-gated ion channel (Barns and Sharp, 1999; Chameau and van Hooft, 2006). Thus, unlike all other 5-HT receptors, which are G-protein-coupled receptors, 5-HT$_3$Rs are associated with fast excitatory synaptic potentials in the BLC and other brain regions (Sugita et al., 1992; Chameau and van Hooft, 2006). Although several distinct 5-HT$_3$R subunits have been described, only the 3A subunit is found in significant concentrations in the rodent CNS (Morales and Wang, 2002; Chameau and van Hooft, 2006). Receptor autoradiographic studies performed in both rat (Waeber et al., 1990; Barnes et al., 1990; Gehlert et al., 1991; Laporte et al., 1992; Steward et al., 1993) and human brains (Bufton et al., 1993; Abi-Darghem, 1993) demonstrate very high levels of 5-HT$_3$R binding in the BLC. Injections of 5-HT$_3$R antagonists into the rat amygdala are anxiolytic in most experimental tests of anxiety (Costall et al., 1989; Higgens et al., 1991; Nevins and Anthony, 1994; Gargiulo et al., 1996).

Since different subpopulations of BLC neurons exhibit specific connections (Muller et al., 2003, 2005, 2006a, 2006b), understanding the effects of serotonergic neurotransmission on information processing by the BLC will require knowledge of the expression of 5-HT$_3$R, and other serotonin receptors, by distinct cell types. Previous studies have shown that there are two major cell classes in the BLC, pyramidal neurons and nonpyramidal neurons. Although these cells do not exhibit a laminar organization, their morphology, synaptology, electrophysiology, and pharmacology are remarkably similar to their counterparts in the cerebral cortex (Carlsen and Heimer, 1988; McDonald, 1992a; Washburn and Moises, 1992; Rainnie et al., 1993; Paré et al., 2003). Thus, pyramidal neurons in the BLC are projection neurons with spiny dendrites that utilize glutamate as an excitatory neurotransmitter, whereas most nonpyramidal neurons are spine-sparse interneurons that utilize GABA as an inhibitory neurotransmitter. Recent dual-labeling immunohistochemical studies suggest that the BLC contains at least four distinct subpopulations of GABAergic interneurons that can be distinguished on the basis of their content of calcium-binding proteins and peptides. These subpopulations are: 1) parvalbumin +/calbindin+ neurons, 2) somatostatin+/calbindin+ neurons, 3) small bipolar and bitufted interneurons that exhibit extensive colocalization of vasoactive intestinal peptide, calretinin, and cholecystokinin, and 4) large multipolar cholecystokinin+ neurons that are often calbindin + (McDonald and Betette, 2001; McDonald and Mascagni, 2001a, 2002, Mascagni and McDonald, 2003; Kemppainen and Pitkänen, 2000).

The neuronal localization of 5-HT$_3$R in the rat forebrain has been studied using in situ hydridization techniques (Tecott et al., 1993; Morales and Bloom, 1997). These studies have mainly focused on the neocortex and hippocampus, but have also commented on the amygdala. In both the cortex and BLC, mRNA for 5-HT$_3$R is mainly expressed in a subpopulation of GABAergic interneurons that are immunoreactive for CCK (Morales and Bloom, 1997). However, the exact extent of overlap of 5-HT$_3$R mRNA expressing neurons with GABA+ and CCK+ neurons in the BLC was not determined. It is also not clear to what extent BLC neurons that express 5-HT$_3$R mRNA also express 5-HT$_3$R protein.

The results of immunohistochemical studies of 5-HT$_3$R protein localization are largely congruent with in situ hybridization studies of 5-HT$_3$R mRNA (e.g., 5-HT$_3$R+ neurons are nonpyramidal cells in cortex and BLC), although there are some discrepancies (Morales and

Bloom, 1997; Morales et al., 1998). It has been suggested that the expression of 5-HT$_3$R mRNA, but not 5-HT$_3$R protein, in some neurons may be due to differences in rates of gene transcription, mRNA translation, or protein transport (Morales et al., 1998). Although the identification of neurons expressing 5-HT$_3$R protein in the BLC is of obvious importance in understanding electrophysiological and behavioral aspects of serotonergic neurotransmission in this region, there is no detailed information regarding the exact distribution of 5-HT$_3$R-immunoreactive neurons in the amygdala, or their overlap with interneuronal subpopulations identified using immunohistochemical techniques (Mascagni and McDonald, 2003). The present investigation is the first study to use dual-labeling immunohistochemistry to address these questions.

EXPERIMENTAL PROCEDURES

Tissue Preparation

A total of 13 male Sprague-Dawley rats (250–350g; Harlan, Indianapolis, IN) were used in this study. All experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Use and Care Committee (IACUC) of the University of South Carolina. All efforts were made to minimize animal suffering and to use the minimum number of animals necessary to produce reliable scientific data.

Rats were anesthetized with chloral hydrate (350 mg/kg) and perfused intracardially with phosphate buffered saline (PBS; pH 7.4) containing 0.5% sodium nitrite (50 ml) followed by 4.0% paraformaldehyde (or a 4.0% paraformaldehyde-0.2% glutaraldehyde mixture in two rats used for dual localization of 5-HT$_3$R and GABA) in 0.1 M phosphate buffer at pH 7.4 (500 ml). Following perfusion, all brains were removed and postfixed for 3.5 hours in 4.0% paraformaldehyde. Brains were sectioned on a vibratome at a thickness of 50 μm in the coronal plane. Sections were processed for immunohistochemistry in wells of tissue culture plates. All antibodies were diluted in 0.1M PBS containing Triton X-100 (0.5%) and 1% normal goat serum.

Immunoperoxidase experiments

Localization of the 5-HT$_{3A}$ receptor subunit (5-HT$_{3A}$R) was performed in 8 rats using the avidin-biotin immunoperoxidase (ABC) technique. A rabbit polyclonal antibody to the 5-HT$_{3A}$R was raised against amino acids 444–457 of the large putative intracellular loop of the receptor (1:4000; antiserum 0165; generously donated by Dr. Marisela Morales, National Institute on Drug Abuse, Baltimore, Maryland). Sections were incubated in primary antibody overnight at 4° C and then processed for the avidin-biotin immunoperoxidase technique using a rabbit Vectastain ABC kit (Vector Laboratories, Burlingame, CA). Nickel-enhanced DAB (3, 3′-diaminobenzidine-4HCl, Sigma Chemical Co., St. Louis, MO) was used as a chromogen to generate a black reaction product (Hancock, 1986). Following the immunohistochemical procedures, sections were mounted on gelatinized slides, dried overnight, dehydrated in ethanols, cleared in xylene, and coverslipped in Permount (Fisher Scientific, Pittsburgh, PA). In some animals adjacent sections were counterstained with cresyl violet to identify nuclear borders. Sections were analyzed using an Olympus BHA and Nikon Eclipse E600 microscopes. Digital light micrographs were taken with a QImaging MicroPublisher 5.0 CCD camera (QImaging Corp., Burnaby, British Columbia). Brightness and contrast were adjusted using Photoshop 6.0 software.

Immunofluorescence experiments

Dual localization of 5-HT$_{3A}$R with GABA or protein/peptide interneuronal markers (parvalbumin [PV], calbindin [CB], somatostatin [SOM], calretinin [CR], cholecystokinin

[CCK], and vasoactive intestinal peptide [VIP]) was investigated in 6 rats. In the two rats fixed with the paraformaldehyde/glutaraldehyde mixture, only dual localization of 5-HT$_{3A}$R and GABA was investigated. In the remaining four rats, which were fixed with paraformaldehyde, localization of 5-HT$_{3A}$R with 4–5 different protein/peptide interneuronal markers were investigated in parallel series of equally-spaced sections at 200–250 μm intervals. Sections were incubated in a cocktail of the rabbit 5-HT$_{3A}$R antibody (1:1000) and one of the following monoclonal interneuronal marker antibodies overnight at 4° C: mouse anti-PV (1:2000, Sigma Chemical Co., St. Louis, MO), rat anti-SOM (1:400; Chemicon International, Temecula, CA), mouse anti-CR (1:3000, Chemicon), mouse anti-CCK (1:500, antibody 93903 generously donated by Dr. John H. Walsh, UCLA), mouse anti-VIP antibody (1:6000; antibody CURE-V55 generously donated by Dr. John H. Walsh, UCLA), mouse anti-CB antibody (1:8000, Chemicon), or mouse anti-GABA antibody (1:50; antibody 115-AD5-A9, generously donated by Dr. Ismo Virtanen, Univ. of Helsinki). After incubation in the primary antibody cocktail, sections were then rinsed in 3 changes of PBS (10 min each), and then incubated in a cocktail of species-appropriate Alexa-568 and Alexa-488 labeled secondary antibodies (1:400; Molecular Probes, Eugene, OR), for 3 hrs at room temperature. All secondary antibodies were highly cross-adsorbed by the manufacturer to ensure specificity for primary antibodies raised in particular species. In each of the confocal immunofluorescence cases some control sections were processed with one of the two primary antibodies omitted. In all cases only the color of the corresponding secondary fluorescent antibody was observed, and only on the appropriate channel. These results indicated that the secondary antibodies were specific for rabbit or mouse IgGs and that there was no "crosstalk" between the red and green channels (Wouterlood et al., 1998).

Sections were then rinsed in 3 changes of PBS (10 min each), mounted on glass slides using Vectashield mounting medium (Vector Laboratories, Burlingame, CA), and examined with a Bio-Rad MRC-1024 confocal laser scanning system equipped with an argon-krypton laser attached to a Nikon Optiphot fluorescence microscope. Fluorescence of Alexa 488 (green) and Alexa 568 (red) dyes was analyzed using filter configurations for sequential excitation/imaging via 488 nm and 568 nm channels. Since GABA-like immunoreactivity was only seen on the surface of the section, analysis of the colocalization of GABA with the 5-HT3A receptor subunit was accomplished by focusing on the upper and lower surfaces of the section using the lowest iris aperture of the confocal microscope (which results in the thinnest "optical section"). This step was not necessary for the other experiments since in those cases immunostaining extended throughout the entire thickness of the section. Digital images were adjusted for brightness and contrast using Photoshop 6.0 software.

The rat BLC consists of the lateral, basolateral, and basomedial nuclei. Each nucleus can be divided into two or more subdivisions. We studied distinct subdivisions of two of the main nuclei of the BLC, the anterior subdivision of the basolateral nucleus (BLa) and the ventromedial subdivision of the lateral nucleus (Lvm) (Paxinos and Watson, 1997), to determine if the phenotypic organization of 5-HT receptor-expressing neurons might differ in different regions of the BLC. Bilateral counts of single-labeled and double-labeled neurons in these nuclei were pooled from 2 animals for each colocalization combination studied (approximately 5–7 sections for each subdivision in each animal). At 200X magnification, cell counts were made from the image of a 400 μm X 400 μm field displayed for merged (red/green) channels on the computer screen (double-labeled cells appear yellow). Images of the non-merged red and green channels were also displayed. Depending on the size of the nuclear subdivision at different levels of the amygdala, counts were made from either one such field positioned in the center of the subdivision (and involving about 80–90% of its cross-sectional area), or two adjacent non-overlapping fields. Only somata of nonpyramidal neurons were counted. Although some pyramidal cells contain low levels of CR or CB, they were easily distinguished from the intensely-stained nonpyramidal neurons at the antibody dilutions used

in this study (McDonald and Mascagni, 2001a). Since dendrites of BLC nonpyramidal neurons are less than 3 μm wide, it was not difficult to distinguish somata from dendrites.

Antibody Specificity

The polyclonal antibody to the 5-HT$_{3A}$ receptor (antibody 0165 of Morales et al., 1996) is an affinity purified antibody raised in rabbit against a synthetic peptide containing amino acids 444–457 of the receptor, coupled to bovine serum albumin using glutaraldehyde. This amino acid sequence is located in the large intracellular loop between transmembrane regions M3 and M4. This antibody immunolabeled both recombinant and neuronal 5-HT$_{3A}$R protein at a band of 50 kDa in western blots. Studies on tissue sections combining immunohistochemistry and in situ hybridization revealed extensive colocalization of 5-HT$_{3A}$R immunolabeling and 5-HT$_{3A}$R transcripts (Morales et al., 1996).

The interneuronal marker antibodies used in this investigation have been characterized and used extensively in previous studies of the cerebral cortex and basolateral amygdala (Szabat et al., 1992; Conde et al., 1994; Kawaguchi and Kubota, 1996; McDonald, 1996; Wong et al., 1996; Sloviter et al., 2001; McDonald and Betette, 2001; McDonald and Mascagni, 2001a, 2002, 2004; McDonald et al. 2004; Mascagni and McDonald, 2003). Each antibody produced the characteristic pattern of labeling for each of the interneuronal subpopulations seen in previous studies.

RESULTS

5-HT$_{3A}$R staining in the forebrain at the level of the amygdala appeared to be identical to that obtained in previous studies using this antibody (Morales et al., 1998). Thus, there was a moderate density of nonpyramidal 5-HT$_{3A}$R+ neurons in the neocortex, hippocampus, and striatum, as well as in the basolateral nuclear complex (BLC) of the amygdala (Fig. 1). In addition, immunostained cells were also seen in anterior portions of the cortical and medial amygdalar nuclei. 5-HT$_{3A}$R+ neurons were of similar morphology in all of these regions. Most immunostained neurons in the BLC had medium-sized somata (14–20 μm long and 8–12 μm wide) that were ovoid or fusiform in shape (Fig. 2). Perikaryal immunostaining varied from intense to very light. Some 5-HT$_{3A}$R+ nonpyramidal neurons in the BLC exhibited 1–4 lightly stained proximal dendrites radiating from the soma, but in most immunostained neurons no staining was detectable in dendrites. There was also very light staining in many pyramidal neurons (Fig. 2), but it was not clear if this staining was specific or represented non-specific background.

As in previous studies (Morales and Bloom, 1997; Morales et al., 1998), the morphology of 5-HT$_{3A}$R+ neurons in the BLC suggested that these neurons were GABAergic interneurons. Dual-labeling confocal immunofluorescence studies were conducted to determine if this was the case, and to identify which interneuronal subpopulations were represented. Although virtually all 5-HT$_{3A}$R+ neurons in the BLa and Lvm were GABA+, very few expressed neuropeptide or calcium-binding protein markers for individual subpopulations (Fig. 3; Table 1). The main subpopulation-specific interneuronal marker expressed by 5-HT$_{3A}$R+ neurons was CCK, but only 16% of 5-HT$_3$R+ neurons in the BLa, and 8% of 5-HT$_{3A}$R+ neurons in the Lvm, were CCK+. Most of these CCK+/5-HT$_{3A}$R+ double-labeled neurons appeared to belong to the subpopulation of larger type L CCK+ interneurons, rather than the subpopulation of small type S CCK+ subpopulation (Mascagni and McDonald, 2003; Fig. 3E, F). Very few 5-HT$_3$R+ neurons expressed CR, VIP or PV, and none expressed SOM or CB.

Additional cell counts were performed in the two 5-HT$_{3A}$R/GABA preparations and in two 5-HT$_{3A}$R/CCK preparations to determine the percentage of GABA+ and CCK+ interneurons that were 5-HT$_{3A}$R+. These analyses revealed that 5-HT$_{3A}$R+ neurons constituted 6.7%

(18/269) of GABA+ neurons in the BLa, and 12.2% (32/262) of GABA+ neurons in the Lvm. $5\text{-HT}_{3A}R+$ neurons constituted 10.7% (9/84) of CCK+ neurons in the BLa, and 6.3% (5/80) of CCK+ neurons in the Lvm.

DISCUSSION

This is the first study to use double-labeling immunohistochemistry to study the phenotypes of $5\text{-HT}_{3A}R+$ neurons in the BLC. As suggested in a previous immunohistochemical study (Morales et al., 1998), $5\text{-HT}_{3A}R+$ neurons in the amygdala were nonpyramidal interneurons that were mainly located in the BLC. The present investigation demonstrated that virtually all neurons in the BLC that expressed $5\text{-HT}_{3A}R$ protein were GABA-immunoreactive, consistent with a previous report that 70–95% of telencephalic neurons expressing mRNA for $5\text{-HT}_{3A}R$ were GABAergic (Morales and Bloom, 1997). Surprisingly, however, the present study found that there was little overlap of $5\text{-HT}_{3A}R+$ neurons with the four main subpopulations of immunohistochemically-identifed GABAergic interneurons in the BLC (Mascagni and McDonald, 2003). These results contrast with recent studies of GluR1, $GABA_A$, $GABA_B$, substance P, and cannabinoid receptors in the BLC, which demonstrated differential expression of these receptors among one or more of the four main interneuronal subpopulations (McDonald, 1996; McDonald and Mascagni, 2001b, 2004; McDonald et al., 2004; Levita et al., 2003). Thus, the great majority of neurons expressing $5\text{-HT}_{3A}R$ protein appear to constitute a previously unrecognized subpopulation of medium-sized interneurons in the BLC.

The only BLC interneuronal subpopulation to exhibit significant $5\text{-HT}_{3A}R$ protein expression was the subpopulation of large CCK+ interneurons (Type L CCK+ neurons, Mascagni and McDonald, 2003); 16% of $5\text{-HT}_{3A}R+$ neurons in the basolateral nucleus and 8% of 5-HT_{3A} + neurons in the lateral nucleus were CCK+. This is consistent with a previous study which reported colocalization of CCK peptide and $5\text{-HT}_{3A}R$ mRNA, but the extent of colocalization was not quantitated in the BLC (Morales and Bloom, 1997). It is of interest that no calbindin-positive (CB+) neurons in the BLC were $5\text{-HT}_{3A}R+$, since approximately one-third of type L CCK+ interneurons are CB+. Likewise, Morales and Bloom (1997) found little colocalization of $5\text{-HT}_{3A}R$ mRNA and CB in the BLC, with the exception of an occasional neuron in the basomedial nucleus. There is colocalization of CCK with calretinin (CR) and VIP in a subpopulation of small CCK+ cells in the BLC (type S CCK+ cells); the finding in the present study of little CR/$5\text{-HT}_{3A}R$ or VIP/$5\text{-HT}_{3A}R$ colocalization provides further evidence that it is the type L CCK+ neurons that express $5\text{-HT}_{3A}R$ protein. Dual-labeling immunohistochemical studies have shown that many of the large type L CCK+ interneurons, but none of the small type S CCK+ cells, exhibit CB1 cannabinoid receptor immunoreactivity (McDonald and Mascagni, 2001b; Katona et al., 2001). Consistent with these immunohistochemical findings, there is extensive colocalization of $5\text{-HT}_{3A}R$ and CB1 receptor mRNA in neurons of the BLC (Hermann et al., 2002; Morales et al., 2004). However, when plots of neurons expressing 5-HT_{3A} and CB1 receptor mRNA in the BLC (see Fig. 1 of Morales et al., 2004) are compared with plots of neurons expressing 5-HT_{3A} and CB1 receptor protein (see Fig. 1 of the present study; Fig. 3 of McDonald and Mascagni, 2001b), it is clear that the density of neurons expressing mRNA for these proteins is much higher than that of neurons expressing the actual proteins. This discrepancy may be due to a variety of factors including low rates of mRNA translation or rapid transport of these proteins from the cell body in some neurons.

Since immunohistochemical studies in the rat neocortex have demonstrated interneuronal subpopulations that are very similar to those in the BLC (Kubota et al., 1994; Kubota and Kawaguchi, 1997), it is of interest to compare patterns of the $5\text{-HT}_{3A}R$ distribution in these two regions. As in the BLC, there is colocalization of $5\text{-HT}_{3A}R$ mRNA with CCK immunoreactivity in the cortex (Morales and Bloom, 1997). However, unlike the BLC, the

degree of colocalization has been quantitated for the cortex. Although the extent of colocalization varies in different cortical areas, on average about 50% of neurons expressing 5-HT$_{3A}$R mRNA are CCK-immunoreactive. This greater degree of expression of 5-HT$_{3A}$R mRNA expression in CCK+ cortical neurons, compared with the extent of 5-HT$_{3A}$R protein expression in CCK+ BLC neurons, may in part be related to the overall greater density of neurons expressing 5-HT$_{3A}$R mRNA versus protein (see above). One major difference in the neocortex was the extensive colocalization of 5-HT$_{3A}$R mRNA with calbindin (CB) immunoreactivity; between 33 and 63% of CB+ cortical neurons expressed 5-HT$_{3A}$R mRNA, depending on the cortical area (Morales and Bloom, 1997). This was not seen in the BLC (see above). A dual-labeling study of the primate neocortex using the same 5-HT$_{3A}$R antibody used in the present study demonstrated 5-HT$_{3A}$R immunoreactivity in subpopulations of small interneurons exhibiting GABA, CR, CB and substance P receptor immunoreactivity, although no quantitative data was collected and colocalization with CCK was not attempted (Jakab and Goldman-Rakic, 2000); the findings of colocalization of 5-HT$_{3A}$R with GABA, CR, and CB is consistent with the 5-HT$_{3A}$R mRNA colocalization findings in rat neocortex (Morales and Bloom, 1997).

The 5-HT$_{3A}$R antibody used in the present study primarily stains cell bodies, where immunoreactivity is mainly confined to the nuclear envelope and granular endoplasmic reticulum (Morales and Bloom 1996). This pattern of staining has suggested that the antibody mainly labels unassembled subunits of the receptor rather than the assembled functional receptor in the plasma membrane (Morales et al., 1998). Thus, while the antibody provides a useful marker for identifying neurons that express the 5-HT$_{3A}$R protein, it cannot be used to precisely localize the site of the functional receptor. Using another 5-HT$_{3A}$R antibody that does appear to localize the assembled receptor, Miquel et al. (2002) found that 5-HT$_{3A}$R immunoreactivity was distributed in both dendritic and axonal processes in the amygdala. This localization pattern is consistent with electrophysiological studies showing both postsynaptic and presynaptic actions of 5-HT at 5-HT$_{3A}$ receptors in the BLC (see below).

Although previous immunohistochemical and in situ hydridization investigations suggested that only GABAergic interneurons in the BLC express 5-HT$_{3A}$Rs (Morales and Bloom 1996, Morales and Bloom 1997, Morales et al., 1998) electrophysiological studies indicate that pyramidal cells in the BLC may also possess these receptors (but see Koyama et al., 2000). Thus, it has been reported that 40% of neurons in the lateral nucleus exhibited postsynaptic excitation mediated by 5-HT$_3$Rs (Sugita et al., 1992), whereas GABAergic interneurons are thought to constitute less than one-quarter of BLC neurons (McDonald, 1985, 1992b). Likewise, in another study 53% of neurons in the BLC and cortical nuclei were excited by a putative 5-HT$_3$R mechanism, suggesting that pyramidal neurons as well as interneurons in the BLC might express 5-HT$_3$Rs (Stein et al., 2000). In the present study, and also in a previous study using the same antibody (see Fig. 10 of Morales et al., 1998), there was light staining of pyramidal cells. However, it remains to be determined whether this represents light immunostaining or non-specific background.

To date there have been no studies of 5-HT$_3$R-mediated postsynaptic responses in identified interneurons in the BLC. However, there is abundant evidence in the neocortex (Ferezou et al., 2002; Xiang and Prince, 2003; Puig et al., 2004) and hippocampus (Ropert and Guy, 1991; Kawa, 1994; McMahon and Kauer, 1997; Sudweeks et al., 2002) that 5-HT can indirectly inhibit pyramidal cells by activating postsynaptic interneuronal 5-HT$_3$Rs. Since electron microscopic studies in the BLC indicate that PV+, VIP+, and somatostatin+ interneurons in the BLC provide a robust inhibitory innervation of pyramidal cells (Muller et al., 2003a, 2003b, 2006), it seems likely that the CCK+ and non-CCK+ interneurons in the BLC that express 5-HT$_{3A}$Rs (present study) have similar connections and will inhibit BLC pyramidal cells subsequent to 5-HT$_{3A}$R-mediated postsynaptic excitation of these interneurons. There is also

evidence that 5-HT can increase GABA release from BLC interneurons via a 5-HT$_3$R-mediated presynaptic action on interneuronal axon terminals synapsing with pyramidal cells (Koyama et al., 2000). Thus, much of the 5-HT$_{3A}$R protein found in the cell bodies of BLC interneurons may ultimately be transported to the axon terminals of these neurons, consistent with electron microscopic studies (Miquel et al., 2002). For the type L CCK+ neurons in the BLC that exhibit 5-HT$_{3A}$R and CB1 receptor immunoreactivity, this is just one of several possible receptor-mediated mechanisms modulating release from these terminals, since activation of CB1 cannabinoid receptors on these terminals decreases GABA release (Katona et al., 2001). Moreover, it is possible that 5-HT$_3$Rs on these terminals also increase CCK release, since this effect is seen in the neocortex (Paudice and Raiteri, 1991; Raiteri et al., 1993).

In summary, some of the neurons expressing 5-HT$_{3A}$R subunit protein in the BLC belong to a subpopulation of large GABAergic interneurons that are CCK+ (type L CCK+ interneurons), but most appear to constitute a previously unrecognized subpopulation of medium-sized GABAergic interneurons. In the awake state typical serotonergic neurons in the raphe fire in a clock-like manner at about 1–2 Hz, depending on the state of behavioral arousal (Jacobs and Azmitia, 1992). Thus, 5-HT$_{3A}$R+ interneurons in the BLC would presumably be rhythmically excited at these frequencies and ultimately inhibit neighboring pyramidal neurons. Although inhibition of BLC pyramidal neurons is usually associated with reduction in fear and anxiety (Davis et al., 1994), previous behavioral studies suggest that activation of 5-HT$_3$Rs has the opposite effect in most experimental tests of anxiety (Costall et al., 1989; Higgens et al., 1991; Nevins and Anthony, 1994; Gargiulo et al., 1996). This suggests that interneurons expressing 5-HT$_{3A}$Rs may have inhibitory connections with other GABAergic interneurons, similar to VIP and PV subpopulations in the BLC (Muller et al., 2003, 2005). Activation of these interneuronal networks via a 5-HT$_{3A}$R-mediated mechanism could then result in disinhibition of pyramidal cells. Further study of the basic electrophysiological properties of 5-HT$_3$R+ interneurons, and how these characteristics, as well as the network properties of the BLC (e.g., Rainnie, 1999) are altered by 5-HT will be required to understand the role of amygdalar 5-HT$_3$ receptors in emotion.

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Abbreviations

5-HT

serotonin

5-HT$_3$R

serotonin type 3 receptor

5-HT$_{3A}$R

serotonin type 3A receptor subunit

AHA

amygdalohippocampal area

BLa

anterior subdivision of the basolateral amygdalar nucleus

BLp

posterior subdivision of the basolateral amygdalar nucleus

BLC

basolateral nuclear complex of the amygdala

BMA

anterior basomedial nucleus

BMP

posterior basomedial nucleus

CB

calbindin

CCK

cholecystokinin

CL

lateral subdivision of the central nucleus

Coa

anterior cortical nucleus

Copl

posterolateral cortical nucleus

Copm

posteromedial cortical nucleus

CR

calretinin

GABA

gamma aminobutyric acid

Ldl

dorsolateral subdivision of the lateral amygdalar nucleus

Lvm

ventromedial subdivision of the lateral amygdalar nucleus

M

medial nucleus

PV

parvalbumin

SOM

somatostatin

ST

stria terminalis

VIP

vasoactive intestinal polypeptide
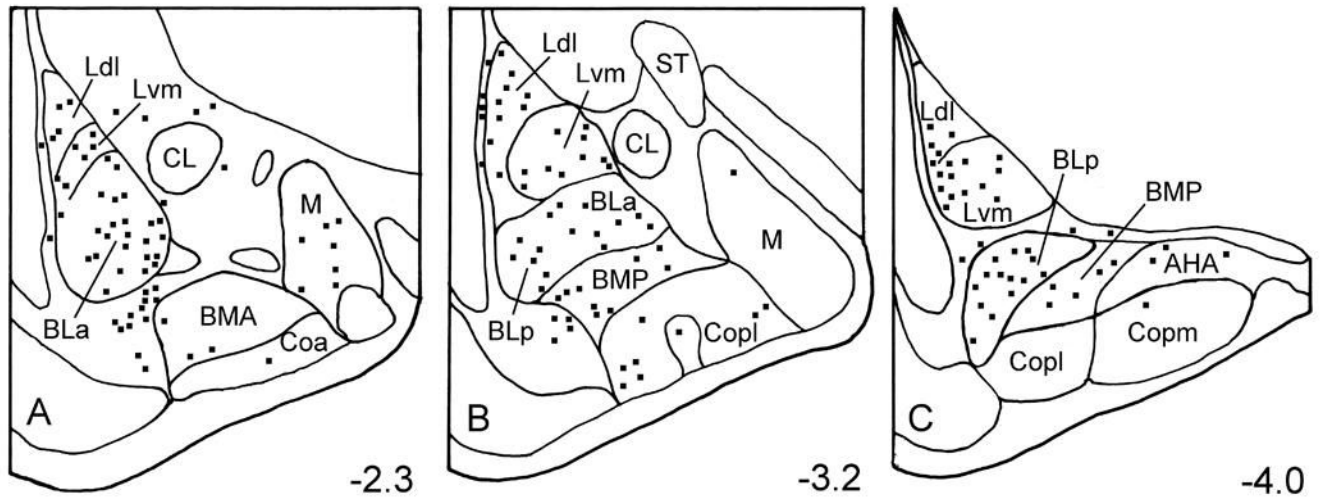
Fig. 1.
Distribution of 5-HT$_{3A}$R+ neurons in the rat amygdala. Neurons were plotted from two 50 μm thick sections at each level of the amygdala (Bregma −2.3, −3.2, and −4.0 of Paxinos and Watson, 1997). A third section located between the two sections was stained with cresyl violet to identify nuclear borders. Abbreviations: AHA, amygdalohippocampal area; BLa, anterior subdivision of the basolateral nucleus; BLp, posterior subdivision of the basolateral nucleus; BMA, anterior basomedial nucleus; BMP, posterior basomedial nucleus; CL, lateral subdivision of the central nucleus; Coa, anterior cortical nucleus; Copl, posterolateral cortical nucleus; Copm, posteromedial cortical nucleus; Ldl, dorsolateral subdivision of the lateral nucleus; Lvm, ventromedial subdivision of the lateral nucleus; M, medial nucleus; ST, stria terminalis.
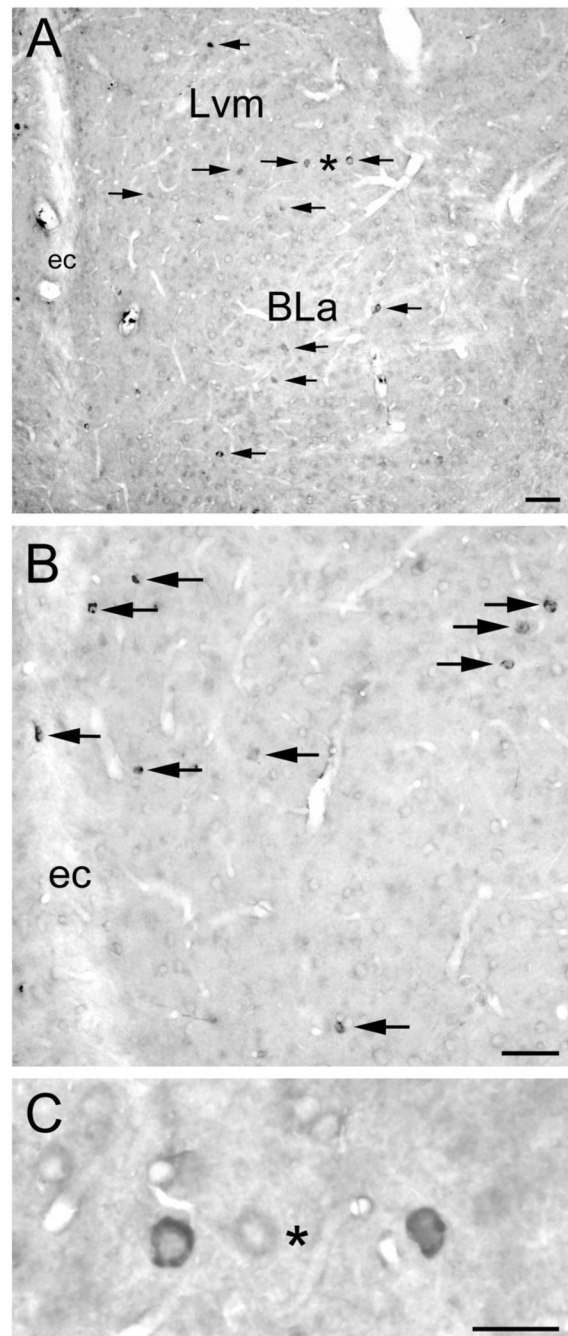
Fig. 2.
5-HT$_{3A}$R immunoreactivity in the BLC. Arrows indicate immunostained cells, all of which appear to be nonpyramidal. A) Low power photomicrograph of the anterior subdivision of the basolateral nucleus (BLa) and the ventromedial subdivision of the lateral nucleus (Lvm) at the bregma −2.6 level (Paxinos and Watson, 1997). Asterisk is located between two immunostained neurons shown at higher magnification in C. ec, external capsule. Scale bar = 50 μm. B) Higher power photomicrograph of the Lvm at the bregma −3.3 level. Scale bar = 50 μm. C) High power photomicrograph of the two immunostained neurons indicated by an asterisk in A, but mediolateral orientation is flipped. Scale bar = 25 μm.
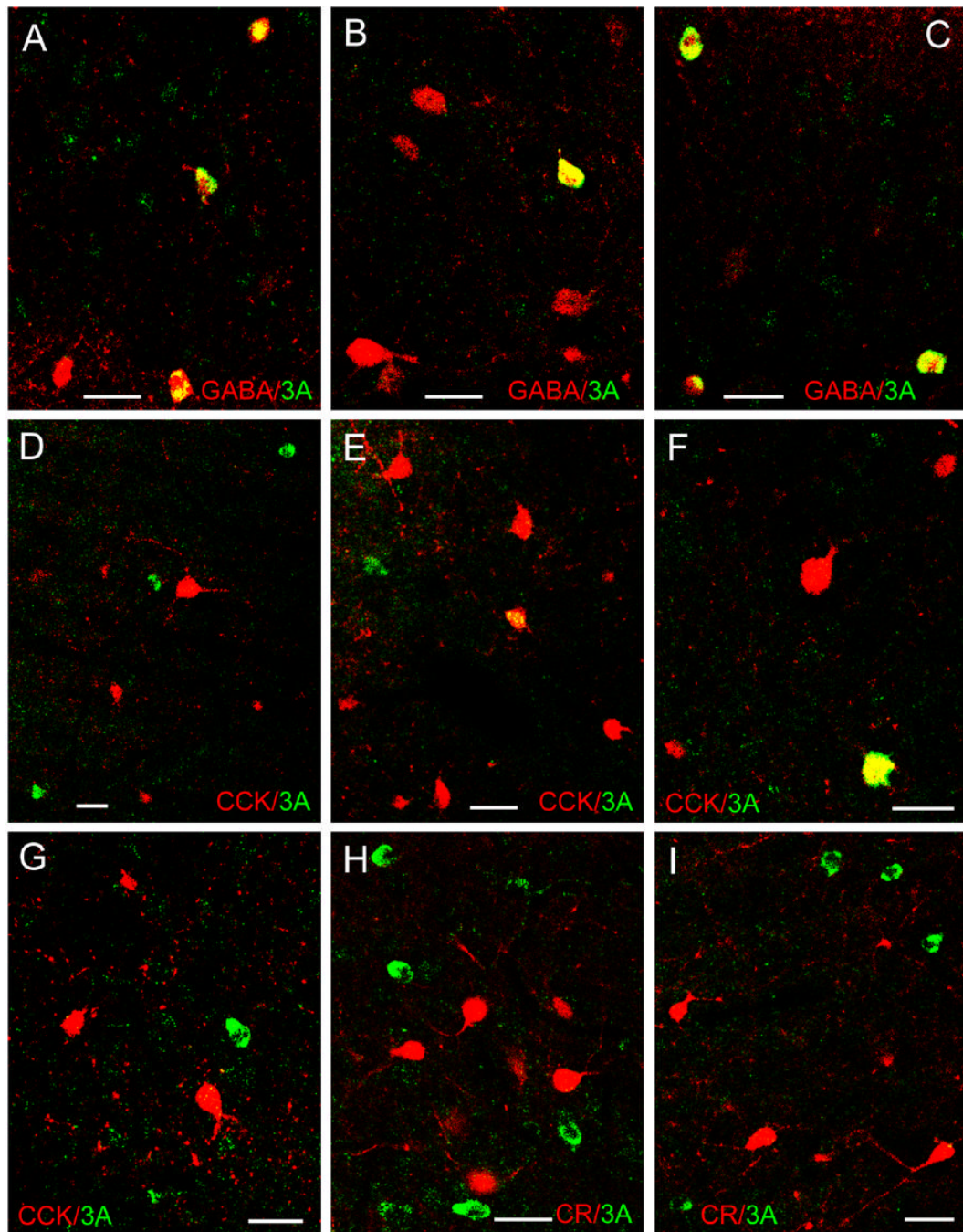
Fig. 3.
Dual localization of 5-HT$_{3A}$R immunoreactivity (green) and interneuronal marker immunoreactivity (red) in the BLC using immunofluorescence confocal laser scanning microscopy. All images are merged images. A–C) Dual localization of 5-HT$_{3A}$R (green) and GABA (red) immunoreactivity in the Lvm (A, C) and BLa (B). Note that all 5-HT$_{3A}$R+ neurons in these fields are double-labeled (yellow). D–G) Dual localization of 5-HT$_{3A}$R (green) and CCK (red) immunoreactivity in the Lvm (D, E) and BLa (F, G). Note that most CCK+ neurons are either large (type L neurons) or small (type S neurons). Most single-labeled 5-HT$_{3A}$R+ neurons (green) are medium-sized or small, whereas double-labeled 5-HT$_{3A}$R+/CCK+ neurons (yellow) are large. (H–I) Dual localization of 5-HT$_{3A}$R (green) and CR (red) immunoreactivity

in the BLa (H) and Lvm (I). Although both 5-HT$_{3A}$R+ neurons and CR+ neurons are robustly labeled, there are no double-labeled 5-HT$_{3A}$R+/CR+ neurons in these fields. All scale bars = 25 μm.
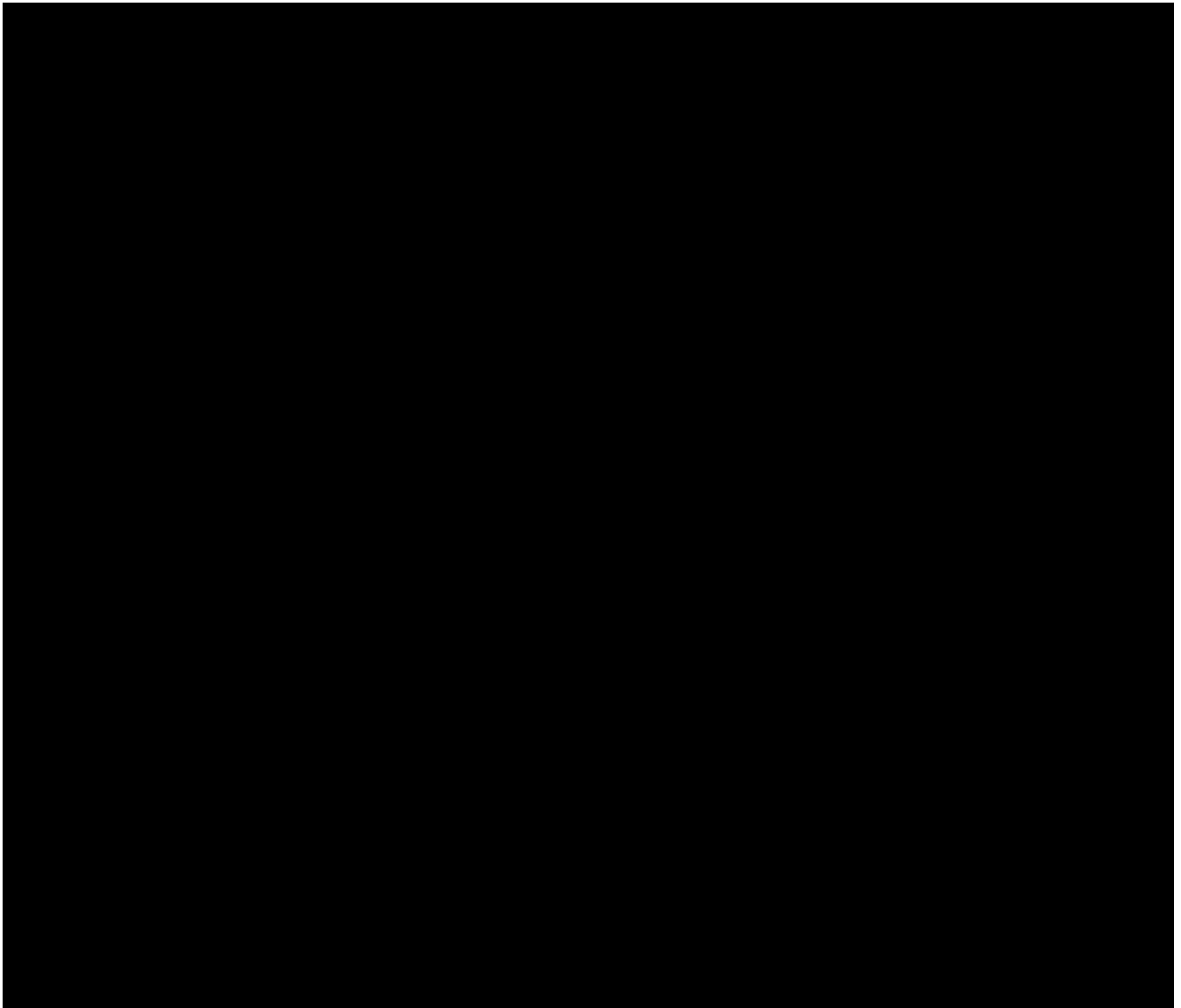
Fig. 4.
Diagram showing the overlap and relative proportions of neuronal populations containing calcium-binding proteins, neuropeptides, and 5-HT$_{3A}$R in the anterior subdivision of the basolateral nucleus (BLa). The relationship of the 5-HT$_{3A}$R+ subpopulation to the other interneuronal subpopulations is similar in the lateral nucleus. Thick lines indicate the 5-HT$_{3A}$R + subpopulation. Lines of intermediate thickness indicate two populations of calcium-binding proteins (CB+ and CR+ neurons), each of which is comprised of several subpopulations. Data on the relationships among SOM+, PV+, CB+, CR+, VIP, and CCK+ neurons is taken from previous quantitative studies (for a review see Mascagni and McDonald, 2003); the relative sizes of the rectangles representing these subpopulations are depicted in relation to the total population of GABA+ neurons in each nucleus (see Fig. 3 of McDonald and Mascagni, 2002). Note that the 5-HT$_{3A}$R+ subpopulation is relatively small and slightly overlaps the subpopulation of large type L CCK+ neurons (CCK$_{L)}$ that do not contain CB. In the present study it appeared that none of the 5-HT$_{3A}$R+ neurons corresponded to the subpopulation of small type S CCK+ neurons (CCK$_S$).

Table 1

Colocalization of 5-HT$_{3A}$R and interneuronal markers in the basolateral amygdala.

Marker	Nucleus	5-HT$_{3A}$R+Single-labeled Neurons	5-HT$_{3A}$R+/Marker +Double-labeled Neurons	Percentage of 5-HT$_{3A}$R+ Neurons Double-labeled
GABA	BLa	0	32	100% (32/32)
	Lvm	1	31	96.9% (31/32)
CCK	BLa	47	9	16.1% (9/56)
	Lvm	57	5	8.1% (5/62)
CR	BLa	49	1	2.0% (1/50)
	Lvm	48	2	4.0% (2/50)
PV	BLa	50	4[*]	7.4% (4/54)
	Lvm	30	0	0.0% (0/30)
SOM	BLa	30	0	0.0% (0/30)
	Lvm	30	0	0.0% (0/30)
CB	BLa	30	0	0.0% (0/30)
	Lvm	30	0	0.0% (0/30)
VIP	BLa	30	0	0.0% (0/30)
	Lvm	57	1	1.7% (1/58)

[*] All four neurons were very lightly stained for 5-HT$_{3A}$R