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Research report

Distribution of secretagogin-containing neurons in the basal forebrain of mice, with special reference to the cholinergic corticopetal system

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ABSTRACT

Cholinergic and GABAergic corticopetal neurons in the basal forebrain play important roles in cortical activation, sensory processing, and attention. Cholinergic neurons are intermingled with peptidergic, and various calcium binding protein-containing cells, however, the functional role of these neurons is not well understood. In this study we examined the expression pattern of secretagogin (Scgn), a newly described calcium-binding protein, in neurons of the basal forebrain. We also assessed some of the corticopetal projections of Scgn neurons and their co-localization with choline acetyltransferase (ChAT), neuropeptide-Y, and other calcium-binding proteins (i.e., calbindin, calretinin, and parvalbumin). Scgn is expressed in cell bodies of the medial and lateral septum, vertical and horizontal diagonal band nuclei, and of the extension of the amygdala but it is almost absent in the ventral pallidum. Scgn is co-localized with ChAT in neurons of the bed nucleus of the stria terminalis, extension of the amygdala, and interstitial nucleus of the posterior limb of the anterior commissure. Scgn was co-localized with calretinin in the accumbens nucleus, medial division of the bed nucleus of stria terminalis, the extension of the amygdala, and interstitial nucleus of the posterior limb of the anterior commissure. We have not found co-expression of Scgn with parvalbumin, calbindin, or neuropeptide-Y. Retrograde tracing studies using Fluoro Gold in combination with Scgn-specific immunohistochemistry revealed that Scgn neurons situated in the nucleus of the horizontal limb of the diagonal band project to retrosplenial and cingulate cortical areas.

1. Introduction

Cortically projecting cholinergic neurons of the basal forebrain are located in the medial septum (MS), the nucleus of the horizontal, vertical and lateral limbs of the diagonal band (HDB/VDB/LDB), the ventral pallidum (VP), in the basal part of substantia innominata (SIB), and in the extension of amygdala (EA) (Zaborszky et al., 2012).[1] This cholinergic space is surrounded by structures that are derived from the ventral telencephalic anlage, forming the subpallium, and feature very similar neuronal populations in terms of neurotransmitter and calcium-binding protein (CBP) content. Such anatomical formations include the accumbens nucleus (Acb), the caudate putamen (CPu), and the globus pallidus (GP) – structures that derive from the medial and lateral ganglionic eminences. Additional regions surrounding the cholinergic projection neurons

Abbreviations: ac, anterior commissure; aca, anterior commissure, anterior part; Acb, accumbens nucleus; AcbC, accumbens nucleus, core; AchSh, accumbens nucleus, shell; acp, anterior commissure, posterior part; AHA, anterior hypothalamic area; CaBP, calcium binding protein; Cb, calbindin; CeM, central amygdaloid nucleus, medial division; ChAT, choline acetyl transferase; CPu, caudate putamen (striatum); Cr, calretinin; EA, extension of the amygdala; HDB, nucleus of the horizontal limb of the diagonal band; ic, internal capsule; IPAC, interstitial nucleus of the posterior limb of the anterior commissure; LA, lateroanterior hypothalamic nucleus; LDB, nucleus of the lateral limb of the diagonal band; LPO, lateral preoptic area; LS, lateral septum; mfb, medial forebrain bundle; MPO, medial preoptic nucleus; MS, medial septum; Pa, paraventricular hypothalamic nucleus; Pe, periventricular hypothalamic nucleus; Pv, parvalbumin; Scgn, secretagogin; ST, bed nucleus of the stria terminalis; StHy, stirohyothalamic nucleus; STLD, bed nucleus of the stria terminalis lateral division, dorsal part; STLV, bed nucleus of the stria terminalis, lateral division, ventral part; STM, bed nucleus of the stria terminalis, medial division; STMV, bed nucleus of the stria terminalis, medial division, ventral part; Tu, olfactory tubercule; VDB, nucleus of the vertical limb of the diagonal band; VP, ventral pallidum.

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[1] The term 'extension of the amygdala' as defined by Paxinos and Franklin in The Mouse Brain in Stereotaxic Coordinates Fourth Edition (2013).

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include the base of the septum at the anterior commissure, the lateral preoptic area, and the internal capsule, structures that are derived from the telencephalic stalk (Garcia-Lopez et al., 2008). The CBP neurons, containing parvalbumin (Pv), calbindin (Cb) and calretinin (Cr) seems to form twisted bands along the longitudinal axis of a central dense core of cholinergic cells and do not seem to define well described anatomical areas (Zaborszky et al., 2005). In rodents, a small percentage of CBP-containing neurons project to the cerebral cortex (Gritti et al., 2003; Zaborszky et al., 1999).

Secretagogin is an EF-hand CBP, containing a helix-loop-helix structural domain that is expressed in the forebrain, including the neocortex in both developing and adult mice (Mulder et al., 2009). It binds four Ca^{2+} ions with low affinity, undergoes Ca^{2+}-induced conformational changes, and interacts with synaptosomal-associated protein 25 (Rogstam et al., 2007). Secretagogin is considered as a Ca^{2+} sensor protein that regulates cellular activity.

Calcium-binding proteins in the brain play important roles in the initiation and maintenance of long-term potentiation (LTP), hence in learning and memory (Martin et al., 2012).

Regarding the functional importance of the CBPs, single cell RT-PCR in basal forebrain tissue cultureindicates that cholinergic neurons contain calretinin mRNA (Murchison and Griffith, 2007). It was also showed that the Ca^{2+} buffering capacity of basal forebrain cholinergic neurons robustly increases in aged, cognitively impaired rats, indicating a strong correlation between Ca^{2+}-dependent signaling, neuronal plasticity, and synaptic function (Murchison et al., 2009). Disturbance of neuronal Ca^{2+} homeostasis is considered to be important in precipitating age-related cognitive impairments (Burke and Barnes, 2010). Scgn is expressed in the forebrain, including the neocortex in both developing and adult mice (Mulder et al., 2009). Furthermore, it has been suggested that Scgn has a neuroprotective effect in a transgenic model of Alzheimer's disease (Attems et al., 2011). Cholinergic basal forebrain corticopetal neurons degenerate in Alzheimer's disease with concomitant decrease of cholinergic markers in the cerebral cortex (Potter et al., 2011). However, no immunocytochemical study has confirmed the presence of Scgn in corticopetal cholinergic or non-cholinergic neurons in mice.

The aim for this study is to identify the precise expression pattern of the Scgn-containing neurons in the basal forebrain cholinergic space, and define the cholinergic or non-cholinergic cell populations that express Scgn. We describe the distribution of Scgn-containing neurons in the basal forebrain and investigate the co-localization between Scgn and choline acetyltransferase (ChAT; a cholinergic marker), other CBPs (Pv, Cb, and Cr), and neuropeptide-Y (NPY). Finally, we examine whether Scgn-containing neurons of the basal forebrain project to the neocortex.

2. Materials and methods

2.1. Animals

Experiments were performed on male adult (3 months old) C57BL/6J, ChAT(BAC)-eGFP transgenic mice (JAX 007902, B6.Cg-Tg(RP23-268L19-EGFP)2Mik/J; stock number 007902; Jackson Laboratory, Maine, USA) ($n = 10$) and NPY-*gfp* (B6.FVB-Tg(Npy-hrGFP)1Lowl/J; stock number 006417; The Jackson Laboratory, Maine, USA) mice ($n = 4$) weighing 20–30 g. Mice were housed in a temperature-controlled environment, with a normal 12:12 h light–dark cycle, with free access to food and water. All mice were monitored daily. All procedures were in accordance with the regulations of the Animal Care and Ethics Committee of the University of New South Wales (07/108B, 08/48B).

2.2. Scgn-staining for bright fieldmicroscopy

Wild-type mice ($n = 4$) were euthanized with sodiumpentobarbitone (0.1 ml, 200 mg/ml) injected intraperitoneally and transcardinally perfused with ice-cold saline (0.9% NaCl in ddH_2O) followed by 4% depolymerizedparaformaldehyde in 0.1 M PBS (100 mL with 4 mL/min rate). Brains were removed and stored in the same fixative at room temperature for 6 h. Brains were immersed into 30% sucrose in 0.1 M PBS overnight at room temperature and were sectioned at 40 μm with a cryostat the next day. Sections were collected from +0.5 mm to −2.5 mm from the Bregma. Sections were treated in 1% hydrogen peroxide (in PBS, 10 min) to reduce non-specific background staining, prevent any possible cross-reaction, and reduce intrinsic hydrogen-peroxide activity during the subsequent immunocytochemical procedures. Sections were incubated in normal serum [2% normal donkey serum (Jackson Immunoresearch Laboratories, West Grove, PA) in PBS, 2 h] to prevent non-specific binding. To visualize Scgn-positive neurons for bright-field microscopy analysis, the sections were incubated with a rabbitanti-secretagogin antibody (1:1000 for 48 h at 4 °C) with 0.1% TritonX-100 followed by a biotinylated, donkey anti-rabbit IgG (Jackson Immunoresearch Laboratories; 1:200 in the antibody diluent, overnight). At the end of the incubation with the secondary antibody, Vectastain peroxidase ABC (1:500 in PBS, 2 h) was employed. The peroxidase reaction was carried out using a developer solution containing 0.4 mg/ml DAB and 0.0006% hydrogen peroxide dissolved in TBS that resulted in the development of brown deposits in Scgn-containing profiles. The sections were mounted onto glass microscope slides, air-dried overnight, cleared in xylenes (Fisher Scientific; 2 × 10 min), and cover slipped with DPX (Electron Microscopy Sciences). As for negative control, the rabbitanti-secretagogin antibody was eliminated from the incubation solution and the same procedure was carried out as described above. We found no DAB labeled neurons or processes. For positive control, we systematically checked the areas of the indusium griseum, that contains well-characterized and strongly labeled Scgn-containing neurons (Mulder et al., 2009). The Scgn antibody by Sigma–Aldrich was first described and characterized by the same group in the telencephalon (Mulder et al., 2009, 2010).

2.3. Fluorescent double labeling

Tissue was obtained from wild type ($n = 5$), ChAT(BAC)-eGFP ($n = 3$) and NPY-GFP ($n = 3$) transgenic mice prepared for immunohistochemisty. Fluorescent double staining was performed on series of sections, to visualize Scgnin neurons expressing ChAT, Pv, Cr, Cb, or NPY. Series of sections were immersed into the mixture of the following antibodies (Table 1). For negative control, the primary antibodies were omitted and the same procedure was carried out that resulted in no fluorescent signal. The tissue was incubated without the primary antibody, followed by incubation with secondary antibodies.

For the double labeling of Scgn/Cr-Pv-Cb forebrain series of sections were incubated in a cocktail of Scgn-and Pv-, Cb-, or Cr-specific primary antibodies with 0.1% Triton X-100 for 48 h at 4 °C, followed by the secondary antibodies for each primary antibody for 2 h. For double labeling of Scgn with ChAT-EGFP and NPY-GFP, sections from the transgenic brains were incubated in Rb-α-Scgn primary antibody for 48 h at 4 °C, followed by the secondary antibody against Scgn (Alexa Fluoro GtαRb IgG 594) for 2 h at room temperature. The sections were then mounted on gelatin-coated slides and immediately cover slipped with Dako fluorescence mounting medium (Dako Australia Pty. Ltd., Victoria, Australia) (Table 1). To visualize and photograph eGFP, Alexa Fluoro 488 and FITC containing neurons a Zeiss filter set #13 was used with excitation 470 nm, emission 505–530 nm with beam splitter at 495 nm. To visualize Alexa Fluoro 594, we used a filter set (#00) suggested by Zeiss with excitation at 530–589 nm, emission at 615 nm with a beam splitter at 600 nm.

Fluorescent images were captured under a Zeiss AxioImager. M2 upright research microscope with ApoTome System, and also equipped with a monochrome digital CCD microscopy camera (Hamamatsu Orca-R2).

2.4. Retrograde tracing

Wildtype ($n = 6$) and ChAT-gfp ($n = 6$) mice were anesthetized by using ketamine–xylazine mixture (80 mg/kg and 10 mg/kg, respectively) injected intraperitoneally and were subjected to surgery when there was no behavioral response to tail pinch. Animals were fixed in a stereotaxic instrument (TSE Systems, Bad Hamburg, Germany) and a sagittal incision was made along the midline of the head to expose the skull. A small hole was drilled according to stereotaxic coordinates measured from the Bregma above the cortical areas. Retrograde tracer Fluoro-Gold™ (FG, Fluorochrome, Denver, CO, USA) was diluted to 5% with distilled water and was injected to the areas of the retrosplenial cortex (M/L: 0.5 mm, A/P: −1.8 mm, D/V: 0.51 mm) and the cingulate cortex (M/L: 0.4 mm, A/P: −0.62 mm, D/V: 1.0 mm), using a Hamilton syringe and applying pressure injection. Coordinates were based on The Mouse Brain in Stereotaxic Coordinates (Paxinos and Franklin, 2007). After one week of survival time, the mice were euthanized and perfused using the method described above. Brains were removed and sectioned as described above.

All the treatments described below were carried out at room temperature unless otherwise specified. Series of sections were collected and stained to visualize Scgn-containing neurons by using rabbitanti-secretagogin primary antibody (HPA006641, Sigma–Aldrich, Sydney, Australia; 1:1000) for 48 h at 4 °C with 0.1% Triton X-100 followed by the secondary antibody Alexa Fluor® goatanti-rabbit 594 IgG (Invitrogen, Mulgrave, Australia, 1:200 for 2 h). Sections were mounted on gelatin-coated slides and cover slipped with Dako fluorescence mounting medium for fluorescence microscopy analysis.

2.5. Stereology

The areas of interest, the nucleus of the horizontal limb of the diagonal band and the extension of the amygdala, were traced in Nissl-stained sections at

Table 1
List of antibodies used to study the co-localization of Scgn with ChAT, Pv, Cr, and Cb.

Primary antibodies	Antibody raised in	Dilution	Source	Catalog number
Anti-secretagogin (Scgn) (polyclonal)	Rabbit	1:1000	Sigma–Aldrich	HPA006641
Anti-calretinin (Cr) (polyclonal)	Goat	1:500	MerckMillipore	AB1550
Anti-parvalbumin (Pv) (monoclonal)	Mouse	1:2000	Sigma–Aldrich	P3088
Anti-calbindin (Cb) D-28k (monoclonal)	Mouse	1:1000	Sigma–Aldrich	C9848

Secondary antibodies with fluorochrome	Flurochrome	Dilution	Source	Catalog number
Goat anti-rabbit IgG (H+L)	Alexa Fluor® 594-AffiniPure (A = 591, E = 614)	1:200	Jackson Immuno	111-585-003
Donkey anti-goat IgG (H+L)	Alexa Fluor® 488-AffiniPure (A = 493, E = 519)	1:200	Jackson Immuno	705-545-003
Goat anti-mouse IgG FITC	Fluorescein isothiocyanate (A = 495, E = 520)	1:200	Sigma–Aldrich	F0257

4× magnification in 3 animals, on 6 sections per animal. For stereology, the total Scgn cell number in the EA and FG and the number of Scgn neurons in the HDB were estimated using the Optical Fractionator principle (West et al., 1991) with StereoInvestigator software 8.0 (MBF Bioscience, Williston, VT, USA) on a Zeiss AxioImager.M2 research microscope equipped with ApoTome (Carl Zeiss AG, Oberkochen, Germany). The size of the counting frame was 100 × 100 μm for the EA and 50 × 50 μm for the HDB. The area counted (XY) was 10000.0 μm^2 in the case of the EA and 2564.0 μm^2 in the case of the HDB (Table 2). Neurons were only counted if the neuron nucleolus was inside the counting frame and the neuron was not touching the excluding borders. Section thickness was measured at every sampling site. Cell counts were performed using a 63× oil immersion objective. This approach combines fractionator sampling with the optical dissector and returns an unbiased estimate of the total cell number regardless of shrinkage or other types of tissue deformation (Gundersen et al., 1988). Additionally, we measured the perimeter (the total distance around the edge of the cell body) of at least 20 neurons from each sampling site to compare the cell sizes. The groups were compared using one-way analysis of variance (ANOVA) and Tukey post hoc test was applied for all pair-wise multiple comparisons. The level of significance was set at $p < 0.001$.

2.6. Image analysis

In case the overall lightness or darkness of the image or the contrast between the stained structures and background was not satisfactory, contrast and brightness was adjusted digitally to provide easy viewing. All groups of pictures were assembled and lettering was added using Adobe Photoshop CS6 and Lightroom3. Sections were analyzed and mapped with MBF StereoInvestigator (MBF Bioscience, VT, USA).

3. Results

3.1. Distribution of secretagogin-containing neurons in the cholinergic basal forebrain

Secretagogin-containing neurons are located in close proximity of the cholinergic neurons in the areas of the HDB (Fig. 1A–D), especially the lateral and dorsal parts of the HDB accommodated numerous Scgn-positive cell bodies (Fig. 1A, inset). A few Scgn-containing neurons were found in the medial septum (MS) (Fig. 1A). The nucleus of the vertical limb of the diagonal band (VDB) only sporadically contained Scgn positive cells (Fig. 1A). Moderate numbers of labeled cells were found in the interstitial nucleus of the posterior component of the anterior commissure (IPAC). In the medial part of the extension of amygdala (EA), a substantial number of large Scgn-positiveneurons were found (from −0.30 mm to −1.10 mm from the Bregma), forming a prominent cell ensemble occupying the center of the basal forebrain (Fig. 1D). The areas of the ventral pallidum (VP) contained cells only occasionally. The paucity of Scgn-labeled cells makes it easy to differentiate the VP from the IPAC, EA, SIB, and the LPO, whereas in the case of the HDB it becomes more distinguishable from the LDB and the nuclei of the amygdala.

3.2. Localization of secretagogin-containing neurons in other forebrain regions surrounding the cholinergic basal forebrain

We did not find Scgn-positive neurons in the areas of the septofimbrial nucleus (SFi), although this area had strong neuropil labeling. In the areas of the lateral septal nucleus (LS) and the septohypothalamic nucleus (Shy), we found a moderate number of Scgn-positive neurons, along with a moderately strong labeling of the neuropil. A few, sporadically distributed neurons were located in the lambdoid septal zone (Ld), lateral to the medial septum (Fig. 1A).

More posteriorly, in the median preoptic nucleus (MPO), we found Scgn-positive neurons in relatively large number, while the remaining preoptic nuclei, such as the lateral, anteroventral, ventrolateral, and ventromedial parts, were free of labeled cells (Fig. 1B and C). The lateral preoptic area (LPO) was not labeled for Scgn (Fig. 1C). The medial part of the bed nucleus of the stria terminalis (STM) expressed numerous, small Scgn-positive cells, as well as dense local axon arborizations (Fig. 1D). The extension of amygdala (EA), as well as the medial interstitial nucleus of the posterior limb of the anterior commissure (IPAC) contained a large number of Scgn-positive neurons (Fig. 1D–F).

3.3. Co-localization of Scgn with ChAT, NPY, and CBPs

3.3.1. Choline acetyltransferase (ChAT)

We investigated whether Scgn is co-localized with the cholinergic marker (ChAT) in the basal forebrain by using double

Table 2
Parameters of the unbiased cell counting results by using the Optical Fractionator workflow in StereoInvestigator in the extension of the amygdala and the nucleus of the horizontal limb of the diagonal band. Number of $Scgn^+/FG^-$, $Scgn^-/FG^+$ and $Scgn^+/FG^+$ containing neurons were counted in the EA and HDB. We included the estimated number of Scgn containing neurons in these areas.

Area	Animal ID	Cut section thickness (μm)	Mean measured section thickness (μm)	Evaluated series	Counting frame size/area (μm^2)	Dissector size	Number of Scgn neurons counted	Number of FG neurons counted	Number of FG/Scgn neurons counted	Estimated population of Scgn neurons
EA	FG3	40	35.3	1:3	100 × 100/10,000	150 × 150	178	5	2	2191
	FG5	40	31.7	1:3	100 × 100/10,000	150 × 150	198	7	3	2164
	FG6	40	35.6	1:3	100 × 100/10,000	150 × 150	136	4	3	1633
HDB	FG3	40	32.6	1:3	50 × 50/2500	100 × 100	35	212	17	178
	FG5	40	26.3	1:3	50 × 50/2500	100 × 100	33	196	17	159
	FG6	40	24.7	1:3	50 × 50/2500	100 × 100	24	214	15	126

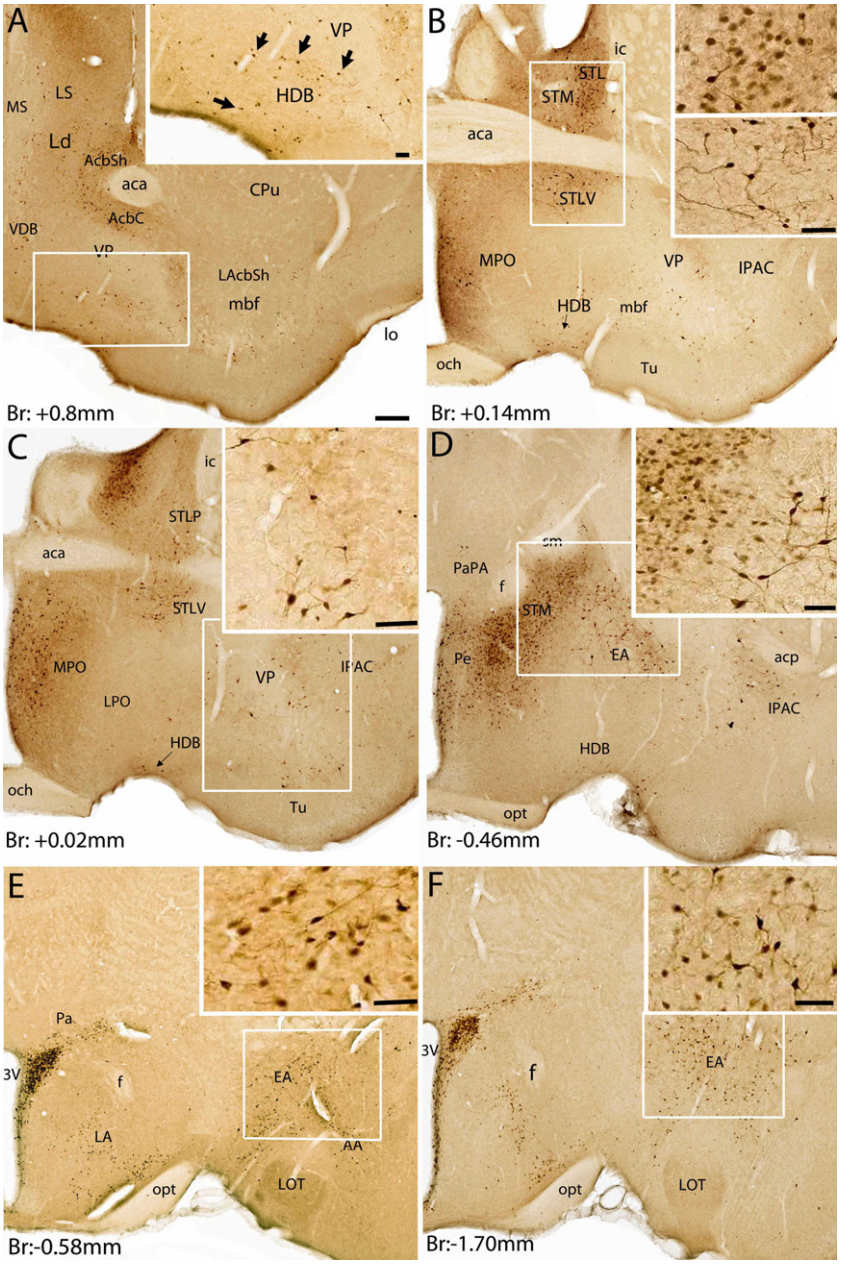


Fig. 1. Secretagogin containing neurons in the cholinergic space at various levels of the brain. (A) Scgn neurons were found in the lateral part of the MS but not in the central part. They were also found in the AcbSh, AcbC, and in the LS. Neither cell or neuropil positivity was not found in the VP. (B) Scgn is strongly expressed in the STM, STL and HDB. (C) Scgn is expressed in the STLP and the STLV, as well as in the IPAC. No Scgn-containing profiles can be found in the LPO. (D) Large Scgn-containing neurons are found in the EA and in the StHy. (E and F) There was no labeling in the LDB but the EA showed strong Scgn expression at every level. Scale bar represents 250 μm in the main figures and 100 μm in the insets.

immunohistochemistry. Significant co-localization was found in the areas of the medial part of the extension of amygdala (Fig. 3A3), the IPAC (Fig. 2A4), the bed nucleus of stria terminalis (Fig. 2A2), and occasionally in the dorsal part of the HDB. Most of double-labeled neurons were located in the more caudal, dorsal part of the extension of the amygdala that is in close proximity of the bed nucleus of stria terminalis. These results were also confirmed by using Scgn immunohistochemistry on ChAT(BAC)-eGFP transgenic mouse sections in the same areas.

3.3.2. *Parvalbumin, calbindin, calretinin*

Out of the three classical calcium binding protein markers, Cr was the only one that showed co-localization with Scgn and this was principally in the accumbens nucleus (Fig. 2B1), the bed nucleus of stria terminalis (STMP; Fig. 2B2), the extension of amygdala (Fig. 3B3), and IPAC (Fig. 3B4).

3.3.3. *Neuropeptide-Y*

We found no co-localization of Scgn and NPY in any of the Scgn populated areas in the basal forebrain (Fig. 3E1–E4).

3.4. *Corticopetal Scgn-containing neurons in the basal forebrain*

The retrosplenial/cingulate cortical areas receive significant projections from the basal forebrain areas, especially from the HDB (Gonzalo-Ruiz and Morte, 2000; Senut et al., 1989). In the present experiments, the retrograde tracer Fluoro-Gold (FG) was delivered in the retrosplenial/cingulate cortex and FG-labeled

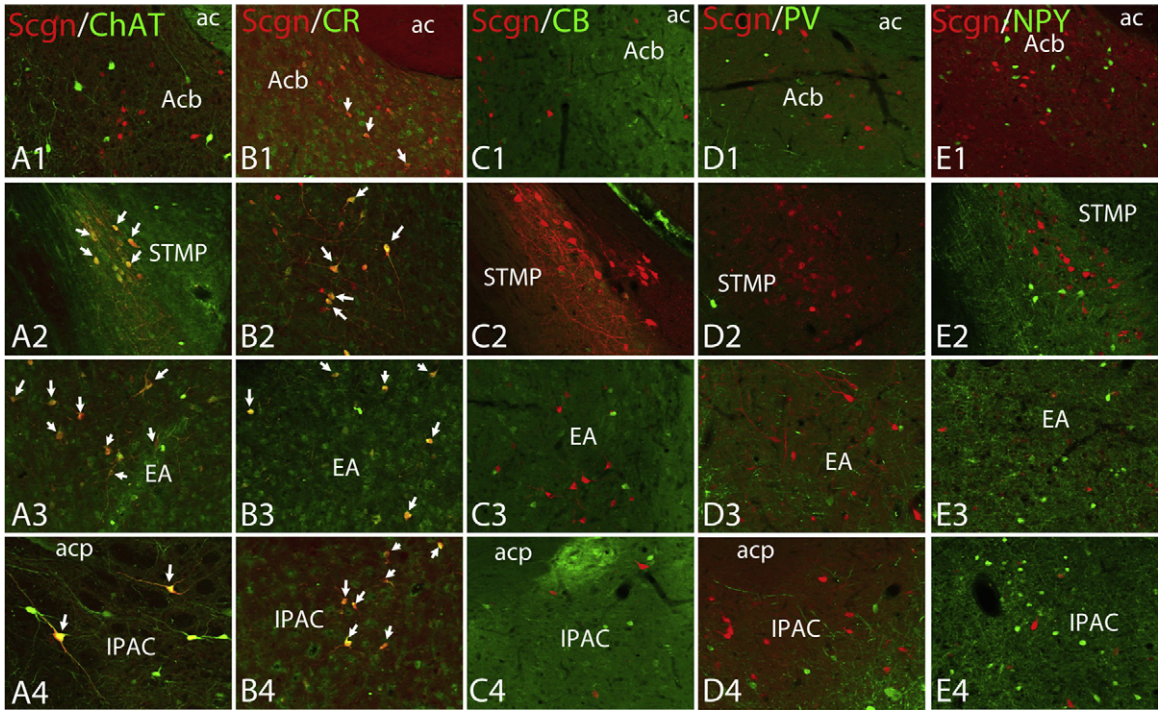


Fig. 2. Co-localization of Scgn with ChAT, Pv, and Cb in neurons of the basal forebrain. A1–A4: Scgn/ChAT: B1–B4: Cr; C1–C4 Cb; D1–D4 Pv; E1–E4 NPY-GFP. Horizontal rows: A1–E1: nucleus accumbens; A2–E2: STMP; A3–E3: extension of the amygdala; A4–E4; IPAC. We found strong co-localizations with ChAT positive neurons in the EA (A–C) as well as in the IPAC regions. In contrast, Scgn seems to be present in completely non-overlapping populations with other CBPs, such as Pv (D–F) and Cb (G–I). Arrows point to cells that contain both Scgn and the relevant marker. Co-localization of the red Scgn with the other green fluorochrome resulted in yellow color in the merged photos. Scale bar represents 100 μm.

neurons were mapped. In the basal forebrain areas, FG significantly labels neurons in the HDB and less prominently the EA (Fig. 3A–H). FG-labeled neurons were counted in the entire HDB (extending between +0.86 mm and −0.30 mm from the Bregma (Paxinos and Franklin, 2007); by using the Optical Fractionator workflow with StereoInvestigator. The estimated number of Scgn containing neurons in the HDB was 154.3 ± 26.31 while FG-labeled cells in the HDB was 414.6 ± 19.7 (n = 3; number of sections analyzed = 7/animal). However, only 16.3 ± 1.15 (3.93%) of those FG labeled neurons co-expresses Scgn. In other words, only 10.5% of the all the Scgn neurons located at the dorsal part of the HDB project to the retrosplenial cortex. While the number of Scgn containing neurons in the EA is significantly larger than what we found in the HDB (the average estimated Scgn cell number in the EA is 1996 ± 314.65) the number of both $FG^+/Scgn^-$ and $FG^+/Scgn^+$ were too small to estimate by using the Optical Fractionator workflow (Table 2). We demonstrate representative photographs showing FG-labeled neurons in the areas of the HDB (Fig. 3I1–J1), Scgn-containing neurons in the same area (Fig. 3I2–J2) along with their overlay images (Fig. 3I3–J3).

3.5. Size comparison of Scgn-containing neurons

The synopsis of the size comparison measurements is shown in Fig. 4. While the Scgn neurons in the EA are large, with well-distinguished and strongly labeled dendrites that can be followed for several tens of micrometers, the Scgn neurons in the bed nucleus of stria terminalis (ST) and accumbens nucleus (Acb) are smaller, lack Scgn-positive processes, and they form strongly aggregated cell clusters. We measured and compared the perimeter of randomly sampled neurons from the accumbens nucleus shell regions (AcbSh), the dorsal and ventral part of the lateral bed nucleus of stria terminalis (STLD and STLV), the extension of the amygdala, and nucleus of the horizontal limb of the diagonal band. The average Scgn neuron perimeter in the AcbCh was 33.2 ± 5.63 μm (n = 79), in the STLD it was 35.6 ± 4.76 μm (n = 91), and in the STLV it was 38.5 ± 6.46 μm. These values are significantly smaller ($p < 0.001$) than the average perimeter of Scgn neurons in the basal forebrain areas, including the EA (47.6 ± 7.01 μm (n = 98)) and HDB (47.2 ± 7.05 μm (n = 55)). The average perimeter of the cholinergic neurons (considering all of them and not only those that expressed Scgn) in the basal forebrain was 62.2 ± 11.4 μm (n = 123). This proved to be significantly larger ($p < 0.001$) than that of any other Scgn neuronal groups (Fig. 4).

4. Discussion

Secretagogin expression has been reported to be present in the rodent basal forebrain areas (Mulder et al., 2010). However, the exact expression pattern regarding its anatomical localization had not been thoroughly examined. Since other CBPs, such as Pv, Cb and Cr were shown to be abundant in basal forebrain areas rich in cholinergic projection neurons, including the medial septum, vertical and horizontal diagonal band nuclei and in the extension of the amygdala (Zaborszky et al., 2005); we expected to find a high number of Scgn containing neurons in these areas as well. While Scgn is strongly expressed in the extension of the amygdala, and sparsely in the medial and lateral septum, vertical, horizontal and lateral diagonal band nuclei, their abundance is not comparable of Pv, Cb and Cr in these regions, as described by Zaborszky et al. (Zaborszky et al., 2005). In addition, Scgn cells were only occasionally observed in the ventral pallidum, compared to Pv or Cr, that are more prominently present (Zaborszky et al., 2005). On the other hand, in the areas neighboring the cholinergic basal forebrain space, we found strong Scgn expression in the bed nucleus of stria terminalis and in the shell of the accumbens nucleus, and moderate labeling in the striatal areas, such as the IPAC and the core of the accumbens nucleus. Since, the average perimeter of the Scgn

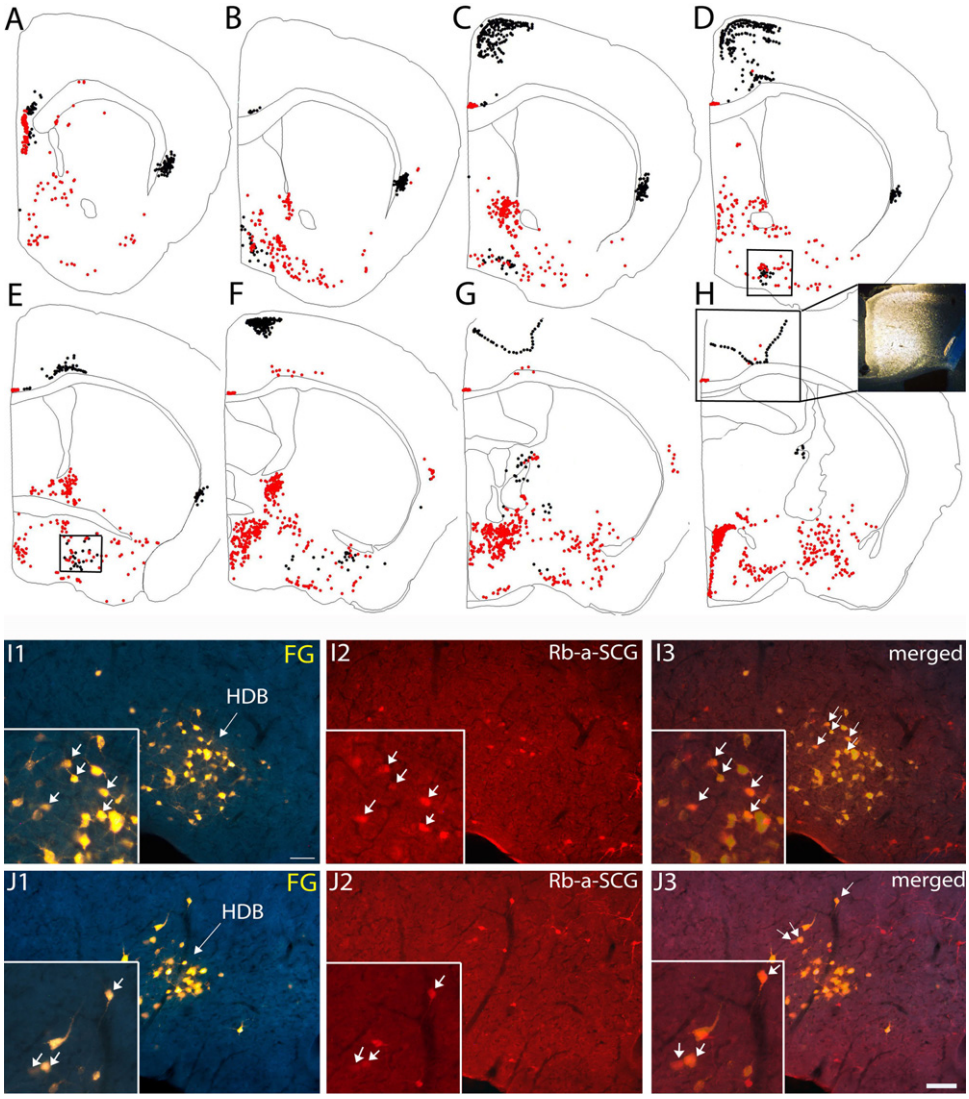


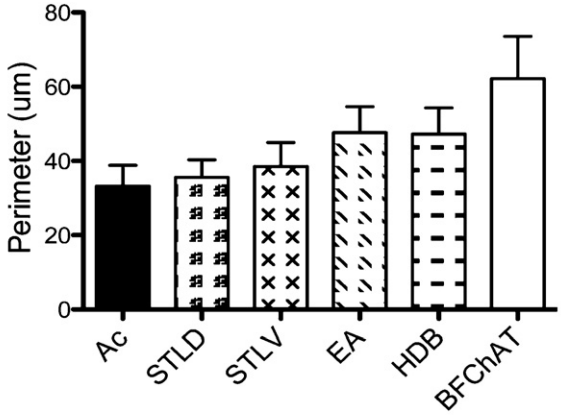
Fig. 3. (A–H) Distribution of FG- and Scgn-containing neurons in the cholinergic space. The inset demonstrates the FG injection site in the retrosplenial cortex. FG-containing, retrogradely labeled neurons were found in the MS, HDB, and EA areas in the basal forebrain (labeled with black dots), as well as in the IPAC and various thalamic nuclei at the levels examined (Bregma: +0.8–1.2 mm). Scgn-containing neurons are overlapping with the FG-labeled neurons in the HDB and EA (labeled with red dots). (I–J) Photographs presented in rows I and J were taken from the area represented by a black rectangle in panels D and E. A small but consistent ratio of Scgn-containing neurons (8.0%) accommodated by the HDB is proved to be projection neurons, sending their axons to the retrosplenial cortex. Double-labeled ($Scgn^+/FG^+$) neurons tend to be localized at the dorsal part of the HDB (marked by white arrows). These neurons are very likely to be cholinergic as well, based on their size and location. Scale bar represents 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

neurons located in the EA and the HDB is significantly larger than those in the accumbens nucleus and the bed nucleus of stria terminalis, suggesting the existence of at least two different Scgn neuronal populations. These findings indicate that Scgn might play a role in the functions of the bed nucleus of the stria terminalis, such as basic autonomic responses (Moga et al., 1989), sets of motor behaviors (Dong and Swanson, 2006a,b,c), circadian rhythmicity (Amir et al., 2004), stress- and anxiety-related behaviors (Ventura-Silva et al., 2012), and in alcohol abuse (Kash, 2012). The accumbens nucleus is implicated in neuropsychiatric disorders, such as schizophrenia and certain calcium binding protein levels (Cb1, Cb2 and hippocalcin) were shown to be altered due to developmental vitamin D deficiency (McGrath et al., 2008). Since high expression of Scgn is evident in the accumbens nucleus, it would be important carefully examine its functional role in psychiatric disorders.

Scgn is sparsely co-localized with ChAT in the medial part of the bed nucleus of the stria terminalis (STMP), the EA, the IPAC, and in the HDB, and is also co-localized with Cr in the accumbens nucleus. This indicates that in terms of their CBP content, these neuron populations are rather heterogenic. While the CBP content does not define the main functional role of the cells, it can modulate their firing properties and the timing of the neurotransmitter release by eliminating intracellular Ca^{2+} from the cytosol.

Cortically projecting neurons in the basal forebrain were characterized as cholinergic, GABAergic and peptidergic (Fisher et al., 1988; Mascagni and McDonald, 2009; Zaborszky et al., 1999). A few Cb- and Cr-containing neurons were also described in rat to send their axons to the neocortex but their neurotransmitters were not identified (Gritti et al., 2003; Zaborszky et al., 1999). Our findings show that a small percentage of cortically projection neurons contain Scgn, however their neurotransmitter identity is unknown. The functional significance of Scgn in different neuronal population in the basal forebrain areas and the rare combination of $ChAT^+/Scgn^+$ is yet to be investigated.

Calcium binding proteins are known to play a crucial role in the elimination of increased calcium ion concentration. Excessive amount of Ca^{2+} accumulation causes the mitochondria to induce



	Ac	STLD	STLV	EA	HDB	BFChAT
Ac		ns	**	***	***	***
STLD	ns		ns	***	***	***
STLV	**	ns		***	***	***
EA	***	***	***		ns	***
HDB	***	***	***	ns		***
BFChAT	***	***	***	***	***	

Fig. 4. Average perimeter of Scgn-containing neurons in the shell of the nucleus accumbens (AcbSh; $n = 79$), in the dorsal (STLD; $n = 91$), ventral (STLV; $n = 60$) part of the bed nucleus of stria terminalis, the extension of the amygdala (EA; $n = 98$), and in the horizontal limb of the diagonal band (HDB; $n = 55$) compared to the average cell-size of the cholinergic neurons in the basal forebrain (ChAT; $n = 123$). One-way analysis of variance: $p < 0.0001$; Tukey's multiple comparison test: $p < 0.001$. The table presents the levels of significance when the average perimeters of the Scgn-containing neurons in the areas of the accumbens (Ac), laterodorsal bed nucleus of stria terminalis (STLD), lateroventral bed nucleus of stria terminalis (STLV), the extension of the amygdala (EA), and the nucleus of the horizontal diagonal band (HDB) were compared with the perimeters of cholinergic neurons of the basal forebrain.

oxidative stress that can eventually lead to cell damage and eventually cell death (Weber et al., 2010). During normal aging or Alzheimer's disease (AD), there is a significant loss of cholinergic neurons in the basal forebrain, and while CBPs, such as calretinin, calbindin and parvalbumin do not co-localized with ChAT in rodents, in primates, including humans, cholinergic neurons were found to express Cb-D28 (Celio and Norman, 1985; Smith et al., 1994). The cholinergic cell loss is accompanied with concomitant decrease of calbindinin the neocortex and with a reduction of calbindinin the basal forebrain (Bu et al., 2003; Geula et al., 2003a,b; Wu et al., 2003). Since cholinergic neurons, that display a loss of calbindinin AD, show immunoreactivity for the apoptotic signals and for abnormally phosphorylated tau protein, the loss of calbindin and simultaneous increase of intracellular Ca^{2+} may be an important process in the pathologic cascade leading to degeneration of basal forebrain cholinergic neurons in this disease (Wu et al., 2005). Calbindin was suggested to have a neuroprotective role during neurodegeneration (Riascos et al., 2011). Although Scgn is presented in the human forebrain in adults and in the areas of hippocampus, subiculum, thalamic territories and germinal layers during development (Attems et al., 2007; Mulder et al., 2010), there is little known about its definite functional role.

Outside of the basal forebrain, a loss of parvalbumin and calretinin immunoreactivity was reported in the hippocampus in the CA1-2 sectors and in the dentate gyrus and hilus of APP(SL)/PS1 KI transgenic mice, and this loss was also found in the parvalbumin- and calretinin-immunoreactivities in neurons of the dentate gyrus of postmortem brain specimens obtained from patients with AD (Geula et al., 2003a). Attems et al. (2011) reported a reduced Scgn expression in the hippocampus of P301L tau transgenic mice. These results suggest a link between tau and Scgn expression in the rodent hippocampus (Attems et al., 2011). Besides the loss of parvalbumin and calbindin expression during neurodegeneration, recently Attems et al. described the loss of Scgn containing neurons in the aged human olfactory bulb (Attems et al., 2012). These results clearly indicate that Scgn containing neurons in the human olfactory system represents a well-differentiated neuron population, however the function of Scgn in the olfactory areas remains unknown. One of the appealing hypothesis regarding the function of CBPs, as Ca^{2+} sensors, is that they contribute to the synaptic responsiveness of the cells through intermolecular actions. It is known that Scgn binds four Ca^{2+} ions and unlike other CBPs, interacts with only one Scgn interacting protein, called the 25 kDa synaptosome-associated protein (SNAP-25). Since SNAP-25 is involved in Ca^{2+}-induced exocytosis in neurons and in neuroendocrine cells, it is likely that through their interaction, Scgn also plays a regulatory role in membrane fusion and neurotransmitter exocytosis (Rogstam et al., 2007). These findings indicate that calcium binding proteins, including Scgn play an important role to regulate intracellular calcium and CBP dysfunction can lead to calcium toxicity, apoptosis, and eventually to neurodegeneration during normal aging and neurodegenerative disorders (Wu et al., 2005).

In addition to its possible role in neurodegeneration, Scgn might also have a link to type II diabetes mellitus. In the pancreas of the rat, Cb, Cr and Scgn are located in the islets of both α- and β-cells (Geula et al., 2003b; Wu et al., 2003). In type II diabetes, rats showed altered levels of CBP in the pancreatic islets (Bu et al., 2003). Since insulin can protect β-amyloid-induced synaptic loss in an AD model, the presence of Scgn in pancreatic β-cells points to common function in endocrine and the nervous systems, which may underlie the pathogenesis of AD and type II diabetes (De Felice et al., 2009).

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