

Secretagogin is a Ca²⁺-binding protein specifying subpopulations of telencephalic neurons

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Contributed by Tomas Hökfelt, October 30, 2009 (sent for review October 9, 2009)

The Ca²⁺-binding proteins (CBPs) parvalbumin, calbindin, and calretinin are phenotypic markers of terminally differentiated neurons in the adult brain. Although subtle phylogenetic variations in the neuronal distribution of these CBPs may occur, morphologically and functionally diverse subclasses of interneurons harbor these proteins in olfactory and corticolimbic areas. Secretagogin (*scgn*) is a recently cloned CBP from pancreatic β and neuroendocrine cells. We hypothesized that *scgn* is expressed in the mammalian brain. We find that *scgn* is a marker of neuroblasts commuting in the rostral migratory stream. Terminally differentiated neurons in the olfactory bulb retain *scgn* expression, with *scgn* being present in periglomerular cells and granular layer interneurons. In the corticolimbic system, *scgn* identifies granule cells distributed along the dentate gyrus, indusium griseum, and anterior hippocampal continuation emphasizing the shared developmental origins, and cytoarchitectural and functional similarities of these neurons. We also uncover unexpected phylogenetic differences in *scgn* expression, since this CBP is restricted to primate cholinergic basal forebrain neurons. Overall, we characterize *scgn* as a neuron-specific CBP whose distribution identifies neuronal subtypes and hierarchical organizing principles in the mammalian brain.

cortex | development | interneuron | neurogenesis | stem cell

The ability to release neurotransmitters at chemical synapses, to integrate the activity of diverse synaptic inputs and trigger molecular mechanisms underlying neuronal adaptation, as well as to maintain excitability in neurons rely on the refined spatial and temporal control of momentary changes in cytosolic [Ca²⁺] (1, 2). Ca²⁺-binding proteins (CBPs) represent a means to effectively regulate intracellular Ca²⁺ dynamics (3). Members of the EF-hand family of CBPs invariably contain a 3-D motif to bind Ca²⁺ at its physiological cytosolic concentrations (4). Some ancestral representatives of this protein family, such as calmodulin, are ubiquitously expressed with a high degree of evolutionary conservation and are involved in the control of fundamental cellular functions ranging from the cell cycle, cell motility and axon polarization to synaptic signaling (3). In contrast, the parvalbumin (PV) and calbindin subfamilies of CBPs, the latter including the vitamin D-dependent 28 kDa isoform of calbindin (CB) and calretinin (CR), exhibit restricted tissue-specific expression patterns in vertebrates (5, 6). During the past decades, PV, CB, and CR received significant attention because of their exquisite developmentally regulated cell type-specific expression in the mammalian nervous system (6–8).

CBPs show a unique association with newly generated neurons in the adult brain (9, 10). Neural progenitors that are born in the subependymal zone and migrate in the rostral migratory stream (RMS) to differentiate into interneurons in the olfactory bulb (OB) commonly express CR at their neuroblast stage with select subpopulations becoming CB⁺ upon their arrival to their final position in the OB. Similarly, neurons generated in the subgranular zone of the dentate gyrus (DG) express CBPs, CR

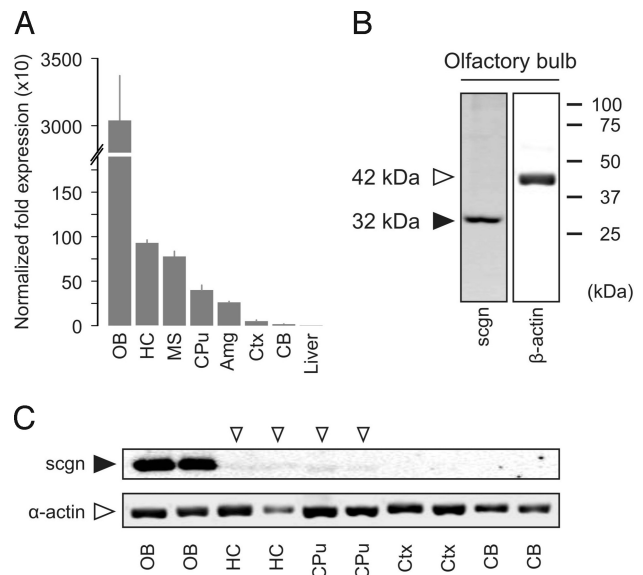


Fig. 1. *scgn* is expressed in the OB and corticolimbic territories of the adult brain. (A) *scgn* mRNA expression profiling of microdissected mouse brain tissues as measured by qPCR relative to *gapdh*. (B) An anti-*scgn* antibody (HPA006641) selectively recognizes a single protein band corresponding to *scgn*'s calculated molecular weight. (C) Regional differences in protein expression in adult mouse brain as determined by Western analysis. Open arrowheads indicate scant protein expression levels. Abbreviations are listed in Table S1.

transiently and CB permanently, during terminal differentiation into granule cells and integration into adult neuronal networks (10, 11). Transient or permanent CBP expression in new neurons of the adult brain can be of functional significance as CBPs can effectively modulate intracellular Ca²⁺ signaling to achieve optimal temporal control of cell motility, neurite outgrowth, and synaptogenesis (3).

Although their quantitative distribution can vary greatly, the laminar organization and cellular identity of PV, CB, and CR expression in the mammalian corticolimbic system remain phylogenetically preserved (3). These CBPs define largely nonover-

Author contributions: T.Hökfelt and T. Harkany designed research; J.M., M.Z., L.S., and G.T. performed research; M.U., Y.Y., and F.A. contributed new reagents/analytic tools; and T. Hökfelt and T. Harkany wrote the paper.

The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0912484106/DCSupplemental.

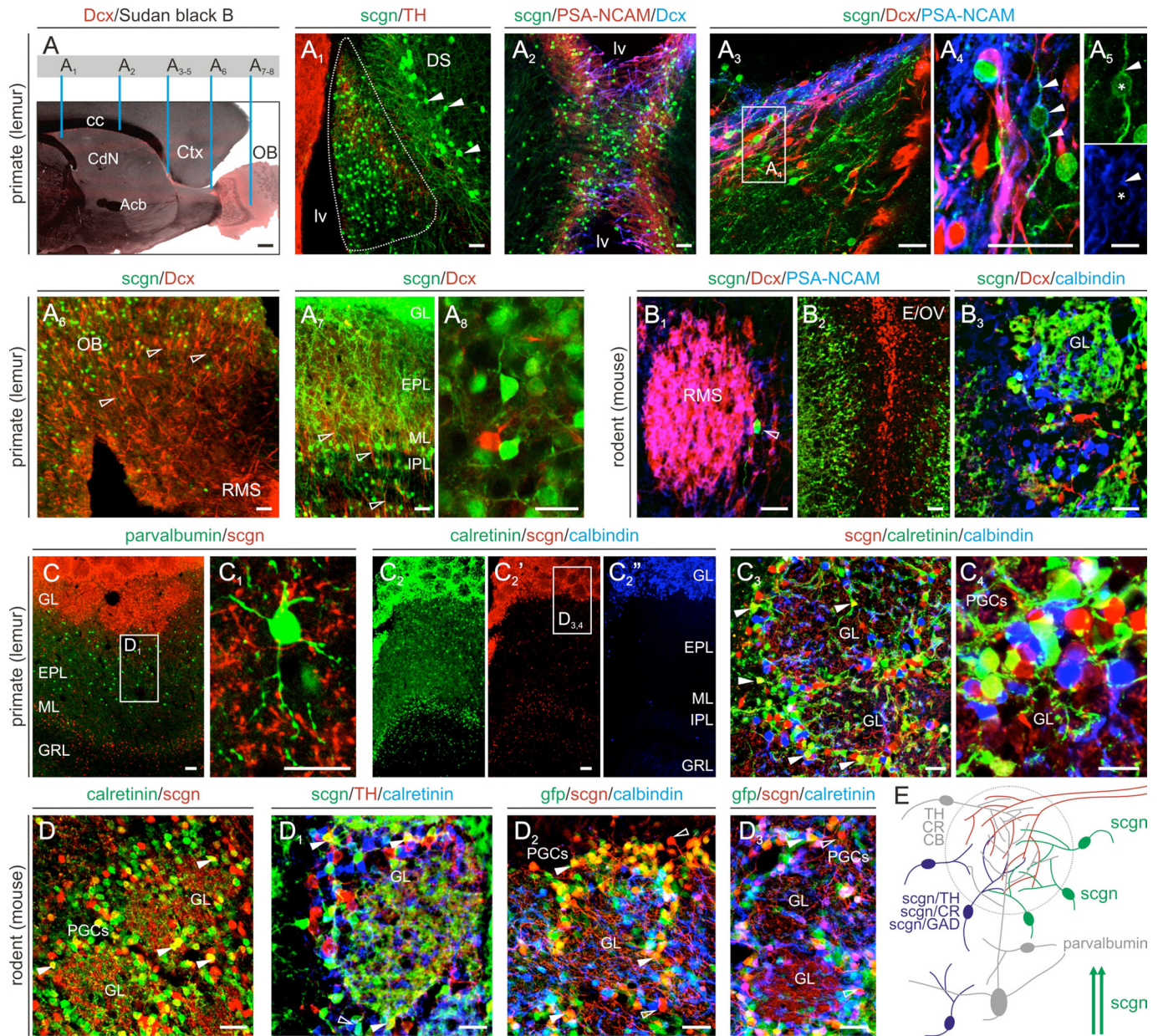


Fig. 2. *scgn* in the RMS and OB. (A) Neurons are continuously committed to the OB through the RMS. Vertical bars indicate the location of panels A_1 – A_8 . In primate brain, *scgn* labels migrating neuroblasts at their birth places (A_1), en route in the RMS (A_2 – A_3), upon invading the OB (A_6), and at their final positions (A_7 and A_8). Arrowheads in panel A_1 identify *scgn*⁺ AHC neurons. Arrowheads in panels A_4 and A_5 denote doublecortin (*Dcx*)[−]/*scgn*⁺/PSA-NCAM⁺ processes. In contrast, *scgn* is absent from rostrally migrating neuroblasts in mouse brain (B_1 – B_3). (C–C₄) In the primate OB, *scgn*⁺ neurons are present in the granular (GRL), internal/external plexiform (I/EPL), and glomerular (GL) layers and are distinct from PV⁺ or CB⁺ PGCs. CR⁺/*scgn*⁺ cells are marked by solid arrowheads. (D–D₃) In the mouse OB, *scgn* often coexists with CR and GAD67 (as identified by *gfp* expression), rarely with tyrosine hydroxylase (TH, solid arrowheads) but not CB in PGCs. (E) Overview of the neurochemical diversity of PGCs. *scgn*⁺ PGCs belong to GAD⁺/TH⁺ and CR⁺ PGCs (blue) or to a separate subclass of PGCs (green). Arrows indicate migrating *scgn*⁺ neuroblasts. Open arrowheads invariably point to a lack of colocalization of histochemical markers. Open boxes denote the positions of insets. Abbreviations are referred to in Table S1. [Scale bars, 22 μ m (A_1 – B_3 , C₁, and C₃–D₃), 60 μ m (C and C₂), and 100 μ m (A).]

lapping and morphologically distinct subpopulations of GABAergic interneurons in rodent, primate, and human cortices (5, 6, 12) with the exception of CB, which, although at low levels, is also expressed by cortical (layer 2/3 and 4) and hippocampal (CA1–CA3) pyramidal cells and DG granule cells (GCs) (5, 12).

Secretagogin (*scgn*) is a recently discovered EF-hand CBP cloned from β cells of the pancreatic islets of Langerhans and endocrine cells of the gastrointestinal tract (13, 14). *Scgn* harbors six putative EF hand motifs (13) and has been functionally

implicated as a Ca²⁺ sensor in insulin synthesis and secretion (13). However, the precise expression pattern of this CBP, the neurochemical identity of *scgn*⁺ nerve cells in neurogenic niches and in the corticolimbic system of the adult mammalian brain and its subcellular targeting in neurons remain unexplored. Here, we report that *scgn* characterizes precursor cells committed to the RMS and terminally differentiated periglomerular and granular layer interneurons in the OB. We find that *scgn* is a selective marker for GCs in the DG and the anterior hippocampal continuation (AHC). We also uncover striking phylogenetic

differences of scgn expression in the RMS and in basal forebrain cholinergic neurons projecting to the hippocampal formation. Cumulatively, our results classify scgn as a fourth major cell type-specific CBP in the mammalian corticolimbic system and reveal an unexpected neurochemical signature unifying GCs in the DG, AHC, and indusium griseum (IG).

Results and Discussion

Expression Profiling in Adult Brain. Although scgn mRNA expression has been shown in cortical areas of the human brain (15), subtle regional variations of scgn expression in rodent brain remain unknown. We assessed scgn mRNA levels in OB, telencephalic areas and cerebellum of the adult mouse by real-time quantitative PCR (qPCR) analysis. We establish that scgn mRNA expression is robust in the OB, moderate in the hippocampus (HC), medial septum (MS), and striatum (CPu), and low in amygdaloid nuclei, neocortex, and cerebellum (Fig. 1A and Fig. S1A and B).

Within the framework of the Human Protein Atlas program (16), we have generated antibodies to >3,000 proteins, including a powerful polyclonal antibody recognizing a phylogenetically conserved scgn epitope (Fig. S2A and B) and tested these on rat and mouse nervous tissues (17). Here, we confirmed that this antibody selectively recognizes scgn in Western blot applications (Fig. 1B and Fig. S2C), and it visualizes a scgn expression pattern in primate and rodent brain and peripheral tissues that corresponds to previously established scgn mRNA expression profiles (13) (Fig. S1B and Fig. S2D–G). To further explore whether scgn expression is prominently associated to the corticolimbic system at the protein level, we detected scgn protein in OB, HC, and CPu (including pallidal territories) upon loading whole cell lysates (20 μ g) on denaturing SDS-PAGE (Fig. 1C). Overall, these data demonstrate regional specificity of scgn expression in the mammalian brain with the OB and corticolimbic system exhibiting pronounced mRNA and protein concentrations of this CBP (Figs. S1B and S3 and Tables S1 and S2).

The RMS and OB. Neurogenesis occurs throughout life in the subependymal zone with cells traveling in the RMS (Fig. 2A) to populate the OB where neuroblasts differentiate into periglomerular interneurons (9). However, the rate at which neurons are generated, the distribution of migrating cells along the RMS, and the molecular control of their phenotypic differentiation are substantially different between primates and rodents (18). We find small (6–8 μ m) scgn⁺ cells in the MS region of the subventricular zone of the gray mouse lemur brain (*Microcebus murinus*; Primates; Fig. 2A₁) that is otherwise known as the birthplace of CR⁺ OB interneurons in rodents (9). scgn identifies doublecortin (Dcx)⁻, but PSA-NCAM⁺ neuroblasts throughout their chain migration in the RMS (Fig. 2A₂–A₅). Scgn⁺ neuroblasts, like Dcx⁺ ones, penetrate the primate OB and contribute cells to the granular, plexiform, and periglomerular layers (Fig. 2A₆–A₈). A fundamental difference between primate and rodent brain is that no scgn⁺ neuroblast is seen in the mouse RMS (Fig. 2B₁ and B₂). Instead, scgn selectively marks postmigratory granular and periglomerular OB neurons (Fig. 2B₂ and B₃).

The laminar distribution of neurochemically diverse GABAergic interneurons in the OB underpins olfactory processing (19). Glutamic acid decarboxylase (GAD)/GABA, tyrosine hydroxylase (TH), CB, and CR are phenotypic markers of periglomerular cells (PGCs) (20). In the primate and rodent OB, scgn⁺ neurons populate the glomerular, internal plexiform/mitral, and periglomerular layers (Fig. 2C–D and Fig. S4). We observe that scgn⁺ cells are invariably distinct from PV⁺ short axon cells (Fig. 2D₁), CB⁺ Blanes cells, or CB⁺ PGCs (Fig. 2C₂ and D₂) in both species. Instead, scgn may coexist with CR, TH, and GAD in PGCs (Fig. 2C₃–D₂). Ramifying dendrites of scgn⁺

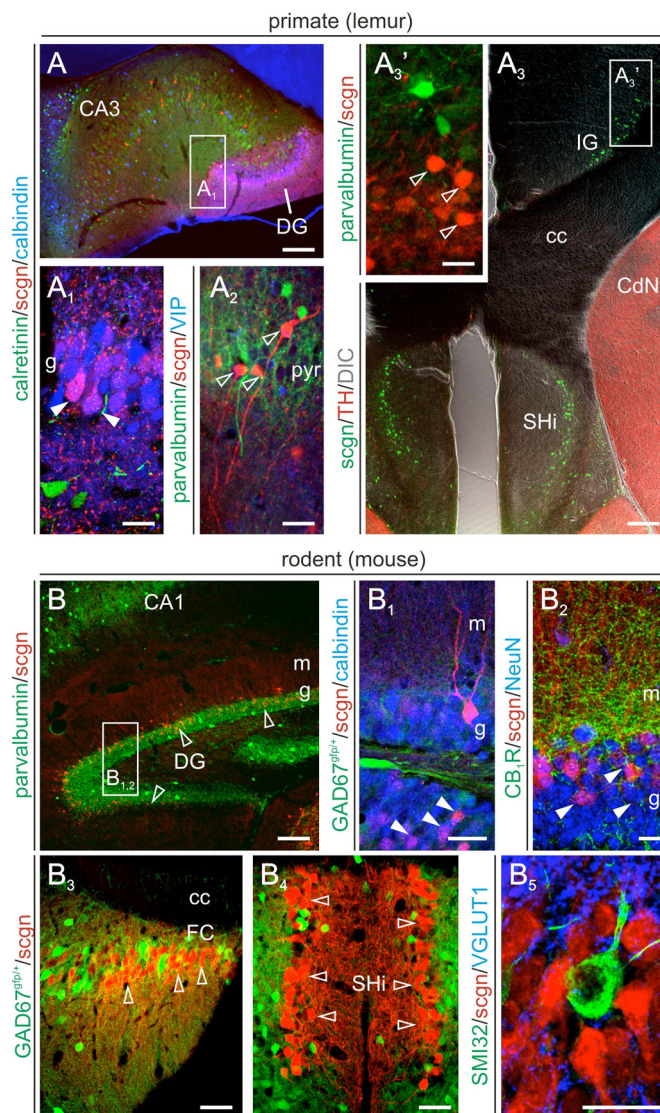


Fig. 3. Scgn identifies GCs in the hippocampal formation. (A) In the primate hippocampus, scgn labels CB⁺ dentate GCs (A₁) and nonpyramidal cells in CA1–3 subfields (A₂). In the AHC/SHI and IG of monkeys, scgn expression is restricted to GCs but is absent in GABAergic, e.g., PV⁺, interneurons. In rodent brain, scgn marks GCs mapped onto the superficial layers of the DG (B₁–B₂). In areas developmentally related to the DG, such as the FC (B₃) and AHC/SHI (B₄ and B₅) scgn reveals a continuum of GCs. Solid and open arrowheads mark double- and single-labeled structures, respectively. Abbreviations are referred to in Table S1. [Scale bars, 25 μ m (A₁–A₃' and B₁–B₅) and 100 μ m (A, A₃, and B).]

PGCs extend into the glomeruli and receive both inhibitory and excitatory inputs (Fig. S4C and D).

Overall, we identify scgn as a specific marker of primate RMS neuroblasts. In contrast, we demonstrate that scgn is invariably expressed in interneurons undergoing terminal differentiation in the OB of both primates and rodents. These findings reinforce that species-specific spatial and temporal differences exist in the onset of neurochemical specification of newly born OB interneurons in the adult mammalian brain (18). Only approximately 40% of PGCs have been neurochemically accounted for and subdivided into (i) GAD/GABA⁺, (ii) CB⁺, and (iii) CR⁺ subclasses (20). While a subset of scgn⁺ PGCs belongs to GAD/GABA⁺ and CR⁺ subclasses, the remainder of scgn⁺ PGCs appears to make up a separate subclass (Fig. 2E). This finding may have important functional ramifications, as contin-

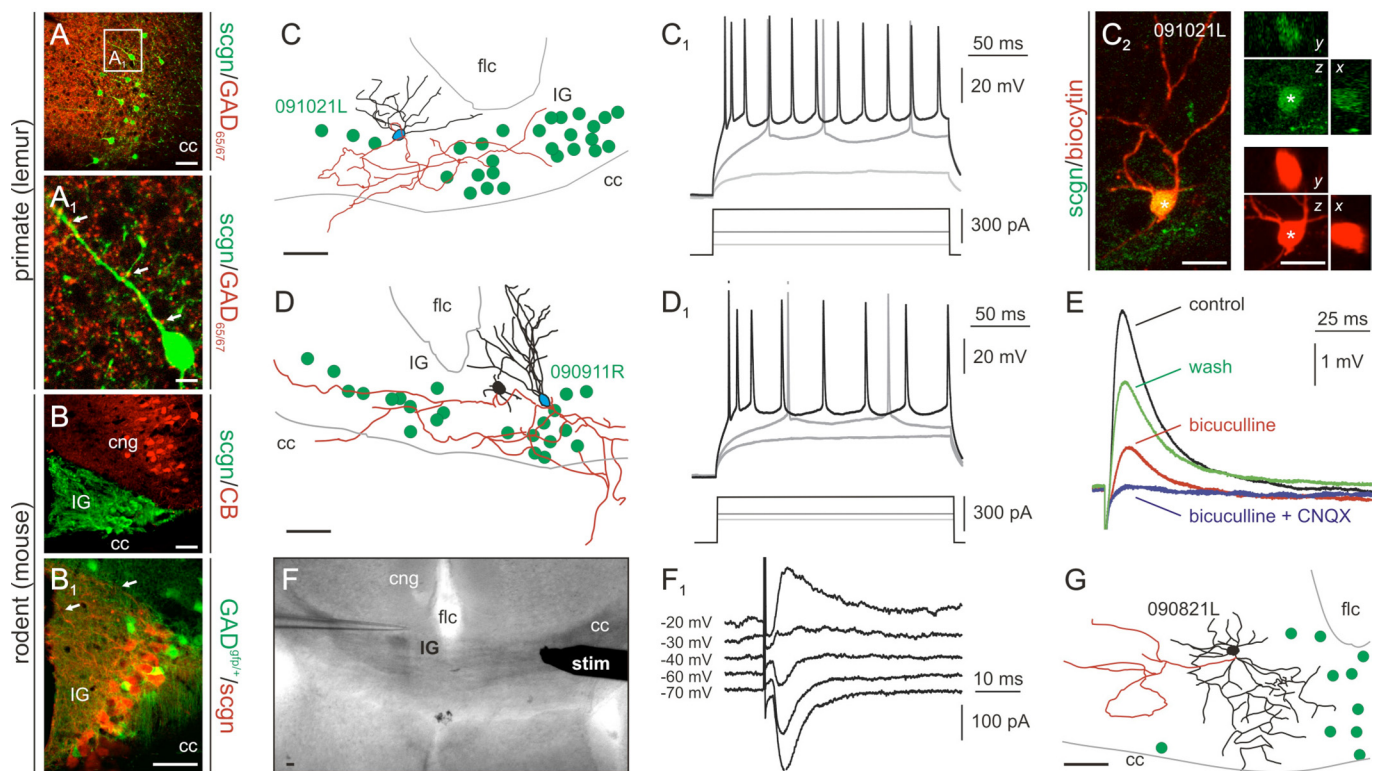


Fig. 4. Cellular characteristics of *scgn*⁺ neurons in IG. (A and A₁) *scgn*⁺ cells receive inhibitory inputs (arrows, A₁) and are non-GABAergic (B and B₁) with apical dendrites targeted to the subpial surface (arrows, B₁). The dendritic morphology (C and D) and action potential waveform (C₁ and D₁) of *scgn*⁺ neurons resembles those of dentate GCs. Data are from biocytin-filled cells unequivocally harboring *scgn* (C₂). (E) *scgn*⁺ GCs receive both inhibitory (bicuculline-sensitive) and excitatory (CNQX-sensitive) local synaptic inputs. (F and F₁) cc stimulation evokes IPSCs in GCs. (G) *scgn*⁺ cells are intermingled with morphologically diverse *scgn*⁻ interneurons in this region. Abbreviations are referred to in Table S1. [Scale bars, 10 μ m (A₁) and 25 μ m (all others).]

ued identification of PGCs will help elaborating the OB wiring diagram and refining the structural backbone of local neuronal subnetworks modulating olfactory perception.

Hippocampal Formation. The extended hippocampal formation is comprised of the interlocking gyri of the Ammon's horn (CA1–3 subfields) and DG, and a continuum of nuclei engulfing the corpus callosum (cc): The fasciola cinereum (FC), IG, AHC, and ventral tenia tecta (vTT) (Figs. S3 and S5). The neurochemical and cytoarchitectural identity of IG and AHC has been a subject of long debate with conflicting views linking these areas to either the OB or DG (21, 22).

Scgn labels GCs at varying intensities in all layers of the primate DG (Fig. 3A and A₁). In addition, *scgn*⁺ nonpyramidal cells with morphologies reminiscent of bistratified cells (12) were found in CA3 stratum pyramidale (Fig. 3A₂), while immunoreactive cells with variable morphologies were infrequently encountered in stratum oriens. Although *scgn* also labeled GCs in the mouse, we find that mostly mature GCs in superficial DG laminae receiving dense innervation in the inner molecular layer contain this CBP (Fig. 3B–B₂). Notably, *scgn*⁺ GC-like neurons form a continuum along the FC (Fig. 3B₂), IG (Figs. 3A₃ and 4A–B₁), AHC (Fig. 3A₃ and B₃–B₅), but not vTT in both species.

The characteristic GC-like morphology of *scgn*⁺ neurons in IG (Fig. 4A–B₁), the segregated subcellular targeting of their excitatory (Fig. 3B₂) and inhibitory (Fig. 4A₁) synaptic inputs, and their lack of CB expression (Fig. 4B) prompted us to determine the cytoarchitecture, discharge properties, and afferentation of these cells. We show by correlated patch-clamp electrophysiology and morphological reconstruction that *scgn*⁺ IG cells have GC-like dendritic complexities (Fig. 4C, C₂, and D), and their action potential pattern in response to somatic

depolarization (Fig. 4C₁ and D₁) together with passive membrane properties ($V_{rest} = -74.3 \pm 0.5$ mV; $R_i = 210 \pm 48$ M Ω) also resemble those of dentate GCs (23). Ipsilateral extracellular stimulation (200–500 μ m from the soma) triggered both inhibitory and excitatory inputs (Fig. 4E), while cc stimulation resulted in monosynaptic PSCs that reversed close to the calculated value for E_{GABA} (Fig. 4F and F₁). We also show that the IG contains various subclasses of interneurons (Fig. 4B₁ and G) with a sudden increase in neuronal heterogeneity at the anatomical boundaries of this nucleus (Fig. S6).

Similarities in the ontogeny, lamination pattern, cellular physiology, and origins of synaptic afferents (22) argue for a close relationship between the IG/AHC and DG. Our findings support this concept by recognizing *scgn* as a unifying GC marker in these territories and by demonstrating fundamental common features in their intrinsic electrical properties. The fact that GCs in IG but not in DG are CB⁻ may suggest that the CBP composition of GCs reflects regional differences in the developmental program and turnover of these cells in adult brain.

Phylogenetic Differences in *scgn* Expression by Cholinergic Neurons. Cholinergic basal forebrain neurons primarily innervate principal cells in both CA1–3 subfields and DG (6) to drive hippocampal network oscillations (24). Although cholinergic projection neurons in rodents appear largely homogenous, striking phylogenetic differences concerning the expression of nitric oxide synthase, neurotransmitter receptors, and CBPs exist between primates and rodents (25). Therefore, we studied *scgn* expression in lemur and mouse cholinergic neurons including both projection cells and interneurons (Fig. 5A and B). We determined that *scgn* coexists in the vast majority of choline acetyltransferase (ChAT)⁺ projection neurons of the primate MS (Fig. 5A₁),

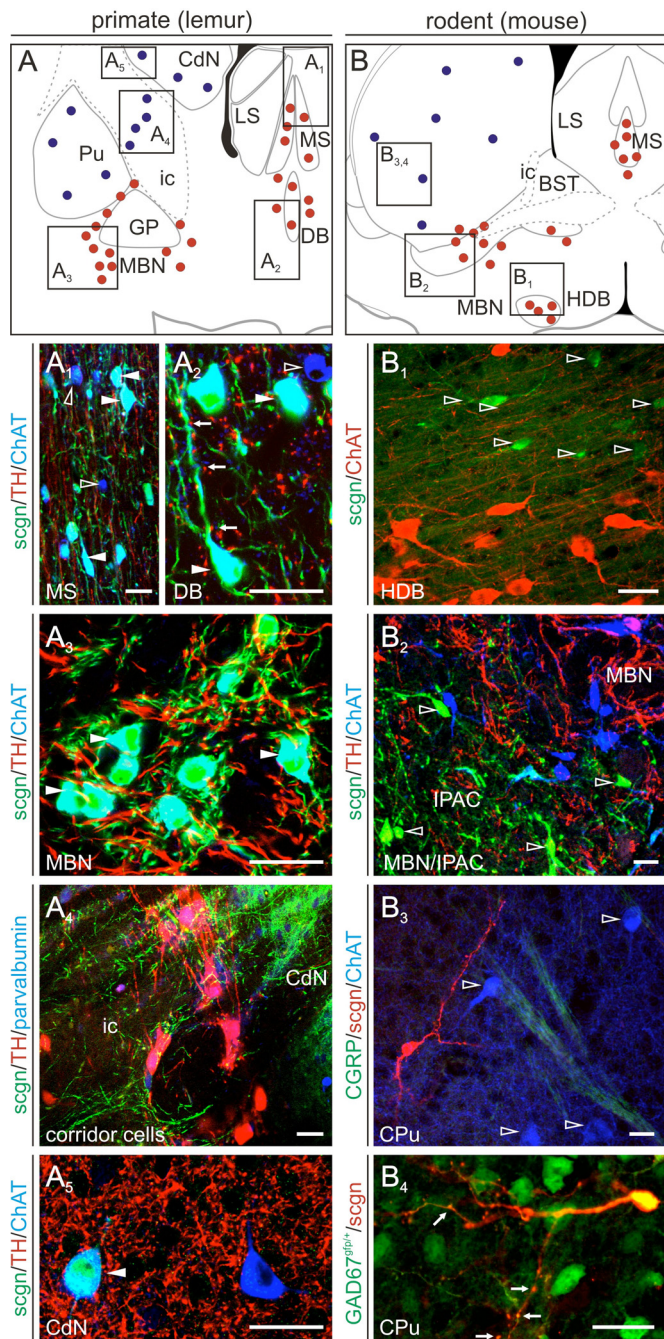


Fig. 5. Phylogenetic differences in *scgn* expression of cholinergic basal forebrain neurons. (A–A₅) Cholinergic projection (A₁–A₃) and interneurons (A₄ and A₅) in the primate basal forebrain frequently contain *scgn*. Arrows (A₂) identify putative TH-positive afferents to cholinergic neurons. (B–B₄) In contrast, neither cholinergic projection neurons (B₁ and B₂) nor striatal cholinergic interneurons (B₃ and B₄) express *scgn* in mouse forebrain. Note that *scgn* is, however, present in spiny (arrows) striatal GABAergic interneurons in mouse (B₄). Solid and open arrowheads point to double and single labeled structures, respectively. Open rectangles in (A and B) identify the general position of particular images. Red and blue circles mark cholinergic projection and interneurons, respectively. (Scale bars, 25 μ m.)

diagonal band complex (Fig. 5A₂), and magnocellular basal nucleus (Fig. 5A₃ and Fig. S7) (26). As an exception to this rule, ChAT[−]/*scgn*⁺ neurons were found scattered in the substantia innominata of the lemur brain (Fig. S7A₃). In contrast, *scgn* exclusively identified a spatially segregated noncholinergic cell

population in mouse basal forebrain territories (Fig. 5B₁ and B₂). Next, we assessed *scgn* expression in cholinergic interneurons of both species and found that *scgn* immunoreactivity decorates a heterogeneous neuron population in the nuclei putamen (Pu) and caudatus (CdN) and connecting cell columns in the capsula interna (Fig. 5A₄) in primates with approximately 50% of cholinergic interneurons being *scgn*⁺ in Pu (Fig. 5A₅), but not CdN (\approx 10%) or ventral pallidum (VP) (Fig. S7). In contrast, *scgn* is absent from cholinergic interneurons of the mouse CPu (Fig. 5B₃). Instead, *scgn*⁺ cells are GABAergic with elaborate, spiny dendritic tufts in this (Fig. 5B₄) and also in pallidal areas (Fig. S7). These findings are significant as the presence of *scgn* in primate but not rodent cholinergic neurons may add a facet of understanding to explain vast interspecies differences in the sensitivity of these cells to noxious stimuli through differential control of intracellular Ca²⁺ signaling.

Conclusions

scgn is a neuronal CBP whose cell-type specificity is distinct from those of other known CBPs. Besides that *scgn* uniquely labels chain-migrating RMS neuroblasts in primate brain, its OB distribution allows us to propose that *scgn*⁺ cells otherwise lacking known classification markers of PGCs represent a distinct periglomerular neuron subclass. *Scgn* is also expressed by newly born dentate GCs and is enriched in GC dendrites targeted onto the subpial surface in the IG. Since *scgn* is expressed in (entero-)endocrine cells and in neuroendocrine cells at the periphery (Fig. S2) and in the brain (Fig. S3), respectively, and has been implicated in the control of hormone secretion, we propose that *scgn* may regulate the release of diverse factors from neuroblasts and dendrites of differentiated PGCs and GCs that are required for new neurons to survive or to develop. Our hypothesis may also help establishing a functional link between the expression of basic fibroblast growth factor and neurotrophin-3 being restricted to the IG and FC in adult brain (27) and the activity-dependent release of these essential substances into the liquor space.

Materials and Methods

Expression Profiling. qPCRs were performed as described in ref. 28 with custom designed primers (Fig. S1A₃). RNA isolated from tissues microdissected from adult C57BL/6N mouse brains ($n = 2$) were subjected to *scgn* expression analysis after validating RNA integrity (Fig. S1A). Results from qPCR experiments were subsequently compared to *scgn* mRNA distribution as determined by in situ hybridization (Fig. S1B).

Tissues and Histochemistry. Young adult C57Bl6/N ($n = 4$) and heterozygous GAD67-GFP (Δ neo) mice (GAD67^{9fl/+}; $n = 6$) (29), and gray mouse lemurs born in a laboratory breeding colony ($n = 5$; Brunoy, France) (30) were maintained, transcardially perfused, and had their brains processed as described in ref. 26. Free-floating coronal and sagittal cryostat sections (40 μ m) from animals of both sexes were used for histochemistry. Experiments in rodents and primates confirmed to the 86/609/EEC directive and were approved by the Home Office and local French authorities (#962773), respectively.

We generated rabbit anti-*scgn* antibodies by designing protein epitope signature tags (PrESTs) of 100–150 aa (17) based on the human protein sequence by using bioinformatic tools (Fig. S2A). PrESTs were recombinantly produced in *Escherichia coli* and used to immunize rabbits. Mono-specific polyclonal antibodies were generated by immunoaffinity purification of ensuing antisera. Multiple immunofluorescence histochemistry with cocktails of primary antibodies (Table S3) was performed according to published protocols (31). Sudan black-B counterstaining of primate tissues was applied to quench tissue autofluorescence (26).

Imaging. Single x-y plane or orthogonal z image stacks were captured by laser-scanning microscopy (510META; Zeiss). Colocalization was defined as immunosignals being preset without physical signal separation in <1.0- μ m optical slices (31). Reconstruction of identified neurons was performed as described in ref. 32.

