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GABAergic cell types in the superficial layers of the mouse superior colliculus

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Abstract

To begin to unravel the complexities of GABAergic circuits in the superior colliculus (SC), we utilized mouse lines that express green fluorescent protein (GFP) in cells that contain the 67 kilodalton isoform of glutamic acid decarboxylase (GAD67-GFP), or Cre-recombinase in cells that contain GAD (GAD2-cre). We used Cre-dependent virus injections in GAD2-Cre mice and tracer injections in GAD67-GFP mice, as well as immunocytochemical staining for GABA and parvalbumin (PV) to characterize GABAergic cells that project to the pretectum (PT), ventral lateral geniculate nucleus (vLGN) or parabrachial nucleus (PBG), and interneurons in the SGS that do not project outside the SC. We found that approximately 30% of SGS neurons in the mouse are GABAergic. Of these GABAergic neurons, we identified 3 categories of potential interneurons in the GAD67-GFP line (GABA+GFP ~45%, GABA+GFP+PV ~15%, and GABA+PV ~10%). GABAergic cells that did not contain GFP or PV were identified as potential projection neurons (GABA only ~30%). We found that GABAergic neurons that project to the PBG are primarily located in the SGS and exhibit narrow field vertical, stellate, and horizontal dendritic morphologies, while GABAergic neurons that project to the PT and vLGN are primarily located in layers ventral to the SGS. In addition, we examined GABA and GAD67-containing elements of the mouse SGS using electron microscopy to further delineate the relationship between GABAergic circuits and retinotectal input. Approximately 30% of retinotectal synaptic targets are the presynaptic dendrites of GABAergic interneurons, and GAD67-GFP interneurons are a source of these presynaptic dendrites.

Graphical Abstract

We used Cre-dependent virus injections in GAD2-Cre mice and tracer injections in GAD67- green fluorescent protein (GFP) mice, as well as immunocytochemical staining for gamma amino butyric acid (GABA) and parvalbumin (PV) to characterize GABAergic cells of the stratum griseum superficiale (SGS) via confocal and electron microscopy. The Venn diagram illustrates the approximate proportions of GABAergic SGS neurons listed to the right. GABAergic neurons that contain GFP in the GAD67-GFP line and/or are labeled with a PV antibody are identified as interneurons. The remaining cells are potential projection neurons that include narrow field

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The data that support the findings of this study are available from the corresponding author upon reasonable request.

vertical (NFV), stellate, and horizontal cells that project to the PBG. The GAD67-GFP mouse line labels intrinsic interneurons in the SGS which are a source of GABAergic presynaptic dendrites that receive retinal input and contact nonGABAergic dendrites.

Keywords

retinotectal; electron microscopy; parabigeminal; pretectum; ventral lateral geniculate nucleus; interneuron; presynaptic dendrite; parvalbumin; GAD67; RRID:AB_10015246; RRID:AB_91337; RRID:AB_477652; RRID: nif-000-30467; RRID:SCR_002798; RRID: AB_144695; RRID: AB_2534077; RRID: AB_141431; RRID: AB_477329; RRID: AB_2298767; RRID:AB_1769132; RRID:AB_23136061

Introduction

The superficial layers of the superior colliculus (SC) receive input from nearly every retinal ganglion cell (Ellis et al. 2016). Retinotectal axons enter the rostral SC via the stratum opticum (SO), and travel in the SO to innervate the stratum griseum superficiale (SGS) and stratum zonale (SZ) in a spatially precise, retinotopic, fashion (Xu et al. 2011). A laminar arrangement of retinotectal axons has also been noted: axons originating from specific ganglion cell types occupy different dorsal to ventral positions within the SGS (Cang et al. 2018; Triplett et al. 2014). The precision of retinotectal signaling is also highly dependent on intrinsic GABAergic circuits (Binns and Salt 1997; Kaneda and Isa 2013; Gale and Murphy 2016). The SGS contains one of the highest densities of GABAergic neurons in the brain (Mize 1992), and approximately one third of the postsynaptic targets of retinotectal terminals are GABAergic (Boka et al., 2006).

A number of different GABAergic SC cell types have been described. The SC contains GABAergic projection cells that innervate the pretectum (PT), lateral geniculate nucleus (LGN) and parabigeminal nucleus (PBG; Born and Schmidt, 2007; Born and Schmidt, 2008; Gale and Murphy, 2014, 2018). In addition, a number of different intrinsic GABAergic cell types have been described based on axon projections, orientation of dendritic arbors, grouping of synaptic vesicles within dendritic appendages or shafts, and/or the co-localization of a variety of immunocytochemical markers (Mize et al., 1982; Appell and Behan, 1990; Lane et al., 1996; Olivier et al., 2000; Behan et al., 2002; Boka et al., 2006; Gale and Murphy, 2014, 2018; Zingg et al., 2017; Villalobos et al., 2018). However, the structural and functional organization of GABAergic SC circuits is still largely unknown.

The mouse provides an excellent model system to begin to unravel the complexities of SC GABAergic circuits and their relationship with synaptic terminals originating from the retina. These efforts are aided by a variety of transgenic lines that can be used to specifically label and manipulate GABAergic cell types. Most notably, a mouse line that expresses green fluorescent protein (GFP) in cells that contain the 67 kilodalton isoform of glutamic acid decarboxylase (GAD67-GFP), and a line that expresses Cre-recombinase in cells that contain GAD (GAD2-cre), have been used to isolate GABAergic cells of the SC (Gale and Murphy 2014; Gale and Murphy 2016; Masterson et al. 2019; Endo et al. 2003; Villalobos et al. 2018; Zingg et al. 2017). Such studies have revealed that GABAergic SC cells can exhibit

a variety of morphologies, physiological properties, synaptic inputs and outputs, as well as the presence or absence of the calcium binding protein parvalbumin (PV). In the present study, we utilize virus and tracer injections in these 2 mouse lines, as well as immunocytochemical staining for GABA and PV, to characterize GABAergic SGS cells that project to the PT, LGN and PBG, as well as interneurons that do not project outside the SC. In addition, we examine GABA and GAD67-containing elements of the mouse SGS using electron microscopy to further delineate the relationship between GABAergic circuits and retinotectal input.

Materials and Methods

Animals

All breeding and experimental procedures were approved by the University of Louisville Institutional Animal Care and Use Committee. Experiments were carried out using mice, of either sex, of C57BL/6J (Jax Stock No: 000664), a line in which neurons that contain glutamic acid decarboxylase (GAD) express Cre-recombinase (Jax Stock No: 010802; GAD2), or a line in which neurons that contain the 67KD isoform of GAD (GAD67) express green fluorescent protein (GFP; Jax Stock No: 007677, G42 line).

AAV injections in the SC

To label SC projections that originate from GABAergic cells, GAD2-cre mice were deeply anesthetized with a mixture of ketamine (100-150 mg/kg) and xylazine (10-15 mg/kg). The analgesic meloxicam (1-2 mg/kg) was also injected prior to surgery. The animals were then placed in a stereotaxic apparatus (Angle Two Stereotaxic, Leica, Wetzlar, Germany). An incision was made along the scalp, and a small hole was drilled in the skull overlying the SC. An adeno-associated virus (AAV) (Flex-rev-oChIEF-tdTomato (plasmid 30541, Addgene, packaged using AAV serotype 9) was injected unilaterally into the SC. Virus was delivered via a 34-gauge needle attached to a Nanofil syringe inserted in an ultramicropump. A volume of 60 nl was injected at each of two sites at a rate of 15nl/minute. After removal of the needle, the scalp skin was sealed with tissue adhesive (n-butyl cyanoacrylate), lidocaine was applied to the wound, and the animals were placed on a heating pad until mobile. Post-surgery, animals were carefully monitored for proper wound healing, and oral meloxicam (1-2 mg/kg) was administered for 48 hours.

Cholera toxin subunit B (CTB) injections in SC projection targets

To label SC projection neurons via retrograde transport, GAD67-GFP adult mice were anesthetized as described above and placed in a stereotaxic apparatus. An incision was made along the scalp, and a small hole was drilled in the skull overlying the lateral geniculate nucleus (LGN), pretectum (PT), parabigeminal nucleus (PBG), or the ventral tegmental area (VTA). Using a Nanofil syringe and 34-gauge needle as described above for the AAV injections in SC, CTB-Alexafluor 555 was injected into each of the above locations. Injection volume varied by injection location as follows: 120 nl (LGN; 3 dorsal-ventral sites), 80 nl (PT), 20-50 nl (PBG and surrounding areas), 300 nl (VTA, 3 dorsal-ventral sites). After removal of the needle and sealing of the scalp with tissue adhesive, the same post-surgical protocol was followed as described for the AAV injections.

Herpes simplex virus (HSV) injections in SC projection targets

To label GABAergic SC projection neurons via retrograde transport, GAD2-cre adult mice were anesthetized as described above and placed in a stereotaxic apparatus. An incision was made along the scalp, and a small hole was drilled in the skull overlying the LGN, PT, or PBG. Using the same equipment as described for CTB and AAV injections, heF1 α -LS1L-EYFP HT (MIT viral vector core) was injected into each of the locations listed above. Injection volume varied by injection location as follows: 120 nl (3 dorsal-ventral sites; LGN), 50 nl (PT), 50 nl (PBG).

Histology

Two days following injection of CTB, or 10-14 days following the injection of viruses, mice were deeply anesthetized with Avertin (0.5mg/gm) and transcardially perfused with a fixative solution of 4% paraformaldehyde, or 2% paraformaldehyde and 2% glutaraldehyde in 0.1M phosphate buffer (PB). Additional C57BL/6J or GAD67-GFP mice that were not injected were also perfused for immunocytochemistry. In each case, the brain was removed from the skull and 70 μ m thick coronal sections were cut using a vibratome (Leica Microsystems, Buffalo Grove, IL). Sections that contained fluorescent labels were mounted on slides and imaged using a confocal microscope (Olympus FV1200BX61), or additionally stained using antibodies as described below.

Selected sections were incubated overnight in a 6-12 μ g/ml concentration of a mouse monoclonal antibody against parvalbumin (Sigma, catalogue #P3088, RRID:AB_477329, immunogen was frog muscle parvalbumin), a 0.5 μ g/ml concentration of a mouse monoclonal antibody against NeuN (Chemicon, catalogue #MAB377, RRID:AB_2298767, immunogen was purified cell nuclei from mouse brain.), and/or a 0.5 μ g/ml concentration of a rabbit polyclonal antibody against GABA (Sigma-Aldrich, St. Louis, MO, catalogue #A2052, RRID:AB_477652, immunogen was GABA conjugated to bovine serum albumin using glutaraldehyde; the GABA antibody shows positive binding with GABA and GABA-keyhole limpet hemocyanin, but not bovine serum albumin, in dot blot assays; manufacturer's product information). In mouse tissue, the GABA antibody stains most neurons in the thalamic reticular nucleus and a subset of neurons in the dorsal thalamus and cortex. This labeling pattern is consistent with other GABAergic markers used in a variety of species (Houser et al. 1980; Fitzpatrick, Penny, and Schmechel 1984; Montero and Singer 1984; Montero and Zempel 1985). In addition to a subset of neurons in the SC, the parvalbumin antibody stained a dense population of neurons in the thalamic reticular nucleus as previously described (Clemente-Perez et al. 2017; Villalobos et al. 2018). The NeuN antibody stains all neurons (identified by nuclei that have less condensed chromatin) and not glial cells (identified by nuclei that contain condensed chromatin; Wei et al., 2011).

The following day, the sections were incubated in a 1:100 dilution of goat-anti-mouse and/or goat-anti-rabbit antibody(s) directly conjugated to fluorescent compounds (Alexafluor 546 Cat. No. A11030/ A11010 [RRID: AB_144695/AB_2534077], or Alexafluor 633 Cat. No. A21050 [RRID: AB_141431], Invitrogen, Carlsbad, CA). The sections were then mounted on slides and imaged using a confocal microscope.

Quantification of GABAergic and parvalbumin cell populations in the SC

Selected tissue sections from GAD67-GFP mice were imaged using a confocal microscope (Olympus FV1200BX61) with a 40x objective. Stacks of 1 μ m optical images of the SGS were obtained in regions that contained a dense distribution of CTB-labeled cells. Cells labeled with CTB and/or antibodies were counted using Neurolucida 360 software (version 2018.2.1; Microbrightfield, Williston, VT). Labeled cells were only counted in regions of antibody penetration. Only one image stack was obtained per section to avoid overlap between sampling regions and to prevent bleaching of fluorescent labels.

Analysis of HSV-labeled projection cells

The distribution of HSV-labeled cells relative to the surface of the SC was measured from confocal image stacks. Prism (**RRID:SCR_002798**) was used for statistical analysis of these depth measurements (Kruskal-Wallis test). Confocal images of HSV-labeled cells that project to the PBG were additionally categorized based on their morphological characteristics. Three categories were used, adapted and slightly modified from a study by Villalobos et al. (2018): narrow-field vertical, horizontal, and stellate. The vertical or horizontal orientation of the dendritic arbors was defined relative to the dorsal surface of the SC in coronal sections. Vertically-oriented dendrites were located within 30 degrees of a line perpendicular to the dorsal surface of the SC. Horizontally-oriented dendrites were located within 30 degrees of a line parallel to the dorsal surface of the SC. The primary dendrites of narrow-field vertical cells were oriented in the vertical direction and the width of their dendritic arbor was less than 250 μ m (measured parallel to the SC surface). The primary dendrites of horizontal cells were oriented in the horizontal direction (as defined above). Stellate cells included any neuron with a dendritic arbor that exhibited no clear orientation, or neurons with arbors that were roughly symmetrical in all directions. Only cells labeled in SGS or SO were analyzed for these morphological characteristics. Additionally, only cells that were labeled beyond their secondary dendrites were included in the analysis. For presentation of confocal stacks, images were imported into Adobe Photoshop software (San Jose, CA), the brightness and contrast were adjusted, and tissue imperfections were removed using the clone stamp tool.

Electron microscopy

Selected sections from GAD67-GFP mice were incubated overnight in a 0.1 μ g/ml concentration of a rabbit polyclonal anti-GFP antibody (Millipore, Billerica, MA, catalogue #AB3080, RRID:AB_91337, created with highly purified native GFP from *Aequorea victoria* as an immunogen). Sections were then incubated in a 1:100 dilution of a biotinylated goat-anti-rabbit antibody (Vector Laboratories, Burlingame, CA, catalogue #BA-100, RRID:AB_23136061, 1 hour), followed by avidin and biotinylated horseradish peroxidase (ABC solution, Vector Laboratories, 1 hour) and reacted with nickel-enhanced diaminobenzidine (DAB). All GFP antibody binding was confined to cells and terminals that contained GFP, as determined by their fluorescence under blue epifluorescent illumination; no staining was detected in sections that did not contain GFP.

SC sections that contained DAB-labeled GFP, and additional SC sections obtained from C57BL/6J mice, were postfixed in 2% osmium tetroxide, dehydrated in an ethyl alcohol

series, and flat embedded in Durcupan resin between two sheets of Aclar plastic (Ladd Research, Williston, VT). Durcupan-embedded sections were first examined with a light microscope to select areas for electron microscopic analysis. Selected areas were mounted on blocks, ultrathin sections (70-80 nm, silver-gray interference color) were cut using a diamond knife, and sections were collected on Formvar-coated nickel slot grids. Selected sections were stained for the presence of GABA. A postembedding immunocytochemical protocol described previously (Bickford et al. 2010; N. Zhou et al. 2018; Masterson et al. 2019) was employed. Briefly, we used a 0.25 µg/ml concentration of a rabbit polyclonal antibody against GABA (Sigma-Aldrich, St. Louis, MO, catalogue #A2052, RRID:AB_477652). The GABA antibody was tagged with a goat-anti-rabbit antibody conjugated to 15-nm gold particles (BBI Solutions USA, Madison, WI, catalogue# GAR12/0.25, RRID:AB_1769132). The sections were air dried and stained with a 10% solution of uranyl acetate in methanol for 30 minutes before examination with an electron microscope.

Ultrastructural analysis

For analysis of retinotectal synaptic connections, ultrathin sections obtained from C57BL/6J mice were stained for GABA and examined using an electron microscope. Retinotectal terminals were identified by their unique “pale” mitochondria with widened cristae; RLP profiles, (Boka et al., 2006; Bickford et al., 2010, 2015; Masterson et al., 2019), and those involved in a synapse were imaged. The areas of the pre- and postsynaptic profiles were measured using Image J, RRID: nif-000-30467, or Maxim DL © 5 software) and the gold particles were counted to calculate the gold density overlying each profile. The presence or absence of synaptic vesicles in postsynaptic profiles was also noted. As previously described (Masterson et al. 2019), profiles were identified as GABAergic if the gold particle density overlying them was greater than the maximum density overlying RLP profiles ($n = 124$; average 9.27 ± 8.05 gold particles/µm²). This analysis revealed that in the C57BL/6J tissue, GABAergic profiles could be identified by a density of > 30 gold particles/µm².

For analysis of GFP-labeled synaptic connections, ultrathin sections obtained from GAD67-GFP mice were stained to reveal GFP and GABA and examined using an electron microscope. GFP was identified by the DAB reaction product (Zhou et al. 2018; Masterson et al. 2019), and profiles involved in a synapse were imaged ($n = 94$). The areas of the pre- and postsynaptic profiles were measured using Image J, RRID: nif-000-30467) and the gold particles were counted to calculate the gold density overlying each profile. For each synaptic partner, the presence or absence of DAB reaction product, synaptic vesicles, and dark or pale mitochondria was noted. As described above, profiles were identified as GABAergic if the gold particle density overlying them was greater than the maximum density overlying surrounding RLP profiles ($n = 31$; average 6.8 ± 4.2 gold particles/µm²). This analysis revealed that in the GAD67-GFP tissue, GABAergic profiles could be identified by a density of > 17 gold particles/µm². For presentation of ultrastructural features, electron microscopic images were imported into Adobe Photoshop software (San Jose, CA), and the brightness and contrast were adjusted.

Results

The SGS contains at least four categories of GABAergic neurons

To aid in our study of the GABAergic neurons of the SGS, we used the GAD67-GFP mouse line which labels a dense population of neurons in the SGS (Figure 1a). First, to determine what proportion of SGS cells contain GFP and/or GABA, we stained sections from transgenic mice with antibodies against both GABA (Figure 1b-d) and NeuN (Figure 1e-g, to stain all SGS neurons). In confocal image stacks within the zone of antibody penetration (9 image stacks, tissue from 3 animals), we quantified cells that contained NeuN, NeuN/GABA, or NeuN/GABA/GFP ($n = 1982$ NeuN-stained cells, 616 GABA-stained cells, and 417 GFP-labeled cells). This analysis revealed that $31.08 \pm 3.71\%$ of SGS neurons are GABAergic, and $67.70 \pm 6.54\%$ of these GABAergic cells contain GFP in the GAD67-GFP line.

To determine whether cells that contain GFP and/or GABA in the GAD67 line also contain PV, we stained sections from the transgenic mice with antibodies against PV and GABA and quantified labeled cells in confocal image stacks within the zone of GABA antibody penetration (52 image stacks, tissue from 5 animals, $n = 4089$ GABAergic cells, 2175 GFP-labeled cells, 1560 PV-labeled cells). This more extensive tissue analysis revealed that $53.19 \pm 10.62\%$ of the GABAergic cells contained GFP. This analysis also revealed that approximately one quarter of the cells labeled with GFP in the GAD67-GFP line could also be labeled with an antibody against PV ($n = 497$, $22.85 \pm 11.29\%$; Figure 1h-j, white arrows). Similar proportions of GABAergic cells contained PV ($n = 1043$, $25.51 \pm 8.35\%$). This was also the case for the GABAergic cells that did not contain GFP ($n = 1914$); of these, approximately one quarter contained PV ($n = 546$, $28.53 \pm 4.86\%$). The proportion of antibody-labeled PV cells that also contained GFP ($n = 497$; $31.86 \pm 12.51\%$) was similar to that reported in a previous study of PV cells labeled in transgenic lines (Villalobos et al 2018; PV cells that contain GFP = $43 \pm 4\%$). However, the proportion of antibody-labeled PV cells that were also stained with the GABA antibody ($n = 1043$, $66.86 \pm 14.20\%$) was higher than that found for PV cells labeled in a transgenic line (PV cells that contain GABA = $36 \pm 3\%$; Villalobos et al., 2018).

Taken together, our immunocytochemical experiments indicate that the GAD67-GFP line labels approximately 60% of the GABAergic neurons in the SGS. Of these, approximately one quarter contain PV, indicating that GAD67-GFP cells are themselves not a homogenous group. Furthermore, our results indicate that 40% of GABAergic cells in the SGS do not contain GFP and of these, approximately one quarter contain PV, indicating that GABA, GAD67-GFP and PV labeling can be used to divide the GABAergic neurons of the SGS into four groups: 1) GABA+GFP (~45%), 2) GABA+GFP+PV (~15%), 3) GABA+PV (~10%), and 4) GABA only (~30%). In the following sections, we describe experiments which suggest that groups 1-3 are interneurons and group 4 includes projection neurons.

Extrinsic SC projection targets labeled in GAD2-Cre mice

Cre-dependent AAV injections placed in the SC of GAD2-Cre mice (Figure 2a) labeled axon projections to a variety of extrinsic targets. The primary extrinsic targets of GAD2 cells are

the parabigeminal nucleus (PBG; Figure 2b), the pretectum (PT; Figure 2c) and the ventral lateral geniculate nucleus (vLGN; Figure 2d). Sparse projections were also observed in the opposite SC, but since previous studies have identified these as originating from neurons ventral to the SGS (Bickford and Hall, 1989; Appell and Behan 1990; Olivier et al. 2000), we did not examine these connections further.

GABAergic SGS cells that project to the PBG

HSV injections in the PBG of GAD2-Cre mice (Figure 3a) labeled a population of cells primarily located in the SGS (Figure 3b; somata located $319.6 \pm 184.4 \mu\text{m}$ from the dorsal surface of the SC, $n = 181$; Figure 6). To characterize their morphology, we used a procedure modified from Villalobous et al. (2018), described in detail in the methods section. Cells that exhibited extensive dendritic labeling ($n=65$) were characterized in each section ($n = 5$) as narrow field vertical (NFV; $32.3 \pm 11.5\%$), stellate ($43.1 \pm 13.5\%$) and horizontal ($24.6 \pm 9\%$). These 3 cell types cells were differentially distributed within the SGS (Figure 6), with NFV closest to the SC surface ($207 \pm 89.9 \mu\text{m}$), stellate cells slightly deeper ($256.6 \pm 118.7 \mu\text{m}$) and horizontal cells farthest from the SC surface ($350.9 \pm 164.1 \mu\text{m}$). However, these depth differences were not found to be statistically significant (Kruskal Wallis test). As expected, no wide field vertical cells (matching characteristics described in Masterson et al., 2019) were labeled by these injections as these cells are glutamatergic cells that innervate the pulvinar nucleus (Gale and Murphy 2014; Gale and Murphy 2016; Gale and Murphy 2018; Zhou et al. 2017).

To determine whether any GABAergic PBG-projecting cells contain PV, we stained HSV-injected tissue with a PV antibody (Figure 3c); we examined 181 HSV-labeled cells within the zone of antibody penetration and found no cells that were labeled with both the virus and the PV antibody. Next, to determine whether any SGS cells with ipsilateral descending projections are labeled with GFP in the GAD67-GFP mouse line, we injected CTB into PBG and surrounding areas (Figure 3d,e). In these cases we examined 490 CTB-labeled cells (Figure 3f) and found that none of these contained both CTB and GFP. We also stained this tissue with the PV antibody which labeled a subset ($16.5 \pm 6.8\%$) of SGS cells with ipsilateral descending projections (Figure 3g). Thus, the SGS GABA and PV cells that project to the PBG and surrounding areas form separate populations, and neither are labeled in the GAD67-GFP line.

GABAergic SC cells that project to the PT or LGN

To determine whether any SGS cells labeled in the GAD67-GFP mouse line project to the PT or LGN, we injected CTB into the PT (Figure 4a) or LGN (Figure 4b) in GAD67-GFP mice; we examined 859 CTB-labeled cells in the SGS (739 in PT-injected cases and 120 in LGN-injected cases) and found just 4 cells (0.48%) that contained both CTB and GFP. Thus, the vast majority of SGS cells that project to the PT or LGN are not labeled in the GAD67-GFP line. We also stained this tissue with the PV antibody. The PV antibody labeled a subset of SC-PT cells ($27.2 \pm 15.2\%$; Figure 4c) and SC-LGN cells ($11.7 \pm 5.2\%$) labeled via CTB injections.

HSV injections in the PT (Figure 5a) or vLGN (Figure 5d) of GAD2-Cre mice labeled cells that were primarily distributed in SC layers ventral to the SGS (SC-PT somata located $508.5 \pm 212.3 \mu\text{m}$ from the dorsal surface of the SC, $n = 343$, Figure 5b,c, Figure 6; SC-vLGN somata located $531.5 \pm 263.7 \mu\text{m}$ from the dorsal surface of the SC, $n = 811$; Figure 3e,f; Figure 6). The distribution of cells that project to the PT or vLGN were significantly different from those that project to the PBG ($p < 0.0001$), but there was no significant difference between the distribution of cells that project to the PT versus vLGN (Kruskal Wallis).

To determine whether any GABAergic PT- or vLGN-projecting cells contain PV, we stained HSV-injected tissue with a PV antibody; we examined 1154 HSV-labeled cells within the zone of antibody penetration and found two cells (0.17%) that contained both virus and PV. Thus, GABAergic and PV-containing SC cells that project to the PT or LGN form separate populations.

Proportions of GABAergic projection neurons and interneurons in the SGS

A recent study identified SC projections to the ventral tegmental area (VTA) following Cre-dependent virus injections in vesicular GABA transporter (vGAT)-Cre mice (Zhang et al. 2019), although we were unable to detect projections to this region using the GAD2-Cre mouse line. To determine whether any SGS cells labeled in the GAD67-GFP mouse line project to the VTA, we placed large injections of CTB into the VTA (Figure 7a) in GAD67-GFP mice. SC cells labeled following these injections were located in the ipsilateral SC in layers deep to the SGS (Figure 7b). Similarly, all crossed descending projections from the SC arise from cells deep to the SGS (Bickford and Hall 1989). Thus, our retrograde CTB tracing studies indicate SGS cells labeled in the GAD67-GFP line do not contribute to the ascending or descending projections of the SC (although we cannot rule out that they may project to targets that we did not inject with CTB, such as the periaqueductal gray). Furthermore, our retrograde HSV tracing combined with PV immunocytochemistry indicate that GABAergic projection neurons in the SGS do not contain PV. Thus, of the 4 categories of cells that we identified above, we can identify 3 groups as potential interneurons (GABA +GFP ~45%, GABA+GFP+PV ~15%, and GABA+PV ~10%), and the fourth group as potential projection neurons (GABA only ~30%).

GAD67-GFP SGS cells include intrinsic interneurons with presynaptic dendrites

Our results described above suggest that SGS neurons labeled in the GAD67-GFP line are interneurons. Thus, we were interested in determining whether these cells are a source of GABAergic presynaptic dendrites within the SGS. In the rat SC, we previously found that 27% of profiles postsynaptic to retinotectal terminals contained GABA, and 71% of these postsynaptic GABAergic profiles contained vesicles (Boka et al., 2006). We obtained similar results in C57BL/6J mice. We examined retinotectal terminals in C57BL/6J tissue stained to reveal GABA with gold particles. As illustrated in Figure 8, retinotectal terminals (pseudo colored green) can be identified by their unique “pale” mitochondria with widened cristae (asterisks); these profiles are referred to as RLP profiles (large profiles with round vesicles

and pale mitochondria). RLP profiles are embedded within complex synaptic arrangements of GABAergic profiles that contain vesicles (Figure 8a-d, pseudo colored red) and nonGABAergic dendrites that do not contain vesicles (Figure 8a-d, pseudo colored blue). RLP profiles make synaptic contacts with both GABAergic (Figure 8a-d, red arrows) and nonGABAergic dendrites (Figure 8a-c, blue arrows). We examined the synaptic contacts of 124 RLP profiles and found that 39 (31%) of the postsynaptic profiles could be classified as GABAergic based on a gold particle density that exceeded the maximum gold particle density overlying RLP profiles (average gold particle density overlying RLP profiles 9.3 ± 8.0 gold particles/ μm^2 ; average gold particle density overlying GABAergic profiles 72.5 ± 25.2 gold particles/ μm^2). Of the GABAergic profiles that received retinotectal input, 34 (87%) contained synaptic vesicles (Figure 8a-c, shown at high magnification in 8d), and these GABAergic profiles could occasionally be seen to contact nonGABAergic dendrites (Figure 8d, blue arrow). Thus, in the rodent SC, approximately one third of retinotectal synapses target GABAergic neurons, and the vast majority of these postsynaptic neurons form intrinsic connections via presynaptic dendrites.

We next examined tissue obtained from GAD67-GFP mice. The GFP was identified via antibody binding revealed with a DAB reaction product (Figure 8e-f) and GABA content was assessed with an antibody tagged to gold particles as described above. We examined 94 GFP-labeled profiles involved in synaptic connections. As previously reported (Masterson et al. 2019), the gold particle density overlying these GFP-labeled profiles was significantly higher than that overlying RLP profiles (GFP 57.7 ± 25.1 gold particles/ μm^2 ; RLP 6.8 ± 4.2 gold particles/ μm^2 ; paired t test, $p < 0.0001$). Of the 94 synaptic connections formed by the GFP-labeled profiles, 31 were with RLP profiles (Figure 8e-f, red arrows). Although the vesicle content of GFP-labeled profiles was occasionally obscured by the DAB reaction product, all profiles postsynaptic to RLP profiles exhibited the ultrastructure of presynaptic dendrites identified in the C57BL/6J mice. Thus, GAD67-GFP interneurons in the SGS are at least one source of GABAergic presynaptic dendrites in the SGS. The remaining synaptic partners of the GFP-labeled profiles were nonGABAergic dendrites ($n = 54$), nonGABAergic profiles with vesicles ($n = 3$), GABAergic dendrites ($n = 4$) and GABAergic profiles with vesicles ($n = 2$). These remaining synaptic connections may also be formed by interneurons of the SGS, although it is possible that some may be contributed by GFP-labeled axon terminals that originate from neurons outside the SC.

Discussion

Our results support the following conclusions: 1) Approximately 30% of SGS neurons in the mouse are GABAergic. 2) GABAergic projection neurons comprise up to 30% of the GABAergic neurons in the SGS, and none of these cells contain PV. 3) GABAergic neurons that project to the PBG are primarily located in the SGS and exhibit NFV, stellate, and horizontal morphology. 4) GABAergic neurons that project to the PT and vLGN are primarily located in the deeper layers of the SC. 5) The GAD67-GFP mouse line primarily labels intrinsic interneurons in the SGS, which make up approximately 60% of the GABAergic SGS neurons. 6) Approximately 45% of GABAergic SGS cells are GAD67-GFP interneurons that do not contain PV. 7) Approximately 15% of GABAergic SGS neurons are GAD67-GFP interneurons that contain PV. 8) Approximately 10% of

GABAergic SGS cells are interneurons that contain PV but are not labeled with GFP in the GAD67-GFP line. 9) Approximately 30% of retinotectal synaptic targets are the presynaptic dendrites of GABAergic interneurons and 10) GAD67-GFP interneurons are a source of GABAergic presynaptic dendrites in the SGS (summarized in Figure 9).

Projections from the SGS to the PBG

Previous studies in a variety of species have shown that projections to the PBG arise almost exclusively from the superficial layers of the SC (Diamond, Fitzpatrick, and Conley 1992; Bennett-Clarke et al. 1989) and recent studies in the mouse indicate that these projections arise from both GABAergic neurons (Gale and Murphy 2014) and glutamatergic neurons that contain PV (Shang et al. 2015, 2018). Our results corroborate these findings; we found that PBG projections arise from GABAergic SGS cells and none of these cells stained with a PV antibody. We also found that $16.5 \pm 6.8\%$ of SGS cells with ipsilateral descending projections contained PV. Whether the remaining cells are GABAergic neurons that project to the PBG, or whether ipsilateral descending projections arise from additional subtypes of SGS cells, remains to be determined. Gale and Murphy (2014) showed that glutamatergic projections to the PBG arise from cells labeled in the Grp-KH288-Cre line, but it is not known whether these cells contain PV. In other rodents, projections from the SGS to the PBG have also been shown to express substance P (Bennett-Clarke et al. 1989), but it is unknown whether these also contain PV or GABA. Nevertheless, since we found that other GABAergic projections from the SC arise primarily from deeper layers, it is likely that the majority of GABAergic projection cells in the SGS are those that innervate the PBG.

Using the retrograde transport of CTB and subsequent intracellular filling, Gale and Murphy (2014) found that SGS-PBG cells exhibit NFV and stellate morphology. Additionally, virus injections in the PBG of GAD2-cre mice labeled horizontal cells in the SGS. Similarly, we found that SGS-PBG cells exhibited NFV, stellate and horizontal dendritic fields. Moreover, since our virus tracing was carried out in GAD2-cre mice, we found that GABAergic SGS-PBG cells exhibit this variety of morphologies. Finally, we also found that these different morphological types were located at slightly different depths within the SGS. NFV cells were located closest to the SC surface; since these cells extend dendrites vertically they can reach the dorsal most regions of the SGS. In contrast, horizontal cells were generally located farther from the dorsal surface of the SC and extend dendrites parallel to the SC surface. Thus these cells could potentially be innervated by different types of retinal ganglion cells that target different dorsal-ventral locations within the SGS.

Transsynaptic labeling studies (Zingg et al., 2017) indicate that inputs from both the retina and V1 innervate SC cells that project to the PBG. However, transsynaptic labeling in GAD2-Cre mice indicates that V1 innervates GABAergic interneurons that do not project outside the SC. Therefore, GABAergic SGS-PBG cells may receive direct input from the retina, but not V1, and nonGABAergic SGS-PBG cells receive direct input from V1, and potentially the retina. Interestingly, optogenetic activation of PV-containing cells in the SGS, or PV projections to the PBG elicit escape responses (Shang et al., 2015, 2018), but activation of PBG terminals originating from SC cells that receive V1 input does not (Zingg et al., 2017). Therefore, key questions for future studies will be to determine whether all nonGABAergic

projections to the PBG contain PV, what inputs target GABAergic and nonGABAergic SGS cells that project to the PBG, and how activation of GABAergic versus nonGABAergic SGS-PBG cells affects behavior.

Projections from the SC to the LGN and PT

Projections from the mouse SC to dLGN have been described as both glutamatergic (Bickford et al. 2015; Gale and Murphy 2018) and GABAergic (Gale and Murphy 2014). SC-dLGN cells are located in the SGS and are topographically organized (Bickford et al. 2015). Following CTB injections in the dLGN of GAD2-Ai9 mice, less than 5% of the SC-dLGN cells could be identified as GABAergic. Moreover, virus injections in the SC of C57BL/6J mice labeled a discrete band of terminals in the lateral shell of the dLGN which, when optogenetically activated, elicited only glutamatergic responses in dLGN cells (Bickford et al. 2015). Finally, large cre-dependent virus injections (AAV serotype 9, chicken β actin promoter) in the SC of GAD2-cre mice label only very sparse projections to the dLGN after survival times of 10-16 days (Bickford et al., 2015; and Figure 2d of the current study). However, in a previous study, after survival times of 4-6 weeks, virus injections (AAV serotype 2.1 synapsin promoter) in the SC of GAD2-Cre mice labeled a dense distribution of terminals in the dLGN which was not restricted to the shell (Gale and Murphy 2014). Furthermore, when activated optogenetically, these terminals could elicit GABAergic responses in dLGN neurons (Gale and Murphy 2014).

Although inconsistencies in the results of the studies described above remain to be resolved, a possible explanation is the finding that AAVs can be transynaptically transported during long survival times, particularly those with synapsin promoters (Zingg et al. 2017). In the current study, we find that GABAergic SC cells project to the PT, and previous studies indicate that GABAergic cells in the PT project to the dLGN (Cucchiari et al., 1991; Wang et al. 2002; Masterson et al., 2016). Thus, after long survival times, virus injections in the SC could label PT-LGN cells via transynaptic transport, resulting in virus-labeled GABAergic axons in the dLGN. Alternatively, since GABAergic cells in the PT project to the SC (Baldauf et al. 2003), GABAergic PT cells could have been labeled via retrograde transport; in fact, the viruses used in this Gale and Murphy (2014) study were reported to label neurons via retrograde transport. Such alternative viral transport routes would explain the differences in previous studies and account for the current findings that GABAergic projections from the SC innervate the vLGN, PT and PBG, but not the dLGN. Furthermore, glutamatergic SC projections to the dLGN arise from the SGS (Bickford et al., 2015), while GABAergic projections to the vLGN and PT arise primarily from cells located ventral to the SGS.

GABAergic interneurons of the SGS

Based on the uptake of [^3H]GABA and immunocytochemistry, Mize (1982,1988) identified 2 main types of GABAergic neurons with intrinsic connections in the cat SGS: horizontal cells and granule cells. GABAergic horizontal cells were defined as cells that have long smooth dendrites oriented parallel to the SC surface, while GABAergic granule cells were defined as cells with stellate or obliquely-oriented dendrites. These two cells types were

found to exhibit different types of synaptic connections. The dendrites of horizontal cells received input from V1 and contained small accumulations of synaptic vesicles along the length of their dendritic shafts, clustered at locations where periodic synaptic contacts were made with nonGABAergic dendrites. In contrast, GABAergic granule cells were identified as the source of presynaptic dendritic appendages that receive input from retinotectal terminals and contact nonGABAergic dendrites within complex “synaptic islands”. In the mouse, there is also evidence for these two general categories of GABAergic cell types. In GAD2-cre mice, V1 injections of a virus that is transported transynaptically primarily labeled horizontal cells that do not project outside the SC (Zingg et al. 2017), whereas few V1 synaptic contacts are found on GABAergic profiles within synaptic islands (Masterson et al., 2019). Moreover, optogenetic activation of terminals that originate from V1 rarely activate GAD67-GFP neurons (Masterson et al., 2019), which in the current study we have shown are a source of presynaptic dendrites that receive input from retinotectal terminals in complex “synaptic islands”.

However, available data from the mouse also suggests that two broad morphological categories of GABAergic interneurons that are differentially innervated by cortical or retinal inputs is insufficient to fully describe the complexity of GABAergic circuits of the SGS. First, GAD67-GFP neurons labeled via the intracellular injection of biocytin exhibit a variety of morphologies (Endo et al. 2005; Villalobos et al. 2018; Masterson et al. 2019). In addition, GABAergic SGS cells may be subdivided based on immunocytochemistry, genetic composition, and/or membrane properties. For example, recordings from GAD67-GFP cells in the mouse SGS revealed 3 distinct types of firing characteristics in response to current injection (Endo et al., 2003), whereas firing characteristics are more uniform in the subset of GAD67-GFP neurons that contain PV (Villalobos et al. 2018). In addition, Rorb-Cre mice identify a subtype of GABAergic interneuron with horizontal characteristics (Gale and Murphy, 2018). Finally, we identified a subtype of interneuron that contains PV and GABA, but is not labeled with GFP in the GAD67-GFP line. Although the features of all GAD67-GFP cells may not be uniform (Villalobos et al. 2018; Endo et al. 2003), our results suggest that they exhibit the unifying feature of being interneurons that do not project outside the SC (although we cannot rule out that they may project to targets that we did not inject with CTB, such as the periaqueductal gray). Our results also indicate that GAD67-GFP cells are a source of the presynaptic dendritic appendages that form complex synaptic arrangements with retinotectal terminals. Thus, this mouse line provides a useful model to investigate how GABAergic dendritic terminals may affect retinotectal visual signals.

A recent in vivo calcium imaging study carried out in the GAD67-GFP line (Kasai and Isa, 2016) has shown that both GFP-positive and GFP-negative SGS cells in the dorsal SGS exhibit receptive fields with small excitatory centers flanked by inhibitory surrounds. These results suggested that GAD67-GFP interneurons contribute to center surround organization via long range lateral connections within the SGS. In contrast, horizontal cells in the mouse SGS have been shown to respond to large visual stimuli and inhibitory circuits formed by these neurons are suggested to underlie the tuning of widefield vertical cells to ensure detection of the movement of small objects (Gale and Murphy 2016). Studies in the optic tectum of the zebrafish also indicate that subsets of GABAergic interneurons respond to large visual stimuli to contribute to the detection of small moving visual stimuli (Del Bene et

al. 2010). Finally studies of the lamprey optic tectum suggest that local feedforward inhibition imposes a visual signal integration time window that restricts excitation buildup, while an interconnected GABAergic network suppresses competitive visual signals to ensure the selectivity of gaze commands (Kardamakis, Saitoh, and Grillner 2015; Del Bene et al. 2010).

Thus, a variety of evidence indicates the existence of both local and global inhibition within the SGS. Key questions for future studies will be to determine whether horizontal and granule cell circuits shape visual signals in the SC in different ways. As a first step, it will be important to determine whether V1 and retinal inputs target different subsets of interneurons, or different segments of the dendritic arbors of individual interneurons. It will also be important to determine whether individual interneurons are targeted by specific subtypes of ganglion cells or integrate several channels of visual information. Finally, since SGS glutamatergic cells also form synaptic connections within the SC (Villalobos et al. 2018; Gale and Murphy 2014; Gale and Murphy 2018), it will be important to determine how the intrinsic connections of both GABAergic and glutamatergic cell types interact to sculpt retinotectal signals.

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Figure 1).

The GAD67-GFP mouse line labels subsets of GABAergic and PV cells in the SGS. a) The GAD67-GFP mouse line labels a dense population of neurons in the SGS. b-d) SGS of a GAD67-GFP mouse stained with an antibody against GABA (b, GFP, c, GABA, d, GFP and GABA). Magenta arrows indicate GABAergic neurons not labeled with GFP. e-g) SGS of a GAD67-GFP mouse stained with an antibody against NeuN (e, GFP, f, NeuN, g, GFP and NeuN). Magenta arrows indicate neurons not labeled with GFP. h-j) SGS of a GAD67-GFP mouse stained with an antibody against PV (h, GFP, i, PV, j, GFP and PV). Magenta arrows indicate PV neurons not labeled with GFP, green arrows indicate GFP neurons not labeled with PV, and white arrows indicate neurons labeled with both GFP and PV. Scale bar in a = 100 μ m. Scale bars in d, g and j = 10 μ m.

Figure 2).

GABAergic extrinsic projection targets of the SC Cre-dependent adeno associated virus (AAV) injections placed in the SC of GAD2-Cre mice (a) labeled axon projections to the parabigeminal nucleus (PBG, b), the pretectum (PT, c) and the ventral lateral geniculate nucleus (vLGN, d). Scale bar = 100 μ m and applies to all panels. SGS, stratum griseum superficiale, SGI, stratum griseum intermediale.

Figure 3).

GABAergic SGS cells that project to the PBG. SC cells labeled by the retrograde transport of Cre-dependent herpes simplex virus (HSV) injections in the PBG (a) of GAD2-Cre mice were primarily distributed in SGS (b). c) None of these virus-labeled SC-PBG cells (green) were stained with a PV antibody (purple). Cholera toxin subunit B (CTB, purple) was injected into the PBG (d) and surrounding areas (e) of GAD67-GFP mice. f) No CTB (purple) was found in GAD67-GFP cells (green), but a subset of CTB-labeled cells were stained with a PV antibody (g, white arrows; $16.5 \pm 6.8\%$ of SC-PBG cells). Scale in a = 100 μm . Scale in b = 100 μm , c = 40 μm . Scale in d = 100 μm and also applies to e. Scales in f and g = 10 μm .

Figure 4). GAD67-GFP cells do not project to the PT or vLGN. Cholera toxin subunit B (CTB, purple) was injected into the PT (a) or LGN (b) in GAD67-GFP mice and the tissue was stained with a PV antibody (blue). c) Virtually no CTB (purple) was found in GAD67-GFP cells (green), but a subset of CTB-labeled cells contained PV (white arrows; $27.2 \pm 15.2\%$ of SC-PT cells, and $11.7 \pm 5.2\%$ of SC-LGN cells). A cell that contains CTB but not PV is indicated with a magenta arrow. Scale in a = 100 μm and applies to b. Scale in c = 10 μm .

Figure 5).

Distribution of GABAergic cells that project to the PT or vLGN. SC cells labeled by the retrograde transport of Cre-dependent HSV injections in the PT (a) or vLGN (d) of GAD2-Cre mice were primarily distributed in SC layers deep to the SGS. b,c) SC-PT cells. e,f) SC-vLGN cells. Scale bar = 100 μ m and applies to all panels.

Figure 6).

Distributions of GABAergic SC projection neurons. SC cells labeled by the retrograde transport of Cre-dependent HSV injections in the PBG, vLGN or PT of GAD2-Cre mice were distributed at different depths relative to the dorsal surface of the SC. The box and whisker plot illustrates the mean depth of labeled cells (X), first and third quartiles (boxes), data 1.5x the interquartile range (lines), and outliers (circles). The distribution of cells that project to the PT or vLGN were significantly different from those that project to the PBG ($p < 0.0001$, ****), but the distribution of NFV, stellate and horizontal cells that project to the PBG were not significantly different from one another. There was also no significant difference between the distributions of cells that project to the PT versus vLGN.

Figure 7). Distribution of SC cells that project to the ventral tegmental area (VTA). Large CTB injections in the VTA of GAD67-GFP mice (a, magenta) labeled SC cells ventral to the SGS (b, magenta). Scale in a = 200 μ m. Scale in b = 100 μ m. SGI, stratum griseum intermediale, SNR, substantia nigra pars reticulata, SO, stratum opticum,

Figure 8).

Retinotectal synaptic connections. Retinotectal terminals (green) can be identified by their unique “pale” mitochondria with widened cristae (asterisks). a-c) Retinotectal terminals are embedded within complex synaptic arrangements of GABAergic profiles that contain vesicles (red) and nonGABAergic dendrites that do not contain vesicles (blue). Retinotectal terminals make synaptic contacts with both GABAergic (red arrows) and nonGABAergic dendrites (blue arrows). d) GABAergic profiles can occasionally be seen to be postsynaptic to retinotectal terminals (green, red arrow) and presynaptic to nonGABAergic dendrites (blue, blue arrow). e-f) In tissue from GAD67-GFP mice, profiles labeled with a GFP antibody (dark reaction product) stain for GABA (high density of gold particles) and are postsynaptic to retinotectal terminals (green, red arrows). Retinotectal terminals that contact

GFP-labeled profiles also make synapses on adjacent nonGABAergic dendrites (blue, blue arrows). Scale in c = 600nm and also applies to a,b,e and f. Scale in d = 200 nm.

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Figure 9).

Summary of GABAergic cells types in the mouse SGS. The Venn diagram illustrates the approximate proportions of GABAergic SGS neurons listed to the right. GABAergic neurons that contain GFP in the GAD67-GFP line and/or are labeled with a PV antibody are identified as interneurons. The remaining cells are potential projection neurons that include NFV, stellate, and horizontal cells that project to the PBG. The GAD67-GFP mouse line labels intrinsic interneurons in the SGS which are a source of GABAergic presynaptic dendrites that receive retinal input and contact nonGABAergic dendrites.