Functional connectivity from zona incerta cells coexpressing dopamine and GABA
to the superior colliculus

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Abstract

The zona incerta is important for anxiety-mediated behaviors. It contains a group of dopaminergic cells that coexpress the vesicular GABA transporter, potentially coreleasing dopamine and GABA. The superior colliculus is a known projection site of the zona incerta and supports anxiety-driven behavior. However, it is not known if dopaminergic zona incerta cells innervate the superior colliculus via dopamine and GABA transmission. We expressed channelrhodopsin in zona incerta neurons of *Th-cre* mice, and their fibres were restricted to the medial motor-related layers of the superior colliculus. The superior colliculus cells received monosynaptic GABA transmission from the zona incerta, and these connected cells were dopamine responsive. These results indicated a direct connection between the zona incerta dopamine-GABA cells and the medial motor layers of the superior colliculus. These results are important in understanding the role of the zona incerta dopamine-GABA cells in the regulation of anxiety-related behaviors in the superior colliculus.

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List of Abbreviations

Abbreviation	Full name
4AP	4-aminopyradine
AAV	Adeno-associated viral vector
ACSF	Artificial cerebrospinal fluid
AHN	Anterior hypothalamic nucleus
AP	Action potential
ARA	Allen reference atlas (www.brain-map.org)
Bic	Bicuculline
ChR2	Channelrhodopsin
Cs	Cesium internal pipette solution
DA	Dopamine
DAB	3,3'-diaminobenzidine
DAT	Dopamine transporter
DMH	Dorsomedial hypothalamic nucleus
DV	Dorsoventral
EGFP	Enhanced green fluorescent protein
GFP	Green fluorescent protein
GABA	Gamma-aminobutyric acid
IHC	Immunohistochemistry
ip	Intraperitoneal
ir	Immunoreactive
KGluc	Potassium-gluconate internal pipette solution
LHA	Lateral hypothalamic nucleus
NDS	Normal donkey serum
NMDG	<i>N</i> -methyl-D-glucamine
oIPSC	Optogenetically-evoked inhibitory postsynaptic currents
PBS	Phosphate buffered saline
PBS-azide	PBS containing 0.05% sodium azide
PBT	PBS containing 0.25% Triton X-100
PBT-azide	PBT containing 0.05% sodium azide
PH	Posterior hypothalamic nucleus
PR	Perireunensis nucleus
PVH	Paraventricular hypothalamic nucleus
Rac	Raclopride
RMP	Resting membrane potential
RT	Reticular nucleus of the thalamus
SC	Superior colliculus
zo	Superior colliculus zonal layer
sg	Superior colliculus superficial gray layer
op	Superior colliculus optic layer
ig	Superior colliculus intermediate gray layer
iw	Superior colliculus intermediate white layer
dg	Superior colliculus deep gray layer

Abbreviation	Full name
dw	Superior colliculus deep white layer
SCH	SCH23390
SMT	Submedial nucleus of the thalamus
TH	Tyrosine hydroxylase
TTX	Tetrodotoxin
vGAT	Vesicular GABA transporter
VM	Ventromedial nucleus of the thalamus
VMH	Ventromedial hypothalamic nucleus
WT	Wildtype
ZI	Zona incerta
ZIda	Zona incerta dopaminergic group
ZI ^{DA+vGAT}	Zona incerta cells that coexpress dopamine and vGAT

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1 Introduction

The zona incerta (ZI), also known as the “zone of uncertainty,” was first described by Auguste Forel in 1877 (Forel, 1877; Romanowski et al., 1985; Mitrofanis et al., 2004; Nieuwenhuys et al., 2008). To date, it has been implicated in visceral activity, arousal, posture, locomotion, shifting attention (Mitrofanis, 2005), and fear-related behaviours (Venkataraman et al., 2021). The ZI is an understudied region in the brain, and most research has focused on characterizing its cytoarchitecture and chemoarchitecture. In contrast, studies looking at the electrical characteristics, connectivity, and functional activity are few and far between. In addition, most research about the ZI has been done with rats, and there are very few reports that have characterized the mouse ZI. To account for this knowledge gap, information is borrowed from the rat ZI to inform the structure and function of the mouse ZI. There is significant work that still needs to be done to characterize the ZI in the mouse brain. As transgenic mouse models can be used to study specific neuronal populations, it is vital to establish the ZI properties in the mouse in order to determine how the different neuronal populations contribute to the overall function of the ZI.

1.1 Structural characterization of the zona incerta

The ZI resides in the area between the thalamus and the hypothalamus. According to the Allen Reference Atlas (ARA) for the mouse brain, the ZI is part of the lateral zone of the hypothalamus and extends from just after the medial preoptic nucleus (level 60) to the ventral tegmental area (level 83; Dong, 2008). Currently, the most common reports for ZI subregions are found in the rat, where the ZI is divided into four subregions described as the dorsal, ventral, rostral, and caudal ZI (Shaw and Mitrofanis, 2002; Nieuwenhuys et al., 2008). These subregions have not yet been identified in the mouse, and the ARA has not divided the ZI into these

subregions. In the mouse, the ZI only has one subdivision known as the zona incerta dopaminergic group (ZIda), which is located medial to the fornix and is restricted to levels 67 to 69 in the ARA, but the rest of the ZI is undivided (Dong, 2008). Despite this, some articles have used the rat nomenclature to classify the area of the mouse ZI they are studying to increase their resolution when describing the area (e.g. Zhang and Van Den Pol, 2017; Chou et al., 2018; Zhao et al., 2019). In addition to these labels borrowed from the rat, researchers often refer to medial and lateral ZI - in relation to the fornix - to describe where in the ZI they are targeting (Wagner et al., 1995).

In this thesis, we will be using the nomenclature and subdivisions of the ZI as defined by the ARA and that includes the ZI and ZIda, as well as refer to the medial and lateral ZI in relation to the fornix.

1.2 Neuronal subpopulations in the zona incerta

1.2.1 Zona incerta GABA cells

The ZI has a dense population of γ -aminobutyric acid (GABA) cells, and they are found throughout the entirety of the ZI (Kolmac and Mitrofanis, 1999). GABA is a major inhibitory neurotransmitter in the adult brain. It is a fast-acting amino acid neurotransmitter that is released directly at the synaptic cleft and diffuses across short, nanometer-long distances to act on its receptors (van den Pol, 2012). This short distance allows for GABAergic neurons to have a direct effect on postsynaptic cells. Studies show that in the rat, ZI neurons exhibit immunoreactivity for glutamic acid decarboxylase, the enzyme that synthesizes GABA from the excitatory neurotransmitter glutamate (Oertel et al., 1982), and immunoreactivity for GABA itself (Ottersen and Storm-Mathisen, 1984). In mice, the ZI has been shown to express mRNA of the vesicular GABA transporter (vGAT), which is required to transport GABA into synaptic

vesicles (Negishi et al., 2020). The ZI also expresses immunoreactivity for GABA transaminase, the enzyme which degrades GABA, in rats (Araki et al., 1984). Thus, the ZI contains the machinery for GABA synthesis, synaptic release, and degradation.

1.2.2 Zona incerta dopamine cells

The dopamine (DA) cells in the ZI are part of the incerto-hypothalamic DA system (Björklund and Nobin, 1973; Arnold et al., 1989; Sita et al., 2003; Nieuwenhuys et al., 2008). These DA cells are localized to levels 67-69 of the ARA (Negishi et al., 2020). Monoamine neurotransmitters were classified in the brain into groups, where catecholamines such as DA are identified as group A. The ZI catecholamine population is referred to as the A13 group (Dahlstroem and Fuxe, 1964).

Tyrosine hydroxylase (TH) is the rate-limiting enzyme in catecholamine synthesis and is used as a marker for catecholaminergic cells, and neurons in the ZI have been shown to express TH (Kolmac and Mitrofanis, 1999; Mitrofanis et al., 2004). Our lab has recently demonstrated that the TH-immunoreactive (ir) neurons in the ZI express immunoreactivity for DA itself and lack the noradrenaline-synthesizing enzyme dopamine beta-hydroxylase (Negishi et al., 2020). Therefore, it is unlikely that ZI neurons can convert DA to other catecholamines like noradrenaline or adrenaline that are downstream of the DA synthesis pathway. Additionally, the ZI catecholamine cells also express the DA-synthesizing enzymes levodopa and aromatic L-amino acid decarboxylase (Sharma et al., 2018). Thus, the catecholamine identity of this group is DA, and TH in this population can be used as a marker for dopaminergic neurons.

For cells to release DA, they must contain the machinery for DA signalling. TH-ir neurons in the ZI were shown to express the vesicular monoamine transporter, which packages monoamines, including DA, into vesicles for synaptic release (Sharma et al., 2018). There are

conflicting reports on whether the ZI cells express the dopamine transporter (DAT), which removes DA from the synaptic cleft after it is released (Lammel et al., 2015; Sharma et al., 2018; Papathanou et al., 2019). The lack of DAT could have implications for DA signalling as dopaminergic populations that do not express DAT (A11, A14, and A15 dopaminergic groups; Koblinger et al., 2014; Lorang, Amara, & Simerly, 1994) or have had DAT knocked out or down show increased DA tone and longer signalling action (Jones et al., 1998). These combined data suggest that the DA cells in the ZI contain the machinery for packaging monoamines into vesicles and the enzymes required to synthesize DA but may not contain the machinery for DA reuptake.

1.2.3 Zona incerta population that expresses both dopamine and GABA

In the past, neurons were assumed to release only one neurotransmitter, and it has since been shown that neurons are able to express multiple neuronal messengers and signal through their corelease (Hökfelt et al., 1986). Corelease may involve the release of two neurotransmitters from the same nerve terminal at the same target site for both neurotransmitters or different target sites for each neurotransmitter (Root et al., 2018). As such, these messengers can work together or in opposition to alter neural activity at the same target site, and each messenger may modulate distinct functions at separate sites (van den Pol, 2012; Chee et al., 2015). Our lab has recently shown that in the mouse ZI, every TH-ir neuron coexpressed vGAT (Negishi et al., 2020). A neuron that expresses vGAT will most likely synthesize and release GABA; therefore, vGAT is a good marker for identifying GABAergic cells. We have shown this TH+vGAT population in the ZI expresses immunoreactivity for DA; hence, these cells are putative dopaminergic and GABAergic ZI neurons (Negishi et al., 2020). This population, herein referred to as the

ZI^{DA+vGAT} cells, is restricted to a 0.2 mm cross-section of the ZI from levels 67–69 of the ARA and is located in the part of the medial ZI.

The GABAergic and dopaminergic cells in the ZI have previously been studied separately. However, as we have identified this novel population of ZI cells that express markers for both neurotransmitters, more research needs to be done to characterize the anatomical projections and functional activity of these combinatorial cells. The release of both GABA and DA from the same cells in the ZI has not been previously studied. It is unknown if the ZI^{DA+vGAT} population mediates known functions of either the GABA cells or DA cells in the ZI or if they have unique functional outputs. Therefore, it will be important to determine if these cells have similar connectivity and functional activity to what has previously been shown.

1.3 Superior colliculus receives projections from the zona incerta

A key feature of the ZI is that it has widespread connectivity throughout the central nervous system (Power and Mitrofanis, 1999; Mitrofanis, 2005). Using nomenclature and organization from the ARA, the main areas of both afferent and efferent projections of the ZI are the cerebrum, interbrain regions such as the thalamus, midbrain regions such as the superior colliculus (SC), and the spinal cord (Mitrofanis, 2005). The SC is one of the established anatomical projections from the ZI, particularly via the GABA cells. This connection is known as the colliculo-incerta pathway, and it has been shown to be involved in attention-directing behaviours (Shaw and Mitrofanis, 2002; Mitrofanis, 2005; Comoli et al., 2012). However, the mechanisms underlying its behavioural outputs are not well known (Essig and Felsen, 2016).

1.3.1 Structure and function of the superior colliculus

The SC is an integration center for sensorimotor inputs, including auditory (Xu et al., 2018), visual and somatosensory stimuli, as well as mediating motor responses (Drager and Hubel, 1976). It is well known for its role in saccadic eye movements (Sparks et al., 1990) and is also involved in avoidance and defensive behaviours (Comoli et al., 2012; Shang et al., 2015).

The SC is a laminar structure divided into two areas: the sensory- and motor-related regions. The sensory-related regions are in the dorsal portion of the SC and are layers 1–3, which are known as the zonal layer (zo), superficial gray layer (sg), and the optic layer (op). The cells in these regions respond to changes in the visual field, and these regions are involved in integrating visual signals from retinal ganglion cells (Dean et al., 1989; Cang et al., 2018). The motor-related regions are more ventral and are layers 4–7 of the SC, which are the intermediate gray layer (ig), intermediate white layer (iw), deep gray layer (dg), and the deep white layer (dw). The motor-related regions are involved in generating what is known as the orienting reflex and other motor responses (Dean et al., 1989). The sensory and motor regions of the SC are interconnected and provide feedback to one another to regulate sensorimotor activity (Ghitani et al., 2014).

1.3.2 The colliculo-incerta pathway

The colliculo-incerta pathway is a reciprocal pathway that includes projections between the ZI and the SC. Although the SC is divided into sensory- and motor-related layers, not all inputs follow these divisions. For example, the medial SC primarily receives input from visual cues, whereas the lateral SC receives somatosensory stimuli. In addition to these, the projections between the SC and the ZI also follow the medial-lateral input pattern rather than a layer-based input pattern. The medial SC is considered the visual area and stimulates the lateral part of the ZI

in response to light cues, whereas the somatosensory-cue responsive area in the lateral SC connects with the medial ZI in response to whisker deflection (Figure 1; Watson et al., 2015).

Figure 1: Schematic of known pathways between the ZI and SC. Sensory input into the medial and lateral zones of the SC mediate approach and avoidance behaviours, respectively.

The SC receives inputs from both the GABA (Kim et al., 1992; Nicolelis et al., 1992) and DA populations in the ZI (Bolton et al., 2015). The ZI GABA population has been shown to have a rostrocaudal topography with the SC, where the rostral GABA cells project to the rostral SC and the caudal ZI GABA cells project to the caudal SC. It also has an inverse mediolateral topography relationship where the lateral ZI cells project to the medial SC and the medial ZI to the lateral SC (Figure 1; Romanowski et al., 1985; Kim et al., 1992). SC behavioural output is also medial-lateral based, where the medial SC is involved in avoidance behaviours, and the lateral SC is involved in approach behaviours (Dean et al., 1989), and are thought to be driven in part by ZI GABAergic projections to these regions (Figure 1, Comoli et al., 2012; Kim et al., 1992)

In addition to the GABAergic pathways, the dopaminergic cells in the medial part of the ZI have also been shown to project to the SC. Injection of retrograde virus directly into the medial SC was shown to be expressed in ZI cells that express TH (Bolton et al., 2015; Essig and Felsen, 2016). The primary source of DA input to the SC is proposed to come from the ZI but evoked DA release from the ZI has yet to be shown to modulate this pathway (Bolton et al., 2015).

1.3.3 Dopamine and GABA receptors present in the superior colliculus

In order for the SC to respond to GABA or DA, it must express its receptors. In the brain, there are three types of GABA receptors: GABA_A, GABA_B, and GABA_C, and all three receptors can be found both pre- and post-synaptically (Owens and Kriegstein, 2002; Bouché et al., 2003). GABA_A and GABA_C are ionotropic, whereas GABA_B is a metabotropic G_i/G_o-coupled receptor. The SC contains GABA_A receptors that are not localized to one area (Kaila, 1994; Owens and Kriegstein, 2002). GABA_B receptors are located in the intermediate gray layer of the SC and were shown to regulate the duration of burst activity in non-GABAergic neurons in the SC (Kaneda et al., 2008). The SC also contains GABA_C receptors in the sensory-related areas (Pasternack et al., 1999; Owens and Kriegstein, 2002).

There are five types of DA receptors, D₁–D₅, and all are metabotropic, G-protein coupled receptors (Kebabian and Calne, 1979; Sokoloff et al., 1990; Sunahara et al., 1991; Van Tol et al., 1991; Civelli et al., 1993). RNA sequencing has shown that predominantly D₁ and D₂ DA receptors are present on postsynaptic cells in the SC (Bolton et al., 2015). G_s-coupled D₁ receptors are expressed more strongly in the sensory-related layers (layers 1–3) of the SC, and G_i/G_o-coupled D₂ receptors are expressed more ventrally in the motor-related layers of the SC (layers 4–5; Kebabian and Calne, 1979; Civelli et al., 1993). In the SC, D₁ receptors, but not D₂,

are primarily located on GABAergic cells. Both D₁ and D₂ receptor activation in the SC produce a net inhibitory effect in the SC (Bolton et al., 2015).

1.4 Rationale, hypothesis, and aims

The primary dopaminergic input to the SC is thought to originate from the ZI, and the SC also receives input from GABA cells in the ZI (Bolton et al., 2015; Zhao et al., 2019). However, it is not known if the ZI^{DA+vGAT} cells we identified contribute both DA and GABA to the SC.

We hypothesize that the ZI^{DA+vGAT} population is functionally connected to the SC through the corelease of GABA and DA. We were able to mark ZI^{DA+vGAT} cells using TH (Negishi et al., 2020), so we used a *Th-cre* mouse line to label this population for our studies. To test this hypothesis, we combined viral tracing, *in vitro* optogenetics, and *ex vivo* pharmacology techniques to achieve the following aims.

Aim 1: Map the distribution of ZI^{DA+vGAT} neuron projections to the SC.

All TH-ir neurons in the ZI coexpress vGAT. These ZI^{DA+vGAT} neurons were identified using a *Th-cre:L10-Egfp* mouse line (herein referred to as TH ZI cells). We performed cre-dependent anterograde tracing from TH ZI neurons to map their fibre distribution across all layers of the SC mediolaterally.

Aim 2: Determine if the ZI^{DA+vGAT} neurons innervate the SC by GABA release.

We transfected *Th-cre* cells in the ZI with a cre-dependent virus encoding channelrhodopsin (ChR2). We performed patch-clamp recordings from SC cells within the fibre field in the SC and photostimulated these ChR2-expressing fibres with a brief light pulse (5 ms) to measure optogenetically-induced inhibitory postsynaptic currents (oIPSC). We analyzed the resulting oIPSCs to determine if the TH ZI neurons innervated the SC by GABA release.

Aim 3: Determine if the $ZI^{DA+vGAT}$ neurons also release DA in the SC.

DA has a net inhibitory effect in the SC. We photostimulated ChR2-expressing TH ZI fibres in the SC with a continuous, high frequency light stimulation (10 Hz for 2 min) and measured changes in membrane potential in order to assess the release of DA from TH ZI fibres in the SC.

2 Methods

2.1 Animals

All animal care and procedures were approved by the Animal Care Committee at Carleton University. Mice had *ad libitum* access to standard mouse chow (Teklad Global Diets 2014, Envigo, Mississauga, Canada) and water. Mice were group-housed in individually vented cages (GM500, Tecniplast, Toronto, ON, Canada) on DGM racks (Tecniplast) at room temperature with a 12:12 light-dark cycle. The temperature of the room was, on average, 21–22°C and the relative humidity was between 40 and 50%.

To visualize catecholaminergic cells, we labelled neurons expressing the catecholamine synthesizing enzyme TH with enhanced green fluorescent protein (EGFP) by generating *Th-cre;L10-Egfp* mice (native-GFP). We generated this mouse by crossing a *Th-ires-cre* (Stock No. 008601, Jackson Laboratory, Bar Harbor, ME) with a C57BL/6 background with a Cre-dependent *R26-lox-STOP-lox-L10-Egfp* reporter mouse (Krashes et al., 2014) which was generously provided by Dr. B. B. Lowell (Beth Israel Deaconess Medical Center, Boston, MA). Cre-negative littermates were used in pharmacology studies and for virus selectivity validation ($n=40$). Both male and female mice were used in all experiments.

2.2 Stereotaxic viral injections

2.2.1 Stereotaxic surgery

All mice undergoing stereotaxic surgery were prepared using this procedure and were anesthetized using 4% isoflurane and 96% oxygen and maintained at 2% isoflurane and 98% oxygen. For pain management, mice received a subcutaneous injection of meloxicam (1 mg/kg) once a day for three days starting on the day of surgery. On the day of surgery, the head of the mouse was shaved from the back of the ears to the eyes and washed with chlorohexidine and

alcohol. The mouse was placed in the stereotaxic apparatus and ear bars so that its head was flat and did not move. Petrolatum ophthalmic gel (Dechra Pharmaceuticals, Pointe-Claire, QC, Canada) was placed on the eyes to prevent them from drying out. The top of the skull was cleaned with betadine to ensure that the incision remained aseptic. An incision was made using a scalpel from anterior to bregma to lambda along the midline, and periosteal connective tissue on the skull was removed using a sterilized cotton swab. A 25 mm long borosilicate glass micropipette (ID 0.30 mm) was pulled using a Flaming/Brown Micropipette puller (Model P-1000, Sutter Instruments, Novato, CA) and was filled with the virus. The glass pipette was used to determine the dorsoventral (DV) coordinates of bregma and lambda, and the head was adjusted so that bregma and lambda were within 0.1 mm. The injection coordinates for both anterograde and retrograde tracing were taken in relation to bregma (see Table 1 for coordinates, viruses, and volumes used), and an electric surgical drill was used to remove the skull at the injection location. The meninges were poked through using a hooked sterile needle to ensure the glass pipette did not bend or break. The glass pipette was lowered to the appropriate DV coordinate, and the virus was pressure-injected at a rate of 25 nl/minute. The glass pipette was not removed for 5 minutes to allow for the virus to disperse. Once the glass pipette was removed, the hole in the skull was sealed with sterile bone wax (Ethicon, Somerville, NJ), and the incision was sutured shut with LOOK silk sutures (4-0 USP, Wyomissing, PA). The mouse was kept on oxygen until they began to regain consciousness. The mouse was removed from the stereotaxic apparatus and single housed in a clean cage. For one-hour post-surgery, the mice were monitored for signs of complications and were continually checked a minimum of three times a day for a minimum of two days.

Table 1: Summary of stereotaxic injections

Experiment	Injection site	Coordinates (mm)	Virus	Volume (nl)	Genotype (n), age
A) Neuroanatomical analysis of anterograde tracing	Zona incerta	AP: -1.2 ML: -0.5 DV: -4.7	AAV2/DJ-Efl α -DIO-ChETA-tdTomato, (AAV1131, Neurophotronics, Quebec City, QC, Canada) AAVDJ/8-Efl α -DIO-hChR2(H134R)-mCherry (AAV1330 Neurophotronics)	25	WT (2) / <i>Th-cre:L10-Egfp</i> (15), 5–10 weeks
B) <i>In vitro</i> optogenetic stimulation	Zona incerta	AP: -1.2 ML: -0.5 DV: -4.7	AAVDJ/8-Efl α -DIO-hChR2(H134R)-mCherry (AAV1330, Neurophotronics)	25–50	<i>Th-cre:L10-Egfp</i> (44), 4–10 weeks
C) Neuroanatomical analysis of retrograde tracing	Superior colliculus	AP: -3.6 ML: -0.1 DV: -1.5	AAV-Retrograde-Efl α -flox-hChR2(H134R)-mCherry-WPRE-HGHpa (20297-AAVrg, Addgene, Watertown, MA)	50–100	<i>Th-cre:L10-Egfp</i> (3), 5–25 weeks

2.2.2 Anterograde tracing

Anterograde tracing experiments were performed by injecting an adeno-associated viral (AAV) vector into the ZI, as described in Table 1, Experiments A and B. Both viruses used for these experiments (AAV2/DJ-Efl α -DIO-ChETA-tdTomato and AAVDJ/8-Efl α -DIO-hChR2(H134R)-mCherry) used a cre-driven elongation factor 1 α (Efl α) promoter. AAV2/DJ-Efl α -DIO-ChETA-tdTomato encoded channelrhodopsin (ChR2) with the E123T mutation

(ChETA)-tdTomato. AAVDJ/8-Ef1 α -DIO-hChR2(H134R)-mCherry encoded the H124R-mCherry variant of ChR2. For each virus, a cre-negative littermate was injected with the virus as a control to ensure the virus was selective for cre-containing cells.

In Experiment A, the virus was incubated for 2–4 weeks before tissue was collected and analyzed as per section 2.5.

In Experiment B, the virus was incubated for 4–8 weeks (primarily 4 weeks) before tissue was collected for electrophysiology as per section 2.3.

2.2.3 Retrograde tracing

Retrograde tracing experiments were performed by injecting a retrograde AAV vector that used a cre-driven Ef1 α promotor and encoded the H124R-mCherry variant of ChR2 into the SC, as described in Table 1, Experiment C. The virus was incubated for six weeks before tissue was collected and analyzed as per section 2.5.

2.3 Electrophysiology

2.3.1 Dissection and slice preparation

In vitro optogenetic experiments were performed 4–8 weeks after AAV injections. Mice were injected intraperitoneally (ip) with 7% chloral hydrate (10 cc/10 g/mL) until anesthetized and did not respond to toe pinch. The mice were transcardially perfused with carbogenated (95% O₂, 5% CO₂), ice-cold N-methyl-D-glucamine (NMDG)-based artificial cerebrospinal fluid (ACSF) solution containing (in mM) 100 NMDG, 2.5 KCl, 20 HEPES, 25 glucose, 30 NaHCO₃, 0.5 CaCl₂, 2 thiourea, 5 Na-ascorbate, 3 Na-pyruvate, and 10 MgSO₄ (285–295 mOsm/L). The NMDG solution was made with 1 mM kynurenic acid (K3375, Sigma Aldrich, Oakville, ON, Canada) to reduce the excitotoxicity of the cells in the superior colliculus. The brain was

removed and was sectioned on a filter paper so that the cortex on the contralateral side to the injection was removed, and the brain was glued onto the metal plate. The brain was sliced coronally into 250 μm sections using a Leica VT1000S vibratome (Leica, Wetzlar, Germany) filled with cold NMDG solution. Slices were collected on nylon mesh and incubated in carbogenated NMDG solution at 37°C in a hot bath (Isotemp 2020, Fisher Scientific, Pittsburgh, PA) for 5 minutes before being transferred to NaCl-based ACSF bath solution containing (in mM) 124 NaCl, 3 KCl, 1.3 MgSO_4 , 1.4 NaH_2PO_4 , 10 glucose, 26 NaHCO_3 , and 2.5 CaCl_2 (285–295 mOsm/L) at 37°C for 5 minutes. Slices were then transferred to room temperature to recover for an hour in carbogenated bath solution.

2.3.2 Whole-cell patch-clamp recording

Glass micropipettes were pulled using a Flaming/Brown Micropipette puller (Model P-1000, Sutter Instruments) with a pipette resistance of 8 M Ω . For whole-cell patch-clamp recordings, individual neurons expressing ChR2-mCherry were assessed with epifluorescence illumination (Examiner.A1; Carl Zeiss) and visualized using infrared differential interference contrast microscopy (Chee et al., 2015). The cesium (Cs) internal pipette solution used for voltage-clamp recordings to record optogenetically-evoked GABA release contained (in mM) 128 CsMS, 11 KCl, 10 HEPES, 0.1 CaCl_2 , 1 EGTA, 4 MgATP, 0.5 NaGTP, and 0.4% biocytin (pH 7.24; 285–295 mOsm/L). The potassium-gluconate (KGluc) internal pipette solution used for current-clamp recordings contained (in mM) 120 K-gluconate, 10 KCl, 10 HEPES, 1 MgCl_2 , 1 EGTA, 4 MgATP, 0.5 NaGTP, 10 phosphocreatine, and 0.4% biocytin (pH 7.24; 285–295 mOsm/L). Recordings were acquired using a MultiClamp 700B amplifier, digitized with a Digidata 1440A, and with pClamp10 software (all from Molecular Devices, San Jose, CA).

2.3.3 Spike fidelity and photostimulation protocols

To determine the spike fidelity of the TH ZI population, whole-cell recordings of *Th-cre* cells that expressed ChR2-mCherry in the ZI in current-clamp were photostimulated with full-field light trains at 1, 5, 10, 20, 50, and 100 Hz and corresponding action potential (AP) firing frequency was recorded (5 ms pulses; Chee et al., 2015). Photostimulation was provided by a blue light-emitting diode (470 nm; power density, 10 mW/mm²; Thorlabs, Quebec, Canada).

Optogenetically-evoked currents from TH ZI terminals expressing ChR2 in the SC were elicited with three light pulses, 500 ms apart, for 20-50 trials every 5 seconds in voltage-clamp (Chee et al., 2015). Evoked currents are averaged over all trials.

To examine optogenetically-evoked dopamine release in the SC, a full field-light train at 10 Hz light frequency, 5 ms pulse width, constant for 2 minutes was used while recording in current-clamp at the RMP of the cell.

2.3.4 Pharmacology

Drugs were prepared immediately before use and were applied to the slice in the bath solution for 5–10 minutes. See Table 2 for a list of drugs used for pharmacology experiments. All drugs were purchased from Sigma Aldrich (Oakville, ON, Canada), except for Raclopride (Rac) which was purchased from Tocris (Oakville, ON, Canada), and tetrodotoxin (TTX) which was purchased from Hello Bio (Princeton, NJ).

Table 2: List and functions of drugs used for electrophysiology studies

Name	Purpose	Cat No.	Concentration (μM)
Dopamine hydrochloride (DA)	DA receptor agonist	H8502	10
Bicuculline (Bic)	GABA _A antagonist	BCBV5862	20
Tetrodotoxin (TTX)	Na ⁺ channel blocker	HB1035	0.500
4-aminopyridine (4AP)	K ⁺ channel blocker	A78403	1000
Raclopride (Rac)	D ₂ + D ₃ + D ₄ antagonist	1810	10
SCH23390 (SCH)	D ₁ + D ₅ antagonist	D054	10

2.4 Antibody characterization

The primary and secondary antibodies that were used for neuroanatomical analysis are listed in Table 3 and Table 4, respectively.

Table 3: List and details of primary antibodies

Antibody	Immunogen	Clonality, Isotype	Source, cat#, lot#	RRID	Dilution
Rabbit anti-DsRed	DsRed-Express full-length synthetic variant of <i>Discosoma sp</i>	Polyclonal	Clontech, 632496, 1805080	AB_10013483	1:2000
Chicken anti-GFP	Isolated from the <i>Aequorea victoria</i> jellyfish	Polyclonal, IgY	Millipore, 06-896, 3022861	AB_11214044	1:2000
Mouse anti-TH	Purified from PC12 cells	Monoclonal, IgG	Millipore, MAB318, 2677893	AB_2201528	1:2000

2.4.1 Rabbit anti-dsRed

This antibody was raised against the full-length dsRed-Express synthetic variant of *Discosoma sp.* red fluorescent protein. It recognizes mCherry and tdTomato, and lysate from DsRed-expressing cells in Western blot had a specific band of 30-38 kDa (Clontech, Certificate of analysis). The selectivity of this antibody has been shown as mice that do not express mCherry, or tdTomato lacked dsRed staining in the presence of the antibody (Bochorishvili et al., 2012; Chee et al., 2013).

2.4.2 Chicken anti-GFP

This antibody was isolated from the *Aequorea victoria* jellyfish as a 29 kDa fluorescent protein and purified from egg yolk (Millipore, Certificate of analysis). Previously, our lab has used this antibody and showed the specificity of expression by performing an IHC stain in wildtype mouse brain tissue and did not observe any GFP-ir neurons (Negishi et al., 2020).

2.4.3 Mouse anti-tyrosine hydroxylase

This antibody was purified from the N-terminus of PC12 cells and recognizes a band of 59-61 kDa (Millipore, Certificate of analysis). The ventral tegmental area and the substantia nigra in rats that were unilaterally lesioned with ibotenic acid and 6-hydroxydopamine hydrobromide respectively did not express TH-immunoreactivity while the contralateral, non-lesioned side expressed TH (Bourdy et al., 2014).

Table 4: List and details of secondary antibodies

Antibody	Isotype	Source, catalogue no., lot no.	RRID	Dilution
Donkey anti-chicken Alexa Fluor 488	IgY (H+L)	Jackson ImmunoResearch, 703-545-155, 139424	AB_2340375	1:500
Donkey anti-mouse Dylight 649	IgG (H+L)	Jackson ImmunoResearch, 715-496-150, 82079	N/A	1:500
Donkey anti-rabbit biotin	IgG (H+L)	Jackson ImmunoResearch, 711-065-152, 101909	AB_2340593	1:500
Streptavidin Alexa 488	N/A	Invitrogen, S32354, 51924A	AB_2315383	1:500
Streptavidin Alexa 647	N/A	Jackson ImmunoResearch, 016-600-084, 135095	AB_2341101	1:500

2.5 Histological Analysis

2.5.1 Tissue processing

Mice that were used for neuroanatomical analysis were injected with 7% chloralhydrate (10 cc/10 g/mL, ip) and transcardially perfused with 0.9% cold (4°C) saline followed by 10% neutral buffered formalin (4°C). Brains were removed and post-fixed in 10% formalin for 12–24 hours and then transferred into 20% sucrose solution containing phosphate-buffered saline (PBS; pH 7.4, 0.01 M) with 0.05% sodium azide (PBS-azide) for 24 hours at 4°C. Brains were sectioned coronally using a Leica SM2000R freezing microtome (Leica) at 30 µm into a series of 4. The tissue was sliced into 9:1 PBS-azide to 10% formalin. Tissue was stored at –20°C in antifreeze (50% formalin, 20% glycerol, and 30% ethylene glycol).

The tissue used for electrophysiological recordings was saved and fixed in 10% formalin for at least 24 hours before being transferred into 20% sucrose in PBS-azide for 24 hours at 4°C. The tissue was resectioned using a Leica SM2000R freezing microtome (Leica), where the 250 µm tissue slice was placed on a platform of optimal cutting temperature compound, coated in sucrose solution, and frozen. The tissue was then sectioned into three 40 µm slices, as well as one 80 µm slice to analyze the biocytin-filled cells. Tissue was stored at –20°C in antifreeze.

2.5.2 Immunohistochemistry

For single and dual immunohistochemical (IHC) staining, an adjacent series to the Nissl was removed from antifreeze and washed six times in PBS for 5 minutes each at room temperature. Tissue was blocked using 3% normal donkey serum (NDS; Jackson ImmunoResearch Laboratories Inc., West Grove, PA) in PBS containing 0.25% Triton X-100 and 0.05% sodium azide (PBT-azide) for two hours at room temperature. The tissue was left to

incubate for 24 hours at room temperature in the primary antibody with blocking serum. Slices were washed six times for 5 minutes in PBS before being transferred to the secondary antibody solution containing 3% NDS made with PBS containing 0.25% Triton X-100 (PBT) for two hours. After the secondary antibody incubation, the tissue was washed three times in PBS for 10 minutes each.

The tissue that was injected with the retrograde virus received a signal amplification for dsRed using a streptavidin-biotin complex. Tissue was removed from antifreeze and washed six times in PBS for 5 minutes each at room temperature and then treated in 0.3% hydrogen peroxide in PBT for 30 minutes. The tissue was then washed three times in PBS for 10 minutes each. Tissue was blocked in 3% NDS for 2 hours at room temperature, then transferred into the primary antibody in blocking solution for 24 hours. The tissue was washed in six 5-minute washes of PBS; then the biotinylated secondary antibody was incubated in 3% NDS in PBT for one hour and then washed off in PBS for 10 minutes three times. The tissue was placed in an avidin-biotin complex (1 μ l avidin, 1 μ l biotin, 833 μ l PBS) for one hour before being washed off in PBS for 10 minutes three times. The tissue was then incubated in the streptavidin secondary antibody for 1 hour, and then the tissue was rinsed in PBS for three ten-minute washes.

After the final washes, all tissue was mounted on SuperFrost Plus glass microscope slides (Fisher Scientific) and coverslipped (#1 thickness) with Pro-Long Gold antifade reagent with DAPI (Fisher Scientific). All primary antibodies and dilutions used are listed in Table 3, and all secondary antibodies are listed in Table 4.

2.5.3 3,3'-Diaminobenzidine (DAB) stain

An adjacent series of tissue to the Nissl stain was washed for 5 minutes in PBS six times. The tissue was then treated in 0.3% hydrogen peroxide in PBT for 30 minutes and then washed

for 10 minutes in PBS three times. The tissue was transferred into the blocking serum composed of 3% NDS in PBT-azide for 2 hours. The tissue was incubated overnight in the primary antibody, Rb-dsRed (Table 3), in the blocking serum and then was washed off in PBS for 5 minutes six times. The biotinylated secondary antibody was incubated in 3% NDS in PBT for one hour and then washed off in PBS for 10 minutes three times. The tissue was then placed in an avidin-biotin complex (ABC; 1:500; 20 μ l avidin, 20 μ l biotin, 9.96 ml PBS) for one hour before being washed off in PBS for 10 minutes three times. The tissue was then incubated in DAB solution (10 ml PBS, 4 drops pH 7.5 buffer, 8 drops DAB, 4 drops 0.3% H₂O₂) for approximately 3 minutes under an Olympus CK2 microscope (Olympus Life Sciences, Tokyo, Japan) until the tissue was appropriately stained. The tissue was rinsed in PBS for 10 minutes three times and mounted on SuperFrost Plus Slides. Mounted tissue was dehydrated in increasing ethanol concentrations (50, 70, and 95%) for 3 minutes each and then twice in 100% ethanol for three minutes. The dehydrated slides were delipidized in 100% xylene overnight and then coverslipped with Richard Allen Scientific mounting medium (VWR, Mississauga, ON, Canada, Cat no. CA84000-112).

2.5.4 Nissl stain

The tissue was mounted on gelatin-coated slides (Globe Scientific, Mahwah, NJ) and left to dry overnight at room temperature so that the tissue remains adhered to the slides. Slides were dehydrated in increasing ethanol concentrations (50, 70, 95 and 100%) for three minutes each and then twice more in 100% ethanol for 3 minutes each. Slides were transferred into two washes of xylenes for 10 and 15 minutes, respectively, before being rehydrated in descending concentrations of ethanol (100, 95, 70, 50%). Slides were then rinsed in distilled water for three minutes before stained with a 0.25% thionin solution (made from thionin acetate salt, pH 4.5,

room temperature, MilliporeSigma, Oakville, Ontario, Canada, Cat no. 861340). Slides were rinsed with water and differentiated in 0.4% glacial acetic acid in deionized water until the gray and white matter are distinguishable. The tissue was then dehydrated in increasing ethanol concentrations (50, 70, 95, 100%) for three minutes each and then twice more in 100% ethanol for 3 minutes. The tissue was cleared with two washes of xylenes for 10 and 15 minutes, respectively, before coverslipped with Richard Allen Scientific mounting medium (VWR).

2.6 Microscopy

All images were taken with a fully motorized Nikon Ti2 inverted microscope (Nikon Instruments Inc., Mississauga, Canada). The NIS Elements software (Nikon) was used to stitch images and adjust brightness and contrast. These files were exported to Tiff files and imported into Adobe Illustrator 24.1.3 (Adobe Systems Inc., San Jose, CA) for assembling figures and analysis. Where applicable, images were stitched using NIS Elements software (Nikon).

2.6.1 Epifluorescent imaging

Epifluorescent images were acquired with the Nikon Ti2 inverted microscope (Nikon) mounted with a Prime 95B CMOS camera (Photometrics, Tuscon, AZ).

To align the nissl-based parcellations onto the fluorescent images for quantitative analysis (outlined in section 2.7), large images of the whole brain section of *Th-cre;L10-Egfp* tissue was acquired using CF160 Plan Apochromat x4 objective lens (0.75 numerical aperture) at 488 nm excitation wavelength. Whole-brain section images showing native-GFP were imaged using the NDS function in NIS Elements. A low magnification (x10 objective lens) scan of the entire microscope slide at 398 nm excitation wavelength was performed, and the whole brain

sections were selected using this tool for high magnification (x20 objective lens) scan at 488 nm excitation wavelengths.

2.6.2 Confocal imaging

A Nikon C2 confocal microscope was used to acquire high-resolution confocal images (2,048 x 2,048 pixels) with two-, three-, or four- colour fluorescent channels with Plan Apochromat x20 (0.75 numerical apertures) objective lenses. Lasers at wavelengths 398, 488, 561, and 640 nm wavelengths were used for excitation of DAPI, Alexa Fluor 488, tdTomato or mCherry, and Dylight 649, respectively. Images were coded as blue (DAPI), green (Alexa Fluor 488 and native-GFP), pseudo-coloured magenta (tdTomato/mCherry), and pseudo-coloured light blue (Dylight 649).

2.6.3 Brightfield imaging

Whole-brain section images of Nissl- and DAB-stained tissue were acquired using the Nikon Ti2 inverted microscope (Nikon) mounted with a Di2 colour camera. The Jobs function in NIS Elements was used to acquire a low magnification (x4 objective lens) scan of the entire microscope slide, and the whole brain sections were selected using this tool for a high magnification (x10 objective lens) scan with the DIA light.

2.7 Neuroanatomical analysis: Nissl-based parcellations, quantification, and mapping

All exported Tiff images were imported into Illustrator (Version 24.1.3 Adobe). Line parcellations were drawn on the Nissl images and were delineated using nomenclature and regional boundaries from the ARA (Dong, 2008; Negishi et al., 2020). Atlas levels from the ARA were then assigned to the parcellated Nissl-stained sections. The parcellations were superimposed and aligned onto the corresponding adjacent fluorescent and/or DAB-stained

series. Using the superimposed parcellations allowed for quantitative and qualitative analysis of cell body and fibre distribution based on regional boundaries and the rostrocaudal location relative to bregma as each atlas level has a corresponding bregma level (Figure 2; Dong, 2008).

Figure 2: Schematic of the process for parcellation-based mapping. *A*, Brightfield photomicrograph of a Nissl-stain to perform Nissl-based parcellations. *B*, Parcellations were superimposed onto an adjacent series of tissue that is DAB-labelled for dsRed. *C*, dsRed-labelled fibres are traces in *A* and transferred onto an ARA atlas plate to generate a standardized set of maps.

Cell bodies were marked with circles using the *Blob Brush* tool, and cell fibres were traced with solid lines using the *Pencil* tool in Adobe Illustrator v25.3.1. Atlas-based maps of cell body or fibre distribution are generated by superimposing the cell and/or fibre representations onto the corresponding atlas level (Negishi et al., 2020). The colours of the circles and the solid lines and what they represent are stated in sections 2.7.1–2.7.3.

2.7.1 Validation of stereotaxic injections into the TH ZI population and mapping the injection site

To determine the accuracy of the injection into the TH ZI population, the percent colocalization of viral expression in *Th-cre;L10-Egfp* mice injected with the cre-dependent virus expressing tdTomato with TH-immunoreactivity was quantified. This analysis provided information on how many of the total number of TH-ir neurons in the ZI had taken up the virus.

Due to the ectopic expression of native-GFP in the *Th-cre* mouse line used in this experiment, we aimed to determine how many cells took up the virus that were positive for native-GFP but did not express TH. We calculated the percent colocalization of viral expression with TH by counting the total number of virus-expressing cells and how many of the virus-expressing cells also expressed TH. These values are reported as mean $\pm$ SEM, as well as the percent colocalization of the virus with TH.

In addition, to validate the accuracy of the injections into the TH ZI population, heat maps were generated to show the distribution of virus-transfected cells in the ZI. Opaque red circles represent virus-transfected cells that express TH, and virus cells that do not express TH are represented by opaque blue circles. The injection sites for multiple cases are combined onto a single atlas map for each atlas level. In areas where there is overlap between two or more injection cases, the colour is darker to show where the greatest density of virus transfected cells was between injections.

2.7.2 Mapping fibres in the superior colliculus

To show that there was a viral expression in the superior colliculus, fibre maps of the SC are generated. DAB-stained fibres (section 2.5.3) are traced in Adobe Illustrator. They are superimposed onto ARA atlas plates where solid red lines represent the shape, density, and directionality of the fibres in the SC. Nissl-based parcellations are used to delineate the cytoarchitectural boundaries of the SC.

2.7.3 Retrograde maps

Solid red circles on ARA maps represent retrograde-labelled cells to show the distribution of hypothalamic catecholaminergic inputs into the medial motor-related layers of the

SC. Nissl-based parcellations were used to delineate the cytoarchitectural boundaries of the ZI and other hypothalamic regions.

2.8 Statistical Analysis

Unilateral and bilateral cell counts were obtained from confocal images for IHC-stained tissue and brightfield images for DAB-stained tissue. All recording data were analyzed using Clampfit 10 software, and all statistical analysis was performed with Prism 6.07 (GraphPad Software Inc., San Diego, CA). Synaptic events were analyzed with MiniAnalysis (Synptosoft, Fort Lee, NJ). Sample traces were obtained using OriginPro (OriginLab, Northampton, MA). Data are represented as mean $\pm$ standard error of mean (SEM), and sample size per group (n) is shown in parentheses in the figures and results. Means were compared using unpaired and paired t-test and were provided at significant differences of $p < 0.05$. Outliers from optogenetic experiments were determined using Prism Outlier Calculator, which uses the Grubb's test at $\alpha = 0.05$ level of significance, which allowed us to exclude outliers that were 1.96 standard deviations away from the mean. Correlational data is analyzed with Pearson Product Moment Correlation at $p < 0.05$.

3 Results

3.1 Viral injections into the ZI target the dopaminergic population

In order to label the distribution of fibres from TH ZI neurons in the SC, we first determined a set of stereotaxic coordinates to consistently target the TH ZI population. These coordinates were designed to maximize viral expression in TH-ir neurons in the ZI while limiting viral spillover into adjacent regions. Using *Th-cre:L10-Gfp* mice, we injected 25–50 nL of a cre-dependent AAV expressing tdTomato into the ZI (ML –0.5 mm, AP –1.3 mm, DV –4.7 mm; Figure 3A). *In situ* hybridization data collected by our lab showed that in the medial ZI, all TH-GFP neurons expressed *Th* mRNA (Bono et al., 2020), and we showed here that all cells that took up the virus also express native GFP (Figure 3B). Additionally, viral injection into the ZI of a cre-negative littermate showed no tdTomato-expression ($n=1$; Figure 3C), indicating the virus was selective for cre-containing cells.

Figure 3: Cre-mediated tdTomato expression of TH ZI neurons. *A*, Schematic of viral injections into the TH ZI cells. *Bi–Bii*, Merged low and high magnification photomicrographs (*ii* outlined from *i*) of the native GFP (green, *Biii*), ZI TH-ir neurons (cyan, *Biv*), and tdTomato (magenta, *Bv*). White arrowheads mark tdTomato-expressing cells that are TH-ir and express TH-GFP, and open arrowheads mark tdTomato-expressing neurons that only express TH-GFP. *C*, Merged low magnification photomicrographs of the ZI of a cre-negative littermate control that was injected with the cre-dependent tdTomato-expressing virus. Scale bars: *Bi*, *Civ* 100 μ m; *Bv* 20 μ m.

Transfected cells were seen throughout the ZI (Figure 4). However, the majority of tdTomato-expressing cells resided within ARA levels 67 to 69 (Figure 4G-I), where the bulk of the TH-ir neurons are located in the ZI (Table 5; Negishi et al., 2020)). There was some spillover of the virus into other hypothalamic and thalamic brain regions, including the paraventricular hypothalamic nucleus (PVH), lateral hypothalamus (LHA), the anterior hypothalamic nucleus (AHN), the posterior hypothalamic nucleus (PH), ventromedial nucleus of the thalamus (VM),

the submedial nucleus of the thalamus (SMT), and the reticular nucleus of the thalamus (RT; Figure 4A).

We then determined if virally transfected cells expressed TH immunoreactivity in the ZI. The total percent colocalization of tdTomato with TH-ir was 70.4% and increased up to 86.59% in the ZI between levels 67–69 (Table 5). The non-TH-ir cells that took up the virus were primarily located in the ZI outside of levels 67–69 (Figure 4C and 4K). Overall, we established a reliable set of stereotaxic coordinates and viral volume for the ensuing experiments.

Table 5: Quantification of virally-transfected cell in the ZI from 6 mice.

ARA	Number of virus-expressing cells (Mean $\pm$ SEM)	Number of virus + TH-ir cells (Mean $\pm$ SEM)	Percent colocalization (%)
63	3.0	0.0	0.0
66	2.3 $\pm$ 1.1	1.0 $\pm$ 0.5	44.4
67	17.7 $\pm$ 10.8	12.7 $\pm$ 9.1	71.7
68	24.2 $\pm$ 5.1	19.5 $\pm$ 5.6	80.70
69	27.3 $\pm$ 5.5	23.7 $\pm$ 4.9	86.6
70	9.3 $\pm$ 2.8	6.5 $\pm$ 2.1	70.3
71	5.5 $\pm$ 2.5	0.5 $\pm$ 0.4	9.1
Total	89.2	63.8	71.6

Figure 4: Distribution of virally-transfected cells throughout the ZI. **A** Summary of the location of virus-transfected cells from six injection cases. **B–L**, Heat maps were generated containing up to 6 injection cases to show the spread of the virus around the injection site, as well as if the tdTomato-expressing neurons are TH-ir (red opaque circles) or not (blue opaque circles). Areas of overlap between separate injections appear brighter, representing the most consistent spread of the virus.

3.2 TH ZI neuron electrical characterization

To study the electrical activity of TH ZI neurons, a cre-dependent ChR2-expressing virus encoding mCherry was injected into the ZI of *Th-cre* and *Th-cre:L10-GFP* mice (Figure 5A). TH ZI neurons have an average resting membrane potential (RMP) of -53.0 ± 3.4 mV ($n=9$) and average rheobase, the minimum current required to fire an action potential (AP), of 8.1 ± 2.3 pA ($n=7$; Figure 5C). At baseline, most TH ZI neurons were constantly firing with an AP frequency of 1.7 ± 0.3 Hz ($n=4$). These neurons sustained steady AP firing up to 15.7 ± 2.5 Hz ($n=5$) and did not accommodate (Figure 5C).

To assess the functional expression of ChR2 in TH ZI cells, we photostimulated ZI cells with 5 ms light pulses at 1–100Hz. We observe that AP firing was time-locked to each light pulse, and the cells could be effectively entrained up to 10 Hz ($n=6$; Figure 5D). Notably, the light-evoked firing of TH ZI cells is consistent with the maximum firing frequency of ZI cells and evoked AP in a similar, steadily firing pattern.

Figure 5: ChR2-mediated entrainment of TH ZI cells. **A**, Schematic of stereotaxic injection of the cre-dependent ChR2 expressing virus in the TH ZI population. Whole-cell patch-clamp recordings from mCherry-labelled ZI cells photostimulation was done using full-field light trains with 470 nm light. **Bi**, Epifluorescent photomicrograph of a coronal *Th-cre* slice injected with ChR2-mCherry in the ZI, and **Bii** shows high magnification photomicrograph of fluorescent patched cells. **C**, Representative traces of current steps at 10 pA and 45 pA inputs (3 s) showing AP firing rate of a TH ZI neuron. **Ciii**, Line graph showing the gradual increase in AP frequency with increasing AP steps. **Di**, Sample traces of AP firing from a ChR2-expressing TH neuron evoked by light trains (5 ms, 470 nm light pulses) at 1, 10, and 20 Hz. **Dii**, Rate of change of TH ZI neuron spike fidelity with photostimulation frequency.

3.3 TH ZI fibres innervate the medial motor-related layers of the SC

To map the distribution of TH ZI fibres in the SC, we injected a cre-dependent virus expressing tdTomato into the ZI and labelled TH ZI projections with dsRed-immunoreactivity. We performed Nissl-based parcellations to delineate the subregions of the SC (Figure 2). Fibres were seen throughout the entire rostrocaudal extent of the SC (Figure 6), with a slight increase in density towards levels 92 to 96 (Figure 6F–I). Interestingly, fibres were rarely observed in the

lateral portion of the SC or the sensory-related layers (i.e., op, zo, and sg; Figure 6A–K). Instead, fibres were mainly restricted to the medial motor-related layers (i.e., ig, iw, dg, and dw; Figure 6A–K). Punctate dsRed-ir boutons can be observed on cells bodies within this region of the SC (Figure 6L) but were not visible in the sensory-related layers or the lateral portions of the SC. These maps focused our following *in vitro* recordings to the medial motor layers of the SC.

To confirm the neuroanatomical connection between the TH ZI cells with the medial motor-related layers of the SC, we injected a cre-dependent retrograde virus into the SC of a *Th-cre* mouse (Figure 7A). We targeted our injection to the medial motor-related layers of the SC, where we observed the highest density of TH ZI fibres (Figure 6). We found retrogradely-labelled cells in the ZI, with a few cells observed in each of the LHA, dorsomedial hypothalamic nucleus (DMH), AHN, ventromedial hypothalamic nucleus (VMH), SMT, perireunensis nucleus (PR), and the PH (Figure 7). Retrogradely labelled cells in the ZI are also TH-ir (Figure 8), and as our lab has shown that all TH-ir neurons in the ZI are also DA-ir (Negishi et al., 2020), this suggests that the ZI is a source of DAergic input to the SC.

Figure 6:
Distribution of TH ZI fibres in the SC.
A–K, dsRed-labelled fibres were traced throughout the rostrocaudal extent of the SC and mapped onto ARA plates using nissl-based parcellations. **L,** High magnification brightfield photomicrographs (outlined in low magnification image in inset) show dsRed-labelled axon boutons (black) located on Nissl-stained SC cells (blue). Black arrowheads mark representative SC soma. Scale bar: 20 μ m (**L**).

Figure 7: Distribution of retrogradely-labelled cells from *Th-cre* neurons in the hypothalamus to the SC. **A**, Schematic of the stereotaxic injection of a cre-dependent retrograde virus encoding mCherry was injected into the medial motor-related layers of the SC of a *Th-cre* mouse.

Inset, shows a representative brightfield photomicrograph of dsRed-labelled *Th-cre* fibres in the medial motor-related layers of the SC at the injection site. SCm, medial SC; SCI, lateral SC. **B–K**, Hypothalamic maps generated from dsRed-labelled retrograde virus using Nissl-based parcellations to show the distribution of catecholaminergic cells that project to the medial motor-related layers of the SC. Scale bar: 100 μ m (**A**).

Figure 8: Retrograde-labelled neurons in the ZI are TH-ir. *A*, Merged high magnification photomicrographs (outlined from inset) of native GFP neurons (*B*), with TH-immunoreactivity (*C*), and retrogradely labelled mCherry-expressing cells (*D*) within the ZI. Scale bars: *A* 100 μ m; *D* 20 μ m.

3.4 SC neurons receive monosynaptic GABAergic input from the TH ZI cells

We previously showed that TH-ir neurons are GABAergic (Negishi et al., 2020); thus, TH ZI neurons may be able to corelease DA and GABA. To evaluate if TH ZI fibres can release GABA in the SC, we photostimulated ChR2-expressing terminals while recording from SC cells in the fibre field (Figure 9A–B). We observed optogenetically-evoked inhibitory postsynaptic currents (oIPSC) that were temporally fixed with single light pulses using Cs-filled electrodes at a V_h of -5 mV. About one-third of recorded SC cells in the medial motor-related layers of the SC were functionally connected with the TH ZI cells (Figure 9C). These oIPSCs had an average amplitude of 12.5 ± 1.7 pA ($n=25$), latency of 4.9 ± 0.2 ms ($n=25$), and the probability of an oIPSC occurring was $75.6 \pm 4.3\%$ ($n=26$). There was no significant difference in oIPSC amplitude ($p=0.779$; Figure 9D), latency ($p=0.191$; Figure 9E), or probability ($p=0.158$; Figure

9Fii) between males and females. As there was no significant difference between sexes, male and female oIPSC data were grouped together from this point.

To elucidate whether these oIPSCs were driven by GABAergic signalling from the TH ZI cells, we applied 20 μ M Bic to block GABA-A receptors. Bic blocked the oIPSCs ($n=6$; $p=0.022$; Figure 9G). Additionally, this GABA-mediated oIPSC persisted in the presence of 500 nM TTX (and 1 mM 4AP; $n=7$, $p=0.515$; Figure 9H; Petreanu et al., 2009), suggesting this was a monosynaptic GABAergic connection (Figure 9G–H).

Figure 9: Analysis of oIPSCs evoked from TH ZI terminals in the medial motor-related layers of the SC. **A**, Schematic of stereotaxic injection of the cre-dependent ChR2-mCherry-expressing virus into the TH ZI cells and whole-cell recordings from the medial motor-related layers of the SC (**B**) in the ChR2-expressing fibre field (inset of **B**). **C**, Pie chart representing the percent of connected cells out of all cells recorded. Bar graphs comparing oIPSC amplitude (**D**) and latency (**E**) in SC cells from male and female mice. **Fi**, Representative raster plot demonstrating synchronicity of IPSC with photostimulation (three 5 ms light pulses, 500 ms interval). **Fii**, Bar graph representing the oIPSC probability in SC cells between males and females. **Gi**, Sample oIPSC trace that disappears in the presence of Bic (**Gii–Giii**). **Hi**, Sample oIPSC trace persists in the presence of TTX and 4AP (**Hii–Hiii**).

To determine if the amplitude of oIPSCs or the percent connectivity was related to the efficacy of TH ZI transfection, we analyzed their relationship with the total number of ZI cells transfected from a subset of injected mice. The percent connectivity was obtained using the number of connected cells out of the total number of cells recorded per mouse. We also classified injections as either a hit or a miss. An injection was considered a hit if the majority of virus-transfected cells were located within the medial ZI between levels 67–69. An injection was considered a miss if the majority of the virus transfected cells resided outside of levels 67–69 or within the lateral portion of the ZI where there were little-to-no TH-ir neurons (Negishi et al., 2020). There was no significant correlation between the total number of TH ZI cells transfected with either oIPSC amplitude (Hit: $n=9$, $p=0.656$; Miss: $n=3$, $p=0.743$; Figure 10A) or the percent connectivity (Hit: $n=6$, $p=0.356$; Miss: $n=2$; Figure 10B). These data suggested that the rate of virus transfection did not contribute to the strength of the GABAergic connection from TH ZI cells to the SC.

Figure 10: Relationships between total TH ZI transfected cells with oIPSC amplitude and percent connectivity. *A*, Correlation between the oIPSC amplitude versus the total cell count. *B*, Correlation between the percent connectivity of the TH ZI cells with the SC from each mouse versus the total cell count. Injections were considered either a hit (filled black dots) or a miss (open gray dots) depending on if the bulk of the injection lay within ARA levels 67–69 in the medial ZI. Pearson's r values were calculated separately for the hit (black line) and missed injections (gray line).

3.5 SC neurons have two opposing responses to dopamine

To identify if TH ZI cells release DA into the SC, we first characterized the effect of pharmacologically-applied DA to SC cells in the medial motor-related layers (Figure 11A). Bath DA application to the slice produced a heterogeneous response. The majority (41.7%) of SC cells recorded hyperpolarized (-11.4 ± 1.3 mV; $n=20$), and a quarter (25.0%) of cells depolarized (9.7 ± 1.3 mV; $n=12$) in the presence of DA (Figure 11B). These two DA-mediated effects were reversible following a washout period (Figure 11Ci–Cii). Additionally, a small percentage (14.6%) of cells did not respond to DA ($n=7$). In 18.8% of cells, the RMP continuously “ramped up” ($n=9$) and did not return to baseline following a washout period; thus, a DA-mediated effect could not be determined (Figure 11B–C). These observations were seen in both males and females, and there was no difference in terms of the magnitude of either the depolarization ($n=11$; $p=0.338$) or hyperpolarization ($n=18$; $p=0.589$) effects between males and females (Figure 11D). Therefore, recordings from male and female mice were analyzed together from this point.

Figure 11: DA-mediated effects on SC cell activity. *A*, Schematic representing the bath application of DA (10 μ M) to the SC during whole-cell current clamp recordings in a WT mouse. *B*, Distribution of DA-mediated effects in all SC cells recorded. Sample traces of depolarized (*Ci*) and hyperpolarized (*Cii*) following DA application (10 μ M), and summary of all DA-mediated effects in all SC cells recorded (*Ciii*). Comparison of the magnitude of the DA-mediated depolarization (*Di*) and hyperpolarization (*Dii*) between males and females. *E*, Correlation plot of the baseline RMP and the DA-mediated change in RMP for cells that depolarized and hyperpolarized in the presence of DA.

Interestingly, there was a significant negative correlation ($n=29$; $r=-0.58$; $p=0.001$) between the baseline RMP of a cell and its DA-mediated response (Figure 11E). Therefore, we may predict that a cell with a more depolarized RMP at baseline would have a hyperpolarization effect in the presence of DA.

To elucidate the mechanisms underlying the hyperpolarization effect, we first pretreated the tissue with 500 nM TTX to determine if DA was acting directly on SC cells. The DA-mediated hyperpolarization persisted in the presence of TTX (DA: -11.5 ± 2.6 mV; DA+TTX: -10.8 ± 2.8 mV; $n=3$; $p=0.797$; Figure 12A), which suggested that DA acted directly on the recorded cell. D₂-like receptors are G_{i/o} coupled receptors and typically have an inhibitory effect, so we pretreated the tissue with 10 μ M raclopride (Rac), a D₂-like receptor antagonist. The application of Rac blocked the DA-mediated hyperpolarization (DA: -11.9 ± 2.1 mV; DA+Rac: -0.6 ± 1.5 mV; $n=5$; $p=0.017$; Figure 12B). These combined data suggested that the DA-mediated hyperpolarization was driven by a postsynaptic D₂-like receptor.

Figure 12: Postsynaptic D₂ receptor-mediated hyperpolarization of SC cells. *A*, TTX (500 nM) application had no significant change in DA-mediated RMP. *B*, Rac (10 μ M) application had a significant blockage of the DA-mediated RMP.

Next, we performed similar experiments for cells that elicited a depolarization response. We pretreated the tissue with 500 nM TTX and blocked the DA-mediated depolarization (DA: 8.8 ± 1.7 mV; DA+TTX: -0.5 ± 1.5 mV; $n=3$; $p=0.056$; Figure 13A). D₁-like receptors are Gs-coupled receptors and typically have an excitatory effect, so we tested if the depolarization is driven by a D₁-like receptor by pretreating the tissue with 10 μ M SCH23390 (SCH), a D₁-like receptor antagonist. Application of SC reduced the DA-mediated depolarization but was not completely blocked (DA: 5.6 mV; DA+SCH: 1.8 mV; $n=1$; Figure 13B). These combined data suggested that the DA-mediated depolarization was at least partially mediated by a presynaptic D₁-like receptor.

Figure 13: DA-mediated depolarization characterization. *A*, TTX (500 nM) application had a non-significant blockage of the DA-mediated RMP. *B*, SCH (10 μ M) application had a decrease in the DA-mediated RMP.

3.6 SC cells that receive GABAergic input from the TH ZI cells are also DA responsive

Given that TH ZI cells can innervate SC cells in the medial motor-related layers via monosynaptic GABA release (Figure 9), we next determined if the TH ZI cells can also regulate these cells by DA release. A high-frequency burst-firing photostimulation protocol is typical for peptide release for in vitro optogenetic experiments (Arrigoni and Saper, 2014). However, TH ZI cells were effectively entrained up to 10 Hz only (Figure 5D). In addition, TH ZI cells were steadily firing, rather than with burst firing (Figure 5C), so we applied a continuous 10 Hz photostimulation protocol over two minutes to evoke DA release.

We used a potassium gluconate (KGluc) internal pipette solution for these recordings to detect potential changes in RMP. The cells we recorded were all shown to receive GABAergic input from TH ZI cells by observing oIPSCs at $V_h = -20$ mV (Figure 14A). The average oIPSC amplitude was 5.9 ± 2.7 pA ($n=10$; Figure 14Bi), which was smaller ($p=0.031$) than that detected

with a cesium (Cs) internal pipette solution (Figure 9D). The oIPSC latency was not altered by the internal pipette solution (5.8 ± 0.5 ms; $n=11$; $p=0.053$; Figure 14Bii).

To isolate the DA effects, we pretreated the slices with 20 μ M Bic to inhibit any GABAergic input from TH ZI fibres that could alter the RMP. We observed a “ramp-up” of the cell RMP during and after photostimulation (10 Hz, 2 minutes; Figure 14C). This observation was similar to the “ramp-up” effect observed with *ex vivo* DA application (Figure 11Ai). Interestingly, we observed the same effect in all six cells on which we performed this photostimulation. This “ramp-up” was not observed when we applied the same photostimulation protocol to wildtype (WT) neurons.

As potential DA-mediated effects may be masked by the instability of the recordings, we analyzed whether SC cells that were functionally connected with the TH ZI were able to respond to DA. Interestingly, every cell that received a monosynaptic GABAergic input from TH ZI cells was DA responsive. DA hyperpolarized 57.1% of these cells (4/7 cells) and depolarized the remaining 42.9% (3/7 cells; Figure 14D). This suggested that SC cells that are functionally connected to the TH ZI cells may also receive DA signals from the ZI as they are able to respond to DA.

Figure 14: DA-mediated effects on connected cells in the SC. *A*, Sample trace of an oIPSC using Kgluc internal solution. oIPSC amplitude (*Bi*) and latency (*Bii*) comparisons between Cs and KGluc internal pipette solutions. *C*, Change in RMP during photostimulation of ChR2-expressing terminals. *D*, DA-mediated changes in RMP in connected cells.

4 Discussion

The SC is one of the main regions that received dense projections from $ZI^{DA+vGAT}$ population that we had identified using a *Th-cre* mouse model (referred to as TH ZI neurons). These projections arriving in the SC are largely restricted to the medial aspect and within the ventral motor-related layers. Interestingly, these TH ZI projections also directly innervated SC cells by GABA release. Furthermore, SC cells that received GABAergic input from ZI projections also responded to dopamine.

Figure 15: Schematic of mechanisms underlying GABAergic and DAergic innervation of the SC. $ZI^{DA+vGAT}$ neurons can release GABA which acts directly on GABA_A receptors on SC cells. DA acts through postsynaptic D₂ receptors to inhibit SC cells. DA acts through presynaptic D₁ receptors to excite SC cells. These D₁ receptors may be present on a presynaptic glutamatergic terminal that project directly to the SC cell to increase excitatory input to the SC. Another potential location of these presynaptic D₁ receptors is on a GABAergic terminal that has a disynaptic connection with the SC via another GABAergic neuron, thereby disinhibiting the SC neurons.

4.1 Neuroanatomical connection from the $ZI^{DA+vGAT}$ cells to the medial motor-related layers of the SC

TH-ir cells in the ZI are dopaminergic and coexpress vGAT, meaning they also have the ability to package and release GABA for synaptic transmission (Negishi et al., 2020). These $ZI^{DA+vGAT}$ cells were located within the medial ZI and sent anterograde projections to the medial SC. Furthermore, we also performed retrograde injections from the medial SC and have confirmed retrogradely-labelled medial ZI cells that were TH-ir. Taken together, these findings indicated that the medially distributed $ZI^{DA+vGAT}$ cells project to the medial SC. However, the GABAergic colliculo-incerta pathway is known to have an inverse medial-lateral topography, where the medial ZI projects to the lateral SC (Mitrofanis, 2005; Comoli et al., 2012). Our $ZI^{DA+vGAT}$ neurons did not follow this projection pattern. Instead, the projections from the $ZI^{DA+vGAT}$ neurons, which are most prominent in the medial ZI, were largely restricted to the medial part of SC. The projections of $ZI^{DA+vGAT}$ cells may thus represent a unique pattern of connectivity that is not observed with the other GABAergic ZI cells (Romanowski et al., 1985; Kim et al., 1992).

Additionally, we found that the projections from $ZI^{DA+vGAT}$ cells concentrated in the motor-related layers of the SC. Given that GABAergic input to the medial SC, albeit from the lateral ZI, mediates avoidance behaviours (Comoli et al., 2012), it suggests that projections from medial $ZI^{DA+vGAT}$ neurons to the medial SC may also contribute to these avoidance behaviours.

4.2 GABAergic connection from the $ZI^{DA+vGAT}$ neurons to the SC

$ZI^{DA+vGAT}$ neurons directly innervated the SC cells by monosynaptic GABA transmission. We were able to photostimulate $ZI^{DA+vGAT}$ fibres in the SC that expressed the light-sensitive ChR2 molecule and found that optogenetically-evoked currents in the SC were abolished by the

GABA_A receptor antagonist, bicuculline, which indicated that the ZI^{DA+vGAT} neurons inhibited SC cells via GABA release. This response is consistent with the literature, as GABA_A receptors can be found throughout the entirety of the SC (Kaila, 1994; Owens and Kriegstein, 2002).

The latency of optogenetically-evoked currents may indicate whether two functionally connected cells are supported by a monosynaptic or polysynaptic event(s), as a shorter latency (4–6 ms) suggests a monosynaptic connection (Arrigoni and Saper, 2014). In our experiments, the latency of the light-evoked GABA current was ~5 ms and suggested a monosynaptic connection. We were able to confirm this as our GABAergic connection persisted in the presence of TTX. These combined data suggest that the ZI^{DA+vGAT} neurons can directly inhibit SC neurons via GABA_A receptor activation (Figure 15). This represents the first account of GABA release from dopaminergic ZI neurons.

We analyzed the relationship between the number of ZI neurons that expressed ChR2 and the magnitude of the GABA-mediated current, as well as with the percent of connected cells we recorded in the SC. We found no relationship between these factors. These observations confirmed our ability to isolate ZI^{DA+vGAT} cells and that we were able to transfect the vast majority of ZI^{DA+vGAT} neurons. Our approach will be relevant for future *in vivo* optogenetic experiments to analyze the behavioural output between ZI^{DA+vGAT} → SC pathway, as our current protocol allowed us to encapsulate the maximum activity of this pathway.

4.3 Excitatory and inhibitory effects of DA in the SC

DA has an overall inhibitory effect in both the sensory and motor-related layers of the SC (Bolton et al., 2015). There are five subtypes of G-protein coupled dopamine receptors, but SC cells predominantly express D₁ (G_s-coupled) and D₂ (G_{i/o}-coupled) receptors only (Kebabian and Calne, 1979; Civelli et al., 1993). Notably, D₁ is localized to the sensory layers, and D₂ is more

populated in the motor layers (Bolton et al., 2015). Consistent with this, we found that SC cells in the medial motor layers were directly inhibited by postsynaptic D₂ receptor activation (Figure 15).

To date, DA activity in the SC has only been shown to elicit inhibitory responses (Bolton et al., 2015). Indeed, we found that inhibitory effects of dopamine were more common in our recordings, but DA could also produce excitatory responses. Dopamine depolarized 25% of cells we recorded from in the motor layer of the SC. This membrane depolarization was blocked by TTX pretreatment and suggested that dopamine-mediated depolarization was activity-dependent. Furthermore, although preliminary, we found that the membrane depolarization was partially blocked by D₁/D₅ receptor antagonism. These findings suggested that the DA-mediated depolarization was driven in part by presynaptic D₁ and/or D₅ receptors. The depolarization response we saw was consistent with the excitatory actions of Gs-coupled receptors like the D₁ receptor. For instance, D₁ receptor activation at a presynaptic GABAergic neuron could mediate feedforward inhibition to produce a net excitation (Figure 15). In support of this, it was shown that D₁ receptor activation could produce a delayed increase in GABAergic input to SC cells (Bolton et al., 2015). Finally, given that the excitatory effect of dopamine was only partially blocked by D₁/D₅ receptor antagonism, it is possible that presynaptic D₂-like receptors on GABAergic afferent projections, such as those arising from the ZI (Boyson et al., 1986), could disinhibit SC cells and contribute to the excitatory effect of dopamine (Figure 15).

The ZI^{DA+vGAT} population is proposed to be a main source of DAergic input to the SC (Bolton et al., 2015). Given the robust actions of dopamine on SC cells, one of the aims of this project was to determine if the ZI^{DA+vGAT} neurons, which produce dopamine (Negishi et al., 2020), contribute dopamine to the SC. We were unable to detect DA release in the SC; however,

this does not rule out the contribution of $ZI^{DA+vGAT}$ neurons. Interestingly, we noted that *all* SC cells directly innervated by $ZI^{DA+vGAT}$ cells (via GABA) responded to dopamine. The proportion of ZI-SC connected cells that hyperpolarized (57%) or depolarized (43%) was similar to that seen in our initial experiments to characterize the effect of dopamine in the motor SC layer (hyperpolarized, 51%; depolarized, 31%; no effect, 18%). This is interesting because it suggested that $ZI^{DA+vGAT}$ neurons could produce both fast-acting inhibition of SC cells (via GABA release) that is prolonged or aborted by dopamine.

4.4 Functional implications of $ZI^{DA+vGAT}$ cells

The behavioural implications of $ZI^{DA+vGAT}$ have included anxiety-associated behaviours such as avoidance as there is a convergence between the functional output of GABA and DA signalling in the medial SC (Brandão and Coimbra, 2019; Venkataraman et al., 2019). However, the role of $ZI^{DA+vGAT}$ projections in the SC is still unknown. Avoidance behaviours are an adaptive response associated with increased anxiety in humans and rodents (Maner and Schmidt, 2006; Kuleshkaya and Voikar, 2014). ZI GABA transmission in the SC is thought to be involved in mediating avoidance behaviours in the medial SC (Kim et al., 1992; Comoli et al., 2012). Specifically, blocking of $GABA_A$ receptors in the motor-related layers of the SC increased anxiety and avoidance behaviours in rats (Redgrave et al., 1981) as well as primates (DesJardin et al., 2013). Therefore, the $ZI^{DA+vGAT}$ population may be involved in inhibiting avoidance behaviours through GABAergic signalling as we have shown that the $ZI^{DA+vGAT}$ neurons activate $GABA_A$ receptors. Additionally, D_2 receptor antagonism in the motor layers of the SC increases anxiogenic behaviours in rats in the elevated plus maze by avoiding the open arms (Muthuraju et al., 2016), thus suggesting that the D_2 -mediated hyperpolarization we observed at our cells could

be anxiolytic. These combined data suggest that DA and GABA signalling both reduce anxiety-associated behaviours in the SC.

We observed opposing responses to dopamine in the SC, and it is not clear if DA-mediated depolarizations are implicated in anxiety. If this excitatory response is involved in the same behaviours, we could assume that it would work in opposition to the inhibitory DA response, such that the DA-mediated depolarization would increase anxiogenic behaviours in the medial motor-layers of the SC. In summary, the $ZI^{DA+vGAT}$ population may be a modulator of anxiety-associated behaviours such as avoidance in the SC.

4.5 Conclusion

Even though this region is called the zone of uncertainty, there is an increasing body of literature on the behavioural output of this region, particularly on its role in anxiety-associated behaviours. The ZI GABA and DA populations have both been shown to be involved in avoidance and defensive behaviours (Brandão and Coimbra, 2019; Venkataraman et al., 2019). These behaviours have been studied with the ZI connections to not only the SC but to other regions such as the PAG (Muthuraju et al., 2016; Chou et al., 2018), reticular thalamic nucleus (Venkataraman et al., 2021), and the posterior complex of the thalamus (Hormigo et al., 2020) as well. This suggests that $ZI^{DA+vGAT}$ activity mediates these anxiety-driven behaviours not only in the SC but also via its connections to other brain regions that have been previously identified. Therefore, the $ZI^{DA+vGAT}$ population and its projections may be a novel area of study for fear-associated behaviours. Ongoing research on the ZI, particularly the $ZI^{DA+vGAT}$ population, will continue to shed light on this zone of uncertainty.

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