Projection-specific anatomy, physiology and behaviour in the mouse superior colliculus

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I, Thomas Wheatcroft confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in this thesis.

Abstract

The superior colliculus (SC) projects to other brain centres through multiple pathways, which are thought to be important in rapid, visually guided behaviours. Projections to the visual thalamus and the periaqueductal gray have been hypothesised to be important for visually-guided defensive behaviour, however, the extent to which these projections are anatomically and functionally distinct remains poorly understood. Further still, our understanding of the influence of these pathways on visually-guided defensive responses is incomplete. We tested the anatomical localisation and segregation of the SC cells projecting to each target using dual retrograde tracing. Through injecting CTB protein conjugated to different fluorophores into the visual thalamus and periaqueductal gray, we found intermingled yet separate SC populations projecting to each target. We took advantage of the intermingled yet separate nature of the two SC output pathways to measure their functional properties. Injecting retrogradely-transported cre virus into either the visual thalamus or periaqueductal gray, we gated expression of GCaMP7s in the SC. We implanted a lens above the SC and measured the global, calcium-dependent fluorescent signal while presenting visual stimuli to head-fixed mice free to run on a treadmill. Employing different visual stimuli, we observed that while there was shared visual tuning between the two SC output pathways, they exhibited different dynamics and responses to the animal's own movement. Finally, we performed behavioural experiments to better understand the roles each pathway might play in defensive behavioural responses. Our results inform theories about the neural pathways that allow mice to produce defensive responses to visual threats.

Impact Statement

“Aberrant decision-making is central to the majority of psychiatric conditions” (Montague *et al.*, 2012, p. 73). A means of developing treatment for aberrant decision-making is to first establish a theory of how brains make decisions in healthy, control conditions, which can then be used to predict how changes in the brain affect decision-making, or what changes in the brain might explain decision-making aberrations.

On the basis of a theory of how brains make decisions, treatment could be designed to effect the brain in a certain way to alter decision-making. Beyond the therapeutic benefits of a theory of how brains make decisions, such a theory would also speak to fundamental questions, such as why individuals behave the way they do.

As does all work on the neural basis of decision-making, the work in this thesis attempts to advance, to an infinitesimal extent, our knowledge of how brains make decisions.

The anatomical and physiological work adds to the evidence that neurons in a common brain area, but with different outputs, can have different properties, and potentially different behavioural roles. Future projection-specific experiments may be beneficial in building a theory of the neural basis of decision-making. The behavioural work offers evidence for, and extends opportunities to study, the influence of internal state on decision-making. Future behavioural experiments could build on our work to try to understand the neural basis of individual differences, as well as the factors that influence them. The literature review provided in the thesis places the specific circuits and behaviours studied in the context of the larger network of the brain and the larger behavioural repertoire. This review suggests experiments to build on our knowledge of how animals decide between different behaviours. Finally, the technical experiences detailed in this thesis can inform future study of the neural basis of decision-making.

Acknowledgements

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1. Introduction: Functional Channels through the Mouse Superior Colliculus

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1) Introduction

The mammalian brain contains a region named the superior colliculus (SC). The SC of mice, in particular, has recently received much attention. Many ideas have been put forward for what the SC might be used for by mice. The SC seems to be important for the mouse to be able to move towards targets, or for the mouse to make certain movements of the eye or tongue (Stubblefield, Costabile and Felsen, 2013; Wang *et al.*, 2015; Duan *et al.*, 2019). The SC also seems to be important for generating putative defence responses; for escaping, or freezing (Wei *et al.*, 2015; Evans *et al.*, 2018). How the SC is able to play a role in all these behaviours is unclear.

Alongside the diversity of behavioural roles for the SC, there is also a diversity of anatomical connections made by the structure. The SC is connected to cortical, cerebellar, thalamic, hypothalamic, midbrain and medullary areas (Benavidez *et al.*, 2020). The role of this confluence of connections is, again, unclear.

An attempt to begin to explain both the diversity of behavioural roles for, and anatomical connectivity of, the SC was made by a ‘Viewpoint’ article (Dean, Redgrave and Westby, 1989). Dean et al., 1989 hypothesised, primarily on the basis of work in rats, that SC neurons projecting to the ipsilateral hemisphere were involved in producing defence behaviours, whereas the SC neurons projecting to the contralateral hemisphere were involved in producing turns towards objects. The ipsilateral and contralateral projecting SC neurons were

not only hypothesised to project to different hemispheres, but also to different targets within those hemispheres. That these two populations of SC neurons might also get different inputs was also discussed, on the basis of their cell bodies having different spatial distributions within the SC. Thus, Dean et al., 1989 hypothesised that there was a spatial organisation of anatomical connectivity and behavioural function within the SC, offering a means of understanding the richness of both the anatomical and behavioural data.

Over the past decade, a number of papers and resources concerning mice have been published that may allow us to further assess the hypothetical spatial segregation of anatomical connectivity and behavioural function proposed by Dean et al., 1989. Recently, means of assessing the anatomical argument have been provided by the Allen Mouse Brain Connectivity Atlas (Oh *et al.*, 2014) and the Mouse Connectome Project (Benavidez *et al.*, 2020). Various labs have elaborated this substantial anatomical dataset, and nuanced it with investigations of the connectivity of specific SC cell types (Gale and Murphy, 2014; Shang *et al.*, 2015, 2018; Masullo *et al.*, 2019). Meanwhile, the role of the SC in a variety of different behaviours has been studied. Can the expanse of anatomical and behavioural data be better understood through the idea of spatial segregation of function? In this review, I first discuss the specific behavioural roles the SC is thought to play. I then discuss the proposed specific spatial delegation of these roles, and the evidence for this proposition.

2) The SC is involved in approach and escape

2.1 The SC is involved in approach

2.1.1 The SC represents and directs movements towards the contralateral side of the mouse

The superior colliculus (SC) of each hemisphere gets inputs representing objects mainly on the contralateral side of the mouse.

2.1.1.1 The SC gets input representing the contralateral visual field

In the visual system, the SC's input mainly represents objects on the contralateral side of the animal. To represent the contralateral side of the animal, the contralateral SC receives inputs from the contralateral nasal retina as well as the ipsilateral temporal retina: parts of the eyes that observe the contralateral visual field. Primary visual cortex (V1) also gets, indirect, input from the same parts of the retina. V1 projects to the ipsilateral SC, allowing the SC to integrate differently filtered visual signals emanating from the contralateral side of the animal.

2.1.1.2 The SC generates movements towards the contralateral side

The SC seems to be involved in generating movements, but the SC of a given hemisphere does not seem to be involved in generating movements in just any direction. In tasks where animals have to make lateralised movements – movements which are not symmetric about each side of the body – the SC does not seem to have equal numbers of neurons predictive of each direction of movement. We will define turning, or orienting, as rotations of the head, or whole body. More SC neurons are active for turns towards the contralateral side (Wilson *et al.*, 2018; Lintz *et al.*, 2019). Consistent with this, artificial activation of the SC biases the animal towards making contralateral movements (Stubblefield, Costabile and Felsen, 2013; Masullo *et al.*, 2019; Cregg *et al.*, 2020; Essig, Hunt and Felsen, 2020). As one might expect, inhibiting the SC does the opposite, biasing the animal away from contralateral movements (Stubblefield, Costabile and Felsen, 2013; Lee and Sabatini, 2020).

2.1.1.3 The SC generates movements to the contralateral side in head-fixed animals

Results implicating the involvement of the SC in evoking contralaterally-directed movements come from studies of naturalistic behaviours looking at whole body turning (Stubblefield, Costabile and Felsen, 2013; Wilson *et al.*, 2018; Masullo *et al.*, 2019; Cregg *et al.*, 2020), or directed licking (Duan *et al.*,

2019). In studies using more contrived movements, the results, on the face of them, appear to indicate the opposite finding; that SC is involved in making movements to the ipsilateral side. In a task where mice have to turn a wheel left or right, the vast majority of neurons in the SC that are selective for one turning direction prefer ipsilateral turns (e.g. clockwise/rightwards turns for the right SC) (Steinmetz *et al.*, 2019). Consistent with their finding, in a task where mice have to rotate the ball beneath them left or right, unilaterally inhibiting the SC biased the mice away from turning the ball to the ipsilateral side (Huda *et al.*, 2020). These results from experiments with more contrived movements seem in direct contradiction to the results from studies with more naturalistic movements. A possible resolution is that turning ipsilaterally in these contrived behaviours requires pushing down to the ipsilateral side, a movement that mice might normally perform to orient themselves contralaterally, when they are not head-fixed (Huda *et al.*, 2020). If this conceptualisation is correct, then I may still maintain that the SC is involved in directing the mouse, or part of the mouse, towards its contralateral side.

2.1.2 The SC represents the direction of objects to the contralateral side, and directs the mouse towards them

Although the SC gets inputs representing objects on the contralateral side of the animal, and directs movements to the contralateral side of the animal, the SC of one hemisphere is not spatially homogenous in terms of its sensory inputs or it motor outputs. If one imagines a sphere, whose origin is a point between the mouse's eyes, sensory signals emanating from objects occupying different patches on that sphere will converge upon, and activate, different parts of the SC (Fig. 1a-b). The SC of one hemisphere represents patches on the contralateral half of the sphere, and the nasal-temporal azimuthal axis of one half of the sphere is mapped onto the anterior-posterior axis of the contralateral SC. Whereas, the higher-to-lower elevation axis of one half of the sphere is mapped onto the medial-lateral axis of the contralateral SC. Signals from in front of the mouse above its eyeline with converge in the anterior-medial SC, whereas eccentric objects below the mouse's eyeline will converge in the posterior-lateral SC. This is proposed to be true of visual, auditory and somatosensory signals.

2.1.2.1 The SC has a retinotopic map of the contralateral visual field

In the visual system, work found that stimulus patches presented in different parts of the visual field evoked responses that were centred in different parts of the SC, consistent with the proposed map (Mrsic-Flogel *et al.*, 2005). This retinotopic mapping is generated because different parts of the retina project to different parts of the SC (Xu *et al.*, 2011). Impressively, the preferred spatial location of a sound source in the SC also shows that topography (at least for the azimuthal axis) (Ito *et al.*, 2020). Moreover, recent work reports that different parts of the SC get input from the appropriate parts of primary somatosensory cortex (S1); the anterior lateral SC gets input from mouth, nose, upper limb and whisker-related S1, whereas the posterior lateral SC gets input from the lower limb and trunk parts of S1 (Benavidez *et al.*, 2020). To summarise, different parts of the SC are activated by objects in different directions away from the mouse.

2.1.2.2 Activation of different parts of the SC evoke differently-directed contralateral movements

Consistent with the role of the SC in orienting towards objects, artificial activation of different parts of the SC produces movements towards the parts of space represented by their sensory input (Fig. 1c). Work finds that unilateral artificial activation of (PITX2+) neurons in different parts of the SC evoke contralateral movements with differences in their yaw and pitch (Masullo *et al.*, 2019). Activation of more posterior locations in the SC evoke larger contralateral (yaw) rotations, consistent with the more posterior SC being activated by objects situated more temporally in azimuth. Moreover, more medial locations of artificial activation tend to evoke (slightly) larger pitch rotations that the more lateral locations. To conclude, there is evidence that a given part of the SC is activated by, and evokes turning towards, objects a certain direction from the mouse.

Since the SC gets input about objects to the contralateral side of the mouse, and activation of the SC biases movement towards the mouse's contralateral side, the SC seems to be involved in directing the body, or parts of it, towards

these objects. I'll call this *approach*. Approach is thus movement of the body, or its parts, towards an object. Approach is intuitively useful in a variety of circumstances, such as when there is something to one's side that one wants to interact with, or investigate.

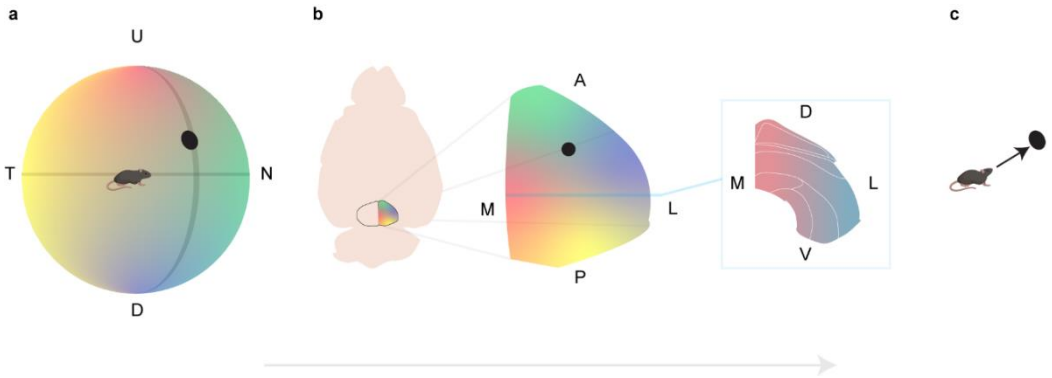


Fig. 1: The SC represents the direction of objects, and directs the mouse towards them. **a**: Schematic illustrating a mouse, and the world to its left, as mapped onto a hemisphere. The direction of objects are defined in terms of visual angle: their azimuth (position along the nasal-temporal axis) and their elevation (position along the up-down axis). Azimuth and elevation axes are depicted as black lines and the black disc represents an example object. N, T, U and D refer to nasal, temporal, up and down respectively. **b**: Schematic representation of the mapping of the world onto different sections of the SC. Left panel shows a horizontal section through the mouse brain, with the superior colliculus of each hemisphere outlined. As seen in the magnified image in the middle panel, the azimuth and elevation axes of the world are mapped onto the anterior-posterior and medial-lateral axes of the SC respectively. Shown is the mapping of the left side of the world onto the right SC. The black disc in the middle panel illustrates the part of the SC that would be activated by the object in a. Right panel illustrates the mapping of the world onto the right hemisphere of the SC, as it would appear in a coronal section taken at the AP position defined by the blue line in the middle panel. A, P, M, L, D and V refer to anterior, posterior, medial, lateral, dorsal and ventral respectively. **c**: Schematic illustration of the effect of the focal activation of the SC described in b. Activation of the relatively anterior-lateral location in the SC described by the disc in b evokes a movement towards the part of the world represented by that part of the SC.

2.2 The SC is involved in escape

Beyond a role in approach, the SC also seems to be involved in a different set of actions; actions involving rapid movement. These movements are thought of as escape behaviours that are directed towards places of relative safety: whether that be away from the current context or towards another. The SC responds during escape behaviour (Evans *et al.*, 2018); unilateral artificial activation of the SC can evoke escape behaviour (Shang *et al.*, 2015); and, at least bilateral, inhibition of the SC can prevent escape behaviour in some circumstances (Shang *et al.*, 2015; Evans *et al.*, 2018).

2.2.1 The role of the SC in escape may be not be in merely orienting away from threat

The SC's role in escape behaviour may be another manifestation of its role in generating movements relative to the direction of a stimulus. Alongside a role in directing the animal towards a stimulus – intuitively useful for approach behaviour – the SC might also be able to direct an animal away from a stimulus: avoidance. Avoidance, or being able to direct the animal away from a stimulus, seems intuitively useful for defence. In rats, work comparing performance on orienting towards versus orienting away tasks finds evidence consistent with the idea that rats find it less demanding to orient towards something (Duan, Erlich and Brody, 2015). Orienting away from a stimulus is therefore likely to be a more demanding task than orienting towards a stimulus, and may involve different neural pathways.

Whether the SC plays a role in directing movements away from stimuli is uncertain. The SC's motor output predominantly produces contraversive, not ipsiversive movements (Masullo *et al.*, 2019). The question arises therefore whether SC can evoke a behaviour whose direction is independent of the direction of the stimulus that evoked it, but there is little work to settle this. Promisingly, unilateral artificial activation of A1-recipient SC neurons evokes escape behaviour whose direction is not obviously biased to the ipsilateral, over the contralateral, side (Zingg *et al.*, 2017)(but see Isa *et al.*, 2020). This is what I would expect if the SC's role in escape behaviour was in evoking escapes independent of the direction of the stimulus.

To summarise, there may be two modes of operation the SC works under. In the first mode, the SC is informed about the presence of something to the contralateral side of the animal, and directs part or all of the body towards that object. In the other mode of operation, the SC is informed about something on the contralateral side of the animal, and pushes the animal to escape, regardless of the direction of the threat.

2.2.2 The SC may play distinct roles in both triggering and directing escapes

Directing the mouse towards another context (e.g. refuge) during escape seems like a natural part of a role for SC in approach. However, whilst the SC may support the approach of another context during escape, it seems to have a more general control in escape than this: a role I'll call 'triggering'.

Artificial activation of the SC is capable of evoking escapes which deliver the animal to a nest (Evans *et al.*, 2018). Given the SC harbours a motor map capable of directing the animal, a nest-directed movement would require a spatiotemporally precise manipulation to activate a certain part of the SC. Nest-directed escapes are nevertheless evoked by large or untargeted activation of the SC, suggesting that the SC triggers the escape behavioural sequence (Vale, Evans and Branco, 2017).

3) Approach and triggering escape may be mediated by the lateral and medial SC respectively: behavioural evidence

3.1 The medial-lateral SC axis represents object elevation

The SC could subserve both approach, and triggering escape, if these behaviours were mediated by different neural populations. Indeed, we already know there is spatial heterogeneity and structure in the sensory inputs to the SC, with topographic mapping of the elevation and azimuth axes onto the medial-lateral and anteriorposterior axes of the SC respectively.

3.2 Elevation as an aversive cue

3.2.1 More elevated objects may predict greater threat

Many authors have pointed out that it is intuitive that the higher the elevation an object, or part of an object, is in the world, the more likely it is to be something aversive, and the less likely it is to be something a mouse would want to approach (Sahibzada, Dean and Redgrave, 1986; Comoli *et al.*, 2012; Savage, McQuade and Thiele, 2017). Being more concerned for greater elevations seems intuitive, but it relies on a lot of assumptions about the experience of mice in evolution and in their own lives. For example, mice are not always on solid ground, and one could imagine threats not extending far in elevation but still being very dangerous (e.g. small poisonous insects). Moreover, it seems likely that mice frequently approach objects above them, such as in rearing up to collect food.

3.2.2 More elevated stimuli may evoke greater defensiveness

There is some evidence that mice find the same visual stimuli differentially threatening depending on the elevation of those stimuli. An expanding black disc on a screen (looming stimulus) seems to evoke defensive behaviours only when the screen is above the mouse, not on the side (Zhou *et al.*, 2019) or below the mouse (Yilmaz and Meister, 2013; Zhou *et al.*, 2019). However, moving dots in the upper *or lower* visual field can both induce freezing (typically considered defensive) (De Franceschi *et al.*, 2016; Procacci *et al.*, 2020).

3.2.3 Activation of the more medial SC may evoke greater aversion

The more lateral part of the SC is activated by objects at low elevations, and might be involved in approach. In contrast, the more medial parts of the SC, that respond to objects at higher elevations, may be involved in triggering escape. This would be a prediction “embedded within the nervous system” (Teufel and Fletcher, 2020, p1) and would reflect constraints on the links between stimuli and actions that mice can make.

Anatomical connectivity may encourage mapping of the elevation axis of the visual field onto the medial-lateral axis of the SC, but such mapping also depends on the elevation of the eye's gaze. If the eye's gaze elevation had a flat distribution, then the exact position of activity along the medial-lateral axis of the SC would not be informative about the true elevation of the visual stimulus. Research however, finds that the gaze elevation of mice clusters around a certain elevation angle (Meyer, O'Keefe and Poort, 2020), permitting the retinocollicular anatomical connectivity to be informative about elevation.

3.3 The lateral SC may have a role in directing escape

The hypothetical delegation of triggering escape and approach to the medial and lateral SC respectively would have us expect that directing oneself towards a nest during escape would require the lateral SC. That is, the medial SC is involved in triggering escape behaviour, whereas the lateral SC would be involved in guiding that escape behaviour towards the nest. Thus, with a threat on the left and a nest on the right, the right medial SC may be activated (by the threat), and the left lateral SC may be activated (to steer the animal towards the nest). If this were true, inhibiting the medial SC would be expected to prevent escape, while inhibiting the lateral SC would be expected to impair the steering of escape towards the nest.

3.4 Manipulations of the medial and lateral SC have different behavioural effects

It is rare for single studies to assess the effects of both medial and lateral manipulations.

3.4.1 Independent studies find that manipulating medial and lateral SC has different effects

Work that has targeted the medial part of the SC has shown that artificial activation of neurons can evoke escape behaviour. Recent work targeted a population of medially-located SC neurons (specifically, primary auditory cortex-recipient neurons) and found that artificial activation of this population evoked escape behaviour (Zingg *et al.*, 2017). Similarly, other work found that artificial activation of the stereotaxically-targeted medial SC also evoked escape behaviour (Evans *et al.*, 2018). Inhibiting a more lateral population of

neurons found to affect the direction of escape as well as instinctive orientation towards sounds (Vale *et al.*, 2020). Unilateral inhibition of the laterally-located (Isa *et al.*, 2020) SC neurons projecting to an area of the pons also impairs some orienting behaviours (Sooksawate *et al.*, 2013) - reaching for a contralateral platform and turning towards a contralateral object.

3.4.2 Studies manipulating both pathways also see different effects

Limited studies have assessed the effects of manipulating populations of SC neurons with different medial-lateral distributions. Shang *et al.* (2019) found that inhibition of a population of lateral SC neurons (specifically subthalamus-projectors) impaired the ability of mice to hunt cockroaches, a putative approach task, but not the *speed* of escape behaviour. Inhibition of a larger SC population (including more of the medial SC) impaired both hunting and escape behaviours. Isa *et al.*, (2020) found that activation of the lateral SC mostly evoked contralateral turning (with some freezing), whereas artificial activation of the medial SC mostly evoked, putatively escape-related backwards walking and fast forwards running.

3.4.3 The medial SC may have an additional role in approach

Whilst I've argued that the medial SC may be alone in its role in triggering escape, is the lateral SC really alone in its role in approach? Or is the medial SC also involved? Masullo *et al.* (2019) found that activating progressively more medial locations of SC evoked larger pitch angles of rotation. Therefore, it may be the case that all of the SC is involved in approach.

The role of the medial SC in approach may be more difficult to observe for a few reasons. First, studies of approach tend to require mice to approach targets at low elevations, and artificial inhibition of the medial SC may not impair performance on such tasks. Second, the role of the medial SC in approach may be more subtle because of the difficulty of measuring pitch rotations when animals are tracked from above/below, and the effect pitch rotations have on making yaw rotations less perceptible. This issue may be made even more problematic by the apparently small dynamic range of pitch rotations artificial activation can evoke (Masullo *et al.*, 2019). To use a phrase

from a discussion of other phenomena in the SC, a concern might be that the differential role of medial and lateral neurons in approach may "…be one of degree, not of kind" (Yin, 2014, p15). Thus, neurons evoking differently directed behaviours may cover the ML axis of the SC, whilst separate, escape-triggering, neurons might concentrate in the medial SC.

In summary, the SC seems to be involved in approach towards, and the triggering of escape by, objects on the contralateral side of the animal. The lateral SC seems to be involved in generating, at least, larger rotations than the medial SC. The medial SC may be involved in evoking smaller rotations for orienting towards objects, but it seems uniquely involved in triggering escape.

4) Approach and triggering escape may be mediated by the lateral and medial SC respectively: anatomical evidence

4.1 The medial and lateral SC have different anatomical connections

The idea that the lateral and medial parts of the SC may evoke approach or escape could be supported by evidence of putative orienting areas being preferentially connected to the lateral SC, and putative triggering escape areas being preferentially connected to the medial SC. The Allen mouse brain connectivity dataset and the mouse connectome project have recently greatly aided one's ability to assess this evidence (Oh *et al.*, 2014; Benavidez *et al.*, 2020).

The mouse connectome project (Benavidez *et al.*, 2020) traced the inputs and outputs of four different parts of SC, segregated mainly along the ML axis. In the following I'll refer to their two more medial parts (medial and central medial), as the medial SC, and their two more lateral parts (central lateral and lateral), as the lateral SC. Interestingly, some brain areas are connected only to the medial SC, or only to the lateral SC (Fig. 2).

If the lateral part of the SC were involved in approach, and if the medial part of the SC were involved in triggering escape, we might expect the areas they are connected to reflect this. Indeed, where there is evidence for their roles in each of the two behaviours, there is intriguing evidence that those role are aligned to the part(s) of the SC they are connected to.

4.2 The lateral SC is connected to areas involved in approach

Some areas only connected to the lateral SC seem to be involved in approach. In some areas this involves turning (of the head or body). In other areas 'approach' involves the production, gating or preparation of body (e.g. tongue) movements needed to interact with objects. And in other areas, 'approach' involves the combination of both turning and interactive movements needed to pursue prey and conspecifics.

It is important to note that the lateral SC being connected to turning-related areas is not necessarily consistent with a role of the lateral SC in approach; these connections may also suggest a role in turning away. Whether their connection to the SC is consistent or inconsistent with the lateral SC's proposed role in approach depends on the specifics of the projection; whether it is ipsi or contralateral, as well as its sign.

4.2.1 The lateral SC is connected to areas involved in approach turning

The lateral SC is connected to many areas involved in producing movements of the body or head, and there is some evidence that the connectivity with the SC supports approach turns. The lateral SC has exclusive projections to the MRN, Gi, Orbital Cortex, basal ganglia and basal ganglia-associated nuclei.

4.2.1.1 Midbrain reticular nucleus

Connectivity with the SC

The midbrain reticular nucleus (MRN) is reciprocally connected to the lateral SC, but not the medial SC (Benavidez *et al.*, 2020).

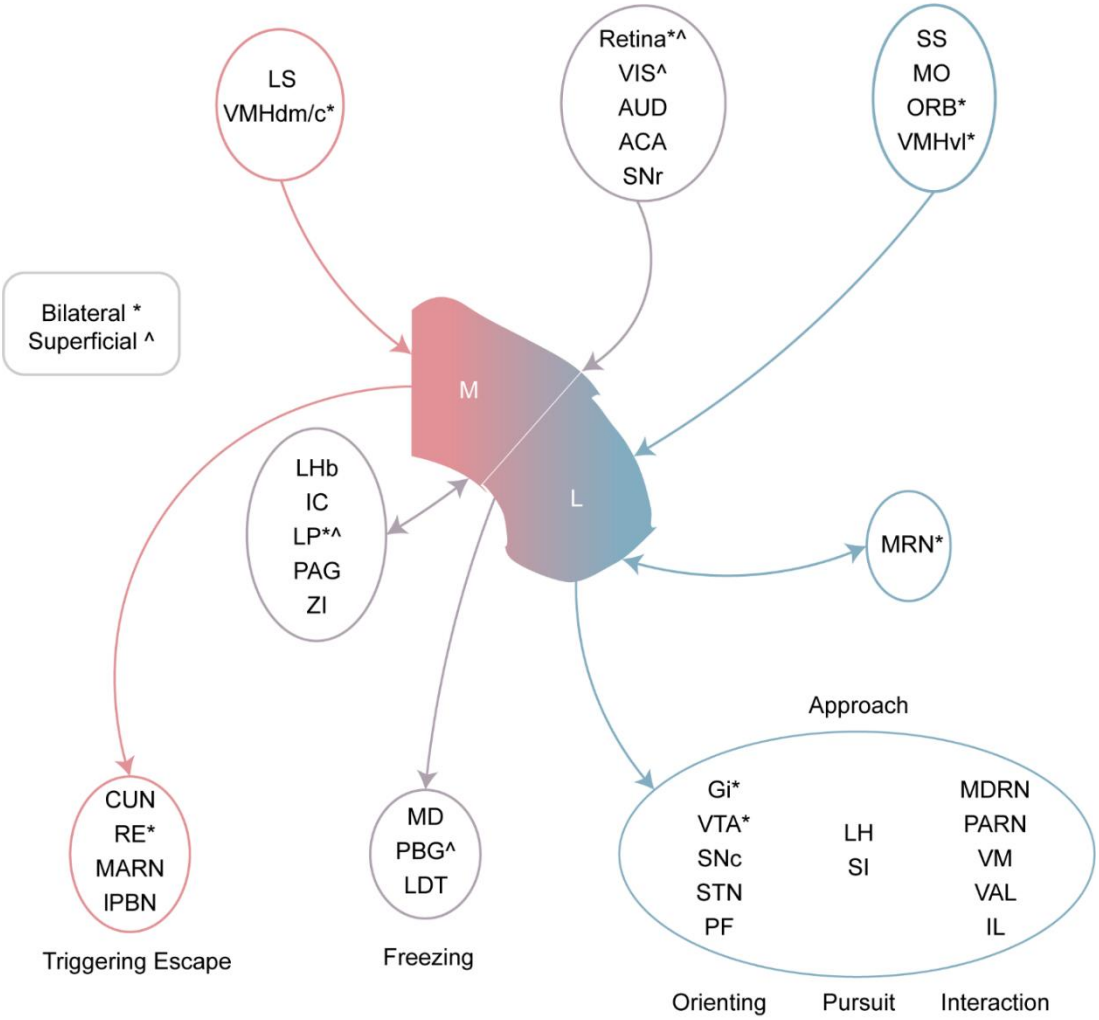


Fig. 2: The medial and lateral SC have different connections. Figure shows schematic outline of one hemisphere of the SC, and the illustrated medial and lateral regions defined by Benavidez *et al.*, 2020. Arrows show connections between parts of the SC and groups of regions with proposedly common functions. Areas are listed as in the Allen brain atlas, albeit with occasional abbreviations or extensions to define superregions and subregions respectively. All connections are with the deep, ipsilateral SC, unless area is suffixed with a symbol given in the key. Data from Benavidez *et al.*, 2020, with modification for Gi and VTA (Zhou *et al.*, 2019; Cregg *et al.*, 2020).

Function

The MRN is reciprocally connected to PITX2+ SC neurons, whose artificial activation was found to be capable of evoking approach orienting, but not escape (Masullo *et al.*, 2019). The MRN contains neurons that respond more when head-fixed mice turn a wheel one way more than the other way (Steinmetz *et al.*, 2019). That the output to the MRN is involved in promoting approach, rather than avoidance movement is suggested by the findings that the SC's projection to the MRN is ipsilateral (Masullo *et al.*, 2019), and that the MRN prefers the same turn direction as the ipsilateral SC (Steinmetz *et al.*, 2019). Thus, the SC's output to the MRN may promote approach turns.

Inagaki *et al.*, 2020 find that the MRN neurons projecting to the thalamus and medulla are separate populations, and that unilateral artificial activation of the thalamus projectors does not bias movement direction. Future work can assess whether medulla-projecting MRN neurons are involved in turning, and whether the SC innervates them.

4.2.1.2 Gigantocellular nucleus

Connectivity with the SC

Both the SC and the MRN also project to CHX10+ neurons in the gigantocellular nucleus (Gi). These Gi neurons that may play a role in orienting, and also receive input from cortical areas which innervate only the lateral SC (M1/2, S1) (Cregg *et al.*, 2020). The SC input is primarily contralateral, and the MRN input is primarily ipsilateral.

Function

Unilateral artificial activation of CHX10+ Gi neurons evokes turning in the ipsilateral direction (Cregg *et al.*, 2020; Usseglio *et al.*, 2020), consistent with the contralateral SC input to these neurons, at least partially from VGLUT2+ neurons (Cregg *et al.*, 2020). Thus, the (e.g. left) SC may functionally excite the contralateral (e.g. right) Gi to promote contralateral (e.g. right) turns, offering an output which could promote approach turns.

4.2.1.3 Orbital Cortex

Connectivity with the SC

The orbital cortex projects only to the lateral SC. The orbital cortex has a large literature associated with it, but manipulations of the orbital cortex can effect behavioural performance in tasks requiring lateralised behavioural responses (e.g. Kuwabara *et al.*, 2020). Thus, the orbital cortex connection suggests a role in turns: future work can assess whether this connection is consistent with a role of the SC in approach, rather than avoidance, turns specifically.

4.2.1.4 Basal ganglia

Connectivity with the SC

The lateral SC alone sends unreciprocated projections to a collection of basal ganglia-associated nuclei and these ipsilaterally-directed connections offer tempered evidence for a role in approach.

Function

Reports from dopaminergic basal ganglia neurons that occupy the ventral tegmental area (VTA) and the substantia nigra pars compacta (SNc) offer some positive support for an approach role for the lateral SC. Consistent with the proposed role of the lateral SC in orienting, Prévost-Solié *et al.*, 2019 find that altering the SC input to the VTA modifies orienting behaviour with respect to conspecifics. The SC projection to the VTA and SNc is ipsilateral and, for the VTA at least, its effect on dopaminergic neurons is primarily excitatory (Prévost-Solié *et al.*, 2019). Therefore the hypothetical role of the lateral SC in approach would have us expect that dopaminergic neurons should prefer contraversive movements. Barter *et al.*, 2015 found that dopaminergic SNc neurons selective for turn direction preferred contraversive movements. Similarly, Parker *et al.*, 2016 find that the terminals of VTA/SNc dopaminergic neurons in the dorsal striatum prefer contraversive over ipsiversive turns (also Tsutsui-Kimura *et al.*, 2020). Interestingly, in head-fixed preparations too, Moss *et al.*, 2020 find that VTA/SNc dopaminergic terminals in the dorsal striatum prefer (putatively) contraversive turns. Therefore there is some evidence that dopaminergic SNc/VTA neurons prefer contraversive turns and that this preference is to be expected from the hypothetical role of the lateral SC in approach.

Further evidence for a role of the SC to SNc pathway in approach comes from Huang *et al.*, 2020, who find that artificially activating and inhibiting the SC inputs to the SNc facilitate and impair hunting behaviour respectively, but not escape responses to visual threats.

Data from other basal ganglia signals offer mixed evidence for a role of the lateral SC in approach. Zhou *et al.*, 2019 find that the vast majority (~85%) of the anatomical SC inputs to GAD2+ VTA neurons are, putatively (i.e. CAMK2+), glutamatergic. However, Hughes *et al.*, 2019 find that unilateral artificial activation of (VGAT+) GABAergic VTA neurons induces ipsiversive head turns, apparently inconsistent with a role of the lateral SC in approach. Furthermore, unilateral artificial activation of other related areas to which the SC sends ipsilateral projections, the parafasicular and subthalamic nuclei, also induce ipsiversive turning (Serra *et al.*, 2020). Finally, the results of Zhou *et al.*, 2019 and Barbano *et al.*, 2020 suggest a role for GABAergic and glutamatergic VTA neurons in escape. Therefore, some of the lateral SC's connections in the basal ganglia suggest a role in ipsiversive, rather than contraversive movements, or even explicit escape behaviours.

A resolution might be found in unravelling the complexity of the SC to VTA connectivity. Some work has found that unilateral artificial activation of GABAergic (GAD2+) SC neurons induces contraversive turning (Essig, Hunt and Felsen, 2020; Sans-Dublanc *et al.*, 2020), like their glutamatergic neighbours, and Zhang *et al.*, 2019 find that GABAergic (VGAT+) SC neurons project to the VTA. Whether sign changes between the SC and downstream basal ganglia populations will offer more evidence for a role of the lateral SC in approach remains to be seen.

4.2.2 The lateral SC is connected to areas involved in object interaction

The SC is not only involved in turning. Its approach role extends to movements of the body parts towards objects.

4.2.2.1 VM, VAL and M2

Connectivity with the SC

The SC receives an ipsilateral projection from M2 and sends an ipsilateral projection to VM and VAL. The SC does not project back to M2, nor do VM or VAL project back to the SC (Benavidez *et al.*, 2020).

Function

VM and VAL are areas whose activity may be greater for contralaterally-directed movements. Guo *et al.*, 2017 inhibited VM and VAL in awake mice performing a behavioural task. Mice had to lick left or right, and the authors found that inhibition induced an ipsilateral licking bias. Similarly, Duan *et al.*, 2019 find that like the SC, unilateral inhibition of M2 biased the mouse towards making ipsilaterally-directed licks. Therefore, VM/VAL/M2 promote contralateral licks and get ipsilateral input from the SC, suggesting a means for the SC to promote contralateral (i.e. approach) licks.

4.2.2.2 Medullary reticular nucleus

The lateral SC is also unreciprocally connected to areas involved in forelimb movements; possibly movements towards targets.

Connectivity with the SC

The medullary reticular nucleus (ventral part) (MDRN) is innervated by the lateral SC but not the medial SC. The MDRN does not send projections back to the SC.

Function

Esposito, Capelli and Arber (2014) found that at least some of the MDRN-projectors in the superior colliculus are VGLUT2+. They found that genetic ablation of VGLUT2+ MDRN neurons does not impair normal locomotion, but did impair performance in a pellet-reaching task. Thus, the MDRN connection suggests a role in reaching towards certain directions: future work can assess whether this connection is consistent with a role of the SC in reaching towards (i.e. approach), rather pulling away (i.e. avoidance), specifically.

4.2.2.3 Parvicellular reticular and spinal trigeminal nucleus

Connectivity with the SC

The lateral SC alone is connected to the parvicellular reticular and the spinal trigeminal nucleus. The lateral SC sends unreciprocated projections to these two downstream areas.

Function

Han *et al.*, 2017 found that neurons in the parvicellular reticular nucleus that target jaw-controlling or neck-controlling motor nuclei. Artificial activation of these neurons prevented 'oromotor activity', and inhibition of these neurons prevented mice from biting prey they'd subdued with their forelimbs.

The parvicellular and neighbouring spinal trigeminal nucleus project to forelimb premotor neurons in the spinal cord (Esposito, Capelli and Arber, 2014), and recordings and manipulations suggest they are important in reaching and handling movements of the forelimb (Ruder *et al.*, 2021).

In summary, the connections to the parvicellular reticular and spinal trigeminal nuclei suggest a role in directed oromotor and forelimb movements: future work can study whether the SC's connections to these areas is consistent with movements towards (i.e. approach), or away (i.e. avoidance), from objects specifically.

4.2.3 The lateral SC is connected to areas involved in pursuit

Areas that have unreciprocated projections from the lateral SC therefore seem to be involved in turning towards and interacting with targets. Other lateral SC-connected areas are also involved in the combinations of these behaviours necessary to pursue and interact with targets. These are the lateral hypothalamus (LH) and the ventromedial hypothalamus's ventrolateral part (VMHvl).

4.2.3.1 Lateral hypothalamus

Connectivity with the SC

The lateral SC alone connects with the lateral hypothalamus: the lateral SC sends unreciprocated projections to the lateral hypothalamus.

Function

GABAergic cells in the LH get input from the SC (Venner *et al.*, 2019). The activity of GABAergic cells in the LH increases during pursuit of prey (Y. Li *et al.*, 2018), and bilateral inhibition of GABAergic LH neurons abolished hunting behaviour, while artificial activation evoked hunting behaviour. Artificial activation of LH GABAergic neurons also evoked attacks on conspecifics. Thus, the lateral SC's output to LH GABAergic neurons suggests a role in pursuit.

In contrast, Li *et al.*, 2018 found evidence for the role of glutamatergic LH neurons in evasion, so it would be interesting to assess if these receive SC input also.

4.2.3.2 Ventromedial hypothalamus (ventrolateral)

Connectivity with the SC

The ventromedial hypothalamus (VMH) projections to the SC are topographically organised. The medial SC gets more input from the dorsomedial VMH (VMHdm/c), whereas the lateral SC gets more input from the ventrolateral VMH (VMHvl). The SC does not project back to the VMH.

Function

The two parts of the VMH seem to have different roles. The lateral SC-innervating VMHvl seems to be involved in approach towards conspecifics and responds during exposure to conspecifics (Li Wang, Talwar, *et al.*, 2019). Unilateral artificial activation in lone mice has no obvious behavioural effect (Lin *et al.*, 2011), but upon the introduction of conspecifics, increases the probability of investigating and attacking the conspecific (Lee *et al.*, 2014). Bilateral inhibition of the VMHvl during close investigation or attack hastens their termination. Thus, the SC's connection to the VMHvl suggests a role in pursuit.

The VMHvl also seems to have a role in defence. When mice are exposed to an aggressive mouse, bilateral inhibition of the VMHvl in the aggressed mouse impairs its ability to defend itself, and artificial activation of the VMHvl can encourage these defensive behaviours during the presentation of a non-aggressive mouse (Li Wang, Talwar, *et al.*, 2019). These results seem at odds

with the argued involvement of the lateral SC in turning towards objects. However, whilst mouse defence does involve “dashing” and “jumping”, it also involves “upright postures” and “pushing”; these latter two behaviours involve movements directed towards the other mouse (Li Wang, Talwar, *et al.*, 2019). Alternatively, Wang *et al.*, 2019 find that these defensive behaviours are associated with the anterior part of the VMHvl, and this part may have different connections to the SC, as it appears to do with some other brain areas. Future work will thus be required to understand whether the SC’s connection to VMHvl can be conceptualised as part of a general role in movements towards objects, or whether it may also suggest a role in avoidance.

4.2.3.3 Substantia innominata

Connectivity with the SC

The substantia innominata is connected only to the lateral SC: the lateral SC sends unreciprocated projections to the substantia innominata.

Function

Like the VMHvl, the substantia innominata is another area innervated by the lateral SC only that is also implicated in aggression. The SI is innervated by the lateral SC and artificial activation of SI neurons induced aggression towards conspecifics (Zhu *et al.*, 2021). Artificial activation of periaqueductal gray (PAG) projecting SI neurons also induced aggression towards pups, crickets and even moving objects. Thus, the SC’s connection to the substantia innominata suggests a role in pursuit.

4.2.4 The lateral SC is connected to areas involved in gating approach

Some of the outputs of the lateral SC may also play a role in gating approach behaviours.

As discussed, the MRN is reciprocally connected to the lateral, but not medial SC, whereas anterolateral motor cortex (ALM)-projecting thalamic areas (VM, VAL, MD and intralaminar thalamus) receive input from the lateral SC alone. ALM-projecting thalamus (besides MD) are not connected to the medial SC.

Inagaki *et al.*, 2020 found the ALM-projecting thalamus underwent large activity changes after the onset of a go cue in their task: a cue which instructs the mouse to produce a lick, a lick whose direction should be determined by a prior stimulus. Artificially activating MRN/PPN terminals in the ALM-projecting thalamus, even unilaterally, did not bias licking in one direction or another, but triggered licking in a direction appropriate to the prior stimulus. This suggests that these lateral SC-recipient areas are involved in gating approach behaviours.

4.2.5 The lateral SC is connected to areas involved in preparing approach

Manipulations of the lateral SC (and some of its connections) do not only affect approach during the manipulation, but can also affect approach behaviours yet to come. In tasks requiring mice lick in a direction governed by a prior stimulus, some lateral SC neurons responded during the choice period, and some lateral SC neurons respond during the delay period, intervening between stimulus and response (Duan *et al.*, 2019). Putative unilateral artificial inhibition of the lateral SC during the delay period, like in the response period, produces an ipsiversive bias (Duan *et al.*, 2019; Hao *et al.*, 2020).

Delay and choice responses are also present in the lateral SC-projecting ALM (Economo *et al.*, 2018), as well as in the ALM-projecting thalamus that the lateral SC (alone) innervates (a region which includes VM, VAL, MD and intralaminar thalamus). Therefore there is some evidence that the lateral SC and its connections support not only the execution, but also the preparation of approach behaviour.

4.2.6 Different lateral SC neurons may evoke different approach behaviours

We are met with a question of how so many different behaviours can involve one part of the brain, the lateral SC. At one extreme, each neuron in the lateral SC might project to all of its outputs, and the role of the lateral SC may only be to represent direction - the rest of the brain may be responsible for choosing which behaviour should be produced, for example turning towards or licking towards. At the other extreme, each lateral SC neuron may project to only one

target. In this scenario, which specific SC neurons are activated would influence which specific directed behaviour would result. If the pattern of activity favours SC neurons projecting to licking-related areas, licking may be more likely to result, whereas if the pattern of activity favours SC neurons projecting to turning-related areas, turning may be more likely to result. Different lateral SC neurons inducing different approach behaviours is intuitively similar to the action-selection model of basal ganglia function (Friend and Kravitz, 2014). Whether different lateral SC neurons induce different approach behaviours is unclear, but there are some circumstantial hints.

First, dual retrograde tracing experiments suggests that, for many pairs of targets, different SC neurons provide input to each target. Lateral posterior nucleus of the thalamus (LP) projectors are separate from parabigeminal nucleus (PBGN) projectors (Shang *et al.*, 2018); zona incerta (ZI) projectors are separate from PAG-projectors, and from mesencephalic locomotor region (MLR) projectors (Shang *et al.*, 2019); SNc-projectors are separate from VTA-projectors, and from ZI-projectors (Huang *et al.*, 2020). Different SC neurons may therefore be able to facilitate different approach behaviours.

Second, some of the inputs to SC seem to be action, but not direction-specific. For example, the VMHvl, that projects to the lateral SC, is an area without a known representation of stimulus or movement direction. Instead the VMHvl is thought to represent actions: some neurons in the VMHvl are more active during some actions than others (Li Wang, Talwar, *et al.*, 2019). If different lateral SC neurons facilitated different actions, VMHvl neurons could encourage the actions they represent by innervating the appropriate lateral SC neurons. Alternatively, projections to SC from the VMHvl and similar areas may play more of a gating role, facilitating the occurrence of approach actions without arbitrating between them: which of these explanations remain to be seen.

Third, there is some evidence of topographic mapping of different approach behaviours in one of the (indirect) inputs to the SC: the striatum. The dorsomedial to ventrolateral axis of the striatum, through the intermediary of the substantia nigra pars reticulata, is mapped onto the medial-lateral axis of

the SC. Peters *et al.*, 2021 found that in a head-fixed task requiring putative orienting behaviour followed by licking to receive reward, the more central striatum responded around the orienting behaviour, whereas the more ventrolateral striatum responded when the mouse received reward via licking. Similarly, Lee, Wang and Sabatini, 2020 found that artificial activation of the medial striatum evoked more turning relative to licking, whereas artificial activation of the lateral striatum evoked more licking relative to turning. Therefore, there is some evidence that the more medial parts of the lateral SC may be involved in approach turns, whereas the more lateral parts of the lateral SC might be involved in approach licking. This data also suggests that different neurons in the lateral SC might preferentially encourage some approach behaviours over some others.

That different lateral SC neurons might contribute to different aspects of approach is also suggested by segregation in its outputs. Guo *et al.*, 2018, found that two inputs to the lateral SC, ALM and M1, are connected to largely different parts of an output of the lateral SC, VM. Similarly, Guo *et al.*, 2020 found that largely different parts of the posterior nucleus of the thalamus (PO), a nucleus which receives unreciprocated input from the SC (medial and lateral), are connected to S1 and M1, two inputs to the lateral SC. Finally, the fastigial and dentate nuclei of the cerebellum, which both send unreciprocated inputs to the lateral SC, connect more to the VM and VAL respectively: two thalamic nuclei innervated by the lateral SC (Gao *et al.*, 2018). Which lateral SC neurons project to which of these downstream thalamic nuclei and their subregions remains to be seen.

A final suggestion that different lateral SC neurons might be responsible for different aspects of approach behaviour comes from Economo *et al.*, 2018. The authors found that two separate ALM populations project to the SC: one which sends collaterals to the thalamus, and one which sends collaterals to the medulla. Interestingly, the paper found evidence for a role of the thalamus-projectors in choice preparation, and for the medulla-projectors in choice execution. It will be interesting for future work to study whether these two ALM inputs different SC populations. Again, segregation of different aspects of

approach in the input hints that different lateral SC neurons may support different roles in approach.

4.3 The medial superior colliculus is connected to areas involved in triggering escape

Only the medial SC projects to the nucleus reuniens, the cuneiform nucleus, the magnocellular reticular nucleus and the lateral part of the parabrachial nucleus: the SC does not receive input from these areas. The medial SC alone also receives input from the VMHdm/c: the SC does not project back to the VMH. These are areas that might play a role in triggering escape behaviour. If the medial and lateral SC are associated with escape triggering and approach respectively, areas connected to only the medial SC should be involved in generating the high speed running part of the escape response, but perhaps not the orienting towards a nest.

4.3.1 Cuneiform nucleus

Connectivity with the SC

The medial SC alone is connected to the cuneiform nucleus. The medial SC sends unreciprocated ipsilateral projections to the cuneiform nucleus.

Function

Isa *et al.*, 2020 found that artificial unilateral activation of the SC neurons that project to the cuneiform nucleus triggered fast forwards running which the authors termed flight, or retreat. Similarly, Caggiano *et al.*, 2018 found that unilateral artificial activation of cuneiform glutamatergic neurons evoked locomotion that could reach high speeds (~60cm/sec). Moreover, bilaterally inhibiting these neurons reduced the probability of fast locomotion. Interestingly, these movements were encouraged by delivering an airpuff, and the response may therefore be considered a non-directed escape, suggesting that the cuneiform is involved in mediating some fast movements, including escape responses. Similarly, activating GABAergic terminals in the mesencephalic locomotor region (MLR, which includes the cuneiform nucleus) from the central amygdala impairs (undirected) active avoidance in head-fixed mice by reducing running (Roseberry *et al.*, 2019). Thus, the cuneiform

nucleus appears to promote fast locomotion, at speeds utilised by escape, suggesting a role for the medial SC in triggering escape.

4.3.2 Magnocellular nucleus

Connectivity with the SC

The medial SC alone is connected to the magnocellular reticular nucleus (MARN). The medial SC sends unreciprocated projections to the MARN (Benavidez *et al.*, 2020).

Function

The MARN (Esposito, Capelli and Arber, 2014) can be split into the lateral paragigantocellular nucleus (LPGi), and the alpha and ventral parts of the gigantocellular nucleus – the latter two being separate from the gigantocellular reticular nucleus (Capelli *et al.*, 2017). Capelli *et al.*, 2017 found that the SC innervates glutamatergic LPGi neurons, whose unilateral artificial activation evoked locomotion.

Interestingly, the paper also reports that the glutamatergic LPGi neurons receive input from the cuneiform nucleus. Indeed, the speed of locomotion induced by artificial activation of the MLR is reduced by genetic ablation of LPGi glutamatergic neurons. Thus, the LPGi glutamatergic neurons represent a direct and indirect target of the medial SC which could play a role in generating the running needed for escape.

4.3.3 Lateral parabrachical nucleus

Connectivity with the SC

Whilst the lateral superior colliculus projects to the medial part of the parabrachial area, the medial superior colliculus projects to the lateral parabrachial area (Palmiter, 2018).

Function

The medial part of the parabrachial nucleus seems to be less studied, though it has been hypothesised to be involved in “taste-guided behaviour” (Chiang *et al.*, 2019). In contrast, the lateral parabrachial area has been studied more (Palmiter, 2018), and neurons there respond to a variety of aversive cues.

Chiang *et al.*, 2020 found that the lateral parabrachial nucleus projects to the lateral PAG, and ventromedial hypothalamus and that artificial activation of the terminals in either of these two output areas evoke high speed locomotion. Thus, the lateral parabrachial nucleus could be an output of the medial SC which could play a role in generating the running needed for escape.

4.3.4 Nucleus reuniens

Connectivity with the SC

The nucleus reuniens is connected to only the medial SC. It receives unreciprocated inputs from the medial SC.

Function

Consistent with its connectivity, cfos activity is the reuniens is higher following presentation of aversive visual stimuli than it is after the presentation of non-aversive visual stimuli (Salay, Ishiko and Huberman, 2018). Moreover, artificial activation of these neurons increased tail-rattling responses to the aversive stimuli (Salay, Ishiko and Huberman, 2018), suggesting a role in defence. Future work can investigate whether the role of the nucleus reuniens in defence extends into triggering escape.

4.3.5 Lateral septum

Connectivity with the SC

The lateral septum is connected to only the medial SC. It sends unreciprocated input to the medial SC.

Function

Azevedo *et al.*, 2019 find that a population of lateral septum neurons (neurotensin-expressing) respond when the mouse escapes from threats, such as footshocks or a robot simulating a predator. Thus, the input to the medial SC from the lateral septum suggests a role in triggering escape.

4.3.6 Ventromedial Hypothalamus (dorsomedial/central)

Connectivity with the SC

As discussed above, ventrolateral parts of the VMH project more to the lateral SC, whereas the dorsomedial/central part of the VMH projects more to the medial SC. The SC does not innervate the VMH.

Function

Neurons in the VMHdm/c have been found to respond to rat urine, and to aversive sounds originally designed to simulate rat vocalisations (Kennedy *et al.*, 2020). Rats are mouse predators. Bilateral inhibition of these neurons lead mice to spend more time in close proximity to rats (Kunwar *et al.*, 2015). Mice exposed to rats typically exhibit defensive behaviours such as freezing and fleeing (Silva *et al.*, 2013), and artificial unilateral activation of VMHdm/c neurons has been found capable of evoking these behaviours also (Kunwar *et al.*, 2015). Note also that medial amygdala neurons have been hypothesised to encourage defence through activating the VMHdm/c (Miller *et al.*, 2019). The VMHdm/c thus appears to play a role in transforming predatory cues into defensive behaviour, such as the triggering of escape: that the medial SC gets input from the VMHdm/c may be consistent with the role of the former in triggering escape.

4.4 Areas with balanced connectivity to the medial and lateral SC may be involved in both approach and triggering escape.

Some areas are connected to both the medial and lateral SC and may be involved in both approach and triggering escape. There are many of these areas. I will focus on the PAG, ZI, IC, AUD and SNr.

4.4.1 Periaqueductal gray

Connectivity with the SC

The periaqueductal gray (PAG) is reciprocally connected to medial and lateral SC.

Function

It is common to divide the PAG into 4 subregions; the dorsomedial, dorsolateral, lateral and ventrolateral PAG (dm,dl,l,vlPAG) (Bandler and Shipley, 1994). The literature on the roles of different subregions of the PAG is large, and studies often combine the four PAG regions to form undivided superregions such as the 'dorsal PAG' (dPAG) and the 'lateral/ventrolateral PAG' (l/vlPAG).

Dorsal PAG

The dorsolateral PAG is often thought to be involved in escape responses. Activation of terminals of CAMK2+ neurons in SC evokes running in head-fixed mice (Haitao Wang, Chen, *et al.*, 2019a) and also evoke escape in freely-moving mice (Wei *et al.*, 2015). Moreover, inhibition of these terminals reduces the speed of running evoked by loud noises in head-fixed mice (Haitao Wang, Chen, *et al.*, 2019a).

Consistent with the role of activation of the dlPAG in evoking escapes is work looking at inhibitory inputs to the area (Chou *et al.*, 2018). Rostral GABAergic ZI neurons project to the dlPAG where they innervate VGLUT2+ neurons. Bilateral artificial activation of these ZI terminals in the dlPAG mildly reduces the speed of the running response to loud noises in head-fixed mice.

Artificial manipulation of glutamatergic dorsal PAG neurons (dPAG, including both the dorsomedial and dosolateral subdivisions) tends to modify escape behaviour. Unilateral activation of the CAMK2+ dPAG neurons evokes running (Deng, Xiao and Wang, 2016), and bilateral artificial activation of VGLUT2+ dPAG neurons evoke nest-directed escapes (Evans *et al.*, 2018) or high-speed running (Tovote *et al.*, 2016). Similarly, inhibiting the dPAG reduces the probability of escape responses to looming visual stimuli (Evans *et al.*, 2018). Therefore, the dPAG appears to be involved in triggering escape.

The dPAG might, however, also be involved in evoking freezing. Although Tovote *et al.*, 2016 find that artificial bilateral activation of VGLUT2+ dl/lPAG neurons evokes high-speed running, they also report that this running was often interrupted by freezing. Kunwar *et al.*, 2015 similarly report that artificial activation of VGLUT2+ dPAG neurons induces "freezing and activity bursts", whereas whilst Deng, Xiao and Wang, 2016 find that unilateral artificial

activation of CAMK2+ dPAG evokes running, when the activation finished, freezing was often observed. The strongest evidence for a role in freezing comes from Wang, Chen and Lin, 2015, who report that unilateral artificial activation of the terminals of VMHdm/c neurons in the dlPAG evokes freezing. However, what the postsynaptic targets are of these VMHdm/c terminals is unclear, and, interestingly, Montardy *et al.*, 2019 find that VMH input to the dPAG is biased towards GABAergic neurons rather than glutamatergic (VGLUT2+) neurons. The role of glutamatergic and GABAergic dPAG neurons in freezing remains to be better understood.

lPAG and vlPAG

More ventrally-located regions of the PAG, specifically the lPAG and vlPAG, are less well understood but may receive SC input (Shang *et al.*, 2019).

vlPAG alone: freezing

Experiments reporting manipulations of the vlPAG in isolation – as opposed to manipulations in tandem with the lPAG – tend to suggest its role in freezing. Tovote *et al.*, 2016 report that bilateral artificial activation of glutamatergic vlPAG neurons evokes freezing, whereas bilateral inhibition of these neurons reduces freezing in the circumstance of cue or contextual fear conditioning, or in the case of exposure to a remote-controlled toy snake. Consistent with this role of glutamatergic vlPAG neurons in freezing, (Vaaga, Brown and Raman, 2020) find that unilateral artificial activation of CHX10+ vlPAG neurons evokes freezing and Tovote *et al.*, 2016 find that manipulations of GABAergic vlPAG neurons, which inhibit the glutamatergic neurons, have the opposite effect. Thus, the activity of vlPAG glutamatergic neurons appears to promote freezing.

Pursuit

Whilst activation of the glutamatergic vlPAG tends to evoke freezing, activating inhibitory inputs to a mix of the lPAG and the vlPAG tends to evoke pursuit. GABAergic neurons in the medial zona incerta (ZI) (Zhao *et al.*, 2019), the lateral hypothalamus (LH) (Y. Li *et al.*, 2018; Hao *et al.*, 2019), and the central amygdala (CeA) (Han *et al.*, 2017) project to the vlPAG, where they make

functional (inhibitory) connections. Artificial activation of these inhibitory terminals facilitates the hunting of crickets (ZI in Zhao *et al.*, 2019, LH in Y. Li *et al.*, 2018; (Rossier *et al.*, 2020); CeA in Han *et al.*, 2017). The postsynaptic targets of this inhibition are uncertain, but there is some evidence that the results may be explained by through the inhibition of glutamatergic l/vlPAG.

Y. Li *et al.*, 2018 find that artificial activation of l/vlPAG glutamatergic neurons abolishes cricket hunting. Consistent with this, the facilitation of hunting evoked by artificial activation of LH (Y. Li *et al.*, 2018) or CeA (Han *et al.*, 2017) GABAergic terminals in the l/vlPAG can be annulled by (the presumably counterbalancing) simultaneous artificial activation of l/vlPAG glutamatergic neurons. In contrast, Han *et al.*, 2017 found that CeA's facilitatory input was not annulled by activation of GABAergic l/vlPAG neurons. At this stage it is notable that these studies artificially activating l/vlPAG glutamatergic neurons do not report that freezing was evoked – the apparent discrepancy between this and the result of (Tovote *et al.*, 2016) may be due to the activation method (opto- versus chemo-genetic). Thus far therefore, we have seen suggestions that the inhibition of l/vlPAG glutamatergic neurons, including by activation of their inhibitory inputs, facilitates hunting.

Fascinatingly, these papers manipulating the input to the l/vlPAG found that attacks were not limited to just crickets. Activating the inhibitory input from the ZI, LH, or CeA induced attacks on artificial prey, whereas activating the input from CAMK2+ MPOA drove interactions with 3D objects. This suggests the role of the l/vlPAG in mediating orienting interactions beyond merely in the case of prey. Consistent with this, Y. Li *et al.*, 2018 found that artificial activation of LH GABAergic terminals in the l/vlPAG also encourages attacks on conspecifics. Falkner *et al.*, 2020 also report a role for the lPAG in conspecific attacks, however, they find that bilateral inhibition of glutamatergic lPAG, rather than facilitating intraspecific attack, impaired it. It will be interesting how these results will be reconciled, but differences in context and the type of attack might be explanatory.

To summarise: the activity of glutamatergic neurons in the dPAG, lPAG and vlPAG is pro-defence, with the dlPAG (and possibly the lPAG) encouraging

escape, and the vlPAG (and possibly the dmPAG) encouraging freezing. In contrast the activity of glutamatergic l/vlPAG neurons appears to be anti-pursuit: inhibition of l/vlPAG glutamatergic neurons encourages pursuit. As a result of this, I'd expect the medial SC to functionally excite dl/lPAG glutamatergic neurons to trigger escape, and the lateral SC to functionally inhibit the l/vlPAG, to encourage approach. Is there evidence for or against this functional connectivity? Interestingly, this pattern of (anatomical) connectivity would respect the topographic connectivity pattern shown in Benavidez *et al.*, 2020, whereby subcortical areas with topographic connections to the SC have their more dorsal parts connected to the medial SC, and their more ventral parts connected to the lateral SC.

The SC has been reported to project to the dlPAG (Haitao Wang, Chen, *et al.*, 2019b), and the l/vlPAG (Shang *et al.*, 2019). Are there anatomical or functional differences in the inputs these PAG areas get from the medial and lateral SC respectively? Evans *et al.*, 2018 find that the dPAG (an undivided region including the dlPAG but also the dmPAG) gets more input from the medial than the lateral SC. The connections to the l/vlPAG are less understood. Besides direct connections to the PAG, the SC can modulate the PAG disynaptically, through its outputs to the LH and the ZI. In terms of the LH, as previously discussed, Benavidez *et al.*, 2020 report that the lateral SC alone projects to the LH, and Li *et al.*, 2018 report that artificial activation of GABAergic LH terminals in the l/vlPAG facilitates hunting. Whether the lateral SC functionally excites LH GABAergic neurons remains to be seen. Besides the LH route, as will be discussed below, there is compelling evidence that the lateral SC functionally inhibits the l/vlPAG glutamatergic neurons through the medial ZI (Shang *et al.*, 2019).

To summarise, we expect the medial SC to functionally excite glutamatergic neurons of the dlPAG and the lateral SC to functionally inhibit glutamatergic neurons of the l/vlPAG. In terms of monosynaptic connections, there is compelling evidence that the medial SC monosynaptically excites glutamatergic neurons of the dlPAG (Evans *et al.*, 2018). Whilst there is little evidence either way with regards to monosynaptic connections to the l/vlPAG, there is compelling evidence of disynaptic pathways through which the lateral

SC can functionally inhibit l/vlPAG glutamatergic neurons. Thus, there is some evidence that the functional connectivity between the SC and the PAG is consistent with the argument that the medial and lateral SC are involved in triggering escape and approach respectively.

4.4.2 Zona incerta

Connectivity with the SC

The Zona Incerta is ipsilaterally reciprocally connected to the medial and lateral SC and these connections are topographically-organised.

Function

The lateral SC is connected to the ventromedial part of the ZI, whereas the medial SC is connected to the dorsolateral part of the ZI. We therefore expect the activity of the more medial ZI to encourage approach, and the activity of the more lateral ZI to encourage defence. Whilst it is rare for a paper to target their study of the ZI to the medial or lateral portions specifically, Zhao *et al.*, 2019 found that bilateral artificial activation of anterior-medial GABAergic ZI neurons facilitated hunting, whereas bilateral inhibition of these neurons opposed hunting. GABAergic medial ZI neurons project to the l/vlPAG, where activation of their terminals facilitated hunting, suggesting a means for the lateral SC to functionally inhibit the l/vlPAG and promote approach.

The ZI has also been implicated in defensive behaviours (X. Wang *et al.*, 2019). Whether the defence-promoting neurons are preferentially innervated by the medial SC remains to be seen.

4.4.1 Substantia Nigra Pars Reticulata

Connectivity with the SC

The substantia nigra pars reticulata (SNr) is reciprocally connected to lateral and medial SC (Benavidez *et al.*, 2020).

Function

The connectivity of the SNr with the SC predicts a role for it in both approach and triggering escape. A role in triggering escape is suggested by the finding that artificial facilitation of the activity of SNr neurons impairs the triggering of

escape by conditioned stimuli predicting footshocks (Hormigo, Vega-Flores and Castro-Alamancos, 2016). Similarly, a role in approach is suggested by, Rizzi and Tan, 2019, who find that unilateral artificial activation of SNr neurons induces ipsiversive turning, consistent with the idea that the SNr provides unilateral inhibitory input to the SC.

4.4.2 Inferior Colliculus & Auditory Cortex

Connectivity with the SC

Benavidez *et al.*, 2020 report that both the medial and lateral SC are innervated by primary auditory cortex (A1) and the inferior colliculus (IC): this connection is reciprocal in the case of the IC.

Function

Xiong *et al.*, 2015 found that inhibition of A1 or the IC reduces the speed of the running response to loud noises, whereas artificial activation of either evokes running. The effect of IC inhibition has been corroborated (Haitao Wang, Chen, *et al.*, 2019b). Therefore, there is evidence of the involvement of the IC in triggering escape behaviour.

Although audition can aid approach behaviour (Hoy *et al.*, 2016; Inagaki *et al.*, 2018), whether there is a role for the inferior colliculus or auditory cortex in such behaviour remains to be seen.

5) The medially-biased SC is involved in freezing

5.1 The SC is involved in freezing

So far, I've argued that the lateral and medial SC respectively are involved in approach and triggering escape. However, artificial activation of the SC can also evoke locomotor arrest (Wei *et al.*, 2015). Locomotor arrest is often interpreted as a defence response, which I'll call "freezing". Since arrest occurs for a variety of reasons, it is important to look for other indications that the arrest is part of a defensive response (Roseberry and Kreitzer, 2017).

If the SC does have a role in freezing, is this role preferentially associated with the medial SC? If the medial SC (rather than the lateral SC) is preferentially involved in freezing, I could generalise the role of the medial SC to defence responses in general, including both the triggering of escape and freezing.

The evidence for the role of the medial SC in freezing comes from the effects of activity manipulations, and from connections which preferentially target the more medial SC.

5.2 Manipulation experiments suggest that freezing is associated with the medially-biased SC

Wei *et al.*, 2015 found that artificial activation of (CAMK2+) SC neurons in the medial SC evoked arrest, the report found that artificial activation of more lateral sites failed to do so.

5.3 Freezing-related areas connect to the medially-biased SC

Further evidence for the role of the more medial parts of the SC in freezing comes from study of the connectivity. I'll argue that medially-biased connections with V1, retina, PBGN, and MD may all be involved in freezing.

5.3.1 Primary visual cortex

Connectivity with the SC

V1 sends ipsilateral projections to the more medial parts of the SC. The SC does not project back to V1.

Function

Artificial activation of V1 terminals in the SC (Liang *et al.*, 2015) evokes arrest behaviour, as does artificial activation of V1-recipient SC neurons (Zingg *et al.*, 2017). Interestingly, the retina also projects to the three more medial columns of the SC, and artificial activation of retinorecipient SC neurons also evokes arrest (Zingg *et al.*, 2017). Whether these arrest behaviours may be called freezing remains to be seen. Thus, the input from V1 suggests a role for the medially-biased SC in arrest, and potentially freezing.

Interestingly, Zingg *et al.*, 2017 report two extrinsic outputs of the V1/retinorecipient SC; the PBGN, and the LP, both of which may be involved in freezing.

5.3.2 Parabigeminal nucleus

Connectivity with the SC

The PBGN is innervated by the three most medial columns of the SC.

Function

Artificial activation of the PBGN evokes arrest (Shang *et al.*, 2018) (but see Zingg *et al.*, 2017) and artificially inhibiting the PBGN reduces freezing to a looming stimulus. Therefore, the output of the medially-biased SC to the PBGN suggests a role of the former in freezing.

Interestingly, freezing evoked by artificial activation of the PBGN is preceded by running, and the PBGN is also innervated by the A1-recipient SC neurons whose artificial activation also evoked running (Zingg *et al.*, 2017). Whether this running would be nest-directed in the presence of a nest remains to be seen, and nest-directed escape has been found to remain in the presence of PBGN inhibition (Evans *et al.*, 2018). The PBGN connection will therefore require further study, though it may suggest that a role in triggering escape may extend beyond the medial SC to the medially-biased SC.

5.3.3 Laterodorsal tegmental nucleus

Connectivity with the SC

The laterodorsal tegmental nucleus (LDt) gets unreciprocated input from the three most medial columns of the SC.

Function

In the LDt, the SC innervates both PV+ and SOM+ neurons (Xiaomeng Wang *et al.*, 2019). Artificial activation of PV+ induces arrest, whereas artificial activation of SOM+ LDt opposes arrest to aversive odours (Yang *et al.*, 2016). The SC is biased towards PV+ neurons (Xiaomeng Wang *et al.*, 2019). Thus, the output of the medially-biased SC to the LDt, including arrest-promoting PV+ LDt neurons, suggests a role for the former in arrest.

5.4 The lateral posterior nucleus of the thalamus connects to both the medial and lateral SC, and may play a role in both freezing and approach

Connectivity with the SC

The LP projection from the SC also likely plays a role in arrest behaviour. However, the LP receives inputs from medial and lateral SC and the LP pathway also seems to play a role in approach. The LP may also project back to the SC.

Function

NTSR1+ SC neurons project only to the LP, and bilateral inhibition of these neurons impairs hunting behaviour (Hoy, Bishop and Niell, 2019). In contrast, Shang *et al.*, (2018) find that bilateral inhibition of LP neurons reduces the probability of freezing in response to a visual looming stimulus. Bilaterally increasing the excitability of LP neurons does the opposite. Thus there is evidence that projection to the LP is important for both approach and freezing.

Further suggestion that the SC-LP pathway contributes to both approach and freezing comes from the connectivity of the postsynaptic LP. The SC mainly projects to the posterior half of the LP (Beltramo and Scanziani, 2019; Bennett *et al.*, 2019; Hu *et al.*, 2019), the half that projects to the basolateral amygdala (BLA) (Bennett *et al.*, 2019).

Artificial activation of inputs to the BLA potentiates the freezing response to a visual threat (Salay, Ishiko and Huberman, 2018), a behaviour abolished by inhibition of the SC (Evans *et al.*, 2018), suggesting that the SC-LP pathway, through the BLA, is involved in the generation of freezing. Is the BLA also involved in approach?

The BLA contains PPP1R1B+ and RSPO2+ neurons (Kim *et al.*, 2016). Artificial unilateral activation of RSPO2+ neurons increases freezing and induced real-time place aversion, whereas artificial unilateral activation of PPP1R1B+ neurons does not change freezing, and induces real-time place preference. PPP1R1B+ are more numerous in the posterior BLA, whereas RSPO2+ neurons are more numerous in the anterior BLA. Thus the BLA

supports both freezing and putative approach behaviours, with these behaviours being preferentially associated with the anterior and posterior BLA respectively. There is topography in the SC input to the LP (Bennett *et al.*, 2019), and, remarkably, Benavidez *et al.*, 2020 report that the lateral SC-recipient LP projects to the posterior BLA, whereas the medial SC-recipient LP projects to the anterior BLA. Thus, the SC-LP-BLA pathway appears consistent with the association between the medial and lateral SC with freezing and approach respectively.

Another SC-recipient structure that targets the BLA is the MD. This area receives input from medially-biased SC, and artificial activation of these inputs modifies freezing behaviour (Baek *et al.*, 2019). However, unlike previous connections, the SC to MD pathway reduces freezing to a fear conditioned cue. Despite this, again the evidence suggests a role for the medially-biased SC in freezing.

In summary, many areas connected to the medially-biased SC may play a role in freezing, whereas the LP pathway, which also arises from the most lateral SC column, may additionally play a role in approach.

5.5 Pathways from the SC that may allow freezing

A site of convergence for many of the aforementioned areas is the central amygdala (CeA). The LP and MD are indirectly connected to the CeA through the BLA (Fadok *et al.*, 2018), whereas the PBGN projects to the CeA directly (Shang *et al.*, 2015). Artificial activation of medial SC NTSR1+ or CAMK2+ neurons (but not PV+ or GAD2+ neurons) activates the CeA and induces freezing (Sans-Dublanc *et al.*, 2020), and manipulating the CeA alters freezing responses to loom stimuli (Zelikowsky *et al.*, 2018). The CeA may promote freezing via several potential pathways, perhaps even through its projection to the PAG (Tovote, Fadok and Lüthi, 2015).

Other SC-recipients may also be involved in arrest behaviour. Activation of terminals in the pontine reticular formation, which includes the SC target PRNc, induces arrest (Giber *et al.*, 2015). Moreover, artificial activation of GABAergic neurons in the MLR, which are innervated by the SC (Roseberry

et al., 2016), also induces arrest. Future work can investigate which outputs the SC promotes arrest through.

To summarise, there is some evidence that the medial SC is involved in freezing, whilst there is less evidence for a role of the lateral SC in the same behaviour. This may represent a shift from approach towards defensive behaviours (freezing, followed by escape) as one moves from lateral to medial SC. Isa *et al.*, 2020 find that whilst artificial activation of the lateral SC evokes approach orienting, and the medial SC evokes putative escape behaviour, artificial activation of a more central region of the SC evoked freezing.

6) Discussion

To conclude, I have argued that the lateral and medial SC are respectively associated with approach and defence (triggering of freezing or escape). Consideration of the evidence for the hypothesis has revealed many gaps, predictions and suggestions for future experiments, particularly in conjunction with questions about the involvement of the SC in avoidance (ipsiversive movement).

First, activation of some of the ipsilateral projection targets of the lateral SC has been shown to evoke ipsiversive turning. The suggestion of a role for the SC in ipsiversive turning recalls the idea of contralateral SC ('crossed') projections, arising mainly from the lateral SC, evoking contraversive turns, and ipsilateral SC ('uncrossed') projections, arising mainly from the medial SC, evoking ipsiversive behaviour (Dean, Redgrave and Westby, 1989). Moreover, whilst most SC neurons prefer contraversive movement, a minority prefer ipsiversive movement (see section below). A role in ipsiversive turning would undermine the idea of the SC playing a role purely in approach orienting, and not in avoidance orienting. Indeed, although evidence for the unilateral SC in avoidance orienting is limited, Isa *et al.*, 2020 report that the fast movements evoked by artificial activation of CUN-projecting SC neurons had an ipsiversive bias (but see Zingg *et al.*, 2017). The apparent role of SC targets such as the parafascicular nucleus in ipsiversive orienting remains to be understood.

Second, some areas do not have the predicted connectivity. The retrosplenial cortex seems to play a role in escape guidance (Vale *et al.*, 2020), yet Benavidez *et al.*, 2020 report that it projects to the medial, or medially-biased SC. The VTA has been implicated in escape triggering behaviour, yet apparently is connected to only the lateral SC. Moreover, the lateral SC alone projects to the subparafasicular nucleus, for which there is evidence of a role in freezing (Kang *et al.*, 2020).

Third, some of the areas connected to *both* the medial and lateral SC have, so far, only been implicated in one behaviour or the other. The IC, ACx, dorsal raphe (Huang *et al.*, 2017) and locus coeruleus (L. Li *et al.*, 2018) all connect to both the medial and lateral SC, but have only been implicated in the triggering of escape. Whether they also play roles in approach or freezing remain to be seen.

6.1 SC neurons preferring ipsiversive movement

I have argued that the major role of the lateral SC is to generate a contraversive movement, towards the part of the world that that part of the SC represents. While the SC as a whole promotes contraversive movement, a minority of SC neurons prefer ipsiversive over contraversive movements. Whether ipsiversive-preferring neurons actually promote ipsiversive movements remains to be seen, but does their presence permit a role for the SC in avoidance?

There is some evidence that ipsiversive-preferring SC neurons may receive different input. For example, Huda *et al.*, 2020 found that the ACC sends glutamatergic ipsilateral projections to the SC, and these SC-projecting ACC neurons prefer ipsiversive movements. Inhibition of the terminals of ACC neurons in the SC shifted the balance in favour of contraversive movements, also suggesting that the ACC input to the SC promotes ipsiversive behaviour. Similarly, Lee and Sabatini, 2020 found that artificial activation of striatal neurons modulated only the activity of contraversive-preferring SC neurons. One route which the striatal neurons could use to influence the SC is their (indirect) connection to the SNr. Interestingly, in other species, SNr inputs to the SC terminate in patches (Graybiel, 1978), while patches of contraversive

movement promoting (PITX2+) neurons have been found in the mouse SC, and these PITX2+ neurons also receive input from the SNr (Masullo *et al.*, 2019). Furthermore, some lateral SC outputs, such as the VTA, are active during ipsiversive as well as contraversive movements (Prévost-Solié *et al.*, 2019)

Local inhibition might control the ipsiversive/contraversive balance in SC. Work does indeed find that unilateral artificial activation of GABAergic SC neurons (GAD2+ and VGAT+ respectively) biases the mouse towards ipsiversive licks (Duan *et al.*, 2019; Hao *et al.*, 2020). However, in contrast, other work finds that GABAergic (GAD2+) SC neurons tend to prefer contraversive, over ipsiversive turns, and that unilateral artificial activation of such GABAergic SC neurons biases the mouse towards contraversive turns (Essig, Hunt and Felsen, 2020; Sans-Dublanc *et al.*, 2020). The extent of the overlap between ipsiversive-preferring and GABAergic SC neurons (or a subset of the latter), remains to be seen.

6.2 Future behavioural experiments

It will be interesting to see whether visual threats presented at different elevations do generate different behaviours. If behavioural responses vary with stimulus elevation, it will be interesting to know the exact relationship between the two, and to relate this to the parts of the visual field represented by the medial and lateral regions identified by Benavidez *et al.*, 2020.

Even if there are different roles in escape triggering for the medial and lateral SC, it will also be interesting to test the proposed lack of contraversive role for the medial SC, especially given that Masullo *et al.*, 2019 find that medial PITX2+ SC neurons evoke contraversive turns as their lateral neighbours do. Thus, it will be useful to study to what extent the azimuthal position of an elevated visual threat (i.e. one activating the medial SC) guides the animal's escape trajectory.

6.2.1 The triggering of escape vs orienting

The SC has a role in both the triggering of escape, and orienting: both of these behaviours involve directed movement. Unilateral activation can be useful - if unilateral activation evokes running without a particular egocentric bias, this

suggests a role in escape triggering; otherwise it may suggest a role in orienting. Bilateral inhibition of a population involved in triggering escape might be expected to interfere with the probability and latency of escape, whereas bilateral inhibition of a population involved in escape guidance might be expected to interfere with the exact escape trajectory mice take (Evans *et al.*, 2018; Vale *et al.*, 2020).

6.2.2 Directed movement vs gating

Artificial activation of a unilateral population might produce a lateralised response, even if the underlying signal is primarily involved in gating. For example, Inagaki *et al.*, 2020 found that unilateral artificial activation of MRN/PPN terminals in ALM-projecting thalamic regions prompted contraversive licking, but only when the stimulus prior to the manipulation had cued such a lick direction. Similarly, Lee and Sabatini, 2020 found that unilateral artificial activation of striatal neurons of the indirect pathway produced ipsiversive licking, but only when mice had had licks in that direction rewarded in the past.

6.2.3 Motor vs stimulus-based definitions of approach

In this review, I've argued that the SC is involved in promoting contraversive movements. Since the SC also represents the contralateral side of the world, I've argued that the SC promotes approach: i.e. movements towards the part of the world the SC represents. For example, in a task where mice have to approach a stimulus, the activity of the contralateral SC would be expected to facilitate that 'approach' behaviour, however, the activity of the ipsilateral SC would be expected to oppose 'approach'.

That the motor output of the SC instructs contraversive movement, rather than approach towards an arbitrarily-located stimulus is indicated by the work of Huda *et al.*, 2020. In this paper, the authors trained head-fixed mice to make movements which would, if they were freely-moving, orient them towards, or away from, a stimulus respectively. Regardless of the contingency used, the authors found that the activity of the unilateral SC promoted contraversive movement. This is as we would expect from the hemisphere-based, rather than the stimulus-based, definition of approach.

This leaves open the possibility of contexts in which there is competition between ipsiversive and contraversive movement, in which the SC might affect the probability of ipsiversive movements more indirectly, through biasing competition.

6.2.4 Running vs triggering escape

An area might play a role in running whilst not playing a role in escape. For this reason, it is useful to have a putative refuge: artificial activation should direct the animal towards this refuge if driving an escape behaviour. Moreover, it can be useful to study the effects of artificial activation when the animal is within the refuge. Auditory threats presented whilst the mouse is in a shelter do not evoke further escape (Vale, Evans and Branco, 2017).

More generally, aversive responses such as escaping or freezing may be split from less obviously aversive responses such as running, approach, or arrest by measuring conditioned behaviours such as conditioned place avoidance. If the artificial activation simulates an aversive experience, I might expect the animal to avoid the context in which it was experienced in the future. Procacci *et al.*, 2020, for example, find that a putative threat stimulus evoked arrest without evoking conditioned place avoidance, casting doubt on whether such arrests can be considered aversive.

6.2.5 Triggering escape vs freezing

Escape and freezing are distinct behaviours, but whether a neural population shows a role in one or both can depend on context. Vale, Evans and Branco, 2017 show that the same visual threat stimulus can evoke either escape or freezing depending on the presence or absence of a nest respectively. Moreover, Wei *et al.*, 2015 found that artificial activation of the medial SC could evoke either freezing or escaping depending on the presence or absence of a nest respectively.

The outputs of the SC involved in freezing (LP and PBGN) tend to be more synapses upstream of the muscles than those involved in triggering escape (e.g. dPAG, CUN and MARN), which may have functional consequences. dPAG (Tovote et al., 2016) and CUN (Caggiano et al., 2018) project to premotor neurons (Tovote et al., 2016), and MARN projects to motor neurons

directly (Tovote et al., 2016). In contrast, LP (Bennett *et al.*, 2019), and PBGN (Shang *et al.*, 2015) are not thought to innervate premotor neurons. The greater number of synaptic stages between the SC and freezing than between the SC and the triggering of escape may enable the freezing pathway to be more sensitive to context. Likewise, since each stage can have multiple outputs, freeze-promoting SC neurons may have more widespread effects (e.g. on learning). Relatedly, connections of SC involved in freezing and the triggering of escape tend to be associated with the sensory- and motor-related layers respectively. Altogether, evidence suggests that freeze-promoting SC neurons may signal a sensory event with context-dependent and widespread behavioural effects. In contrast, SC neurons promoting the triggering of escape represent a relatively context-independent and narrowly-routed motor command. Future behavioural work should assess whether freeze generation is more context-dependent than the triggering of escape, and whether freeze-promoting areas have more widespread behavioural effects than escape triggering areas.

7) Outputs to the LP and PAG in visually-guided freezing and escape

Artificial activation of SC terminals in the LP can evoke freezing (Wei et al., 2015; Zingg et al., 2017; Shang et al., 2018), whereas artificial activation of SC terminals in the PAG can evoke escape (Wei et al., 2015; Evans et al., 2018; Haitao Wang et al., 2019a). Recent work has also found that inhibition of the LP or PAG prevents freezing or escape responses respectively to certain visual threats (Evans et al., 2018; Shang et al., 2018). These results suggest that visual threats may be able to evoke freezing through a retina to SC to LP pathway, or escape through a retina to SC to PAG pathway. However, this hypothesis makes many predictions that are untested. Are LP and PAG projectors separate populations in the SC? Do LP-projectors favour the kinds of stimuli that tend to induce freezing, and PAG-projectors the kinds of stimuli that tend to induce escape? Does inhibition of LP and PAG-projectors impair freezing and escape responses to visual threats respectively?

In this thesis I detail the experiments I performed to address each of these questions, with the aim of testing a hypothesis about the neural circuits underlying defensive responses to visual threats. Our results suggest that the two pathways are anatomically segregated, and show different response dynamics, which may support their putative roles in different defensive responses. However, the two pathways also exhibited similar visual tuning, which may facilitate the influence of internal state on behavioural responses. Indeed, our behavioural experiments suggest a heavy influence of internal state on responses to common visual stimuli.

2. Anatomy: LP and PAG projectors are partially intermingled SC populations

Introduction

The mouse superior colliculus (SC) has many outputs with which to influence behaviour. For example, anterograde tracing from the SC labels its axons in the ipsilateral lateral posterior nucleus of the thalamus (LP) (Beltramo & Scanziani, 2019; Bennett et al., 2019; Fang et al., 2020; Montardy et al., 2021; Shang et al., 2018; Wei et al., 2015; Zhou et al., 2017; Zingg et al., 2017) and the ipsilateral dorsal periaqueductal gray (dPAG) (Zingg *et al.*, 2017; Evans *et al.*, 2018; Wang *et al.*, 2019; Montardy *et al.*, 2021). Artificial activation of SC terminals in the ipsilateral LP (Wei *et al.*, 2015; Zingg *et al.*, 2017; Shang *et al.*, 2018) and the ipsilateral dPAG (Evans *et al.*, 2018; Wang *et al.*, 2019) can evoke freezing and escape responses, respectively. Results from manipulation experiments therefore suggest that the SC's outputs to the LP and PAG might encourage freeze and escape responses, respectively. However, the extent to which LP and PAG projectors are separate populations of neurons in the SC is not known.

Previous work shows that neurons projecting to the ipsilateral LP are found in the superficial SC. Anterograde tracing from NTSR1+ SC neurons, which are concentrated in the optic layer, labels the ipsilateral LP, demonstrating that at least some of the SC input to the LP emerges from these cells (Gale and Murphy, 2014, 2018). Retrograde tracing from the LP primarily labels the ipsilateral SC: mostly the optic layer, but also parts of the SC superficial to the optic layer (Zhou *et al.*, 2017; Shang *et al.*, 2018; Benavidez *et al.*, 2020). Results from anterograde and retrograde tracing therefore suggest that the ipsilateral SC input to the LP emerges from the superficial layer: particularly the optic layer, but also the parts of the SC superficial to it.

Some neurons projecting to the LP might be found deeper than the optic layer. Wei *et al.*, 2015 found that injections of anterograde tracers that targeted the intermediate layers of the SC labelled axons in the LP, and that lesioning the LP reduced intermediate layer SC labelling following an injection of a transynaptic retrograde tracer into the amygdala. LP input from the intermediate layer of the ipsilateral SC has also been reported elsewhere (Huang *et al.*, 2017; Benavidez *et al.*, 2020). These results therefore suggest that LP-projecting SC neurons might extend deeper than the optic layer, into the intermediate layers ventral to it. In addition, some SC neurons project to the contralateral LP. Anterograde tracing from the SC labels the contralateral LP (Gale and Murphy, 2014, 2018; Zhou *et al.*, 2017). Where exactly in the SC the neurons projecting to the contralateral LP are is not known. Thus, there is evidence and potential for LP-projectors to be found deeper than the optic layer.

SC neurons projecting to the dPAG are generally found in the deeper layers of the SC. Retrograde tracing from the dPAG labels the intermediate and deep layers of the SC (Evans *et al.*, 2018; Shang *et al.*, 2018). However, Evans *et al.*, (2018) also found labelling in the superficial SC, and retrograde tracing from the adjacent lateral/ventrolateral PAG labels both the superficial and deeper layers of the SC (Shang *et al.*, 2019).

The extent of the segregation of LP and dPAG projectors in the SC is therefore not resolved by the extant literature. While most work suggests that neurons projecting to ipsilateral LP and dPAG occupy the superficial and deeper layers of the SC respectively, there is potential for overlap between these populations. In addition, which SC neurons project to the contralateral LP have not been clearly identified. To assess the degree of segregation of LP and PAG projectors in the SC we used retrograde tracing from each of these two targets.

Methods

All experiments were performed in accordance with the Animals (Scientific Procedures) Act 1986 (United Kingdom) and Home Office (United Kingdom) approved project and personal licenses. The experiments were approved by the University College London Animal Welfare Ethical Review Board under Project License 70/8637.

Animals

33 C57BL6/J mice, aged 8-24 weeks, were used in total: UMT1-4, and M19021-6 were female, whereas the rest were male. 6/33 underwent terminal surgeries to refine injection site parameters (UMT12-17). The remaining 27 mice underwent recovery surgeries. 11/27 used injection coordinates which were subsequently refined (UMT1-11) and only UMT4 & 7 from these animals are retained in the results. Of the remaining 16 mice; four received unilateral injections into either the LP or PAG (UMT24-7); six mice received unilateral injections into *both* the LP *and* PAG (M19021-6); six mice received *bilateral* injections into both the LP and PAG (UMT18-23). 8/16 mice did not contribute to the current results since they lacked SC labelling (UMT18, UMT21-24, UMT26 & M19021). In mice with bilateral injections, injection sites in the hemisphere contralateral to that shown in the results were less successfully targeted. Some of the labelling observed in mice in which bilateral injections were attempted could be due to the attempted contralateral injection, though labelling was not conspicuously different from mice with unilateral injections.

Injections

Mice were anaesthatised with 3% isoflurane, head-fixed, and had their scalps shaved and then cleaned with betadine and ethanol prior to incision. The coordinates of Bregma and lambda were measured with a 23G needle and head-fixation was adjusted until the measured DV offset between the two was less than 0.05mm. The needle was then used to mark points on the skull where the craniotomies were to be centred.

In all but four mice, 130nl of 0.1% w/v of CTB-488 or CTB-555 in PBS was injected at 100nl/min (Shang *et al.*, 2018) into targets. In the remaining four mice (UMT24-7), the LP or PAG was unilaterally injected with a mix of CTB-

488 and pAAV-Ef1a-mCherry-IRES-Cre virus: this mix was also injected into contralateral primary visual cortex (UMT24-7). Since SC does not project to cortex (e.g. Zingg *et al.*, 2014), these mice were included in the results.

The LP injection was made 2.46mm posterior, 1.5mm lateral, and 2.53mm ventral of Bregma. The PAG injection was aimed at the dorsal PAG (specifically the boundary between the dorsomedial and dorsolateral subdivisions): 4.6mm posterior, 0.24mm lateral, and 2.1754mm ventral of Bregma, but was achieved by means of an angled injection. Taking the dorsoventral axis of the brain as 0^{o}, the injection was angled at a pitch of 30^{o} in the posterior direction, requiring a craniotomy at 5.855mm posterior of Bregma, and an injection 5.855mm posterior and 2.625mm ventral of Bregma. The PAG injection was angled to reduce risk of the leak of tracer into the SC along the injection tract: this was unnecessary in the LP, where the overlying structures (cortex and hippocampus) aren't innervated by the SC (e.g. Benavidez *et al.*, 2020).

The LP and PAG are roughly cylindrical structures whose long axes stretch millimetres along the AP axis of the brain. The injection parameters chosen covered large portions of the ML and DV extents at the given AP, but were necessarily limited in terms of their AP spread. In the PAG, the injection was centred at 4.6mm posterior of Bregma to align with the part of the PAG in which Evans *et al.*, (2018) found artificial activation of SC terminals evoked escape. In the LP, 2.46mm posterior of Bregma was chosen to align with a part of the LP in which retrograde tracing labels the SC (Hu *et al.*, 2017; Huang *et al.*, 2017), and in which artificial activation of SC terminals evoked freezing (Wei *et al.*, 2015; Zingg *et al.*, 2017). Subsequent work has found that SC axons are substantially denser in the posterior half of the LP than the anterior half (Beltramo and Scanziani, 2019; Bennett *et al.*, 2019; Hu *et al.*, 2019; Fang *et al.*, 2020). Our injection site is in the anterior part of the posterior half of the LP: more posterior LP sites receive denser SC input, but 2.46mm posterior of Bregma receives prominent SC input nonetheless.

Mice received subcutaneous injections of 5% w/v carpofen in saline prior to the surgery and received 20ul of metacam in condensed milk for 3 days post-surgery.

Histology

Mice were perfused 3-4 weeks after surgery. Mice were anaesthetised with 3% isoflurane, injected with pentobarbital intraperitoneally, and transcardially perfused with 0.9% NaCl in 0.1M phosphate buffer (PB), followed by 4% paraformaldehyde (PFA). The brain was extracted and placed in 4% PFA overnight at 4ºC, and subsequently cryoprotected with 30% sucrose solution. A coronal section was made to cut away the cerebellum, following which brains were frozen in O.C.T. Compound (Sakura FineTek). Coronal sections of 40µm thickness were then sliced on a cryostat (Leica, CM1850 UV). Slices were mounted using Vectorshield with DAPI (Vector Labs) and imaged with a standard fluorescence microscope (Leica DMi8). 2.5x images were taken of the slice with the largest injection site.

Conspicuous anatomical reference landmarks were chosen to infer AP positions. For the SC, the slices that were 4.04, 4.24, 4.48 and 4.72mm posterior of Bregma were inferred using the distance from the boundary between the superior and inferior colliculi, and then imaged under either a 20x or 40x objective. The AP position of the PAG injection site image was also inferred using the distance from the intercollicular boundary, whereas the AP position of the LP was inferred using the distance of that slice from the anterior emergence of the dorsal third ventricle. Boundaries in the SC were overlayed based on the visible outlines of the SC and PAG of the relevant hemisphere; in the PAG based on the visible outlines of the PAG of the relevant hemisphere; and in the LP based on the visible outline of the subcortex of the relevant hemisphere.

Imaging

Slices were also imaged under a Leica TCS SPE1 confocal microscope with 40x magnification. A subset of the slices with potentially overlapping labelling from the LP and the PAG, were imaged. Only the hemisphere with labelling from both the PAG and the LP was imaged, and 2-5 images were taken

along the long axis of the region of intermingled LP and PAG labelling. Non-overlapping fields of view (approximately 180μm) along this axis were chosen according to the presence numerous LP and PAG-labelled cells. Z-stacks were taken with a z-step size of 2μm. Labelling was manually judged to be cellular if it was roughly circular, with a diameter approximately between 10-25μm, had DAPI in its centre and was present for at least 3 serial sections in the Z-stack. Cells were only counted in Z-stacks which contained at least one cell of each (single-labelled) population. Different tracers were visible in different channels: ROIs were drawn over cells in each channel, before labelling in that ROI was checked for the other channel.

Quantitative analysis

For quantitative analysis, from each of the four mice with retrograde labelling from the both the LP and the PAG, of the four coronal sections imaged (Fig. 1-4), we chose the section with the greatest combined labelling from each target (these were also sections that were confocally imaged). We then rotated each image to make the dorsal surface of the SC run parallel. A rectangle was defined which fit the dorsoventral extent of the SC (0.9683mm high), and was limited to the medial half of the SC (0.7419mm wide). The rectangle was aligned in each animal such that its dorsal and medial limits were the surface and midline of the SC respectively, and used to crop out a region of the SC (Fig. 7a). To compare dorsoventral distributions across mice, for each animal and each channel, we averaged fluorescence along the mediolateral axis, and normalised by dividing by the sum (Fig. 7b-c).

Results

Distribution of projections from SC to LP and PAG

To investigate the spatial distribution of LP-projecting SC neurons, we injected a retrograde tracer (CTB conjugated to a fluorophore) into the LP (Fig. 1a-b). We found that these retrograde tracers tended to label neurons along an extended stretch of the anterior-posterior (AP) axis of the ipsilateral SC (Fig. 2). Neurons were labelled in the optic layer, including the region around its interface with the intermediate layers, but also the more superficial

parts, of the ipsilateral SC (Fig. 1c). Our results therefore suggest that neurons projecting to the ipsilateral LP emerge from the superficial SC, including at the interface between the superficial and deep SC.

In some animals, retrograde tracing from the LP also labelled SC neurons in the contralateral hemisphere (Fig. 1d). Like the labelling in the other hemisphere, contralaterally labelled neurons were found in the superficial SC, but appeared more concentrated in the optic layer, as opposed to the parts of the SC superficial to it. In the optic layer, labelling included the interface with the intermediate layers. Retrograde tracing therefore suggests that the superficial SC, including the superficial-deep interface region, projects to the LP of the contralateral hemisphere.

We studied the locations of PAG-projecting SC neurons by injecting a retrograde tracer into the PAG (Fig. 3a-b). Retrograde tracing labelled the ipsilateral SC: labelling was observed in the deep layers, but also the intermediate layers (Fig. 3c). This labelling pattern was observed across different mice (Fig. 4). Labelling in the intermediate layers included labelling at the interface between the optic and intermediate layers. Results from our retrograde tracing suggest that the deeper layers of SC, including the superficial-deep interface region, projects to the ipsilateral PAG.

Labelling from the PAG injections tended to appear patchy in the intermediate layer. Labelling along the axis parallel to the SC layer boundaries tended to appear as columns or stripes, rather than homogenous. I return to the relationship between this patchy PAG labelling along the mediolateral axis of the SC and the mediolateral distribution of other variables, such as other anatomical inputs/outputs, in Discussion.

Overlap in projections from SC to LP and PAG

We performed dual retrograde tracing to investigate the segregation of LP and PAG projectors, particularly at the interface between the superficial and deeper layers of the SC. CTB protein conjugated to discriminable fluorophores (Alexa-488 and Alexa-555) was injected into the LP and PAG, respectively (Fig. 6a-b). In four mice, retrograde labelling from both the LP and PAG was observed in the SC. In two of these mice, the LP injection was

contralateral to the hemisphere of the SC studied (UMT19-20) – in the other two mice, retrograde labelling in the SC was observed from the ipsilateral LP and PAG (M19022, M19025). In all four mice, widefield microscopy exhibited overlapping label at the interface between the superficial and deep SC (Fig. 6c, 7). Whilst individual cells were clearly marked from the LP injection, the PAG injection also produced a large amount of neuropil labelling.

We used the greater resolution of confocal microscopy to understand whether LP and PAG projectors are separate populations at the SC's superficial-deep interface (Fig. 6d). Imaging the interface between the superficial and deep SC, we observed neurons labelled from the LP injection, neurons labelled from the PAG injection, and very few neurons labelled from both (2% of labelled neurons). Despite LP projectors being more superficial, on average, than PAG projectors, the two populations were interfaced, with some PAG projectors being more dorsal than some LP projectors. Dual retrograde tracing results therefore indicate that LP and PAG projectors are separate SC populations, which spatially intermingle at the interface between the superficial and deep SC.

Discussion

In this study, we used retrograde tracing to investigate the spatial distribution of LP and PAG projectors in the SC. We observed that LP projectors were found in the superficial SC, and the PAG projectors were generally found in deeper SC. LP and PAG projectors were, however, both present at the interface between the superficial and deep SC, where they formed spatially intermingled but largely separate populations. These results support the possibility, relevance, and implementation of future projection-specific experiments to investigate the behavioural roles of these SC output pathways.

Retrograde tracer injections into the LP labelled the SC. Subcortical regions neighbouring the LP are, however, also innervated by the SC (Zhou et al., 2017). The anterior pretectal nucleus (APN), the dorsal lateral geniculate nucleus (dLG) (Bickford *et al.*, 2015), and the posterior thalamus (Po) (Gharaei *et al.*, 2020) all are innervated by the SC (Benavidez *et al.*, 2020).

Whilst the input to the Po comes from the deep SC, the input to the dLG comes from the superficial SC (the APN output has not been much studied). Bickford *et al.*, (2015) found that SC input to the dLG emerges primarily from the layers superficial to the optic layer, raising the possibility that some of the retrogradely labelled SC neurons we saw project to the dLG, rather than the LP. However, the pattern of SC labelling we observed was consistent across multiple mice, and our injections were generally away from the dLG. Furthermore, LP projectors in the optic layers and layers superficial to it have been reported previously (Zhou *et al.*, 2017; Shang *et al.*, 2018). The retrograde tracing we observe, therefore, is likely to reflect the presence of neurons projecting to the LP.

Retrograde tracing from the LP labelled a substantial portion of the AP extent of the SC: the axis along which azimuth is mapped in the SC. That relatively restricted injection sites labelled so much of the AP axis of the SC suggests that the SC-to-LP projection is not strongly topographic, at least along the visual field azimuth. Consistent with this, in previous work, anterograde tracing from different AP positions in the SC produced poorly segregated axons in the downstream LP, and receptive field mapping failed to reveal a topographic mapping of azimuth in the SC-recipient LP (Bennett *et al.*, 2019). That common parts of the LP receive input from both hemispheres of the SC would further impede a topographic map of azimuth in the SC-to-LP projection. Whether there are functional implications of this lack of topography remains to be understood.

Retrograde labelling from the LP and PAG each labelled potential subpopulations of SC neurons. Retrograde tracing from the PAG tended to label the more superficial parts of the intermediate layers, along with the deep layers. Since some inputs to the SC target specifically the intermediate or deep layers respectively, transsynaptic anterograde tracing from such inputs could be used to target intermediate or deep PAG projectors, respectively (Zingg *et al.*, 2017). Otherwise, the intermediate and deep layers of the SC show differential expression of markers such as choline acetyltransferase (Bednárová, Grothe and Myoga, 2018), suggesting the possibility of finding a promoter to target a certain subpopulation. Likewise,

retrograde tracing from the LP labelled the optic layer, but also the parts of the SC more superficial to it. At least some of the optic layer LP-projectors are NTSR1+ (Gale and Murphy, 2014), and possibly substance P+ (Zhou *et al.*, 2017): a proportion of the LP projectors located more superficially are NTSR1-/PV+ (Shang *et al.*, 2018). Genetic techniques may therefore be able to access and study each population separately. Whilst the SC neurons projecting to the ipsilateral and contralateral LP hemispheres may be partially segregated populations, dual retrograde tracing from each LP hemisphere does double-label a small number of SC neurons (Zhou *et al.*, 2017), suggesting the two populations are not entirely separate. Future experiments can investigate the possibility of functional subpopulations within the LP and PAG projecting SC neurons.

Strong neuropil labelling was observed in the SC following CTB injection into both the LP and the PAG. Conte, Kamishina and Reep, (2009) observed small amounts of anterograde tracing using CTB. Chen and Aston-Jones, (1995) suggested that anterograde tracing may occur by uptake of CTB by axons passing through the injection site and Conte, Kamishina and Reep, (2009) suggest that such tracing may be made more likely by damage at the injection site. Some axons projecting to the SC in the injection sites may be provided by LP and PAG neurons, since some research suggests that both the LP and the PAG (Montardy *et al.*, 2021) project back to the SC (Benavidez *et al.*, 2020). Therefore neuropil labelling may reflect the axons of PAG neurons in the SC.

Neuropil labelling from the PAG had a patchy organisation along the axis parallel to the SC layer boundaries. Patchy organisation is interesting because of the patchy organisation of other variables in the SC. Wallace and Fredens, (1989) found a patchy organisation of intermediate layer SC labelling following the injection of an anterograde tracer into the PAG. The same study found that the labelling from the PAG aligned with the labelling of NADPH-diaphorase, which itself aligns with labelling of acetylcholinesterase, suggesting that the PAG-recipient SC neurons may be selectively targeted by other inputs also. Future work could also look at how labelling from the

PAG aligns with the patchy distribution of contraversive movement-promoting (PITX2+) cells in the intermediate layers of the SC.

Our confocal imaging suggested that LP and PAG projectors were almost entirely separate populations, even in the regions of SC where they were likely to be intermingled. In the superficial-deep interface region, confocal imaging found very few double labelled cells (2%). Other studies using dual retrograde tracer injections into SC targets found a similar percentage of double labelled SC cells and concluded that the sources of these projections were separate. Shang *et al.*, (2018) concluded that LP projectors are separate from parabigeminal projectors; Shang *et al.*, (2019) concluded that zona incerta-projectors are separate from PAG-projectors and midbrain locomotor region-projectors; Huang *et al.*, (2020) concluded that substantia nigra-projectors are separate from ventral tegmental area-projectors and zona incerta-projectors. A much higher percentage of double labelled neurons are found in the SC if the CTB proteins conjugated to different fluorophores are both injected into the substantia nigra (Huang *et al.*, 2020), for example. Similarly, a much higher percentage of double-labelled cells are found in areas in which neurons tend to send collaterals to the two injected downstream structures, as in the case of the ventromedial hypothalamus neurons which project to the PAG and the anterior hypothalamic nucleus (Wang, Chen and Lin, 2015).

A precedent for segregation of different SC projection populations has recently been observed for other targets. LP projectors are separate from parabigeminal projectors (Shang *et al.*, 2018); zona incerta-projectors are separate from PAG-projectors, from midbrain locomotor region-projectors (Shang *et al.*, 2019) and from substantia nigra-projectors (Huang et al., 2020). Substantia nigra-projectors are also separate from ventral tegmental area-projectors (Huang *et al.*, 2020). The adaptive benefit of having multiple independent parallel pathways streaming out from the SC remains to be understood.

Dual retrograde tracing suggested that LP and PAG-projectors form intermingled populations at the interface between the superficial and deep

SC. Recently, other separate SC projection populations have been shown to exhibit a very high degree of spatial intermingling (Shang *et al.*, 2018, 2019; Huang *et al.*, 2020). The intermingling that occurs in partially intermingled (interfaced) populations may support local interactions between different SC outputs. Intermingling may also allow inputs to a certain part of the SC to influence neurons projecting to different outputs. If projection populations are spatially intermingled, then it would be challenging to record or manipulate them separately without cell-type specificity. The study of the SC may be a field in which cell type-specific technology is particularly useful, therefore.

In conclusion, we used retrograde tracing to understand the distributions of LP and PAG projectors in the SC. We found that LP and PAG projectors have different spatial distributions which interface between the superficial and deep SC. These results allow, facilitate, and motivate future studies of the physiological properties and behavioural influence of the LP and PAG output pathways of the SC.

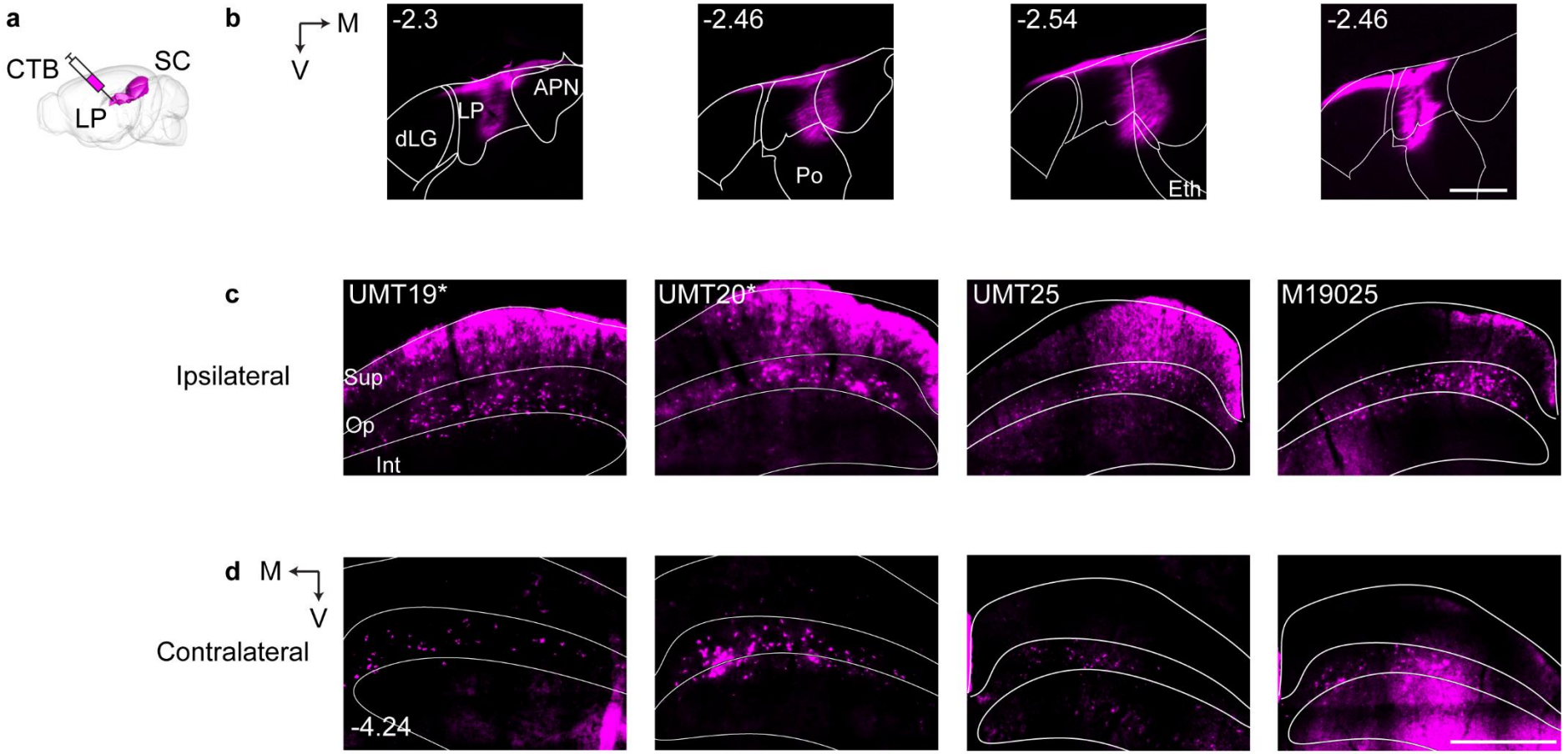
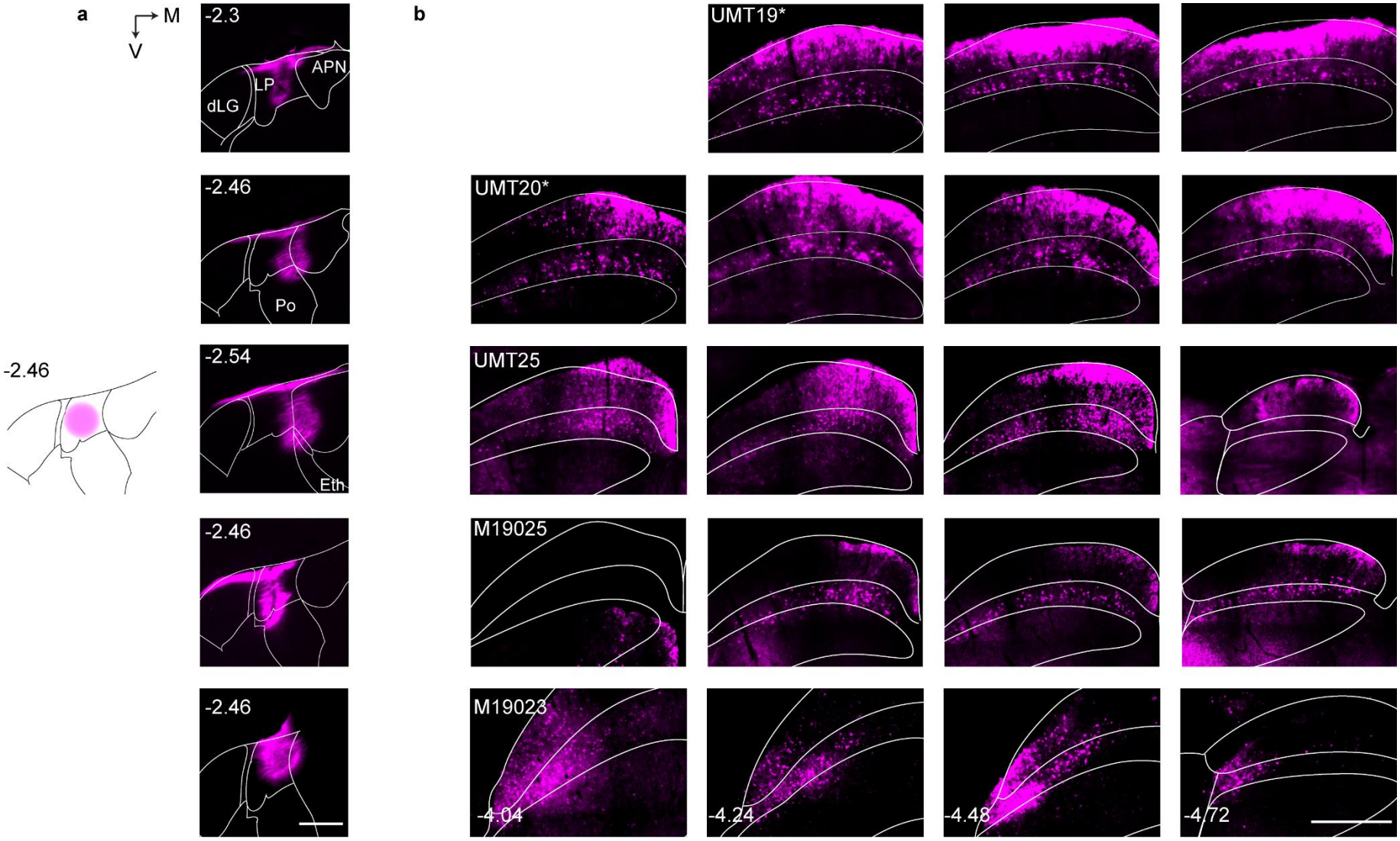


Fig. 1: LP-projectors are found in the superficial SC, including at the boundary of the deep SC. **a**. Schematic illustration of the CTB injection into the LP, downstream of the SC. **b**. Injection sites in the LP in each of four animals. M and V refer to medial and ventral

respectively, and numbers in top left refer to AP locations of images. dLG, APN, Po and Eth refer to the dorsal lateral geniculate nucleus, the anterior pretectal nucleus, the posterior thalamus, and the ethmoid thalamus nucleus, respectively. **c**. Labelling in ipsilateral SC occurs in the optic layer, and the layers superficial to it. Op, Int and Dp refer to the optic, intermediate and deep layers of the SC respectively: Sup refers collectively to the superficial gray and zonal layers layers of the SC respectively **d**. Labelling in the contralateral SC occurs mainly in the optic layer. Scale bars are 500μm.


a
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Eth
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b
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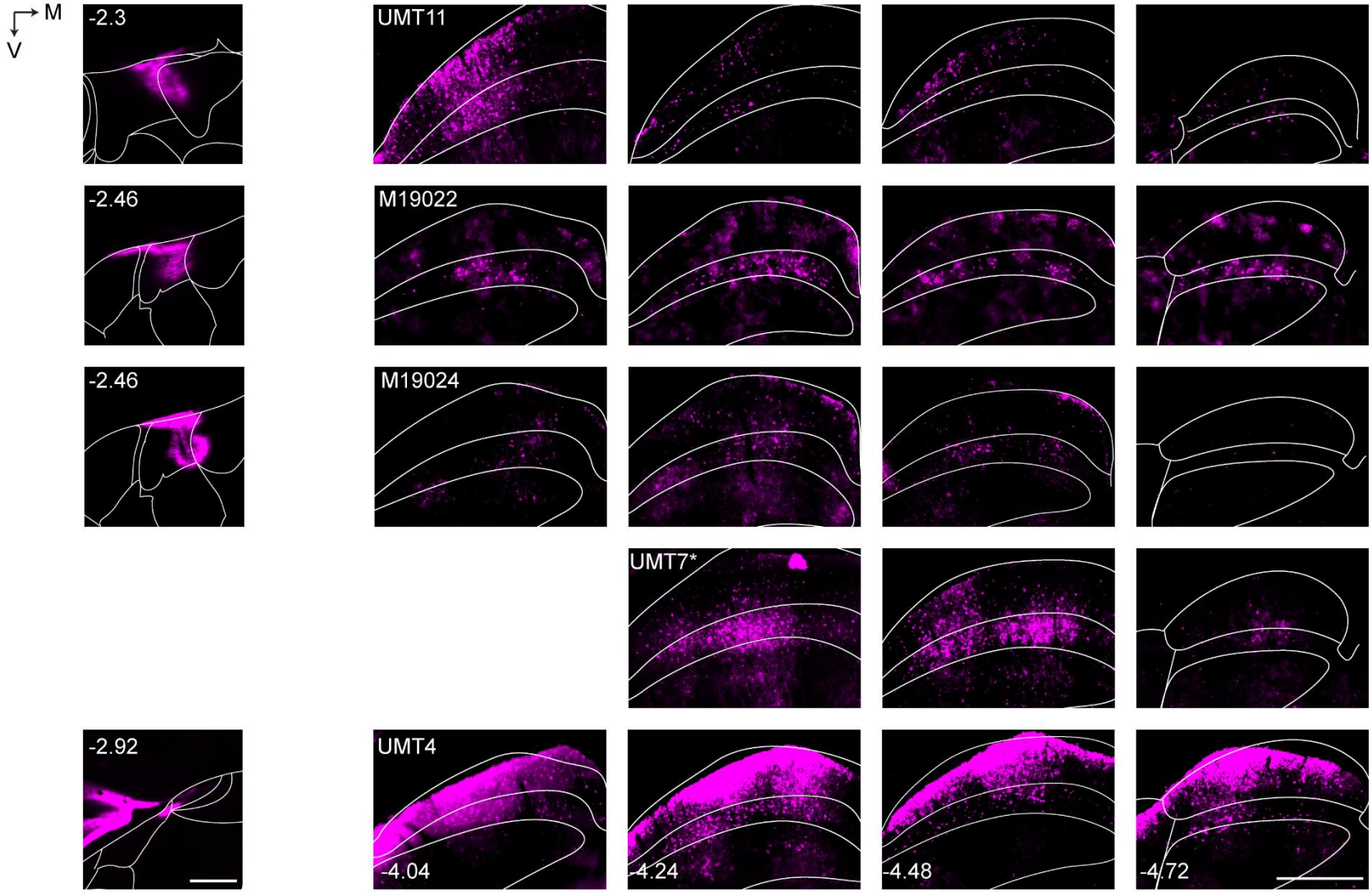
M
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UMT11
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M19022
-2.46
M19024
UMT7*
-2.92
UMT4
-4.04
-4.24
-4.48
-4.72

Fig. 2: LP-projectors were were labelled in the superficial SC of multiple animals. **a**. LP injection sites in each animal. M and V refer to medial and ventral respectively, and numbers in top left refer to AP locations of images. Left inset indicates the intended injection site. dLG, APN, Po and Eth refer to the dorsal lateral geniculate nucleus, the anterior pretectal nucleus, the posterior thalamus, and the ethmoid thalamus nucleus respectively. **b**. Labelling in ipsilateral SC occurs in the optic layer, and the layers superficial to it, across a large range of AP positions across mice. Scale bars are 500μm. Op, Int and Dp refer to the optic, intermediate and deep layers of the SC respectively: Sup refers collectively to the superficial gray and zonal layers.

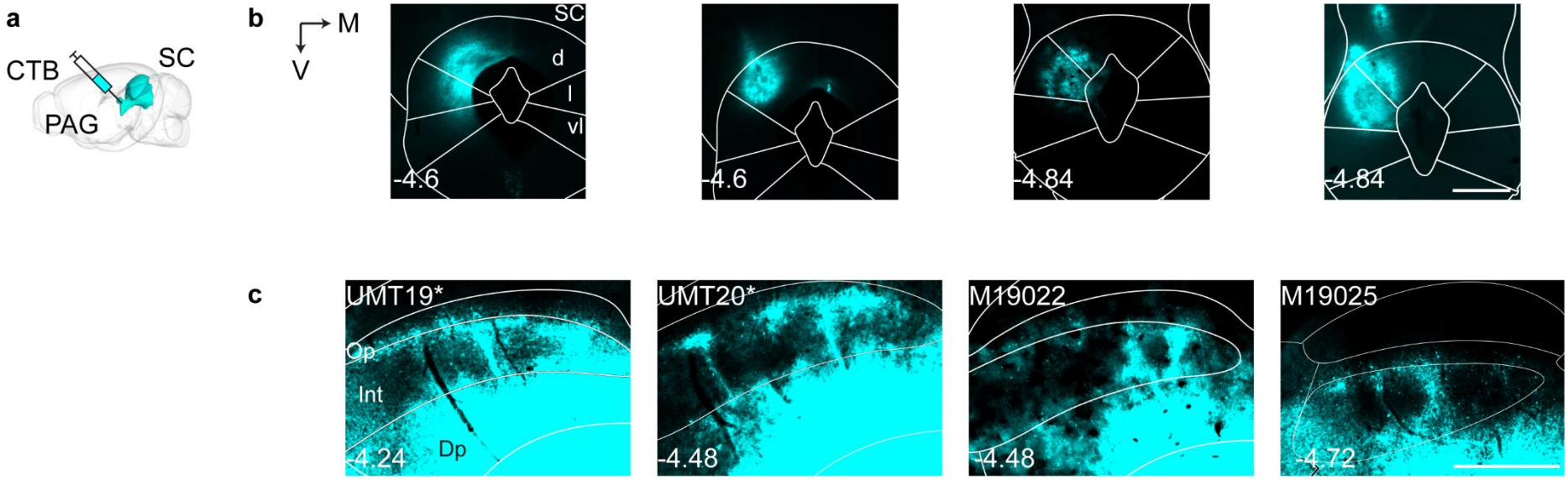
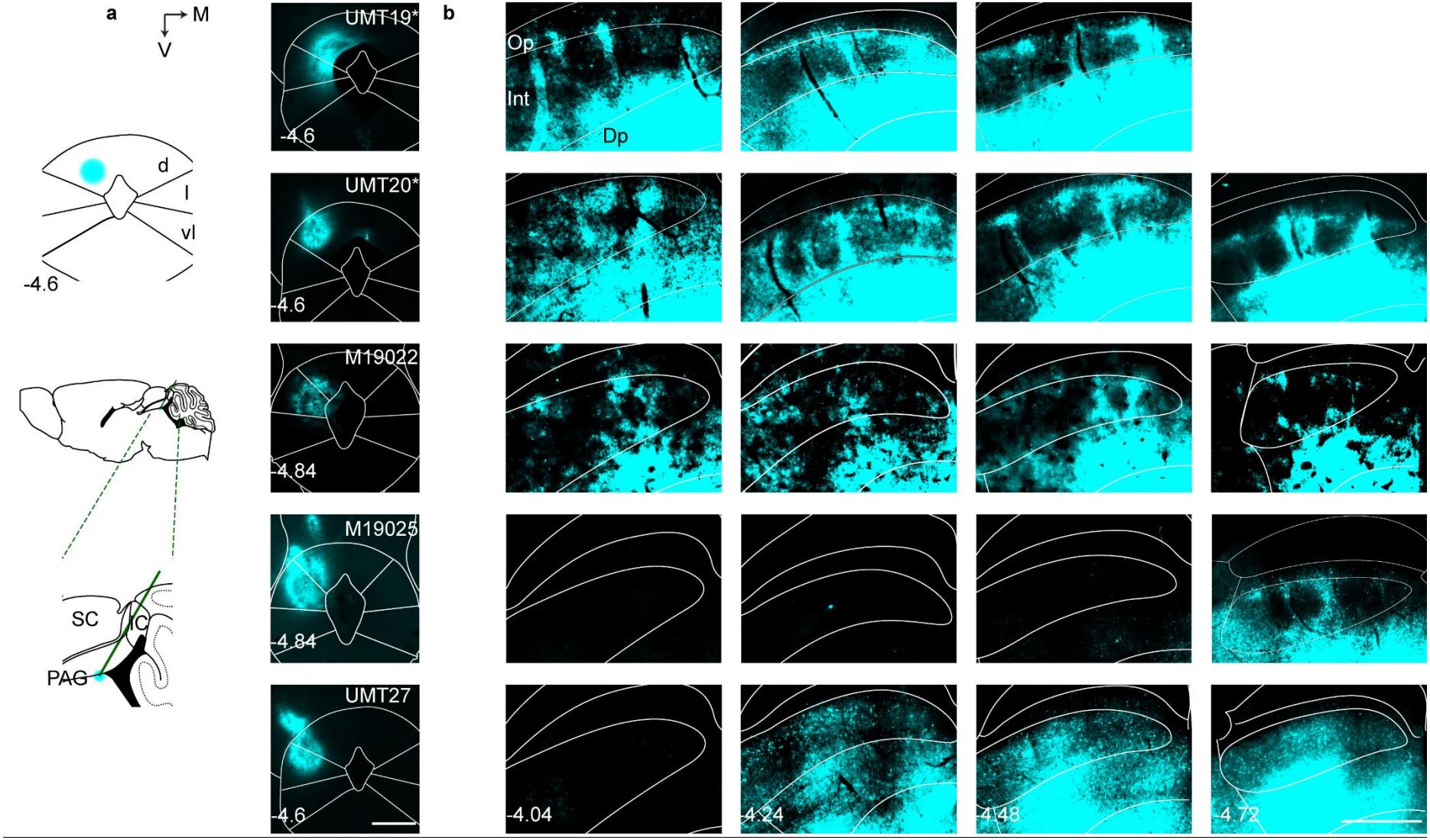


Fig. 3: PAG-projecting neurons are found in the deep SC, including at the boundary of the superficial SC. **a**. Schematic illustration of the CTB injection into the PAG, downstream of the SC. **b**. Injection sites in the PAG in each of four animals. M and V refer to medial and ventral respectively, and numbers in top left refer to AP locations of images. d, l, and vl refer to the dosral, lateral and ventrolateral subregions of the PAG. **c**. Labelling occurs in the ipsilateral deep SC: in the intermediate layers, and in the deep layers. Op, Int and Dp refer to the optic, intermediate, and deep layers of the SC respectively. Scale bars are 500μm.


a
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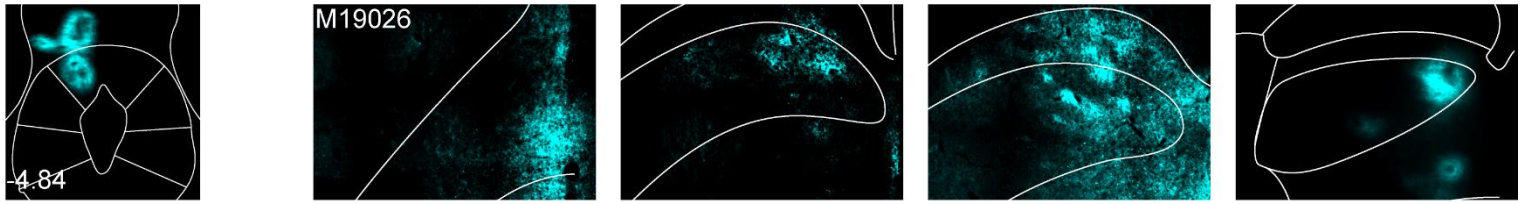

Fig 4: PAG-projecting neurons were labelled in the deep SC in multiple animals. **a**. PAG injection sites in each animal. M and V refer to medial and ventral respectively, and numbers in top left refer to AP locations of images. Left inset indicates the intended injection site, including the intended injection trajectory. d, l, and vl refer to the dosral, lateral and ventrolateral subregions of the PAG. **b**. Labelling occurs in the ipsilateral deep SC across different APs and different animals. Scale bars are 500μm

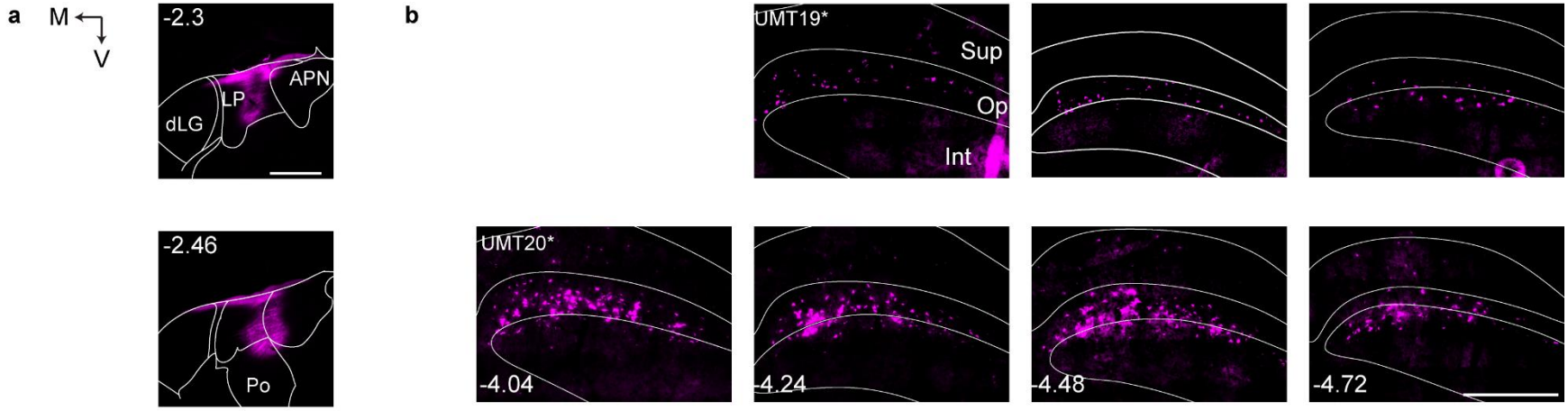

Fig. 5: LP-projecting SC neurons occupy the optic layer of the contralateral SC. **a**. LP injection sites in each animal. M and V refer to medial and ventral respectively, and numbers in top left refer to AP locations of images. dLG, APN and Po refer to the dorsal lateral geniculate nucleus, the anterior pretectal nucleus, and the posterior thalamus respectively. **b**. Labelling occurs in the contralateral superficial SC across different APs and different animals. Scale bars are 500μm

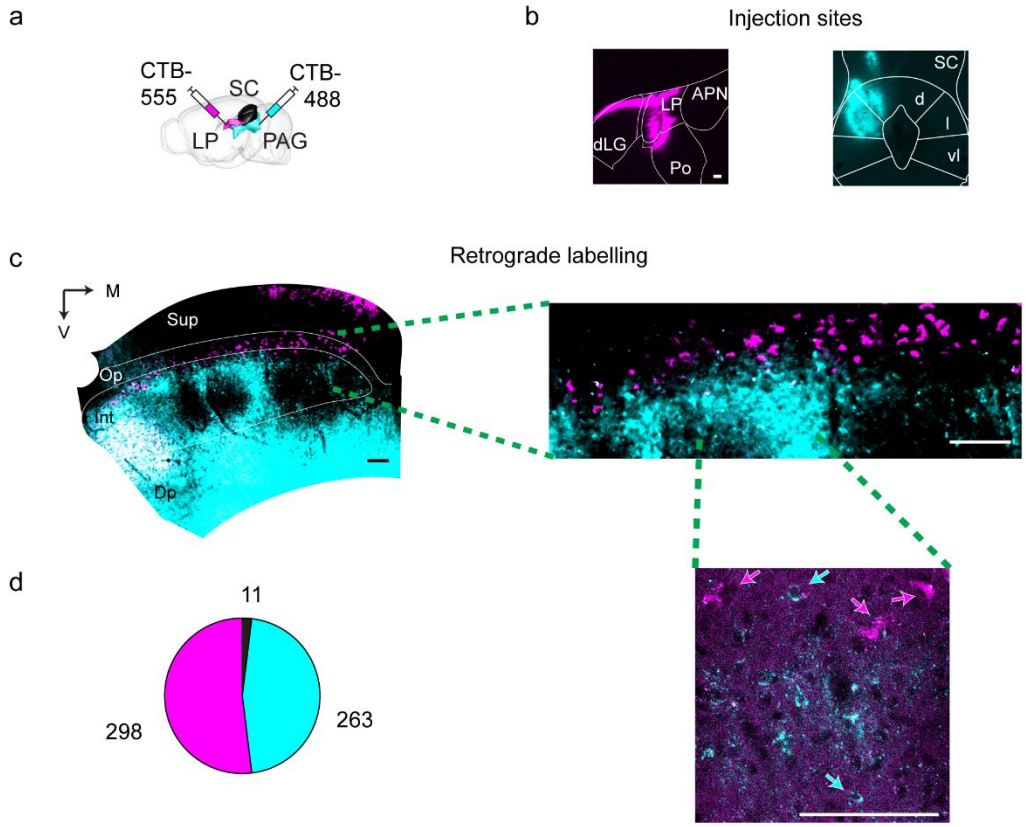

Fig 6. LP and PAG-projectors are intermingled yet separate populations at the boundary between the superficial and deep SC. **a**. Schematic illustration of the CTB injection into the LP and PAG, downstream of the SC. **b**. Injection sites in the LP (-2.46 AP) and PAG (-4.84 AP) respectively. **c**. Left panel shows retrograde labelling in the superficial and deep SC from the LP and PAG respectively, but interfaced labelling at the superficial-deep boundary. Right panel shows magnified interface region. **d**. Right panel shows confocal image of cellular labelling in the interface region. Left panel shows the number of cells labelled from the LP and PAG projections, and the number labelled from both. All scale bars are 100μm. Op, Int and Dp refer to the optic, intermediate and deep layers of the SC respectively: Sup refers collectively to the superficial gray and zonal layers.

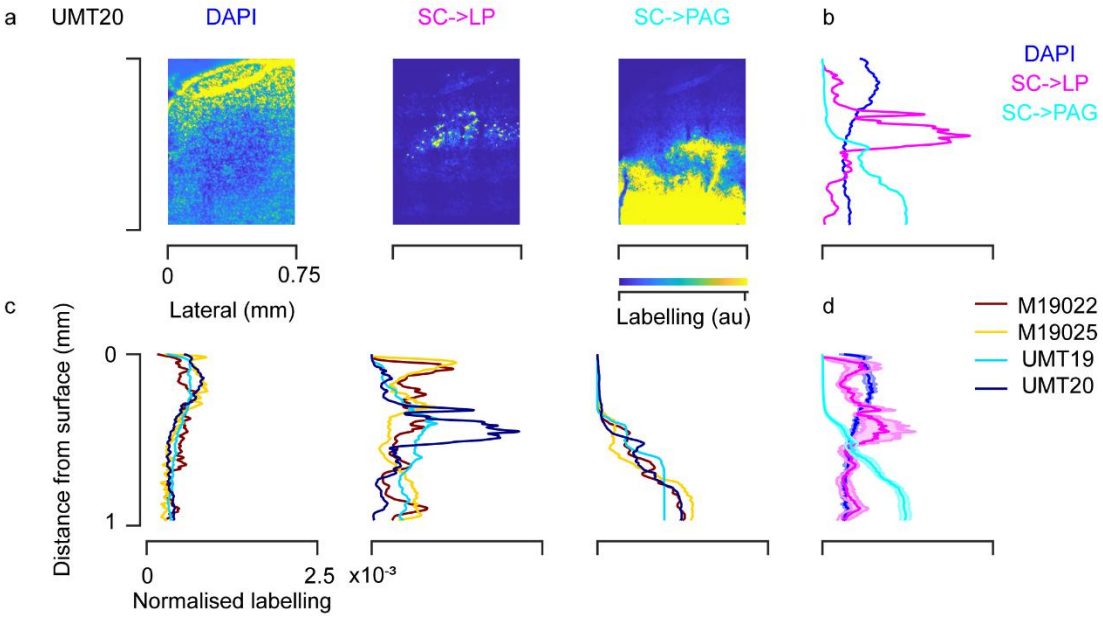

Fig 7. LP and PAG-projectors are intermingled yet separate populations in the SC. **a**. Retrograde tracing from LP and PAG in an example animal. Images show fluorescence in the DAPI channel, from LP-projectors and from PAG-projectors respectively. **b**. Dorsoventral distribution of labelling in each channel in an example animal. **c**. Dorsoventral distribution of labelling across animals. Panels show fluorescence in the DAPI channel, from LP-projectors and from PAG-projectors respectively. **d**. Dorsoventral distribution of labelling in each channel, averaged across animals.

3. Physiology: SC to LP and SC to PAG pathways share visual tuning but have different dynamics

Introduction

When presented with certain visual stimuli, mice choose between freezing and escape (De Franceschi *et al.*, 2016). De Franceschi *et al.*, (2016) found that slowly translating dots, called sweeping stimuli, tend to evoke freezing, whereas expanding dots, called looming stimuli, tend to evoke escape. Similarly, faster translating dots tend to evoke a brief freeze and then an escape. The neural pathways that allow mice to respond appropriately when they are presented with these freeze and escape-inducing visual stimuli are not understood.

Some suggestions as to the neural pathways underlying visually-guided freeze and escape behaviour are present in the literature. A synapse away from the retina, in the superior colliculus (SC), there are responses to both sweeping and looming stimuli, among other visual stimuli. Downstream of the SC, artificial activation of SC terminals in the lateral posterior nucleus of the thalamus (LP) tends to evoke freezing. In contrast, artificial activation of SC terminals in the dorsal parts of the periaqueductal gray (PAG) tends to evoke escape. The anatomical experiments described in the previous chapter suggest that the cells in the SC that contribute to each output pathway form separate yet intermingled populations. An explanation for the behavioural observations is therefore that visual stimuli may be able to evoke freezing through a retina to SC to LP pathway, and that visual stimuli may be able to evoke escape through a retina to SC to PAG pathway.

The hypothetical association of the LP and PAG output pathways from the SC with freezing and escape respectively raises lots of questions about their functional properties. First, there is a question of whether these pathways respond to potentially threatening visual stimuli, and whether they prefer them

to less behaviourally-relevant stimuli. Second, there is a question of how selective each pathway is. At one extreme, each pathway might respond equally well to both the types of stimuli that tend to induce freezing, and the types of stimuli that tend to induce escape. At this end of the spectrum, each pathway is relatively unselective. At the other end of the spectrum, each pathway might have a strong preference for freeze-inducing over escape-inducing stimuli, or vice versa. Beyond visual tuning and response strength, there is also a question of what the dynamics in each pathway look like, and how those dynamics might influence behaviour. Finally, a generalisation of the idea of freeze and escape pathways is the idea that different pathways may suppress or promote movement. As such, there is a question of whether the two pathways respond differently around the onset of movement bouts.

We assessed the physiology of the SC outputs to the LP and PAG using virus-mediated calcium imaging. We assessed the visual tuning of response strength in each pathway and observed that the two pathways show similar tuning. However, the LP-projectors in the SC showed more sustained responses. Our results inform theories of how mice decide how to respond when presented with visual threats.

Methods

All experiments were performed in accordance with the Animals (Scientific Procedures) Act 1986 (United Kingdom) and Home Office (United Kingdom) approved project and personal licenses. The experiments were approved by the University College London Animal Welfare Ethical Review Board under Project License 70/8637. 16 male C57BL6/J mice (M19054-5, M19126-31, M20053-60) were used, aged 8-10 weeks at the first surgery.

Surgery

1ul of 0.06mM CTB-647 Hydrazide (henceforth CTB) was added to 5ul of AAVrg-Ef1a-mCherry-IRES-Cre (henceforth referred to as ‘retro-cre virus’) (>7 x 10^{12} vg/ml) to give a final CTB concentration of 10uM and a final retro-cre virus concentration of 5.8^12 vg/ml (the mix of retro-cre virus and CTB is

henceforth called retro-cre-CTB). AAV9-pGP-Syn-FLEX-GCaMP7s (>1 x 10^{13}vg/ml) (henceforth referred to as 'GCaMP virus') was also used.

Mice were anaesthetised with 3% isoflurane, head-fixed, and had their scalps shaved and then cleaned with betadine and ethanol prior to incision. Following incision, the skull was treated with hydrogen peroxide and activator and then scored with a 0.6mm drill bit to provide a better surface for subsequent adhesion. The coordinates of Bregma and lambda were measured with a 23G needle and the head-fixing was adjusted until the measured DV offset between the two was less than 0.05mm. Then, the needle was used to mark points on the skull where the 2-4 craniotomies were to be centred.

In SC->LP mice (M19126-31), two marks were made over the right LP at 2.46 posterior and 1.5mm lateral of Bregma (as in retrograde tracing experiments) and 2.54mm posterior and 1.585mm lateral of Bregma respectively. In SC->PAG mice (M20053-60), a mark was made over the PAG at 3.64mm posterior, and 0.17mm lateral of Bregma. In all mice, one mark was made over the right SC at 3.64 posterior and 0.91mm lateral to Bregma, and one was made at an appropriate point in the left hemisphere for the insertion of a skull screw. A 1mm diameter circle was marked centred on the right SC mark using a 1mm biopsy punch, and the area inside of this circle was drilled using a 0.6mm drill bit. The craniotomy was cleaned using cotton buds and surgi-tips. Craniotomies were also made at the marks for the skull screw and, if necessary, the LP/PAG injection sites using the same drill bit. ~1ul of retro-cre-CTB was taken up into a micropipette.

In SC->LP mice, the micropipette was then moved to 2.46 posterior, 1.5mm lateral and 2.53mm ventral of Bregma (as in retrograde tracing), before 260nl was injected at 50nl/min. Then the same micropipette was used to make the same injection at 2.54mm posterior, 1.585mm lateral and 2.65mm ventral of Bregma. In SC->PAG mice, only one retro-cre-CTB injection was made, 350nl at 30nl/min at 3.61 posterior, 0.17mm lateral and 2.476mm ventral of Bregma. We waited 10 minutes after each injection before the micropipette was removed from the brain at a rate of 0.1mm every 10s.

In all mice, ~1ul of the cre-dependent GCaMP virus was taken up into a micropipette. The micropipette was then moved to the centre of the craniotomy, and 520nl was injected at 50nl/min (c.f. Evans *et al.*, (2018) for same injection volume and same magnitude injection speed for GCaMP in the SC) 1.675mm ventral of the skull at bregma. 10 minutes after the injection had finished, the micropipette was removed, and a blunt, hollow 26G needle was attached, and the DV coordinate of the skull at the right-hand edge of the craniotomy was measured. Then, the needle was moved to the centre of the craniotomy, and inserted to 1.575mm ventral of the skull level at the right hand edge, and then left for 5 minutes (c.f. Da Silva *et al.*, (2018) for use of needle). Then, a 0.5mm diameter 6.1mm long lens was moved to be centred upon the mark made by the needle (or the centre of the craniotomy) and then lowered to 1.675mm. Both the lens, and the needle before it, were lowered until they entered the brain, and then lowered in 0.1mm steps every 10s (c.f. Jimenez *et al.*, (2018) for a similar lowering process). The lens was cemented in place, and then released from the lens holder. The severed tip of an Eppendorf cap was cemented over the lens for protection until the next surgery (as in Vander Weele *et al.*, (2018)). A headplate was implanted anterior of the cap. Mice received subcutaneous injections of 5% w/v carpofen in saline prior to the surgery and received 20ul of metacam in condensed milk for 3 days post-surgery.

2, 5, or 22 weeks after the first surgery SC, SC->LP and SC->PAG mice respectively received a second surgery. Mice were anaesthetised as in the first surgery and headfixed using their implanted headplate. The cap covering the implanted lens was removed using a drill and a UCLA miniscope (Ghosh et al., 2011) with a baseplate attached was positioned over the lens until the best field of view of GCaMP fluorescence was observed. The baseplate, now appropriately positioned, was cemented to the skull before the miniscope was detached and the mouse allowed to recover.

Experimental setup

Around the time of the second surgery, the mice were habituated to head-restraint while presented with a blank gray screen on the monitor (described

below). On the first day the restraint lasted 15 minutes, the next day 30 minutes, and the final day 45 minutes.

During recordings, mice were headfixed with a monitor to their left (Iiyama ProLite EE1890SD), the monitor rotated 45º in yaw so that its screen was normal with respect to the line of sight of the mouse's left eye. The screen was 19cm away from, and its centre 2.5cm above, the mouse. The screen was 30.7cm high and 37.6cm wide, so the middle of the screen spanned from 44º to -34º in elevation, and the screen spanned from 0 to -90º in azimuth. A square, which changed from black to white when a stimulus was being shown, was presented in the bottom left of the screen and monitored by a photodiode to record stimulus timings. Another screen was present on the right hand side, orthogonal to, but otherwise the same as the first. It was held at the mean luminance.

The monitors were controlled by BonVision (Lopes *et al.*, 2020), which also performed 'gamma calibration', and spatially warped the stimuli that were presented. Gamma calibration was performed by measuring the output of each of the red, green and blue 'guns' on the monitor with a photometer, at each of 12 linearly spaced intensity levels. These measurements were used to define a RGB look-up table. Spatial warping was used to compensate for the relatively small distance between the stimulus and the monitor, which has the effect that the visual angle subtended by a pixel depends on where it is on the monitor. Without warping, an object of a fixed pixel dimensions might subtend an appropriate visual angle at the centre of the screen, but a smaller visual angle at the edges of the screen. Warping allows objects to maintain their visual angle size regardless of their position on the screen. BonVision uses the dimensions and position of the monitor with respect to the mouse to calculate the warping necessary to preserve visual angle.

Mice stood on a wheel on which they could run, and the wheel speed was recorded using a rotary encoder. Pupils were imaged using an infrared camera (DMK 22BUC03, ImagingSource; 30 Hz) focused on the left eye through a zoom lens (Computar MLH-10X Macro Zoom Lens).

During sessions with miniscope recordings, mice were headfixed in front of a gray screen for 5 minutes before the miniscope was attached. We left approximately 2 minutes before the onset and after the offset of each stimulus set to record baseline activity.

Visual stimuli

Within a stimulus set, trials were presented in a pseudo-random fashion: all stimuli were presented in a random order once, and the same stimulus set then repeated several times. The exception was the dot speed stimulus set (Fig. 7), where the stimulus order was random instead. For the approach angle experiments, the order of stimuli within each stimulus set was also randomised. For the SC->PAG animals, in alternate sessions of the drifting grating speed, dot speed and sweep loom adaptation experiments the first block of trials was swapped with another block. The motivation for swapping stimulus orders across sessions was to mitigate any monotonic changes in the responses over the course of blocks.

Sparse Noise

In sessions with drifting grating orientation stimuli, mice were first presented with sparse noise. Sparse noise stimuli consisted of a 5^{o} wide black and white squares presented at a grid of positions on an otherwise gray screen. Square centre locations varied from -81 to -6^{o} in azimuth and -23.5 to 31.5^{o} in elevation. Each stimulus frame lasted 500ms and there was no interframe interval; three randomly chosen locations were chosen on each frame, and the colour (white, black) of each square was also assigned randomly. 1800 stimulus frames were presented in total.

In sessions with dot speed, sweep loom adaptation or approach angle stimuli, the sparse noise consisted only of black squares, and only 600 frames were shown. Receptive field position was then inferred using MATLAB analysis of this sparse noise data, and integrated with previous sparse noise results from the same animal (see below). The position of the receptive field centre was then used to position the subsequent stimuli in that session appropriately.

Drifting Grating Orientation

The grating pattern was a square wave of 0.0625 cycles/^{o}, drifting at 2 Hz, that occupied the full elevation of the screen, and extended from -3.5 to -83.5^{o} in azimuth. Responses were measured at each of the four cardinal directions (0, 90, 180 and 270 degrees).

Drifting Grating Speed

The grating pattern was a horizontal sinusoid wave drifting upwards. Each of the 25 combinations of 5 different spatial (0.016, 0.032, 0.066, 0.132, 0.26 cycles/^{o}), and temporal (0.5, 1, 2, 4, 8 Hz) frequencies was shown. Gratings occupied the full elevation of the screen, and extended from -3.5 to -83.5^{o} in azimuth.

Dot Speed

Moving dot stimuli were black 5^{o} diameter discs which started from below the bottom of the screen and then moved upwards beyond the top edge of the screen at various speeds, crossing a total path length of 81.325^{o} of the elevation axis. Seven different speeds were presented: 4, 8, 15, 31, 61, 122 and 246^{o}/s. The azimuth position of each disc was offset from the inferred receptive field centre by ± 5^{o} to ensure the receptive field was not missed, yielding 14 types of stimuli in total. Given that path lengths were constant, if an equal number of trials was assigned to each speed, then there would be large differences between the time different stimulus speeds were shown during the session. To mitigate this, the number of trials was scaled by a factor of 1.25 from the slowest to fastest speed, such that the number of trials per speed was 10, 12, 15, 19, 24, 30, and 38 respectively.

Sweep Loom Adaptation

Both looming and sweeping stimuli were centred on the receptive field. Loom stimuli were black discs whose diameter linearly increased from 2 to 50^{o} in 250ms, and then remained on screen for 100ms. Sweeping stimuli were 5^{o} diameter discs which moved nasally 21^{o} in 1s. Both sets of stimuli were half contrast. Each trial was composed of two blocks separated by a 1s pause: in

each block, one stimulus (e.g. sweep), was presented once per second, 15 times. On different trials, different blocks were combined in different orders, yielding four types of trials.

Approach Angle

Approach angle stimuli simulated the appearance of 1.4cm black spheres moving along line paths in the real world and were present for ~5.4s each. 14 different paths were simulated. These spheres would appear as a 5 degree disc when translating across the screen.

Paths #1-7 were 18cm long. Path #1 ran along the line beginning at the mouse, and running through the measured receptive field centre: e.g. -45^{o} azimuth and +10^{o} elevation. The sphere moved along path#1 closer to the mouse (-25.5 to -7.5cm), making it a looming stimulus, the diameter of which increased exponentially from approximately 3 to 11^{o}. Paths #2-7 were rotated at various angles in elevation relative to path #1.

Paths #2-3 were perpendicular to path#1. Paths #2-3 were approximately 16.5cm away from the mouse (paths were chords, so distance varied slightly over the path) and thus a sphere moving along them subtended an angle of ~6.8^{o} diameter, similar to 'sweep' stimuli presented in other experiments. Spheres on paths #2-3 moved at ~10.6 ^{o}/s: in the range of speeds that tend to evoke freezing in mice (De Franceschi *et al.*, 2016).

Paths #2-3 involved approach angles of +90 and -90^{o} respectively, moving from above the receptive field to below it, and below the receptive field to above it, respectively. Spheres along the remaining four paths (paths #4-7), instead of moving either directly towards (0^{o} approach angle), or perpendicularly to (±90^{o} approach angles), the mouse, moved at intermediate angles: -60, -30, +30 and +60^{o}.

Three of the remaining 7 'paths' (paths #8-10) had the same starting position as paths #1-3, but the spheres did not move from their starting positions. Spheres on these paths were thus static presentations of the starting position of these paths and had the same duration as other stimuli.

Path #11 was the same as path #1 except that it was 30cm rather than 18cm long, extending from 31.5cm to 1.5cm away from the mouse. Finishing at 1.5cm from the mouse allowed the sphere to reach ~50º in diameter, similar to the stimuli which evoke escape in freely moving mice (De Franceschi *et al.*, 2016). Since path #11 was traversed in the same duration as all other paths, spheres moving along path#11 simulated an object looming at a faster speed, producing a 'fast loom' stimulus.

The final three paths (paths#12-14) were point-like, beginning and ending at the end of sphere#11's path, yielding static stimuli. The opacity of spheres along paths #12-14 varied dynamically to mimic the brightness changes evoked by the movement of spheres #1, 2 and 11 respectively.

Stim. Set	# Stim.	s/Stim.	ISI (s)	Trials/Stim.	Set Time (min)	Blockwise	#Sess.
SN	1800	0.5	0	1	15	N	3
DG	4	2	6	30	16	Y	3
DGS	25	4	6	10	41.67	Y	5
SN	600	0.5	0	1	5	N	5
DS	14	Various	6	>10	~47	N	5
SN	600	0.5	0	1	5	N	5
SLA	4	31	30	10	40.67	Y	5
SN	600	0.5	0	1	5	N	5
AA	14	5.3955	6	20	53.18	Y	5

Table 1: Parameters of different stimulus sets. SN, DG, DGS, DS, SLA, and AA refer to sparse noise, drifting grating orientation, drifting grating speed, dot speed, sweep loom adaptation, and approach angle stimulus sets respectively. Alternating gray and white row pairs segregate different session types.

Data Analysis

Signals were collected from the camera monitoring the eye, the wheel, a photodiode monitoring the screen and the miniscope and synchronised into a '*csv'* output using a single instantiation of Bonsai (preview version 2.4). The miniscope signal was the summed intensity of pixels on each recorded frame. The running speed and pupil size were recorded. A separate Bonsai program presenting the visual stimuli also outputted a *csv* detailing the relevant timing of the stimuli that were presented.

Offline analyses were performed in MATLAB (Mathworks, NA, Release 2020b). The onset time of each stimulus was inferred from the photodiode signal, and used to split the recorded signals into peri-onset time windows (trials) (Fig. 1). For movement bout-triggered responses (Fig. 10-11), timepoints were extracted where the mouse's movement speed went over 1.5cm/s (similar to the 1cm/s used by Savier et al., 2019), excluding occasions where the speed had not returned to 0cm/s following the previous bout.

On each trial, the average activity in the 200ms prior to stimulus onset was subtracted from the rest of the trace, then the activity at each timepoint was divided by the average activity in the 200ms prior to stimulus onset, yielding a df/f signal (Fig 1). To average across sessions, the responses in each session were then normalised by their average, yielding a normalised df/f signal (this normalisation was not used in the analysis of drifting grating orientation, sparse noise, or movement bouts).

In the dot speed and approach angle experiments, response timecourses were registered to the time of peak response. To do this, within a given session, for each stimulus separately (e.g. for each of the dot speeds), the peak of the average response timecourse to the stimulus was calculated, and all trials with that stimulus had their timecourses shifted so that the calculated peak time now occurred at time zero 'relative to peak'.

For sparse noise analyses, response maps were computed as the average response in the 500ms following the stimulus onset, upsampled 10x using bilinear interpolation, and then Gaussian smoothed using a sigma of 0.09º.

Maps were computed for each session, and then averaged across sessions within animal.

For analysis of stimulus-evoked changes in running speed, we extracted wheel speed in the same peristimulus time window as for the neural signal.

Histology

Following recordings, mice were anaesthetised with 3% isoflurane and injected with pentobarbital intraperitoneally. Mice were transcardially perfused with 0.9% NaCl in 0.1M phosphate buffer (PB), followed by 4% paraformaldehyde (PFA). The brain was extracted and placed in 4% PFA overnight at 4^{o}C, and subsequently cryoprotected with 30% sucrose solution. A coronal section was made to cut away the cerebellum, and brains were frozen in O.C.T. Compound (Sakura FineTek). Coronal sections of 40µm thickness were sliced on a cryostat (Leica, CM1850 UV), between the start of the hippocampus and the end of the superior colliculus (-1.82 and -4.96mm AP). Three out of every four slices were mounted using Vectorshield with DAPI (Vector Labs) and slices were imaged with a standard fluorescence microscope (Leica DMi8). 20x images of the SC were taken where there was the greatest evidence of lens damage. 10x images of the LP and PAG: for the PAG, the slice with the greatest degree of PAG retro-cre-mcherry labelling was chosen, whereas, for the LP, the slice with the greatest degree of LP GCaMP+ axons was chosen. As in the Anatomy chapter, the AP position of the SC and PAG images was inferred using the distance of that slice from the boundary between the superior and inferior colliculi. Likewise, the AP position of the LP image was inferred using the distance of that slice from the anterior emergence of the dorsal 3rd ventricle.

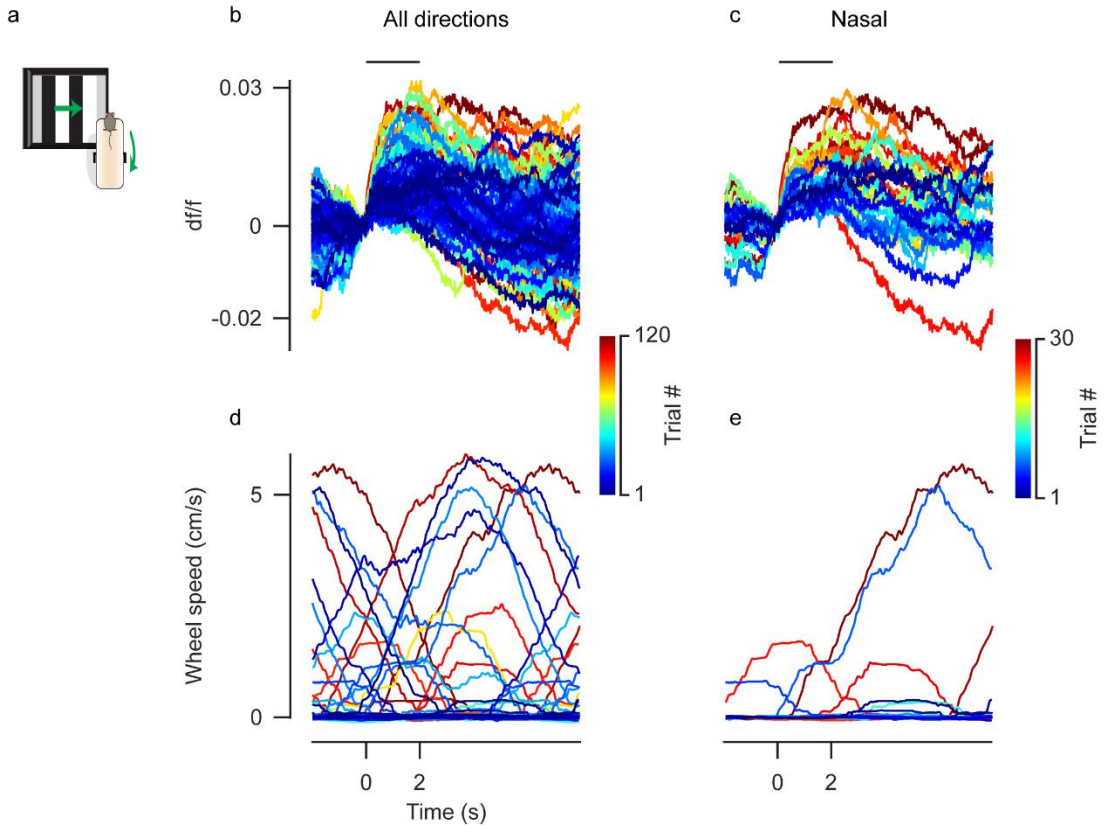

Fig. 1: Example session data from an SC->LP mouse. **a**. Schematic illustration of mouse capable of running on a wheel while viewing a drifting grating stimulus. **b**. Peristimulus response timecourses, across all stimulus directions, of the miniscope (top) and wheel signals (bottom) from an example session. Colour indicates the trial number. **c**. Same as (b), but for the subset of trials with nasally-directed motion. Data comes from the first session of the drifting grating orientation stimulus set from M19129.

Results

To measure the functional properties of SC output pathways, we used virus-mediated calcium imaging. The logic of the experiments is outlined in Fig 2. Fig 2a-c recapitulate the main observations from the previous chapter. Discriminable retrograde tracers were unilaterally injected into the LP and PAG (Fig. 2a-b). Both tracers labelled cells in the ipsilateral SC, but the cells retrogradely labelled from the LP had a different distribution to those retrogradely labelled from the PAG (Fig. 2c). LP projectors were found in the superficial SC, while PAG projectors were found in deeper layers. Labelling from both targets was also observed at the interface between the superficial and deep SC.

To record from LP-projecting SC neurons, we unilaterally injected one virus (retro-cre) into the LP, and injected another virus into the ipsilateral SC (forming 'SC->LP' mice) (Fig. 2e). The 'retro-cre' virus is retrogradely transported from the terminals in LP to the cell body in SC, where it produces Cre. The virus injected into SC allowed for the expression of a calcium indicator (GCaMP7s) in those neurons expressing Cre. In SC->PAG mice, surgeries were the same except that the retro-cre was injected into the PAG (Fig. 2f). GCaMP7s expression patterns in the SC mimicked the results of our retrograde tracing experiments. To provide a comparative data-set, in 'SC mice', we co-injected both the Cre virus and the Cre-dependent GCaMP7s virus near to the optic layer in SC, to express GCaMP7s across the population of neurons there, and found GCaMP7s expression in each SC layer (Fig. 2d). In SC->LP mice, we observed GCaMP7s axons in the LP, and in SC->PAG mice we observed GCaMP7s axons in the PAG (Fig. 3-4).

In each mouse, we recorded the population fluorescence emitted by indicator-expressing SC neurons by means of an implanted lens in awake, headfixed mice free to run on a wheel.

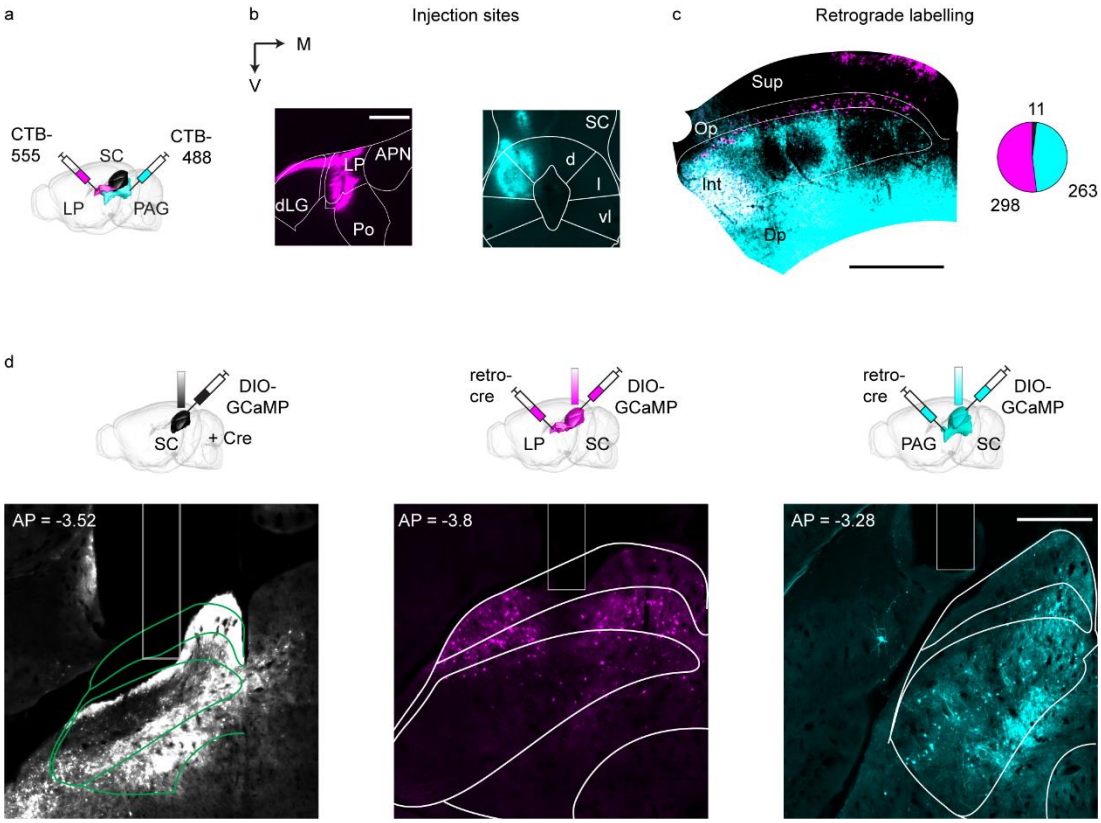

Fig. 2: SC->LP and SC->PAG form separate populations, allowing projection-specific population calcium imaging. **a**. Schematic of injection of discriminable retrograde tracers into the LP and PAG, showing also the upstream SC. **b**. Injection sites in LP and PAG. **c**. Retrograde labelling from the LP and PAG in the SC. Pie chart shows counted cells in the interface region from four mice, labelled by each tracer. **d**. GCaMP expression and lens location in mice in which the general SC, the LP-projecting SC and the PAG-projecting SC populations were recorded. Scale bars are 500μm.

d, l, and vl refer to the dosral, lateral and ventrolateral subregions of the PAG. dLG, APN and Po refer to the dorsal lateral geniculate nucleus, the anterior pretectal nucleus, and the posterior thalamus respectively. Op, Int and Dp refer to the optic, intermediate, and deep layers of the SC respectively: Sup refers collectively to the superficial gray and zonal layers.

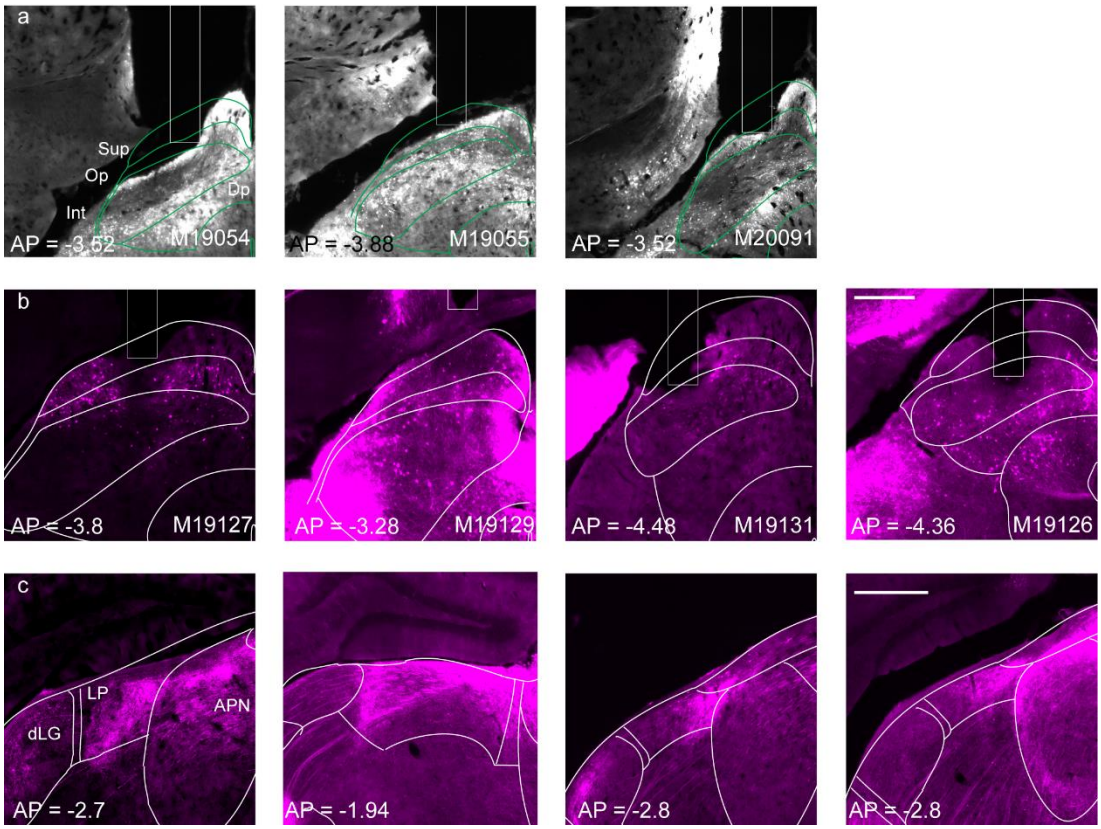

Fig. 3: GCaMP expression across the general SC population and in LP projecting SC neurons in different mice. **a**. GCaMP expression and lens location in mice from which the general SC population was recorded. **b**. GCaMP expression and lens location in the mice in which the LP-projecting SC population was recorded. **c**. Axonal labelling in the downstream LP in different mice. Scale bars are 500μm. Op, Int and Dp refer to the optic, intermediate and deep layers of the SC respectively: Sup refers collectively to the superficial gray and zonal layers. dLG and APN refer to the dorsal lateral geniculate nucleus and the anterior pretectal nucleus respectively.

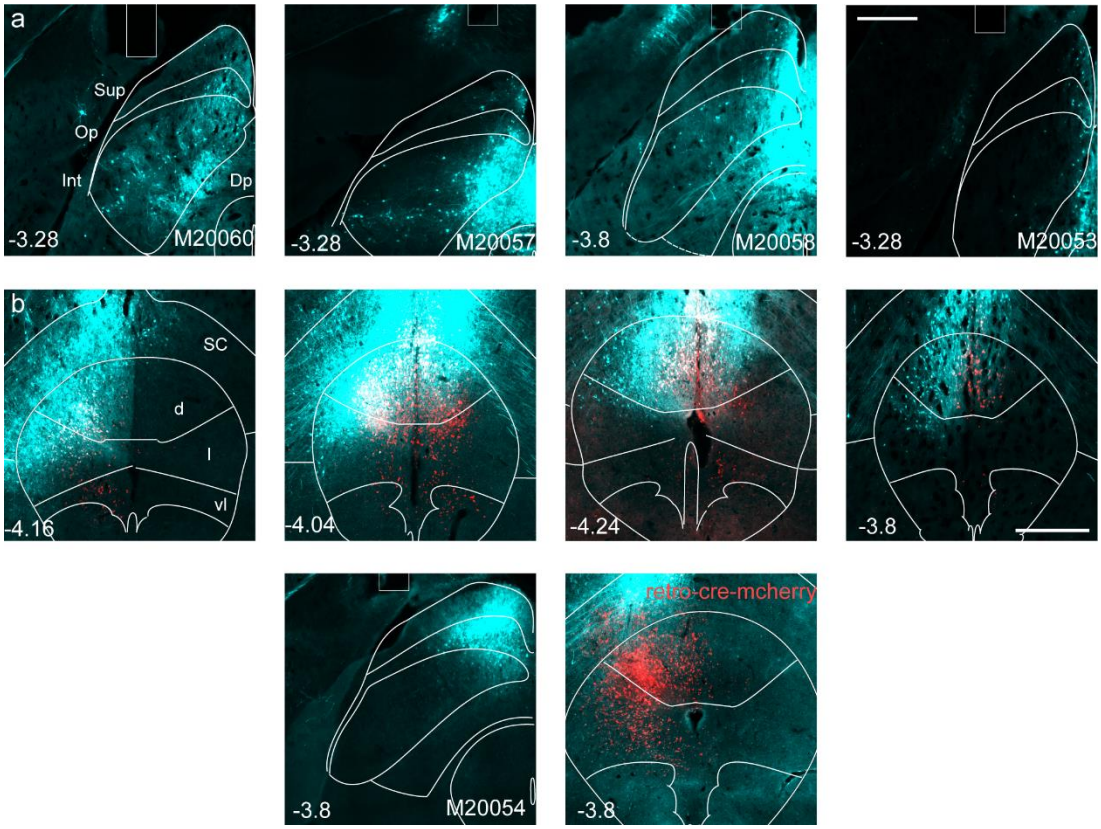

Fig. 4: GCaMP expression in PAG-projecting SC neurons in different mice. **a**. GCaMP expression and lens location in mice from which the PAG-projecting SC population was recorded. **b**. Axonal labelling and retro-cre-mcherry expression in the downstream PAG in different mice. Scale bars are 500μm. Op, Int and Dp refer to the optic, intermediate and deep layers of the SC respectively: Sup refers collectively to the superficial gray and zonal layers. d, l, and vl refer to the dorsal, lateral and ventrolateral subregions of the PAG.

Responses to sweeping and looming stimuli

To record responses to the types of stimuli that typically evoke freezing and escape, we presented sweeping and looming stimuli respectively, both centred on the measured receptive field for each animal (Fig. 5a). To extract population response timecourses we normalised responses within session, and then averaged across sessions and mice. In mice in which we expressed GCaMP7s across the general SC population, we observed bell-shaped responses to sweeping stimuli, as the stimulus passed into, and then out of, the receptive field (Fig 6a). Looming stimuli, by contrast, were present in the receptive field from their onset, and the result was an initial onset response, which then decayed. Subsequently, the response increased as the stimulus increased in size, and decayed after the stimulus offset.

We observed similar response profiles in both groups of projection-specific mice. In both SC->LP and SC->PAG mice, we observed responses to both sweeping and looming stimuli, suggesting that there is not a simple segregation of responses between the pathways (Fig 6a). We therefore asked whether there may be more subtle differences in the preferences of the SC->LP and SC->PAG pathways. Our sweeping stimulus simulated the appearance of an object moving along an axis tangential (orthogonal) relative to the mouse, and our looming stimulus simulated the appearance of an object moving directly towards the mouse. We therefore presented intermediate stimuli, between these two extremes, which simulated the appearance of objects moving along intermediate axes. Responses to these stimuli were also similar in all mice. Response amplitude varied little as a function of directness of approach, suggesting that SC->LP and SC->PAG are both broadly tuned for the direction at which an object moves relative to the animal (Fig. 6b). Response width increased when objects approached the mouse, probably because approaching stimuli are more likely to be in the receptive field throughout the stimulus presentation (Fig. 6c). However, different populations showed different response widths. SC->LP appeared to have broader responses, whereas the SC->PAG appeared to have narrower responses, though this was not significant ($p = 0.061$, unpaired t test). Our results

therefore suggest that SC->LP and SC->PAG share broad tuning for approach angle, but that SC->LP shows responses which are broader in time, or space, than SC->PAG.

We asked whether selectivity would be observed in either pathway if we used other examples of visual stimuli that evoke freezing and escape in freely-moving mice. De Francheschi et al. (2016) observed that whilst slower moving dots, such as the sweeping stimuli discussed so far, typically evoke freezing, faster-moving dots typically evoke a brief freeze then an escape. We therefore presented dots moving upwards at a variety of speeds through the measured receptive field centre (Fig. 7a). Average response timecourses showed bell-shaped responses, as the stimulus moved into, and then out of, the receptive field (Fig. 7b). Response amplitude declined at faster speeds (Fig 7d). Response width narrowed as the speed increased (Fig 7b), expected as faster stimuli spend less time in a receptive field. We quantified response width as the full width at half height (Fig. 7e). Responses appeared broader in the SC->LP than in the SC->PAG but this was not significant ($p = 0.123$, unpaired t test). Responses to moving objects are also influenced by spatial receptive field, so we plot the data in terms of space, as well as time (Fig. 7c). These suggest that the SC->LP responds over a larger area of the visual field than SC->PAG, suggesting that differences in spatial receptive field size between the pathways might influence responses to these small moving stimuli (Fig. 7f).

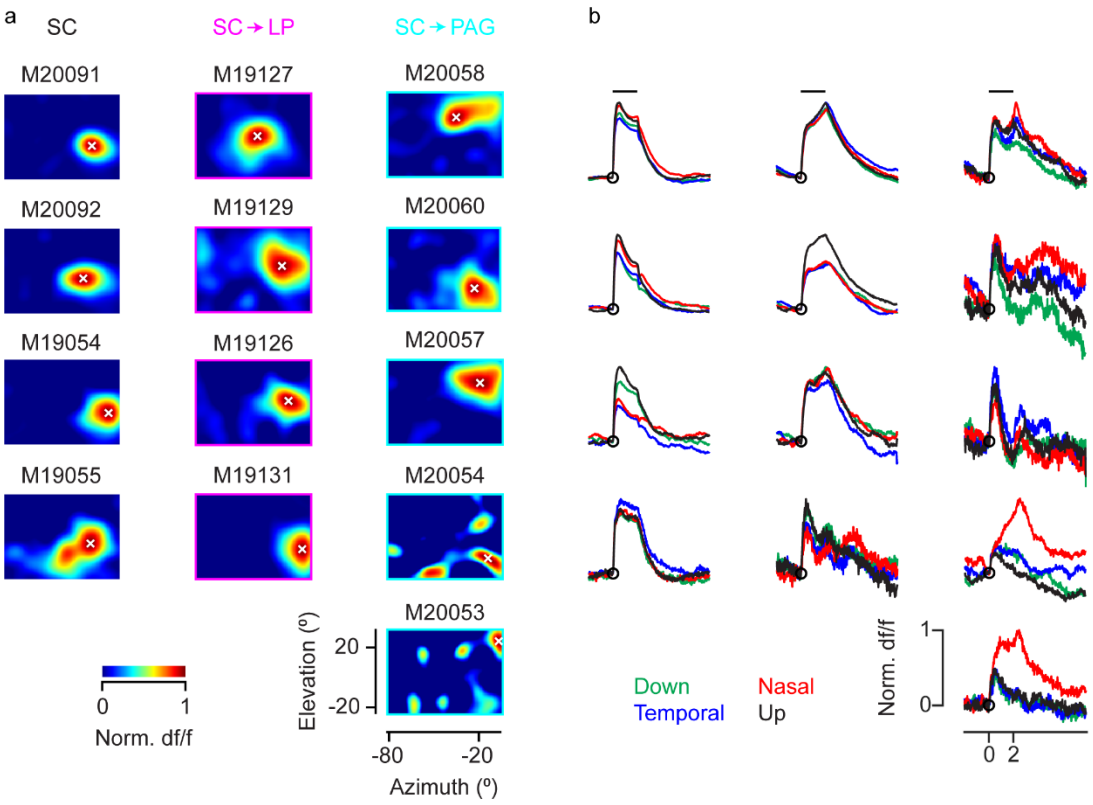

Fig 5: Most mice show population receptive fields, and population responses tended to lack direction selectivity **a**. Each animal's data is an average over 7-18 sessions. **b**. Each animal's data is an average over 3 sessions (except M19054-5, M19126 and M19131, which had 1, 4, and 6 sessions respectively).

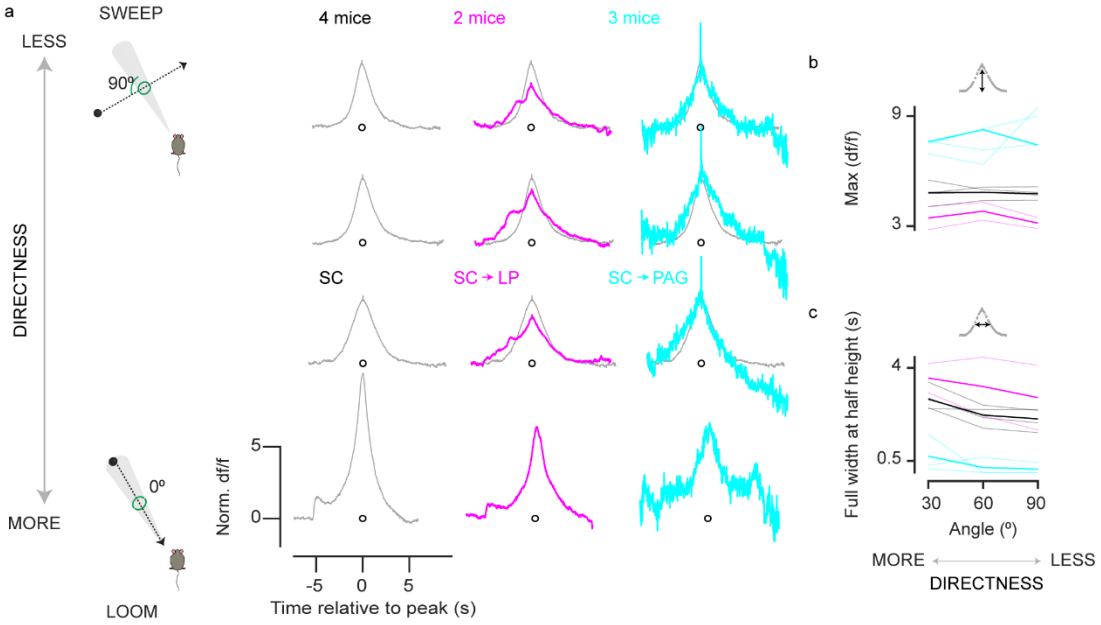

Fig. 6: SC->LP and SC->PAG share broad tuning for directness, but SC->LP shows broader responses in time. **a**. Group average response timecourses to stimuli with varying directness. The SC group had four mice, whereas the SC->LP and SC->PAG had two and three respectively: responses in each mouse were averaged over 4-5 sessions. Inset shows schematic illustration of stimuli with varying directness: stimuli simulating the appearance of black spheres moving more or less directly towards the animal. Object trajectory is indicated by the dotted black arrow, and the measured receptive field of the recorded population is indicated by the gray cone and green circle. Stimuli at the extremes of this dimension are similar to sweeping and looming stimuli. **b**. Variation in response peak height as a function of directness. **c**. Variation in response width as a function of directness. Thin lines indicate individual mice and thick lines indicate group averages.

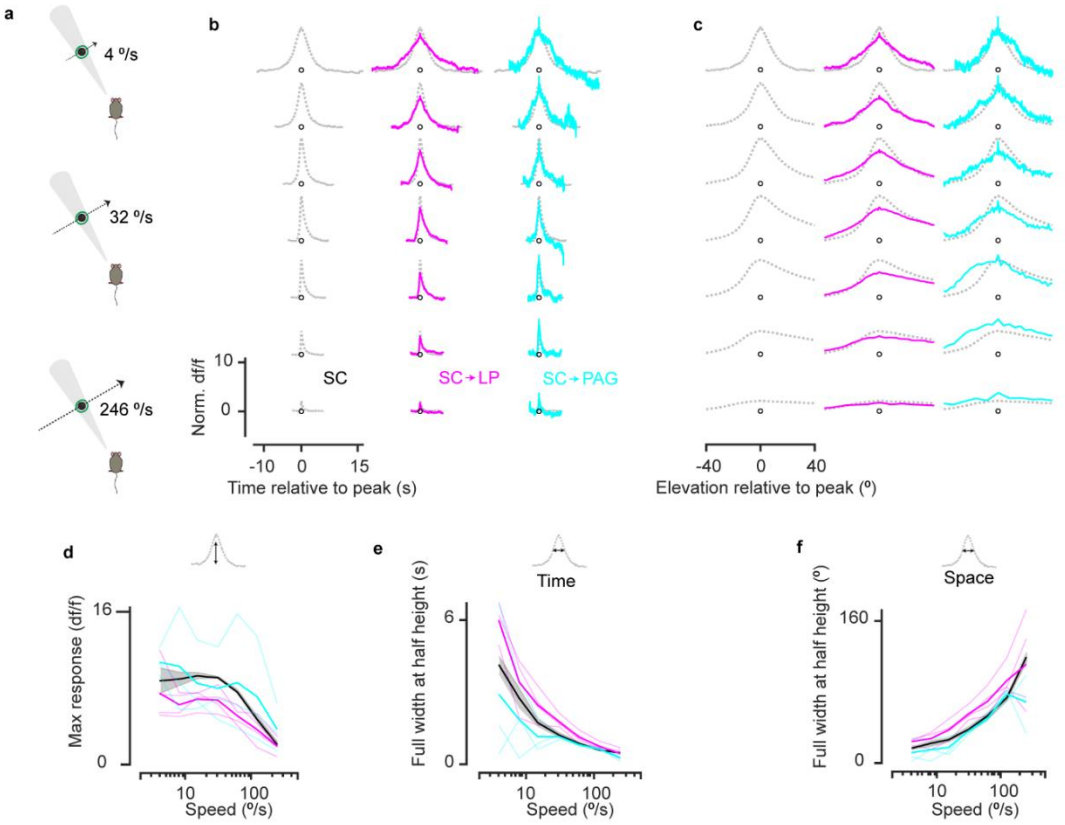

Fig. 7: SC->LP and SC->PAG share a preference for slower speeds. **a**, Schematic illustration of dots moving at various speeds, similar to Fig. 1. **b**. Group average response timecourses to dots moving at different speeds. Gray lines in the SC->LP and SC->PAG traces signal the response in the general SC population as a reference. The SC and SC->LP groups had four mice, whereas the SC->PAG had three: responses in each mouse were averaged over 5 sessions (except in one mouse which observed only 3 sessions). **c**. Group average responses as a function of space, rather than time. **d**. Response peak height as a function of stimulus speed. **e.** Response width in time as a function of stimulus speed. **f**. Response width in space as a function of stimulus speed. Faint lines indicate individual animals: solid lines indicate group averages. For the mice in which the general SC population was recorded, shaded region indicates the standard error of the mean.

Habituation of responses

Behavioural responses to sweeping and looming stimuli habituate (Wei et al., 2015; Shang et al., 2018; Tafreshiha et al., 2021), and the responses of SC neurons are also thought to be stronger for unexpected or novel stimuli, than familiar or repeated stimuli. We therefore asked whether responses to sweeping and looming stimuli might change over multiple stimulus repetitions (Fig. 8a). We presented trains of 15 stimuli, each 1s in duration, of each type. Average response timecourses for each group oscillated at the frequency of the presented stimuli (Fig. 8a). The oscillations are weaker for sweeping stimuli, which likely reflects the fact that the sweep stimulus will sequentially activate subunits of the global signal, while the looming stimulus is likely to activate them simultaneously . Response amplitude declined over the first half of the stimulus train. To characterise this, we took the maximum response in the first and last three seconds of the stimulus train and compared them (Fig 8b). For both sweeps and looms, responses were larger to stimuli at the start of the train than at the end ($p<0.001$ for both sweeps and looms respectively, paired t test) (Fig. 8b).

We therefore asked whether selectivity for sweeps or looms might emerge over multiple stimulus repetitions. We compared the responses to sweeps versus looms at the start of the stimulus train, and at the end of the stimulus train (Fig. 8c). At both the start and end of the stimulus train, responses were similar for both sweeps and looms ($p = 0.718$ and $p = 0.209$ at the start and end of the train respectively, paired t test). Our results suggest that responses in both the SC->LP and SC->PAG habituate, but that this habituation is similar for sweeping and looming stimuli.

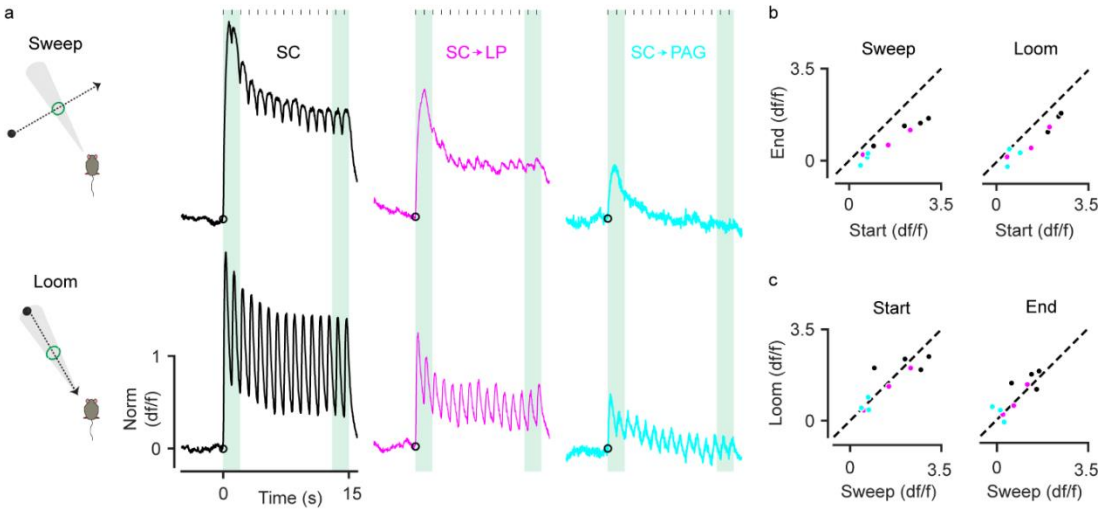

Fig. 8: SC->LP and SC->PAG responses habituate but retain a balance between sweeps and looms. **a**. Group average response timecourses to sweeping and looming stimuli. The SC group had four mice, whereas the SC->LP and SC->PAG had three: responses in each mouse were averaged over 5 sessions (except in one mouse which observed only 4 sessions). Inset shows schematic illustration of sweeping and looming stimuli, similar to Fig. 6. **b**. Responses to both sweeping and looming stimuli are larger at the start of the stimulus train than at the end, across mice. **c**. Responses at the start and end of the stimulus train retain a balance between sweeps and looms. Different points are different mice.

Independent habituation of sweep and loom responses

If the neurons responding to sweeps and looms are separate populations of neurons, then the population response to a loom, for example, should not be afected by a preceding train of sweeps. We therefore sequentially presented trains of either the same, or different stimuli (Fig. 9a). In the general SC population, the initial response to a train of sweeping stimuli was smaller if that train was preceded by a train of sweeps rather than a train of looms. Likewise, the initial response to a train of looming stimuli was smaller if it was preceded by a train of looms rather than a train of sweeps. To characterise this we compared responses to trains of stimuli preceded by a gray screen, a train of the same stimulus, or a train of different stimuli (Fig. 9b). If the train was preceded by gray screen, or by a train of the opposite stimulus, responses appeared similar. In contrast, if the train was preceded by a train of the same stimulus, responses early on in the second train were weaker. We extracted the peak response in the three seconds following train onset and compared the amplitudes (Fig. 9c). Responses were larger if the preceding stimulus was different to that which followed it, but this was only significant for looms (sweep: $p = 0.053$; loom: $p = 0.002$, paired t test). The independent habitation of sweep and loom responses suggests that these two stimuli are represented by independent sets of neurons, either in the SC, or in its afferent input. Similar independence was seen in the general SC population, and in both projection populations, suggesting that independent sweep and loom pathways are located before these projections diverge.

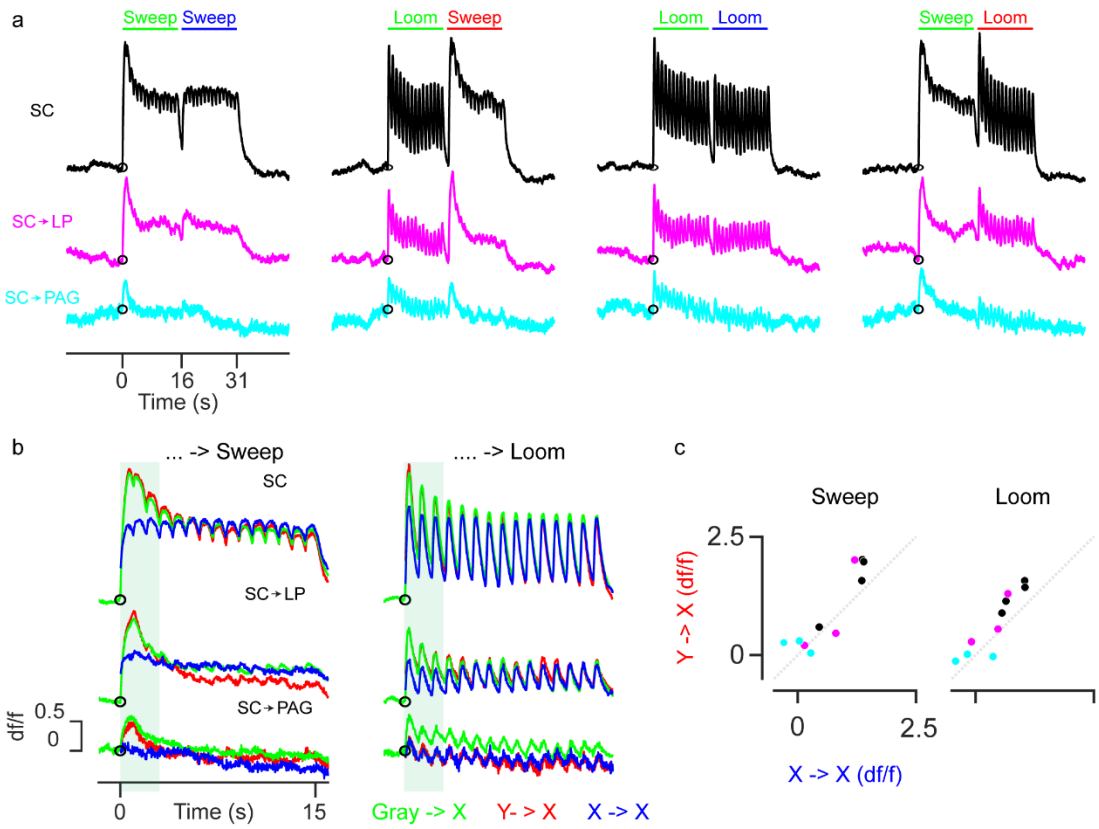

Fig 9. Evidence that independent pathways may mediate sweep and loom responses. **a**. Group average response timecourses to trains of sweeping and looming stimuli, preceded by trains of sweeping or looming stimuli. The SC group had four mice, whereas the SC->LP and SC->PAG had three: responses in each mouse were averaged over 5 sessions (except in one mouse which observed only 4 sessions). **b**. Comparison of response timecourses to trains preceded by gray screen, the same, or opposite stimulus **c**. Response at the start of the second stimulus train if the preceding train consisted of the same, or opposite stimulus. Different points are different mice.

Responses to drifting gratings

Most work in visual neuroscience has explored the response to simple patterns, such as drifting gratings. These stimuli have less obvious behavioural relevance than sweeping or looming stimuli, but they have the advantage of being able to vary visual features parametrically, such as contour orientation, direction and speed, while avoiding potential confounds between time- and space that arise for discrete stimuli, such as sweeping or looming dots.

In initial experiments we measured the tuning for orientation and direction of a drifting grating. Orientation- and direction selectivity are prominent in SC neurons (Wang et al., 2010) but our measurements of global calcium signal will only show tuning to these dimensions if there are large scale biases across the population under study. We found no obvious preference for motion direction or contour orientation in any of the populations (Fig. 5b). We therefore used the responses to drifting gratings of a particular orientation and direction (moving upwards) to assess response dynamics and tuning for spatial and temporal frequency, and visual speed (Fig. 10a-b).

In mice in which GCaMP7s was expressed in the general SC population, responses were step-like in shape, but also showed distinct transient and sustained components (Fig. 10c). We defined the transient response as the maximum response within 1s of stimulus onset, and the sustained response as that in the last 1s of the stimulus presentation (the stimulus was presented for 4s). In contrast, responses in the SC->PAG were more transient, and in the SC->LP more ramp-like and sustained.

The relative strength of the transient and sustained components varied in different parts of the stimulus space. To characterise this, we calculated the strength of the transient and sustained components, as well as the difference between them, across the spatiotemporal frequency space (Fig. 10d). The transient and sustained components were generally balanced across spatiotemporal frequencies in the general SC population (Fig. 10e). In SC->LP mice, however, the sustained response dominated over most spatiotemporal frequencies, and in SC->PAG mice the transient dominated (Fig 10d). Indeed,

averaged responses (Fig. 10e) showed that responses in SC->LP were more sustained than responses in SC->PAG ($p = 0.004$, unpaired t test).

Gratings with the same ratio of spatial to temporal frequency have the same speed (Fig. 10b), so we averaged across iso-speed stimuli to produce a speed tuning curve. For both the transient and sustained component, speed tuning curves suggested that the SC->LP and SC->PAG both prefer slower speeds (Fig. 10f-g). The speed tuning observed in drifting grating experiments are similar to those observed in the moving dot experiments, suggesting that the preference for lower speeds observed in both SC->LP and SC->PAG is not solely due to differences in time spent in receptive field.

Modulation of calcium activity by movement bouts

To assess whether movement response strengths differed between pathways, we investigated responses around the onset of movement bouts. Animals spontaneously exhibited bouts of movement throughout our experiments, both in the presence and absence of visual stimuli (Fig. 10a). In some mice we observed changes in the miniscope fluorescence signal around the onsets of these bouts. To extract bout-related activity changes, we defined bout onsets in baseline periods and used them to produce peri-bout neural response traces, and produce averages across bouts (Fig 10b).

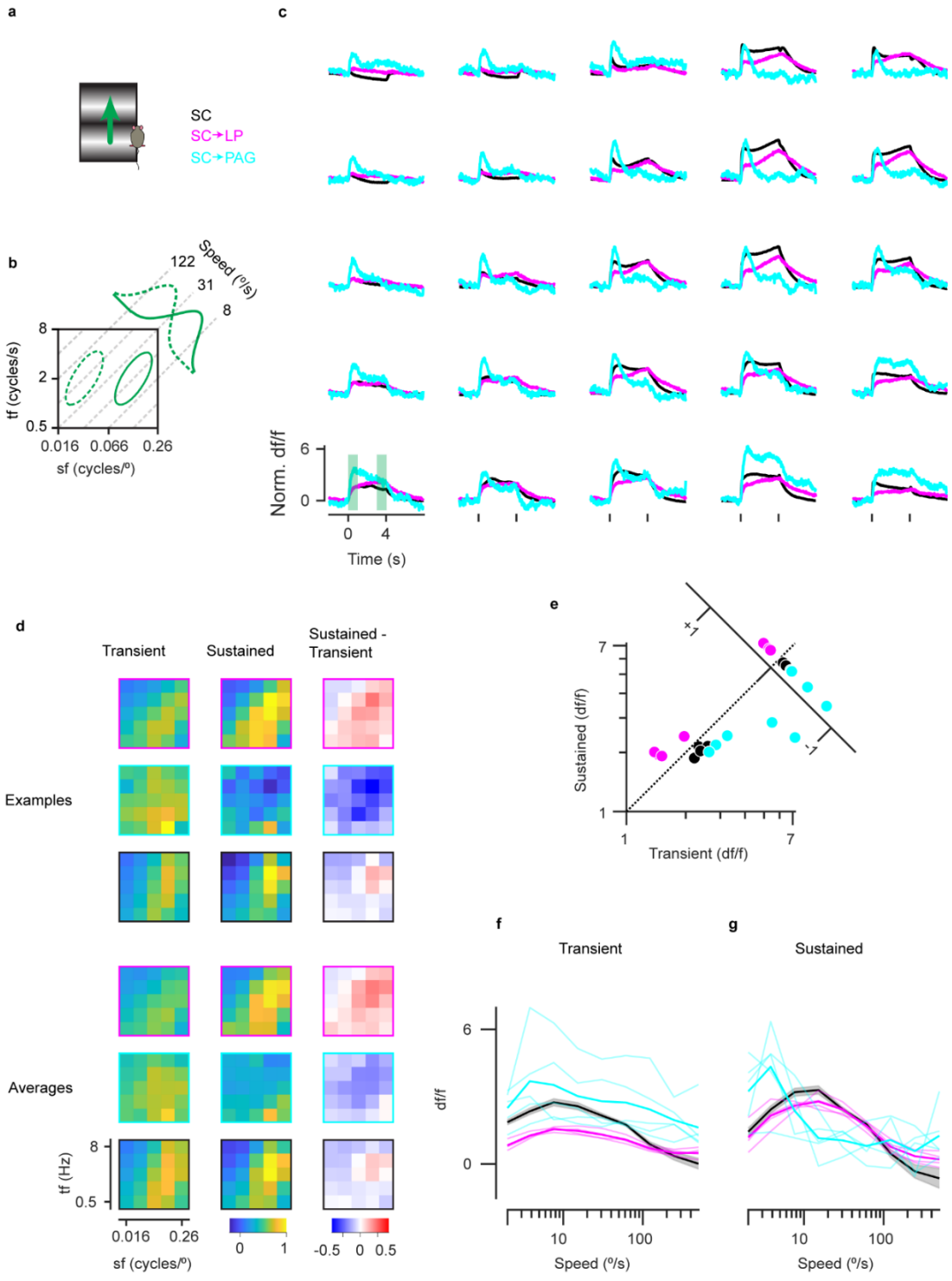

Fig. 10: SC->LP and SC->PAG share a preference for slower speeds, and SC->LP responses are more sustained. **a**. Schematic illustration of drifting grating stimuli. **b**. Schematic illustration of the spatiotemporal frequency stimulus space used. Green ovals indicate areas of the space in which two example populations might respond. Curves indicate the speed tuning that might result

from the example populations. **c**. Group average response timecourses across the stimulus space. Four mice were used for the SC and SC->LP groups and five mice were used for the SC->PAG: responses in each mouse were averaged over 5 sessions (except in two mice which observed only 4 sessions). Ticks on the x axis indicate the onset and offset of the stimulus. Green bars indicate the transient and sustained response windows. **d**. Peak responses in each window across the stimulus space, and the difference between the two. Top ratemaps indicate data from example animals. Bottom ratemaps indicate data averaged within group. **e**. Balance between transient and sustained in different mice. Different points represent different mice, and the transient and sustained strengths reflect averages across the transient and sustained heatmaps respectively seen in d. **f-g**. Speed tuning curves for the transient and sustained responses. Faint lines indicate individual animals: solid lines indicate group averages. For the mice in which the general SC population was recorded, shaded region indicates the standard error of the mean.

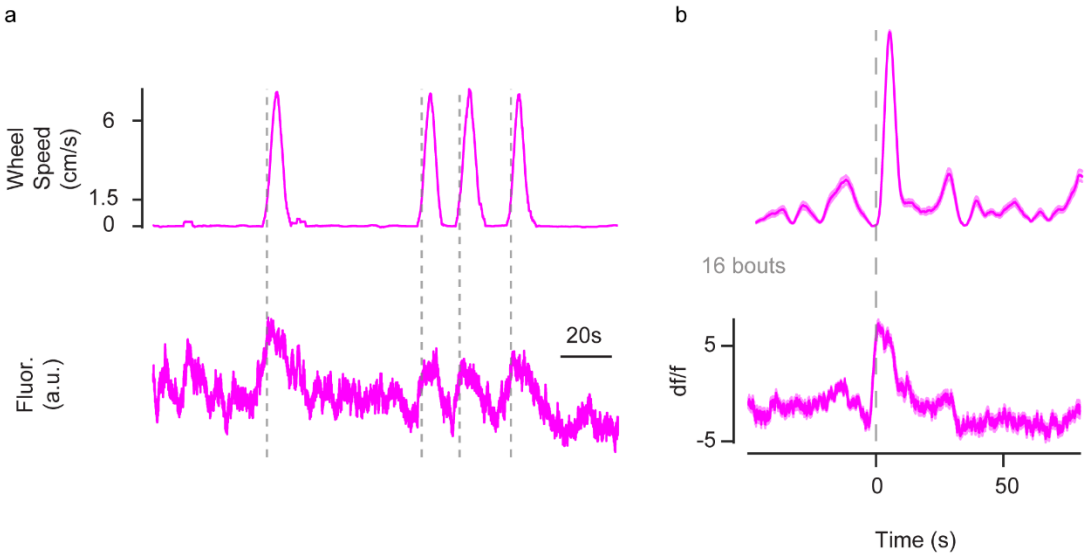

Fig. 11: Spontaneous movement bouts modulate calcium signals in the absence of visual stimuli. **a**. Data from an example session showing bouts of animal movement and concurrent changes in the fluorescence signal. Bouts were extracted using 1.5cm/s as a threshold. **b**. Extracting bouts across sessions within mouse allowed the production of peri-bout running and fluorescence signals. Data is from an SC->LP mouse: M19126.

To investigate whether there are pathway differences in movement response strengths, we compared the relative modulation of the calcium signal by visual stimuli, and by movement bouts (Fig. 6). Population responses did not show strong selectivity for motion direction (Fig. 5b), so we averaged responses across directions for each mouse to acquire a measure of visual response strength. In each mouse in which we recorded responses from the general SC population, we observed strong visual responses (Fig. 12a). In contrast, responses in the general SC population around the onset of movement bouts were relatively weak. Comparison of peak responses from the peri-stimulus and peri-bout timecourses show much stronger visual responses (Fig. 12c). Relatively stronger movement related activity was seen in both projection-specific populations ($p = 0.014$ SC->LP, $p < 0.001$ SC->PAG, unpaired t test), particularly in SC->PAG responses (although this was not significant: $p = 0.099$, unpaired t test). Our results suggest that the SC neurons projecting to the LP or PAG are particularly active around the onset of movement bouts.

To investigate whether movement bout responses might influence responses, we studied wheel speed changes around the onset of behaviourally-relevant visual stimuli. Average wheel responses in each animal to each trial type in the behaviourally-relevant stimulus sets did not show modulation of wheel speed by stimulus onsets (Fig. 13a-c). Therefore our results speak against the idea that stimulus-related changes in neural activity were influenced by stimulus-evoked changes in animal movement.

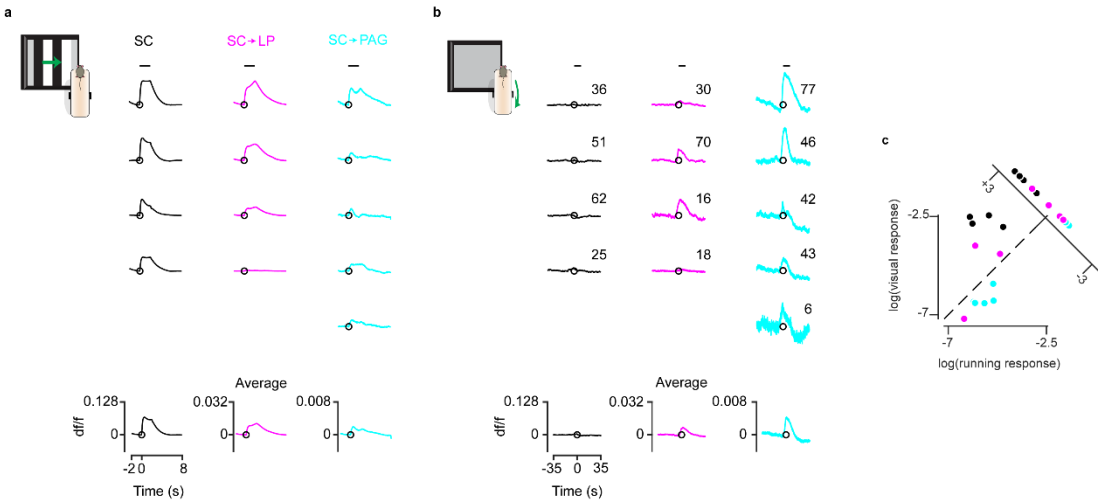

Fig. 12: SC->LP and SC->PAG are particularly active around the onset of movement bouts. **a**. Average response timecourses in each mouse to a drifting grating. Different traces show responses in different mice to the stimulus whose duration is indicated by the black lines. In each mouse, data was averaged over at least three sessions. Inset shows schematic illustration of the stimulus. **b**. Average response timecourses in each mouse around the onset of a movement bout. Different traces show responses in different mice aligned to the onset of bouts, the average duration of which is indicated by the black lines. Numbers alongside traces indicate the number of bouts being averaged over. **c**. Visual versus movement bout response peak heights for each mouse. Visual and movement response strengths were the peaks of the peri-onset timecourses shown in a and b.

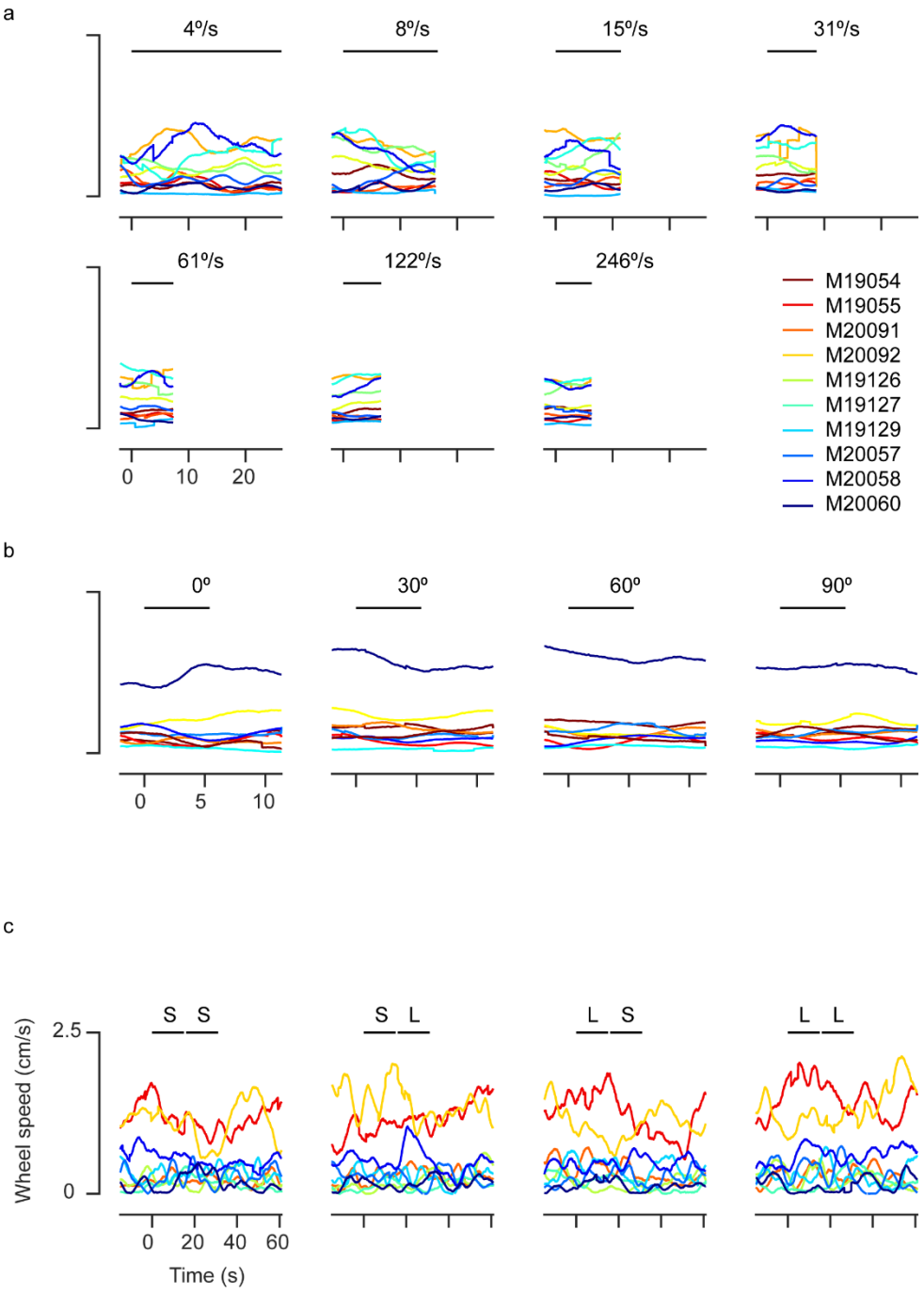

Fig. 13: Behaviourally-relevant visual stimuli did not elicit wheel movements in head-fixed mice. **a-c**. Average peri-stimulus wheel speed timecourses for each mouse across trials and sessions. Lines atop panels indicate duration of stimulus **a**. Responses to different speeds of moving dots. **b**. Responses to objects approaching at different angles. **c**. Responses to multiple repetitions of sweeping and looming stimuli.

Discussion

In this study, we used projection-specific calcium imaging techniques to study the functional properties of two output pathways from the SC: that to the LP and that to the PAG. The SC outputs to the LP and PAG both respond to threatening visual stimuli, and exhibit similar visual tuning. However, the output to the LP shows more sustained responses, and the output to the PAG is particularly active around movement bout onsets.

The SC->LP and SC->PAG both respond to threatening visual stimuli

Both the SC population innervating the LP and that innervating the PAG respond to threatening visual stimuli, and robust responses to sweeping and looming stimuli were observed in each pathway. In the literature, looming responses have been observed in SC neurons projecting to the LP, as well as their terminals (Gale and Murphy, 2016; Shang *et al.*, 2018; Bennett *et al.*, 2019). Neural responses to sweeping and looming stimuli habituated over the course of several seconds; similar habituation of responses has been seen for looming stimuli in the intermediate layers of the SC (Lee *et al.*, (2020). Future work could look for behavioural correlates of this fast neural habituation. Habituation of behavioural responses to looming stimuli has been observed, though generally over longer interstimulus intervals, or between sessions (Yilmaz and Meister, 2013; Wei *et al.*, 2015; Shang *et al.*, 2018; Tafreshiha *et al.*, 2021). We did not see clear signs of neural habituation persisting over several minutes (i.e. between presentations of the train of habituating stimuli) so the neural basis of this long term behavioural habituation might be downstream of the SC populations we measured from.

The SC->LP and SC->PAG show similar visual tuning

The SC projections to the LP and the PAG show similar visual tuning to a variety of stimuli. Both SC->LP and SC->PAG showed broad tuning for directness of approach and both preferred slower speeds. That the SC->LP and SC->PAG respond both to the types of stimuli that typically evoke freezing *and* the types of stimuli that typically evoke escape is not consistent with the

hypothetical association between those pathways and freezing and escape respectively. However, broad tuning in putative freeze and escape pathways might also be adaptive. For example, if the mouse encounters a stimulus which typically evokes escape, but does not or cannot escape, then coactivation of a freeze pathway might allow a 'backup' freezing response. Indeed, whilst artificial inhibition of the PAG impairs escape responses to looming stimuli, freezing responses occur in their stead (Evans *et al.*, 2018). Likewise, artificial inactivation of the LP shifts the balance of responses to a looming stimulus in the absence of a nest towards escape (Shang *et al.*, 2018). More generally, in the absence of a manipulation, that freeze-inducing stimuli sometimes induce escape and that escape-inducing stimuli sometimes induce freezing (especially in the absence of a nest) suggests that there is at least some overlap between the pathways (Wei *et al.*, 2015; De Franceschi *et al.*, 2016).

Independent habituation of sweep and loom responses suggests that these stimuli might be represented by different SC neurons or different SC inputs. Single cell recordings could assess whether individual SC neurons in the general population, or the LP and PAG pathways, show more of a preference for sweeping or looming stimuli than the population averages do (see Conclusion chapter). If single cells do exhibit more of a preference, future work could look at correlates of this, including anatomical correlates. For example, the SC innervates the dorsal PAG, where manipulations tend to affect escape behaviour (Deng et al., 2016; Evans et al., 2018), but also the lateral/ventrolateral PAG (Shang et al., 2019), where manipulations tend to affect freezing (Tovote et al., 2016). Alternatively, single cells may respond to both sweeping and looming stimuli, suggesting that the representation of these stimuli is more segregated upstream of the SC. For example, LP-projecting SC neurons are innervated by retinal ganglion cells which might be expected to prefer looming stimuli, and retinal ganglion cells which might be expected to prefer sweeping stimuli (Reinhard et al., 2019). Future work could investigate the segregation of sweep and loom responses in different SC neurons and their inputs.

Where the behavioural decision between freezing and escape becomes manifest in neural activity remains to be seen. That the two pathways respond to both freeze- and escape-inducing stimuli suggests that some stimulus selectivity is added within the LP and the PAG. Indeed, both the LP and the PAG host a convergence of inputs from many interconnected brain regions, and the LP has been found to show substantial responses to looming stimuli (Shang *et al.*, 2018). More work will be needed to find neurons which, for example, both prefer freeze-inducing stimuli and whose activity promotes freezing.

The SC->LP shows more sustained responses than SC->PAG

The SC->LP showed responses which were more sustained than the SC->PAG. Differences in the dynamics of response in the different SC output pathways may relate to differences in the durations of the SC's role in different behaviours. If the role of the SC is to represent the visual stimulus, the SC to LP pathway might be particularly important for behaviours which need to be guided by the visual stimulus over longer periods of time. Freezing, for example, might be a behaviour whose duration is tightly linked to the duration the stimulus is present for. The SC to LP pathway may also be important for representing the presence of a prey stimulus (Hoy et al., 2019). Sustained responses might also permit computations requiring longer durations. In contrast, the relative transiency of responses in the SC to PAG pathway might limit its ability to provide a sustained representation of the stimulus. Once an escape behaviour is triggered, it may become principally guided by the nest, rather than the stimulus that triggered it, making transient responses sufficient.

The SC->LP and SC->PAG are particularly active during movement

In the SC->LP and SC->PAG, responses around the onset of movement bouts were particularly large relative to visual responses compared with the general SC population. Responses in the SC->PAG appeared more biased towards movement than those in the SC->LP, but this was not significant. Movement bout onset responses in the SC->PAG may be consistent with the notion that the SC->PAG has a general role in promoting movement. However, that the

SC->LP shows detectable movement responses suggests that activity in this pathway is not merely inversely related to the probability of movement. .

That the SC->LP and SC->PAG pathways have stronger movement responses than the general SC population suggests that neighbouring SC populations have weaker movement responses. Although the collateral targets of LP and PAG-projectors remain to be seen, the SC has a large number of extrinsic (and intrinsic) targets beyond the LP and PAG (Benavidez et al., 2020), suggesting the presence of other SC projection populations. Indeed, recordings from the general SC population find that some neurons are more modulated by arousal than others (Savier et al., 2019; Schroder et al., 2020).

Inclusion of information about the mouse's own behaviour might aid analysis of the spatial relationship between the mouse and objects in its visual field (Parker et al., 2020). Sweeping and looming stimuli simulate the appearance of objects moving relative to the mouse. Visual motion caused by the mouse's own movement could be better discriminated from visual motion caused by the movement of the objects themselves through information about the mouse's own movement. During defensive behaviour, the mouse's task may be limited to detecting movement (sweeps) and detecting movement towards the mouse (loom). However, if the stimulus also guides escape trajectory, as it guides approach trajectories during hunting (Hoy et al., 2016; Hoy et al., 2019), the mouse's task may extend to calculating the displacement of a moving object. Given the evidence for the role of the PAG (Zhao et al., 2019) and the SC to LP pathway (Hoy et al., 2019) in hunting, future work can look into the specific effects of movement on the activity in these pathways, as well as looking for behavioural evidence of visually-guided escape trajectories.*Experimental caveats*

Some caveats must be kept in mind when interpreting results from these experiments. Firstly, population properties observed in global measurements of calcium activity might not reflect the properties of the individual single units. Moreover, given the neuropil (e.g. axonal) expression of GCaMP in our mice, different cells are likely to have different areal extents in the field of view, exacerbating bias in sampling. Properties may also differ between different

parts of a neuron, complicating matters further: our recordings are not solely of cell bodies, as is achieved in single cell calcium imaging.

Even if the global signals recorded here are representative of the average properties of the relevant single units, there is also a question of how representative the average is of the distribution. For example, larger population receptive fields in the SC to LP pathway might result from bigger, or less overlapping, single unit receptive fields. Population tuning curves might be broad and unimodal, but underlying single unit tuning curves may be narrower, with varied means. Single cell recordings can look at how homogenous properties are within neurons projecting to a common target (see Conclusion chapter).

Beyond the issues associated with global measurements, additional issues are introduced by our use of miniscopes. Photometric recordings tend to use dual fluorescent channels to discount ratiometrically observed changes in GCaMP fluorescence that are actually due to changes in background (Cui *et al.*, 2014). We did not have an additional fluorescence channel to use in this manner. Single cell recordings allow experimenters to perform spatial background subtraction, but since we could not reliably identify cells in our recordings, this option was also unavailable. Thus, there is a concern that our responses may partially reflect changes in background fluorescence, rather than changes in GCaMP fluorescence. This is a particular concern around the onset of movement bouts, and in the SC to PAG animals, where the lenses were further from GCaMP-expressing cells. Future work could use ratiometric photometry or single cell recordings (e.g. with better targeted lenses) to address these concerns.

That different populations were recorded in different mice is also not ideal. Future experiments could exploit the partially intermingled nature of the LP and PAG projection populations to record simultaneously each population using dual colour calcium imaging (Meng *et al.*, 2018).

Beyond the issues encountered with our recordings, further issues are arise through our use of retro-cre virus. We coinjected CTB to attempt to visualise

the injection sites of our retro-cre injections, but could not visualise the CTB in our histology, likely due to the lengthy duration of the experiments. Since we saw GCaMP axons in the targets, we can be confident that at least some of the recorded neurons in our SC to LP animals are, for example, LP-projectors. However, SC axons must travel to the LP through intermediate brain regions, so there are inevitably axons labelled outside of the LP, including its neighbours. Whether and where LP-projectors send collateral axons is also not known. Thus, it is difficult to rule out the possibility that some of the SC neurons we are recording project to parts of the injection site outside of the LP, rather than the LP itself. Future work could use coinjections of fluorogold or retrobeads to mark injection sites more successfully (Tervo *et al.*, 2016).

4. Behaviour: Internal state influences behavioural responses to common visual stimuli

Introduction

The hypothesis that the LP and PAG outputs of the SC are responsible for freezing and escape respectively makes predictions for behavioural experiments. Work has found that artificial activation of the LP or PAG, or SC terminals in these targets, can induce freezing and escape respectively (Wei *et al.*, 2015; Deng, Xiao and Wang, 2016; Zingg *et al.*, 2017; Evans *et al.*, 2018; Shang *et al.*, 2018). Recently, work has also found that the probability that looming stimuli can evoke freezing and escape is reduced by artifical inhibition of the LP (Shang *et al.*, 2018) and PAG (Evans *et al.*, 2018) respectively, or the SC terminals in these targets. Left outstanding from behavioural tests of the hypothesis is whether inhibiting the SC, LP, or the connection between them, impairs freezing to sweeping stimuli. Also outstanding is a study of the behavioural effects of inhibiting PAG-projecting SC neurons but also a study which employs inhibition of each pathway in the same experiment.

Attempts to test the behavioural predictions of the pathways hypothesis are made more challenging by the substantial behavioural variability that has recently been observed in responses to looming stimuli (Shang *et al.*, 2018; Lecca *et al.*, 2020). We define different 'internal states' as existing when there are different behavioural responses to common visual stimuli. Why there might be such considerable variability in responses is not known.

In this chapter, we attempted to test a hypothesis about the neural circuit basis of visually-guided freeze and escape behaviour. We observed a surprising mix of freezing and escape to looming stimuli, that was not explained by our attempted manipulation. Instead, different mice showed different responses to looming stimuli. We looked for behavioural correlates of this inter-animal

variability in loom responses and found some evidence that the mice that escaped more appeared more anxious.

Methods

TW mice

12 female C57Bl/6J mice aged 8-12 weeks underwent injection surgeries (Thomas Wheatcroft's or 'TW' mice). The mice came from 3 cages of 4 mice, and were generally housed post-surgery in cages of 2-3, although two mice were individually-housed.

Injections in general

All injections were of 260nl and were made at a rate of 50nl/min. A single pipette was used for all injections of a single area of the same animal, and 10 minutes was waited after each injection finished before the pipette was removed from a given site. All coordinates are relative to skull level at bregma.

SC Injections

In each mouse, 4 injections were made in the SC, 2 per hemisphere, with within-hemisphere injections separated in the AP axis. All SC injections were made ±0.55mm lateral of Bregma. The anterior injection site was targeted to 3.649mm posterior and 1.759mm ventral of Bregma (similar to the GCaMP7s injection site in Chapter 3). The posterior injection site was targeted to 4.250mm posterior and 1.504mm ventral of Bregma. Our previous experiments showed that retrograde labelling from the LP and PAG was found in the SC at both of these AP coordinates (see Chapter 2). SC injections were angled 45^{o} posteriorly in pitch to avoid the sinus and overlying neocortex. The coordinates, in the angled reference frame, of the anterior SC injections were therefore -5.35 posterior, and 2.405mm ventral of Bregma. Likewise, the coordinates, in the angled reference frame, of the posterior SC injection were -5.71 posterior, and 2.065mm ventral of Bregma. In 4 out of the 12 mice which received SC injections, 8 were injected with AAV5-hSyn-DIO-hM4D(Gi)-mCherry (henceforth hm4di-mcherry) and 4 were injected with a control virus AAV5-hSyn-DIO-mCherry (mcherry only).

LP and PAG Injections

Each of the 12 mice received AAVrg-Ef1a-mCherry-IRES-Cre (retro-cre-mcherry) injections in the LP or PAG at locations found to be innervated by the SC in Chapter 2.

In 6 of the 12 mice in which SC injections were made, 2 injections were made in the LP, one per hemisphere. Injections into the LP were not angled, and were targeted to 2.46 posterior, ± 1.5 lateral and 2.53mm ventral of Bregma.

In the other 6 mice in which SC injections were made, 2 injections were made in the PAG, one per hemisphere. The PAG injection was targeted to 4.555 posterior, ± 0.3mm lateral, and 2.273 ventral of Bregma. Injections into the PAG were angled 30^{o} posteriorly in pitch to avoid the sinus. In the angled reference frame, the coordinates of the anterior injection site was -5.855 posterior, ± 0.3 lateral and 2.625mm ventral of Bregma.

Behaviour

The first day of behavioural testing for each mouse occurred ~8 weeks (57-60days) post-surgery. Each day is called a session, and each mouse was tested in 6 sessions, with 3-4 days between consecutive sessions (intersession interval similar to Evans *et al.*, (2018)). At the start of the session, the mouse was placed in the environment for 15 minutes to acclimate (De Franceschi *et al.*, 2016). In all but the first 5 sessions of 3 mice, and the first session of 4 more mice, before the mouse was placed in the arena, 6 small drops of 10% condensed milk in tap water were placed in the centre to encourage the mouse to visit this region. Following the 15 minutes of acclimation, the mouse was removed and placed in a cage by itself in another room. It then received a 10ml/kg intraperitoneal injection (e.g. 0.21ml for a 21g mouse). In 3 of the 6 sessions, this injection was 0.9% saline. In the other 3 sessions, 1 mg/ml of (10mg/kg, c.f. Evans *et al.*, (2018) for the same dose) CNO 2HCl was injected instead. The order of saline and CNO sessions was counterbalanced across mice. In the 30 minutes following injection, the mouse was left alone in this room, singly-housed. 30 minutes post-injection, (c.f. Evans *et al.*, (2018) and Salay, Ishiko and Huberman, (2018) for the same lag)

the mouse was entered into the environment once more, and allowed 5 minutes to re-acclimate. After the 5 minutes of re-acclimation ended, when the mouse occupied the centre of the arena moving at a sufficiently slow speed, a stimulus was delivered. Following this stimulus, 3 more stimuli were, in turn, delivered, provided that the mouse met the aforementioned behavioural criteria, and that at least 2 minutes had passed since the presentation of the last stimulus. The mice and sessions mentioned above that did not involve baiting the arena during acclimatisation with condensed milk used a different order: sweep, sweep, loom, loom. Subsequently sessions used the following order of stimuli; sweep, loom, sweep, loom. Once the 4 stimuli had been delivered, two minutes were waited and the mouse was removed. If 4 stimuli had not been delivered 65mins after the injection, the mouse was removed. After removal from the environment, the mouse was housed alone in a cage in another room, along with the other mice being tested on the same day. The mice were held here until all the mice had been tested, whereafter they were returned to their homecages.

Stimuli were delivered manually via a keypress through custom scripts using MATLAB and PsychToolBox, whilst the mouse's position within the environment was recorded with Blackfly S Mono camera (Clear View Imaging, UK) and tracked using Bonsai at 60Hz. An Optoma GT760ST projector projected stimuli onto a rear projection screen above the mouse. Stimulus timing was recorded by delivery of an offscreen 'strip' stimulus, which was obscured from the animal yet visible to the camera.

The loom was a black disc that expanded from 2 to 50^{o} diameter in 250ms and then stayed onscreen for a further 500ms (De Franceschi *et al.*, 2016). The sweep was a 5^{o} diameter black disc that travelled for 3.9s at 21^{o}/s.

The inside of the box was 45.8 x 39.3cm and was made out of 4 infrared-transmitting plastic sheets which had been glued together. The bottom of the arena and the bottom of the walls was lined with white card (~7.7cm high) to aid tracking of the mouse, and help make the nest more visually obvious to the mouse. On one wall a 5 x 8cm hole was placed to give access to the nest, an infrared plastic box 20 x 12cm. The floor was 33cm from the screen, and the

screen extended beyond the region visible to the mouse. Infrared light was provided via a strip of IR LEDs surrounding the screen.

Making CNO 2HCl

CNO 2HCl was stored at -20ºC until made into solution. 5mg of CNO 2HCl was mixed with 5000ul of filtered PBS to make 1mg/ml solution. The solution was vortexed, and then split into 10, 0.5ml aliquots, which were labelled, sealed with parafilm, and stored in labelled falcon tubes at -20ºC. Aliquots were removed from the -20ºC and kept at room temperature for at least an hour before injection, and after storage at room temperature, were kept in the fridge for no longer than a day. Aliquots in the -20ºC were kept for no longer than a month.

Perfusions

Mice were perfused 12-13 weeks after surgery. Mice were anaesthetised with 3% isoflurane and injected with pentobarbital intraperitoneally, transcardially perfused with 0.9% NaCl in 0.1M phosphate buffer (PB), followed by 4% paraformaldehyde (PFA).

MFM data

12 1-3 month-old C57Bl/6J mice were used: 6 females and 6 males. These mice received 1 session a day for 5 days. Within a session, mice were placed in the environment for a period in which no stimuli were delivered, to allow them to acclimate (10 min on the first day, 5 min thereafter). The session ended after 4 stimuli were presented, or 20 minutes after the acclimation period finished: whichever happened first. Bonvision (Lopes *et al.*, 2020) was used to present stimuli in the order of sweep, loom, sweep, loom. The environment, projector and screen had the same properties as in the manipulation experiment, except the entirety of the walls was covered with white card. MFM data was collected in a different institute to that in the manipulation experiment, and handling of MFM mice was minimised through the use of transfer tubes, otherwise the procedure was the same.

Control experiment data

The effects of IP injections of saline and CNO in the absence of surgically delivered viruses was tested in 8 female C57Bl/6J mice, 8 weeks old at the start of experiments (see black and gray circles in Fig. 2b). Environment was the same as in MFM's data, except that visual stimuli were presented using an LCD screen. Experiments were the same as in the manipulation experiment, except that in some sessions, a lower dose of CNO (1mg/kg) was administered instead of that used in the manipulation experiment. Each animal received four (rather than six) sessions, including at least one saline, at least one low CN dose, and at least one standard CNO dose. Within each session, up to two stimuli were presented, rather than up to 4: sweep, then loom. Behavioural responses were manually defined from recorded videos.

Analysis

The image distortions in the behaviour videos were estimated by calculating the requisite polynomial transformation matrix from daily calibration images using the function cp2tform in MATLAB (De Franceschi *et al.*, 2016). The inverse of this matrix was used to transform positional estimates from image space to arena space, using the function tforminv. We used the xy coordinates (and thus the instantaneous speed) of the animal on each frame, and the time of each stimulus to compute more refined variables.

To look at stimulus responses, we isolated the XY coordinates and instantaneous speed of the animal on each frame in the 5s following the onset of a stimulus. Instantaneous speed in these windows was then smoothed with a moving average filter of width 83ms (De Franceschi *et al.*, 2016). Freezing was defined to occur if the speed reduced to ≤2cm/s for over 200ms (500ms was used by De Franceschi *et al.*, (2016)). Escape was defined if the animal's speed reached 40cm/s, and, thereafter, the nest was reached in less than 2s (1s was used by De Franceschi *et al.*, (2016)). Both freezing and escape could occur on an individual trial. We calculated escape probability and freeze probability for each stimulus (sweep or loom) for each animal. We also calculated an escape index for each stimulus and animal by calculating the

contrast between escape and freeze probability for each stimulus and animal: that is, the escape index for sweeps for a given animal = (probability of escape to sweeps – probability of freezing to sweeps) / (probability of escapes to sweeps + probability of freezing to sweeps).

We measured latency of freezing and escape responses as the time from the onset of the stimulus to the time freezing or escape events began. Peak escape speed was defined as the maximum speed achieved between the initiation of escape and the reaching of the nest. Latency and peak speed values were pooled across trials and sessions within an animal.

To characterise ongoing behaviour, we examined the first 5 minutes of each session, before any stimuli had been presented. We defined subregions of the environment using a series of rectangles (Fig. 5a). The environment was split into nest and arena by defining two abutting rectangles: a third rectangle (nest opening) covered part of the interface between them to cover the entry to the nest region. A fourth rectangle defined the centre of the arena. Additional rectangles define the corners and edges of the arena. The environment is therefore described by 9 parameters: the x and y limits of the nest, the minimum y values of the nest opening and the arena, the width of the nest opening and centre, and the y offset of the centre. Only the minimum y value of the arena was changed between datasets. We used these rectangles to define times in which the animal was in the nest vs the arena; in the centre of the arena vs its edge; and in the corridors vs the corners. Time in a region (e.g. the arena) was calculated by summing the amount of time spent in that region across sessions within animal, and then dividing this by the amount of time spent in the appropriate super-region (e.g. the arena or nest), summed across sessions within animal.

Spontaneous escapes were defined in the same manner as escapes in stimulus response windows. The average frequency of spontaneous escapes, the average arena trip duration and the average arena speed were calculated by averaging within each session, and then averaging across sessions within animal.

Statistics

All r and p values are given to 3 significant digits. Correlations and tests are based on Pearson's correlation coefficient.

Results

We studied the effects of neural activity manipulations in freely-moving mice (Fig. 1a-b). Overall, responses to sweeping and looming stimuli showed some similarities, but also some differences, with previous work. As in previous work, both sweeping and looming stimuli could evoke both freezing and escape responses (Fig. 1c) (De Franceschi *et al.*, 2016). Freezing and escape responses to sweeping stimuli were similar to previous work (89% freeze and 15% escape, versus 84 and 22% in De Franceschi *et al.*, (2016)). In response to looming stimuli however, we observed a surprising preference for freezing. On average, mice froze on 75%, and escaped on 32%, of loom trials (much smaller than the 88% escape rate in De Franceschi *et al.*, (2016)). Thus, whilst responses to sweeping stimuli were similar to previous work, mice were more likely to respond to looming stimuli by freezing.

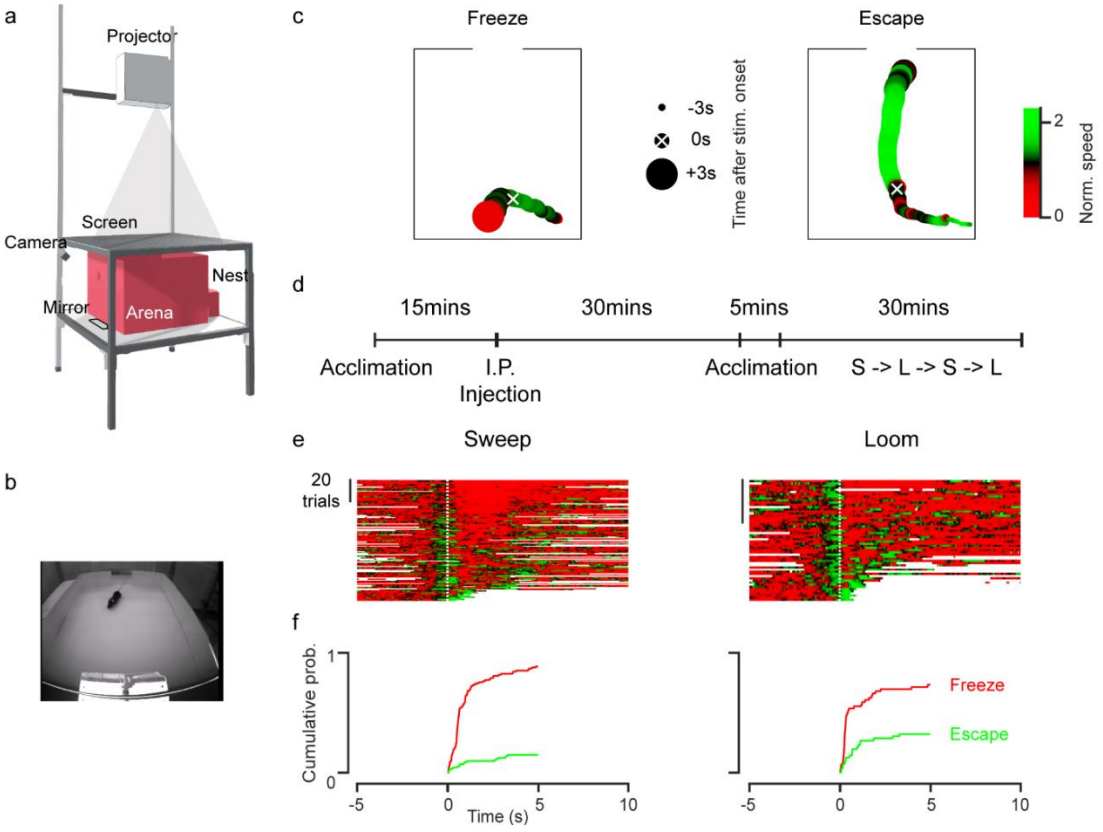

Fig. 1: Mixed behavioural responses to looming stimuli. **a**. Schematic illustration of the behavioural setup. **b**. View from the perspective of the camera. **c**. Example freeze and escape trajectories: spot colour indicates the instantaneous speed of the animal and spot size indicates the time of the frame relative to the stimulus onset (larger indicates later, the X marks the time of stimulus onset). **d**. Timeline of an individual session. IP injection occurred between acclimatisation periods, after which up to four stimuli were presented, usually in the order of sweep (S), loom (L), sweep, loom. **e**. Heatmaps showing the instantaneous speed of animals (N = 12) in a peristimulus time window (each row indicates one trial). Trials are sorted by the average speed between 0 and 5s. **f**. Cumulative response probabilities in the response window of 0-5s).

In these mice, we used an intersectional viral approach to perturb specific cell types (Fig. 2a). We used retrogradely-transported Cre virus to gate expression of an inhibitory DREADD (hM4Di) in the SC. By co-injecting a Retro-Cre virus into the LP and PAG we hoped to inhibit LP and PAG projecting SC neurons respectively. However, our attempted manipulation did not obviously effect behavioural responses. The behaviour of DREADD mice was no more different between CNO and saline sessions than was the behaviour of control mice (Fig 2b). For example, in terms of freezing responses to sweeping stimuli (first panel in Fig 2b), in terms of animals in which the SC to LP pathway was targeted (magenta circles), mice with putative DREADD-mcherry expression (filled circles) did not appear further from the unity line than animals with putative mcherry expression alone (hollow circles). Thus, if CNO effected behaviour, it did not affect the DREADD-mcherry-expressing mice more than the mice expressing mcherry alone. This phenomenon was observed too for the other groups (e.g. the mice in which the SC to PAG pathway was targeted), for escape responses, and responses to looming stimuli also. These results suggest that our attempted manipulation did not affect behavioural responses, or explain the surprising responses to looming stimuli.

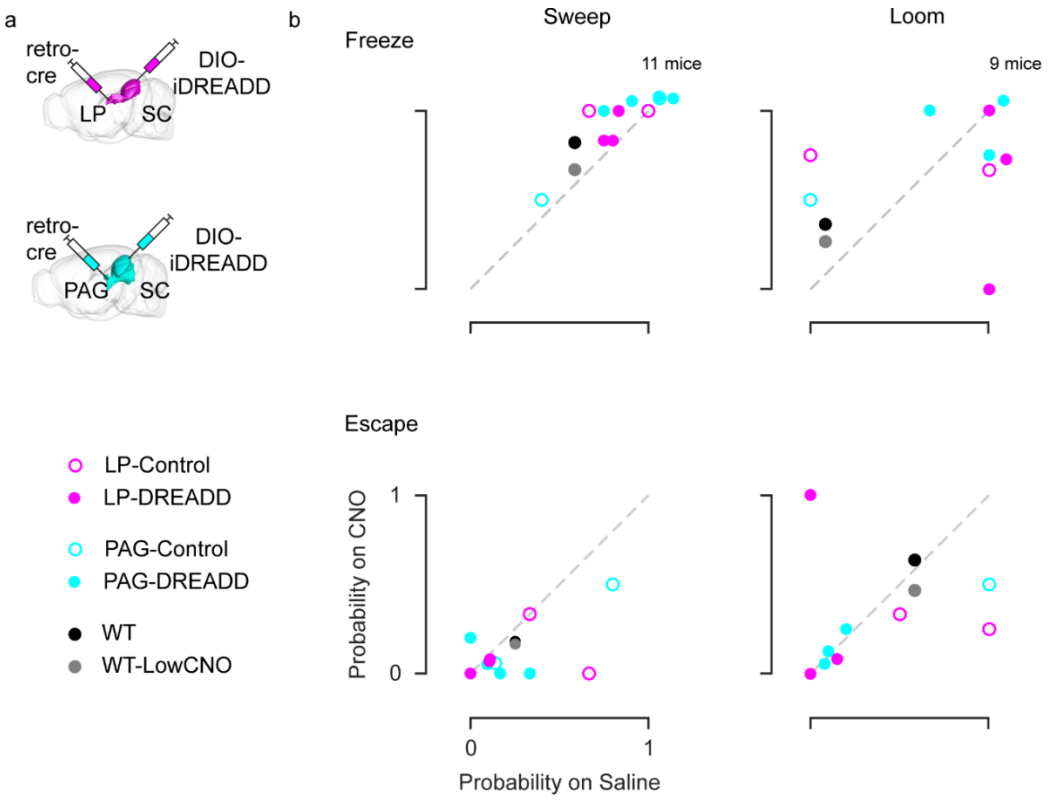

Fig. 2: Viral expression had no clear effect on behaviour. **a**. Schematic illustration of intersectional viral approach aiming to express inhibitory DREADD protein in LP and PAG-projecting SC neurons respectively. **b**. Plots of response probabilities on saline vs on CNO for each stimulus (sweep and loom) and response (freezing and escape) combination. Coloured data points indicate individual animals. Black and gray points indicate average data from a control experiment in mice which had not undergone surgeries (wild type mice, WT). For visibility, overlapping points were jittered by a distance less than the diameter of individual points.

Idiosyncratic responses to threatening stimuli

The data in Figure 2 suggest strong inter-animal variability in behaviour (freeze, escape) that is not simply due to the expression of virus or the injection of CNO. To further understand the contribution of inter-animal variability to the mixture of freeze and escape responses to looming stimuli observed at a population level, we included data from additional experiments performed by another PhD student (Monica Freitas Fernandes Martins, 'MFM'), using the same experimental apparatus but in a different animal unit, and with re-derived WT mice. The mice ('MFM Mice') did not undergo surgery or injection. Figure 3 shows the responses of these animals. At the population level, MFM mice showed predominantly freezing responses to sweeping stimuli (71% trials led to freezing and 42% led to escape) (Fig. 3a). Looming stimuli produced escape responses on a larger fraction of trials than in Fig 2, but less than that seen in De Franceschi *et al.*, (2016).

We used the combined dataset to assess potential contributions to inter-animal variability in behavioural responses. Each animal appeared to show different combinations of freeze and escape probabilities in response to looming stimuli (Fig 3b), and freezing and escape probabilities were negatively correlated. The negative correlation between freezing and escape probability meant that most of the variability in response occurred along an 'escape index' line (slope = -1). Positive escape indices indicate an animal escaped more and froze less, whereas negative escape indexes indicate an animal escaped less and froze more (Fig 3c). To establish the consistency of this metric we compared the escape index between halves of the experiment: i.e. the first three sessions versus the last 2-3 (Fig 3d). We observed that escape indices were consistent between the first and second halves of the experiment, suggesting that different animals show idiosyncratic responses to potential threats.

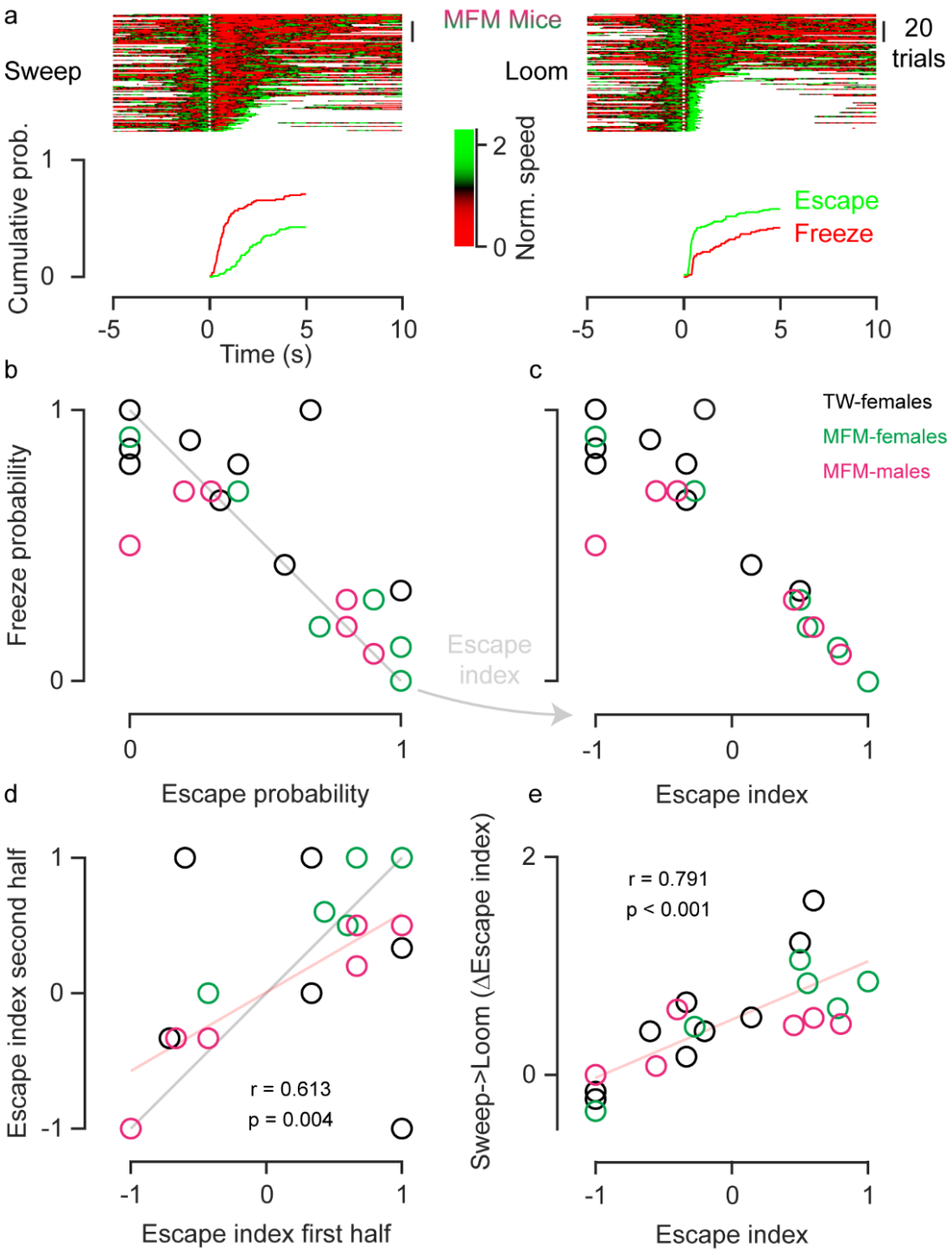

Fig. 3: Some mice escaped more than others. **a**. Response heatmaps and cumulative response distributions for MFM data. Conventions as in Fig 1e-f. **b**. Relative probability of escape and freeze for loom stimuli. An escape index is defined as the y = 1-x line in this space. **c**. Larger escape indices are associated with fewer freezes. **d**. Escape indices are consistent between halves of the experiment. **e**. Mice with higher escape indices are more likely to show different behavioural responses to sweeping and looming stimuli.

We looked for correlates of inter-animal loom escape index variability in terms of other parameters describing stimulus responses. To understand why different mice might be showing different escape indices to looming stimuli, we first looked at whether the freezing and escape responses of different mice discriminate better, or worse, between sweeping and looming stimuli (Fig 3e). We found bigger changes in escape index between sweeping and looming stimuli in mice that were more likely to escape, suggesting that mice with higher escape indices were more also more discriminating in their choice of responses to different potential threats. We further found that mice that were more likely to escape showed faster escapes to looming stimuli (Fig 4a-c), and ran faster during that escape (Fig 4d) (see also Evans *et al.*, (2018)).

We next asked if the general aspects of behaviour, before the onset of threat stimuli, might predict defensive responses. We divided the environment in which the animal was tracked into nest and arena subregions (Fig 5a). We found that animals that escaped more also spent less time in the arena, and more time in the nest (Fig 5b). These animals spent less time in the arena because they made briefer trips to the arena (Fig 5c), and not because they made less frequent trips to it (data not shown). In the time they were in the arena, these mice also moved faster (Fig5d) and showed a higher frequency of spontaneous escapes (Fig5e). Our results therefore suggest that the mice that escape more to looming stimuli spend less time in the arena, because they make briefer trips there, in which they move faster, and make more spontaneous escapes.

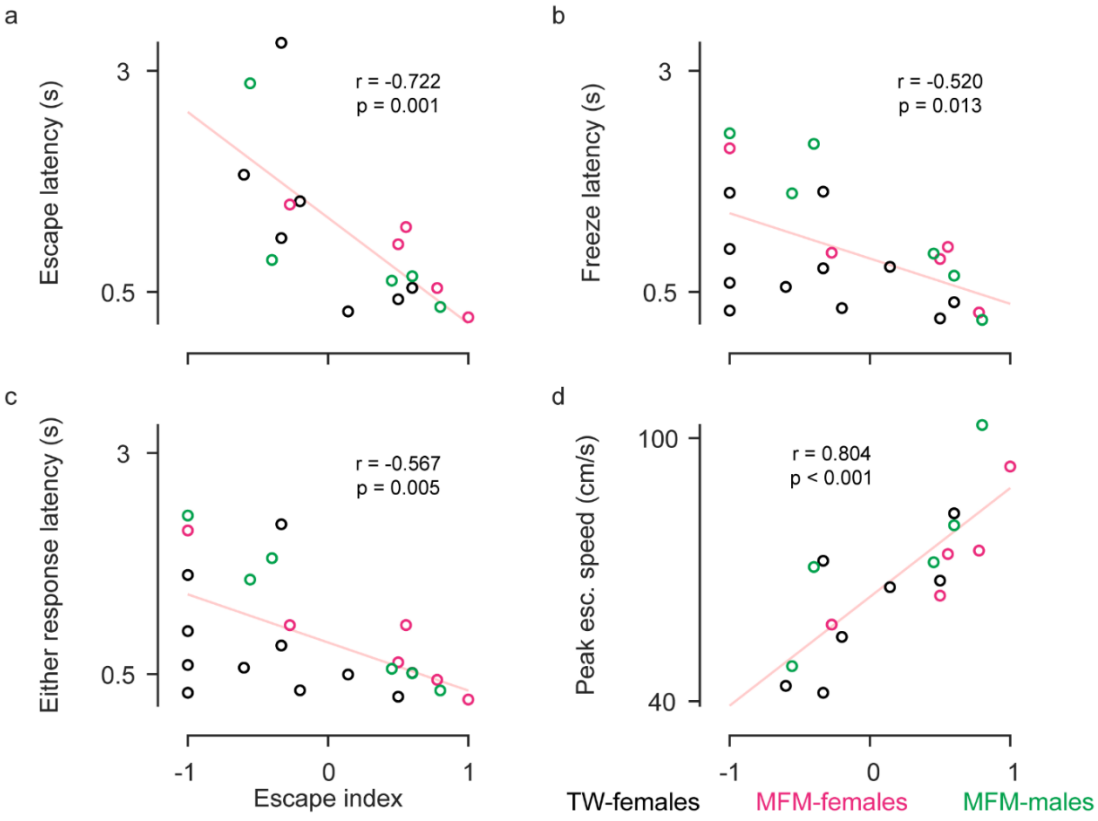

Fig. 4: Mice that escape more respond quicker and escape faster. **a**. When mice escape, the onset of the escape is shorter in mice that escape more. **b**. When mice freeze, the onset of the freeze is shorter in mice that escape more. **c**. The onset of the earliest response (freeze or escape) is shorter in mice that escape more. **d**. When mice do escape, the peak speed of that escape is higher in the mice that escape more.

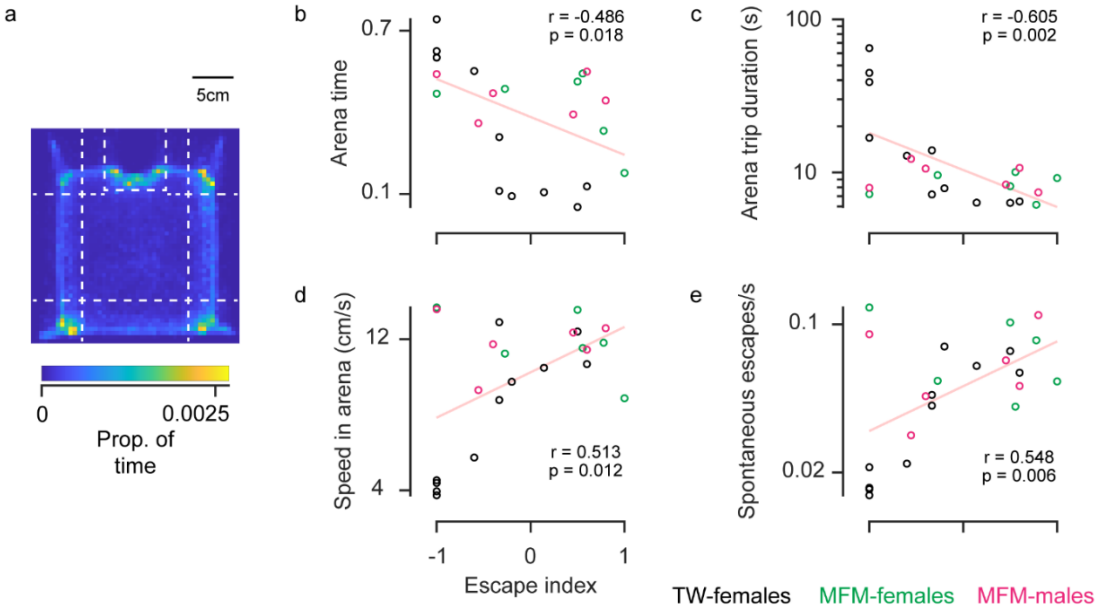

Fig. 5: Mice that escape more spend less time in the arena, making briefer trips with more frequent spontaneous escapes. **a**. Average occupancy map across sessions and mice in the MFM data. Birds-eye-view of environment where the colour of a pixel indicates how long mice spent in that part of the environment. White lines indicate the hierarchical splitting of the environment into subregions. **b**. Mice that escape more spend less time in the arena. **c**. Mice that escape more make briefer trips to the arena. **d**. Mice that escape more move faster in the arena. **e**. Mice that escape more make more frequent spontaneous escapes when they are in the arena.

Discussion

In this study, we attempted a neural activity manipulation to test a hypothesis about the neural pathways that may underlie freezing and escape responses to visual threats. We found no evidence that our attempted manipulation affected how mice respond to visual threats. In addition, while responses to sweeping stimuli were similar to those observed previously, we found a surprising mix of freezing and escape to looming stimuli, where previous work had found a dominance of the escape response. The mix of freezing and escape to looming stimuli at the population level was also seen in individual animals, which showed idiosyncratic behaviours. We tried to understand why different mice might be responding differently to the same stimulus, and found several correlates of this response variability.

No clear effect of chemogenetic manipulation

We attempted a chemogenetic manipulation, but saw no effect. Many factors may contribute to a null result in a bilateral projection-specific chemogenetic manipulation experiment. One possibility is that the manipulation worked as intended but did not affect behaviour. However, recent work has found that inhibiting the SC/PAG (Evans *et al.*, 2018) or the LP-projecting SC neurons (Shang *et al.*, 2018) impairs escape and freezing responses to looming stimuli respectively. Another possibility is that there was insufficient DREADD expression in the neurons of interest, because there were inadequate injections of cre-dependent DREADD virus into the SC, or of retro-cre virus into the LP/PAG. In the subset of animals in which histology was performed (2 SC->LP and 2 SC->PAG), mcherry expression was observed in the injection sites and SC, suggesting that, at least in a subset of mice, cre was being expressed in at least some of the neurons intended. Since both cre and DREADD protein expression were linked with expression of mcherry, it was not possible to assess here whether the DREADD protein was being expressed. A third possibility is that DREADD protein was expressed sufficiently, but that neural activity was not affected, because of DREADD-expressing neurons encountering insufficient concentrations of CNO: though similar IP injection concentrations have been used for inhibitory DREADD

experiments in the SC (Evans *et al.*, 2018). To understand which factors have contributed to the null result, we would be need improved experimental design and further histological analysis.

Variable responses to potential threats

Even were the chemogenetic manipulation working as intended, the behavioural variability we see, even in control conditions, makes observing effects more challenging. The less certain one is about how a mouse will respond to a stimulus in the absence of a manipulation, the greater the importance of either within-animal controls, or larger sample sizes. In the study in which extensive variability in mouse responses to looming stimuli was first shown, the authors countered this variability by using large numbers of mice in their manipulation experiment (~50 with the appropriate expression patterns) (Shang *et al.*, 2018). Other studies have managed to increase consistency across animals by increasing the probability of escape through the use of repetitive looming stimuli, 3-15 repetitions in quick succession (Yilmaz and Meister, 2013; Wei *et al.*, 2015; Huang *et al.*, 2017; Evans *et al.*, 2018; Liu *et al.*, 2018; Salay, Ishiko and Huberman, 2018; Shang *et al.*, 2018, 2019; Zhou *et al.*, 2019; Tseng *et al.*, 2020; Montardy *et al.*, 2021). However, this makes interpretation more difficult (in these experiments the animals initial response is often to freeze, then escape to a subsequent repetition). Another means of increasing escape probabilities might be through varying the parameters of the looming stimulus. Although studies tend to use very similar start diameters, expansion durations and durations on screen post-expansion, looming stimuli tend to expand to half the diameter we used, yielding slower expansion speeds (Yilmaz and Meister, 2013; Wei *et al.*, 2015; Huang *et al.*, 2017; Shang *et al.*, 2018; Lecca *et al.*, 2020) (but see De Franceschi *et al.*, (2016) and Evans *et al.*, (2018)). Whilst changing a single parameter like expansion speed might potentially change responses, such studies used multiple stimulus repeats, making it difficult to predict the changes that would occur if these parameters were used for single stimuli. An exponential loom expansion profile, rather than the linear profiles used here and elsewhere in mouse behaviour, might also increase escape probability, as this visual pattern

may be more naturalistic. Boosting escape probability may also increase the possibility of observing an effect of a manipulation.

Loom responses in our study may differ from work from other groups due to variability in stimulus parameters, but it is surprising that our results were different from previous work in our lab (De Franceschi *et al.*, 2016). Although previous work used the same stimuli, an arena with similar dimensions, and included mice of the same sex and age as we did, there were some differences between the experiments. Previous work used a nest with a bigger entrance, presented visual stimuli with a monitor instead of a projector, and only presented one stimulus per session. Any of these differences might contribute to our different results. However, even the original study reporting escape responses to looming stimuli observed more of a mix of freezing and escape when they did the same experiments in a different institute (Yilmaz and Meister, 2013), suggesting that factors outside of the experiments are also likely to have an influence.

The escape indices of MFM mice were larger than in the manipulation mice. This might be due to the manipulation mice being older, having undergone surgeries and recent IP injections. The walls in the MFM experiments were also entirely covered with white card, which may serve to reduce reflections and influence responses. During the manipulation experiments, the arena was baited during the baseline period, the memory of which may also distract the animal from the screen during stimulus presentation. Finally, the two experiments were performed in different institutes with different handling and housing practices, which could contribute to differences.

Idiosyncratic responses to potential threats

We characterised animal responses using escape index, since most of the response variation occurred along this axis. Freezing and escape probabilities were negatively correlated, possibly because freezing and escape are behaviours which take time to execute, after which the animal may be less disposed to produce a second behaviour.

Interanimal variability in escape likelihood might be due to different animals occupying different positions on a threat imminence spectrum. As detailed by Perusini and Fanselow, (2015), threat imminence theory hypothesises that escape in rats tends to occur when an imminent threat is presented, whereas freezing tends to occur when a less imminent threat is presented. Applying this theory to mice suggests that the mice that escaped more tended to see looming stimuli as representing a more imminent threat than the mice that escaped less. An explanation of why some mice might escape more than others might be that looming stimuli induce a fixed increase in threat imminence, but that prior to stimulus onset, different mice might occupy different positions on the threat imminence spectrum. In the mice with higher baseline levels of belief about the imminence of threat, the threshold for escape might be more frequently crossed. Thus hypothetically, variation in escape index might be explained by different baseline levels on the threat imminence continuum.

We found several behavioural predictors of variability in escape index that might be consistent with a threat imminence model. Threats more distal than those evoking freezing have been hypothesised to evoke behaviours thought to reflect anxiety (Perusini and Fanselow, 2015), and tend to evoke a reluctance to leave the nest. We found that the mice that escaped more escaped faster, made faster runs, and spent longer in the nest. Similarly, mice manipulated to show increased behavioural signs of anxiety start escaping earlier after the onset of a looming stimulus, reach the nest faster and subsequently spend longer in the nest (Li *et al.*, 2018), while mice with reduced anxiety show the opposite changes in their responses (Liu *et al.*, 2018).

We found that the mice that escaped more made more frequent spontaneous escapes. Evans *et al.*, (2018) found that associating part of an environment with threat increased the frequency of spontaneous escapes made from the associated region, suggesting again that the mice that escaped more occupied a higher position on the threat imminence continuum. Similarly, if freezing occurs when a threat imminence threshold is crossed (albeit a threshold lower

than that required for escape), then this would offer an intuitive explanation as to why mice that escape more also freeze at a lower latency.

We also found that the mice that escaped more moved faster around the arena (Fig. 5d), spending a greater proportion of their arena time in its centre, and less in the corners (data not shown). Movement speed, and time in centre are usually thought to be negatively correlated with anxiety when observed in environments without a nest. In the presence of a nest, however, these parameters might be less useful indicators of anxiety. We found that the duration of a trip to the arena was negatively correlated with that trip's movement speed, time in centre, and time in corners (data not shown), possibly resulting, in part, from the centrally located nest. Since the mice that escaped more made briefer trips to the arena, the statistical effects observed using the open field parameters might result from a reluctance to leave the nest for extended periods. Thus, these surprising open field parameter relationships might, too, be explainable under a threat imminence model. Future work could test whether mice that escape more to looming stimuli also show greater signs of anxiety in standardised assays such as the open field, and elevated plus maze, tests.

5. Conclusion

In this thesis, we found evidence that LP and PAG projectors form anatomically and physiologically separate populations in the SC. Despite the segregation of LP and PAG projectors, and some neurophysiological dissimilarities, we found evidence that the two have similar visual tuning, possibly facilitating the influence of internal state which we observed in our behavioural experiments. In this chapter, I will discuss possible hypotheses and future experiments to test them based on the observations made in this thesis.

I will discuss how observed dynamics in each pathway might relate to the dynamics of different behaviours, and the guidance of such behaviours by the visual stimulus. Observed properties of a pathway might reflect the combined properties of a more heterogeneous underlying population: I will discuss the space for this phenomenon and future experiments to investigate it. The similar visual tuning we observed in each pathway might facilitate the influence of internal state on behavioural responses, as will be discussed. Finally, I will discuss changes that might have improved experimental design: changes which could be utilised in future studies.

Dynamic stimulus-guided behaviour through the SC

We found evidence that LP and PAG projectors are both anatomically and physiologically different. Specifically, LP projectors showed more sustained responses than PAG projectors. If the role of the SC is to represent the visual stimulus, the more sustained responses in the LP pathway might permit freezing responses to be guided by the visual stimulus over a longer period. The link between dynamics and behaviour predicts a relationship between the duration of the SC to LP response and the duration of freezing in freely-moving mice. An alternative is that the SC to LP pathway initiates, but does not sustain, freezing behaviour. These alternatives could be tested by recordings of the LP projectors during the behaviour and with temporally-precise inhibition of the pathway after freezing has begun. Work could also look into the relationship between visual stimulus parameters and freezing durations, as well as

behavioural predictors of freezing duration, to better understand the dynamics of this behaviour. A better understanding of the relationship between stimulus parameters and freezing dynamics would yield more specific predictions about response dynamics in the SC to LP pathway.

Besides the presence or absence of a stimulus, the SC to LP pathway might also convey additional information. Firstly, the SC to LP pathway might represent information about stimulus identity and history. Freezing behaviour to a given visual stimulus is sensitive to the animal's experience with that stimulus, discriminating between different stimuli (Procacci *et al.*, 2020; Tafreshiha *et al.*, 2021). The part of the LP innervated by the SC is also part of a network involving the ventral visual stream and the amygdala which are areas thought to be involved in visual cue association learning (Burgess *et al.*, 2016; Ramesh *et al.*, 2018; Bennett *et al.*, 2019). Freely-moving recordings could assess whether the SC to LP pathway carries information about this experience and discriminates between stimuli with different histories, or whether this information is included downstream. The SC to LP pathway might also convey information about the location of the stimulus. Means of observing the sensitivity of defensive behaviour to visual stimulus location remain to be found, but hunting behaviour has been shown to be guided by the spatial location of visual stimuli (Hoy *et al.*, 2016). Interestingly, the ability of mice to initiate pursuit of prey is impaired if the SC to LP pathway is inhibited (Hoy, Bishop and Niell, 2019). As discussed in Chapter 2, our work and that of others (Bennett *et al.*, 2019) suggests that there is not a strong topographic organisation of azimuth in the SC-recipient LP, consistent with the idea that the SC to LP pathway might be more important in detection, than in spatial localisation (Hoy, Bishop and Niell, 2019). Freely moving recordings could investigate response dynamics during such visuospatial behaviour.

On the other hand, the relative transiency of responses in the SC to PAG pathway might limit the duration over which the SC can represent the stimulus. As discussed, besides vigour, the extent to which escape behaviour (e.g. its direction) is guided by the specific properties of the visual stimulus is uncertain. Thus, the SC may not need to represent the direction of the visual stimulus

over a prolonged period, or at all; it may be sufficient for the SC to trigger an escape, which, thereafter, is guided by the location of shelter. These ideas could be tested by recording the duration of PAG-projector responses during freely-moving escape behaviour. Work could also test the influence, and dynamic influence, of stimulus direction on escape behaviour (e.g. escape direction) by varying the visual field position of the looming stimulus in freely-moving mice. Similarly, temporally-precise inhibition could attempt to inhibit the SC to PAG pathway in different phases of escape, to test whether this pathway is necessary once escape is in progress.

More generally, the relatively sustained responses in the SC to LP pathway may reflect a more widespread behavioural role of this pathway. Our anatomical experiments suggest that LP-projectors are more associated with the sensory-related SC, whereas the PAG-projectors are more associated with the motor-related SC. As discussed in Chapter 1, the LP is thought to be more synapses from the muscles than the PAG, suggesting that the LP could have more widespread downstream effects. Therefore, relatively sustained responses in the SC to LP pathway may reflect that this pathway is used for more than a mere motor signal, with more sustained responses providing more sensory information which could be used for other systems (e.g. learning).

Heterogeneity within each projection pathway

Our physiological experiments yielded an estimate of the average properties of each SC projection population. Future work could investigate how homogenous the LP and PAG projectors in the SC are respectively. LP and PAG projectors had a spread along the mediolateral and anterior-posterior axes of the SC. PAG-projectors seemed to stratify along the dorsoventral axis also, with some occupying the intermediate and some occupying the deeper layers. Moreover, neurons projecting to a common hemisphere of the LP could occupy different hemispheres of the SC. Spatially/genetically targeted experiments (discussed in Chapter 2) could look for heterogeneity of anatomical connectivity, neurophysiological properties or behavioural roles across differently located LP and PAG projectors in the SC. In terms of anatomy, work could investigate whether different parts of the mediolateral

axis of the SC (and potentially different clusters along it) project to different parts of the PAG, for example.

The evidence for roles of the lateral and medial SC in approach and triggering escape respectively (Chapter 1), suggest that the more medial LP and PAG projectors might have different behavioural roles to more lateral LP and PAG projectors. Future work could assess the behavioural effects of artificially activating each population across the mediolateral axis of the SC (Masullo *et al.*, 2019). Likewise, work could look into whether there is variation in visual tuning in these projections along the mediolateral axis. More medial neurons would be expected to have more elevated receptive fields. Future behavioural work could assess whether there is a relationship between freeze/escape probability, or vigour, and the elevation of visual threats. If visual stimuli which are threatening in the upper visual field aren't threatening in the lower visual field, then this would suggest different behavioural roles for laterally-located LP and PAG-projectors.

Even in a restricted part of the SC, projection-specific single cell recordings, or looking at variations in the widefield signal across the field of view, can extend population recordings and assess how homogenous neurons projecting to a common target are.

Similar visual tuning and the influence of context

LP and PAG projectors in the SC showed similar population tuning for visual properties. An alternative circuit organisation might have been for LP-projectors to be activated only by the types of stimuli that tend to induce freezing, and for PAG-projectors to be activated only by the types of stimuli that tend to induce escape. If these properties were fixed, and if the SC was the only source of visual information guiding these behaviours, it would be difficult for a looming stimulus to evoke freezing, for example. The co-activation of both pathways by a common stimulus allows for a balance between them, and possibly resultant behaviour, to be altered by internal state. In our behavioural experiments we found that different mice appear to have persistently different internal states (Chapter 4). The influence of internal state

may be mediated by dopaminergic and noradrenergic inputs from the locus coeruleus (Li *et al.*, 2018; Montardy *et al.*, 2021). It will be interesting for future work to study how such inputs effect LP and PAG projectors and animal behaviour. Future work could also study whether animals that escape more shown greater signs of anxiety in standard tests, such as the open field and elevated plus maze tests.

The presence or absence of a nest is also known to influence the balance between freezing and escape to looming stimuli (Vale, Evans and Branco, 2017). Likewise, head-fixed mice in our experiments didn't appear to attempt escape, suggesting the influence of context on responses to common stimuli. The multitude of inputs the SC receives suggests the possibility of contextual modulation of the balance between the LP and PAG pathways on the basis of context. Future work could look at whether looming responses in the SC to LP and SC to PAG pathways differ in freely-moving mice in the presence or absence of a nest, or whether information about the availability of refuge is represented elsewhere. That contextual information might be conveyed to the SC is suggested by the evidence that both the SC (and the PAG) respond during escapes evoked by contextual fear (Evans *et al.*, 2018). The SC is innervated by the anterior cingulate cortex (Benavidez *et al.*, 2020), which is thought to be involved in contextual fear, for example (de Lima *et al.*, 2021).

Alternatively, the pathways not exhibiting the predicted preferences of freeze and escape pathways might imply the need for a more thorough revision of hypotheses. From the literature, it appeared that there was some evidence for the following idea: that visual threats may evoke freezing through a retina to SC to LP pathway, or escape through a retina to SC to PAG pathway. Inhibition of the SC prevents freezing or escape responses to looming stimuli (Evans *et al.*, 2018) but other visual pathways may be used for sweeping stimuli. Manipulations of the PBGN (another target of the SC) show (albeit mixed) evidence of a role in escape-like behaviour (Shang *et al.*, 2015, 2018; Evans *et al.*, 2018), and future work could assess whether the visual tuning of PBGN-projecting SC neurons is what one would expect from an escape pathway. Future work could also look into how the PBGN mediates its effects on

behaviour: the PBGN may be an intermediary between retinorecipient SC neurons and PAG-projecting SC neurons (Evans *et al.*, 2018). To summarise, other pathways may mediate responses to sweeping stimuli, and preferences for visual stimuli that tend to induce escape might be more prominent in other SC output pathways.

To summarise, having more of a balance between responses to sweeping and looming stimuli in the SC may permit greater modulation of behavioural responses, such as by anxiety or availability of refuge, or suggest a revision of hypotheses about the neural basis of responses to visual threats.

Experimental design improvements

The experience of performing the research involved in this thesis revealed numerous experimental design improvements which would have benefitted our experiments and should benefit future studies.

Preliminary experiments to refine and establish consistently successful injection parameters, and a focus on unilateral over bilateral injections would have permitted experiments to progress faster and go further. Earlier establishment of a pipeline for assessing anatomical data would have also helped. Analysis of publicly available anatomical datasets (Oh *et al.*, 2014; Zingg *et al.*, 2014) would not have been able to answer the same questions as our data, but might have aided and contextualised injection parameters (Bennett *et al.*, 2019).

Other means of targeting LP and PAG-projectors respectively would have improved experiments. Better choices of viral reporters and injection site markers would have aided interpretation of physiological and behavioural data. Awareness of the trans-synaptic potential of certain serotypes in the SC (Zingg *et al.*, 2017) would have benefitted some of the experiments not presented in this thesis. Using a transgenic line to target LP projectors might have increased the success rate of physiology and behavioural experiments (Gale and Murphy, 2014; Hoy, Bishop and Niell, 2019), but may have introduced inter-line variability in behavioural responses – it is also unclear what percentage of LP-projectors are labelled by such lines. Using a

retrograde virus directly expressing GCaMP, rather gating its expression, might also have increased efficiency, but would have introduced a concern that we were recording from different neurons to those manipulated in our behavioural experiments.

A more temporally-precise method of manipulating neural activity might have been easier to confirm the effects predicted based on the neurophysiology. However, this would likely have meant tethering the mice during freely-moving behaviour. Wireless optogenetics (e.g. Anpilov *et al.*, 2020), or genetically-targeted neural ablation (Esposito, Capelli and Arber, 2014) might be good options for manipulations whose effects would be detectable with extracellular recordings or histology.

A better appreciation of the challenges associated with miniscope imaging might have benefitted experiments. Improved lens targeting might have made consistent single cell recordings more likely. However, only one study has performed single cell one photon calcium imaging from the SC so far (Evans *et al.*, 2018), so the utility of the current technology in recording this structure remains uncertain. In the absence of spatial background subtraction, background subtraction could also have been performed by different means. Photometry recordings would have permitted background subtraction using ratiometry (Cui *et al.*, 2014), would likely have been easier to establish, perform in each animal, and combine with freely-moving behaviour. Photometry recordings would also have made recording from each pathway simultaneously in individual mice easier. Non-projection-specific extracellular recordings with the stimuli we used would have complemented the data from the mice in which we recorded from the general SC population, and would have identified the space of response properties within which LP and PAG projectors would sit. Extracellular recordings could also have looked at responses at different depths as a proxy of projection target, given the finding that LP and PAG projectors have different dorsoventral positions, on average. Recordings from the LP and PAG directly, rather than the SC inputs to them, would likely have been easier to perform and would have yielded a comparative dataset.

Work studying the effects of manipulation of visual stimulus parameters (not presented in this thesis) or neural activity (Chapter 4) on behavioural responses would have been more powerful without the increase in loom response variability relative to previous work in the lab (De Franceschi *et al.*, 2016). The reason for the increase in variability is unclear, but might partially relate to changes in the rig: changes which were designed to minimise the effect of viewing angle on the presented stimulus, and make the environment more naturalistic and capable of being manipulated. The variability observed was surprising: experiments to confirm, better understand, and potentially reduce variability would have useful to perform prior to manipulations. They would also have provided more extensive data for our study of internal state. If variability could not be reduced by changing stimulus or environmental parameters, the consistency and predictors of behavioural responses exhibited in Chapter 3 could have been used to preselect animals in which the effects of certain manipulations might have been more identifiable. Alternatively, greater sample sizes could have been used.

To summarise, performing the experiments involved in this project has exposed many ways of extending those experiments and executing them more effectively in the future.

Summary

In conclusion, the work in this thesis attempted to understand the role of the SC in behaviour, and the ability of the SC to partake in different behaviours through the diversity of its connections. Experimental work focussed on testing a hypothesis about how the SC might partake in visually-guided freezing and escape behaviours through its projections to the LP and the PAG. We found evidence that LP and PAG projectors are partially intermingled yet separate populations, with different response dynamics. Yet, the SC outputs to the LP and PAG shared visual tuning properties, which might facilitate the influence of internal state on responses to visual threats. Indeed, we found evidence that internal state influences behavioural responses to visual threats, and that different animals have persistently different internal states. Future work could corroborate these findings, record different cells within these projections in

head-fixed and freely-moving animals, and test the effects of manipulating them.

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