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A striatal circuit balances learned fear in the presence and absence of sensory cues

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Summary

During fear learning, defensive behaviors need to be finely balanced, to allow animals to return to normal behaviors after the termination of threat-indicating sensory cues. Nevertheless, the circuits underlying such balancing are largely unknown. Here, we investigate the role of direct (D1R+) - and indirect (Adora+) pathway neurons of the amygdala-striatal transition zone (AStria) in fear learning. *In-vivo* Ca²⁺ imaging revealed that fear learning increased the responses of D1R+ AStria neurons to an auditory CS, given that the animal moved. In Adora+ neurons, fear learning also induced a differential activity during freezing and movement, albeit with little influence of the CS. *In-vivo* optogenetic silencing during the training day showed that plasticity in D1R+ AStria neurons contributes to auditory-cued fear memories, whereas Adora+ neurons suppressed learned freezing when no CS was present. Circuit tracing experiments identified cortical input structures to the AStria, and recording of optogenetically-evoked EPSCs at the corresponding projection revealed different forms of long-term plasticity at synapses onto D1R+ and Adora+ AStria neurons. Taken together, direct- and indirect pathways neurons of the AStria show differential signs of *in-vivo* and *ex-vivo* plasticity after fear learning, and balance defensive behaviors in the presence and absence of aversively motivated sensory cues.

Introduction

Fear learning is an evolutionary conserved behavior, critically important for animals to detect signs of threats in an ever-changing environment (Phelps and LeDoux, 2005; Janak and Tye, 2015). As such, fear learning is relevant for the survival of animals. Nevertheless, learned defensive behaviors need to be finely balanced, so that animals can return to their normal behaviours after cessation of threat-indicating sensory cues allows (Zanette et al., 2011). Furthermore, a pathological overexpression of defensive behaviours is a hallmark of several anxiety-related disorders in humans (Dunsmoor et al., 2011). Therefore, it is important to understand the neuronal circuits that balance the expression of learned defensive behaviors during and after the presence of threat-predicting sensory cues.

The study of the neuronal mechanisms of fear learning has been strongly facilitated by employing auditory-cued fear learning in model animals like rodents (Davis, 1992; LeDoux, 2000). In fear learning, subjects learn to associate an initially neutral auditory cue, the conditioned stimulus (CS), with an aversive or painful outcome like a footshock (the unconditioned stimulus, US). After associative learning, an animal will develop a defensive behavior when the CS is later presented alone (Fanselow, 2018). The defensive behavior that is mostly studied in the context of fear learning in rodents is behavioral arrest, also called freezing (Fanselow, 1994). Studies spanning several decades have firmly established that the amygdala has an important role in fear learning (see Davis, 1992; LeDoux, 2000; Duvarci and Paré, 2014; Tovote et al., 2015, for reviews). In the lateral (LA)- and basal amygdala (LA and BA respectively), auditory (CS) and somatosensory (US) information are integrated (Romanski et al., 1993; Quirk et al., 1995; Amano et al., 2011; Grewe et al., 2017), while the execution of learned freezing depends on a central amygdala to midbrain (periaqueductal gray) projection (LeDoux et al., 1988; Tovote et al., 2016). Nevertheless, given that freezing is a strong change in the behavioral pattern of an animal, it is likely that further neuronal circuits beyond the BLA - central amygdala - midbrain axis are involved in fear learning.

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59 The striatum is part of the basal ganglia motor system, a neuronal system with important roles in
60 movement regulation (for reviews, see Hikosaka et al., 2000; Graybiel, 2005; Grillner et al., 2005;
61 Redgrave et al., 2010; Nelson and Kreitzer, 2014; Klaus et al., 2019). The principal neurons of the
62 striatum are inhibitory projection neurons of two types: First, striato-nigral neurons that project in a
63 direct pathway to output structures like the Substantia nigra pars reticularis (SNR) and others; these
64 neurons selectively express D1-dopamine receptors. Second, striato-pallidal neurons which project
65 in a more indirect pathway towards the basal ganglia output structures; these neurons selectively
66 express D2-dopamine receptors, and also adenosine-2A receptors (Adora) (Gerfen et al., 1990;
67 Schiffmann and Vanderhaeghen, 1993). Different sub-areas of the striatum have different roles in
68 motor control and motor learning. The dorsal striatum is involved in the learning of motor
69 sequences and in the selection of appropriate actions (Nelson and Kreitzer, 2014; Klaus et al.,
70 2019), as well as in habit formation (Redgrave et al., 2010; Burguiere et al., 2015). The ventral
71 striatum is implicated in reward-based learning (Humphries and Prescott, 2010; Cox and Witten,
72 2019). Recently, based on a systematic study of the cortical inputs to the mouse striatum, a further
73 sub-area of the striatum was identified; a posterior area called tail striatum (Hintiryan et al., 2016;
74 Hunnicutt et al., 2016). Interestingly, early *in-vivo* recordings in the LA have found US-responsive
75 neurons also in the amygdala - striatal transition zone (AStria; Romanski et al., 1993). The AStria
76 is adjacent to the LA and part of the tail striatum, and is similarly composed of D1R-expressing
77 (DR1+) and D2R- and adenosine-2A-receptor expressing (Adora+) neurons (Gangarossa et al.,
78 2019). Nevertheless, a possible role of the AStria in fear learning has not been explored in a
79 systematic fashion.

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81 Here, we use *in-vivo* miniature microscope Ca^{2+} imaging, as well as *in-vivo* and *ex-vivo* optogenetic
82 approaches and circuit tracing, to investigate the role of the two subtypes of AStria neurons in
83 auditory-cued fear learning.

Results

Fear learning causes interdependent coding for tones and movement in D1R+ AStria neurons

The AStria has been known to respond to auditory- and footshock stimulation (Romanski et al., 1993), and the tail striatum, which partially overlaps with the AStria, is innervated by dopamine axons which code for salient sensory stimuli (Menegas et al., 2018). Nevertheless, the *in-vivo* activity of AStria principal neurons themselves, and a possible role of this structure in fear learning, are unknown. We started by recording the activity of AStria neurons during a three-day fear learning paradigm (Figure 1A). To access the AStria, a brain area deeply nested in the posterior-ventral striatum close to the basolateral - and central nuclei of the amygdala, we used miniature-microscope Ca^{2+} imaging (Figure 1B, C; Ghosh et al., 2011). The AStria, similar to other striatal circuits, contains D1-receptor expressing neurons, and D2-receptor, or Adora (adenosine2A-receptor)-expressing neurons (Gerfen et al., 1990; Gangarossa et al., 2019). Therefore, we used either D1R^{Cre}, or Adora^{Cre} mice, to unambiguously and separately target the expression of GCamp6m to each subpopulation of AStria neurons, combined with a Cre-dependent AAV vector (Fig. 1B; Materials and Methods). In a first series of experiments (Figure 1, 2), D1R+ AStria neurons were continuously imaged during each day of the fear learning protocol (day 1 - habituation; day 2 - training; day 3 - retrieval).

D1R+ AStria neurons showed, in general, a low frequency of Ca^{2+} events, especially on the habituation day ($\sim 0.01 - 0.02$ Hz average frequency across the population; Figure 1D; see also Figure 2T). On the habituation day of the fear learning protocol, 30-s CS stimulation blocks consisting of 7 kHz tone beeps were applied (0.1s duration, repeated 30 times at 1 Hz; see Figure 1, Figure Suppl. 1 for the fear learning protocol). To this auditory stimulation, some D1R+ neurons responded, albeit with a low response fidelity (Figure 1D). During the training day, when each of the six CS blocks was followed by a 1-s footshock, many D1R+ AStria neurons robustly responded

to the footshocks (Figure 1E, G, H). During the fear memory retrieval day, when the CS was presented alone in a different context, the activity of D1R+ AStria neurons seemed to increase during the CS presentation (Figure 1F).

We performed a deconvolution analysis to derive the action potential (AP) activity of AStria neurons, taking into account the timing and amplitude of individual Ca^{2+} events (Yaksi and Friedrich, 2006). We then constructed peri-stimulus time histograms (PSTHs) and analyzed the significance of the responses with receiver-operated characteristic analysis (ROC; Britten et al., 1992); see Materials and Methods). About one-third of the imaged D1R+ AStria neurons showed a significant footshock response on day 2 (Figure 1G, H; ROC; see horizontal dashed line; 57 out of 167 neurons). Regarding the responses to individual tone beeps, the tone-driven activity of the most active D1R+ AStria neurons was clearly time-locked (see one example neuron in Figure 1I). ROC analysis showed that the number of neurons with a significant response to tones increased on day 2, and further increased on day 3 (Figure 1J, K; $p = 0.0001$, Chi^2 test for trend; 184, 167 and 193 neurons were imaged on days 1, 2, 3, respectively; $N = 8$ D1R^{Cre} mice). Averaging the PSTHs over all imaged neurons similarly suggested an increased tone response in the population of D1R+ neurons during fear learning (Figure 1L). Thus, *in-vivo* Ca^{2+} imaging showed that a significant fraction of D1R+ neurons in the AStria responds to footshocks, and furthermore, that D1R+ neurons increase their responsiveness to the CS.

During fear learning, rodents acquire a defensive behavior in response to a CS, in the form of freezing (LeDoux, 2000; Fanselow, 2018). In our experiments, freezing bouts of mice typically lasted a few seconds, and were interrupted by movement re-initiation (see e.g. Figure 1 D-F). Interestingly, visual inspection of Ca^{2+} population data suggested that D1R+ AStria neurons were preferentially active during the CS blocks, and within these at times when the animal moved (see e.g. Figure 1F). We therefore first asked whether during the CS, transitions from freezing to

movement would drive activity in D1R+ AStria neurons. PSTHs of the Ca^{2+} - events using the movement onset for the alignment revealed Ca^{2+} events in response to movement onset in a subpopulation of D1R+ AStria neurons, as analyzed during the CS epochs (Figure 1M; called "movement-ON" responses). ROC analysis showed that a significant movement-ON response was present in ~ 5% of the neurons on the habituation day; the percentage of responding neurons increased to ~ 20% on day 2 and 3 of the fear learning protocol (Figure 1M, N; $p = 0.003$; Chi-square test for trend). The average PSTH for the entire population of imaged D1R+ neurons also indicated a stronger population Ca^{2+} response during day 2 and day 3 as compared to day 1 (Figure 1O). The response fidelity of individual neurons to movement onset showed a highly skewed distribution, with significantly more non-zero values during the training day and the fear memory retrieval day (Figure 1P, $p < 0.0001$, Kruskal-Wallis [KW] test followed by Dunn's multiple comparisons [MC] test; Hab vs. Train, $p = 0.0002$; Hab vs. Ret, $p < 0.0001$). The estimated AP content, derived from the amplitude of the Ca^{2+} events (see Materials and Methods), was changed less substantially (Figure 1Q, $p = 0.0159$, KW test followed by Dunn's MC test; Train vs. Ret, $p = 0.0155$). We next analyzed the movement-ON responses in the absence of the CS, and found that these were substantially smaller than during the CS (Figure 1R, and Figure 1, Figure Suppl. 2). Finally, when we aligned the Ca^{2+} event traces to *freezing* onset, we found a decreased Ca^{2+} event frequency (Figure 1, Figure Suppl. 3, 4). These experiments thus show that D1R+ AStria neurons increase their AP firing activity during the onset of movements after a period of immobility. Furthermore, the movement-ON responses were more pronounced during the CS than in the absence of a tone, and fear learning causes an increase in the number of D1R+ AStria neurons which showed movement-ON responses during the CS.

These results indicate that the representation of movement-ON transitions, and the coding for sensory events (tones) in the AStria positively influence each other. We therefore next validated whether the tone responses, which we had analyzed above irrespective of movement state (Figure

11-L), would depend in their intensity on the latter. This analysis showed that during fear retrieval, the response fidelity to tone beeps was significantly higher during movement than during freezing (Figure 1S, left, $p < 0.0001$, KW test followed by Dunn's MC test; Hab_Frz vs Hab_Move, $p < 0.0001$; Train_Frz vs Train_Move, $p = 0.0004$; Ret_Frz vs Ret_Move, $p = 0.0027$). Furthermore, the estimated AP content of the Ca^{2+} events was significantly higher during movement than during freezing (Figure 1S; $p = 0.0012$, KW test followed by Dunn's MC test; Train_Frz vs Train_Move, $p = 0.0007$). Therefore, similarly to the movement-ON responses which were potentiated by the presence of the CS, the response to auditory stimuli during the CS were potentiated during movement as compared to freezing. These results thus reveal an inter-dependency of CS-driven responses, and movement driven responses of D1R+ AStria neurons.

For a more comprehensive analysis of how the Ca^{2+} -event frequency depends on the movement state of the animal *and* on the presence or absence of a CS, we analyzed the average Ca^{2+} event frequency for the four combinations of movement states / tone presentations, for each day of the fear conditioning paradigm (Figure 1T). The data was significantly different across conditions ($p < 0.0001$; KW test). Pairwise comparisons showed that during day 1 (habituation), tones did not change the activity of D1R+ during the movement state (Dunn's MC test; Mov_noCS_Hab vs. Mov_CS_Hab, $p = 0.286$); we did not statistically compare the data involving the freezing state because of a paucity of freezing events on the habituation day.. On days 2 and days 3 of the fear learning paradigm, this pattern of activity of D1R+ neurons profoundly changed (Figure 1T). Thus, the CS now induced a significant *increase* of activity in D1R+ neurons *given* that the animals moved (Dunn's MC test; Mov_noCS_Train vs. Mov_CS_Train, $p = 0.0008$), whereas in the freezing state, the CS induced a *decreased* activity (Dunn's MC test; Frz_noCS_Train vs. Frz_CS_Train, $p = 0.0001$). As a consequence, during the CS, the activity of D1R+ neurons was significantly higher when the mice moved, as compared to when they froze (~ 2.5 fold increase; Dunn's MC test; Frz_CS_Train vs. Mov_CS_Train, $p < 0.0001$), whereas the activity was

unchanged between movement and freezing state in the absence of a CS (Dunn's MC test, Frz_noCS_Train vs Mov_noCS_Train, $p = 0.99$). This activity pattern was maintained during the fear memory retrieval day (day 3), when again the CS increased the activity of D1R+ neurons *given* that the mice moved (Dunn's MC test; Mov_noCS_Ret vs. Mov_CS_Ret, $p = 0.013$), and the CS *decreased* their activity given that the mice froze (Dunn's MC test; Frz_noCS_Ret vs. Frz_CS_Ret; $p < 0.0001$). Thus, the activity of the D1R+ AStria neurons, during and after fear learning, is strongly increased when two conditions are met: Movement, and the presence of an aversively-motivated sensory cue.

To determine the location of the imaged neurons and to compare them across mice, we semi-automatically aligned the center of the GRIN lens to a mouse brain atlas in each mouse, and generated a common cell map based on the cell coordinates relative to the lens center (Materials and Methods). This revealed a hotspot of footshock- and tone-responding D1R+ neurons in the posterior - ventral region of the tail striatum medial to the LA (Figure 2A, B). D1R+ neurons with movement-ON responses (as measured during the retrieval day) were located in roughly the similar area, and were in addition found in more anterior regions (~ -1.0 mm AP; see Figure 2C). An analysis of the overlap of neurons with the various types of responses showed that on the habituation day, neurons with responses to the CS, and to movement did not strongly overlap, and represented only a small proportion of the imaged neurons on this day (Figure 2D). During the training day of the fear learning protocol, the neurons with movement-ON responses increased in numbers and substantially overlapped with a larger sub-population of footshock-responsive neurons. On the retrieval day, the populations of both tone- and movement responders had increased, and their overlap became more substantial as compared to the habituation day (Figure 2D). Taken together, *in-vivo* Ca^{2+} imaging with a miniature microscope shows that a subpopulation of D1R+ AStria neurons, located in a posterior - ventral hotspot of the tail striatum, responds to

footshocks. During the course of fear learning, these neurons increasingly code for an aversively-motivated CS, and for freezing to movement transitions, in a highly inter-dependent fashion.

Coding for footshocks and movement by Adora+ AStria neurons

The other large population of principal neurons in the AStria are Adora+ neurons which, in analogy to other striatal areas, represent neurons of an "indirect" pathway through the basal ganglia (Gerfen et al., 1990; Gangarossa et al., 2019). We next investigated the *in-vivo* activity of this population of AStria neurons throughout the three-day fear learning protocol, using an Adora^{Cre} mouse line to target the expression of GCamp6 to Adora+ neurons (Figure 3A-D; see Materials and Methods). More than half of the Adora+ AStria neurons responded to footshocks on day 2 (148/251 neurons; Figure 3C; 3E-F). A smaller subpopulation (~ 10%) responded to individual tone beeps during the CS (see Figure 3G for an example neuron). However, different from the D1R+ neurons, the percentage of tone (CS) - responsive neurons did not change during fear learning, and the population response to tones was modest throughout the three days of fear learning (Figure 3 H, Chi² test for trend, $p = 0.698$) (see also Figure 3I; Figure 3, Figure Suppl. 1). The response fidelity was found to be different between days (Figure 3J, KW test, $p = 0.011$), with a slight decrease from the training - to the retrieval day (Figure 3J, Dunn's MC test, $p = 0.012$), whereas the estimated AP content of the Ca²⁺ events showed no change across the fear learning days (Figure 3K, KW test, $p = 0.457$). Thus, a subpopulation of Adora+ neurons in the AStria responds to footshocks, but the number of Adora+ neurons that responds to tones (CS) is relatively small, and remains largely unchanged during fear learning.

We next analyzed whether the activity of Adora+ AStria neurons was modulated by the movement- or freezing state of the mice. We first restricted the analysis to the 30-s intervals of CS stimuli, and found for these times that a sub-population of Adora+ neurons responded to movement onset (Figure 3L). ROC showed that the number of Adora+ neurons with a movement response increased

during day 2 and day 3 of the fear learning protocol (Figure 3M, $p = 0.0016$, χ^2 test for trend). The increase in movement-ON responses was also visible in PSTHs over the entire population of imaged Adora⁺ neurons (Figure 3N). The response fidelity of movement-ON responses was significantly different between the three days of the fear learning protocol, with a significant increase between day 1 and day 2, and between day 2 and 3 (Figure 3O, $p < 0.0001$, KW test followed by Dunn's MC test; Hab_RF vs. Train_RF, $p < 0.0001$; Hab_RF vs. Ret_RF, $p < 0.0001$), while the estimated AP content was unchanged (Figure 3P, $p = 0.281$, KW test). Corresponding to an increased activity at movement onset, aligning the activity of Adora⁺ AStria neurons to the onset of freezing revealed a decreased activity (Figure 3, Figure Suppl. 2). Finally, an analysis of the movement-ON responses of Adora⁺ neurons in the absence of a CS revealed only a small response (Figure 3Q). Taken together, these experiments reveal that during the course of fear learning, an increasing percentage of Adora⁺ AStria neurons coded for movement onset.

We next analyzed whether vice-versa, the responses to tones might be modulated by the movement state of the mice. Indeed, the response fidelity of Adora⁺ neurons to tone beep stimulation was significantly higher when the mice moved, as compared to when the mice froze, for all three days of the fear learning protocol (Figure 3R, $p < 0.0001$ KW test followed by Dunn's MC test; Hab_Frz vs. Hab_Mov, $p < 0.0001$; Train_Frz vs. Train_Mov, $p < 0.0001$; Ret_Frz vs. Ret_Mov, $p = 0.0003$). On the other hand, the estimated AP content of Ca²⁺ events evoked by tone stimulation was not modulated by the movement state of the mice (Figure 3R; $p = 0.545$, KW test). These data thus show, similar to D1R⁺ AStria neurons, that the transient movement-ON activity of Adora⁺ neurons is potentiated in the presence of a CS, and, vice-versa, that tone-driven activity is potentiated by the movement of the mouse.

In order to assess the interaction between movement-state dependent activity, and CS-driven activity in Adora⁺ AStria neurons more comprehensively, we next analyzed their average Ca²⁺

event frequencies for the four combinations of movement states / tone representations, separately for each day of the fear memory protocol (Figure 3S). A KW test showed that the Ca^{2+} event frequencies differed across categories (Figure 3S; $p < 0.0001$). Given that the mice moved, the activity of Adora⁺ neurons was unchanged by a CS (Dunn's MC test, Mov_noCS_Hab vs. Mov_CS_Hab, $p = 0.99$); as in the data for the D1R⁺ neurons, the paucity of freezing events on the habituation day did not allow us to make statistical comparisons with data obtained in the freezing state on day 1. During the training day, the average Ca^{2+} event frequencies were ~2-fold lower during the freezing state as compared to the movement state, both in the presence- and in the absence of the CS (Figure 3S, Dunn's MC test, Frz_noCS_Train vs. Mov_noCS_Train, $p < 0.0001$; Frz_CS_Train vs. Mov_CS_Train, $p < 0.0001$). On the other hand, for a given movement or freezing state, the presence or absence of the CS did not significantly change the activity of Adora⁺ neurons, and the same was true for the retrieval day (Dunn's MC test, $p > 0.05$ for all comparisons, see statistics Table for Figure 3 for exact p values). Furthermore, on the retrieval day, the activity of Adora⁺ neurons was again higher in the movement - than in the freezing state, both in the absence and presence of the CS (Figure 3S, Dunn's MC test, Frz_noCS_Ret vs. Mov_noCS_Ret, $p < 0.0001$; Frz_CS_Ret vs. Mov_CS_Ret, $p < 0.0001$). This analysis revealed that overall, the neuronal representation of movement in Adora⁺ AStria neurons is less influenced by fear learning than in the D1R⁺ neurons (Figure 1); the learning - induced activity change might consist in a downregulation of the activity of Adora⁺ neurons in the freezing state. Another difference to the D1R⁺ neurons is the finding that the CS did not have a primary importance for the activity of Adora⁺ neurons (Figure 3S).

Taken together, a large population of Adora⁺ AStria neurons shows robust responses to footshocks. Only a small fraction of these neurons, however, responds to the CS and these responses are not modulated by fear learning (Figure 3H-K). Adora⁺ neurons, on the other hand, show a transient response to movement onset given that a CS is present, and fear learning increases the number of

Adora⁺ neurons with such a response. The spatial distribution of footshock- and movement-ON responding Adora⁺ neurons was broader, and the responding neurons were located more anteriorly as compared to the responding D1R⁺ neurons (Figure 3T, U; compare to Figure 2A-C). Finally, we found that the percentage of active Adora⁺ neurons on the training day was high (~ 75%; Figure 3V), primarily because a large percentage of the Adora⁺ neurons is activated by footshocks. However, only a relatively small population of Adora⁺ AStria neurons maintained their activity in response to the CS and to movement-ON events on the fear memory retrieval day (Figure 3V). Thus, *in-vivo* Ca²⁺ imaging reveals similarities, as well as differences in the response types and in the plasticity of responses during fear learning between D1R⁺ and Adora⁺ neurons in the AStria.

D1R⁺ and Adora⁺ AStria neurons do not instruct freezing or movement

Our *in-vivo* Ca²⁺ imaging experiments showed that the two main populations of AStria principal neurons code for footshocks, for the CS (in the case of the D1R⁺ neurons) and for movements, and that many of these representations were changed during the course of fear learning (Figure 1-3). We next wished to investigate whether, and how D1R⁺ and Adora⁺ AStria neurons might contribute to fear learning. A classical model of basal ganglia function postulate that D1R⁺ neurons in the direct pathway initiate movement, whereas Adora⁺ (or D2R⁺) neurons in the indirect pathway suppress movements (Kravitz et al., 2010; Nelson and Kreitzer, 2014; but see Klaus et al., 2019). Thus, one possible straightforward hypothesis is that activity of Adora⁺ neurons of the AStria instructs an arrest of movement, or freezing, and vice-versa, that D1R⁺ neurons might instruct movement re-initiation. We wished to test this hypothesis by optogenetic activation of either D1R⁺ - or Adora⁺ AStria neurons, at times when the mice are engaged in regular exploratory behavior. For this, we used the channelrhodopsin variant Chronos, which we expressed bi-laterally, and Cre-dependently in D1R⁺, or Adora⁺ neurons of the AStria, using AAV1:hSyn:FLEX:Chronos-eGFP in the respective Cre-mouse line (Figure 4A). Three to four weeks later, the mice were allowed to explore the fear conditioning chamber, and trains of

optogenetic stimuli were applied (50 light pulses of 1 ms length at 25 Hz, repeated six times for each mouse). Optogenetic stimulation did not lead to changes in the movement activity of the D1R^{Cre} mice, nor of the Adora^{Cre} mice (Figure 4B, C; N = 8 and 8 mice, respectively; p = 0.621 and 0.163 respectively, paired t-test). These experiments suggest that in naive mice, the activity of Adora⁺ neurons is not sufficient to instruct freezing. Similarly, the activity of D1R⁺ AStria neurons was not sufficient to modulate the movement activity of the mice, although we cannot exclude that a role of the D1R⁺ neurons was masked by ongoing movement.

Plasticity of the D1R⁺ AStria network contributes to fear learning

We found above that many D1R⁺ AStria neurons respond to footshocks, and that following fear learning, a partially overlapping population of D1R⁺ neurons becomes activated during the CS, given that the animal moved at the same time (Figures 1, 2). This suggests that fear learning drives plasticity in D1R⁺ AStria neurons. To investigate the role of this aversively-motivated plasticity in fear learning, we next silenced the activity D1R⁺ AStria neurons during footshock presentation, and observed the effects of this manipulation on freezing behavior during the training day, and one day later during fear memory retrieval. The rationale behind this manipulation is that a strong activity of D1R⁺ AStria neurons during the footshock (Figure 1) is likely a major drive for plasticity at input synapses onto these neurons, and that blocking the footshock-evoked activity likely also blocks plasticity.

For optogenetic silencing, we expressed the light-sensitive proton pump Archaeorhodopsin (Arch; Chow et al., 2010) in a Cre-dependent manner bilaterally in the AStria of D1R^{Cre} mice, and implanted optic fibers over each AStria (Figure 5A, B). Mice in a control group were injected with an AAV vector driving the expression of eGFP (Materials and Methods). Four weeks later, both groups of mice underwent auditory-cued fear learning, and on the training day yellow laser light (561 nm) was applied for 3s, starting 1s before each footshock, with the aim to suppress footshock-

evoked activity in Arch-expressing D1R+ neurons (Figure 5C, D). We found in *ex-vivo* experiments that yellow light strongly hyperpolarizes Arch-expressing D1R+ and Adora+ AStria neurons, suppresses action potential (AP) firing, and does not evoke rebound APs when the light is switched off (Figure 5, Figure Suppl. 1).

During the habituation day of the fear learning protocol the mice showed little freezing, as expected (Figure 5E). On the training day, during which mice received a 1s footshock after each tone block, mice in both groups showed increasing levels of freezing throughout the training period (Figure 5F; CS periods and footshocks are indicated by blue and yellow vertical lines). Freezing levels were not different between the Arch- and the control group, neither for the CS- nor for the no-CS epochs (Figure 5G, left, for the CS epochs; $p = 0.386$; Figure 5G right, for the no-CS epochs; $p = 0.249$; 2-way RM-ANOVA). Thus, optogenetic silencing of the footshock-evoked activity of D1R+ AStria neurons did not change the freezing behavior of mice during the training day.

On the third day, we tested for fear memory retrieval by applying tone stimulation (CS) alone in a different context (see Materials and Methods). The time-resolved freezing analysis revealed a gradual increase of freezing levels when mice entered the conditioning chamber (to ~20%; Figure 5H, arrow). This baseline level of freezing has been observed before (Cummings and Clem, 2020), and likely represents a residual contextual fear memory, despite the change of the context between the training day and the retrieval day (see Materials and Methods). The 30-s CS blocks caused a vigorous increase in freezing in eGFP-expressing control mice, whereas in the Arch - expressing mice, the CS was less efficient in driving freezing (Fig. 5H, black- and green average traces, $N = 9$ eGFP- and $N = 11$ Arch -expressing D1R^{Cre} mice). Averaging and statistical analysis revealed a significant difference in CS-driven freezing between control - and Arch -expressing D1R^{Cre} mice (Figure 5I, closed data points; $p = 0.023$, two-way RM- ANOVA). On the other hand, freezing during the no - CS epochs was unchanged between the two groups (Figure 5I, open data points; $p =$

0.541, 2-way RM-ANOVA). Thus, optogenetic inhibition of D1R⁺ AStria neurons during the aversive footshocks on the training day causes a diminished auditory-cued recall of fear memory one day later. This suggests that D1R⁺ AStria neurons contribute to auditory-cued fear learning.

The Adora⁺ AStria network suppresses learned contextual fear

We next investigated the role of footshock-driven activity in the Adora⁺ AStria network for auditory-cued fear learning. For this, we silenced the activity of Adora⁺ AStria neurons during the footshocks on the fear memory training day (Figure 6A-C), in an approach analogous to the one used above for the D1R⁺ neurons. During the habituation day, we observed low freezing as expected (Figure 6D). On the training day, mice in both the eGFP - expressing control group and in the Arch group showed a gradual increase in freezing with successive CS - US pairings, interrupted by low freezing activity immediately following the footshocks, caused by running and escape behavior (Figure 6E). The analysis of freezing during the CS - and no-CS epochs revealed no difference in the freezing levels between the Arch - and the eGFP groups (Figure 6F; $p = 0.375$ and $p = 0.196$ respectively; 2-way RM ANOVA). Thus, similar as for the D1R⁺ neurons, footshock-driven activity in Adora⁺ AStria neurons is not necessary for freezing behavior that develops during the training day.

On the fear memory recall day, the dynamics of the fear behavior differed between the eGFP and the Arch group (Fig. 6G, H). While the eGFP - expressing control mice displayed an increased freezing during each CS epoch followed by a relaxation to lower freezing levels, mice in the Arch group showed a delayed relaxation of freezing following the CS epochs (Figure 6G; black and red traces respectively). To quantify these effects, we analyzed the average time spent freezing during the CS and during a late no-CS epoch (Figure 6G, blue vertical bar, and grey stripes). This showed that freezing during the no-CS epochs was *increased* in the Arch group as compared to eGFP controls (Figure 6H, right; $p = 0.041$; 2-way RM ANOVA), whereas freezing during the CS epochs

was unchanged between the Arch - and the control group (Figure 6H, left; $p = 0.548$; 2-way RM-ANOVA). Thus, surprisingly, footshock - driven plasticity of Adora⁺ AStria neurons causes a suppression of learned freezing in the *absence* of the auditory cue during fear memory retrieval. This finding, together with the results obtained from silencing D1R⁺ AStria neurons (see above), establishes separate, but functionally synergistic roles of the direct- and indirect pathway neurons of the AStria in fear learning. In fact, the action of both sub-systems together increases the difference between the strength of a defensive behavior in the presence, and absence of a learned aversive sensory cue.

Brain-wide screening of presynaptic inputs to the AStria

Our *in-vivo* Ca²⁺ imaging and optogenetic experiments have revealed a differential role of D1R⁺ - and Adora⁺ AStria neurons in auditory-cued fear learning. To start investigating the role of synaptic afferents to AStria neurons in these plasticity processes, we next used monosynaptic retrograde rabies virus tracing to identify the presynaptic neuron pools that provide input to D1R⁺ and Adora⁺ neurons in the AStria (Fig. 7A; Wickersham et al., 2007; Wall et al., 2013); see Materials and Methods).

Mice were injected into the AStria of either D1R^{Cre}, or Adora^{Cre} mice with a mix of AAV-helper viruses to render subsets of Cre-expressing neurons competent for EnvA-pseudotyped rabies virus uptake, and to drive eGFP expression (Wickersham et al., 2007); see Materials and Methods for the specific viruses). Three weeks later, a pseudotyped delta-G rabies vector driving the expression of dsRed was injected at the same coordinates. In this way, starter cells at the injection site in the AStria could be identified by GFP- and dsRed co-labelling (red) (Fig. 7B, cells appearing yellow in the overlay); retrogradely-labelled neurons were identified based on their single dsRed expression. The analysis of trans-synaptically labelled neurons was restricted to dsRed positive neurons outside of the striatum. We placed all presynaptic neurons found in N = 2 D1R^{Cre} and N = 2 Adora^{Cre} mice

into brain-wide models, with cells from the two mice arbitrarily positioned on different brain sides (Figure 7E, F; red and blue dots, respectively), and analyzed the number and density of back-labelled neurons in each brain area. In both D1R^{Cre} and Adora^{Cre} mice, we found the highest number and density of dsRed-expressing cells in the dorsal (granular and dysgranular) part of the posterior insular cortex (pInsCx), and in the adjacent ventral part of the secondary somatosensory cortex (S2; see also Figure 7 Table Suppl. 1 for a list of all brain structures with detected presynaptic neurons)(Figure 7C, D, G, H). A sizeable number of back-labelled neurons was also found in various areas of the primary somatosensory cortex (S1), in the globus pallidus externa (GPe), and in the thalamic parafascicular nucleus (PF; Figure 7C, D, G, H; Figure 7, Figure Suppl. 1). The GPe and the PF stood out by having high densities of presynaptic neurons to both D1R- and Adora-MSNs, even though their absolute numbers were not large (Figure 7G, H; black bars). Although the brain-wide distribution of presynaptic input neurons to D1R⁺ and Adora⁺ AStria neurons was overall similar, we detected differences on a finer scale (see Figure 7, Figure Suppl. 2). Because S2 and the pInsCx provided the largest number of input neurons to the AStria, we analyzed the anterior - posterior localization of these neurons. This showed that presynaptic neurons in the S2 and pInsCx that provide input to D1R⁺ AStria neurons have the highest density at bregma levels of ~ 0, whereas neurons in S2 and pInsCx that provide input to Adora⁺ AStria neurons are located at somewhat more posterior positions (Figure 7I, J). Taken together, rabies-virus mediated circuit tracing shows that the AStria receives its main cortical input from the S2 and the pInsCx, followed by primary somatosensory areas. Thalamic areas like PF and VPM, and basal ganglia like GPe, as well as limbic areas like CeA and BLA for the Adora⁺ neurons, also contain sizeable numbers of neurons that provide input to the AStria.

The posterior insular cortex provides strong excitatory drive to the AStria

Retrograde rabies-virus labelling showed that the pInsCx, and the adjacent ventral S2 provide a primary cortical input to the AStria (Figure 7). We next wished to functionally validate the putative

connection from the pInsCx to the AStria, using optogenetically-assisted circuit-mapping (Petreanu et al., 2007; Little and Carter, 2013). For this, we injected a viral vector driving the expression of Chronos (AAV8:hSyn:Chronos-eGFP; Klapoetke et al., 2014) into the pInsCx (Fig. 8A, B). We used D1R^{Cre} x tdTomato, or Adora^{Cre} x tdTomato mice, to target patch clamp recordings to either of the two principle neuron populations in the AStria four weeks later. Blue light pulses (1 ms) at maximal light intensity evoked robust optogenetically-evoked EPSCs of 8.8 ± 1.6 nA in D1R+ AStria neurons (n = 12 cells), and of 2.5 ± 0.5 nA in Adora+ AStria neurons (n = 19; p = 0.002, Mann Whitney test; Figure 8C-E). At both connections, gradually increasing the stimulus light intensity led to a smooth increase of the EPSC amplitude, which shows that many axons from pInsCx neurons converge onto each type of AStria neuron (Figure 8C, D). With high light intensities, the optogenetically-evoked EPSC amplitudes saturated, suggesting that a maximal number of input axons was activated (Figure 8C, D). The paired-pulse ratio (PPR) did not differ between the two neuron types, whereas the excitation/inhibition (E/I) ratio was significantly smaller in Adora+ AStria neurons as compared to D1R+ neurons (Figure 8, Figure Suppl. 1; PPR: p = 0.208, unpaired t-test; E/I: p = 0.0054, Mann Whitney test). Taken together, optogenetically-assisted circuit mapping shows that both D1R+ and Adora+ neurons of the AStria receive robust excitatory inputs from neurons in the pInsCx. This, together with the monosynaptic rabies tracing (Figure 7), identifies the pInsCx, together with the ventral S2, as main cortical input area to the AStria.

Fear learning causes opposite plasticity at synapses on D1R+ and Adora+ AStria neurons

The *in-vivo* imaging data showed that many D1R+ and Adora+ AStria neurons robustly respond to footshocks, and that during the course of fear learning, subpopulations of these neurons become responsive to the CS and to movement-ON transitions (Figures 1-3). Furthermore, silencing each AStria neuron population during the footshocks led to characteristic temporal changes in defensive behavior during the fear memory retrieval day (Figures 5, 6). These findings suggest that plasticity

might take place at the synapses that drive D1R⁺ and Adora⁺ AStria neurons. We therefore tested whether fear learning induces plasticity at the glutamatergic synapse from the pInsCx to the D1R⁺ and Adora⁺ AStria neurons. Measurements of the AMPA/NMDA ratio can be used as an assay for postsynaptic forms of long-term plasticity following experience-dependent learning (Yin et al., 2009; Shan et al., 2014; Rothwell et al., 2015). Similarly, changes in paired-pulse ratio can be used to test whether presynaptic forms of long-term plasticity have occurred (Lucas et al., 2016). Thus, we used optogenetically-assisted circuit mapping to ask whether fear learning induces signs of plasticity at the synapses from the pInsCx to either D1R⁺ or Adora⁺ AStria neurons.

We injected D1R^{Cre} x tdTomato mice, and in a separate series of experiments Adora^{Cre} x tdTomato mice, with an AAV vector to drive the expression of Chronos in neurons of the pInsCx. Three to four weeks later, the mice were subjected to auditory-cued fear learning (Figure 8F). Following the fear-memory retrieval session on day 3, which was performed to validate that mice had successfully learned the CS, optogenetically-evoked EPSCs were recorded in slice preparations. A control group of mice underwent the same protocols, but no footshocks were applied ("CS only" group; Figure 8F). Optogenetically evoked EPSCs at the pInsCx to D1R⁺ AStria connection showed a significant increase of the AMPA/NMDA ratio in the CS + US group, as compared to the CS-only group (Figure 8G; $p = 0.001$, unpaired t-test), suggesting that at this connection, a postsynaptic form of LTP had occurred during fear learning. The paired-pulse ratio, however, was unchanged (Figure 8G; $p = 0.974$, unpaired t-test; Figure 8, Figure Suppl. 2). Conversely, at the pInsCx to Adora⁺ AStria connection, the AMPA/NMDA ratio was unchanged (Figure 8H, $p = 0.162$, unpaired t-test; see also Figure 8, Figure Suppl. 3), but interestingly, the paired-pulse ratio was increased in the CS+US group as compared to the CS-only group (Figure 8H; $p = 0.001$, Mann Whitney test). The latter finding suggests that fear learning might induce a presynaptic form of LTD at the pInsCx to Adora⁺ AStria synapse. Thus, auditory-cued fear learning drives long-term plasticity with opposite directions at cortical synapses onto D1R⁺ and Adora⁺ neurons in the AStria.

These differential forms of long-term plasticity might contribute to the complementary roles which the two types of AStria neurons play during fear learning.

Discussion

We describe a role of a posterior striatal circuit, the amygdala-striatal transition zone (AStria) in the formation of auditory-cued fear memories. *In-vivo* Ca^{2+} -imaging revealed that sub-populations of D1R+ and Adora+ neurons in the AStria respond to tones, footshock stimulation, and the movement state of the animal. During the course of fear learning both neuron populations increasingly code for movement-ON transitions, and D1R+ neurons increased their responsiveness to the CS, showing that neuronal coding in the AStria is changed in a plastic manner after fear learning. *In-vivo* optogenetic silencing during the footshock presentations revealed a differential role of each subsystem in fear learning. Whereas plasticity in the D1R+ neurons contributes to the retrieval of a cue-dependent fear memory, the Adora+ network acts to suppress freezing in the *absence* of a sensory cue. We used molecularly defined retrograde tracing approaches and *ex-vivo* optogenetic circuit mapping to study the role of defined synaptic afferents onto each neuron class of the AStria in fear learning. We found that neurons in the dorsal pInsCx, and in the adjacent ventral S2 provide the main cortical input to AStria neurons. Measurements of optogenetically-evoked EPSCs at the synapses arising from the pInsCx showed that fear learning induced opposite forms of long-term plasticity at synapses onto D1R+ versus Adora+ AStria neurons. Taken together, we find that direct and indirect pathway neurons in the AStria balance the amount of learned fear behavior in the presence, and absence of relevant sensory cues.

Plasticity of *in-vivo* responses of AStria neurons

In-vivo Ca^{2+} imaging with a miniature microscope in freely moving mice showed commonalities and differences in the *in-vivo* activity of D1R+ and Adora+ neurons of the AStria. In both neuron

types, footshocks evoked robust responses; the percentage of footshock-activated neurons was higher in Adora⁺ than in D1R⁺ neurons. Following fear learning, the number of D1R⁺ neurons that coded for the CS as well as for movement-ON transitions increased. Intriguingly, the responses to the sensory stimulus (CS) and to the movement-state change potentiated each other, such that following fear learning, D1R⁺ neurons responded best when the animals moved in the presence of a CS (Figure 1T). Thus, the activity of D1R⁺ AStria neurons following fear learning might be seen as a signal of heightened alertness, given that under natural conditions, the decision to move in the presence of an aversive CS might expose the animal to harm. Movement - correlated activity has been observed in striatal neurons (Markowitz et al., 2018), as well as in several types of sensory cortices and also in a more brain-wide fashion (Niell and Stryker, 2010; Keller et al., 2012; Stringer et al., 2019). Regarding the pInsCx, which as we show sends strong afferents to the AStria (Figure 7, 8), previous studies have shown somatosensory and auditory processing in this cortical area (Rodgers et al., 2008; Sawatari et al., 2011; Gogolla et al., 2014), as well as state-dependent signaling relating to physiological needs and to the aversive state of the animal (Livneh et al., 2017; Gehrlach et al., 2019), although movement - ON responses have not been reported for the pInsCx. Nevertheless, it is possible that the CS- and movement - state dependent activity of D1R⁺ neurons is computed in an upstream cortical area like the pInsCx and/or the ventral S2. Alternatively, movement-ON activity, and auditory (CS) information could be conveyed to D1R⁺ AStria neurons from largely separate brain areas via different synaptic inputs, and summation of EPSPs would then lead to a positive interdependence between both type of inputs. For example, based on previous anatomical evidence and evidence from *in-vivo* stimulation experiments, it seems likely that the auditory thalamus provides input to the AStria (Rogan and LeDoux, 1995; Polepalli et al., 2020). Thus, the possibility that D1R⁺ AStria neurons extract information about the movement state and a CS via distinct excitatory inputs which positively interfere with each other, could be investigated in future studies.

The *in-vivo* activity of Adora+ AStria neurons and their changes after fear learning were different in several respects from the one of D1R+ neurons. Fear learning did not induce a measurable plasticity of the CS representation in Adora+ neurons (Figure 3). Furthermore, in Adora+ neurons fear learning induced a difference in neuronal activity between the freezing - and the movement state, but this difference was mainly caused by a *reduced* activity during the freezing state, apparent both during the CS and in the absence of the CS (Figure 3S). This activity pattern was quite different from the one of D1R+ neurons which showed an increased activity given that the animal moved in the presence of a CS (see above). Thus, *in-vivo* Ca²⁺ imaging showed that population coding of the CS and of movement states were differentially modulated by fear learning in the direct-, and indirect pathway neurons of the AStria.

Differential roles of direct- and indirect pathway AStria neurons in fear learning

To investigate the role of the AStria in fear learning, we optogenetically inhibited D1R+ and Adora+ AStria neurons during the presentation of footshocks on the training day. The rationale for this manipulation was that significant populations of each neuron type robustly responded to footshocks on the fear learning training day (Figures 1, 3). Therefore, silencing the footshock - driven activity of these neurons should reduce an important driving force for aversively motivated plasticity. This manipulation, applied to D1R+ neurons, led to a reduced amount of CS-driven freezing during fear memory retrieval (Figure 5), showing that plasticity of D1R+ neurons contributes to the formation of an auditory cued fear memory. Many aspects of plasticity in D1R+ AStria neurons, and their contribution to an auditory-cued fear memory, can be seen as analogous to the role of LA principal neurons in fear learning. Thus, D1R+ neurons integrate US- and CS- information (Figure 1), similar to LA neurons (LeDoux et al., 1990; Romanski et al., 1993). Furthermore, D1R+ AStria neuron develop a sensitivity to the CS which is, in principle, similar to the enhanced CS responses in the LA (Quirk et al., 1995; Amano et al., 2011; Grewe et al., 2017; Zhang and Li, 2018), with the difference that the D1R+ AStria neuronal CS response is coupled to

the movement state of the animal, whereas movement -onset responses were not reported in the above studies in the LA (note that more recent studies in the BA revealed additional coding for behavioral states and behavioral decisions; (Kyriazi et al., 2018; Gründemann et al., 2019). Finally, D1R+ AStria neurons show signs of postsynaptic LTP at a major cortical input after fear learning (Figure 8); signs for postsynaptic LTP have been observed in LA- and/or BA neurons following fear learning, when studying electrically stimulated thalamic - and cortical inputs (McKernan and Shinnick-Gallagher, 1997; Tsvetkov et al., 2002; Rumpel et al., 2005; Namburi et al., 2015). Thus, D1R+ AStria neurons contribute to the formation of an auditory - cue dependent fear memory, in an analogous fashion as LA principal neurons. One difference between both systems are, however, their different output structures: While LA neurons signal to the BA- and to the central amygdala (see Duvarci and Paré, 2014 for a review), our preliminary evidence suggests that D1R+ AStria neurons have a classical basal ganglia output to the Substantia nigra pars reticularis (Figure 8, Figure Suppl. 4). Therefore, future work could investigate how D1R+ AStria neurons influence downstream structures of the basal ganglia (Roseberry and Kreitzer, 2017), and how these are ultimately merged with the amygdala - periaqueductal gray pathway (Tovote et al., 2016), to exert an integrated control of fear behavior.

Intriguingly, optogenetic silencing experiments revealed a different role for the Adora+ AStria neurons in fear learning. Silencing the Adora+ neurons during the footshock led to an *increased* freezing, given that *no* CS was present during fear memory retrieval (Figure 6). This suggests that following fear learning, footshock-driven plasticity of the Adora+ AStria neurons normally *suppresses* freezing when no threat - predicting sensory cues are present. The differential role of the D1R+ - and Adora+ AStria subsystems in fear memory is further apparent by the different types of CS coding *in-vivo*, and by the different signs of long-term plasticity at the cortical input synapses to each neuronal subsystem. We currently have only little direct evidence how the *in-vivo* activity of Adora+ neurons in the AStria relates to defensive behavior. Nevertheless, our data is consistent

with the idea that a decreased activity after fear learning, given that the animal freezes (Figure 3S), signals the absence of danger after the cessation of the learned CS, and might then "liberate" the decision to move. Consistent with a model of a decreased activity of Adora+ neurons is the observation that the pInsCx to Adora+ AStria synapse shows a presynaptic form of LTD after fear learning (Figure 8). Interestingly, the finding that the AStria Adora+ subsystem signals the absence of danger, and therefore safety, resembles previous work which has correlated the *in-vivo* activity of AStria neurons with safety learning, albeit without distinguishing between direct- and indirect pathway neurons (Rogan et al., 2005). Taken together, our study shows differential roles for the direct and indirect pathway neurons in the AStria: D1R+ neurons signal danger given that the animal moves during a CS, whereas Adora+ neurons signal safety in the absence of a learned CS.

Subtle, but functionally important differences in the brain areas that project to D1R+ versus Adora+ AStria neurons might determine their different *in-vivo* responses, and the different role each type of AStria neuron plays during fear learning. In addition, the different responses might also be caused by different effects of dopamine in D1R+ versus Adora+ neurons (Gerfen et al., 1990; Hjorth et al., 2020). Interestingly, recent work has shown that a specific class of midbrain dopamine neurons innervates the posterior tail striatum, and that these dopamine projections are activated by salient, but not by rewarding stimuli (Menegas et al., 2015; Menegas et al., 2018). Although footshock stimuli were not tested in these studies, it is possible that aversive stimuli would similarly cause the release of dopamine in the AStria. Indeed, footshock-driven activity of VTA dopamine neurons that project to the BA was recently shown to contribute to fear learning; furthermore, the same study has obtained anatomical evidence for a projection of VTA DAT+ neurons to the AStria (Tang et al., 2020). Therefore, dopamine signaling in the AStria might contribute to the differential plasticity observed here for the two types of AStria neurons, and to their roles in auditory-cued fear learning.

Our optogenetic activation experiments, performed in the naive state of the mice, did not reveal significant effects on the movement - or freezing behavior of mice (Figure 4). Earlier work has shown that bilateral stimulation of D1R+ neurons in the dorsomedial striatum causes an increase in movement, whereas activation of D2R+ neurons increases freezing (Kravitz et al., 2010). On the other hand, optogenetic activation of neurons in a more posterior region of the dorsal striatum did not induce movement, but modulated the performance in an auditory learning task (Guo et al., 2018). Our findings in the likewise posteriorly located AStria agree with Guo et al. (2018), showing that neuronal activity in the AStria is not sufficient for driving movement or freezing directly. Rather, the activity of AStria neurons, after plasticity induced by fear learning, seems to play a role during the retrieval of associative, sensory-cue driven fear memories.

Conclusion

In summary, we show a role for the AStria in temporally balancing the expression of learned fear in the presence, and absence of aversive sensory cues, thus balancing cue-specific reaction with a more generalized fear response. Adaptive fear discrimination is critically important for animal survival, but is dys-regulated in anxiety disorders (Sangha et al., 2020). Indeed, it was found that during fear generalization, PTSD patients exhibited stronger responses in the striatum, amygdala and insular cortex amongst other areas (Morey et al., 2020). Because fear expression in the absence of aversive sensory cues is downregulated by Adora+ neurons in the AStria, it is an intriguing possibility to harness the differential pharmacology of direct- and indirect pathway neurons together with *in-vivo* manipulations of plasticity (Creed et al., 2015), in an attempt to mitigate the effects of fear generalization.

Methods

Animals

All experimental procedures with laboratory animals (*Mus musculus*) were performed under authorizations for animal experimentation by the veterinary office of the Canton of Vaud, Switzerland (authorizations VD3274 and VD3518). The following mouse lines were used: 1) D1R^{Cre} (B6.129-Tg(*Drd1a-cre*)EY217Gsat/Mmucd; see Gong et al., 2007; MMRRC: 030778-UCD; 2) Adora^{Cre} (B6.FVB(Cg)-Tg(*Adora2a-cre*)KG139Gsat/Mmucd; see Gerfen et al., 2013; MMRRC: 036158-UCD; 3) Cre-dependent tdTomato reporter line/Ai14 (B6.Cg-Gt(ROSA)26Sor^{tm9(CAG-tdTomato)Hze}/J; JAX stock #007909; Madisen et al., 2010). All animals used for experiments were male mice without prior contact to female conspecifics. Animals were group-housed under a 12/12 h light/dark cycle with food and water ad libitum until separated into single cages one day before surgery. For behavioral experiments (Figure 5, 6), mice from 1-2 litters were randomly assigned to control - (GFP - expressing) or effect group (Arch-expressing).

Viral vectors and injection coordinates

For *in-vivo* Ca²⁺-imaging experiments (Figures 1 -3), we injected AAV1:hSyn:FLEX:GCaMP6m (200nl; 6.8 x10¹² vg[vector genomes]/ml; cat. 290-1; viral vector facility University of Zürich, Switzerland), into the left AStria of D1R^{Cre} or Adora^{Cre} mice, using the following coordinates: medio-lateral (ML) 3.2 mm; anterior-posterior (AP) -0.8 to -1.0 mm; dorso-ventral (DV) -4.3 mm (from bregma). For the optogenetic activation experiments (Figures 4), we injected AAV1:hSyn:FLEX:Chronos-eGFP (200nl; 2.80x10¹² vg/ml; University of North Carolina - UNC vector core, Chapel Hill, NC, USA) bi-laterally into the AStria of D1R^{Cre} or Adora^{Cre} mice. For the optogenetic inhibition experiments (Figures 5, 6), we injected AAV1:CAG:FLEX:Arch-eGFP (200nl; 2.05x10¹² vg/ml; UNC vector core) bi-laterally into the AStria of D1R^{Cre} or Adora^{Cre} mice. In earlier experiments AAV1:CBA:FLEX:Arch-eGFP (200nl;5.48x10¹² vg/ml; AV-1-PV2432;

University of Pennsylvania vector core, Philadelphia, PA, USA) was used; both Arch constructs correspond to the initially described "Arch" (Chow et al., 2010). Mice in the control group received AAV1:CAG:FLEX:eGFP (200nl; 4.4×10^{12} vg/ml; UNC vector core).

For the *ex-vivo* optogenetically-assisted circuit-mapping experiments (Figure 8), D1R^{Cre} x tdTomato mice, or Adora^{Cre} x tdTomato mice were injected unilaterally into the posterior insular cortex with AAV8:hSyn:Chronos-eGFP (200nl; 6.5×10^{12} vg/ml; UNC vector core), at the following stereotaxic coordinates: ML 4.2 mm; AP -0.55 mm, DV -3.8 mm (from bregma). In some experiments, D1R^{Cre}- or Adora^{Cre} mice were used, and an AAV8:CAG:FLEX:tdTomato (200nl; 6.5×10^{12} vg/ml; UNC vector core) was additionally injected into the AStria (coordinates as above), to visualize D1R^{Cre} - or Adora^{Cre} - positive neurons for subsequent patch-clamp recordings.

Surgery for virus injection, GRIN lens and optical fiber implantation

The surgery for stereotactic injection of all viral vectors and fiber implantation was performed using male mice of the indicated genotype, of 42-56 days of age. The procedures were similar as described in (Tang et al., 2020). In short, a mouse was anesthetized with isoflurane (induction with 3%, maintained at 1%) and the head was fixed in a Model 940 stereotactic injection frame (David Kopf Instruments, Tujunga, CA, USA) using non-rupture ear bars (Zygoma Ear cups, Kopf Instruments Model 921). Local anesthesia was applied subcutaneously using a mix (50 μ l) of lidocaine (1mg/ml), bupivacaine (1.25mg/ml) and epinephrine (0.625 μ g/ml). After the skull was exposed, small craniotomies were drilled above the indicated coordinates for injection and fiber insertion. For fiber implantation, an additional craniotomy was made close to Lambda to insert an anchoring micro screw. Virus suspension was injected using pulled glass pipettes and an oil hydraulic micromanipulator (MO-10, Narishige, Tokyo, Japan) with an injection speed of ~ 60 nl/min. For *in-vivo* Ca²⁺-imaging (Figures 1 - 3) or optogenetic experiments (Figures 4 - 6), a GRIN lens (600 μ m / 7.3 mm ProView™ integrated lenses; cat. 1050-004413; Inscopix Inc, Palo Alto,

CA, USA) or custom made optical fiber implants, respectively, were implanted 350 μm above the virus injection site(s). Prior to GRIN lens implantation, a 25G medical injection needle was slowly inserted and retracted to facilitate the later insertion of the blunt-ended lens. To reduce deformation of brain tissue due to continuous vertical pressure, we lowered the GRIN lens with alternating down (150 μm) and up (50 μm) movements until the last 200 μm before the final position. Optic fiber implants for *in-vivo* optogenetic experiments were custom-made of a 200 μm core / 0.39 NA / 230 μm outer diameter optic fiber (FT200EMT; Thorlabs Inc, Newton, NJ, USA) inserted into 1.25 mm outer diameter ceramic ferrules (CFLC230; Thorlabs) as described in (Sparta et al., 2012). GRIN lens or optical fiber implants were treated with a light-curing adhesive iBond Total Etch (Kulzer GmbH, Hanau, Germany) and secured in place by blue-light curing dental cement (Tetric EvoFlow, Ivoclar Vivadent, Schaan, Liechtenstein). The open end of the GRIN lens at the integrated docking platform was sealed using a Kwik-Sil silicone compound (World Precision Instruments, Sarasota, FL, USA). After stitching, the skin was covered with Bepanthen® Plus cream (Bayer AG, Leverkusen, Germany), the drinking water was supplemented with 1 mg/ml paracetamol and the animals were monitored for the following 6 days to ensure proper post-surgical recovery.

Behavior

Auditory-cued fear learning was tested three to four weeks after the surgery. Mice were handled by the experimenter and habituated to the procedure of attachment/detachment of the optical patch cords (before optogenetic experiments), or a dummy mini-microscope (Inscopix Inc.) to the GRIN lens platform (before Ca^{2+} -imaging experiments), for 10-15 min on five consecutive days. An auditory-cued fear learning paradigm was performed in a conditioning chamber of a Video Fear Conditioning Optogenetics Package for Mouse (MED-VFC-OPTO-M, Med Associates Inc., Fairfax, VT, USA) under control of VideoFreeze® software (Med Associates Inc). On day 1 (habituation), a mouse at a time was connected to the optic fiber patch cords, or a nVista3.0 mini-

microscope (Inscopix Inc.) was attached, and the animal was placed in the conditioning chamber. The latter was a rectangular chamber with a metal grid floor, cleaned with 70% ethanol. Six tone blocks (CS), each consisting of 30 tone beeps (7 kHz, 80 dB, 100 ms long, repeated at 1 Hz for 30 s), were applied 90 s apart. On day 2 (training), the mouse was placed in the same chamber and presented with six CS blocks pseudo-randomly spaced 60-120 s apart, each followed by a 1s foot shock US (0.6 mA, AC) delivered by a stimulator (ENV-414S, Med Associates Inc). On day 3 (retrieval), the mouse was placed in a conditioning chamber within a different context, consisting of a curved wall and a smooth acrylic floor, cleaned with perfumed general-purpose soap. Four CS blocks as on days 1 and 2 were applied for fear memory recall (see Figure 1, Figure Suppl. 1 for the timing of CS - and US stimuli).

For optogenetic silencing experiments with Arch (Figures 5, 6), light was delivered during 3s starting 1s before the footshock via 200 μ m core / 0.22 NA optic fiber patch cords (Doric Lenses, Canada) from a 561 nm diode-pumped solid state laser (MGL-FN-561-AOM, 100 mW, Changchun New Industries Optoelectronics Technology, Changchun, China). The laser was equipped with an AOM and an additional mechanical shutter (SHB05T; Thorlabs). For optogenetic activation experiments with Chronos (Figure 4), 1-ms light pulses were delivered at 25 Hz, 2s duration from a 473 nm diode-pumped solid-state laser (MBL-FN-473-150mW, Changchun New Industries Optoelectronics Technology). The intensity of each laser was adjusted before the experiment to deliver 10 mW light power at the fiber tip.

The behaviour of animals was recorded at video rate (30 Hz) by the VideoFreeze® software (Med Associates Inc). Based on the behavioural videos, a movement trace was generated using ezTrack software (Pennington et al., 2019); see e.g. Figure 1F-H, red trace). The experimenter, and the person analyzing the data were blinded to the assignment of each mouse to the control - or test group. The movement index trace from ezTrack was used to compute a binary freezing trace using

custom procedures in IgorPro 7 (WaveMetrics Inc, Lake Oswego, OR, USA). The animal was considered to be immobile (freezing state) if the movement index was below a threshold of 40 arbitrary units (without cable attachment) or 120 a.u. (with cable attachment), for a minimum duration of 0.5 s. The binned trace of percent time spent freezing (10s bin size; see e.g. Figures 4G, J; 5F, H; 6E, G) was calculated as a time-average of the freezing state from the binary trace, and then averaged across mice in each group. If mice from any experimental group showed low freezing, they were excluded from the analysis; we used a threshold of 20% time spent freezing during the fifth and sixth CS-blocks on day 2 (7 out of 59 mice for the behavioral experiments were excluded).

Microendoscopic Ca^{2+} -imaging data acquisition and analysis

We used the nVista 3.0 system (Inscopix Inc.) for imaging the activity of either D1R⁺ or Adora⁺ neurons in the AStria over the three-day fear conditioning paradigm. Fluorescent images were acquired at 30 Hz sequentially from three focal planes, resulting in an effective sampling rate of 10 Hz per plane. The intensity of excitation LED in the nVista3.0 miniature microscope was set to 1 – 1.5 mW/mm², and the camera gain was adjusted to achieve pixel values within the dynamic range of the CCD.

The initial processing of *in-vivo* Ca^{2+} -imaging data was done using the Inscopix Data Processing Software (IDPS; Inscopix Inc). This included: 1) deinterleaving of the videos into the frames taken at individual focal planes; 2) spatial filtering; 3) motion correction; 4) manual selection of ROIs; 5) removal of duplicates (i.e. cells from the neighboring focal planes if their intensity traces strongly correlated with correlation coefficient > 0.9); 6) export of the absolute fluorescence traces for each ROI and of individual timestamps into .csv files. TTL pulses delivered from the behavioral setup (Med Associates) were used to align the Ca^{2+} -traces to CS or US events. The data were then analyzed using custom routines in IgorPro 7 (WaveMetrics) as follows. From the absolute intensity

traces for each i -th cell ($F_i(t)$), Z-score traces were calculated as $Z_i(t) = \frac{F_i(t) - \langle F_i(t) \rangle}{\sigma(F_i(t))}$, where $\langle F_i(t) \rangle$ and $\sigma(F_i(t))$ are the mean and the standard deviation of the fluorescence intensity, respectively, calculated from the whole trace. $Z_i(t)$ traces were denoised with a low-pass finite impulse response filter (101 coefficients; end of the pass and start of the reject bands, 2 and 4 Hz respectively). To quantitatively analyze cellular responses, filtered Z-score traces $\hat{Z}_i(t)$ were deconvolved using Fourier transform with a kernel function approximating a unitary fluorescence response to a single AP, similar to the approach used in (Yaksi and Friedrich, 2006). For the kernel, a function $z(t) = (1 - e^{-t/\tau_{rise}}) \cdot e^{-t/\tau_{decay}}$ was assumed with the time constants $\tau_{rise}=0.08s$ and $\tau_{decay}=0.5s$, respectively, with the peak amplitude set to 2.0. Deconvolution yielded a trace proportional to the AP firing rate $R_i(t) = FFT^{-1} [FFT[\hat{Z}_i(t)]/FFT[z(t)]] / \Delta t$ measured in [a.u./s] where $\Delta t = 0.1s$, sampling interval. The $R_i(t)$ traces were smoothed with a 3-points boxcar filter, and the local peaks exceeding a given amplitude threshold (generally set to 2 a.u./s) were detected using a first-derivative method. The time location (limited to 100 ms by sampling rate) and the amplitude of the peaks thus defined discrete Ca^{2+} events $E_i(t_j)$, which corresponded to the start of the rising phase of the j -th fluorescent transient seen in $F_i(t)$ and $Z_i(t)$. The amplitudes of the events from deconvolution (referred to as estimated AP content, EAC [a.u./s]) were proportional to the amplitude of the Ca^{2+} -transients (e.g. see Figure 1 - Figure Supplement 1E). With this method, we did not aim to infer exact AP spiking rates of cells (for which we lack temporal resolution), rather we interpret each "event" as a burst of several APs starting at a given timepoint, with the frequency of APs in a burst being proportional to EAC value.

Based on the timing and amplitudes of discrete events $E_i(t_j)$, we performed a receiver operating characteristic (ROC) analysis for sorting the cells by their strength of responses to a given sensory input (CS or US), or to a movement state transition (e.g. freeze - movement, or movement - freeze). For this, we calculated an auROC value for each cell (Britten et al., 1992) by comparing the

distribution of cellular responses taken as a sum of $E_i(t_j)$ amplitudes during test and baseline time windows of the same duration. Baseline was chosen during periods irrelevant for the cell activity. For tone responders, the baseline window was (-200;0) ms and the test window (0;200) ms relative to tone beep onset at $t = 0$. For move-on transitions, the baseline window was a 600 ms window close to the middle of a freezing bout outside the CS epoch; the test window was a (-300;300) ms window centered on movement onset during CS tone blocks. To estimate the significance of the calculated auROC value for each cell for a given stimulus, we compared these values with auROC values obtained by random shuffling (1000x) of the test and baseline time windows. A neuron was considered negatively or positively discriminative, if its auROC was below or exceeded, respectively, 5% or 95% quantile of the shuffled auROCs (Li et al., 2017). For convenience, we converted auROC values into the response strength values defined as $RS = (2 \cdot auROC - 1)$ (Li et al., 2017). The cells were sorted according to their activity based on their RS values, and were classified into responding or non-responding groups by the 5% / 95% quantile thresholds as described above (e.g. see separation by the dashed line in Figures 1J, 1M, 3L). To construct PSTHs, the event amplitudes $E_i(t_j)$ [a.u./s] were cumulatively counted in a given time bin and multiplied with its duration (100 ms), resulting in [a.u.].

Rabies tracing

For rabies tracing experiments (Fig. 7) D1R^{Cre} or Adora^{Cre} mice were injected into the AStria (see above for coordinates) with a mix of AAVs (AAV8:hSyn:FLEX:TVA-2a-oG and AAV8:hSyn:FLEX:H2B-GFP-2a-oG; 250 nl; 1:1), to render cells competent for EnvA-pseudotyped rabies virus uptake (Wickersham et al., 2007; Wall et al., 2013). In later experiments, we used a tricistronic vector; AAV1:hSyn:FLEX:TVA-2a-eGFP-2a-oG (250 nl; 5.3×10^{12} vg/ml; cat. 243-1; viral vector facility University of Zürich) to transduce the starter population. Three weeks later, the rabies vector EnvA:deltaG-RV-dsRed (250 nl; viral vector core Salk Institute for Biological Studies, La Jolla, CA, USA) was injected at the same coordinates. The animals were

sacrificed 7 days later, and a histological analysis was performed on every second 40 μ m thick coronal section of the entire brain, from the level of the prefrontal cortex up to the end of the cerebellum. The resulting images were analysed in a semi-automatic fashion, i.e. dsRed positive neurons were marked manually, brain sections were registered to the Allen brain atlas and marked neurons were automatically mapped onto the resulting brain regions using Matlab-based software (SHARP-Track; (Shamash et al., 2018)). The positioning of all long-range projecting cells was analysed and plotted on a 3D brain model using the python-based Brainrender software (Claudi et al., 2020).

Electrophysiology

For whole-cell patch-clamp electrophysiology in slices, mice expressing Chronos in the insular cortex (see above) were deeply anesthetized with isoflurane. After decapitation, the brain was quickly removed from the skull and placed in ice-cold slicing buffer containing (in mM): 110 NMDG (N-methyl-D-glutamine), 2.5 KCl, 1.2 NaH_2PO_4 , 20 HEPES, 25 Glucose, 5 Na- ascorbate, 2 Thiourea, 3 sodium pyruvate, 10 MgCl_2 , 0.5 CaCl_2 , saturated with carbogen gas (O_2 95%/CO₂ 5%), pH of 7.4 adjusted with HCl. Coronal slices (300 μ m) containing the ventral tail striatum were cut using a Leica VT1200S slicer (Leica Microsystems, Wetzlar, Germany). Slices were stored for 7 min at 34°C in the slicing buffer and thereafter placed in a chamber containing a storage solution containing (in mM): 92 NaCl, 2.5 KCl, 30 NaHCO_3 , 1.2 NaH_2PO_4 , 20 HEPES, 25 glucose, 5 sodium ascorbate, 2 Thiourea, 3 Na-pyruvate, 2 MgCl_2 and 2 CaCl_2 , pH 7.4 at room temperature, saturated with carbogen (Ting et al., 2014). Whole-cell patch-clamp recordings were performed with a standard extracellular solution containing (in mM): 125 NaCl, 2.5 KCl, 25 NaHCO_3 , 1.2 NaH_2PO_4 , 25 glucose, 0.4 Na-ascorbate, 3 Myo-Inositol, 2 Na-pyruvate, 1 MgCl_2 and 2 CaCl_2 , pH7.4, saturated with carbogen gas. The set-up was equipped with an EPC10/2 patch-clamp amplifier (HEKA Elektronik GmbH, Reutlingen, Germany), and an upright microscope (BX51WI; Olympus, Tokyo, Japan) with a 60x / 0.9 NA water-immersion objective (LUMPlanFI, Olympus).

Patch-clamp experiments including E/I-ratio and AMPA/NMDA-ratio measurements (Figure 8) were performed using a Cs⁺-based intracellular solution (in mM): 140 Cs⁺-gluconate, 10 HEPES, 8 TEA-Cl, 5 Na-phosphocreatine, 4 Mg-ATP, 0.3 Na-GTP, 5 EGTA, pH 7.2 adjusted with CsOH. AMPA/NMDA - ratio experiments were done in the presence of 5μM GABA_A receptor antagonist gabazine (SR-95531; Abcam, Cambridge, UK). Experiments to test the archaerhodopsin (Arch) properties (Figure 5 - Figure Supplement 2) were performed using a K⁺-based solution with (in mM): 8 KCl, 145 K-gluconate, 10 HEPES, 3 Na-phosphocreatine, 4 Mg ATP, 0.3 Na-GTP, 5 EGTA, pH 7.2 adjusted with KOH. All electrophysiological experiments were conducted at near-physiological temperature (34°C) using an inlet heater SHM-6, a heated recording chamber RC-26GL/PM-1 and a thermostatic control unit TC-344B (all from Warner Instruments, Holliston, MA, USA). All chemicals, unless indicated, were from Sigma-Aldrich (St. Louis, MO, USA).

For activation of the excitatory and inhibitory opsins in slice experiments, and for visualization of fluorophores in brain slices, high-power LEDs (CREE XP-E2, 480 nm and 530 nm; Cree Inc., Durham, NC, USA) were custom-coupled into the epifluorescence port of the microscope. Illumination was controlled by the EPC10 amplifier DAC board connected to the LED driver (BLS-1000-2, Mightex Systems, Toronto, Canada). Irradiance was measured by a photodiode (DET36A/M, Thorlabs) coupled into the illumination light path, whose readings were calibrated by the light power measured under the 60x objective using a power-meter model 1918-R equipped with a 818-UV detector (NewPort, Irvine, CA, USA). Electrophysiological recordings were analyzed in IgorPro (WaveMetrics) using the NeuroMatic plug-in (Rothman and Silver, 2018).

Histology

For anatomical analysis of optic fiber - or GRIN lens positions, mice were transcardially perfused with a 4% paraformaldehyde (PFA) solution. The brains were post-fixed in PFA overnight and then

transferred to 30% sucrose in phosphate - buffered solution for dehydration. Coronal brain sections of 40 μm thickness were prepared using a HM450 sliding microtome (Thermo Fisher Scientific, Waltham, MA, USA). Slices were mounted on Superfrost® Plus slides (Thermo Fisher Scientific) and embedded in Fluoroshield mounting medium containing DAPI to stain cell nuclei (Sigma-Aldrich). Slices were imaged with a Slide Scanner VS120-L100 (Olympus) with a 10x/0.4 NA objective, or with an upright Leica SP8 confocal microscope. Brain atlas overlays are taken from (Franklin and Paxinos, 2016). The majority of brain structure names and their abbreviations follows (Franklin and Paxinos, 2016); their correspondence to the Allen brain atlas are given in Figure 7, Table Suppl. 1.

Statistical analysis

Statistical analysis was performed in GraphPad Prism 9 (GraphPad, San Diego, CA, USA). Before choosing the main statistical test, the distribution of the data was tested for normality using a Shapiro-Wilk test. When normality was confirmed, we used a paired or unpaired version of the two-tailed Student's t-tests for two-sample datasets, as indicated. For the comparison of relative datasets, we used a one-sample t-test. When the data was not normally distributed, we used two-tailed non-parametric tests: a Wilcoxon matched-pairs signed-rank test for paired comparisons, or Mann-Whitney U test for unpaired comparisons of two-sample datasets, as indicated.

For datasets with more than two samples (*in-vivo* Ca^{2+} imaging data; Figures 1, 3), the data showed skewed distributions which did not pass the normality test, therefore we used a non-parametric version of a one-way ANOVA, a Kruskal-Wallis test (KW test). If the KW test detected significant differences, it was followed by Dunn's post-hoc test for multiple comparisons (called Dunn's MC test). For datasets influenced by two factors such as the optogenetic silencing/control group and the time of the experiment (Figures 5, 6), we used a repeated-measures two-way ANOVA (RM-

ANOVA) separately for the training and fear retrieval days. If RM-ANOVA reported significance, it was followed by Šidák's post-hoc tests for the respective factor.

The change in number of neurons responding *in-vivo* to different events across training (Figures 1K, 1N, 3H, 3M) was statistically assessed using a Chi-square test for trend. For the optogenetic silencing experiments, we determined the sample size *a priori* using a G*Power software (Faul et al., 2007) for an RM-ANOVA test (two groups, 4 replicate measurements for the fear retrieval day) assuming an average change in freezing of 20% (from 60 to 40%) and a standard deviation of 15% (resulting in an effect size of 0.667), significance level $\alpha=0.05$, power $1-\beta = 0.85$, and the correlation between repeated measures of 0.5. The resulting total sample size was $N = 16$ ($N = 8$ mice per group), with a critical $F_{crit} = 4.6$. There was no sample size estimation made for other experiments.

Each specific statistical test is mentioned in the Results text, and the input data along with statistical test summary (such as p-values, values of test statistics, degrees of freedom, etc.) and the main descriptive statistics are given in the statistics Tables for each relevant figure. The data are expressed as mean $\pm$ SEM. Statistical significance, if applicable, is indicated in the Figures using asterisks as $p \leq 0.05$ (*), $p \leq 0.01$ (**) and $p \leq 0.001$ (***).

Data availability

The essential raw data leading to the conclusions of this paper will be made available at 10.5281/zenodo.4003654.

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Figure Legends

Figure 1. Miniature Microscope Ca²⁺-imaging of D1R⁺ neurons in the AStria reveals interdependent coding for tones and movement during fear learning.

(A) Outline of the 3-day fear conditioning protocol. On the training day, after the tone a 1s, 0.6 mA footshock was applied (see inset on the *right*). (B) Experimental scheme of the injection of an AAV vector and placement of the GRIN lens in the AStria of D1R^{Cre} mice. (C) Posthoc fluorescence microscopy image from an injected D1R^{Cre} mouse expressing GCaMP6m (green). The black region indicates the position of the GRIN lens. The putative imaging area is depicted with a red rectangle. Scalebar, 500 μ m. (D-F) Z-scored Ca²⁺ traces for three example neurons (black traces); movement traces of the mouse (red traces); freezing-state trace of the mouse (blue); and the color-coded z-scored Ca²⁺ traces for all neurons in one example mouse (bottom). Data are from the second CS presentation of day 1 (D), the fourth CS-US presentation of day 2 (E), and the second CS presentation on day 3 (F). (G) Six consecutive footshock responses of an example neuron (black) and the average trace (red). (H) PSTH of the footshock responses of all neurons (n = 167) imaged on day 2 in all D1R^{Cre} mice (N = 8). The fraction of ROC - identified responders is depicted by the horizontal dashed line (n = 57). (I) Ca²⁺-traces of an example neuron in response to all 120 tone beeps applied on day 3 (grey and black lines), and the average response (red). (J) PSTHs of tone responses of all cells on the training days, sorted by response strength. A significant response as determined by ROC was found in the neurons above the horizontal dashed line. (K) Percentage of neurons with a significant response, as determined by ROC, to tone stimulation for each day. (L) The PSTH of the tone beep response, averaged over the entire population of imaged neurons for each day. Note the increased tone responsiveness especially on the fear memory retrieval day (red trace). Blue and green traces depict data from the habituation- and training days, respectively. (M) PSTHs of the movement-onset responses in a similar display as above for tone responses (J), analyzed for times within CS presentations. (N) Percentage of neurons with a significant response to movement onset after freezing. (O) The PSTH of the movement onset response, averaged over

the entire population of imaged neurons for each day. Color code is as in (L). **(P, Q)** Response fidelities (P), and estimated AP content of the Ca^{2+} events (Q) (see Materials and Methods) in response to movement onset, for the entire population of imaged neurons on day 1, 2 and 3 (blue, green, and red data, respectively). **(R)** The population PSTH of the movement onset response for each day, now analyzed in the *absence* of the CS ("no CS"). Note the smaller responses as compared to the movement - onset response in the presence of the CS (O, same y-scale). **(S)** Response fidelities and estimated AP content of the Ca^{2+} events for the tone responses, analyzed both during the freezing state of the mouse ("freeze") and when mice moved ("move"). Note the significantly higher response fidelity on day 3, and the significantly higher estimated AP-content on day 2 when mice moved as compared to the freezing state (see Results for statistical significance). **(T)** The frequency of Ca^{2+} events was analyzed separately for each of the four combinations of CS/no CS, and movement/Freezing states of the mice, for habituation day ("Hab"), training day ("train"), and fear memory retrieval day ("Ret"). The presence of a CS is indicated by the blue bars. The p - values for statistical significance are reported in the Results text, and can additionally be found the statistic Table for Figure 3.

Figure 2. Spatial localization of the imaged D1R+ AStria neurons, and overlap of neurons coding for sensory events and movement state.

(A-C) Maps with the position of all imaged D1R+ AStria neurons, plotted as horizontal projection. Shown are the footshock-responses (A), the tone (CS) responses as imaged on day3 (B), and the movement - onset responses imaged on day 3 (C). Neurons are color - coded based on their respective ROC classification (red, responders, blue, non-responders) and size - coded based on their response amplitudes. For a coarse orientation, outlines of the amygdalar nuclei (basolateral amygdala; "BLA" and central amygdala, "CeA") and cortex ("Ctx") are shown. **(D)** Venn diagrams showing the overlap of neuronal populations within the D1R+ AStria neurons that respond to tones (green), to movement - onset transitions (blue), and to footshock stimulation on day 2 (red). Note the increased number of neurons responding to tones and movement - onset during fear memory retrieval, and the overlap of these sub-populations (right panel).

Figure 3. Adora+ AStria neurons code for footshocks and movement onset, but CS coding is less relevant than in D1R+ AStria neurons.

(A) Experimental scheme of the injection of an AAV vector and placement of the GRIN lens in Adora^{Cre} mice. **(B-D)** Ca²⁺-traces for three example neurons (black); movement trace (red); freezing - state trace of the mouse (blue); and the color-coded Ca²⁺ traces for all neurons in this example mouse. Data are from the second CS presentation of day 1 (D), the fourth CS-US presentation of day 2 (E), and the second CS presentation on day 3 (F). **(E)** Six footshock responses of an example neuron (black) and the average trace (red). **(F)** PSTH of the footshock responses of all neurons (n = 251) imaged on day 2 in the D1R^{Cre} mice (N = 8). **(G)** Ca²⁺-traces of an example neuron in response to all 120 tone beeps applied on day 3 (grey and black lines), and the average response (red). **(H)**. Percentage of neurons with a significant response to tone stimulation for each day. **(I)** The population PSTH to tone beep stimulation, for habituation day (blue), training day (green), and fear memory retrieval day (red). **(J, K)**. Response fidelities (J), and estimated AP content of the Ca²⁺ events (K) in response to tone stimulation for all neurons on day 1, 2 and 3 (blue, green, and red data, respectively). **(L)** PSTHs of the movement-onset responses of all imaged neurons, analyzed for times within CS presentations. **(M)** Percentage of neurons with a significant response to movement onset after freezing. **(N)** The population PSTH of the movement onset response in the presence of the CS. Colour code is as in (I). **(O, P)** Response fidelities (O), and estimated AP content of the Ca²⁺ events (P) in response to movement onset for days 1, 2 and 3 (color code as in J, K). **(Q)** The population PSTH of the movement onset response in the absence of the CS. **(R)** Response fidelities and estimated AP content of the Ca²⁺ events for the tone responses, analyzed both during the freezing state of the mouse ("freeze") and when mice moved ("move"). Note the significantly higher response fidelities on all three days when the mice moved, as compared to the freezing state (see Results for statistical significance). **(S)** The frequency of Ca²⁺ events, analyzed separately for the four combinations of CS/no CS, and movement/Freezing states of the mice, for habituation day ("Hab"), training day ("train"), and fear memory retrieval day

("Ret"). The presence of a CS is indicated by the blue bars. The p-values for the indicated statistical comparisons are reported in the Results text. (T, U) Maps with the position of all imaged Adora+ AStria neurons from N = 8 mice, plotted in the same way as in Figure 2A-C for the D1R+ neurons. Shown are the footshock-responses (T), and the movement responses imaged on day3 (U). (V) Venn diagrams showing the overlap of neuronal populations within the Adora+ AStria neurons that respond to tones (green), to movement - onset transitions (blue), and to footshock stimulation on day 2 (red).

Figure 4. Optogenetic stimulation of D1R+ and Adora+ AStria neurons has no direct effect on movement.

(A) Scheme showing the bilateral placement of optic fibers over each AStria, and the injection of an AAV vector driving the Cre-dependent expression of Chronos. **(B)** Adora^{Cre} mice were allowed to explore the fear conditioning chamber, and trains of blue light stimuli (50 pulses of 1 ms length, 25 Hz, duration of 2s) were applied. The average movement indices for N = 8 mice are shown, either centered around the time of light stimulation (red trace), or taken from a control time when no light was applied ("ctrl TP"). **(C)** Analogous experiment to the one in (B), now performed for D1R^{Cre} mice (N= 7). Note that optogenetic stimulation of neither Adora+ neurons (B), nor of D1R+ neurons led to notable changes in the movement of mice.

Figure 5. Optogenetic inhibition of D1R+ AStria neurons during footshock reduces auditory-cued fear memory retrieval one day later.

(A) Scheme showing the bi-lateral injections in D1R^{Cre} mice of an AAV vector driving the expression of either Arch or eGFP (for controls), and the placement of optic fibers over each AStria. **(B)** Image of a coronal brain section at the indicated Bregma levels, illustrating the fiber placement (black arrows at fiber tip) and Arch expression (green). **(C)** Behavioral paradigm outlining the 3-day fear-learning protocol. **(D)** Scheme of the tone - footshock pairing, ending with a 1 s footshock. The time of the 3s yellow light application is indicated (yellow bar; a 3s constant light pulse of 561 nm and 10 mW at the fiber tip). **(E)** Freezing levels during 30s CS presentations on the habituation day for the control mice (expressing eGFP; black circles), and for Arch - expressing mice (green circles). **(F)** Time - binned average percent freezing traces for control mice (black data) and Arch - expressing mice (green data; bin width is 10 s in both cases). Light blue boxes indicate the 30s CS presentation periods. Grey bars indicate epochs of "no CS" analysis. Vertical orange lines indicate the times of footshock presentation. **(G)** Quantification of freezing during the training day for the control mice and Arch - expressing mice (black and green data, respectively). Freezing was averaged during the 30s CS presentations (filled circles, *left*) and for the no CS epochs (open circles, *right*). **(H)** Time-resolved freezing during the cued retrieval on day 3. Note the phasic increases in freezing caused by each CS presentation (blue) in control mice (black trace, average of N = 9 eGFP expressing mice), which were smaller in amplitude in Arch - expressing mice (N = 11, green trace). **(I)** Quantification of freezing during the 30s CS presentation (*left*) and during the no CS epochs (*right*). Note the significant reduction of freezing during the CS in the Arch group (see Results for statistical significance).

Figure 6. Optogenetic inhibition reveals a role of Adora+ AStria neurons in suppressing learned freezing in the absence of a learned CS.

(A) Scheme of the experimental approach. **(B)** Example of a post-hoc validation of Arch expression (green) and the fiber placement (red dashed line) in the AStria of an Adora^{Cre} mouse. **(C)** Scheme of the tone - footshock pairing on the training day (left), and of the time of yellow light application. **(D)** Freezing levels during CS presentations on the habituation day for controls mice (expressing eGFP; black data) and Arch - expressing mice (red filled circles). **(E)** Time-resolved freezing analysis for control mice (black trace, average of N = 8 mice) and Arch - expressing mice (red trace, N = 10), displayed for 10 s bins. Bars and boxes have the same meaning as in Figure 5F. **(F)** Quantification of freezing during the 30s CS presentations (left), and during the 30s "no CS" epochs (right), both for control mice (black) and Arch - expressing mice (red). **(G)** Time-resolved freezing during the fear memory retrieval day. Color codes are as in (E). **(H)** Average percent of time spent freezing, analyzed during the 30s CS presentations (left), and during the no CS epochs (right) of the fear memory retrieval day, for both groups of mice. Note the significantly enhanced freezing in the Arch group at times when no CS was present (right; for statistical significance see Results).

Figure 7. Retrograde transsynaptic tracing of brain-wide inputs to both D1R+ and Adora+ AStria neurons.

(A) Scheme of the experimental protocol for rabies-virus mediated transsynaptic tracing. **(B)** *Left*, Example confocal images of the injection site in a D1R^{Cre} mouse, *Right*, confocal images at a higher magnification taken from the boxed area of the left image. Green, red and blue channels are eGFP-labelled cells expressing the helper viruses (green), rabies-virus expressing cells (red) and DAPI labelling (blue, only shown in the overlay images). Scale bars, 200 μ m (*left*) and 50 μ m (*right*). **(C, D)** Example widefield epifluorescence images of rabies virus - labelled presynaptic neurons in coronal sections, from a D1R^{Cre} mouse (C) and an Adora^{Cre} mouse (D). The abbreviations of brain areas are shown in Figure 7, Suppl. Table 1. Scalebars, 250 μ m. **(E, F)** Localizations of presynaptic neurons plotted in a 3D brain model, both for D1R^{Cre} (E) and Adora^{Cre} (F) mice. Each brain shows the results of two animals (blue and red dots) with one dataset being artificially mirrored on the other hemisphere for each genotype. For orientation, some brain areas are highlighted (S2 – green, InsCtx – yellow, PF – red, BLA – pink). **(G, H)** Quantification of labelled neurons for each brain area. Upwards and downward data signify cell density and absolute number of cells, quantified for N = 2 mice of each genotype. Data from single animals are plotted as filled grey circles and triangles **(I, J)** Distribution of presynaptic neurons along the anterior - posterior axis in the insular cortex (I), and in the secondary somatosensory cortex (J). The dashed line indicates the border between the anterior and posterior insular cortex according to (Franklin and Paxinos, 2016).

Figure 8. pInsCx provides strong excitatory inputs onto D1R⁺ - and Adora⁺ AStria neurons which show differential plasticity after fear learning.

(A) Experimental scheme of injection of an AAV vector driving the expression of Chronos into the pInsCx, and subsequent slice electrophysiology in the AStria. **(B)** *Left*, example fluorescence images of the injection site in the cortex expressing Chronos-eGFP (scalebar, 500 μ m); *middle*, overview brightfield image with the position of the patch pipette (black dotted lines) in the AStria; *right*, higher magnification images examples of a recorded example cell (brightfield, *top*; and tdTomato fluorescence, *bottom*). **(C, D)** *Left*, EPSCs recorded by stimulating with 1 ms light pulses of increasing intensities (blue, photodiode-recorded trace), and *right*, the resulting input-output curve of EPSC amplitude versus light intensity, with data from all recorded cells overlaid. The example cell shown on the left is highlighted in black in the right panel. Data is shown for D1R⁺ AStria neurons (C, n = 12 recordings) and Adora⁺ AStria neurons (D, n = 19 recordings). **(E)** Quantification of the maximal amplitude of EPSCs (*left*), and of the EPSC delay (*right*) measured in D1R⁺ - and Adora⁺ AStria neurons (n = 12 and n = 19, respectively). **(F)** Timeline of the *ex-vivo* patch-clamp plasticity experiments. See Results for details. **(G)** Example traces for NMDA-EPSCs recorded at +50 mV (top traces), and for AMPA-EPSCs at -70 mV (bottom traces), recorded in D1R⁺ neurons in each experimental group (*left*). The *right* panel shows the quantification of AMPA/NMDA ratios and paired-pulse ratios (50 ms inter-stimulus interval) recorded in each group of D1R^{Cre} mice ("CS only", n = 11 recordings; "CS+US", n = 12). **(H)** Example AMPA-EPSC traces for the measurement of paired-pulse ratio (50 ms inter-stimulus interval) recorded in Adora⁺ neurons in each experimental group, as indicated. *Right*, quantification of AMPA/NMDA ratios, and paired-pulse ratios recorded in each group of Adora^{Cre} mice ("CS only", n = 12 recordings; "CS+US", n = 12).

Figure Legends for Figure Supplements

Figure 1, Figure Suppl. 1.

Exact timing of tone blocks (CS) and footshocks on each day of the fear learning protocol. Each tone block (CS presentation) consisted of $n = 30$ tone beeps (7 kHz, 80 dB) of 100 ms length, repeated at 1 Hz. On the training day, each CS was terminated by a 1 s footshock (0.6 mA). For further details of the fear learning protocol, see Materials and Methods.

Figure 1, Figure Suppl. 2.

(A) PSTH of movement - onset responses of all imaged D1R⁺ AStria neurons, in the *absence* of a CS.

(B) Corresponding PSTH of the movement - onset responses, averaged over the entire population of imaged neurons. Note the only small population response to movement onset in the absence of a CS (compared with Figure 1L, during the CS).

Figure 1, Figure Suppl. 3.

(A, B) PSTHs of Ca²⁺ event frequency for individual D1R⁺ neurons (A), and the population PSTH (B), aligned to freezing onset, for events that had occurred *during* the CS. Note the decreased Ca²⁺ event frequency at the onset of freezing on the training day and on the fear memory retrieval day (B, green and red traces).

(C) A plot of the averaged event frequency, normalized to pre-freezing baseline, revealed a statistically significant reduction in Ca²⁺ event frequency during day 2 and day 3 ($p < 0.001$ for both; one-sample t-test).

Figure 1, Figure Suppl. 4.

(A, B) PSTHs of Ca²⁺ event frequency for individual neurons (A), and the population PSTH (B), aligned to freezing onset in the *absence* of the CS.

Figure 3, Figure Suppl. 1.

PSTH of Ca^{2+} event frequency for individual Adora⁺ neurons, aligned to tone onset, irrespective whether the mouse moved, or froze. See Figure 3I for the corresponding population PSTH.

Figure 3, Figure Suppl. 2.

(A, B) PSTH of Ca^{2+} event frequency for individual Adora⁺ neurons (A), and the corresponding population PSTH (B), aligned to the onset of freezing, for events that had occurred during the CS. **(C)** A plot of the averaged event frequency, normalized to pre-freezing baseline, revealed a significant reduction in event frequency during the fear memory retrieval day ($p < 0.001$; one-sample t-test).

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1416 **Figure 5, Figure Suppl. 1.**

1417 Properties of light-activated archaerhodopsin, as measured in D1R+ - and Adora+ AStria neurons
1418 in slice electrophysiology at 34°C.

1419 **(A)** An Adora^{Cre} x Ai14 mouse was injected with AAV1:CAG:FLEX:Arch-eGFP AAV vector into
1420 the AStria, and *in-vitro* patch-clamp recordings of Arch - expressing neurons were performed 3-6
1421 weeks later. Example of light-induced currents evoked by 300 ms exposures with increasing light
1422 (530 nm) intensities in an Adora+ neuron expressing Arch. Maximal light intensity was 15
1423 mW/mm²; the holding potential was -70 mV.

1424 **(B)** Quantification of the maximal plateau current measured at the end of the light pulse (grey
1425 dashed box in A) for D1R+ - (n = 7) and Adora+ AStria neurons (n = 13) expressing Arch.

1426 **(C)** Example traces recorded in current-clamp showing that light exposure (3 s) was sufficient to
1427 block APs generated by a 1s current injection (no light, black trace; with light, red trace). This
1428 observation was made in all neurons recorded under current-clamp.

1429 **(D-F)** Repeated light exposures (300 ms pulses at 1 Hz, 30 times) were applied to test for the
1430 efficiency of repeated hyperpolarizations, and for possible rebound APs after light offset.

1431 Hyperpolarization (E) and rebound potentials (F) were quantified for D1R+ (n = 6) and Adora+
1432 AStria neurons (n=13).

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Figure 7, Figure Suppl. 1.

Additional example images for transsynaptically retrogradely labelled neurons after injection of an AAV helper virus, and a rabies virus, into the AStria of a D1R^{Cre} mouse (A) and into the AStria of an Adora^{Cre} mouse (B). The images on the left sides illustrate a weak density of labelled neurons in the amygdalar region (left images, CeA, BLA, and LA, see also Figure 7 G, H for the quantifications). The images on the right sides illustrate labeling in a confined region of the PF (right images), which led to a notably high *density* of back-labelled neurons in the PF (see also Figure 7 G, H for the quantifications, and Figure 7, Figure Suppl. 1 - Table for the abbreviations of brain areas).

Figure 7, Figure Suppl. 2.

Analysis of the number of back-labelled neurons in Adora^{Cre} mice *relative* to D1R^{Cre}, according to the analysis of cell numbers shown in Figure 7G, H. The numbers were sorted according to relatively stronger back-labelling of brain areas observed in Adora^{Cre} mice than in D1R^{Cre} (beginning on the left hand side). Note that Adora+ AStria neurons showed larger proportions of back-labelled presynaptic neurons in the lateral amygdala (LA), basolateral amygdala (BLA), central amygdala (CeA), temporal association cortex (TeA), and auditory cortex (AUD), as compared to D1R+ neurons; interestingly, these areas represent limbic - auditory areas with roles in associative learning (LeDoux, 2000; Weinberger, 2007; Sacco and Sacchetti, 2010; Dalmaï et al., 2019). On the other hand, a higher proportion of neurons providing input to D1R+, as compared to Adora+ AStria neurons, was found in the S1, in the somatosensory thalamus (VPM), motor cortex (MO) and piriform cortex (PIR).

Figure 7, Suppl. Table 1.

Definition of brain area abbreviations, and correspondence of the names across the Allen Brain Atlas and the Mouse Brain Atlas of Franklin and Paxinos (2016).

Figure 8, Figure Suppl. 1

(A) *Left* panel, example traces of paired-pulse ratio measurements at 50 ms inter-stimulus interval.

Traces are from a D1R^{Cre} and Adora^{Cre} mouse (green and red traces, respectively). *Right*,

quantification of the paired-pulse ratio from all recorded cells (n = 12 D1R+ and n = 18 Adora+

AStria neurons).

(B) Excitation-inhibition (E/I) ratio was measured by recording optogenetically-evoked EPSCs at -

70 mV, and subsequently recording optogenetically-evoked IPSCs at the reversal potential for the

EPSCs (+5 to +15 mV across experiments). *Left* panel shows example traces, the quantification of

E/I ratio (*middle*), and the quantification of latency between the start of the light pulse and the

respective PSC (*right*, for n = 12 D1R+ and n = 14 Adora+ AStria neurons). The statistical

information for E/I ratios is given in the Results text. The delays for EPSCs were significantly

shorter than the delays for IPSCs (D1R: p = 0.001, paired t-test; Adora: p < 0.0001, Wilcoxon

matched-pairs signed-rank test). Data are shown as average ± SEM.

Figure 8, Figure Suppl. 2

EPSC traces illustrating the measurement of paired-pulse ratios in a D1R+ neuron from a mouse

that had undergone the "CS only" protocol (left), and in a D1R+ neuron from a mouse that had

undergone the "CS+US" protocol. Relates to the dataset shown in Figure 8G.

Figure 8, Figure Suppl. 3

EPSC traces illustrating the measurement of AMPA/NMDA ratios in a Adora+ neuron from a

Adora^{Cre} x Ai14 mouse that had undergone the "CS only" protocol (left), and in a Adora+ neuron

from a Adora^{Cre} x Ai14 mouse that had undergone the "CS+US" protocol. Relates to the dataset

shown in Figure 8H.

Figure 8, Figure Suppl. 4

Anatomical evidence that D1R⁺ and Adora⁺ AStria neurons are analogous to striato-nigral, and striato-pallidal neurons of a direct, and indirect pathway, respectively. The images show slide scanner images of the fluorescence of eGFP, after bi-lateral injection of AAV1:CAG:FLEX:eGFP into the AStria of D1R^{Cre} - and Adora^{Cre} mice (B, C, respectively). Note that eGFP expression in D1R⁺ neurons of the AStria leads to axonal labelling in the GPe which we interpret as axons of passage, and to labeling in the GPi and SNR which is confined to the lateral parts of these nuclei. On the other hand, eGFP expression in Adora⁺ neurons of the AStria leads to labeling in the GPe. The experiments were taken from the control data sets of *in-vivo* optogenetic experiments of Figures 5, 6.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8

Figure 3, Figure suppl. 1

Figure 3, Figure suppl. 2

Figure 5, Figure suppl. 1

Figure 7, Figure suppl. 1

Figure 7, Figure suppl. 2

Figure 8, Figure Suppl. 1

Figure 8, Figure Suppl. 2

Figure 8, Figure Suppl. 3

Figure 8, Figure suppl. 4

Figure 7, Suppl.Table 1

Abbreviation	Allen Brain atlas	Franklin and Paxinos
AI	Agranular insular area	Agranular insular cortex (dorsal, ventral and posterior)
AUD	Auditory areas (dorsal and ventral)	Auditory cortex (dorsal, primary and ventral)
BLA	Basolateral amygdalar nucleus (anterior, posterior and ventral)	Basolateral amygdaloid nucleus (anterior, posterior)
CeA	Central amygdalar nucleus (capsular, medial and lateral)	Central amygdaloid nucleus (capsular, medial and lateral)
Ect	Ectorhinal area	Ectorhinal cortex
ENT	Entorhinal area	Entorhinal cortex
EP	Endopiriform nucleus	Clastrum (dorsal, ventral and endopiriform)
GPe	Globus pallidus, external segment	Globus pallidus
GPi	Globus pallidus, internal segment	Entependuncular nucleus
InsCtx	Gustatory areas and Visceral area	Insular cortex (granular and dysgranular)
LA	Lateral amygdalar nucleus	Lateral amygdaloid nucleus
MO	Primary motor area	Primary motor cortex
PF	Parafascicular nucleus	Parafascicular thalamic nucleus
PIR	Piriform area	Piriform cortex
PO	Posterior complex of the thalamus	Posterior thalamic nuclear group
S1 barrel	Primary somatosensory area, barrel field	Primary somatosensory cortex, barrel field
S1 mouth	Primary somatosensory area, mouth	Primary somatosensory cortex, upper lip/barrel field
S1 nose	Primary somatosensory area, nose	Primary somatosensory cortex, upper lip
S1 unassigned	Primary somatosensory area, unassigned	Primary somatosensory cortex, dysgranular zone
S1 upper limb	Primary somatosensory area, upper limb	Primary somatosensory cortex, shoulder region
S2	Supplemental somatosensory area	Secondary somatosensory cortex
SNR	Substantia nigra, reticular part	Substantia nigra, reticular part
TeA	Temporal association areas	Temporal association cortex
VPM	Ventral posteromedial nucleus of the thalamus	Ventral posteromedial thalamic nucleus