LETTER

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Ventral tegmental area GABA projections pause accumbal cholinergic interneurons to enhance associative learning

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The ventral tegmental area (VTA) and nucleus accumbens (NAc) are essential for learning about environmental stimuli associated with motivationally relevant outcomes. The task of signalling such events, both rewarding and aversive, from the VTA to the NAc has largely been ascribed to dopamine neurons[1–3]. The VTA also contains GABA (γ-aminobutyric acid)-releasing neurons, which provide local inhibition[4,5] and also project to the NAc[6,7]. However, the cellular targets and functional importance of this long-range inhibitory projection have not been ascertained. Here we show that GABA-releasing neurons of the VTA that project to the NAc (VTA GABA projection neurons) inhibit accumbal cholinergic interneurons (CINs) to enhance stimulus–outcome learning. Combining optogenetics with structural imaging and electrophysiology, we found that VTA GABA projection neurons selectively target NAc CINs, forming multiple symmetrical synaptic contacts that generated inhibitory postsynaptic currents. This is remarkable considering that CINs represent a very small population of all accumbal neurons, and provide the primary source of cholinergic tone in the NAc. Brief activation of this projection was sufficient to halt the spontaneous activity of NAc CINs, resembling the pause recorded in animals learning stimulus–outcome associations[8–12]. Indeed, we found that forcing CINs to pause in behaving mice enhanced discrimination of a motivationally important stimulus that had been associated with an aversive outcome. Our results demonstrate that VTA GABA projection neurons, through their selective targeting of accumbal CINs, provide a novel route through which the VTA communicates saliency to the NAc. VTA GABA projection neurons thus emerge as orchestrators of dopaminergic and cholinergic modulation in the NAc.

We took advantage of optogenetic projection targeting to visualize and selectively activate VTA GABA projections neurons (GPNs) to the NAc, which represent about 25% of all VTA GABA cells[7]. We stereotactically injected GAD-Cre mice (65-kDa isoform of the *Gad2* locus[4,13]; also called Gad2-CreER) with adeno-associated virus (AAV) containing floxed ChR2-eYFP into the VTA and prepared sagittal slices of the NAc 3 weeks later (Fig. 1a). Enhanced YFP expression allowed the visualization of axonal projections (Fig. 1b). At higher magnification, these revealed corkscrew-like terminal arborizations that were sparsely distributed throughout the NAc (Fig. 1c). When such images were merged with immunohistochemical identification of choline acetyltransferase (ChAT) to label CINs in fluorescence and light microscopy, the axons of the VTA GPNs closely apposed the soma and proximal dendrites of CINs (Fig. 1c, d). Parvalbumin-positive neurons in the NAc, a subclass of GABA interneurons, did not show any axonal appositions (Supplementary Fig. 1). To examine whether the appositions onto CINs gave rise to synaptic contacts, we next prepared slices for electron microscopy to perform an ultrastructural characterization of the appositions. CINs were identified by immunoreactivity for ChAT revealed by diaminobenzidine (DAB) using a pre-embedding immunoperoxidase reaction, as well as by distinctive invaginations of their nuclei. VTA GABA axons (DAB revelation of anti-GFP antibodies) surrounding the CINs gave rise to multiple symmetrical synaptic contacts typical of GABA synapses (Fig. 1e). Conversely, VTA GABA axons apposed to medium spiny neurons (MSNs), the predominant cell type in the NAc, almost never formed synaptic contacts (Fig. 1f). In fact, we found 8 synapses with eYFP-stained synaptic boutons on 132 cross-sections or approximately 6,300 μm of analysed membrane of CINs and

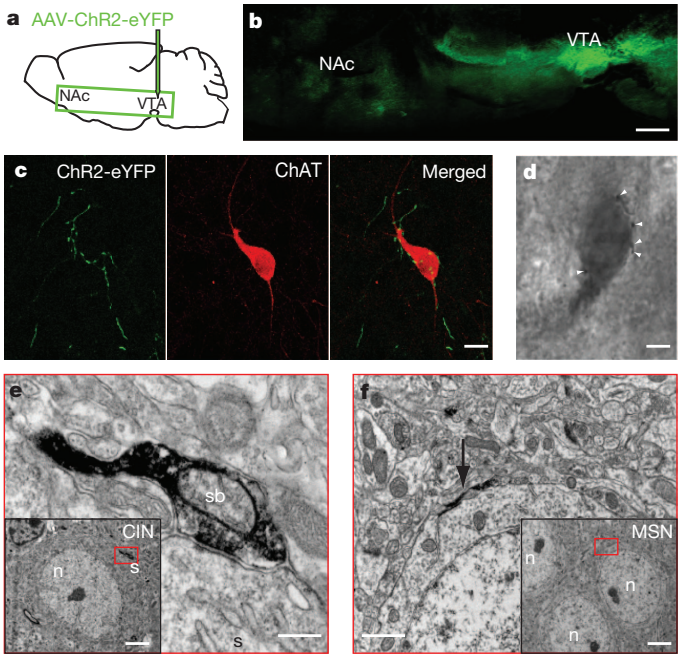

Figure 1 | Long-range GABA projections from the VTA form synaptic contacts selectively with CINs in the NAc. **a**, Schematic diagram showing the injection site and region (green rectangle) used for the sagittal section. **b**, Sagittal section of a GAD-Cre$^+$ mouse brain showing eYFP$^+$ axons coursing from the VTA to the NAc. Note fibre bundle projecting elsewhere, probably to the prefrontal cortex[7]. **c**, Confocal images of an example NAc CIN immunoreactive for choline acetyltransferase (ChAT) enwrapped by an eYFP$^+$ axon. **d**, Light microscopy image of pre-embedding immunoperoxidase double labelling of ChAT$^+$ cell body and eYFP$^+$ axons (arrow heads). **e**, Electron microscopy image of a synaptic contact formed between an eYFP$^+$ synaptic bouton (sb) and a CIN. The CIN exhibited a lightly DAB-labelled soma (s, inset), and possessed a typical indented nucleus (n, inset). **f**, An example image of an eYFP$^+$ axon apposing (arrow), but not forming a synapse with, an MSN (smaller cell body, round nucleus typical of MSNs with clear cytoplasm (inset)). Scale bars: **b**, 500 μm; **c**, **d**, 20 μm; **e**, 0.3 μm and 3 μm (inset); **f**, 1 μm and 3.5 μm (inset).

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only one such synaptic contact on 1,100 cell cross-sections or more than 35,000 µm of MSN membrane (Mann–Whitney *U*-test, $P < 0.0001$), indicating a selective targeting of NAc CINs by VTA GPNs.

To examine transmission from VTA GPNs onto accumbal neurons further, we performed whole-cell recordings in CINs, MSNs and parvalbumin interneurons while activating VTA GPN axons (Fig. 2a). CINs and MSNs could readily be distinguished by their distinct appearance and electrophysiological properties (Fig. 2b–d; see also refs 14, 15). For the identification of the parvalbumin interneurons we crossed a line that expresses the Cre element in parvalbumin cells with a line expressing a floxed tomato construct (fTom^{PVALBcre}). A brief flash of blue light yielded robust inhibitory postsynaptic currents (IPSCs) in 100% of CINs; these were abolished by the $GABA_A$ receptor blocker picrotoxin (Fig. 2c, right panel). In contrast, recording from neighbouring MSNs and parvalbumin interneurons with the same stimulation protocol failed to elicit a response in 96.6% and 80% of cases, respectively, and yielded only small IPSCs in responding neurons (Fig. 2d, e). On average, current amplitudes recorded from CINs were markedly larger than those currents recorded from MSNs and parvalbumin interneurons, if they showed any at all (CINs: 360 ± 124 pA, $n = 17$ of 17 neurons; MSNs: 52 ± 12 pA, $n = 2$ of 60 neurons; parvalbumin interneurons: 20 ± 3 pA, $n = 3$ of 14 neurons; Fig. 2f). The combination of structural imaging as well as electrophysiological evidence *in vitro* demonstrates that VTA GPNs selectively target CINs of the NAc, which is remarkable considering that CINs account for less than 1% of the total number of cells in this region[16].

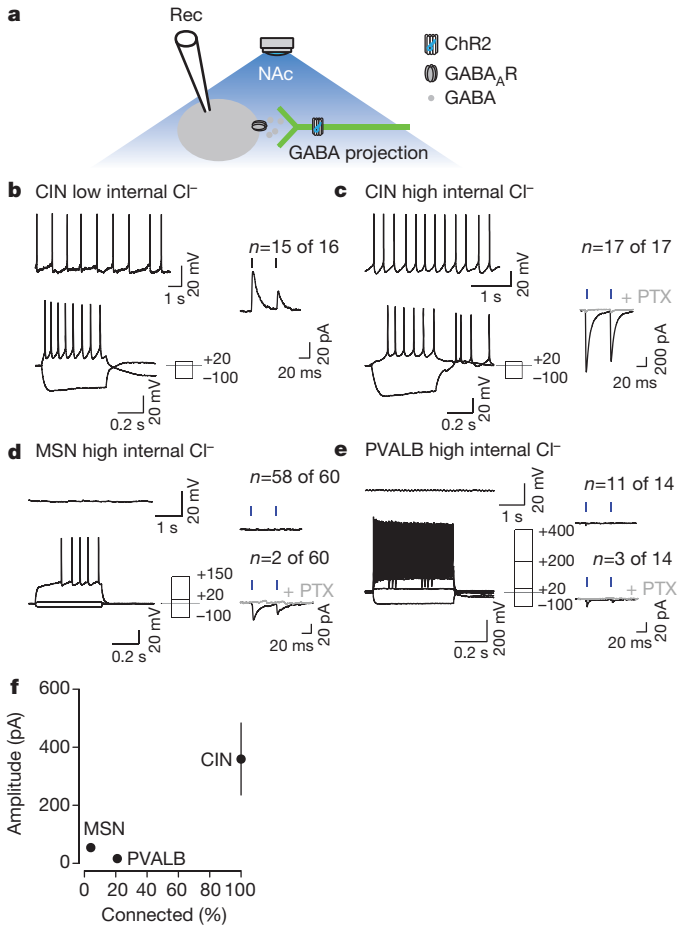

Figure 2 | Inhibitory currents can be selectively elicited in CINs by activating VTA GABA axons in the NAc. **a**, Schematic showing the preparation used to obtain *in vitro* whole-cell patch-clamp recordings of NAc neurons. **b**, Example traces of whole-cell current-clamp recordings of CIN spontaneous activity (left, top panel) or responses to current steps (left, bottom panel) with low internal chloride concentration. An example trace (right) of voltage-clamp recordings of a CIN during a paired-pulse light-flash protocol (4-ms pulses, 20 Hz; representing $n = 15$ of 16) is shown. **c**, Same as in **b** but with high internal chloride concentration ($n = 17$ of 17). IPSCs were blocked by picrotoxin. **d**, Same as in **c** but for an MSN. Most MSNs ($n = 58$ of 60) exhibited no response to light flashes (right, top panel; example trace). In a minority of cases a picrotoxin-sensitive current was observed (right, bottom panel; example trace, $n = 2$ of 60) that was smaller to that observed in CINs. **e**, Same as in **c** and **d** but for identified parvalbumin interneurons. NAc slices were prepared from non-floxed ChR2-infected fTom^{PVALBcre} mice in the VTA. Most parvalbumin interneurons ($n = 11$ of 14) exhibited no IPSCs in response to blue-light stimulation and in a few cases ($n = 3$ of 14), a picrotoxin-sensitive current of much smaller amplitude than in CINs was recorded. Note that in this preparation IPSCs were still selectively recorded in CINs (data not shown). **f**, Graph showing average amplitude (± s.e.m.) of IPSCs recorded in CINs, MSNs and parvalbumin interneurons as a function of the percentage under the inhibitory synaptic control of VTA GPNs.

The connectivity observed here is intriguing because it correlates with the observed *in vivo* responses of CINs and VTA GABA cells to salient events. That is, despite heterogeneity in VTA GABA neuron responses, many are phasically activated[17], whereas striatal CINs pause in response to motivationally important outcomes and associated stimuli[8–12], irrespective of their valence. Although the firing pattern of CINs in the NAc has not been well described in animals learning discrete stimulus–outcome associations, our next series of experiments addressed the question of whether activation of VTA GPNs is sufficient to induce a pause in the activity of NAc CINs. We first observed that, in the slice preparation, CINs exhibited tonic firing with an average spontaneous discharge of about 1 Hz. Optogenetic activation of VTA GPN axons was sufficient to transiently silence tonic activity *in vitro*, which was followed by rebound excitation (Fig. 3a). The pause–rebound was abolished in the presence of the $GABA_A$ receptor blocker bicuculline but remained unaffected by either dopamine or acetylcholine (ACh) receptor antagonists (Fig. 3a and Supplementary Fig. 2). We then aimed an optical fibre at the VTA of anaesthetized mice and delivered a light stimulation protocol that was efficient in driving action potentials in VTA GABA neurons *in vivo* (Supplementary Fig. 3) and mimicked physiological activity of these cells (for example, in response to foot shock[4]). When recording from NAc CINs during this same protocol (Fig. 3b), we found that blue light was sufficient to silence spontaneous CIN activity for the entirety of the stimulation (Fig. 3c, e). Again, this pause was followed by rebound excitation, similar to the CIN response observed in the dorsal striatum of behaving monkeys[9]. To verify the accuracy of the electrophysiological criteria used to identify CINs *in vivo* (Supplementary Fig. 4[18]), we labelled a number of these recorded neurons with the juxtacellular method to later confirm ChAT expression (Fig. 3d). Together, these data demonstrate that VTA GPN activation is sufficient to pause spontaneous firing of NAc CINs through $GABA_A$ receptor-mediated inhibition.

In correlative studies using non-human primates a 'conditioned pause response' of CINs in the dorsal striatum develops as animals learn about a stimulus (for example, a light or tone) that predicts an important outcome (for example, a punishment or a reward[8–12]). The appearance of the conditioned pause requires several stimulus–outcome pairings and follows a time course that mirrors the occurrence of stimulus-guided anticipatory responses (for example, licking at the spout where a liquid reward will be delivered[19]). Given the role of the ventral striatum in associative learning[1], we predicted that activation of VTA GPNs to force a pause response in NAc CINs from the very first stimulus–outcome pairing would facilitate learning about the stimulus–outcome association.

To test this we combined a discrete cue and contextual conditioning task with optogenetically generated pause responses of NAc CINs (Fig. 4a) and compared learning between GAD-Cre$^+$ and GAD-Cre$^-$ mice, both of which were injected in the VTA with AAV-ChR2-eYFP. Mice were first trained across two successive days to associate an auditory cue (conditioned stimulus, CS+; for example,

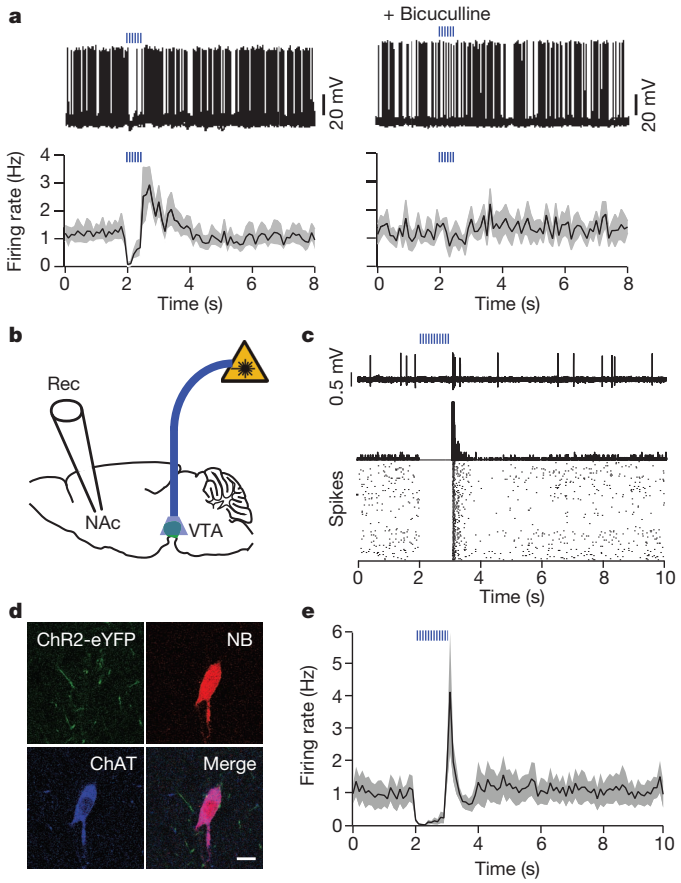

Figure 3 | Pausing NAc CINs through selective VTA GABA projection neuron activation. **a**, Twenty superimposed traces of an example CIN recording *in vitro* in whole-cell current-clamp configuration (top left) shows the blocking of spontaneous action potential firing by VTA GABA axon activation (4-ms pulses, 20 Hz, 0.5 s). This inhibition was often followed by a rebound excitation, as seen in the average response of all recorded neurons (bottom left, $n = 14$). Bath application of 10 μM bicuculline abolished this inhibition, as shown in the example cell (top right) and the averaged neuronal response (bottom right, $n = 6$). **b**, Schematic showing implantation of a fibre optic through a guide cannula in the VTA and *in vivo* recordings in the NAc. **c**, Single-unit recording of a neuron inhibited by the light-flash protocol (4-ms pulse, 20 Hz, 1 s) evident in the single sweep (top), the summed peri-stimulus time histogram (PSTH) (middle, 5-ms bins) and the raster plot of all sweeps (bottom, 5-ms bins). **d**, This neuron was labelled with the juxtacellular method and later confirmed as cholinergic using immunohistochemical processing. **e**, Average responses (mean ± s.e.m., 100-ms bins) of all recorded NAc CINs, some of which were labelled and later confirmed as CINs ($n = 4$ of 11; see also Supplementary Fig. 4).

white noise) with an aversive outcome (a brief foot shock). Mice were also trained with a second stimulus (CS−; for example, pure tone), but had no associated outcome. A light guide aimed at the NAc (Supplementary Fig. 5) was used to stimulate VTA GABA projections, which would induce a pause response in CINs during each CS+ presentation (20 Hz of 10-ms pulses for a duration of 300 ms, starting 80 ms after CS+ onset), reproducing the conditioned pause that is known to develop to salient stimuli[9]. During training, GAD-Cre$^+$ and GAD-Cre$^-$ mice developed freezing behaviour, but this did not differ between groups (Fig. 4b). Freezing to the context where foot shocks were delivered was also evident in both groups, but again did not differ between groups (Fig. 4c). Thus, mice were able to learn that the training environment was associated with an aversive event, but forcing a pause of NAc CINs did not affect the associative strength of contextual cues. After training, we placed the mice in a new context and, in the absence of further foot shocks or optogenetic manipulation, asked what had been learned about the CS+ and CS− presentations by measuring freezing responses to the two stimuli. Because multiple stimulus–foot-shock pairings were trained, GAD-Cre$^-$ mice showed generalized freezing, being unable to discriminate between the CS+ and the CS− stimuli (Fig. 4d). Notably, GAD-Cre$^+$ mice were able to discriminate effectively between the two stimuli, showing higher levels of freezing to the CS+ than the CS– stimuli. Forcing a pause of NAc CINs during the learning of a stimulus–outcome association therefore enhanced detection of the CS+ as the motivationally important stimulus, allowing GAD-Cre$^+$ mice to respond appropriately to the CS+ and avoid generalizing to the CS− during the subsequent test session.

To examine the possibility that antidromic activation of VTA GABA neurons could inhibit dopamine neurons, thereby influencing our behavioural findings, we first recorded VTA GABA neurons while activating terminals in the NAc. Antidromic action potentials were found in 1 out of 13 GABA neurons of the VTA (Supplementary Fig. 3). Because even a small fraction of interneurons could inhibit dopamine neurons via divergent collaterals, we also monitored VTA dopamine neuron firing while shining light into the NAc. A total of 6 out of 23 dopamine neurons showed responses ranging form inhibition to excitation (Supplementary Fig. 6). Importantly, however, the average dopamine neuron response did not significantly differ from baseline activity. We also performed a behavioural experiment to ensure that blue-light stimulation was not aversive per se, by replacing the foot shock with blue light (Fig. 4e). Contextual and cued freezing did not differ between GAD-Cre$^+$ and GAD-Cre$^-$ mice, and freezing was not controlled by cue presentations (Supplementary Fig. 7). Taken together, these data show that GPN activation in the NAc does not produce an aversive state.

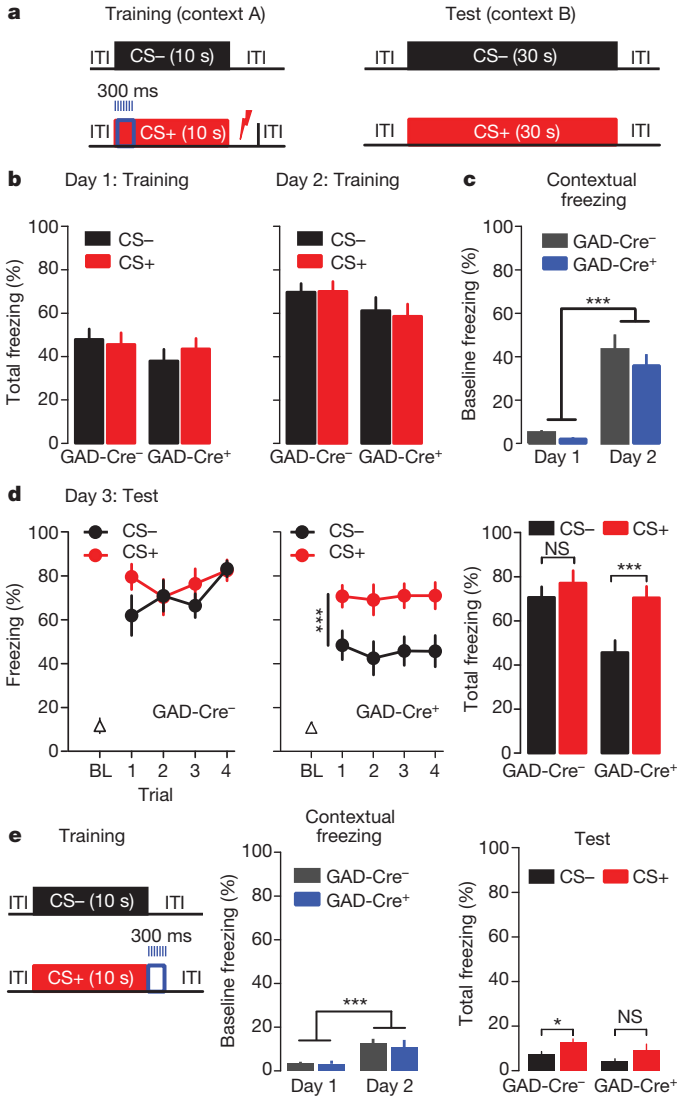

Figure 4 | Activation of VTA GPN axons in the NAc enhances stimulus–outcome learning in an aversive paradigm. **a**, For training, GAD-Cre$^+$ ($n = 15$) and GAD-Cre$^-$ ($n = 12$) mice were presented with an auditory stimulus (CS+, 10 s) followed by a foot shock (2 s, 0.6 mA) and a second auditory stimulus (CS−, 10 s) associated with no outcome (context A). Each stimulus trial was separated by a variable inter-trial interval (ITI; mean = 100 s). 80 ms after CS+ onset, the blue-light laser was pulsed (300 ms, 20 Hz) to activate VTA GPN axons in the NAc. For the test, stimulus-generated freezing responses were assessed in a new context (context B). **b**, On both training days, GAD-Cre$^-$ and GAD-Cre$^+$ mice showed equal levels of freezing to the CS− and CS+ presentations. **c**, Contextual freezing was assessed by comparing baseline freezing (%) between the two training days. Contextual freezing was evident (day, $^{***}P < 0.001$), but did not differ between GAD-Cre$^+$ and GAD-Cre$^-$ mice. **d**, In the test, GAD-Cre$^-$ mice froze equally to the CS+ and CS− presentations across trials (left panel). This response pattern differed in GAD-Cre$^+$ mice (stimulus × group, $P < 0.01$; group, $P < 0.05$), which discriminated between the two stimuli across trials (stimulus, $^{***}P < 0.001$; middle panel). A comparison of total freezing (%) to the stimuli confirmed that GAD-Cre$^+$ mice, but not GAD-Cre$^-$ mice, discriminated between the CS+ and CS− (post-hoc, two-tailed t-test, $^{***}P < 0.001$; right panel). NS, not significant. **e**, In a control experiment, GAD-Cre$^+$ ($n = 7$) and GAD-Cre$^-$ ($n = 7$) mice received blue-light stimulation in place of a foot shock during training (left panel). Freezing increased between training days (day, $^{***}P < 0.001$), but did not differ between GAD-Cre$^+$ and GAD-Cre$^-$ mice (note that contextual freezing responses were lower compared with mice that received foot shocks). In the test (right panel), freezing during CS+ and CS− presentations did not differ between GAD-Cre$^-$ and GAD-Cre$^+$ mice. In GAD-Cre$^-$ mice, freezing was increased during CS+ versus CS− presentations (post-hoc, two-tailed t-test, $^{*}P < 0.05$; see also Supplementary Fig. 7). Error bars show s.e.m.

The pathways responsible for CIN pausing in the dorsal striatum and NAc may be different[15,20,21]. For example, a well-established connection between certain thalamic nuclei and CINs[20] can drive a pause after an initial excitation burst[15]. This burst–pause depends on dopamine and ACh receptor activation, which was not the case in the NAc (Supplementary Fig. 2) in line with anatomical reports that thalamic inputs to the NAc preferentially target MSNs[22]. Moreover, the responses of CINs recorded in behaving animals are heterogeneous; often a CIN pause occurs in the absence of any initial excitation, but is nearly always followed by rebound excitation[9]. The direct inhibition that we observe between the VTA and NAc could reflect a common route between the midbrain and striatum to explain such CIN pause responses.

The downstream effects of pausing CIN activity on accumbal processing are not fully known, but may provide a salience window in which temporally coincident dopamine signals can influence accumbal neurons[23]. Notably, a rebound excitation was observed immediately after light-induced silencing of CIN activity in both our *in vitro* and *in vivo* preparations, which mirrors that observed in electrophysiological recordings of CINs in the dorsal striatum of non-human primates learning stimulus–outcome associations[8–12] and after direct inhibition of NAc CINs with halorhodopsin[14]. The rebound excitation of CINs may directly stimulate dopamine release[24,25], and is likely to form a crucial part of the intra-NAc processing of information[14].

The present study examined learning related to an aversive stimulus–outcome association. Studies in monkeys suggest that CINs pause in response to a cue that predicts a salient event, regardless of the valence[10]. Moreover, some VTA GABA neurons are activated in similar conditions[17]. We now show that the VTA GPNs and NAc CINs are connected, which makes it likely that appetitive learning may also be under the control of the VTA GPN–NAc CIN projection. A wealth of literature has demonstrated the importance of NAc cholinergic tone in regulating appetitive behaviours for both food[26] and drug reward[27,28]. However, as activation of VTA GPNs in the NAc does not alter reward consumption in mice[5], the function of the pathway may be specialized for learning about stimuli that predict rewarding events.

Disruption of saliency processing could result in failure to isolate and recognize motivationally relevant stimuli efficiently, which may cause responses to develop to non-relevant signals (that is, stimulus generalization). Alternatively, perturbed processing could result in some stimuli becoming overly salient, pulling attention away from otherwise important signals. Both possibilities could feature in neuropsychiatric disorders, for example in generalized anxiety in post-traumatic stress disorder or in drug seeking and relapse in addiction. There is already evidence to show that VTA GABA neurons are affected by drug experience[29,30], and it will be of great importance to understand how traumatic events or drug exposure may cause neuroadaptive synaptic plasticity of GABA transmission onto CINs.

We identify a selective projection from VTA GPNs onto NAc CINs. Activation of this projection is sufficient to pause ongoing CIN activity through direct inhibition and thus modify behavioural responses to conditioned stimuli, enhancing contrast between cues with differing motivational relevance. In addition to their established role in controlling dopamine function, our anatomical and functional analysis demonstrates that VTA GABA neurons also control the cholinergic system of the NAc.

METHODS SUMMARY

All experiments were reviewed by the institutional ethics committee and approved by the relevant authorities of the Canton of Geneva. GAD-Cre mice were injected bilaterally into the VTA with AAV-floxed-ChR2(H134R)-eYFP (200–500 nl), and fTom^{PVALBcre} mice injected with a non-floxed construct, using standard surgical procedures. Mice were perfused using 2% paraformaldehyde (containing 0.2% glutaraldehyde for electron microscopy experiments). Immunohistochemical procedures on 50 µm sections were used to reveal immunoreactivity for ChAT, visualized with CY3 on a confocal microscope. Pre-embedding immunoperoxidase staining with DAB revelation was performed for ChAT and GFP, on ultrathin (60 nm) NAc sections. Images were taken at ×3,900–46,000. For *in vitro*

experiments, acute slices were prepared as previously described[4] and whole-cell patch clamp recordings performed during varying blue-light stimulation protocols. For *in vivo* electrophysiology experiments, mice were anaesthetized and placed in a stereotactic frame with a fibre optic aimed at the VTA. Single-unit recordings of CINs were performed in the NAc during blue-light VTA stimulation and in some cases the neuron was labelled with 1.5% neurobiotin in the recording electrode (the juxtacellular method) to confirm its identity. Auditory cued and contextual conditioning was performed in AAV-floxed-ChR2(H134R)-eYFP-injected GAD-Cre^{+} and GAD-Cre^{-} mice with fibre-optic implants directed at the NAc using standard surgical procedures. Freezing responses were continuously assessed using automated software.

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