Segregated cholinergic transmission in the ventral tegmental area

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Abstract
Dopamine neurons in the ventral tegmental area (VTA) receive cholinergic innervation from brainstem structures associated with either movement or reward. While cholinergic neurons of the pedunculopontine nucleus (PPN) carry an associative/motor signal, those of the laterodorsal tegmental nucleus (LDT) convey limbic information. Here we used optogenetic methods combined with in vivo juxtacellular recording and labeling to dissect the influence of brainstem cholinergic innervation of distinct subpopulations of neurons in the VTA. We found that LDT cholinergic axons selectively enhance the bursting activity of mesolimbic dopamine neurons that are excited by aversive stimulation. In contrast, PPN cholinergic axons activate and change the discharge properties of VTA neurons that are integrated in distinct functional circuits and are inhibited by aversive stimulation. While both structures conveyed a reinforcing signal, they had opposite roles in locomotion. Our results demonstrate that two modes of cholinergic transmission operate in the VTA and segregate neurons involved in different reward circuits.
Introduction

Dopamine neurons (DA) of the ventral tegmental area (VTA) are implicated in goal-directed behaviors and reinforcement learning. They change their discharge mode from tonic to phasic in response to sensory events that predict a reward outcome. This phasic activation produces synchronous bursts and encodes a prediction error signal which is crucial for reinforcement learning. Thus, the switch in the firing mode of DA neurons, triggered by excitatory drive, is critical for the expression of reward-oriented behavior.

The VTA receives excitatory inputs from several regions including the prefrontal cortex, amygdala, lateral hypothalamus, subthalamic nucleus and mesopontine tegmentum in the brainstem. While all of these afferent systems provide a glutamatergic input, the mesopontine tegmentum, composed of the pedunculopontine nucleus (PPN) and the laterodorsal tegmental nucleus (LDT), also provides the main source of cholinergic innervation of DA neurons. Previous reports have shown the involvement of glutamatergic mechanisms following PPN stimulation that lead to increases in the number of DA neurons bursting. Similarly, activating a predominantly glutamatergic projection from the LDT produces burst firing in DA neurons and elicits conditioned place preference in behaving rodents. However, less clear is the influence of cholinergic afferents over the activity of DA neurons in vivo. Nicotinic and muscarinic acetylcholine receptors are widely expressed in the VTA and their activation in brain slices causes depolarization and burst firing in DA neurons. Furthermore, behavioral experiments have consistently shown a prominent role for VTA acetylcholine receptors in goal-directed behavior and addiction, presumably through the activation of DA neurons. It is thus likely that cholinergic afferents, derived from the mesopontine tegmentum, play a role in tuning the activity of DA neurons in the VTA.

The PPN and the LDT share similar connections and neurochemistry but differ in the functional networks to which they contribute. Whereas PPN is connected to sensorimotor and associative structures, as well as those involved in the regulation of arousal, the LDT is connected to limbic systems. Because DA neurons are heterogeneous in terms of their firing
properties, connectivity and functionality \(^{21-26}\), it is likely that brainstem cholinergic pathways produce different effects within the VTA and differentially affect sub-populations of DA neurons.

In order to address these issues, we characterized the innervation of the VTA by PPN and LDT cholinergic neurons by retrograde and anterograde labeling. Using an optogenetic approach in ChAT::Cre+ rats combined with \textit{in vivo} juxtacellular single cell recording and labeling, microiontophoretic drug delivery, and retrograde labeling, we characterized the effect of the activation of PPN or LDT cholinergic afferents on the spontaneous activity of identified DA and non-DA neurons in the VTA. We then identified the targets of a subset of DA neurons and correlated their responses to cholinergic modulation with the functional circuit in which they participate. Finally, we characterized the functional role of these cholinergic afferents in a reward-related behavior and in locomotion.

\textbf{Results}

\textbf{Cholinergic neurons modulate the activity of DA and non-DA neurons of the VTA}

In order to define topographical relationships between cholinergic neurons of the brainstem and the VTA we first made deposits of two retrograde tracers in the VTA (rostral and caudal) to define the topographical organization of the brainstem cholinergic innervation of the VTA. We observed that cholinergic neurons of the caudal PPN and LDT innervate both rostral and caudal regions of the VTA, arising from similar numbers of cholinergic neurons in the PPN and LDT (\textbf{Supplementary Fig. 1} and \textbf{Supplementary Table 1}). Next we used a transgenic rat line that expresses Cre recombinase under the choline acetyltransferase (ChAT) promoter (ChAT::Cre+; \(^{27}\)). We stereotaxically injected into the PPN or LDT an AAV vector that incorporated a transgene coding for a fluorescent reporter (enhanced yellow fluorescent protein, eYFP) and a light-activated ion channel (channelrhodopsin-2, ChR2) (AAV2-EF1a-DIO-hChR2-YFP; \textbf{Fig. 1a} and \textbf{2a}). Appropriate controls were used to verify the specificity of
the vector for cholinergic neurons both in the brainstem and other brain regions using a vector that encodes only the fluorescent reporter (Supplementary Fig. 2 and Supplementary Table 2; see online Methods). In addition, we injected the same vector in each cholinergic cell group (Ch1-Ch8) and confirmed that only PPN and LDT provide cholinergic innervation of the VTA (Supplementary Fig. 3). In both PPN- and LDT-injected rats, we detected fluorescently-labeled axons in the VTA, the borders of which were defined by the distribution of tyrosine hydroxylase-immunopositive (TH+) neurons (Fig. 1b and 2b). Labeled axons were mapped across the full extent of the VTA and showed a relatively homogeneous distribution whether derived from the PPN or LDT, although LDT axons had a greater overall length than PPN axons (albeit not significant; Supplementary Fig. 4) and with an area of higher density in the dorsal part of the parabrachial pigmented area (Fig. 1c and 2c). Consecutive sections were processed to reveal the YFP by a permanent peroxidase reaction product and processed for electron microscopy to characterize the synapses formed by YFP-expressing, cholinergic axons. Gray’s Type 1 synapses (asymmetrical) were formed by PPN cholinergic axons with DA dendrites, whereas LDT cholinergic axons formed asymmetrical synapses with both DA and non-DA dendrites (40/60%, respectively). In contrast, Gray’s Type 2 synapses (symmetrical) from both structures were formed predominantly in non-DA structures (80% for PPN and 100% for LDT). Furthermore, the synapses derived from the PPN made contacts with a larger proportion of DA processes (Fig. 1d and 2d), whereas those derived from the LDT showed a greater preference for non-DA processes (Fig. 1e and 2e). The combined results from the anatomical characterization thus reveal that cholinergic axons originating in the PPN and LDT are intermingled within the VTA and contact both DA and non-DA neurons. In addition, while the number of VTA-projecting cholinergic neurons is similar between PPN and LDT, there is an indication from the axonal mapping that the latter has a higher level of collateralization that gives rise to a larger number of synaptic contacts with non-DA neurons. Next we tested the effect of optogenetic stimulation of the brainstem cholinergic axons on neurochemically identified VTA neurons recorded in vivo and juxtacellularly labeled. Following
the post hoc histological identification of the recorded neurons, we classified them as DA (TH+; \( n = 60 \)) or non-DA (TH-; \( n = 36 \)) ([Fig. 1f, 1g, 2f and 2g]). The firing rate and pattern of each neuron during the light stimulation was compared to their firing characteristics during the preceding baseline period (10 s). Two to four trials were carried out for each neuron and combined to calculate the average response ([Supplementary Fig. 5]; see online Methods). Following light stimulation of cholinergic axons from either the PPN or LDT we observed both excitatory and inhibitory responses in both DA and non-DA neurons, although the proportions varied slightly. No responses were observed when cholinergic neurons were transduced only with the fluorescent reporter ([Supplementary Fig. 6]). Stimulation of axons from the PPN produced predominantly excitation in responding DA neurons ([Fig. 1h]), while only a small fraction showed inhibition. This activation was maintained throughout the period of the laser stimulation. The excitation of responding non-DA neurons followed similar patterns of activation during the stimulation period however a larger proportion of non-DA neurons were inhibited ([Fig. 1i]). The stimulation of LDT cholinergic axons also produced predominantly excitation in responding DA neurons, but the proportion of responding neurons was larger than following PPN axon stimulation (50% vs 38%; [Fig. 2h]). In contrast to PPN axon stimulation, we observed a proportion of ‘late-responding’ neurons, whose firing rate increased once the light stimulation stopped. The effects of stimulation of LDT cholinergic axons on non-DA neurons were more variable: the excitation was not as prominent as for PPN axon stimulation and a slightly larger proportion of non-DA neurons were inhibited ([Fig. 2i]).

The activation of DA neurons showed a slow response that increased as a function of the number of pulses and reached its maximum towards the end of the light stimulation period ([Fig. 3]). These dynamics were observed following both PPN- ([Fig. 3a]) and LDT-cholinergic axon stimulation ([Fig. 3b]), but the magnitude of the response was slightly greater for the PPN axon stimulation, albeit not significant (see legend). This contrasts with the short-latency responses observed in nigral neurons following electrical stimulation of the PPN output \(^9,^{28,29}\).

In a separate set of experiments we tested the effects of in vivo electrical stimulation of the
PPN/LDT region on identified VTA DA and non-DA neurons. In line with the above reports, we observed short-latency excitatory and inhibitory responses in DA and non-DA neurons in the VTA (Supplementary Fig. 7). This suggests that electrically stimulating the output of the PPN/LDT leads to a combined response mediated by glutamatergic, GABAergic and cholinergic transmission, whereas the optogenetic manipulation dissects out the cholinergic terminals.

In order to ensure that the responses to light stimulation were mediated by released acetylcholine, in a separate set of experiments we attached a glass pipette for the microiontophoresis of drugs to the juxtacellular recording electrode (Fig. 4a). This allowed us to test in vivo the local effects of acetylcholine receptor antagonists on the responses of individual VTA neurons to the stimulation of cholinergic axons from either the PPN or LDT. The excitatory responses to the laser in both DA (Fig. 4b) and non-DA neurons following the stimulation of either PPN and LDT cholinergic axons were abolished during the iontophoretic administration of nicotinic and muscarinic antagonists in the vicinity (~100µm above) of the recorded neurons (Fig. 4c, 4d and 4e), and were quickly reversed within a few minutes of stopping the microiontophoretic current for the drug delivery. In order to further identify the acetylcholine receptors involved, we also performed ex vivo whole-cell recordings of VTA neurons and measured the response to the local application of the cholinergic agonist carbachol. We observed excitatory responses in DA neurons that were eliminated following the administration of mecamylamine but not methyllycaconitine or DHβE, suggesting a mechanism mediated by nicotinic type III receptors and independent of GABAergic and glutamatergic transmission (Supplementary Fig. 8). These results thus demonstrate that the observed effects on VTA neurons following stimulation of the cholinergic axons are a consequence of the release of acetylcholine and not the release of glutamate or GABA. The specificity of the viral expression for cholinergic neurons is further supported by the absence of glutamate vesicular transporter-2 in PPN/LDT axons expressing the fluorescent reporter within the VTA (Supplementary Fig. 9). Thus, in contrast to the short-latency excitatory
(presumably glutamatergic) effects of the electrical stimulation, our findings demonstrate a slow cholinergic modulation of VTA DA neurons arising from both PPN and LDT.

**Bursting activity is enhanced by LDT stimulation**

In anesthetized rats, DA neurons fire in regular, irregular or bursting mode. Here we analyzed the spike trains of DA neurons that showed bursting activity during the baseline and/or during optogenetic stimulation of PPN and LDT cholinergic axons. We detected a switch from non-bursting to bursting mode and *vice versa* only following PPN stimulation. Thus, 27% of neurons that showed bursting activity during the stimulation, did not have any bursts during the baseline (*Fig. 5a, left*). On the other hand, 18% of neurons that were spontaneously bursting during the baseline stopped bursting during the stimulation. This contrasts with LDT axon stimulation that did not elicit any switch to or from bursting activity. Nevertheless, following LDT axon stimulation, neurons that were already firing in bursts during baseline increased their bursting activity (*Fig. 5a, right*), detected as an increase in the number of bursts episodes (*Fig. 5b*) and a higher burst probability (*Fig. 5c*). Accordingly, we observed a tendency for an increase in the proportion of spikes within bursts only after LDT axon stimulation (*Fig. 5d*). In addition, stimulation of LDT axons led to a decrease in the number of inter-burst spikes when compared to PPN stimulation ($U = 41$, Mann-Whitney; $P = 0.027$; PPN $n = 13$; LDT $n = 13$; only neurons bursting during laser stimulation). Further differences between PPN and LDT effects were observed: whereas LDT axon stimulation decreased the ratio of spikes outside bursts to spikes inside bursts (decreasing the burst entropy) in the majority of cases, PPN stimulation tended to produce the opposite effect, disrupting the burst organization (*Fig. 5e*). These data suggest that the effect of PPN cholinergic axon activation is heterogeneous, such that it is able to switch the activity of some DA neurons to bursting mode while disrupting the burst organization in DA neurons that were already bursting. On the other hand, LDT cholinergic axon stimulation reorganizes the spiking into bursts. These data
show that cholinergic neurons in the PPN and LDT modulate the activity of DA neurons in the VTA by different mechanisms.

**Mesolimbic DA neurons are selectively activated by LDT cholinergic axons**

Since cholinergic neurons of the PPN and LDT are components of functionally distinct forebrain circuits, we tested the hypothesis that they may innervate functionally distinct subsets of VTA neurons. We injected a tracer into the nucleus accumbens (NAcc) shell to retrogradely label the so-called, mesolimbic neurons in the VTA (Fig. 6a). These injections led to labeling predominantly in the dorsal half of the VTA (Fig. 6b) and included both DA and non-DA neurons. We obtained a sample of 17 DA and 6 non-DA neurons that were recorded and labeled using the juxtacellular method and in which the retrograde tracer was also detected (Fig. 6c and 6d). We observed that mesolimbic DA neurons had a greater excitatory response following LDT axon stimulation than following PPN axon stimulation (Fig. 6e and 6f). Those DA neurons that increased their firing rate following PPN axon stimulation lacked the retrograde tracer (Fig. 6e), suggesting that they innervate a subset of neurons that project to other targets of VTA. In contrast, mesolimbic non-DA neurons were inhibited by LDT, but not PPN, axon stimulation (Fig. 6g).

**Cholinergic axons differentiate between DA neurons involved in distinct functional circuits**

Midbrain DA neurons have been classically associated with reward mechanisms 1, 4. While the majority of them increase their firing in response to motivating stimuli and are consequently inhibited by aversive stimuli, a proportion of them are excited by noxious (aversive) stimuli 30, 31. This can be emulated in the anesthetized rat by a hind-paw pinch 32 or foot-shock 23. Such differences in responses have been proposed to be associated with the functional pathways in which dopamine neurons are integrated 11, 22. From all the neurons that were activated by
the aversive stimulation, we identified nearly half of them as NAcc-projecting (47%, 7 out of 15), which contrasts with the lower of number of neurons inhibited by the stimulation that were identified as NAcc-projecting (27%, 7 out of 26). We then correlated the responses of DA neurons to the hind-paw pinch with their responses to the optogenetic activation of the cholinergic axons arising in either the PPN or the LDT (Supplementary Fig. 10). We observed an opposite trend in the effect of PPN and LDT drive: whereas PPN axon stimulation tended to modulate more consistently those neurons that showed a greater inhibition to the pinch (Fig. 7a), LDT axon stimulation more robustly modulated the neurons that were excited by the pinch (Fig. 7b). Indeed, 75% (6/8) of aversive stimuli-excited DA neurons increased the number of spikes within the bursts during LDT axon stimulation, in contrast 85% of aversive stimuli-excited DA neurons (6/7) decreased their number of spikes within bursts or switched to non-bursting mode during PPN axon stimulation. These results further support the notion that subsets of DA neurons that receive cholinergic afferents from the PPN and LDT are organized into functionally distinct pathways arising in the VTA.

**Cholinergic neurons convey reinforcing signals but dissociate locomotor effects**

Neurons of the PPN and LDT have been proposed to facilitate reward-related behavior through the modulation of midbrain DA neurons \(^{33,34,35}\). In addition, activation of DA neurons elicits locomotion presumably mediated by dopamine release in striatal circuits \(^{36}\). We tested the consequences of differentially activating cholinergic axons in freely-moving rats with chronically implanted optic fibers above the VTA in an open field and during an instrumental lever-pressing task (Fig. 8a). Optogenetic activation of cholinergic axons from PPN and LDT in an open field produced contrasting effects: PPN transiently increased motor activity whereas LDT decreased locomotion (Fig. 8b). However, the overall effect from LDT stimulation facilitated movement, reflected by the increased cumulative distance over the full testing session. PPN stimulation did not produce a cumulative effect on locomotion (Fig. 8c). All the locomotor effects elicited by optogenetic stimulation were abolished by the systemic
administration of acetylcholine receptor antagonists (as used in our previous experiments; Supplementary Fig. 11). Separately, rats were trained to lever-press in order to obtain a sugar pellet. Following the acquisition, sugar pellets were replaced by optogenetic stimulation of cholinergic axons during lever presses. Activation of either PPN or LDT cholinergic axons was sufficient to significantly slow-down lever-press extinction compared to wild-types (Fig. 8d). Interestingly, post hoc analysis revealed that PPN and LDT groups were not different from each other. These results suggest that while both PPN and LDT cholinergic neurons produce similar effects during reinforcement, they induce distinct motor effects that may reflect the differential output of DA neurons.

Discussion

In this study we demonstrate that two brainstem cholinergic nuclei, one typically associated with motor/arousal functions and the other with reward, have differential effects on subsets of neurons in VTA. First, we observed that cholinergic neurons of the PPN and LDT project extensively throughout most of VTA. Second, stimulation of either of the two cholinergic pathways produces a slow modulation of the firing rate of DA and non-DA neurons; effects that are mediated by acetylcholine. Third, cholinergic modulation of DA neurons takes on two different forms depending on the source of innervation: PPN axon stimulation switches the firing pattern to bursting mode in a proportion of DA neurons and increases the level of entropy in the spike train of most neurons, whereas LDT axon stimulation enhances the organization in bursting spike trains. Fourth, LDT axons predominantly target mesolimbic DA neurons that are excited by aversive stimulation, whereas PPN axons target a distinct subset of DA neurons which are predominantly inhibited by aversive stimulation. Fifth, PPN axons transiently induce motor activity whereas LDT axons have the opposite effect. Our findings thus suggest that functional segregation of brainstem cholinergic neurons is maintained at the level of the VTA and is underpinned by differential modulation of sub-populations of DA and non-DA neurons.
**Functional microcircuits in the VTA**

Dopamine neurons receive excitatory afferents from several regions of the brain including the prefrontal cortex, the lateral hypothalamic and lateral preoptic area, and the brainstem. The projections originating in the PPN and LDT are heterogeneous and consist of cholinergic, glutamatergic and GABAergic components. Although the PPN and the LDT are structurally and neurochemically similar, and indeed share some of their afferent and efferent connections, they differ in the functional circuits to which they contribute. For example, the PPN is connected to structures involved in motor (including most regions of the basal ganglia) and arousal functions. On the other hand, LDT is connected to cortical and thalamic regions associated with the limbic system. The VTA, in turn, is heterogeneous, with distinct distributions of dopamine neurons that project to different targets involved in different functional pathways. We thus hypothesized that the two cholinergic pathways would have different effects in the VTA and possibly differentially target subpopulations of DA neurons. The anatomical data shows that the projections from both the PPN and LDT innervate most of the VTA and indeed, DA and non-DA neurons that were modulated by PPN axons were intermingled in the same VTA regions as those modulated by LDT axons. Nevertheless, LDT cholinergic axons selectively targeted mesolimbic DA neurons, whereas PPN axons had little influence on them. In contrast, PPN axons primarily modulated DA neurons that are components of different circuits and whose targets are yet to be determined (e.g. amygdala, hippocampus). This suggests that neighboring DA neurons can be differentially modulated by cholinergic afferents that encode either motor or limbic signals. The motor - limbic segregation is maintained in other brainstem cholinergic targets, most notably the striatum: cholinergic LDT neurons that innervate the NAcc send collaterals that innervate the midline thalamus and the VTA, both of which in turn also project to the NAcc. This suggests that the cholinergic LDT neurons that modulate mesolimbic DA neurons also
target postsynaptic structures in the NAcc and potentially converge with the axons of the same mesolimbic DA neurons that they modulate within the VTA.

We also observed that cholinergic LDT axons show a higher degree of collateralization in the VTA and contact a higher proportion of non-DA neurons than PPN cholinergic axons. Thus, LDT cholinergic neurons may have a greater influence on non-DA neurons than PPN cholinergic neurons. This suggestion may be consistent with some of our observations in the LDT optogenetics experiments: first, the ‘late-responding’ DA neurons (Fig. 2h) may represent a rebound excitation following the excitation of GABAergic interneurons, and second, mesolimbic non-DA neurons (putative GABAergic) that are inhibited by cholinergic activation (Fig. 6d) may act in coordination with DA neurons to reinforce dopamine transmission in the NAcc (e.g. by inhibiting cholinergic neurons in NAcc 45). Overall, our data demonstrate multiple functional mechanisms by which the cholinergic brainstem neurons may influence the activity of limbic circuits.

The involvement of cholinergic brainstem neurons in salience and reward

Cholinergic neurons of the brainstem have been conceptually associated with the reticular activating system. Sensory stimulation triggers a phasic activation of PPN cholinergic neurons that, in turn, increase the responsiveness of their target neurons enabling them to bind other modalities of stimuli. This suggests a role in salience, where cholinergic neurons signal the presence of potentially relevant cues that in turn, increase the level of behavioral arousal. Recent evidence, however, points to a more specific role of the PPN in coding reward and reward prediction error suggesting that PPN neurons also encode motivational value. Our data support the notion that both PPN and LDT provide a reinforcing signal to DA neurons. Nevertheless, cholinergic neurons only constitute a fraction of the PPN and LDT, and indeed glutamatergic neurons may also modulate the activity of DA neurons, either directly or indirectly through cholinergic neurons. Glutamatergic neurons, however, have different
connectivity and dynamic properties to cholinergic neurons, suggesting that they are likely to have different, if not complementary, effects on VTA neurons.

Our findings also demonstrate that activation of brainstem cholinergic axons changes the bursting behavior of DA neurons. Activation of PPN axons produced bursting in some neurons while increasing the ratio of spikes outside bursts in the majority of responding DA neurons. In contrast, LDT afferents reorganized the spike train into bursts. These differences may give clues to the specific functions of these cholinergic neurons. By switching the discharge mode of DA neurons, PPN afferents may be triggering a state change where neurons disengage from their preceding activity and increase their responsiveness to other inputs, in line with the notion of both, an arousal system that generates orienting or attentional responses, and a motor system that facilitates motor output, as observed in our experiments. In contrast, by increasing the number of spikes within bursts, the LDT increases the amount of information contained within each burst, equivalent to increasing the value associated with a reward prediction. Stimulation of LDT axons produced an overall kinetic effect that is likely the result of inducing dopamine release from mesolimbic neurons, triggered by a slow dynamics that resemble the time course in the response of DA neurons (Fig. 2h). Interestingly, we detected a fast and transient inhibitory effect on motor activity that may be associated with a shorter latency in the response of non-DA neurons to cholinergic activation; an observation that deserves further investigation. Thus, the differences observed here may underlie the neuronal basis of saliency and reward at the level of the cholinergic brainstem and VTA neurons (see also).

In summary, cholinergic neurons of the brainstem provide a functionally-segregated modulation of DA and non-DA neurons of the VTA, consistent with their connectivity with other structures within motor and limbic circuits in the basal ganglia and thalamus. Our findings thus demonstrate the importance of the cholinergic inputs for the modulation of DA neuron function. It remains to be established how brainstem cholinergic neurons work in concert with brainstem
glutamatergic neurons at the level of both the midbrain and the striatum to shape behavior and to determine an organism’s response to reward-related stimuli.

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Figure Legends

Figure 1. Optogenetic activation of PPN cholinergic axons modulates DA and non-DA neurons of the VTA. (a) An AAV vector (AAV2-DIO-E1Fa-YFP-ChR2) was injected into the caudal PPN of ChAT::Cre+ rats. (b) YFP-positive axons were detected in the VTA and (c) mapped using 250-µm² grids (n = 6). (d, e) PPN cholinergic axons (b, bouton) make synaptic contacts (arrows) with both TH+ and TH- dendrites (d). (f) Individual TH+ neurons (n = 34) were recorded in vivo during optogenetic stimulation of PPN axons (8 s, 10 Hz, 50 ms pulses) and were subsequently labeled with neurobiotin. (g) The same protocol was followed for TH- neurons (n = 19). (h) Normalized firing rate (z-score along the whole trial period) for each TH+ neuron around the laser stimulation in the VTA showing three categories of neurons: excited (E, 38%), non-responsive (NR, 56%), and inhibited (I, 6%) (cluster based permutation test, P < 0.05; 200 permutations). (i) Normalized firing rates of TH- neurons show similar proportions in each group in relation to their response (E, 37%; NR, 47%; I, 16%). Scale bars (in µm): b, 70; d and e, 0.5; f and g, 50.
Figure 2. Optogenetic activation of LDT cholinergic axons modulates DA and non-DA neurons of the VTA. (a) Virus injections were delivered into the LDT. (b) YFP-positive axons were observed more frequently than in PPN-injected animals. (c) The total axonal length was higher for the LDT, suggesting a higher level of collateralization (n = 6; see Supplementary Fig. 3c for a comparison). (d, e) LDT cholinergic axons (b, bouton) make synaptic contacts (arrows) with TH- processes more often than with TH+ processes (d, dendrite). (f, g) Protocol for recording, stimulation (8 s, 10 Hz, 50 ms pulses) and labeling of TH+ (n = 26) and TH- (n = 17) neurons. (h) Neurons were separated into three categories according to their responses to the laser stimulation: excited (E, 50%), non-responsive (NR, 42%), and inhibited (I, 8%) (cluster based permutation test, $P < 0.05$; 200 permutations). (i) There was more variability in the responses of TH- neurons to the LDT stimulation (E, 35%; NR, 41%; I, 24%). Scale bars (in µm): b, 70; d and e, 0.5; f and g, 50.

Figure 3. Activation of cholinergic axons produces a slow and robust excitation of DA neurons. The normalized firing rate of all TH+ neurons that were excited by the laser stimulation show a similar slow modulation when cholinergic axons of either PPN (a; n = 15) or LDT (b; n = 15) were stimulated. The responses following PPN cholinergic axon stimulation were greater in magnitude, no significant differences were observed (cluster based permutation test, $P = 0.715$; 200 permutations). Data are depicted as mean ± CI.

Figure 4. Cholinergic antagonists block the response to laser stimulation in DA and non-DA neurons. (a) Individual TH+ and TH- neurons were recorded in vivo during optogenetic stimulation of PPN axons and local microiontophoretic administration of nicotinic and muscarinic antagonists (methyllycaconitine 20 mM, dihydro-β-erythroidine 40 mM, atropine 40 mM and mecamylamine 100 µM). (b) Neurons were subsequently labeled with
neurobiotin and their neurochemical profile identified. (c, i) Example of a neuron that was recorded during a baseline response to the optogenetic activation of LDT cholinergic axons. (ii) Following the iontophoretic application of the acetylcholine antagonist cocktail, the same laser stimulation failed to produce a response, but (iii) the responsiveness to the laser stimulation recovered following drug wash-out (2 min after). (d, e) Excitatory responses to laser stimulation of both PPN and LDT cholinergic axons were blocked in DA ($n = 14$; $F_{1, 12} = 21.3$, $P = 0.0006$, 2-way mixed ANOVA) and non-DA ($n = 12$; $F_{1, 9} = 20.26$, $P = 0.001$, 2-way mixed ANOVA) neurons following the administration of acetylcholine antagonist cocktail and recovered following wash-out. No significant effects in the axon source (PPN/LDT) factor or in the interaction (stimulation x source) were observed. Bars represent mean $\pm$ SEM. Scale bar in b: 50 $\mu$m.

**Figure 5. Laser stimulation of cholinergic axons modifies the bursting activity of DA neurons.** (a) DA neurons modified their bursting activity following optogenetic activation of PPN or LDT cholinergic axons. Red numbers represent the percentage of spikes within a burst before, during and after laser stimulation. While PPN stimulation tended to switch the pattern of activity of DA neurons, LDT axon stimulation did not change the bursting regime but increased the number of spikes within bursts (red) of already bursting neurons. (b) LDT axon stimulation significantly increased the number of bursts in those neurons already bursting when compared to the baseline ($F_{1, 12} = 7.18$, $P = 0.02$, 1-way RM ANOVA, $n = 13$) and to PPN axon stimulation ($U = 47.5$, $P = 0.02$, Mann-Whitney, $n = 15$). (c) Increased burst probability during LDT-axon stimulation ($t = 2.18$, t-test, $P = 0.039$). (d) LDT axon stimulation produced more spikes within bursts during the stimulation ($P = 0.06$) whereas PPN stimulation resulted in fewer spikes within bursts when compared to LDT ($t = 1.76$; $P = 0.09$). (e) Ratio of spikes outside:inside bursts during baseline and laser stimulation. During PPN axon stimulation, in all but 2 cases (light gray), there was a disruption in the bursting activity characterized by a larger number of spikes outside bursts. In contrast, during LDT axon
stimulation, in all but 2 cases (gray) there was an increase in the concentration of spikes within bursts \((P = 0.006\text{ between PPN and LDT})\). This change in the ratio was significantly different between PPN \((n = 15)\) and LDT \((n = 13; t = 2.72; P = 0.011)\). Group means are depicted in black.

**Figure 6. LDT cholinergic axons preferentially target mesolimbic DA and non-DA VTA neurons.** (a) Schematic of the experimental design. Fluorogold (FG) was injected into the nucleus accumbens (NAcc) of ChAT::Cre+ rats that also received a virus injection into the PPN or LDT. (b) FG-labeled neurons were observed throughout the VTA, most prominently in the dorsal regions. (c) Example of a FG+/TH+ neuron that was excited by LDT-axon stimulation. (d) Example of a FG+/TH- neuron that was inhibited by LDT axon stimulation. The basal firing rate and action potential duration of mesolimbic neurons was not significantly different to that of neurons that did not contain the tracer \((TH+, n = 43; TH-, n = 30; \text{basal firing rate: } TH+, U = 322, P = 0.854; TH-, U = 138, P = 0.495; \text{action potential duration: } TH+, U = 397.5, P = 0.649; TH-, U = 165.5, P = 0.171, \text{Mann-Whitney})\). (e) DA neurons that project to the NAcc were preferentially excited by the optogenetic stimulation of LDT cholinergic axons \((n = 11)\). In contrast, PPN-axon stimulation did not activate NAcc-projecting neurons \((n = 6; t = -1.84, \text{one-tailed } t\text{-test, } P = 0.04\text{ between PPN and LDT for NAcc-projecting neurons})\). Control experiments, in which animals were transduced with YFP only (no ChR2, green, \(n = 5\)), did not show a response to the laser. (f) Normalized firing rate (mean ± CI) of all TH+/NAcc-projecting neurons following PPN or LDT cholinergic axon stimulation. Black line in the bottom panel represents the time points during which response to LDT stimulation was significantly greater than PPN (cluster-based permutation test; \(P = 0.02, 200 \text{ permutations})\). (g) Non-DA neurons that project to the NAcc were inhibited by LDT axon stimulation but not by PPN axon stimulation. Black boxes represent means ± SEM of NAcc-projecting neurons in e, or only means in g. Scale bars (in µm): b, 500; c and d, 30.
Figure 7. Cholinergic axon stimulation differentially modulates functionally distinct DA neurons. (a, b) Significant correlations were observed between the change in the firing rate of DA neurons during the hindpaw pinch (aversive stimulus) and their responses to the laser activation of PPN (a; n = 25) and LDT (b; n = 19) axons. Thus, DA neurons that are more inhibited by the pinch tend to respond more to PPN stimulation (7 out of 13) and less to LDT stimulation (3 out of 11), whereas DA neurons that were excited by the pinch are more strongly modulated by the LDT (5 out of 8) and less by the PPN (1 out of 7). Means and SEM for positive or negative values in the change to the aversive stimulus are indicated by black (PPN) and white (LDT) circles with error bars.

Figure 8. Optogenetic activation of cholinergic axons in the VTA in behaving rats. (a) Cholinergic neurons of the PPN and LDT were transduced with YFP and ChR2, and an optic fiber was chronically implanted above the VTA. (b) Optogenetic activation of cholinergic axons in the VTA produced different effects on stimulation-locked locomotor activity (10Hz, 50ms, 80 pulses, 13 stimulations): PPN axon stimulation (n = 12) increased motor activity whereas LDT axon stimulation (n = 10) decreased it; no changes were observed in control animals (n = 10) (2-way ANOVA; stimulation effect $F_{(2, 58)} = 3.569, P = 0.035$; group effect $F_{(2,58)} = 0.325$, $P = 0.725$; interaction $F_{(4,58)} = 16.58, P < 0.001$; post hoc comparisons PPN vs WT: $P = 0.029$, PPN vs LDT: $P < 0.001$, LDT vs WT: $P = 0.044$). Asterisks represent significantly different time-points following laser stimulation based on a nonparametric cluster-based comparison ($P = 0.002$; $n = 500$ permutations). (c) Cumulative distance over a 30 min recording with pulses delivered every 2 min show significant differences for LDT, but not PPN, axon stimulation; control animals do not show any changes (1-way ANOVA: $F_{(2,31)} = 9.353, P = 0.001$; post hoc comparisons: PPN vs control: $P = 0.877$, PPN vs LDT: $P = 0.001$, LDT vs control: $P = 0.003$). A nonparametric, cluster-based t-test analysis ($n = 500$ permutations) shows that there is a significantly higher value for movement in LDT stimulated animals compared to control (green bar) or PPN (gray bar) animals ($P = 0.002$). (d) Following training in a Pavlovian lever-press...
task in a progressive random interval schedule (see also Supplementary Fig. 11), sugar pellets delivery was replaced by optogenetic stimulation of either PPN or LDT cholinergic axons. Control animals also received laser pulses. Conditioned extinction was recorded during 4 consecutive days. PPN and LDT cholinergic axons stimulation produced a slowing in extinction compared to the control group, as shown by a significantly higher number of lever presses in PPN and LDT groups (2-way ANOVA; day effect: $F_{(3, 27)} = 15.747$, $P < 0.0001$; group effect: $F_{(2,29)} = 13.781$, $P < 0.0001$; interaction: $F_{(6,54)} = 0.785$, $P = 0.586$; post hoc comparisons: PPN vs LDT: $P = 0.839$, PPN vs control: $P = 0.000392$, LDT vs control: $P = 0.000148$).

References


