Dorsal tegmental dopamine neurons gate associative learning of fear

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Functional neuroanatomy of Pavlovian fear has identified neuronal circuits and synapses associating conditioned stimuli with aversive events. Hebbian plasticity within these networks requires additional reinforcement to store particularly salient experiences into long-term memory. Here we have identified a circuit that reciprocally connects the ventral periaqueductal gray and dorsal raphe region with the central amygdala and that gates fear learning. We found that ventral periaqueductal gray and dorsal raphe dopaminergic (vPdRD) neurons encode a positive prediction error in response to unpredicted shocks and may reshape intra-amygdala connectivity via a dopamine-dependent form of long-term potentiation. Negative feedback from the central amygdala to vPdRD neurons might limit reinforcement to events that have not been predicted. These findings add a new module to the midbrain dopaminergic circuit architecture underlying associative reinforcement learning and identify vPdRD neurons as a critical component of Pavlovian fear conditioning. We propose that dysregulation of vPdRD neuronal activity may contribute to fear-related psychiatric disorders.

The brain uses predictors of important events to optimize future behavioral responses. Pavlovian learning pairs a stimulus with an emotionally salient experience to form emotional memories that can be stored for life. Deconstructing the neuronal basis of storage and recall of such associative memories and the underlying learning models promises insight into fundamental and biomedically relevant brain functions.

The primary neuronal representation of associations between conditioned stimulus (CS) and unconditioned stimulus (US), the CS–US pairing, is stored as synaptic memory traces in neuronal circuitry. While Hebbian plasticity (coinciding pre- and postsynaptic activity) accounts for the primary CS–US pairing, additional processes are required to link associative plasticity to particularly salient events and to the progress in learning itself. Reinforcement signals coupled to prediction errors (PE; a central element in learning models representing the discrepancy between the value of actual and predicted events) can serve that purpose. However, neuronal circuit motifs encoding all necessary components for associative learning, i.e., CS–US integration, PE-coupled reinforcement signals, and synaptic memory traces, remain largely uncharted.

In the mammalian brain, Pavlovian fear-related neuronal plasticity in the amygdala is the canonical model for storage of associative memory traces. Within the amygdala, the central amygdala (CE) operates as central hub that reshapes neural responses and synaptic connectivity during learning. In this regard, neurons in the lateral part of the CE (CEl) can be functionally divided into several classes of distinguishable inhibitory neurons that receive excitatory input from the basolateral amygdala (BLA). Fear learning leads to postsynaptically expressed long-term potentiation (LTP) of the input onto SST+ neurons, whose activity correlates with aversive fear states in various fear-related behavioral assays.

Dopamine (DA) is the canonical link between PE and synaptic reinforcement signals modulating Hebbian plasticity rules of CS–US associations. Foremost identified as a key mediator of reward learning, DA neurons may also drive aversive learning, suggesting a general role in both negative and positive reinforcement learning. DA modulates neural activity in the CEl via D1-like and D2-like receptors (D1R and D2R), making it a promising candidate for experience-dependent rewiring of amygdala connectivity. Although evidence for aversive signaling in ventral tegmental area (VTA) DA neurons (the midbrain DA neurons for appetitive reinforcement) has been reported, the majority of studies observed inhibition or no response at all in response to aversive signals. This raises the obvious question of whether other DAergic neurons outside the VTA reward system might provide DA driven aversive teaching signals for CE fear learning. Notably, a relatively neglected group of dorsal tegmental DA neurons in the ventral periaqueductal gray (vPAG) and dorsal raphe (vPdRD neurons) represent a particularly promising candidate: although optogenetic stimulation of vPdRD neurons modulates social behavior, it does not reinforce operant responses, functionally separating vPdRD neurons from VTA DA neurons. Moreover, the vPAG itself can encode aversive teaching signals, as it integrates afferent, aversive somatosensory, and nociceptive information while being an output structure for various fear-conditioned responses.

Taken together, the CE and the midbrain DA system emerged as promising entry points in our search for circuit motifs integrating Hebbian memory traces and reinforcement signals in associative learning. Synaptic tracing and circuit mapping revealed vPdRD neurons as a major source of DAergic projections to the CE. Suppression of vPdRD neuron activity diminished fear learning, accompanied by a reduction in experience-dependent potentiation of the

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Fig. 1 | vPAG/DR–CE circuitry is a major DAergic component in the fear pathway. a–c. Representative images of (a) CE as a target of TH immunopositive terminals in the temporal lobe, (b) TH-immunonegative neurons in the midbrain and (c) identifying CE DA sources via CTB retrograde tracing. Cpu, caudate putamen. Inset: fraction of CE-projecting vPdRD neurons. d. CTB retrogradely labeled neurons in the vPAG/DR and co-localized with TH terminals in the temporal lobe, (e) TH-immunopositive neurons in the midbrain and (f) TH-immunopositive neurons in the substantia nigra. e–f, Anterior-to-posterior distribution of TH+CTB+ PAG neurons after CE CTB injection. g. Combined CTB+ChR2 optogenetic circuit mapping of vPdRD neurons in AAV::DIO-ChR2, AAV::DIO-GFP–injected TH::Cre/ PKCδ::Cre double-transgenic animals. h–l, ChR2+ fibers of (h) infected vPdRD neurons (i) innervate the CE (cf. TH+ terminals in a). j, Postsynaptic currents recorded in whole-cell patch-clamp configuration from CE neurons (red, average trace; n = 10 neurons) induced by optogenetic activation of ChR2+ fiber terminals of vPdRD neurons (i). EPSC in artificial cerebrospinal fluid (aCSF) can be blocked by application of 10 μM CNQX + 50 μM APV (black, average trace; n = 10 neurons). k, Fraction of CE SST+ and PKCδ+ neurons responding to optogenetic activation in CE. l, EPSC amplitude of responding CE SST+ and PKCδ+ neurons (n = 5 neurons; two-sided unpaired t test, t 0.0281 = 0.6799). m, Spatial distribution of a subset of responding (i) and nonresponding cells in CE (cf. distribution of TH+ (a) and ChR2+ (i) terminals). Representative images from at least 3 independent experiments (3 animals). Bars show mean ± s.e.m. Significance levels between groups at *P < 0.05.

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Fig. 2 | Modulation of vPAG/DR–CEl circuitry by shock and DA. a, Deep-brain Ca2+ imaging of vPAG/DR neurons. b, Bulk Ca2+ imaging of vPAG/DR neuronal activity of freely moving mice in response to footshock (n = 2 animals). Inset: bulk imaging of vPAG/DR neurons expressing GCaMP6m. Circle indicates region of interest (ROI) used to calculate the bulk signal. c, Top left: representative example of vPAG/DR neuronal units expressing GCaMP6m (n = ROIs from 2 animals). Bottom: fraction of neuronal units responding (expressing GCaMP6m). Circle indicates region of interest (ROI) used to calculate the bulk signal. d, Microdialysis of DA release in the rat amygdala upon footshock (n = 4 animals with shock session, n = 3 animals without shock session; re-exposure; two-way RM ANOVA; interaction, F₁,₁₁ = 3.141, P = 0.0005; time, F₀,₁₀ = 6.798, P < 0.0001; columns, F₁,₁₁ = 2.325, P = 0.0847; Holm–Sidak post hoc tests). d, Microdialysis of DA release in the rat amygdala upon footshock (n = 4 animals with shock session, n = 3 animals without shock session; re-exposure; two-way RM ANOVA; interaction, F₁,₁₁ = 4.26, P = 0.0009; time, F₀,₁₀ = 2.461, P = 0.0286; groups, F₁,₁₁ = 2.633, P = 0.1656; Holm–Sidak post hoc tests). e, CEl LFP recordings from acute slices with HFS (100 Hz, 1 s, three times, every 30 s) + 20 µM DA (aCSF control group, n = 8 cells; DA group, n = 6 cells; tests for LTP: two-way RM ANOVA; interaction, F₁,₁₁ = 20.2, P = 0.0007; time, F₀,₁₀ = 81.69, P < 0.0001; groups, F₁,₁₁ = 23.35, P = 0.0004; Holm–Sidak post hoc tests). f, LTP recordings of CEl neurons in the presence of (f) 20 µM DA (SST⁺ cells, n = 12; PKCδ⁺ cells, n = 7; tests for LTP: two-way RM ANOVA; interaction, F₁,₁₁ = 9.052, P = 0.0094; time, F₀,₁₀ = 3.807, P = 0.0713; groups, F₁,₁₁ = 11.6, P = 0.0043; Holm–Sidak post hoc tests) and (g) 20 µM DA + 50 µM SCH23390 DA (SST⁺ cells; n = 9, PKCδ⁺ cells; n = 6; tests for LTP: two-way RM ANOVA; interaction, F₁,₁₁ = 0.001787, P = 0.9896; time, F₀,₁₀ = 0.0008886, P = 0.9768; groups, F₁,₁₁ = 0.01172, P = 0.9158; Holm–Sidak post hoc tests). Lines with shaded regions represent means ± upper and lower bounds (b) or s.e.m. (c,d). Error bars show mean ± s.e.m. Significance levels between groups (*) and from baseline (BL) or pre-HFS (#) at */**P < 0.05, ***/**/**/P < 0.001, ****/*****/P < 0.0001 and ###/####/P < 0.0001.

Collectively, these data establish a potential link between vPdRD neurons, Pavlovian fear conditioning, and DA modulation of CEl circuitry.

We therefore investigated the synaptic connectivity of vPdRD neuron projections to SST⁺ and PKCδ⁺ cells, two major neuronal types in the CEl, in acute brain slices. We injected PKCδ⁺:Cre/TH:Cre double-transgenic mice with AAV::DIO-GFP into the CEl and AAV::DIO-ChR2-YFP into the vPAG and DR (vPAG/DR; Fig. 1g–i). Targeted electrophysiological recordings of postsynaptic CEl GFP- and GFP⁺ cells, considered identical with SST⁺ and PKCδ⁺ neurons (Supplementary Fig. 3a), respectively, revealed targets in the temporal lobe (Fig. 1a). Although projections to CEl from canonical DAergic midbrain sources (i.e., substantia nigra and VTA; compare Fig. 1b) have been reported⁸⁻¹³, combined CE cholera toxin B (CTB; retrograde synaptic marker) injections (Fig. 1c) and TH IHC (Fig. 1d) mapped the majority of DAergic afferents to vPdRD neurons (Fig. 1e). Moreover, a large fraction of these neurons throughout the rostrocaudal extension of vPAG and dorsal raphe (DR; Fig. 1f) projects to the CEl, and particularly to CEl, with surprising specificity (Supplementary Fig. 1), whereas DAergic projections from substantia nigra and VTA to the amygdala in general appear to be rather sparse (Supplementary Fig. 2). Collectively, these data establish a potential link between vPdRD neurons, Pavlovian fear conditioning, and DA modulation of CEl circuitry.
direct excitatory postsynaptic currents in response to optogenetic presynaptic stimulation of CEi vPdRD neuron terminals (Fig. 1j), blocked by bath application of the AMPA receptor antagonist CNQX and the NMDA receptor antagonist APV. Together with recent reports18,19, these data suggest that vPdRD neurons innervate and potentially co-release glutamate and DA21 into the CEi in vivo. While the fraction of responding cells (Fig. 1k) and the signal amplitude in responding neurons (Fig. 1l) were similar between cell types, the overall location of responding neurons was spatially biased to the medial part of the CEi at the CEi–medial CE transition boundary (Fig. 1m), congruent with the vPdRD neuron-innervation pattern (Fig. 1a,1). The vPdRD–CE axis features characteristics of a learning circuit. We reasoned that this specific innervation from the vPdRD to CEi, a multimodal brain region involved in pain processing, might directly reinforce fear learning in the CE circuitry through glutamate and DA co-release in response to aversive experiences. Indeed, fiber-endomicroscopic Ca2+ imaging in freely moving animals injected with AAV-expressing GCaMP6m in the vPdRD (Fig. 2a and Supplementary Fig. 4a) showed strong bulk Ca2+ responses to the Pavlovian shock US (Fig. 2b) in this region, with neuronal subsets directly responding to the US (Fig. 2c).

These responses were accompanied by a shock-US-specific rise in intra-amygadal DA levels as observed through targeted microdialysis in freely moving animals during Pavlovian fear conditioning (Fig. 2d and Supplementary Fig. 5a). Note that for better feasibility the microdialysis was performed in rats, assuming a (gross) fear neuropatomy similar to mice. These experiments must be interpreted with caution with respect to the stereospecificity of sampling site (CEi versus BLA) and source (VTA versus vPdRD neurons). First, the method sampled amygdala DA across CEi and BLA. However, given the steep gradients in amygdala DA innervation (Fig. 1a and Supplementary Fig. 2), we believe that the majority of the sampled DA originated from CEi. Second, these experiments also sampled CE DA from VTA. However, most CE (and amygdala) DAergic innervation stems from vPdRD neurons and not from VTA (Fig. 1e and Supplementary Fig. 2). Combined with the fact that vPdRD neurons are active during the shock (Fig. 2c) and have been shown to increase CE DA21, it seems likely that a large part of the shock-induced DA observed (Fig. 2d) originated from vPdRD neurons and CE. Notably, the increase in amygdalar DA was absent during re-exposure to the shock context (CS) 24 h later (Fig. 2d), consistent with a role for CE DA in aversive reinforcement learning. To determine whether DA re-shapes CE synaptic connectivity, we examined DAergic modulation of BLA to CE synaptic weights, which potentiate during Pavlovian fear conditioning22. We first probed glutamatergic synapses onto CEi neurons for activity-dependent plasticity in response to high-frequency stimulation (HFS) of BLA inputs using acute slice electrophysiology (Fig. 2e). HFS-induced synaptic plasticity of evoked LFPs in the CEi increased in the presence of DA27. To assess the cell-type specificity of this potentiation, we performed whole-cell patch-clamp recordings on single cells and filled recorded neurons with biocytin for post hoc classification of neuronal subtypes (Supplementary Fig. 3a). Under basal conditions, the major CEi cell types (SST+ and PKCδ+) failed to undergo LTP after HFS stimulation of BLA inputs (Supplementary Fig. 3b). However, application of DA specifically gated LTP of excitatory BLA inputs onto CEi SST+ cells (Fig. 2f) but not PKCδ+ neurons. This effect was blocked by the D1R antagonist SCH23390 (Fig. 2g). Population-specific transcripational profiling of fluorescence-activated cell-sorted neurons from Cre-dependent tdTomato reporter mouse lines crossed to either SST::Cre or PKCδ::Cre animals revealed higher expression of D1Rs in SST+ neurons (Supplementary Fig. 6). These data suggest that cell-type-specific DA-dependent LTP (Fig. 2f) may be mediated by postsynaptic D1R signaling. The asymmetric distribution of D1Rs could specifically sensitize SST+ neurons for fear-related associations and map aversive states asymmetrically on genetically and functionally predefined SST+ neurons. These data might explain how fear conditioning could teach SST+ neurons to respond to tone Csa23,24, selectively strengthen their responses to BLA input1, and ultimately link them to aversive states25.

Taken together, DA, likely released from vPdRD neuron afferents, contributes to cell-type-specific potentiation of a BLA-to-CE fear synapse to gate associative learning of Pavlovian fear. Therefore, we examined whether successful acquisition, storage, and/or expression of fearful experiences of animals may require vPdRD neuronal activity. vPdRD neurons control associative learning of fear. We injected Cre-dependent adeno-associated virus (AAV) into the vPAG/DR of TH::Cre animals for selective expression of clozapine-N-oxide (CNO) receptors exclusively activated by designer drugs (M4-DREADD, AAV::DIO-M4) in vPdRD neurons (M4 cohort in Supplementary Fig. 7a). These animals received intraperitoneal CNO injections 30 min prior the conditioning phase. This treatment, expected to hyperpolarize and electrically silence vPdRD neurons, resulted in decreased freezing responses to the CS during training (Fig. 3a, b). Notably, this cohort showed substantially less freezing than controls during (drug-free) recall the next day. These results establish a critical role for vPdRD neurons in fear learning. Silencing vPdRD neurons with M4 did not lead to overt differences in the elevated plus maze or light–dark transition tests (Supplementary Figs. 8a,b), indicating that these cells do not directly modulate anxiety states.

We next tested whether input from vPdRD neurons is required for experience-dependent rewiring of CE circuitry. To this end, we ablated vPdRD neurons with stereotactic injections of the neurotoxin 6-hydroxydopamine (6-OHDA) into the vPAG/DR (Supplementary Fig. 7b) and post hoc determined synaptic weights of BLA–CE connectivity after fear conditioning (Fig. 3c). As expected, ablation of vPdRD neurons impacted fear learning and recall, similarly to the effects observed in our DREADD cohort earlier (Fig. 3d). Note that 6-OHDA neurotoxicity toward vPdRD neurons, which lack dopamine-β-hydroxylase33, provides direct evidence that these cells are indeed DAergic.

Next, we isolated acute slices from these lesioned animals and recorded excitatory postsynaptic currents (EPSCs) in neighboring SST+ and PKCδ+ neuronal pairs after electrical stimulation of BLA inputs. Notably, EPSC amplitudes were increased selectively in SST+ neurons after fear conditioning, resulting in a shift of synaptic weights from BLA-to-PKCδ+ to BLA-to-SST+ synaptic connectivity (Fig. 3e, f). These results are in line with previous studies8 and indicate that fear conditioning rewires BLA–CE connectivity in a cell-type-specific manner.

In 6-OHDA-lesioned animals, in which ablation of vPdRD neurons decreased fear memory formation (Fig. 3c, d), the fear-conditioning-induced shift of synaptic weights was markedly reduced and not significantly different from that of the HC cohort (Fig. 3f). Furthermore, the excitatory drive, determined as frequency and amplitude of spontaneous EPSCs recorded in SST+ and PKCδ+ neurons, resembled the HC state in 6-OHDA lesioned animals (Supplementary Fig. 9). Thus, selectively ablaing vPdRD neurons and thereby eliminating their inputs to the CE resulted in fear memory deficits, which were accompanied by failure to rewire BLA–CE connectivity.

Following the observation of D1R-dependent, cell-type-specific gating of LTP at BLA–CE synapses, we next probed whether experience-dependent synaptic plasticity in the CE and fear memory were dependent on CEi D1R signaling. Indeed, RNA interference (RNAi)-mediated knockdown of CEi D1R, through injection of...
Fig. 3 | vPdRD neurons support fear memory formation and amygdala rewiring. a, AAV-mediated Cre-dependent expression of M4 in vPdRD neurons of TH::Cre transgenic animals. b, Fear-conditioning protocol and freezing responses for systemic CNO injection and subsequent M4-receptor-dependent tonic vPdRD neuron inhibition during conditioning (control group: n = 10 animals; M4 group: n = 11 animals; conditioning: two-way RM ANOVA; interaction, F_{4,80} = 6.276, P = 0.002; time, F_{4,80} = 75.96, P = 0.0001; groups, F_{1,29} = 9.119, P = 0.0068; recall: two-way RM ANOVA; interaction, F_{4,78} = 7.161, P = 0.0149; time, F_{4,78} = 83.32, P < 0.0001; groups, F_{1,29} = 3.717, P = 0.0689; Holm–Sidak post hoc tests). CS1–4, conditional stimuli 1–4. WT, wild type. c, Freezing responses after 6-OHDA lesioning of vPdRD neurons during conditioning (control group: n = 4 animals; 6-OHDA lesion group: n = 4 animals; conditioning: two-way RM ANOVA; interaction, F_{4,80} = 1.619, P = 0.2021; time, F_{4,80} = 34.05, P < 0.0001; groups, F_{1,29} = 6.029, P = 0.0494; Holm–Sidak post hoc tests) and testing (recall: two-way RM ANOVA; interaction, F_{4,78} = 13.44, P = 0.0007; time, F_{4,78} = 40.88, P < 0.0001; groups, F_{1,29} = 7.098, P = 0.0373; Holm–Sidak post hoc tests). d, Averaged whole-cell patch-clamp recordings of postsynaptic currents of CEl SST+ and PKCδ– neurons next to each other (mean 60 µm apart) in response to BLA stimulation of animals that underwent fear conditioning (FC, n = 16 cell pairs), that were naive (HC, n = 23 cell pairs), that received a control virus and underwent fear conditioning (FC + D1R shRNA, n = 10 cell pairs), and that received a control virus and underwent fear conditioning (FC + Renilla shRNA, n = 10 cell pairs). e, Ratio of CEl SST+ to PKCδ– neuron postsynaptic currents after mice underwent a given behavioral protocol as described in a (n = cell pair groups described in e; groups: one-way ANOVA (pairs); groups, F_{1,16} = 4.624, P = 0.0021; Dunnett’s post hoc test). f, Targeted pharmacological inhibition of CEl D1Rs by local infusion of SCH23390. g, Freezing responses after CEl-targeted SCH23390 infusion during conditioning (conditioning: two-way RM ANOVA; interaction, F_{4,80} = 1.106, P = 0.2593; time, F_{4,80} = 14.79, P < 0.0001; groups, F_{1,29} = 0.106; 0.6655; Holm–Sidak post hoc tests) and drug-free testing sessions (recall: two-way RM ANOVA; interaction, F_{4,78} = 6.164, P = 0.0225; time, F_{4,78} = 36.94, P < 0.0001; groups, F_{1,29} = 2.88, P = 0.1060; Holm–Sidak post hoc tests). Error bars show mean ± s.e.m. Significance levels between groups (*) and to BL (#) at */P < 0.05, **/##/***/****P < 0.01 and ****/#####P < 0.0001; n.s., nonsignificant.
**Fig. 4** | vPdRD neurons encode PE-linked teaching signals. 

**a.** Combined CE CTB + ChR2 injections and whole-cell patch-clamp recordings in the vPAG/DR.

**b.** Representative image of biocytin-filled, TH+ and CTB retrogradely labeled (derived from CE-targeted CTB injections) vPdRD neurons.

**c.** Optogenetic activation of CE-originating ChR2 fibers elicits inhibitory post-synaptic currents in vPdRD neurons (red, average trace; n = 8 cells) blocked by application of 50 µM bicuculline (black, average trace; n = 5 cells).

**d.** Expression of GCaMP6 in vPdRD neurons in TH::Cre animals injected with dependent AAV.

**e.** Freezing levels of Ca²⁺-imaged animals during habituation (hab.) fear conditioning (cond. 1 and cond. 2) and recall.

**f.** Bulk imaging of vPdRD neurons expressing GCaMP6f. Circle indicates ROI used to calculate the bulk signal.

**g.** Trial averages of bulk Ca²⁺ signals of vPAG/DR neuronal activity during fear conditioning and recall (n = ROIs from 3 animals; two-way RM ANOVA; interaction, F_{54,56} = 3.204, P < 0.0001; rows, F_{27,28} = 3.434, P = 0.1484; Holm–Sidak post hoc tests).

**h.** Representative example of vPdRD neuronal units expressing GCaMP6f.

**i.** Clustered traces of Ca²⁺ signals from vPdRD neuronal units from experiment shown in g. Top: trial averages of Ca²⁺ event amplitudes of a CS- and/or US-responsive subset of vPdRD neurons (n = units from 3 animals; two-way RM ANOVA; interactions, F_{54,1080} = 3.749, P < 0.0001; time, F_{27,1080} = 15.43, P < 0.0001; column, F_{2,40} = 1.15, P = 0.3270; Holm–Sidak post hoc tests). Bottom: fraction of subsets of single units responding to either CS and/or US (n = units from 3 animals; χ²_{20.05,4}, P = 0.005). Representative images from three independent experiments (n = 3 animals). Bars and lines with shaded regions represent mean ± s.e.m. Significance levels between groups at *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001. Ca²⁺ signals and event amplitudes are derived from per-ROI (g) or per-cell (i). ∆F/F values, standardized over the whole experiment and given as units s.d.
**Fig. 5** | vPdRD neuron reinforcement signals direct associative learning. **a.** Optogenetic targeting of vPdRD neurons in TH::Cre animals injected with AAV for Cre-dependent expression of eArch3.0 or ChR2. **b.** Freezing responses to neuronal inhibition by eArch3.0 during CS–US presentations (control group: *n* = 8 animals, Arch group: *n* = 7 animals; conditioning: two-way RM ANOVA; interaction, *F*\(_{1,13}\) = 1.612, *P* = 0.1853; time, *F*\(_{4,52}\) = 24.97, *P* < 0.0001; groups, *F*\(_{1,13}\) = 4.189, *P* = 0.0615; recall: two-way ANOVA; interaction, *F*\(_{4,52}\) = 2.846, *P* = 0.1137; time, *F*\(_{4,52}\) = 14.57, *P* = 0.0019; groups, *F*\(_{4,52}\) = 3.18, *P* = 0.0962; Holm–Sidak post hoc tests). Opto, optogenetic stimulation. **c.** Quantification of a slow-motion behavioral state (cf. **d**) upon vPdRD neuron activation (control group: *n* = 9 animals, Chr2 group: *n* = 10 animals; two-way RM ANOVA; interaction, *F*\(_{1,13}\) = 5.309, *P* = 0.0341; time, *F*\(_{1,13}\) = 13.01, *P* = 0.0022; groups, *F*\(_{1,13}\) = 4.969, *P* = 0.0396; Holm–Sidak post hoc tests). **d.** Design of associative blocking experiment (control group: *n* = 9 animals, Chr2 group: *n* = 10 animals). Acquisition of fear to CS A during first training session (two-way RM ANOVA; interaction, *F*\(_{1,17}\) = 0.6537, *P* = 0.5843, time, *F*\(_{3,51}\) = 57.27, *P* < 0.0001; groups, *F*\(_{1,17}\) = 2.927, *P* = 0.1053; Holm–Sidak post hoc tests). Fear response and slow-motion postures during compound-conditioning phase (fear response: two-way RM ANOVA; interaction, *F*\(_{2,34}\) = 1.198, *P* = 0.3142; time, *F*\(_{2,34}\) = 0.03155, *P* = 0.9690; groups, *F*\(_{2,34}\) = 7.392, *P* = 0.0146; Holm–Sidak post hoc tests; slow motion: two-way RM ANOVA; interaction, *F*\(_{2,34}\) = 54.57, *P* < 0.0001; time, *F*\(_{2,34}\) = 54.57, *P* < 0.0001; groups, *F*\(_{2,34}\) = 193.7, *P* < 0.0001; Holm–Sidak post hoc tests) with presentation of CS A and CS B. Fear response during testing phase to alternating presentation of CS A and CS B (two-way RM ANOVA; interaction, *F*\(_{2,34}\) = 4.624, *P* = 0.0167; time, *F*\(_{2,34}\) = 15.68, *P* < 0.0001; groups, *F*\(_{2,34}\) = 0.0281; Holm–Sidak post hoc tests). Cond., condition. **e.** Quantification of blocking effect from recall in **c** (two-sided unpaired t test; control vs. Chr2, *t* = 2.58, *P* = 0.0195; one-sample t tests against zero; control, *t* = 2.407, *P* = 0.0427, Chr2, *t* = 1.235, *P* = 0.2482). Error bars show mean ± s.e.m. Significance levels between groups (*) and to BL or between trials or sessions (#) at */* *P* < 0.05, /**/ *P* < 0.01, ####/### *P* < 0.0001.
AAVs expressing GFP-linked short hairpin RNAs (shRNAs) before fear conditioning (Supplementary Fig. 10a–e), resulted in a shift in BLA–CE connectivity similar to that observed in 6-OHDA-lesioned animals (Fig. 3f) and in fear memory deficits at recall (Supplementary Fig. 10f). Together, these results suggest that vPDn neurons and the CE form a learning circuit. As components of DAergic reinforcement systems, vPDn neurons should be most active when important events have not been predicted (large positive PE) and decrease activity with the progress in learning, a notion supported by the fact that the PAG has the capacity to signal aversive PEs in both rats and humans. The most intuitive implementation of such negative feedback from learning would be direct inhibition of DA neurons by CE SST* cells or by medial CE (CEm, the major CE output), the elements that ‘learn’ with conditioning. Indeed, PAG-targeted CTB injections revealed retrogradely labeled neurons in the CE, originating predominantly from SST* neurons (Supplementary Fig. 11). This was supported by bilateral AAV-DIO-ChR2 injections in the CE of SST::Cre and PCK6::Cre mice, showing rather selective vPAG/DR innervation from SST* neurons (Supplementary Fig. 11). Together with the strong retrograde labeling of the CEm (Supplementary Fig. 11), this indicates that SST* neurons, but not PCK6* neurons, and the CEm are the major CE sources for vPDn neuron innervation.

To elucidate the interconnectivity between CE and vPDn neurons in more depth, we co-injected ChR2-YFP and CTB-555 in the CE for optogenetic manipulation of CE-arising fibers connecting to vPDn neurons (retrogradely labeled by CTB) that project back to the CE (Fig. 4a). The DAergic nature of biocytin-labeled recorded neurons was confirmed post hoc by TH IHC (Fig. 4b). Optogenetic stimulation of CE inputs evoked inhibitory postsynaptic currents in CE-projecting DA neurons in the PAG, sensitive to application of the GABA<sub>A</sub> receptor antagonist bicuculline (50 µM; Fig. 4c). Thus, CE SST* neurons and CEm may inhibit further reinforcement from vPDn neurons, in particular after BLA–CE circuitry has learned to respond to, and predict, the fear US<sup>23</sup>. In search of the proposed modulation of vPDn neuron activity during learning progress, we compared deep-brain Ca<sup>2+</sup> signals of vPDn neurons expressing GCaMP6f (Fig. 4d–j and Supplementary Fig. 4b) during a series of two reinforced (Cond. 1, Cond. 2) and one nonreinforced (Recall) fear-conditioning sessions. Freezing levels of animals confirmed that mice developed robust fear memories during CS–US association trials (Fig. 4e).

Bulk Ca<sup>2+</sup> population signals from vPDn neurons showed a strong increase in Ca<sup>2+</sup> signals during CS–US pairings in the first conditioning (Fig. 4f,g and Supplementary Fig. 12c,f). As learning progressed to the second conditioning session, the population Ca<sup>2+</sup> signal of vPDn neurons started to register the previously reinforced CS, whereas the population Ca<sup>2+</sup> signal to the US, which could be predicted by the animal at this timepoint, decreased. During nonreinforced recall 24 h later, after consolidation of fear memories, vPDn neurons showed strong increases in Ca<sup>2+</sup> signals to the CS when compared to the conditioning sessions.

A similar picture emerged at the level of single units (Fig. 4h–j, Supplementary Fig. 12f, and Supplementary Videos 1–3). Analysis of single-unit Ca<sup>2+</sup> signals (Fig. 4i) revealed a shift of the dominant response clusters from US- to CS-driven activity over the course of the experiment. To investigate whether this trend reflected discrete, stimulus-driven neuronal firing (mirroring actual action potentials), we transformed the Ca<sup>2+</sup> signals into neuronal activity events, defined as a rise in Ca<sup>2+</sup> signals> 3 s.d. We then filtered for those units (referred to as ‘responders’) whose firing to either CS and/or US exceeded the 95% confidence interval of the expected mean activity (Supplementary Fig. 12f). Indeed, the population activity of these responders followed a similar pattern (Fig. 4i), with the fraction of responder types shifting from US to CS throughout the experiment (Fig. 4j). Notably, these results are also contained within the vPAG/DR neuronal population as a whole (Supplementary Fig. 12a–d). Thus, vPDn neurons appear to encode a positive PE in aversive reinforcement learning.

Stimulus-associated activity of vPDn neurons gates learning. If vPDn neurons encode PE-linked reinforcement signals, their activity during associations should critically modulate learning. Thus, we examined whether optogenetic inhibition of vPDn neurons during the four 20-s CS–US pairings, a period that corresponds to the highest neuronal activity during fear conditioning (Fig. 4g,4i), would be sufficient to recapitulate the behavioral consequences of M4-DREADD silencing (Fig. 3a,b). Selective optogenetic inhibition of Arch-expressing vPDn neurons (Fig. 5a and Supplementary Fig. 7c), suppressing PE-linked reinforcement signals during associative periods, resulted in less freezing behavior to CS during conditioning and impaired fear responses when tested the next day (Fig. 5b). This established a critical role for vPDn neurons at the time of CS–US pairings and for the conversion of these pairings into short- and long-term fear memory. Similarly to M4-DREADD inhibition, Arch-mediated silencing of vPDn neurons did not influence nociception (Supplementary Fig. 8e–h), functionally dissociating these neurons from a general role of vPAG/DR in gating pain.

Hypothetically, activation of vPDn neurons could be enough to induce plasticity in the CE in the absence of an instructive US (footshock). To investigate this possibility, we performed channelrhodopsin (ChR2)-driven optogenetic activation of vPDn neurons during CS pairings (Fig. 5a and Supplementary Fig. 7d). This optogenetic activation was not sufficient to instruct aversive memories in the absence of real shock USs (Supplementary Fig. 13). Notably, we observed that optogenetic vPDn neuron activation evoked slow continuous movement (Fig. 5c and Supplementary Video 4). A similar observation has been made recently upon activation of a different neuronal population in the ventral vPAG/DR<sup>28</sup>. Overall, these results are in line with a role of vPDn neurons in primarily mediating PE-coupled reinforcement learning without encoding an intrinsic valence per se.
As activation of vPdRD neurons could not replace an instructive US during aversive fear learning by itself, we asked whether it may rather gate fear memory and associative learning in response to those contingencies that are novel and informative. We therefore examined whether vPdRD neuronal activity interferes with associative blocking of compound conditioning, a conditioning variant sensitive to aberrant reinforcement learning\(^5,16\) (Fig. 5d). Under normal conditions, linking a novel CS (CS B) to a US is blocked when the novel CS is co-presented with a CS (CS A) that is already associated with, and thus predicts, the subsequent US. This effect is also evident in our experiment, as controls froze significantly more to CS A than CS B, indicating successful blocking of the association between CS B and the US (Fig. 5d). In contrast, optogenetic activation of vPdRD neurons during compound CS–US presentations had two effects. It significantly increased the previously observed slow motion attend-like behavior (Fig. 5c) at the expense of freezing in that session (Fig. 5d) and inverted the CS–response pattern during recall (Fig. 5e). Thus, artificially increasing PE and reinforcement learning during associative periods resulted in establishing memories that are normally suppressed.

Taken together, these experiments show that manipulation of stimulus-bound vPdRD neuronal activity—effectively simulating a larger- or smaller-than-actual PE at time of association—bidirectionally modulates fear learning. Thus, PE signals in vPdRD neurons and DA reinforcement signals originating from vPdRD neurons positively gate associative learning.

**Discussion**

Learning from aversive experiences is one of the most basic and biomedically important brain functions. Here we describe a circuit motif reciprocally interconnecting vPdRD neurons with CE circuitry (Supplementary Fig. 14). It couples a positive aversive PE signal to DAergic reinforcement of an experience-dependent memory trace at an amygdala fear synapse.

Amygdala nuclei are the canonical substrate for fear memory formation. However, the reinforcing mechanisms that rewire amygdala circuitry during learning are much less understood. Notwithstanding the known interaction between VTA DA and the amygdala, a longstanding missing element in fear learning was a dedicated DA system that allows aversive PE-coupled reinforcement learning to modulate amygdala synaptic memory traces. In identifying vPdRD neurons as a major DA input to CE circuitry, we provide a circuit context that links vPAG/DR, which integrates nociceptive US-related information\(^7\) and encodes PE information\(^26,28,35\), to DA-driven rewiring of BLA–CE connectivity.

In line with US responses of the vPAG region during acquisition of fear learning\(^28\), we find that the response of vPdRD neurons shifts from US to CS as learning progresses with conditioning. This reorientation toward the predictive value of emotionally relevant information is in line with vPdRD neurons encoding PE-coupled teaching signals. While PE-coupled reinforcement signals are integral parts of several Pavlovian fear-learning models, their neuronal implementation has not been fully resolved at the circuit level\(^25\).

We propose that vPdRD neuronal activity and DA signals mirror the underlying mechanism of the fear-experience-dependent reshaping of BLA synapses onto CE SST\(^+\) neurons (Fig. 3e,f). The fact that 6-OHDA lesions of vPdRD neurons can partially revert this effect links aversive teaching signals from vPdRD neurons to DA-mediated synaptic plasticity in the CE. Since D1R potentiates BLA-to-CE synapses\(^11,14\) and learning\(^11,14\) and given that, in our hands, blocking CE D2R signaling showed smaller behavioral consequences in fear conditioning (data not shown), we focused on D1Rs in our circuit model. However, we would like to point out that D2R effects might also contribute. In fact, our sequencing results show D2R expression in both CE cell types (data not shown). Thus, CE D2R signaling may synergize with D1R activation to induce experience- and vPdRD-neuron-dependent rewiring of BLA–CE circuitry. Notably, such a mechanism integrates earlier work\(^26\) into the circuit framework put forward by our study. The complexity of DA-receptor signaling in the amygdala\(^13,34\) demands further study to dissociate the role of D1R- versus D2R-dependent mechanisms.

It has recently been shown that CE feedback to the ventrolateral PAG controls fear learning, stimulus responsibility of ventrolateral PAG neurons, and PE coding in the amygdala\(^1\). Our results extend the possible explanation for this observation, as we find direct inhibitory synaptic connectivity of CE output from CE SST\(^+\) cells and CEm to vPdRD neurons (Fig. 4a–c, Supplementary Fig. 11). During fear learning, this negative-feedback projection could regulate vPdRD neurons and henceforth adapt PE signaling according to the neuronal activity state in CE and CEm (for example, suppression of US responding by a preceding predictive CS after CS learning by SST\(^+\) CE neurons).

We used a combination of fear conditioning and an associative blocking design\(^25\) to assess whether manipulation of vPdRD neuronal activity modulates associative learning. Silencing vPdRD neuronal activity during CS–US pairings, thereby simulating a PE that is smaller than actual, resulted in decreased CS–US associations (Fig. 5b). Conversely, increasing activity during CS–US compound pairings in associative blocking, simulating a PE that is larger than actual, facilitated the association of the novel CS (Fig. 5d,e). Thus, modulation of vPdRD activity, presumably representing PE-coupled reinforcement, gates learning. In contrast to VTA DA neurons, direct activation of which can induce behavioral conditioning\(^45\) or reinforcement, our results demonstrate that this is not the case for sole activation of vPdRD neurons. This dissociates this class of DA neurons from VTA neurons. Moreover, it indicates that, besides vPdRD neuron activity, additional signals are required to write experiences into synaptic long-term memory in CE circuitry. In the context of aversive experiences, these US pain-related signals may come from other brainstem systems\(^46\). Hence, vPdRD neuron activity retains a primarily reinforcing nature but does not encode an intrinsic affective valence.

Regardless, activation of vPdRD neurons in a neutral context led to a mild behavioral switch, manifested as constant slow
movement. This type of behavior has been described recently for the vPAG as ‘slow-motion’ behavior\(^1\). Notably, it was able to override freezing behavior in our associative blocking assay (Fig. 5d), while increasing associative performance. Thus, vPdRD neurons may drive a freestanding, distinct attention-like behavioral state.

A notable aspect of our study, in line with recent findings\(^2\), is the observation that vPdRD neurons can co-release glutamate in the CEI. The co-release of DA together with either of the fast ionotrophic neurotransmitters GABA and glutamate has been observed for VTA DA neurons previously\(^3\), but there is no satisfying theory about how it could affect behavior. Strikingly, blocking DA in the CEI only affected fear testing (Fig. 3h), whereas inhibition of vPdRD neurons, which most likely blocked the majority of DA and glutamate release in the CEI, affected short-term fear memory during the conditioning phase as well (Figs. 3b, 5b). One attractive hypothesis is that glutamate co-release facilitates short-term learning, which DA reinforces to long-term synaptic memory, functionally dissociating these co-released neurotransmitters. Notably, glutamate activates PKC\(\beta\) neurons (Fig. 1k), which could drive short-term learning by increasing attention-like states via the basal forebrain\(^4,5\). The slow-motion behavior evoked with very short delays after vPdRD neuron activation, likely driven largely by fast glutamate signaling from vPdRD neurons (rather than by the more slowly acting DA), may reflect such attention-like states.

Taken together, we identified a circuit motif, interconnecting vPdRD neurons and amygdala, which integrates the main components of associative learning (CS–US information, PE, and synaptic memory) to shape an amygdala fear response. DA release in the CEI acts as a retrograde reinforcement signal by setting synaptic learning rules to control Pavlovian memory traces. An inhibitory feedback loop may inhibit reinforcement signals to prevent excessive associations. This delineates an intuitive model (Supplementary Fig. 14) of how the brain computes a learning problem. In the context of aversive learning, the vPdRD component integrates nociceptive US-related information and, via direct interaction with the CE, signals the PE to reinforce plasticity at a CEI fear synapse.

The dopaminergic identity of vPdRD neurons has previously been suggested\(^6\), and our 6-OHDA lesion results provide further proof of this notion. Thus, our study assigns a defined neuronal circuitry and behavioral function to vPdRD neurons, a hitherto relatively uncharted class of DA neurons in the mammalian brain. In turn, we identify vPdRD neurons as a central component for negative-reinforcement learning. Notably, the CE is a critical component of reward conditioning\(^7,8\), which might point toward a more general role of vPdRD–CE circuitry in positive-reinforcement learning as well. Together with the fact that DAergic neurons in general\(^9\) and the vPAG/DR region\(^10\) play a key role in learning processes, vPdRD neurons might emerge as central gatekeepers of associative reinforcement learning.

From a biomedical point of view, signatures of aversive PE have recently been detected in the human PAG\(^11\). Thus, dysregulation of DAergic vPAG/DR–CE circuitry could lead to inadequate fear memory formation observed in PTSD, and, given the pain sensing properties of vPAG/DR, the comorbidity of PTSD and chronic pain\(^12\).

**Methods**

Methods, including statements of data availability and any associated accession codes and references, are available at [https://doi.org/10.1038/s41593-018-0174-5](https://doi.org/10.1038/s41593-018-0174-5).}

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**Author contributions**

F.G. conceived, designed, performed, and analyzed most of the experiments and wrote the manuscript. T.M. and S.M. performed whole-cell patch-clamp and LFP recordings for LTP experiments. J.K. and P.P. performed and analyzed Ca"'" imaging experiments. J.G. performed mouse surgeries. D.K performed anatomical tracings and designed and tested AAVs for optogenetics, DREADDs, and GCaMP6m. A.R. designed, performed, and analyzed microdialysis experiments. S.B. and K.K. performed and analyzed SCH injections, fear conditioning, acute anxiety assays, and pain tests. J.Z. designed RNAi viral vectors and supervised knockdown experiments. V.L. co-supervised experiments and wrote the manuscript. WH. initiated and conceived the project; designed, analyzed, and supervised experiments; and wrote the manuscript. All authors contributed to the experimental design and interpretation and commented on the manuscript.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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Methods

Subjects. Male mice 2–4 months old were group housed (2–5 animals per cage) in a colony on a 14 h light/10 h dark cycle starting at 07:00 with food and water provided ad libitum. All animal procedures were performed in accordance with institutional guidelines, approved by the respective Austrian (BGA-B 2005/101), ifd BGBI I no. 162/2005) and European authorities ( Directive 86/609/EEC of 24 November 1986, European Community), and covered by the license M58/002220/11/9. C57BL/6J wild-type mice were bred in-house and provided after weaning from the Research Institute of Molecular Pathology (IMP) Breeding facility. Prkcd::GluCl-Cre RAC transgenic mice (PCK::Cre), [1] 7630403G23RikTg(Th-cre)1Tmd transgenic mice (Th-Cre, stock no. 008631, Jackson Laboratory), SOM-IRE5-Cre transgenic mice (SST::Cre; stock no. 013044, Jackson Laboratory), and B6129S6-Gt(Rosa)26Sortm5(Sto)Albino/litters) mice (Rosa::loxP-STOP-loxP-tdTomato, stock no. 007999, Jackson Laboratory) were maintained heterozygous on a C57BL/6J background. Cohort sizes: for neuroanatomical tracing, approximately 2- to 4-month-old male mice; slice electrophysiology, approximately 50 1- to 3-month-old male mice; microdialysis, neuroanatomical tracing, approximately 30 2- to 4-month-old male mice; slice electrophysiology, approximately 50 1- to 3-month-old male mice; microdialysis, neuroanatomical tracing, approximately 30 2- to 4-month-old male mice; slice electrophysiology, approximately 50 1- to 3-month-old male mice; and ChR2, 19 2- to 3-month-old male mice.

Stereotactic surgery for viral/toxin injections and cannula/light fiber implantation. Male mice 2–4 months old were deeply anesthetized with isoflurane (5%, Abbott Laboratories) and placed in a stereotaxic frame (Kopf). Anesthesia was kept constant with 1.5–2% isoflurane supplied per anesthesia circuit. For neuronal modulation of animals expressing DREADDs, clozapine-N-oxide (Sigma, 2.5 mg/kg) was diluted in physiological 1 × PBS and injected intraperitoneally 30 min before the start of the experimental session. Animals that received this treatment in experiments were habituated by PBS injections during handling sessions.

Histological analysis. To verify virus expression (see Supplemental Fig. 1A) and correct locations of optical fiber tips and cannulae, animals were killed by a mixture of 10 mg/mL ketamine (OGRIS Pharma) and 1 mg/mL medetomidine hydrochloride (Domitor, ORION Pharma) in 1 × PBS, and tissue sectioning was performed as described under “Immunohistochemistry” below. Expression of viral constructs and location of optical fiber tips/cannulae were assessed for correct targeting (Supplemental Figs. 4 and 7).

Fear conditioning. Mice were handled on two different days before all behavioral training experiments. Fear Conditioning was separated into habituation, conditioning, and testing phases and conducted on three different consequent days in a large soundproof isolation cubic that contained an adaptive mouse test cage (Coulbourn instruments). The context of the mouse test cage was modified to make the box distinct for different phases of the experiment.

Conditioning (Context A). The mouse test cage was changed to a gray/white striped, symmetric triangular box (15 × 12 × 7 cm); the floor texture consisted of the characteristic checkered base plate; and box walls were swiped with lemon flavor.

Testing (Context B). The test cage was changed to a square white box. The floor consisted of a white flat base plate, and box walls were swiped with ethanol flavor. On day 1, mice individually underwent the habituation phase in context B with each session taking 300 s. On day 2, mice were conditioned individually in context A with four pure tones (3 kHz, 70 dB, 20 s each) delivered at intervals with variable duration (80–120 s), each sound co-terminating with a 1 s, 0.5-mA footshock, delivered by a precision-regulated animal shaker (H13-15, Coulbourn Instruments). Testing of fear memory was performed 24 h after conditioning on day 3 in context B using behavioral cues to four pure tones (3 kHz, 70 dB, 20 s each) delivered at variable intervals (80–120 s). Matlab scripts were programmed to deliver footshocks and tones. The isolation cubic was illuminated in every phase of the experiment and behavior was captured with a CCD camera at 25 fps and stored on a PC. Test cages and test floors were thoroughly cleaned with water and dedicated flavor-alcohol mixtures in between mouse runs on a given day. Behavioral responses of all phases were analyzed offline by Ethovision software or video by an observer blind to the experimental condition. A list of behavioral experiments and the experimental history of different cohorts is provided in Supplemental Fig. 1b.

Blocking experiment. The blocking experiment was performed as described earlier[1] with some modifications. The preparations and equipment were identical to those used in fear conditioning (described above), but based on a different protocol (Fig. 4). All blocking experiment phases took place in the dark without constant illumination. After the habituation phase on day 1 (identical to that described in “Fear Conditioning,” above), mice underwent 3 consecutive days of conditioning in context A on day 2 to day 4, each day consisting of a session during which, in the periods of 1-h long light pulses (CS A) at 15 s intervals were presented four times, each period co-terminating with a 1 s, 0.5-mA footshock. The intervals between these periods randomly varied from 80 to 120 s. The conditioning phase was followed by a 2-d compound-conditioning phase on days 5 and 6, again in context A, during which animals received a compound CS four times in each session/day, composed of CS A accompanied by 1-Hz pulsed white noise (CS B) in random 80–120-s intervals, each presentation co-terminating with one 0.5-mA footshock. The next day (day 7), behavioral responses to 12 CS A and 12 CS B presentations were recorded in one session. The CS presentation in this phase was designed such that two CS A presentations were followed by two CS B presentations, with a constant time interval of 60 s between each CS (CS A, 60 s, CS A, 60 s, CS B, 60 s, CS B, 60 s, 60 s, CS B, 60 s, CS B, 60 s, CS B, 60 s, CS B, 60 s, 60 s, CS B). Systemic injections in behavioral experiments. For neuronal modulation of animals expressing DREADDs, clonazepam-N-oxide (Sigma, 2.5 mg/kg) was diluted in physiological 1 × PBS and injected intraperitoneally 30 min before the start of the experimental session. Animals that received this treatment in experiments were habituated by PBS injections during handling sessions.

Optogenetic manipulation in behavioral experiments. Animals that had undergone stereotactic injection of optogenetic AAV virus for later neuronal modulation during behavior underwent habituation for attaching a fiber-optic patch cord (Doric lenses) on implanted optic fibers. For ChR2 activation, laser trains of blue light (473 nm) were delivered, consisting of 20-m s pulses delivered at 20 Hz (if not noted otherwise) at an intensity of 8–10 mW at the fiber tip. For Arch activation, laser trains of constant yellow light (568 nm) were delivered at an intensity of 5–7 mW. Intensities of all laser stimulations were measured before every experiment at the tip of the optic fiber via a Power Meter (Thorlabs, PM100D). Laser stimulation was controlled by Matlab scripts during conditioning experiments and by Arduino boards running customized scripts executed by AnyMaze software (Stoelting) during pain tests and baseline anxiety tests.

Intracranial drug delivery during behavioral experiments. Intracerebral drug administration was delivered through previously (2–4 weeks) implanted guide cannulae. Animals were handled for 5 min once a day for 3 d. On the day of the experiment, internal cannula that protruded 1 mm beyond the edge of the guide cannula was inserted, and either D1 receptor antagonist SCH23390 (Tocris) in saline or saline vehicle was infused bilaterally. We injected 80-ng doses of SCH23390 in 0.2 μL saline or saline alone (0.2 μL/side) over a period of 5 min using a syringe controlled by an infusion pump (Harvard Apparatus Pump 11). Behavioral tests were started 30 min after infusion.

Automated von Frey test. Touch sensitivity was tested with a dynamic plantar aesthesimeter (Ugo Basile S.R.L., Italy). Mice were habituated to the testing chambers for approximately 2 h before testing. On each behavioral test, mice were tested three times with increasing force ranging from 0 to 10 g, with a 20 s ramp-up time and at least 20 s between each trial on the same mouse. The average of three trials was calculated for each hindpaw. Readout parameters were the force and latency at which the mouse lifted the hindpaw.

Hot plate test. Thermal sensitivity was tested 1 week after the von Frey testing, using a hot plate analgesia meter (IITC Life Science Inc., CA, USA). Mice were put on the hot plate at 45°C and the temperature was increased from 45 to 55°C within 2 min. The experiment was stopped as soon as the mice performed the first jump. Mice were videotaped and the latency and temperature of the first reaction (hind paw shaking or licking) or jump recorded.

Elevated plus maze. Mice were placed in the center zone (6.5 × 6.5 cm), facing an open arm of a custom-built elevated plus maze (elevated 54 cm above the floor) with two open arms (OA, 30-cm length, 7-cm width) and two wall-enclosed arms (closed arms, CA, 30-cm length, 6-cm width, walls 14.5-cm high) allowing the mice to explore freely for 5 min. Their path was video-recorded using Topscan software (CleverSys, Inc., VA, USA), and the amount of time spent and distance traveled in the open arms, closed arms, and center zone were evaluated.
Light/dark box. Mice were placed in the light zone and allowed to explore the light/dark arena (open field arena from TSE-Systems modified with custom-built dark-zone boxes) freely for 20 min. Their path was video-recorded in the light zone using Videomotion software (VTX, TSE-Systems GmbH, Germany) and the amount of time spent in the light versus dark zones and distance traveled in the light zone were evaluated, as well as the latency until they escaped to the dark zone. Lux levels were 150 lx in the light zone and about 0 lx in the dark zone. Each zone (light zone and dark zone) was 24.5 cm × 50 cm in size.

Microdialysis. Experiments were performed by Brains-OnLine (Charles River Laboratories) following established amygdala microdialysis routines for awake, behaving animals. Adult male rats were anesthetized using isoflurane (2%, 800 mL/min O2). Bupivacaine/epinephrine was used for local analgesia and carprofen was used for peri- and postoperative analgesia. The animals were placed in a stereotaxic frame (Kopf Instruments, USA). Rats were implanted with a push–pull microdialysis probe (2 mm exposed surface, PEE membrane, BrainLink, the Netherlands) in the amygdala (AP = −3.3 mm, ML = −4.5 mm, DV = −9 mm). Note that the stereotaxic position between BLA and posterior CE (Supplementary Fig. 5a) and the use of lateral exposed dialysis surfaces allowed us to sample BLA and CE DA while preventing excessive CE tissue damage.

After surgery, animals were housed individually in cages and provided food and water ad libitum. Microdialysis sampling was initiated approximately 24 h after surgery. On the days of the sampling (days 1 and 2), the probes were connected with PAN tubing to a microperfusion pump (Harvard PHD 2000 Syringe pump, Holliston, MA, USA). The micropipettes for microdialysis probes were perfused with aCSF containing 147 mM NaCl, 3.0 mM KCl, 1.2 mM CaCl2, and 1.2 mM MgCl2, at a flow rate of 1.5 µL/min. Microdialysis samples were collected for 15-min periods by an automated fraction collector (820 Microsampler, Univerit, Malta) into polystyrene (300 µL) minivials. All the dialysis samples were stored at −80 °C for later analysis. After habituation, 15-min samples of baseline dialysate were collected prior to the animals’ home cage. Rats were then placed inside the test cages with grid floor shockers (Coulbourn Instruments, Lehigh Valley, PA) and dialysate samples were collected 1 h before two shocks were administered (2 × 1 s shocks at 0.6 mA). Animals remained in the test cage for 30 min before being moved back to their home cages for the remainder of the experiment. The next day, animals underwent the same procedure as on day 1 except that the footshock was omitted. After microdialysis, brains were fixed in 4% PFA. Histological samples were visually inspected for correct probe placement.

Deep-brain Ca2+ imaging. Deep-brain calcium imaging was conducted using the nVista HD 2.0 system (In vivo Rodent Brain Imaging System, Incscopix, Inc.). A microendoscope was implanted directly above the ventral PAG, and a baseplate containing 147 mM NaCl, 3.0 mM KCl, 1.2 mM CaCl2, and 1.2 mM MgCl2, at a flow rate of 1.5 µL/min. Microdialysis samples were collected for 15-min periods by an automated fraction collector (820 Microsampler, Univerit, Malta) into polystyrene (300 µL) minivials. All the dialysis samples were stored at −80 °C for later analysis. After habituation, 15-min samples of baseline dialysate were collected prior to the animals’ home cage. Rats were then placed inside the test cages with grid floor shockers (Coulbourn Instruments, Lehigh Valley, PA) and dialysate samples were collected 1 h before two shocks were administered (2 × 1 s shocks at 0.6 mA). Animals remained in the test cage for 30 min before being moved back to their home cages for the remainder of the experiment. The next day, animals underwent the same procedure as on day 1 except that the footshock was omitted. After microdialysis, brains were fixed in 4% PFA. Histological samples were visually inspected for correct probe placement.

Slices were transferred to 4% PFA in 1× PBS after recording and stained as described above including fluorophore-tagged streptavidin (Sigma) to secondary antibody incubation.

Combined CTB tracing/TH IHC experiments. CTB-Alexa Fluor 555 (Invitrogen) was delivered by stereotaxic injection. Animals were killed for analysis 1 week after injection and brains processed for IHC as described above. Co-localization of CTB back-labeled neurons with either PKCδ (CE) or TH (PAG) labeling was scored by an observer blind to the experimental condition. In the CE, SST+ and PKCδ neurons were identified by the absence or presence of PKCδ immunoreactivity, respectively.

6-OHDA vPAG lesions. 6-hydroxydopamine hydrobromide (Sigma) in saline containing 0.01% (w/v) ascorbic acid was delivered by two stereotactic 100-nL injections into the rostral and caudal ventral PAG region at a concentration of 10 µg/µL.

D1R knockdown. To suppress D1R expression in the CE we constructed an AAV-based vector expressing GFP and miRNA-adapted shRNAs in the optimized miR-e backbone under control of the SFFV promoter (AAV-SFFV-GFP miR; ASGE). Two independent shRNAs targeting D1R (guide sequences: 5’-TACTA[…add 22 mer guides]–3’) were designed based on optimized design rules, cloned into miR-e, and tested for knockdown potency at the protein level using an established two-color reporter assay. In brief, NIH-3T3 cells were stably transfected with a construct expressing a tdTomato transgene harboring target sites of D1R and several control shRNAs in its 3’UTR (name vector). Subsequently, cells were transiently transfected with ASGE vectors harboring D1R and control shRNAs, and tdTomato-reporter knockdown in GFP-reporter cells was quantified using flow cytometry. Percentage of knockdown was calculated as a ratio of the mean tdTomato signal in the GFP+ cell population to the mean tdTomato signal in the GFP− cell population using the formula as follows.

\[
\% \text{ knockdown} = \frac{\text{mean tdTomato GFP}^+ \times 100}{\text{mean tdTomato GFP}^-}
\]

Fiber density analysis. Transgenic SST-Cre and PKCδ-Cre mice received CEi-targeted injections of AAV-ChR2-YFP. Brains were perfused and cut by a vibratome, and slices were immunostained against TH to confirm correct targeting to the vPAG/DR region. The analysis was performed using Definins Developer XD software on maximum intensity projections of the 3D datasets. To segment the axons, a 2D bandpass filter was applied, and the resulting image was thresholded. Objects low in contrast were removed, and the total area of axons per image was measured.

Acute brain slice electrophysiology. Virally infected TH-ce, SST-Cre, and PKCδ-Cre single- or double-transgenic male mice (2–3-month-old) were deeply anesthetized with isoflurane and decapitated, and their brains were quickly chilled in sucrose-based dissection buffer, bubbled with 95% O2/5% CO2 and containing the following (in mM): 220 sucrose, 26 NaHCO3, 2.4 KCl, 10 MgSO4, 0.5 CaCl2, 3 sodium pyruvate, 5 sodium ascorbate, and 10 glucose. Transverse coronal brain slices (300 µm) were cut in dissection buffer using a vibratome (Leica, VT1000S) and immediately incubated for a 15-min recovery phase in oxygenated artificial cerebrospinal fluid (aCSF) containing, in mM: 126 NaCl, 2.5 KCl, 1.25 NaH2PO4, 26 NaHCO3, 2.5 CaCl2, 2.5 MgCl2, and 25 glucose in 95% O2/5% CO2 at 32°C. This was followed by a slice resting phase with oxygenated aCSF for at least 45 min at room temperature (RT; 20–22°C).

Individual brain slices containing CEi were placed on the stage of an upright, infrared-differential interference contrast microscope (Olympus BX51WI) mounted on an X-Y table (Olympus) and visualized with a 40× water-immersion objective by an infrared-sensitive digital camera (Hamamatsu, ORCA-03). Slices were fully submerged and continuously perfused at a rate of 1–2 µL/min per well with oxygenated aCSF.

Optogenetic circuit mapping. PKCδ+ and SST− neurons in the CE were identified by the presence of GFP fluorescence. Patch pipettes were pulled on a Flaming/Brown micropipette puller (Sutter, P-97) from borosilicate glass (1.5-mm outer and 0.86-mm inner diameter, Sutter) to final resistances ranging from 3 to 5 MΩ. Internal solution for voltage-clamp recordings of responses to optogenetic (luminator) in bl_inode (in mM): 135 cesium methanesulfonate, 5 KCl, 10 HEpes, 2 MgCl2, 0.2 EGTA, 1 NaATP, 0.4 NaGTP, and 10 Na+–phosphocreatine for excitatory responses in the CE and 140 KCl, 10 HEpes, 2 MgCl2, 0.2 EGTA, 1 NaATP, 0.4 NaGTP, and 10 Na+–phosphocreatine for inhibitory responses in the PAG (280–290 mOsm). Membrane currents were recorded with a Multiclamp 700B amplifier (Molecular Devices). Electrophysiological signals were
lowpass-filtered at 3kHz, sampled at 10kHz (Digidata 1440 A, Axon Instruments), and stored on a PC for offline analysis with pClamp 10 software (Molecular Devices).

Cells were held at ~70mV. Cells were allowed to reestablish constant activity during the 5-min waiting time after breaking the seal. We delivered 20-ms blue-light pulses through a 40x electrophysiology microscope objective, driven by a 120-W mercury lamp (X-Cite 120 PC Q). The amplitude of 4 pulses, 5 s apart, was averaged as postsynaptic response. Inhibitory responses were identified by adding 10µM bicuculline and excitatory responses identified by adding 10µM CNQX + 40µM D-APV (both from Sigma) to the bath.

**LTP experiments.** Standard procedures were applied to prepare coronal slices from male C57Bl/6j mice (P28–P47). In brief, mice were deeply anaesthetized by inhalation of 4% isoflurane and killed by decapitation. A block of tissue containing the amygdala was rapidly removed and placed in a dissection buffer containing (in mM): sucrose, 195; KCl, 2.4; NaH2PO4, 1.25; NaHCO3, 26; MgCl2, 1; CaCl2, 2; and glucose, 10; bubbled with 95% O2/5% CO2. The amygdala was rapidly removed and placed in a dissection buffer containing (in mM): sucrose, 195; KCl, 2.4; NaH2PO4, 1.25; NaHCO3, 26; MgCl2, 1; CaCl2, 2; and glucose, 10; bubbled with 95% O2/5% CO2.

**Local field potential recordings.** Local field potential recordings were conducted in an interface chamber at 32 °C. In aCSF composed of (in mM): NaCl, 125; KCl, 2.5; NaH2PO4, 0.8; NaHCO3, 25; MgCl2, 1; CaCl2, 2; and glucose; with bubbled with 95% O2/5% CO2. Gabazine (0.1µM) was added to reduce GABAergic inhibition.

**Data exclusion.** For slice electrophysiology, approximately 10 of 50 animals did not reach sufficient virus expression and/or missed injection targets and were excluded. Of the SCH23390 cohort, 9 of 31 animals were excluded from the analysis due to misplaced or blocked infusion cannulae.

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Reporting Summary

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When statistical analyses are reported, confirm that the following items are present in the relevant location (e.g. figure legend, table legend, main text, or Methods section).

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  - The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
  - An indication of whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
  - The statistical test(s) used AND whether they are one- or two-sided
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  - Clearly defined error bars
    State explicitly what error bars represent (e.g. SD, SE, CI)

Our web collection on statistics for biologists may be useful.

Software and code

Policy information about availability of computer code

Data collection

Animal Behavior:
Audacity software (http://www.audacityteam.org)
Matlab (R2015b, MathWorks) - custom script
Ethovision XT 8 (Noldus Information Technology)
ANY-maze (Stoelting Europe)

Histology and Microscopy:
Pannoramic Viewer (3D HISTECH Ltd.)
Developer XD (Definiens AG) - custom script
Fiji ImageJ (https://imagej.net/)
ZEN software (Carl Zeiss Microscopy GmbH)

Electrophysiology:
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Neural Population Sequencing:
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Data

Policy information about availability of data
All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:
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The data that support the findings of this study (Fig. 1–5, Supplementary Fig. 3–5, 7–13) are available from the corresponding author upon reasonable request. Data Supporting Fig. 1,2 are from Allen Mouse Brain Connectivity Atlas (http://connectivity.brain-map.org/). For data Supporting Supplementary Fig. 6 are Accession Codes section.

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size  No statistical methods were used to predetermine sample sizes. Sample size were similar as reported in previous publications4,31,59,60.

Data exclusions  Out of the SCH 23390 cohort, 9 out of 31 animals were excluded from the analysis due to misplaced or blocked infusion cannulae.

Replication  All methods used in the manuscript underwent rigorous testing for reproducibility when established. Histological, electrophysiological and imaging experiments were replicated. Behavioral experiments were replicated using different methodologies.

Randomization  All animals and samples were randomly assigned to the experimental groups.

Blinding  Data collection and analysis were not performed blind to the conditions of the experiments. Data was acquired, processed and analyzed by automated workflows, except Fig. 1e, f, Supplementary Fig. 3a, Supplementary Fig. 4a,b, Supplementary Fig. 5a,b, Supplementary Fig. 7a,b,c,d, Supplementary Fig. 10e, Supplementary Fig. 11b.

Reporting for specific materials, systems and methods

Materials & experimental systems

| n/a | Involved in the study |
|-----|-----------------------|
| ☒  | ☑ Unique biological materials |
| ☑  | ☐ Antibodies |
| ☑  | ☐ Eukaryotic cell lines |
| ☐  | ☑ Palaeontology |
| ☐  | ☑ Animals and other organisms |
| ☑  | ☑ Human research participants |

Methods

| n/a | Involved in the study |
|-----|-----------------------|
| ☐  | ☑ ChIP-seq |
| ☐  | ☑ Flow cytometry |
| ☑  | ☑ MRI-based neuroimaging |
Antibodies

Primary antibodies used:
- mouse antibody to PKCd (BD Biosciences, 610398, 1:500, LOT: n/a) published results for immunohistochemistry in mouse
- chicken antibody to TH (Abcam, ab 7963, 1:1000, LOT: 2387494) published results for immunohistochemistry in mouse
- goat antibody to SST (Santa-Cruz, sc-7819, 1:500, LOT: n/a) antibody was tested rigorously for functionality in mouse central amygdala
- rabbit antibody to DsRed (Living Colors, 632496; 1:1000; LOT: n/a, to optimize visualization of AAV::DIO-M3 and AAV::DIO-M4 expression, both tagged with mCherry fluorophore), published results for immunohistochemistry in mouse

Secondary antibodies used:
- Thermo Fisher Scientific, 1:1000; Alexa Fluor published results for immunohistochemistry; validated binding to primary mouse AB

Validation
- Antibodies were pre-established published commercial antibodies.

Eukaryotic cell lines

Policy information about cell lines

- Cell line source(s): NIH-3T3
- Authentication: The identity of NIH-3T3 line has not been authenticated specifically
- Mycoplasma contamination: The NIH-3T3 cell line has been tested for contamination
- Commonly misidentified lines: No commonly misidentified cell lines were used

Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

- Laboratory animals
  - Male C57BL/6J mice (1-4 months old, Charles River Laboratories)
  - Male TH::Cre mice (1-4 months old, Jax #008601)
  - Male PKCδ::GluCl-CRE BAC transgenic mice (1-4 months old, mmrrc #11559)
  - Male SST-RES-Cre mice (1-4 months old, Jax #13044)
  - Rosa::loxP-STOP-loxP-td-Tomato animals (Jackson #007905) were crossed to either PKCδ::GluCl-CRE BAC transgenic mice or SST-RES-Cre mice for double transgenic male offspring.
  - TH::Cre mice were crossed to Male PKCδ::GluCl-CRE BAC transgenic mice for double transgenic male offsprings.
- Wild animals: No wild animals were used
- Field-collected samples: No field collected samples were used

Flow Cytometry

Plots
- Confirm that:
  - The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
  - The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
  - All plots are contour plots with outliers or pseudocolor plots.
  - A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation: D1R expression in CEI neurons (Supplementary Figure 6):
- PKCd::Cre or SST::Cre mice were crossed to Rosa::loxP-STOP-loxP-td-Tomato animals and the offspring used for neural population sequencing. Males (2-5 months old) were decapitated, brains extracted on ice and 1mm thick brain sections cut in ice-cold Hibernate A Low Fluorescence solution (BrainBits). The central amygdala was extracted using biopsy punchers (1mm
diameter; Integra Miltex) and enzymatically dissociated following standard procedure (Papain Dissociation System, Worthington Biochem). For each animal, the central amygdala was extracted once on each side and then pooled.

RNAi mediated knock-down efficiency (Supplementary Figure 10): NIH-3T3 cells were stably transfected with a construct expressing a td-Tomato transgene harboring target sites of D1R and several control shRNAs in its 3'UTR. These cells were transduced with ASGE vectors harboring either D1R or control shRNAs. Cells were finally collected and td-Tomato reporter knockdown in GFP-shRNA expressing cells was quantified using flow cytometry.

| Instrument | BD FACS Aria III |
| Software   | BD FACSDiVa Software |

**Cell population abundance**

D1R expression in CEl neurons (Supplementary Figure 6):

Approximately 1000 td-Tomato+ cells were retrieved with FACS sorting. Proportionally, SST-cre/tdTomato and PKCd-cre/tdTomato mice yielded approximately 20% positive cells. Note that these numbers underestimate the typical fraction of PKCd+ and SST+ cells in CEA, as conservative gating settings were used, to restrict sampling to unambiguously identified positive cells.

**Gating strategy**

D1R expression in CEl neurons (Supplementary Figure 6):

1st gate: FSC-A vs. SSC-A
2nd gate: FSC-A vs. FSC-W - singlet/aggregate separation.
3rd gate: mCherry (target channel - 610/20 nm) vs. PE-Cy7 (non-target channel - 780/60 nm) - positive/negative separation in aspect of mCherry, while avoiding high auto-fluorescent events.

RNAi mediated knock-down efficiency (Supplementary Figure 10):

Abundance and post-sort fractions are described in Supplementary Figure 8

1st gate: FSC-A vs. SSC-A
2nd gate: FSC-A vs. FSC-W - singlet/aggregate separation.
3rd gate: two channels, tdTomato (target channel - 610/20 nm) vs. GFP (non-target channel - 470/90 nm), were split into four groups: GFP pos/tdTomato neg, GFP pos/tdTomato pos, GFP neg/tdTomato pos, GFP neg/tdTomato neg

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.