Ventral striatal islands of Calleja neurons control grooming in mice

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The striatum comprises multiple subdivisions and neural circuits that differentially control motor output. The islands of Calleja (IC) contain clusters of densely packed granule cells situated in the ventral striatum, predominantly in the olfactory tubercle (OT). Characterized by expression of the D3 dopamine receptor, the IC are evolutionally conserved, but have undefined functions. Here, we show that optogenetic activation of OT D3 neurons robustly initiates self-grooming in mice while suppressing other ongoing behaviors. Conversely, optogenetic inhibition of these neurons halts ongoing grooming, and genetic ablation reduces spontaneous grooming. Furthermore, OT D3 neurons show increased activity before and during grooming and influence local striatal output via synaptic connections with neighboring OT neurons (primarily spiny projection neurons), whose firing rates display grooming-related modulation. Our study uncovers a new role of the ventral striatum’s IC in regulating motor output and has important implications for the neural control of grooming.
genic access to the IC, an irregular and deep neural structure. Whole-brain imaging demonstrates that the IC form a continuous, branched network in the ventral striatum, largely within the OT. Optogenetic activation of OT D3 neurons induces robust grooming even in competition with other ongoing behaviors, while inactivation of these neurons halts ongoing grooming. Genetic ablation of OT D3 neurons reduces the total spontaneous grooming time by reducing both the number of grooming bouts and grooming duration per bout. Retrograde tracing reveals that OT D3 neurons receive direct inputs from other brain regions implicated in grooming (for example, the ventral pallidum, lateral hypothalamus and amygdala), while anterograde tracing and ex vivo electrophysiological recordings indicate that OT D3 neurons influence striatal output by inhibiting neighboring OT neurons including SPNs. In vivo calcium imaging via fiber photometry demonstrates increased OT D3 neuronal activity before and during grooming. Moreover, single-unit recordings reveal that the firing rates (FRs) of OT units display grooming-related modulation in freely behaving mice. Taken together, this study uncovers a new role of ventral striatal circuitry involving OT D3 neurons in grooming control.

Results
Islands of Calleja contain densely packed D3 neurons. To dissect the neural circuitry and function of the IC, we took advantage of a BAC transgenic D3-Cre line (Methods), which allows genetic access to IC neurons characterized by expression of the D3 dopamine receptor. When crossed with a Cre-dependent tdTomato reporter line (Ai9) to generate D3-Cre/tdTomato mice, the IC are readily visible via whole-brain CLARITY imaging (Fig. 1a, Extended Data Fig. 1a–c and Supplementary Video 1). In coronal sections, the IC are identifiable as clusters (‘islands’) of D3-Cre/tdTomato granule cells in the ventral striatum (Fig. 1d,e). Interestingly, in whole brains, these ‘islands’ appear as a contiguous structure with sparser cell bodies and neurites of isolated D3 neurons in between islands (Fig. 1a, Extended Data Fig. 1 and Supplementary Video 2). With the exception of the ‘major island’ in the nucleus accumbens (NAc), all other islands are situated in the OT (Extended Data Fig. 1b). The shape and spatial distribution of the IC showed some variations between the two hemispheres of the same mouse and across different mice (Extended Data Fig. 2). We also observed tdTomato-positive neurons in the piriform cortex, hypothalamus and hippocampus (Extended Data Fig. 3).

To determine whether we can specifically target the IC using this D3-Cre mouse line, we quantified D3-Cre/tdTomato neurons in the OT, NAc and ventral pallidum (Extended Data Fig. 1b), which account for 83.3%, 11.3% and 5.4% of the total tdTomato+ neurons in this region, respectively (Extended Data Fig. 1d,e). Within the OT, although there are some ‘loose’ D3-Cre/tdTomato neurons, the vast majority (~90%) of OT D3-Cre/tdTomato neurons can be categorized as ‘dense’ clusters belonging to the IC network (Fig. 1b,c and Methods). Overall, this transgenic D3-Cre line provides genetic access to OT D3 neurons, which predominantly reside in the IC.

We characterized intrinsic electrophysiological properties of OT D3 neurons using whole-cell patch-clamp recordings in acute brain slices from D3-Cre/tdTomato mice. We recorded IC D3-Cre/tdTomato neurons (Fig. 1c), which had small cell bodies (6–8 µm in diameter) and high input resistances (2.10 ± 0.28 GΩ; n = 10 cells). These neurons displayed no spontaneous action potentials, suggesting that they are under tonic inhibition and/or need external excitatory inputs to fire. Upon current injections, 29.1% (16 of 55) of IC D3 neurons fired a single spike, but the majority (70.9%; 39 of 55) fired persistently with a maximal FR of ~20 Hz (Fig. 1f), which guided the stimulation parameters in the initial optogenetic experiments described below.

Optogenetic activation of olfactory tubercle D3 neurons induces grooming. To manipulate the activity of OT D3 neurons, we generated D3-Cre/ChR2 mice by crossing the D3-Cre line with the Cre-dependent channelrhodopsin-2 (ChR2) line (Ai32). We verified that OT D3-Cre/ChR2 neurons in acute brain slices were reliably activated by blue laser stimulation (latency < 1 ms; Fig. 2a). The persistent firing neurons (as in Fig. 1f) responded with high fidelity to stimulations up to 20 Hz, consistent with our acute slice analysis, and maintained robust firing for up to 20 s. These stimulation parameters were used in the initial optogenetic experiments in D3-Cre/ChR2 mice with an optical fiber unilaterally implanted in the OT (Fig. 2b). Upon blue-light stimulation, mice immediately stopped exploration in the open field and started grooming. During 20–s stimulations, mice groomed for ~10 s before resuming other activities (for example, walking and exploring; Fig. 2b).

To further analyze the light-induced grooming behavior, we videotaped D3-Cre/ChR2 mice in a clean cage from a side view. Blue-light-induced grooming always started with phase I (paw and nose grooming with elliptical bilateral strokes) and about 10% (n = 70 trials from 10 and 20-s stimulation in Fig. 2d) progressed to phase II (face grooming with unilateral strokes) or phase III (head grooming with bilateral strokes; Supplementary Video 3). We never observed blue-light-induced grooming ending with body licking (phase IV), even though the mice exhibited spontaneous grooming bouts with complete four-phase syntax. In this study, if not otherwise stated, ‘grooming’ thus refers to nose–face–head grooming (phase I to III) excluding body licking (phase IV). The beginning of a grooming bout was defined as when both paws were lifted to reach the face and the ending as when both paws returned to the cage floor. Notably, optogenetically induced grooming had a side bias: the forepaw on the stimulated side always lifted first, even though spontaneous grooming could start with lifting of either forepaw (40 bouts examined from the same mice in Fig. 2d).

Next, we quantified grooming behavior upon blue-light stimulation of the OT by varying stimulation parameters. The latency to grooming onset upon light stimulation was short (0.49 ± 0.13 s; n = 175 trials from seven mice) and independent of stimulation duration (Fig. 2e,f). Grooming time gradually increased with stimulation duration (Fig. 2c,d and Supplementary Video 4). For stimulation >10 s, grooming typically stopped before the light turned off, suggesting an internally programmed upper limit of grooming duration per bout (see below). When the frequency of light pulses increased from 1 to 20 Hz, mice showed a significant increase in grooming duration (Fig. 2f). Strikingly, the animals displayed faster strokes at higher stimulation frequencies (Fig. 2g and Supplementary Video 5). Taken together, these results suggest that OT D3 neurons may function in initiation, maintenance and execution of grooming behavior.

We performed several controls to test if blue-light-induced grooming indeed results from optogenetic activation of OT D3-Cre/ChR2 neurons. First, green laser with the same parameters did not elicit grooming behavior in the same cohort of D3-Cre/ChR2 mice (Supplementary Video 3). Green light should excite ChR2 with less efficiency although we did not validate this here. Second, the same blue laser stimulation did not elicit grooming behavior when the optical fiber was implanted in the OT of D3-Cre/tdTomato mice (n = 6; Fig. 2b and Supplementary Video 6). Third, the same blue laser stimulation did not elicit grooming behavior when the optical fiber was implanted in either the NAc (n = 5) or the hippocampus of D3-Cre/ChR2 mice (n = 3 in dentate gyrus and n = 2 in CA3; Supplementary Video 6). Finally, to rule out that blue-light-induced grooming results from stimulation of en passant ChR2+ fibers originating elsewhere (such as the hippocampus; Extended Data Fig. 3c,c′), we focally injected Cre-dependent adeno-associated virus serotype 1 (AAV1)-DIO-ChR2-EYFP virus in the OT of D3-Cre/tdTomato mice. Here, blue-light stimulation in the OT also induced
grooming. In contrast, we never observed grooming upon light stimulation or blue-light stimulation in a different brain region (Supplementary Video 7).

We next asked whether optogenetic activation of OT D3 neurons triggers grooming even when the animals are engaged in other highly motivated or compulsive behaviors (Fig. 3). In a cohort of D3-Cre/ChR2 mice (an optical fiber unilaterally implanted in the OT), we introduced an unfamiliar mouse into the test cage (both sexes used in separate trials). When the resident D3-Cre/ChR2 mouse was engaged in social investigation, blue-light stimulation was triggered. Blue light induced grooming (with a similar latency as described in Fig. 2c) while suppressing social investigation, which was resumed after grooming termination. By contrast, green light, which should cause weaker activation of ChR2, had little effect on social investigation behavior (Fig. 3b,c and Supplementary Video 10). Because a complete grooming bout starts from nose grooming (phase I) and ends with body licking (phase IV), we examined the effect of OT D3 neuron activation on ongoing episodes of body licking. Blue-light stimulation of the OT in D3-Cre/ChR2 mice terminated the ongoing body licking in all trials and reinitiated phase I grooming in more than 70% of the trials (17 of 24), while green light had minimal effects (2 of 24; Fig. 3d,d c' and Supplementary Video 11). Taken together, these findings indicate that optogenetic activation of OT D3 neurons is sufficient to induce robust grooming behavior even in competition with alternative ongoing behaviors.

**Loss-of-function of olfactory tubercle D3 neurons affects grooming.** Because phasic optogenetic activation of OT D3 neurons may impose unusually strong network activity in striatal circuits, we asked whether inactivation of OT D3 neurons affects ongoing grooming behavior. We injected Cre-dependent AAV2/9-DIO-eArchT-EGFP virus unilaterally into the OT of D3-Cre/tdTomato mice so that infected D3 neurons expressed the green-light-activated outward proton pump archaerhodopsin and were thus inhibited upon green-light exposure (Fig. 4a). We verified that D3-Cre/eArchT neurons in acute brain slices were hyperpolarized by green laser pulses without rebound firing (Fig. 4a). For in vivo testing, mice were monitored in clean cages 4 to 6 weeks after virus injection (to allow development of substantial photocurrents upon modest light stimulation c' and fiber implantation). When mice started spontaneous grooming, green light was delivered to inhibit neuronal activity of D3-Cre/eArchT neurons (Fig. 4b and Supplementary Video 12). To rule out that behavioral changes resulted from thermal effects of continuous green-light stimulation,
we always subjected the same mice to continuous blue-light exposure at the same intensity. Green-light inactivation of OT D3-Cre/eArchT neurons halted spontaneous grooming within 3 s in 69% of the trials, while blue light with the same parameters, which should excite eArchT neurons with less efficiency, only coincided with halted grooming in 12% of the trials (Fig. 4b and Supplementary Video 12). As expected, grooming duration in green-light trials was significantly shortened compared to blue-light trials (Fig. 4c,d). Similarly, green-light inactivation of OT D3-Cre/eArchT neurons stopped water-spray-induced grooming in 50% of the trials, significantly higher than the 3% in the blue-light group (Fig. 4e and Supplementary Video 13). These results suggest that OT D3 neuronal activity is necessary to maintain grooming behavior.

As multiple brain regions have been previously implicated in grooming\(^{34,35}\), we asked whether OT D3 neurons play a role in generating spontaneous grooming under normal conditions. For genetic ablation of OT D3 neurons, D3-Cre/ChR2 mice were unilaterally or bilaterally injected with Cre-dependent diphtheria toxin subunit A (DTA) virus (AAV8-mCherry-FLEX-DTA) or control AAV8-TurboRFP virus. DTA-dependent ablation of D3-Cre/ChR2 neurons was verified after completion of behavioral tests (Fig. 5a,b). Note that ChR2 was never stimulated but instead the fused EYFP was used to gauge effectiveness of the genetic ablation. EYFP fluorescence signal was significantly reduced in the ablated side compared to the control side in unilaterally ablated mice (Fig. 5b), and similar efficacy was verified in bilaterally ablated mice (Extended Data Fig. 5). The grooming behavior within 30 min was compared at different time points after viral injection (1 to 4 weeks). Compared to the control group, the total grooming time was reduced by 50% (Fig. 5c), arising from significant decreases in both the number of grooming bouts and the grooming duration per bout (Fig. 5d,e). Consistent with the results of unilateral optogenetic inactivation, unilateral ablation of OT D3 neurons caused similar effects as bilateral ablation of OT D3 neurons. These findings suggest that OT D3 neurons play a critical role in grooming initiation as well as maintenance under normal conditions.

**Olfactory tubercle D3 neurons receive inputs from other grooming centers.** To define the brain regions that exert control over OT D3 neurons, we used the pseudotyped rabies virus (RV) system for retrograde labeling of neurons that are monosynaptically connected to this population\(^{34,35}\). We unilaterally injected Cre-dependent AAVs with bicistronic expression of TVA-mCherry, a required receptor for EnvA-pseudotyped RV, and RV glycoprotein, required for transsynaptic spread, into the OT of D3-Cre mice. After 10 d, EnvA-pseudotyped RV-EFGR virus was injected into the OT. Brains were fixed 7 d later and sectioned coronally (100-μm thickness) for confocal microscopy imaging (Fig. 6a). We first verified that the ‘yellow’ D3 neurons (TVA-mCherry and EGFR double positive; presumptive ‘starter’ cells) were located within the OT, predominantly within the IC (Fig. 6a,b). We then quantified 40, 55 and 68 starter cells in three mice (Methods), and counted presynaptic (EGFP+ only) cells from every other section throughout the brain to calculate the percentage of cells labeled in retrograde in each brain region (Fig. 6c). Interestingly, presynaptic partners of OT D3 neurons were found in several brain regions (that is, the ventral pallidum, lateral hypothalamic area and amygdala; Fig. 6c and Extended Data Fig. 4), which have been implicated in mediating grooming behavior\(^{34,45}\).
**Fig. 3 | Activation of olfactory tubercle D3-Cre/ChR2 neurons induces grooming while suppressing alternative ongoing behaviors.** a–c. Blue light (activation of D3-Cre/ChR2 neurons) or green light (weaker activation of ChR2) was delivered during three ongoing behaviors: social investigation (green light, 74 trials; blue light, 102 trials) (a), feeding after 20-h food deprivation (green light, 69 trials; blue light, 84 trials) (b) and itch-induced scratching (green light, 33 trials; blue light, 42 trials) (c). a’–c’. Summary data from eight mice. Each mouse was tested in 8–15 trials for a’, 6–18 trials for b’ and 4–10 trials for c’, and a percentage was calculated. d–d’. Blue light, but not green light, stopped body licking (n = 24 trials; three trials per mouse in eight mice). Wilcoxon signed-rank test, *P < 0.01. Both blue and green laser stimulations were at 20 Hz with 10-ms pulses. All averaged data are shown as the mean ± s.e.m.

Olfactory tubercle D3 neurons make local synaptic connections. To identify downstream targets of OT D3 neurons, we used multiple parallel approaches. First, we traced the output of OT D3-Cre/tdTomato neurons via CLARITY imaging and found no projections outside the ventral striatum (Extended Data Fig. 1), even though we were able to trace fine projections of D3 neurons in the hypothalamus (Extended Data Fig. 3b,b’). Second, we performed anterograde tracing by injecting Cre-dependent AAV(DJ/8)-FLEX-synaptophysin::EGFP unilaterally into the OT for optogenetic (activation of D3-Cre/ChR2 neurons) or green light (weaker activation of ChR2) was delivered during three ongoing behaviors: social investigation (green light, 74 trials; blue light, 102 trials) (a), feeding after 20-h food deprivation (green light, 69 trials; blue light, 84 trials) (b) and itch-induced scratching (green light, 33 trials; blue light, 42 trials) (c). a’–c’. Summary data from eight mice. Each mouse was tested in 8–15 trials for a’, 6–18 trials for b’ and 4–10 trials for c’, and a percentage was calculated. d–d’. Blue light, but not green light, stopped body licking (n = 24 trials; three trials per mouse in eight mice). Wilcoxon signed-rank test, *P < 0.01. Both blue and green laser stimulations were at 20 Hz with 10-ms pulses. All averaged data are shown as the mean ± s.e.m.

To verify that anatomically identified synaptic connections are functional, we performed whole-cell patch-clamp recordings in acute brain slices. We first examined synaptic connections among OT D3 neurons by injecting Cre-dependent AAV1-DIO-ChR2-EYFP virus into the OT of D3-Cre/tdTomato mice (Fig. 7c). Three to four weeks later, OT D3 neurons were recorded in brain slices. As expected, in ChR2-EYFP+ neurons, blue laser pulses evoked high-fidelity action potentials under current clamp mode (similar to Fig. 2a) and inward somatic optical currents with extremely short latency (<1 ms) under voltage clamp mode. To enhance our detection of GABA A-mediated inhibitory postsynaptic currents (PSCs), we used a high Cl− internal solution to set the reversal potential of GABA A-mediated currents at ~0 mV and recorded inward currents from a holding potential of ~60 mV (Methods). In a subset (41.0%) of D3-Cre/tdTomato-positive, but ChR2-EYFP-negative neurons, blue-light pulses evoked inward PSCs (latency of ~4.7 ms and jitter of ~1.3 ms; Fig. 7c and Extended Data Fig. 6a,b). The variation in latency and polyphasic appearance in some traces during repeated stimuli were likely because some presynaptic D3 neurons fired more than one spike upon light stimulation (for example, the first light pulse induced two spikes in the example shown in Fig. 2a) and not all presynaptic D3 neurons fired/released neurotransmit-
Fig. 4 | Inactivation of D3-Cre/eArchT neurons halts ongoing grooming. a, Left, viral injection strategy. Middle, postmortem verification of viral infection and optical fiber implantation (similar results were observed in seven mice). Scale bars, 1,000 µm (left image) and 100 µm (right image). Right, green light effectively inhibited OT D3-Cre/eArchT neurons in acute brain slices (n = 3 of three neurons). b, Green light (inactivation of OT D3-Cre/eArchT neurons), but not blue light (which potentially excites eArchT with much less efficiency), shortened spontaneous grooming bouts. Left, representative trials. Right, the percentage of spontaneous grooming terminated within 3 s upon stimulation with green or blue light. Wilcoxon signed-rank test, P = 0.016. c, Distribution of grooming durations upon 10-s stimulation of OT D3-Cre/eArchT neurons by green or blue light. d, Average grooming durations upon green-light or blue-light stimulation of OT D3-Cre/eArchT neurons. Student’s t-test, t = 25.505 and P = 2.4 x 10⁻². The same dataset was analyzed for b-d: n = 25 trials from seven mice with 3–6 trials per mouse. e, Green light (inactivation of OT D3-Cre/eArchT neurons), but not blue light, stopped water-spray-induced grooming. Left, representative trials. Right, the percentage of water-spray-induced grooming terminated within 3 s upon green-light or blue-light stimulation of OT D3-Cre/eArchT neurons (n = 7 mice; green light, n = 90 trials with 11-15 trials per mouse; blue light, n = 69 trials with 5-16 trials per mouse). Wilcoxon signed-rank test, P = 0.016. Both green and blue light were delivered continuously with the same intensity for a single mouse. *P < 0.05 and ****P < 0.0001. All averaged data are shown as the mean ± s.e.m.
Two-sample Kolmogorov–Smirnov test was used to compare any two distributions: untreated control versus vector control, $P = 0.512$; untreated control versus bilateral ablation, $P = 0.054$. ** $P < 0.0001$. All averaged data are shown as mean $\pm$ s.e.m.

The results indicate that OT D3 neurons were active before and during grooming, supporting their contribution to grooming initiation and possibly maintenance.

To test how OT neuron firing is modulated during grooming behavior, we implanted five wild-type mice with tungsten multiwire electrode arrays in their OTs (Fig. 8f) and analyzed single-unit activity during a total of 117 spontaneous grooming events (Fig. 8g; see Extended Data Fig. 7 for single-unit verification). As D3 neurons are small granule cells, we assume their activity will not be directly acquired by wire electrode arrays and thus the activity most likely stems from SPNs, the predominant type of striatal neurons. Further supporting this, from the 27 isolated OT single units, most of them had relatively low background FRs (mean $\pm$ s.e.m., $2.0 \pm 1.5$ Hz; Extended Data Fig. 7), which is characteristic of SPNs. We compared the averaged FRs within $\pm 1$ s relative to grooming onset to their background activity. Among all neurons, 14 of 27 were significantly modulated by grooming: 7 displayed significant decreases and 7 displayed increases in FRs (Fig. 8g; see Methods for details on statistical analysis). Because each grooming bout displayed by a mouse may uniquely influence and/or be influenced by local OT neural activity, we generated individual ‘cell–groom pairs’ ($n = 621$) derived from significantly modulated neurons in relation to each bout and classified them as being decreased or increased during the $1 \text{s}$ before grooming ($\Delta \text{Hz FR}_{\text{pre}} = \text{FR}_{\text{background}}$), or within $1 \text{s}$ following the start of grooming ($\Delta \text{Hz FR}_{\text{during}} = \text{FR}_{\text{background}}$). This allowed us to identify both the magnitude and direction of firing changes from all neurons monitored in a given bout relative to grooming. In both time windows, some OT cell–groom pairs (87 and 82 in ‘pre’ and ‘during’, respectively) displayed decreases in FRs while others (90 and 89 in ‘pre’ and ‘during’, respectively) displayed increases (Fig. 8h). This bidirectional change in firing relative to grooming differed from what was observed when sampling from the same neurons yet during pseudorandomly selected grooming-free ‘shuffled’ periods of time, which revealed statistically more cell–groom pairs with increased firing in the period of time before grooming onset (Fig. 8h and Discussion). Together, these findings indicate that firing of OT neurons (primarily SPNs) is altered during grooming.

**Discussion**

In the present study, we investigated the neural circuitry and function of OT D3 neurons mostly concentrated in the IC, a previously understudied cell population of the ventral striatum. By combining optogenetics, ex vivo and in vivo electrophysiology, whole-brain...
CLARITY imaging, viral circuit tracing and in vivo fiber photometry, we discovered that this neuronal population is involved in controlling grooming behavior, highlighting a new function of the IC striatal circuits.

Our study strongly supports that OT D3 neurons play a role in grooming initiation, maintenance and execution. Supporting a role in grooming initiation, optogenetic activation of these neurons was sufficient to elicit robust grooming with a short latency of ~0.5 s (Fig. 2), even when the animals were engaged in other highly motivated or compulsive behaviors including social investigation, feeding and itch-induced scratching (Fig. 3). In addition, genetic ablation of OT D3 neurons reduced the total grooming time and the total grooming bouts by ~50% (Fig. 5), suggesting that these neurons contribute to grooming initiation under normal conditions. Moreover, in vivo calcium recordings from populations of OT D3 neurons via fiber photometry reveal elevated neuronal activity before grooming onset, consistent with the finding that significantly more OT neurons show firing changes before the grooming onset (Fig. 8).

Several lines of evidence support the contribution of OT D3 neurons to grooming maintenance. When OT D3-Cre/ChR2 neurons were optogenetically activated, the duration of induced grooming increased with the stimulation duration from 1 to 20 s (Fig. 2). Furthermore, optogenetic inactivation of these neurons halted
ongoing grooming (Fig. 4), and genetic ablation of these neurons significantly decreased the grooming duration per bout (Fig. 5). Finally, OT D3 neurons were active before the grooming onset and the elevated activity remained for a few seconds during grooming (Fig. 8), although we note the limited temporal resolution of this approach.

The potential involvement of OT D3 neurons in grooming execution is supported by optogenetically induced grooming experiments, wherein stroke frequency increased with optogenetic stimulation frequency from 1 to 10 Hz (Fig. 2). Moreover, initiation of grooming exhibited a side bias (that is, the forepaw on the stimulated side lifted first), suggesting that activation of OT D3 neurons produces a motor rather than motivational signal. Taken together, the current dataset is consistent with involvement of OT D3 neurons in grooming initiation, maintenance, and execution.

While OT D3 neurons are intimately linked to grooming, our data also suggest additional mechanisms for grooming control. For example, during 20-s optical stimulation of OT D3-Cre/ChR2 neurons, the induced grooming typically stops before the light turns off (Fig. 2), suggesting other pathways involved in cessation of grooming. Animals with unilateral ablation of OT D3 neurons still displayed spontaneous grooming (Fig. 5). Because unilateral ablation had similar effects as bilateral ablation, this finding likely results from involvement of other grooming centers rather than incomplete ablation. It is worth mentioning that OT D3 neurons unlikely contribute to programming of the grooming syntax. Although optogenetic activation of OT D3 neurons robustly initiated grooming, the induced grooming was limited to nose–face–head grooming (phase I to III) and almost never progressed to body licking (phase IV). Furthermore, activation of OT D3 neurons during phase IV (resulted

Fig. 7 Olfactory tubercle D3 neurons make local synaptic contacts. a. Left, experimental design of anterograde tracing from OT D3-Cre/tdTomato neurons. Middle, EGFP axonal terminals were visible after 4 weeks within the IC and OT. Right, enlarged image from the dashed rectangle. Note clustered D3 neuron cell bodies (bottom) and neuropils with numerous labeled synapses (top). Similar observations in seven mice. Scale bars, 200 μm (middle) and 20 μm (right). b. D3 neurons filled with Alexa Fluor 488 within an island (left; seven neurons) or in between two islands (right; three neurons) of D3-Cre/tdTomato mice. Scale bars, 200 μm. c. OT D3 neurons inhibit each other. Left, experimental design. Middle, postmortem verification of the viral injection site in the OT. Scale bar, 200 μm. Right, repeated 10-ms blue-light-evoked PSCs in IC neurons (tdTomato reporter). d. D3 neurons inhibited neighboring OT SPNs. Left, IC with densely packed D3-Cre/ChR2 neurons in the OT. Scale bar, 500 μm. Right, repeated 10-ms blue laser pulses evoked PSCs in OT SPNs near the IC. e. Light-evoked PSCs in OT SPNs were blocked by GABA<sub>ₐ</sub> receptor antagonist (10 μM bicuculline) but not changed by glutamate receptor antagonists (50 μM AP5 + 20 μM CNQX). Friedman test: F(3,36) = 18.840, P = 2.95 x 10⁻⁴. f. Light-evoked PSCs in OT SPNs were blocked by TTX (1 μM) and reappeared after co-application of TTX + 4-AP (1 mM), supporting monosynaptic connection. Friedman test: F(2,12) = 10,000, P = 7.72 x 10⁻⁴. n = 10 and 5 cells from five mice in e and f, respectively. Each data point in e and f is an average of 6–10 traces. ***P < 0.001. Holding potential, −60 mV.
from a spontaneous grooming bout) stopped body licking, and in most trials, reinitiated nose grooming (phase I; Fig. 3). These results further support a role of OT D3 neurons in grooming initiation.

While the IC connectivity had been previously proposed, our study directly assesses the inputs and outputs of OT/IC D3 neurons via viral tracing tools. Our findings not only confirm previously identified presynaptic areas (for example, the NAc, piriform cortex, amygdala, and ventral tegmental area), but also extend to new regions (for example, the ventral pallidum, hypothalamus, and midbrain structures; Fig. 6). Some of these regions have previously been implicated in grooming control, including the ventral pallidum, lateral hypothalamus and midbrain structures. Interestingly, optogenetic activation of glatamergic neurons in the lateral hypothalamus and medial amygdala also induce self-grooming, but with a latency of several seconds, longer than the latency period observed upon OT D3 neuron stimulation (Fig. 2). The IC/OT D3 neuron network may thus coordinate with other grooming centers in the brain to generate grooming under different conditions. Dissecting the distinct contributions from each region warrants future investigations.

IC/OT D3 neurons likely influence striatal output and behavior by inhibiting other neurons in the ventral striatum. In contrast to the ‘islands of Calleja’ nomenclature, whole-brain imaging of D3-Cre/tdTomato neurons reveals that these islands actually form a continuous, branched structure throughout the ventral striatum (Fig. 1 and Extended Data Figs. 1 and 2). Via anterograde tracing from OT D3-Cre/tdTomato neurons, we did not find projections outside the ventral striatum, suggesting that these neurons act as local interneurons and exert their function through modulation of other neighboring neurons including both D3 neurons and OT SPNs (Fig. 7). During in vivo optogenetic experiments, presumably only a subset of OT D3 neurons can be directly activated or inactivated optically via a 400-μm fiber, but such manipulations reliably triggered or halted grooming, respectively (Figs. 2–4). In addition, unilateral ablation of OT D3 neurons had a similar effect as bilateral ablation in reducing the number of grooming bouts and grooming duration per bout (Fig. 5), suggesting that loss of function of a partial IC network is sufficient to interfere with the grooming behavior. The effectiveness of these manipu-
lations suggests that either a small subset of D3 neurons is sufficient to mediate grooming or that IC neurons function as a unified network, with local manipulations propagating to distant parts of the structure. The unique geometry and connectivity may enable IC neurons to act as a unified network to simultaneously modulate widespread SPNs in the ventral striatum. In addition to chemical synapses (Fig. 7), densely packed IC D3 neurons may also communicate with each other via gap junctions and/or ephaptic coupling, which may facilitate synchronized activity among the densely packed D3 neurons. Because single-cell recordings from tiny IC neurons situated in the most ventral part of the brain are extremely challenging, we used in vivo fiber photometry to demonstrate elevated neuronal activity from populations of OT D3 neurons during grooming (Fig. 8). Given that OT D3 neurons provide direct GABAergic inputs onto neighboring neurons including SPNs (Fig. 7), it may seem counterintuitive that more OT units show increased firing before the grooming onset (Fig. 8). Several factors possibly contribute to this finding. As all OT neurons are embedded in a largely inhibitory local network, activation of a subpopulation of GABAergic neurons (for example, IC D3 neurons) inhibits monosynaptically connected cells while leading to disinhibition of others. Additionally, type D1 and type D2 SPNs, which are not distinguished in our in vivo recordings, may be differentially modulated during grooming behavior. Moreover, the average FR of OT neurons is relatively low (~2 Hz), which makes it challenging to identify decreased firing. Nevertheless, the results support the notion that IC neurons are uniquely positioned to change striatal output and behavior.

One question remaining is what specific role(s) the D3 receptor in IC neurons may play in grooming control and/or other physiological functions in health and disease. Genetic knockout of the D3 receptor increases the basal level of grooming behavior. However, because the majority (~80%) of IC D3 neurons coexpress the D1 receptor, whereas the remaining likely coexpress the D2 receptor, specific pharmacological reagents targeting the D3 receptor or genetic knockdown or knockout of this receptor in defined cell populations would be required to tease out the function of the D3 receptor in IC D3 neurons.

As the most ventral part of the striatum, the OT receives sensory inputs from the olfactory system among others, and has been implicated in associative learning, reinforcement and reward-related behaviors. The current study reveals a surprising role of IC and OT circuitry in motor control, specifically in mediating grooming behavior, expanding our current understanding of distinct roles of different subdivisions of the striatum. Interestingly, abnormal grooming is frequently observed in animal models of neurological and neuropsychiatric disorders (Tourette syndrome, obsessive compulsive disorder and autism spectrum disorder), which are often accompanied by impairment of striatal circuits. Our study highlights a new role of the IC network in grooming control under normal conditions and suggests their dysfunction in pathological states.

Online content
Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41593-021-00952-z.

Received: 13 May 2020; Accepted: 1 October 2021; Published online: 18 November 2021

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Methods

Animals. The bacterial artificial chromosome transgenic D3-Cre line (stock B6.FVB(Tg)-Drd3-cre) was donated by Dr. N. Heinis (The Rockefeller University, New York) and Dr. C. Gerfen (NIH, National Institute of Mental Health). The D3-Cre line was crossed with the tdTomato reporter line (JAX stock no. 007909 or A9 line B6.Cg-Gt(Rosa)26Sortm32(CAG-COP4*H134R/EYFP)Hze) to generate D3-Cre/tdTomato mice. A subset of D3-Cre/tdTomato mice with unilateral AAV1-DIO-ChR2-EYFP virus injection was used for all experiments except for in vivo unit recordings. Because no sex difference was observed, the data from both sexes were combined. Mice were housed in temperature-controlled and humidity-controlled facilities on a 12-h light–dark cycle with food and water available ad libitum unless otherwise stated. Mice were group housed until the surgery of receiving virus injection and intracranial implantation and singly housed afterwards. All experimental procedures were performed in accordance with the guidelines of the NIH and were approved by the Institutional Animal Care and Use Committees of the University of Pennsylvania and the University of Florida. The number and sex of mice used in each set of experiments are summarized in Supplementary Table 1.

Viruses. Cell-type-specific transsynaptic RV labeling requires expression of Cre-dependent AAVs expressing TVA-2a-mCherry, the receptor for EnvA pseudotyped RV and RV glycoprotein to enable transsynaptic spread. These plasmids (pAAV.CAG.DIOloxP.TVA667a.mCherry; pAAV.CAG.DIO-FlloxP.EGFP; pAAV.CAG.DIO-FlloxP.EGFP; pAAV.CAG.DIO-FlloxP.EGFP) were used as kind gifts from L. Luo and the viruses were packaged in-house with the D5 serotype. The EnvA pseudotyped RV-EGFP was generated both in-house (expanded RV-EGFP virus derived from stock kindly provided by B. Lim) as well as purchased from the Salk viral core. AAV(D1/8)-FLEX-synaptophysin:EGFP (AAV(D1/8)-FLEX-synaptophysin:EGFP-WPRE-hGH, 1 × 10¹³ viral genomes per ml) were from the Neuroconnectivity Core, Baylor College of Medicine, AAV1-DIO-Chr2-EYPF (AAV1-EF1a-Dio-hChr2 (H134R)-EYPF-WPRE-hGH, ≥7 × 10¹³ viral genomes per ml) was a gift from Dr. G. Srinivasan, Addgene viral preparation no. 1011389 (AAV1-FLEX-synaptophysin:EGFP-WPRE-hGH). AAV2/9-DIO-eArchT-EGFP (AAV2/9-DIO-DIO-eArchT-EGFP, ≥1 × 10¹³ viral genomes per ml) were used with a SYS-micro4 controller attachment (World Precision). The tip of the syringe was anesthetized with isoflurane (~3% in oxygen) and secured in a stereotaxic system. Anatomical tracing and confocal imaging. For viral retrograde transsynaptic tracing experiments, a mixture of AAV5.CAG.DIOloxP.TVA667a.mCherry and AAV8.CAG.DIOloxP.AI9 (1:1 ratio; 300 nl) was unilaterally injected into the OT, and after a recovery of 10d, the (EnvA).SAD.G.EGFP virus (300 nl) was injected into the same site to initiate transsynaptic tracing. Seven days later, the mice were anesthetized, and the brains were dissected for fluorescence imaging. Mice were perfused transcardially with 4% paraformaldehyde (PFA) in fresh PBS. The brain was dissected out and postfixed in 4% PFA overnight at 4°C, then transferred into PBS. Coronal slices (100-μm thick) were prepared using a Leica VT1200S vibratome. The slices were treated with glycerol in PBS (volume ratio of 1:1) for 30 min followed by glycerol in PBS (volume ratio of 1:9) for 30 min before being mounted onto SuperFrost slides for imaging. A SPS Leica confocal microscope equipped with LAS AF Lite software and 473-nm, 599-nm and 635-nm lasers was used to obtain images at x10 (for coarse location of fluorescent regions as in Extended Data Fig. 5) and x40 (for cell counting in fluorescent regions as in Fig. 6). For cell counting, we acquired z-stack confocal images for 40 μm (with a z-step of 1 μm) and obtained a projected image for that region. At the injection site, we identified ‘starter’ cells (mCherry and EGFP double positive) from 5 to 6 sections per mouse and counted 16, 22 and 27 cells from the three mice. Because we only imaged 40 μm of 100-μm sections, we estimated the total number of starter cells from the three mice to be 40, 55 and 68, respectively, assuming relative homogeneity throughout. For quantification of presynaptic cells, we counted EGFP-positive (but mCherry-negative) cells from every other section throughout the brain and calculated the percentage of cells in each brain region. Although this approach underestimated the number of labeled cells, it allowed consistent identification of the major input areas in all three mice.

For anterograde tracing, Cre-dependent AAV(D1/8)-FLEX-synaptophysin:EGFP virus (300 nl) was unilaterally injected into the OT, and after a recovery of 10d, the (EnvA).SAD.G.EGFP virus (300 nl) was injected into the same site to initiate transsynaptic tracing. Seven days later, the mice were anesthetized, and the brains were dissected for fluorescence imaging. A subset of mice injected with Cre-dependent AAV1-DIO-Chr2-EYPF or AAV2/9-DIO-eArchT-EGFP viral injection in the OT using a spray bottle with room-temperature pure water. The mice were placed on a turntable at 30 frames per second or an iPhone at 60 frames per second (for accurate measurement of the latency to grooming onset and stroke frequency).

The water-spray-induced grooming was produced in D3-Cre/tdTomato mice with AAV2/9-DIO-eArchT-EGFP viral injection in the OT using a spray bottle with room-temperature pure water. The mice were faced toward the direction of the nozzle (~20 cm away), and water mist was sprayed three times to adequately coat the mouth and facial parts. The effects of green laser (optogenetic inactivation) and blue laser (exciting eArchT with less efficiency) were tested for 3–5 times a day for 3d. For spontaneous grooming, the effects of blue and green lasers were tested for 1–3 times a day for 3d.

The effects of optogenetic activation of OT D3 neurons in D3-Cre/Chr2 mice with unilateral optical fiber implantation were tested in alternative ongoing behaviors. To induce social investigation behavior, the mice were placed in a new cage with fresh bedding for 10min of habituation. Then a stranger mouse (either male or female at the same age) was introduced into the cage. To induce feeding behavior, both the mice were starved for 24h, and then were individually placed in a new cage with fresh bedding with ad libitum access to food pellets. To elicit itch-induced scratching behavior, chloroquine (40 mM; 15 μl) was unilaterally injected into the mouse cheek. In a subset of animals, the alternative behavior included spontaneous grooming that progressed to phase IV (body licking). While the mice were engaged in these behaviors, blue laser (optogenetic activation) or green laser (exciting Chr2 with less efficiency) was delivered.

In vivo optical stimulation and behavioral assays. All behavioral procedures were performed during the light cycle between 9:00 and 12:00. The experimental mice were transferred to the testing room at least 1h before the tests to acclimatize to the environment. Before each test, the mouse was briefly anesthetized via isoflurane and the optical fiber was coupled to the implanted fiber using a 4-way coupling sleeve (Thorlabs). The mice were placed in an open field (40 cm × 40 cm) or a clean new cage when blue laser (473 nm, 10–15 mW per mm², 1–20 Hz of 10-ms pulses for optogenetic activation) or green laser (532 nm, continuous for optogenetic inactivation) was delivered. The interval between two successive optogenetic stimulations was 3–5 min to avoid cumulative effects of the light stimulation. Mice were habituated to the open field for 10min and then placed at 30 frames per second or an iPhone at 60 frames per second (for accurate measurement of the latency to grooming onset and stroke frequency).
respective) and decapitated. The brain was dissected out and immediately placed in ice-cold cutting solution containing 92 mM N-methyl-d-glucamine, 2.5 mM KC1, 1.2 mM NaH2PO4, 30 mM NaHCO3, 20 mM HEPES, 25 mM glucose, 5 mM sodium pyruvate, 0.5% ascorbic acid, 3 mM CaCl2, 0.5 mM MgCl2, 0.5 mM CaSO4, 60 mM Na2SO4, 5.5 mM glucose and 4.47 mM sodium succrose; osmolality - 305 mOsM and pH ~7.3, bubbled with 95% O2 and 5% CO2. Coronal sections (250-μm thick) containing the OT were cut using a Leica VT1200S vibratome. Brain slices were incubated in oxygenated artificial cerebrospinal fluid (124 mM NaCl, 3 mM KCl, 1.3 mM MgSO4, 2 mM CaCl2, 26 mM NaHCO3, 1.25 mM NaH2PO4, 5.5 mM glucose and 4.47 mM sodium succrose; osmolality ~305 mOsM and pH ~7.3, bubbled with 95% O2 and 5% CO2) for ~30 min at 1°C and at least 30 min at room temperature before use. For recordings, slices were transferred to a recording chamber and continuously perfused with oxygenated artificial cerebrospinal fluid. Fluorescent cells were visualized through a ×40 water-immersion objective using an Olympus BX61WI upright microscope equipped with epi-illumination and a 3-wavelength laser (405, 561, 633 nm). Whole-cell patch-clamp recordings were controlled by an EPC-10 amplifier combined with Pulse v8.74 (HEKA Electronik) and analyzed using Igor Pro 6 (WaveMetrics). Recording pipettes were made from borosilicate glass with a Flaming-Brown puller (P-97, Sutter Instruments; tip resistance of 5–10 MΩ). The pipette solution contained 120 mM potassium gluconate, 10 mM NaCl, 1 mM CaCl2, 10 mM EGTA, 10 mM HEPES, 5 mM Mg-ATP, 0.5 mM Na-GTP and 10 mM phosphocreatine. For light-evoked inhibitory PSCs, a high Cl- containing 250 mM KCl instead of potassium gluconate was used so that the reversal potential of [Cl−] was ~ −50 mV and GABAa-receptor-mediated currents would be inwardly directed, allowing all cells to be dendritic. To increase the resting membrane potential kept them healthy for a longer time during recording. Light stimulation was delivered through the same objective via blue laser (473 nm; FTEC473-635YFL, Blue Sky Research) with varying lengths and frequencies. Viral infection in the OT was confirmed in brain slices during recording. Pharmacological drugs AP5, CNQX, bicuculline, TTX citrate and 4-AP were bath perfused during recording.

**CLARITY: tissue clearing, brain imaging and data analysis.** CLARITY-based tissue clearing9,10 and whole-brain microscopy were performed as previously described11–13 with minor adjustments. Briefly, perfusion-fixed brains from adult D3-Cre/tDTomato mice of both sexes were removed from the skull. To maintain structural integrity, tissue was fixed overnight at 4°C in hydrogel fixation solution containing 4% acrylamide, 0.05% bis-acrylamide, 0.25% VA-044 initiator, 4% PFA in Ca2+- and Mg2+-free PBS (pH 7.4). After acrylamide polymerization at 37°C, samples were washed and incubated in 0.5% SDS and 10 mM boric acid (pH 8.5) at room temperature for 24 h and, subsequently, at 37°C for two more months. Next, samples were incubated for 24 h in refractive index matching solution containing 80 g Nycodenz (nRMS80), 20 mM PS (phosphate buffer, pH 7.5), 0.1% Tween 20 and 0.01% sodium chloride. Specimens were kept in a custom-designed imaging chamber that was mounted on the stage of a Leica DMi8 inverted microscope. Imaging was performed with an Andor Dragonfly 500 spinning-disk confocal unit using a 63×-immersion and a HC PL FL 1.30 objective.

For anatomical mapping of D3-positive neurons, data were preprocessed using IMARIS 9.5.1 three-dimensional (3D) rendering software (Bitplane). Surface objects were generated and exported to MeshLab14 to generate STL files. Next, Allen Brain Atlas version 3 (Allen Institute for Brain Science) was merged and manually adjusted to match the specimen surface objects using the mesh deform modifier in Blender 2.78b–2.80 (https://www.blender.org/). The Iterative Closest Point Registration add-on for Blender by N. Klop was used for accurate mesh alignment. Importantly, we exclusively transformed the original 3D brain atlas’ bounding box to match the specimen structure excluding somewhat biased deformations. The resulting sample-matched brain atlas was then sliced into a virtual stack that we superimposed onto the imaging data in IMARIS. Next, OT surfaces were generated and exported using IMARIS and Microsoft 3D Builder 18.0.1931. Virtual volumes were generated using PrusaSlicer 2.1 (Prusa Research). In Blender, the optional overlay was iteratively determined by optimizing the overlap of both volumes.

For categorization and visualization of individual tD Tomato-labeled cells, we first applied a Laplacian-of-Gaussian-Scale Space Maximum Projection (LoGSSMP), corresponding to the seed detection stage of the nucleus detection algorithm TWANG (using the ITK-based C++ application XPIWIT15–17). In brief, the original images were filtered using differently scaled LoG filters and the kernel standard deviations were matched to the manually measured minimum and maximum cell sizes (rmin = 3, rmax = 4 μm). The four-dimensional scale-space was reduced to a 3D image by a maximum intensity projection of the individual LoG-filtered images. Local maxima were then identified in the 3D LoGSSMP image. To minimize false negatives (that is, missed cells), we additionally allowed for detection of multiple seed points on intensity plateaus where no single maximum pixel was present. To coarsely reduce false-positives we determined background regions using the trained D3 classifier that were larger than the global mean plus one standard deviation of the LoGSSMP image intensity was considered. Redundant detections with a distance of less than three voxels in the image space were locally fused to a single detection.

The detection module was parameterized to be very sensitive, that is, to avoid false negatives. This, however, results in increased false-positive detections. Given different signal intensity levels at different tissue depths, using a simple threshold for false-positive rejection was not possible. Instead, we trained a classifier to suppress erroneous detections, that is, to distinguish false positives from true cells. This classifier was trained on 10 manually labelling sets of representative cells using EmbryoMiner for ground truth generation and visual result confirmation18. This interactive software allows superimposing of automatically obtained detections on raw images. Using freehand selection tools, we assigned cells to different groups. For each manually classified detection, we cropped a 32×32 2D patch with the detection located in the center. After the x-slice running through the center of the cell, the resulting training snippets (>5,000 per class) were normalized to zero mean and unit standard deviation and then used to train a small convolutional neural network (CNN) for classification with the following architecture: input layer (32×32×1), conv2D layer (32×32×2), conv2D layer (32×32×4), maximum pooling layer (16×16×4), conv2D layer (16×16×4), conv2D layer (16×16×12), conv2D layer (12×12×32), dropout layer (1), output layer (2). Finally, we trained for 100 epochs using the Adam optimizer. We assessed the performance of the classifier using a fivefold cross-validation and achieved an average classification accuracy of 96.18% on an independent test set. The trained model was then used to classify all remaining cells and was classified as false positive if it lay too far from the target cell. For categorization of OT D3 neurons into either dense or loose populations, we computed a cell density measure by counting the number of detections located in a sphere of radius r = 50 μm surrounding each detection. This measure yields high values if the cell is located in a dense region and low values if few cells are residing in the vicinity. Distribution analysis based on histogram plots of various brain areas confirmed the threshold of r = 5 to separate loose from densely packed cells. Thus, cells with r > 5 neighbors were considered to reside in a dense region. This criterion, however, might not apply to few cells at the edges of dense regions. Consequently, the percentage of truly loose cells is likely an overestimate.

**In vivo fiber photometry.** The AAV9-FLEX-GCaMP7s virus (500 nL) was unilaterally injected into the OT of D3-Cre/tDTomato mice and a 400-μm optical fiber cannula was implanted above the injection site. Based on postmortem histology, the mice were categorized into two groups: (1) optical fiber near IC, where the optical fiber was right above a GCaMP7s-positive island and the vertical distance between the fiber tip and the island was <200 μm, and (2) optical fiber away from IC, where there was no GCaMP7s-positive island right below the fiber tract. In this case, the distance from the fiber tip to the closest GCaMP7s-positive island was measured and used in Fig. 8e. After allowing 3–4 weeks for GCaMP7s expression, mice were attached via an optical fiber (400-μm core, 0.48 NA; Thorlabs, M76L01) to a Doric four-port minicube (FM4C, Doric Lenses). Blue (480-nm wavelength for GCaMP7s stimulation; Thorlabs, M405FP3) and violet (405-nm wavelength for artifact control fluorescence; Thorlabs, M405FP1) LED light was delivered to the brain via 18–15 μW (LED driver; Thorlabs, DC1104). Mice were connected to the optical fiber with the LEDs on for 10–15 min before recording start. Emissions passed through a dichroic mirror, a filter with a cutoff wavelength of 500–550 nm, and were then detected by a femtowatt silicon photoreceiver (Newport, 2151). Analog signals were demodulated and recorded using an RZ processor and Synapse Software (Tucker-Davis Technologies). Time-locked behavioral videos were obtained via a webcam (30 frames per sec) and grooming events were scored. Grooming bouts within 5 s from a previous bout were excluded in the analysis to avoid cumulative effects on the fluorescence signals. A single non-grooming period right before each grooming bout was included as a control. To calculate the fluorescence change ratios (ΔF/F), the raw data were analyzed using MATLAB and downsampled to 9 Hz. For each wavelength, the fluorescence change (ΔF/Fa or ΔF/Fb) was calculated as (F − F0)/F0, where F0 was the baseline fluorescence signal averaged from periods with no grooming. Then behavior recordings were separated into subevents categorized as white, brown or black. This was obtained by subtracting fluorescence from bregma to lowered to 4.9 mm ventral to the brain surface. These implanted mice contributed single-unit data to a prior study13. For the present study, we utilized single-unit data in which time-stamped grooming events were separated in time from behaviors investigated in the prior study involving reward behavior13.
Spontaneous grooming events were captured via a camera as mice explored a familiar 15 × 15 × 30-cm chamber in 1-h sessions. They were connected to a flexible tether and the output of the electrode arrays was amplified with a digital headstage (Instar Technologies). Recordings were acquired at 24.4 kHz (0.3–5 kHz bandpass) using Synapse software (Tucker-Davis Technologies) with one of the electrodes in the OT of each mouse used as a local reference. Following behavior, mice were overdosed with sodium pentobarbital (Fatal Plus, Patterson Veterinary) and transcardially perfused with cold 0.9% saline and 10% phosphate-buffered formalin.

Brains were sectioned at 40 μm and counterstained with DAPI for postmortem histological verification of electrode recording sites (Extended Data Fig. 5b).

We used offline spike sorting (Spike2, Cambridge Electronic Design) with a combination of waveform template matching and k-means-assisted cluster cutting based on principal-component analysis to establish a population of putative single units. Next, among these putative single units, we used a conservative interspike interval threshold to determine possible multiunit data from the population. To accomplish this, we eliminated any putative single units that displayed >2% of their spikes within a 2-ms refractory period to generate a population of confirmed single units (Extended Data Fig. 7). Any instances of a single unit whose activity was observed across more than one channel was removed to prevent oversampling. Among the confirmed single spikes, grooming onset events or aligned to pseudorandomly identified (for shuffled comparison) time markers that did not include grooming events were extracted and imported into MATLAB (MathWorks, R2020a). Spike density functions in 50-ms bins were calculated based on convolving spike trains with a function resembling a postsynaptic potential. Each neuron’s background FR was calculated based on its activity during numerous 2-s windows (mean of 53.5), equally sampled throughout the 1-h sessions that did not include any grooming. Spike density functions for each analysis window were z-score normalized to each neuron’s average background firing.

To identify task-modulated neurons, we compared each neuron’s averaged background firing to its averaged FR ± 1 s relative to grooming onset via t-tests with a false discovery rate correction of 1% using Benjamini and Hochberg method. To examine and identify cell–groom pairs, among significantly modulated neurons defined above, we subtracted background activity from their FRs during individual groom bouts and averaged their FRs across time bins within 1 s before grooming onset (‘pre’), and within 1 s following grooming onset (‘during’). This gave the change in FRs: ΔFR = FRduring − FRpre or FRΔ = FRduring − FRpre. Only cell–groom pairs with ΔFR ± 1 Hz were included. The same approach was used to determine the distribution of FR changes for shuffled, non-grooming events, including ΔFR values within ±1 Hz. Analyses and figures were generated using MATLAB and/or Prism 7 (GraphPad).

Statistics and reproducibility. Sample sizes for individual experiments were determined according to the NIH Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research (sample size determination: https://www.ncbi.nlm.nih.gov/books/NBK43321). Shapiro–Wilk normality tests were used to verify normal distribution of each dataset. Parametric statistical tests were used for normally distributed datasets; otherwise, non-parametric tests were used. All statistical tests and results are reported in Supplementary Table 2. Various controls (for example, genotype, virus, laser wavelength and brain regions) were included in experimental design, and the number of replications for each experiment was included in figure legends as well as in Supplementary Table 1. Animals were allocated randomly in all experiments and various stimulating conditions (for example, blue versus green laser, stimulation duration and frequency) for individual mice were randomized. All animals with verified optical fiber implantation/viral injection sites were included in data analysis. Blinding in data collection and analysis were carried out as much as possible with the following exceptions. For CLARITY imaging and patch-clamp recordings, only mice with desired genotype were used. For optogenetic experiments in behaving mice, blue-light activation of OT D3-Cre/ChR2 neurons always induced grooming behavior, which would reveal the genotype or virus type of the mice, thus preventing complete blinding in data collection and analysis.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability The raw data generated and/or analyzed during the current study are available from the corresponding authors on reasonable request. The Allen Mouse Brain Connectivity Atlas (https://connectivity.brain-map.org/transgenicexperiment/304168043/) was used for Fig. 1d and the Allen Mouse Brain Common Coordinate Framework version 3 (https://scalablebrainatlas.imc.org/mouse/ABA_v3/) was used to outline brain structures in Figs. 2b and 4a and Extended Data Figs. 4 and 5. Source data are provided with this paper.

Code availability All commercial software used to collect and analyze the data in this study are described. Custom code for CLARITY brain imaging has been previously published and is publicly available.

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Acknowledgements We thank Acai Bfi and Ander for providing access to the Dragonfly 500 spinning-disk confocal microscope platform. This work was supported by the NIH (R01NS117061 to D.W.W., M.V.F. and M.M., R01DC006213 to M.M., R01DA049545 and R01DA049449 to M.M. and D.W.W., R01DC016619 and R01DC014443 to D.W.W., R01MH18369 to M.F.V., R21DC019191 to J.F.B., F32DC018452 to K.N.W., F31DC017054 to M. Schreck et al. F131MH124372 to E.J.), by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation; 368482240/GRK2416 to M. Speth and 269953372/GRK2150 to J.M. and J. Spehr) and by the Whitehall Foundation and Foundation for OCD Research to M.V.F. The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

Author contributions Conceptualization, Y.-F.Z., D.W.W., M. Spehr, M.V.F. and M.M.; methodology, all authors; investigation, Y.-F.Z., L.V.C., K.N.W., J.P.B., J.M., D.F., E.J., S.L.C., N.G.,
M. Schreck, A.H.M., Y.Y., J.S. and D.W.W.; formal analysis, data curation and visualization, Y.-F.Z., L.V.C., K.N.W., J.P.B., J.M., D.F., E.J., C.J., J.S., D.W.W., M. Spehr, M.V.F. and M.M.; writing—original draft, Y.-F.Z., D.W.W., M. Spehr, M.V.F. and M.M.; writing—review and editing, all authors; resources, B.R.A., J.N.B., W.L., J.S., D.W.W., M. Spehr, M.V.F. and M.M.; supervision and funding acquisition, D.W.W., M. Spehr, M.V.F. and M.M.

Competing interests
The authors declare no competing interests.

Additional information
Extended data is available for this paper at https://doi.org/10.1038/s41593-021-00952-z.
Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41593-021-00952-z.
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Peer review information Nature Neuroscience thanks Eric Burguiere, Christiane Schreiweis, Xin Jin, and the other, anonymous, reviewer(s) for their contribution to the peer review of this work.
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Extended Data Fig. 1 | Quantification of D3-Cre/tdTomato neurons in the ventral striatum. a, Left, ventral view of location(s) of D3-Cre/tdTomato neurons within a mouse brain mapped onto a sample-adjusted version of the Allen Mouse Brain Atlas. The OT and hippocampus are outlined as light blue volumes. Right, 3D projection of the OT region outlined (black rectangle). Scale bars = 400 µm (upper left), 100 µm (upper right), 150 µm (lower left), and 50 µm (lower right). b, Left, frontal view projection of the location(s) of D3-Cre/tdTomato neurons. Right, 3D projection of the ventral striatum. Scale bar = 300 µm. c, Left, frontal side view projection of the location(s) of D3-Cre/tdTomato neurons. Right, 3D surface rendering of the IC network. Scale bar = 500 µm. d-e, Quantification of D3 neurons in the ventral striatum and ventral pallidum. Absolute numbers of dense versus loose neurons in d, total numbers of neurons in e (left) and cell density in e (right) in the VP, the NAc and the OT. n = 6 hemispheres from 3 mice. All averaged data are shown as mean ± s.e.m. OT, olfactory tubercle. HF, hippocampal formation. IC, islands of Calleja. islm, major island. VP, ventral pallidum. NAc, nucleus accumbens.
Extended Data Fig. 2 | Quantification of the IC between hemispheres and among individuals. **a**, Left, 3D reconstruction of D3-Cre/tdTomato neurons in the OT demonstrating that the IC form a continuous branched network. Similar results were observed in 6 OTs from 3 mice. Right, map of registered neurons categorized as dense or loose neurons, respectively. Scale bars = 600 μm (left) and 500 μm (right). **b**, Top, 3D reconstructions of OT IC structures in individual hemispheres (left and middle panel), and the area of maximal overlap (right panel). Bottom left, IC overlap between the two hemispheres from the same mouse. Mirrored 3D objects were merged and aligned to create maximum overlap (white voxels). Scale bar = 500 μm. Bottom right, maximum volume overlap of the IC network between two hemispheres and among individuals. All averaged data are shown as mean ± s.e.m.
Extended Data Fig. 3 | D3-Cre/tdTomato neurons in the piriform cortex, the hypothalamus and the hippocampus. Location(s) of D3 neurons within the mouse brain are mapped onto a sample-adjusted version of the Allen Mouse Brain Atlas. The OT and hippocampal formation (HF) are outlined as light blue volumes. a, D3 neurons in the piriform cortex do not project to targets outside this region. a’, 3D projection of the piriform cortex region outlined in a (black rectangle). Different areas are shown at higher magnification. Note that no projection fibers are evident; in frontal view, D3 neurons appear to adhere to a layer-specific organization. Scale bars = 500 μm (left), 200 μm (middle), and 100 μm (right). b, Two areas in the hypothalamus harbor D3 neurons. Of these, neurons in the caudal aspect of the hypothalamus exhibit some projections. b’, 3D maximum projection of the hypothalamic region outlined in b (black rectangle). Different areas are shown at higher magnification. Relatively sparse, but consistent tdTomato expression is observed at both a relatively caudal and rostral region within the hypothalamus (close to midline). Note that few fibers are evident at the caudal site, whereas no fibers are found at the rostral site. Scale bars = 1000 μm (left), 300 μm (middle), and 100 μm (right). c, D3 neurons in the hippocampus. c’, 3D projection of the hippocampal region outlined in c (black rectangle). Different areas are shown at higher magnification. Scale bars = 1000 μm (left), 1000 μm (middle, upper panel), 100 μm (middle, lower panel), 200 μm (right, upper panel) and 100 μm (right, lower panel). Similar results were observed in 3 mice for a-c.
Extended Data Fig. 4 | Retrograde tracing of presynaptic partners of OT D3 neurons. Representative images showing labeled presynaptic partners of OT D3 neurons from the anterior (upper left) to posterior brain sections (lower right). Similar results were observed in 3 mice. MOB, main olfactory bulb. ORB, orbital area. AI, agranular insular area. AON, anterior olfactory nucleus. PC, piriform cortex. NAc, nucleus accumbens. VP, ventral pallidum. OT, olfactory tubercle. LS, lateral septal nucleus. NDB, diagonal band nucleus. SH, septohippocampal nucleus. LPO, lateral preoptic area. LHA, lateral hypothalamic area. AMY (AAA), anterior amygdalar area. AMY (CEA), central amygdalar nucleus. AMY (MEA), medial amygdalar nucleus. AMY (LA), lateral amygdalar nucleus. AMY (COA), cortical amygdalar area. PVH, paraventricular hypothalamic nucleus. TU, tuberal nucleus. ARH, arcuate hypothalamic nucleus. PH, posterior hypothalamic nucleus. PMv, ventral premammillary nucleus. TH, thalamus (mostly in the subparafascicular area). AMY (BLA), basolateral amygdalar nucleus. SN, substantia nigra. VTA, ventral tegmental area. MRN, midbrain reticular nucleus. PAG, periaqueductal gray. RAmb (CRN), medbrain raphe nuclei, central part. RM, nucleus raphe magnus. PRNc, pontine reticular nucleus, caudal part. RAmb (DRN), medbrain raphe nuclei, dorsal part. PCG, pontine central gray. PB, parabrachial nucleus. Brain atlas images are modified from Allen Mouse Brain Common Coordinate Framework version 3 (https://scalablebrainatlas.incf.org/mouse/ABA_v3). Scale bars = 500 µm.
Extended Data Fig. 5 | Viral injection/expression sites and optical fiber placements for experiments in Figs. 2–8. Coronal brain sections at the bregma levels showing viral injection sites (dots)/expression areas (gray shadow areas with red borders) and/or optical fiber tracts (vertical bars) for mice included. a–b, Optogenetic experiments (Figs. 2, 3 in a and Fig. 4 in b). c, DTA ablation experiments (Fig. 5). d–e, Retrograde (Fig. 6 in d) and anterograde (Fig. 7 in e) tracing experiments. f, Electrophysiological recordings (Fig. 7 in f), and (g) fiber photometry experiments (Fig. 8). For clarity, an optical fiber (400 µm) is shown as a thin vertical bar at the center of the tract. h, Electrode array locations from in vivo unit recording experiments (Fig. 8). i, Schematic showing viral expression and fiber covered areas for the two coordinates used in the OT. Scale bar = 500 µm. Each dot or line represents one animal except for bilateral AAV8-DTA injection in c. Brain atlas images are modified from Allen Mouse Brain Common Coordinate Framework version 3 (https://scalablebrainatlas.incf.org/mouse/ABA_v3). IC, islands of Calleja. OT, olfactory tubercle. NAc, nucleus accumbens. PVH, paraventricular hypothalamic nucleus.
Extended Data Fig. 6 | Properties of postsynaptic currents (PSCs) upon optogenetic stimulation of D3-Cre/ChR2 neurons in the OT. a and c, Latency to PSC onset in D3-Cre/tdTomato neurons (4.73 ± 0.19 ms) (a) and SPNs (4.63 ± 0.11 ms) (c). b and d, Jitter of PSCs (SD of latencies during repeated light stimuli) in D3-Cre/tdTomato neurons (1.28 ± 0.08 ms) (b) and SPNs (1.14 ± 0.10 ms) (d). Data are quantified in 16 D3-Cre/tdTomato neurons and 38 SPNs (6-10 traces/neuron) showing light-evoked PSCs. All averaged data are shown as mean ± s.e.m.
Extended Data Fig. 7 | Single unit quality control metrics. a, Upper panel, PCA plot of two putative single units recorded from the same electrode. Ellipses denote 2.5x SD of each K-means cluster. Lower panel, overlaid waveforms of the same neurons. b–c, Inter-spike intervals (ISIs, 2 ms bins) for the same two neurons as in (a) indicating significantly different distributions (two-sample Kolmogorov-Smirnov test $D(2493) = 0.22, p < 0.0001$). Insets, ISI distributions (1 ms bins) showing limited numbers (< 2%) of ISI events < 2ms. d, Distribution of the proportion of ISI violations (< 2ms between spikes) among all single units. 100% of units had <2% of their spikes occurring within 2ms of each other. e, Distribution of mean firing rates during entire recording session of all single units (median: 1.97 Hz, typical of spiny projection neurons). f, Distribution of spike amplitude: noise floor values for all single units.
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All commercial software used to collect the data in this study are described, including LAS AF Lite, PULSE v8.74, IMARISTM 9.5.1, and SYNAPSE. Custom code for CLARITY brain imaging is previously published and publicly available.

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All commercial software used to analyze the data in this study are described, including Igor Pro 6, ImageJ, MATLAB, Deep Learning Toolbox, Prism 7, R package, and Spike 2. Custom code for analyzing CLARITY data is previously published and publicly available.

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Due to multiple specialized platforms used to collect the data and the enormous size of the raw data (CLARITY imaging, confocal images of whole-brain sections, behavioral videos, ex vivo and in vivo recordings), it is not feasible for us to make them accessible to readers in a public data depository. However, we will make the raw data available to readers upon reasonable request. Source data are provided for all main and Extended Data figures wherever applicable. Source data are provided for all figures wherever applicable. The Allen Mouse Brain Connectivity Atlas (http://connectivity.brain-map.org/transgenic/experiment/304168043) was used for Fig. 1d and the Allen Mouse Brain Common Coordinate Framework version 3 (https://scalablebrainatlas.incf.org/mouse/ABA_v3) was used to outline brain structures in Figs. 2b, 4a, and Extended Data Figs. 4, 5.
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| Sample size | Sample sizes for individual experiments were determined according to the NIH "Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research" (Sample Size Determination: https://www.ncbi.nlm.nih.gov/books/NBK43321/). |
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Laboratory animals

The bacterial artificial chromosome (BAC) transgenic D3-Cre line (STOCK B6.FVB(Cg)-Tg(Drd3-cre)1Ki198Gsat/Mmucd, RRID:MMRRC_031741-UCD) line was obtained from the Mutant Mouse Resource and Research Centers (MMRRC) at University of California at Davis, an NIH-funded strain repository, and was donated to the MMRRC by Nathaniel Heintz, Ph.D., The Rockefeller University, GENSAT and Charles Gerfen, Ph.D., National Institutes of Health, National Institute of Mental Health. The D3-Cre line was crossed with the Cre-dependent tdTomato reporter line (JAX Stock No: 007909 or Ai9 line: B6.Cg-Gt(Rosa)26Sortm9(CAG-tdTomato)Hze/) or Cre-dependent channelrhodopsin 2 (ChR2)-EYFP line (JAX Stock No: 024109 or Ai32 line: B6.Cg-Gt(Rosa)26Sortm32(CAG-COP4*H134R/EYFP)Hze) to generate D3-Cre/tdTomato or D3-Cre/ChR2 mice, respectively. Double transgenic D3-Cre and D1-tdTomato mice (JAX Stock No.016204, B6.Cg-Tg(Drd1a-tdTomato)6Calak) were bred to achieve identification of D1-type and presumptive D2-type SPNs in slice recordings. Wild-type C57bl/6j mice were offspring from breeders purchased from the Jackson Laboratory. Approximately equal numbers of male and female mice (8-12 weeks old) were used for all experiments except for in vivo unit recordings. Since no sex difference was observed, the data from both sexes were combined. Mice were housed in temperature- and humidity-controlled facilities on a 12 h light/dark cycle with food and water available ad libitum unless otherwise stated. Mice were group-housed until the surgery of receiving virus injection and intra-cranial implantation and singly-housed afterwards. All experimental procedures were performed in accordance with the guidelines of the National Institutes of Health and were approved by the Institutional Animal Care and Use Committees of the University of Pennsylvania and the University of Florida. The number/sex of mice used in each set of experiments are summarized in Supplementary Table 1.
Wild animals

The study did not involve wild animals.

Field-collected samples

The study did not involve samples collected from the field.

Ethics oversight

All experimental procedures were performed in accordance with the guidelines of the National Institutes of Health and were approved by the Institutional Animal Care and Use Committees of the University of Pennsylvania and the University of Florida.

Note that full information on the approval of the study protocol must also be provided in the manuscript.