GLP-1 acts on habenular avoidance circuits to control nicotine intake

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Tobacco smokers titrate their nicotine intake to avoid its noxious effects, sensitivity to which may influence vulnerability to tobacco dependence, yet mechanisms of nicotine avoidance are poorly understood. Here we show that nicotine activates glucagon-like peptide-1 (GLP-1) neurons in the nucleus tractus solitarius (NTS). The antidiabetic drugs sitagliptin and exenatide, which inhibit GLP-1 breakdown and stimulate GLP-1 receptors, respectively, decreased nicotine intake in mice. Chemogenetic activation of GLP-1 neurons in NTS similarly decreased nicotine intake. Conversely, Glp1r knockout mice consumed greater quantities of nicotine than wild-type mice. Using optogenetic stimulation, we show that GLP-1 excites medial habenular (MHB) projections to the interpeduncular nucleus (IPN). Activation of GLP-1 receptors in the MHB–IPN circuit abolished nicotine reward and decreased nicotine intake, whereas their knockdown or pharmacological blockade increased intake. GLP-1 neurons may therefore serve as ‘satiety sensors’ for nicotine that stimulate habenular systems to promote nicotine avoidance before its aversive effects are encountered.

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detected in TH⁺ neurons in the rNTS (Supplementary Fig. 1). However, nicotine increased the numbers of TH⁺ neurons that were Fos⁺ in the ventrolateral medulla (Fig. 1b). These findings suggest that nicotine activates NTS neurons but acts preferentially on GLP-1 neurons rather than catecholaminergic neurons (Fig. 1c). It is noteworthy that a sizable fraction of Fos⁺ NTS neurons were neither GLP-1⁺ nor TH⁺ (Fig. 1c), and we detected Chorda-GFP⁺ neurons that were not immunoreactive for GLP-1. Together these findings demonstrate that nicotine activates GLP-1 neurons and at least one other population of non-TH⁺ neurons in the NTS.

**GLP-1 regulates nicotine intake**

Circulating GLP-1, which is rapidly degraded by the enzyme dipeptidyl peptidase-4 (DPP4), enhances insulin secretion and sensitivity⁸ (Fig. 2a). Drugs that block DPP4, such as sitagliptin (Januvia), or that mimic the actions of GLP-1 by stimulating GLP-1 receptors (GLP-1Rs), such as exenatide (exendin-4; Byetta), are used clinically for the treatment of type 2 diabetes (Fig. 2a). Exendin-4 (Ex-4) (≤10 µg kg⁻¹) is known to attenuate the rewarding effects of nicotine, cocaine, amphetamine and alcohol in rodents, measured using place conditioning procedures⁹–¹¹. Therefore, we investigated the role for GLP-1 transmission in regulating the motivational properties of nicotine.

In mice lever-pressing for nicotine under a fixed ratio 5, time-out 20 s (FR5TO20) schedule of reinforcement (Fig. 2b), we found that Ex-4 (10 µg kg⁻¹) decreased nicotine intake (Fig. 2c) but did not alter responding for food rewards (20-mg chow pellets) (Supplementary Fig. 3). Similarly, sitagliptin (10 mg kg⁻¹) also decreased nicotine self-administration in mice (Fig. 2d). These findings are consistent with an inhibitory action of GLP-1 transmission on nicotine intake. We also found that Glp1r knockout mice consumed more nicotine than their wild-type littermates across a range of nicotine doses, resulting in an upward shift in the dose–response curve (Fig. 2e). By contrast, responding for food rewards was similar between Glp1r knockout and wild-type mice when tested under the same FR5TO20 reinforcement schedule (Fig. 2f). Notably, nicotine (0.25 mg kg⁻¹ subcutaneously) decreased responding for food similarly in wild-type and Glp1r knockout mice (Supplementary Fig. 4), suggesting that GLP-1 is not likely to be involved in the anorectic effects of nicotine. Together, these findings support a role for GLP-1 transmission in regulating the motivational properties of nicotine.
Chemogenetic stimulation of GLP-1 neurons decreases nicotine intake

In addition to brain, GLP-1 receptors are expressed in intestine, liver, lung, pancreas and kidney\(^2\), making it unclear whether GLP-1 influences nicotine intake through actions inside or outside the brain.

To address this issue, we used designer receptors exclusively activated by designer drugs (DREADDs) to place GLP-1 neurons under experimenter control. To selectively target GLP-1 neurons with DREADDs, we used a line of Phox2b-Cre mice in which the Cre transgene in NTS is reported to be expressed selectively in GLP-1+ neurons\(^3\). To confirm
this specificity of expression, we bred the Phox2b-Cre mice with a line of ROSA-tdTomato mice in which the fluorescent protein tdTomato is expressed in a Cre-dependent manner. We found that the majority of tdTomato+ neurons in these mice were immunopositive for GLP-1 and immunonegative for TH (Supplementary Fig. 5). However, we detected occasional TH+ neurons that were tdTomato+ (Supplementary Fig. 5), suggesting that populations of TH+ (and perhaps other non-GLP-1) cells may express Cre in the NTS of these mice.

To compare the effects of selectively activating of GLP-1 neurons with more generalized activation of the NTS, we injected AAV-DIO-hM3Dq-mCitrine (Cre-dependent) or AAV-hM3Dq-mCitrine (non-Cre-dependent) excitatory DREADDs into the cNTS of Phox2b-Cre mice (Fig. 3a). We detected prominent fluorescence in GLP-1 neurons in the DIO-hM3Dq-expressing Phox2b-Cre mice (Fig. 3b). By contrast, we detected widespread transduction of NTS neurons in the non-DIO-hM3Dq-expressing Phox2b-Cre mice (Fig. 3c). Moreover, clozapine-N-oxide (CNO; 1 mg kg⁻¹) markedly decreased nicotine self-administration (0.1 mg kg⁻¹ per infusion) in DIO-hM3Dq-expressing Phox2b-Cre mice relative to saline injection (Fig. 3d), whereas CNO merely tended to decrease nicotine intake in the DIO-hM3Dq-expressing Phox2b-Cre mice compared with saline injection (P = 0.066; Fig. 3e). As expected, CNO had no effects on nicotine intake relative to saline injection in Phox2b-Cre mice that received intra-NTS injections of AAV-EGFP (Fig. 3f). Also, CNO did not alter responding for food rewards in the DIO-hM3Dq- or EGFP-expressing Phox2b-Cre mice (Supplementary Fig. 6). By contrast, CNO decreased food responding in non-DIO-hM3Dq-expressing Phox2b-Cre mice (Supplementary Fig. 5). As small numbers of TH+ neurons in NTS of Phox2b-Cre mice expressed Cre (Supplementary Fig. 5) and Phox2b is reported to be expressed in non-GLP-1+ neurons in NTS and other hindbrain sites, it is possible that CNO may have decreased nicotine intake in the DIO-hM3Dq-expressing Phox2b-Cre mice in part by stimulating non-GLP-1 neurons. Therefore, to further confirm that GLP-1 neurons act to regulate nicotine intake, we used Gcg-Cre mice, in which Cre expression is controlled by the gene promoter of the preproglucagon (Gcg) gene from which GLP-1 is derived. We injected AAV-DIO-hM3Dq-mCherry into cNTS of Gcg-Cre mice and trained them to respond for nicotine infusions as described above (Fig. 3g). As expected, mCherry fluorescence was detected only in GLP-1-immunopositive neurons in NTS of these mice (Fig. 3g), and CNO significantly reduced their nicotine intake compared with saline injection (Fig. 3h). Together, these findings are consistent with a central site of action for GLP-1 in regulating the motivational properties of nicotine.

**GLP-1 stimulates habenular inputs to the interpeduncular nucleus**

Next we investigated the site of action for GLP-1 in the brain in controlling nicotine intake. Central GLP-1 receptors demonstrate a relatively restricted expression pattern, with some of the highest densities of binding sites detected in IPN. Recently, our laboratory established that nicotine-induced stimulation of excitatory inputs from MHb to IPN, and consequent activation of IPN neurons, promotes nicotine avoidance. We therefore tested the possibility that GLP-1 may stimulate IPN activity to decrease nicotine intake. We detected GFP+ fibers in the IPN of mice after injection of AAV-EGFP into the NTS (Fig. 4a,b), consistent with NTS projections to IPN. We also detected GLP-1-immunoreactive fibers in IPN of mice (Fig. 4c), suggesting that at least a portion of this NTS input comes from GLP-1 neurons. CNO increased Fos immunoreactivity in IPN neurons of Phox2b-Cre mice that received cNTS injection of AAV-DIO-hM3Dq-ChR2-GFP into the NTS (Supplementary Fig. 7). This suggests that NTS inputs stimulate local neuronal activity in IPN. To more directly investigate this possibility, we injected Cre-inducible channelrhodopsin-2 (DIO-ChR2-GFP) into the NTS of Gcg-Cre mice and tested the effects of optically stimulating ChR2+ terminals in IPN (Fig. 4d). As expected, GFP from this virus was detected exclusively in GLP-1 neurons in NTS (Fig. 4e) and we detected GFP+ fibers in the IPN of these mice (Fig. 4f). We found that high-intensity optostimulation (20 Hz; Fig. 4g–i) but not low (1 Hz; Fig. 4j–l) of GFP+ terminals markedly increased the frequency of excitatory events in IPN neurons.
**Figure 5** GLP-1 activates IPN neurons by stimulating habenular terminals. (a) Example traces of mEPSCs in IPN neurons before and after bath application of Ex-4 (100 nM). (b,c) Cumulative probability (± s.e.m.) (b) and summarized results (c; mean ± s.e.m.) showing that the relative amplitude of mEPSCs in IPN neurons is not altered by Ex-4. (d,e) Cumulative probability (± s.e.m.) (d) and summarized results (e; mean ± s.e.m.) showing that the relative frequency of mEPSCs in IPN neurons is increased by Ex-4. *P = 0.0165, paired t-test; n = 9 cells from 4 animals. (f) Top: the MHb–IPN circuit (green). Bottom: cholinergic MHb neurons (left) send axonal projections to the IPN (right) as evinced by fluorescence from ChAT-ChR2-eYFP mice. Nuclear DAPI staining is shown in blue. Scale bars, 50 µm. (g) Sample trace showing that the amplitude of light-evoked EPSCs in IPN neurons from ChAT-ChR2-eYFP mice is increased by Ex-4. (h) Summarized results (mean ± s.e.m.) showing that Ex-4 significantly increases the amplitude of light-evoked EPSC in IPN neurons. ***P = 0.0005, paired t-test; n = 13 cells from 9 cells animals. (i) Representative micrographs showing induction of Fos in IPN following nicotine challenge in GLP-1R knockout and wild-type mice. Scale bar, 100 µm. (j) Mean (± s.e.m.) number of Fos-positive neurons per IPN section in GLP-1R knockout (saline, n = 6; nicotine, n = 7) and wild-type (saline, n = 6; nicotine, n = 7) mice following nicotine challenge. Two-way ANOVA, genotype: F(1,22) = 17.69, P = 0.0004; nicotine: F(1,22) = 51.71, P < 0.0001; genotype × nicotine: F(1,10) = 13.36, **P = 0.0014.
but not the amplitude of excitatory post-synaptic currents (EPSCs) in IPN neurons. As high frequency optostimulation (≥20 Hz) is usually required to trigger neuropeptide release from terminals, these data suggest that GLP-1 released from NTS terminals in IPN increases excitatory currents in IPN neurons.

The data described above suggest that GLP-1 likely acts through a presynaptic mechanism to increase glutamatergic transmission onto IPN neurons. In keeping with a presynaptic site of action, bath application of Ex-4 (100 nM) increased the frequency but not the amplitude of miniature EPSCs (mEPSCs) in IPN neurons (Fig. 5a–e). We next sought to identify the source of excitatory input onto IPN neurons that is stimulated by GLP-1. As high-frequency (20 Hz) but not low-frequency (1 Hz) optostimulation increased EPSCs in IPN and 1–2 Hz stimulation is usually sufficient to increase glutamate release from excitatory terminals, it is unlikely that GLP-1 terminals are a major source of glutamatergic drive onto IPN neurons. The IPN receives massive cholinergic innervation from MHb via the fasciculus retroflexus17. MHb cholinergic neurons co-release glutamates and provide the main source of glutamatergic input to IPN17. Therefore, we hypothesized that GLP-1 stimulates habenular terminals to enhance excitatory drive onto IPN neurons. To investigate this possibility, we used Chat-Chr2-YFP mice, in which Chr2 and yellow fluorescent protein (YFP) are expressed in cholinergic neurons under the control of the promoter for the choline acetyltransferase (Chat) gene. Excitatory currents evoked by optical stimulation of the IPN in Chat-Chr2-YFP are derived almost exclusively from MHb terminals.17 In the Chat-Chr2-YFP mice, we observed dense Chr2 and YFP expression in MHb neurons and on MHb terminals in IPN, but not post-synaptically on local IPN neurons (which are GABAergic) (Fig. 5f). As expected, optical stimulation of IPN slices from these mice evoked a robust EPSC in IPN neurons (Fig. 5g). This effect was markedly enhanced by bath application of Ex-4 (100 nM) (Fig. 5h). Finally, we found that nicotine-induced increases in IPN activity in mice, measured using Fos immunoreactivity, were greatly diminished in Glp1r knockout mice compared with wild-type controls (Fig. 5i,j). Together these findings suggest that GLP-1 released from NTS terminals enhances IPN neuron activity by stimulating habenular terminals and that GLP-1 plays a permissive role in the stimulatory effects of nicotine on MHb–IPN circuit activity.

GLP-1 signaling in the MHb–IPN circuit regulates nicotine intake

A plausible explanation for how GLP-1 stimulates excitatory habenular inputs to IPN is by activating GLP-1Rs expressed on the terminals of these neurons. Using bacterial artificial chromosome–translating ribosome affinity purification (bac-TRAP) mouse lines, habenular cholinergic neurons were shown to transcribe modest levels of Glp1r mRNA18. To directly investigate the functional significance of Glp1r+ mRNA on habenular terminals, we tested the effects of knocking down Glp1r transcripts in MHb on nicotine intake. We injected AAV-sh-Glp1r-GFP or a control AAV-GFP vector into MHb of rats and detected robust GFP expression in MHb and GFP+ fibers in IPN (reflecting terminals of MHb neurons) (Fig. 6a), confirming accurate targeting of virus injections to MHb. We also detected robust knockdown of Glp1r transcripts in MHb of AAV-sh-Glp1r-GFP rats compared with AAV-GFP rats (Supplementary Fig. 8). Responding for food reinforcers was similar between rats that received intra-MHb injections of AAV-sh-Glp1r-GFP and AAV-GFP (Supplementary Fig. 9). However, nicotine intake was increased in the AAV-sh-Glp1r-GFP rats compared with AAV-GFP rats (Fig. 6b), an effect apparent at higher doses of the drug (0.09 and 0.12 mg kg−1 per infusion; FR5TO20).

These data are consistent with a role for GLP-1 receptors expressed on habenular terminals in regulating the actions of GLP-1 in IPN to control nicotine intake. We also found that infusion of Ex-4 (0.1 µg) into IPN (Fig. 6c,d), but not 2 mm above the IPN (Fig. 6e), dramatically decreased nicotine intake in rats (0.03 mg kg−1 per infusion; FR5TO20). Ex-4 acting in the IPN did not alter responding for food rewards (Supplementary Fig. 10). GLP-1 receptor activation is known to stimulate production of the intracellular second messengers cAMP and cGMP19. Intra-IPN infusions of 8-bromo-cGMP (0.75 µg or 3.0 µg), a nonhydrolyzable and cell-permeable analog of GMP, had no effects on nicotine intake in rats (Supplementary Fig. 11). By contrast,
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Ex-9 into IPN increased nicotine intake, whereas infusion of Ex-4 into the IPN decreased intake. These findings suggest that GLP-1 controls nicotine intake, at least in part, by regulating excitatory transmission in IPN derived from habenular inputs.

We found that Ex-4 infused into IPN decreased nicotine intake and abolished the ICSS threshold-lowering (rewarding) effects of nicotine. However, Ex-4 infused into the IPN was not intrinsically aversive, as measured by elevation of ICSS thresholds. These are important observations for two reasons. First, they suggest that engaging MHb–IPN circuit activity can override the rewarding properties of nicotine, but precisely how this occurs is unclear. The IPN provides inhibitory GABAergic projections to the VTA39, a brain site known to be critical to regulating the rewarding properties of nicotine2. Therefore, IPN-derived GABAergic transmission onto VTA dopamine neurons could contribute to the inhibitory effects of MHb–IPN circuit activity on nicotine reward. Moreover, it is possible that GLP-1 may also act in the VTA to decrease the rewarding effects of nicotine and other drugs of abuse, and attenuate their effects on mesocumbens dopamine transmission, by stimulating inhibitory GABAergic transmission derived from IPN inputs. Second, the stimulatory effects of nicotine on glutamatergic transmission in IPN is hypothesized to contribute to aversive (ICSS threshold-elevating) effects of the drug30,31. However, we report here that GLP-1 enhances glutamatergic drive onto IPN neurons, mimicking the actions of nicotine (Fig. 4 and ref. 20), and yet is not intrinsically aversive. This suggests that nicotine avoidance and nicotine aversion may be dissociable phenomena explained by different underlying mechanisms in the MHb–IPN circuit, with nicotine avoidance but not aversion related to enhanced glutamatergic transmission from habenular terminals in IPN. In addition to glutamate, nicotine also stimulates acetylcholine release from habenular terminals in IPN17. Different firing patterns of habenular neurons are required to stimulate glutamate versus acetylcholine release17, with brief optogenetic stimulation sufficient to elicit glutamate-mediated excitatory currents but more persistent (tetanic) optogenetic stimulation required to trigger acetylcholine-mediated currents17. Cholinergic transmission in striatum and cortex is known to regulate states of aversion32. Therefore, glutamate released in response to nicotine or GLP-1 may be sufficient to promote avoidance behaviors, whereas acetylcholine released in response to nicotine but not GLP-1 induces an aversive behavioral state. The fact that GLP-1 transmission in the IPN can promote nicotine avoidance without having noxious effects is promising from a translational perspective, as therapeutics that can promote nicotine avoidance without inducing a negative affective state may help promote smoking cessation. Considering that enhancers of GLP-1 transmission, such as DPP4 inhibitors (Januvia) or GLP-1 mimetics (Byetta), are used for the treatment of type 2 diabetes, it will be important to determine whether the motivational properties of tobacco are altered in diabetes patients treated with these drugs.

GLP-1 neurons in the NTS signal satiety states, and GLP-1 receptor agonists decrease food intake33. Hence, it may have been expected that chemogenetic stimulation of GLP-1 neurons would have decreased responding for food and that Glp1r knockout mice would have consumed more food than their wild-type counterparts. However, we observed no effects of manipulating GLP-1 receptor–mediated transmission on food responding in any experiment. This may reflect the fact that animals in our experiments were food restricted and that satiety signals from vagal or higher order inputs to NTS were likely not engaged during the food responding sessions. However, our data are in line with a recent report showing that DREADD-mediated activation of GLP-1 neurons in NTS, using similar experimental approaches to those described here, similarly had no effects on chow intake or body weight34. Also, Glp1r knockout mice are known to consume similar amounts of chow to their wild-type counterparts35. These discrepancies may be explained by the fact that GLP-1 receptor agonists generally decrease cumulative chow intake when consumption is measured over relatively long time periods (2–24 h), instead of the relatively short (60 min) food responding sessions reported here, or when the food used in such experiments is high in hedonic and caloric value. Notably, chemogenetic stimulation of GLP-1 neurons decreases consumption of a palatable high-fat diet without altering chow intake34, and Glp1r knockout mice show differences in body weight when maintained on a high-fat diet36. In light of these findings, it will be interesting to investigate the role for GLP-1 inputs to the MHb–IPN circuit in regulating palatable food consumption and long-term weight gain.

In summary, nicotine and other major drugs of abuse usurp brain reward systems otherwise dedicated to motivating the foraging and consumption of natural reinforcers such as food and water. The data reported here suggest that nicotine also recruits brain systems dedicated to avoiding natural rewards after satiety has been achieved. GLP-1 neurons are activated in response to food intake to block food reward, induce feelings of satiety and trigger meal termination. GLP-1 neurons also mediate malaise and nausea when food is consumed past satiety and contribute to anorectic responses to noxious stimuli such as lithium and cisplatin37–38. As in feeding behavior, the present data show that GLP-1 neurons are activated by nicotine and that GLP-1 receptor transmission in the MHb–IPN circuit can abolish nicotine reward and promote avoidance. GLP-1 neurons may therefore serve as satiety sensors for nicotine that titrate intake at the levels sufficient to stimulate brain reward circuits but below those necessary to encounter aversive properties of the drug.

METHODS

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

L.M.T., A.D., Z.C., C.D.F., B.R.L., X.-A.L., Q.L. and M.I. conducted all experiments. T.M.K., M.C., M.P. and M.R.H. provided essential reagents. L.M.T. and P.J.K. designed the experiments, analyzed the data and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Animals. Male and female mice with null mutation of the Glp1r gene and their wild-type littermates were bred in our animal facilities. The mutant mice had been bred for more than 10 generations onto a C57BL/6J background. Breeding was conducted by mating heterozygous pairs. All mice were housed in cages of 1–3 and were at least 6 weeks of age at the beginning of each experiment. For DREADD experiments we used commercially available 6-week-old Phox2b-Cre mice (016223, Jackson Laboratories) and Gcg-Cre mice (strain 358; MMRRC). To label GLP-1 neurons in NTS we crossed Gcg-Cre mice with commercially available ROSA26-Tomato mice obtained (stock no. 007914, Jackson Laboratories). For electrophysiological experiments we used ChAT-ChR2-YFP mice (P45–100); primers for the wild-type gene were 5′-TACCAATGGGAGGGCCCTA-3′ and 5′-AAGTCATGGGATGTGGCTGGA-3′; for the Glp1r knockout allele were 5′-CTTGGGTGGAGAGGCTATTC-3′ and 5′-AGTTGAGATGACAGAGAAA-3′. Samples were processed for genetic amplification with PCR and subsequently run on a 1% agarose gel with ethidium bromide. The band for the Glp1r wild-type gene was at 180 bp, and the Glp1r mutant gene was at 280 bp.

Drugs. For self-administration experiments in mice and rats, (−)-nicotine hydrochloride tartrate salt (Sigma Chemical Co., St. Louis, MO) was dissolved in 0.9% sterile saline. All doses of nicotine refer to the free-base form. The GLP-1R antagonist Ex-9, agonist Ex-4, PKA inhibitor cAMPS-Rp, and 8-bromo-cAMP (Tocris, Ellisville, MO) were all dissolved in 0.9% saline and microinjected at a volume of 0.5 µL for over 1 min. The injector was held in place for an additional 2 min to allow for diffusion and to prevent backflow into the cannula. Clozapine-N-oxide (CNO, Enzo Life Sciences, Farmingdale, NY) and stigmasterol (TFA salt) was diluted in 0.9% saline for intraperitoneal injection. The pH of all solutions was adjusted to ~7.4.

Intravenous self-administration. Mice and rats were mildly food restricted to 85–90% of their free-feeding body weight and trained to press a lever in an operant chamber (Med Associates, St. Albans, VT) for food pellets (20–mg pellets for mice; 45-mg food pellets for rats; TestDiet, Richmond, IN) under a fixed-ratio 5, time out 20 s (FR5TO20) schedule of reinforcement during 1 h daily sessions before catheter implantation. Once stable responding was achieved (>30 pellets per session in mice; >90 pellets per session in rats), subjects were catheterized. Mice and rats were anesthetized with an isoflurane (1–3%)/oxygen vapor mixture and prepared with intravenous catheters. Briefly, the catheters consisted of a 6-cm (mouse) or 12-cm (rat) length of Silastic tubing fitted to guide cannula (Plastics One, Wallingford, CT) bent at a curved right angle and enced in dental acrylic. The catheter tubing was passed subcutaneously from the animal’s back to the right jugular vein, and a 1-cm (mouse) or 2.5-cm (rats) length of the catheter tip was inserted into the vein and secured with surgical silk suture. Catheters were flushed daily with physiological sterile saline solution (0.9% w/v) containing heparin (10–60 USP units/ml). Catheter integrity was tested with the ultra-short-acting barbiturate anesthetic Brevital (methohexitol sodium, Eli Lilly, Indianapolis, IN).

Thereafter, the animals were allowed at least 48 h to recover from surgery, then permitted to respond for food reinforcers again under the FR5TO20 schedule. Once food responding criteria were re-established, subjects were permitted to acquire intravenous nicotine self-administration by autoshaping during 1-h daily sessions, 5–7 d per week. Nicotine was delivered through the tubing into the intravenous catheter by a Razel syringe pump (Med Associates). Each nicotine self-administration session was performed using two retractable levers (one active, one inactive) that extended 1 cm into the chamber. Completion of the response criteria on the active lever resulted in the delivery of an intravenous nicotine infusion (0.03 ml infusion volume for mice; 0.1 ml for rats). Responses on the inactive lever were recorded but had no scheduled consequences. Animals that did not demonstrate stable responding on the training dose (at least 6 infusions per 60 min session) or showed broken IV catheters were excluded from analysis. For dose-response studies (fixed ratio schedules), animals were presented with each dose of nicotine for at least 5 d (mouse) or 3 d (rats); the mean intake over the last 3 (mouse) or 2 (rats) sessions for each dose was calculated and used for statistical analyses. In between dose, subjects were placed back on the training dose for at least 2 d or until their intake returned to baseline levels before being tested on the next dose.

Microinjections, cannula and electrode implantation. Animals were anesthetized as above and positioned in a stereotaxic frame (Kopf Instruments, Tujunga, CA). Unless otherwise noted, the incisor bar was set to the ‘flat-skull’ position. To test the effects of NTS and GLP-1 neuronal activation on food and nicotine reinforcement, we expressed synthetic receptors (GFP control, Cre-dependent and non-Cre-dependent M3 DREADDs) in mouse NTS. In mice, two bilateral injections (0.375 µL each at a flow rate of 0.375 µL/min) were made at the following coordinates: anterior-posterior (AP) level of the occipital crest; medial-lateral (ML): ±0.5 mm from midline; dorsal-ventral (DV): –4.8 mm from skull surface. To knock down Glp1r transcripts in MHb, rats were injected with AAV1-sh-Glp1r-GFP or AAV1-GFP virus particles (titer = 5 × 1012) according to the following stereotaxic coordinates: flat skull, 10° angle toward midline; AP: 3.2 mm from bregma; ML: ±1.35 mm from midline; DV: –5.3 mm from skull surface. During microinjections, the injector needles extended into MHb and virus particles were administered in a volume of 0.3 µL and at a rate of 0.1 µL per minute. The injector needle remained in place for 2 min after injection. For IPN microinjections in rats, guide cannulae (Plastics One, Wallingford, CT) were implanted as follows: flat skull; 10° angle toward midline; AP: –6.72 mm from bregma; ML: ±1.6 mm from midline; DV: –6.5 mm from brain surface. During injections, the injector needle extended 2 mm below the tip of the cannula for placement into the brain region and virus was administered in a volume of 0.5 µL and at a rate of 0.5 µL/min. The injector needle remained in place for a minimum of 2 min after injection. For the ICSS electrode, a stainless steel bipolar electrode (Plastics One) was implanted into the lateral hypothalamus (AP: –0.5 mm from bregma; ML: ±1.7 mm from midline; DV: –8.3 mm from brain surface; incisor bar was adjusted to 5 mm above the interaural line).

ICSS procedure. Rats were trained to respond according to a modification of the discrete-trial current-threshold procedure of Kornetsky and Esposito22 in an operant box equipped with a wheel manipulandum and ICSS stimulator (Med

New York, NY).
stat, and the floating sections were stored in 0.1 M PBS with 0.01% sodium azide tagged cells in mice, we used a chicken polyclonal IgG that recognizes a 27 kDa immunostaining of GFP, GLP-1, TH and Fos. To localize mCitrine- or GFP-Fluorescence immunolabeling.

at 4 °C until processing for immunohistochemistry. (pH 7.4) for 72h. All brains were cut into 30–40 aorta with 0.9% saline, followed by 4% paraformaldehyde in 0.1 M phosphate pentobarbital (0.1 mg/10 g body weight) and perfused through the ascending tissue dissection.

Mice and rats were euthanized by inhalation of CO2, and brains were rapidly removed and frozen on dry ice. Tissues were stored at −80 °C until dissection.Brains were sliced on a cryostat, and bilateral dissections were made for the hippocampus, habenula, NTS, IPN and/or VTA with a scalpel. Samples were pooled across multiple subjects due to the small size of selected brain areas and stored at −80 °C until processing.

Brain perfusion and fixation. Mice and rats were anesthetized with sodium pentobarbital (0.1 mg/10 g body weight) and perfused through the ascending aorta with 0.9% saline, followed by 4% paraformaldehyde in 0.1 M phosphate buffer saline (PBS; pH 7.4). Brains were collected, postfixed overnight in 4% paraformaldehyde and then stored in 30% sucrose in 0.1 M phosphate buffer (pH 7.4) for 72h. All brains were cut into 30–40 μm coronal sections on a cryostat, and the floating sections were stored in 0.1 M PBS with 0.01% sodium azide at 4 °C until processing for immunohistochemistry.

Fluorescence immunolabeling. Floating sections were processed for fluorescent immunostaining of GFP, GLP-1, TH and Fos. To localize mCitrine- or GFP-tagged cells in mice, we used a chicken polyclonal IgG that recognizes a 27 kDa protein derived from the jellyfish Aequorea victoria. Sections were rinsed in 0.1 M PBS, pH 7.4, with 0.5% Triton X-100 (PBt) and then blocked in 10% normal donkey serum/PBT for 1 h. Thereafter, sections were incubated in the primary antibody in PBt at 4 °C overnight (or, for GLP-1, 1, 2 h). The primary antibodies were as follows: chicken anti-GFP (1:1,500, ab-13970, Abcam), rabbit anti-GLP-1 (1:1,200, T4057, Bachem, Torrance, CA), mouse anti-TH (1:500, SC-25269, Santa Cruz, Santa Cruz, CA), and rabbit anti-Fos (1:1,500, ab-7963, Abcam, Cambridge, MA). On day 2, the sections were rinsed and incubated in two of the following antibodies: Alexa 488 donkey anti-rabbit (1:500, A21206, Invitrogen, Carlsbad, CA), Alexa 488 donkey anti-mouse (1:500, R37114, Invitrogen), Alexa 594 donkey anti-rabbit (1:500, A21207, Invitrogen). Alexa 488 donkey anti-mouse (1:500, R37115 Invitrogen) or DyLight 488 donkey anti-chicken (1:400, 703–485–155, Jackson ImmunoResearch, West Grove, PA). Sections were incubated with secondary antibodies in PBS (in 2% donkey serum) for 1 h. Next, sections were rinsed, mounted on slides with VectaShield (with DAPI) (H-1200, Vector Labs, Burlingame, CA) and coverslipped. Controls included processing the secondary antibodies alone to verify background staining, processing each primary with the secondary antibody to verify laser-specific excitation, checking for autofluorescence in an alternative laser channel with tissue lacking that laser-specific probe, and using sequential scanning. For subsequent fluorescence images, only brightness and/or contrast levels were adjusted after acquisition, and adjustments were imposed across the entire image. All antibodies used have been previously validated for the intended applications, as per manufacturer. For all representative images of qualitative data, the immunolabeling experiment was successfully repeated in at least 3 animals.

Fos procedure. Ninety minutes after saline, nicotine or CNO injections, animals were perfused and brain removed and stored as described above. Brain sections were cut at 40 μm on a cryostat and stored in 0.1 M PBS with 0.01% sodium azide until processing. For Fos immunolabeling, sections were rinsed in 0.1 M PBS (pH 7.4), treated with 0.3% H2O2 in PBS for 15 min, rinsed in PBS, and then blocked in 10% normal goat serum and 0.5% Triton X-100 in PBS for 1 h. Thereafter, sections were incubated in biotinylated rabbit anti-Fos IgG (1:3,000 dilution; Ab-7963, Abcam) in 0.5% Triton-PBS overnight at 4 °C. The following day, sections were incubated at room temperature for 2 h, rinsed in PBS, and then incubated in a 1:300 dilution of goat anti-rabbit secondary IgG (BA-1000, Vector Labs) in 0.5% Triton X-100 in PBS for 2 h. Following rinsing, sections were incubated in ABC Elite (PK-6100, Vector Labs) for 90 min and rinsed in PBS, and immunoreactivity was revealed by using 3-diaminobenzidine with nickel (SK-4100, Vector Labs). To reduce variability in the background and to standardize the staining, sections from subjects across groups were processed concurrently. Sections were mounted and coverslipped with Permount (Fisher Scientific, Waltham, MA). Fos-immunoreactive cells in the IPN were counted for each animal under 20x magnification. The number of Fos-immunoreactive cells in IPN section was added and then divided by the number of IPN sections for that given animal (average 6–8 sections), yielding an individual animal average. All representative Fos images were taken from animals receiving the indicated treatment and were also used for quantification.

Electrophysiology. Before decapitation, ChAT-ChR2-YFP mice (P45–100) were briefly anesthetized with isoflurane and subsequently transcardially perfused with 4 °C NMDG solution: (in mM) 135 N-methyl-D-glucamine (NMDG), 1 KCl, 1.2 KH2PO4, 1.5 MgCl2, 0.5 CaCl2, 20 choline bicitarone, 10 glycine and 295–305 mOsM, equilibrated with 95% O2/5% CO2. The mouse was decapitated and the brain was removed, glued to a block and sliced using a Leica VT1200s vibratome in 4 °C NMDG solution. Coronal slices of 250 μm thickness were cut such that the preparation contained the IPN and then submerged in 36 °C oxygenated standard artificial cerebral spinal fluid ACSF (in mM): 119 NaCl, 2.5 KCl, 1.0 NaH2PO4, 26.2 NaHCO3, 1.3 MgCl2, 2.5 CaCl2, 11 glucose and 285–290 mOsM, equilibrated with 95% O2/5% CO2 for 30 min and then stored at room temperature with constant oxygenation until being transferred to the recording chamber.

Standard whole-cell voltage-clamp recordings were made using a MultiClamp 700B amplifier and a Digidata 1440A digitizer (Molecular Devices) through borosilicate glass electrodes (3–5 Ωm), filtered between 2.6–3 kHz and digitized at 20 kHz using Clampex 10.3 software (Molecular Devices). For all recordings, series resistance was 8 to 14 M2 and was left uncompensated. Series resistance was monitored continuously during all recordings, and a change beyond 15% was not accepted for data analysis.

Voltage-clamp recordings were used to measure miniature excitatory post synaptic currents (mEPSCs) and evoked excitatory postsynaptic currents. During recordings, slices were superfused with picrotoxin (100 μM)-containing aCSF that was heated to 29–33 °C by passing the solution through a feedback-controlled inline heater (Warner Instruments, Hamden, CT) before entering the chamber. Recordings were made under visual guidance (40x differential interference contrast optics) using a Scientifica SliceScope Pro 6000 (Scientifica, UK). For recordings, the membrane potential was held at −70 mV. Patch pipettes were filed with a cesium-based solution (in mM): 140 cesium methanesulphonate, 5 TEA chloride, 0.4 EGTA, 20 HEPES, 2.5 Mg-ATP, 0.25 Na-GTP, 1 RX-314, pH 7.3 and 290 mOsM. Cells were identified within the IPN, ChAT-ChR2 was verified by visualizing the YFP signal.

For miniature recordings, once a cell was patched the perfusion was switched to ACSF containing TTX (1 μM) using the Programmable Perfusion System (Scientifica, UK). Cells were allowed to stabilize after starting toxin-containing solutions for at least 10 min. Due to possible effects of action potential blockade on synaptic properties, all mEPSC recordings were made within 30 min after TTX was applied to the slice. Once a baseline was recorded (~5 min) the perfusion system was switched again to ACSF containing TTX (1 μM) and Ex-4 (100 nM). For miniature event analysis, at least 50 s of events were analyzed from each cell and each condition using Clampfit 10.3 (Molecular Devices). The event threshold was set at 10 pA and the baseline amplitude was adjusted for each event to midsignal at the initiation time of event. For evoked EPSC recordings, all evoked responses were delivered using a SLOC laser (Shanghai Laser and Optics Century Co., Shanghai, China) at...
473 nM, through an 105 μm optic fiber lowered into the bath just above the slice. The duration of the pulse was 1–2 ms, ~10 mW². Once a stable evoked response was achieved, a baseline was recorded (~5 min) then the perfusion was switched to ACSF containing Ex-4 (100 nM) and evoked responses were recorded for 10–20 min. Amplitudes of EPSCs were calculated using Clampfit 10.3 (Molecular Devices) by averaging 15–50 EPSCs by measuring the peak (5 ms window) compared to the baseline (10 ms window). TTX and Ex-4 were purchased from Tocris Bioscience. All other chemicals were purchased from Sigma-Aldrich.

Statistical analyses. Animal sample size was justified by previously published data or preliminary experiments. Data distribution was assumed to be normal, but this was not formally tested. All experiments animals with the same genotype were randomly allocated to experimental groups. For self-administration experiments, animals that did not achieve stable levels of intake (<20% variation in intake across three consecutive days) or that took less than <5 nicotine infusions on average across sessions were excluded from experiments. All data were analyzed by one- or two-way analysis of variance (ANOVA) or t-test using GraphPad Prism software (La Jolla, CA). Significant main or interaction effects were followed by Bonferroni or Newman-Keuls post hoc tests as appropriate. The criterion for significance was set at P < 0.05. For all electrophysiological data, results are shown as the mean ± s.e.m. When appropriate, Grubbs test was used to identify outliers. A Supplementary Methods Checklist is available.

Data availability. The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Corrigendum: GLP-1 acts on habenular avoidance circuits to control nicotine intake

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