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Leptin activates dopamine and GABA neurons in the substantia nigra via a local pars compacta-pars reticulata circuit

Abbreviated title: Leptin activates substantia nigra neurons

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Abstract

Adipose-derived leptin contributes to energy homeostasis by balancing food intake and motor output, but how leptin acts in brain motor centers remains poorly understood. We investigated the influence of leptin on neuronal activity in two basal ganglia nuclei involved in motor control: the substantia nigra pars compacta (SNc) and pars reticulata (SNr). Using a mouse reporter line to identify cells expressing leptin receptors (LepRs), we found that in both sexes, a majority of SNc dopamine neurons express a high level of LepR. Whole-cell recording in ex vivo midbrain slices from male wild-type mice showed that leptin activates SNc dopamine neurons directly and increases somatodendritic dopamine release. Although LepR expression in SNr GABA output neurons was low, leptin also activated these cells. Additional experiments showed that the influence of leptin on SNr neurons is indirect and involves D1 dopamine receptors and TRPC3 channels. Administration of leptin to male mice increased locomotor activity, consistent with activation of dopamine neurons in the SNc coupled to previously reported amplification of axonal dopamine release by leptin in striatal slices. These findings indicate that in addition to managing energy homeostasis through its actions as a satiety hormone, leptin also promotes axonal and somatodendritic dopamine release that can influence motor output.

Significance statement

Dopamine neurons regulate motivated behaviors, but how they are influenced by metabolic hormones, like leptin, is incompletely understood. We show here that leptin increases the activity of substantia nigra (SN) pars compacta dopamine neurons directly, and that this enhances somatodendritic dopamine release. Leptin also increases the activity of GABAergic neurons in the SN pars reticulata, but does so indirectly via D1 dopamine receptors activated by locally released dopamine. Consistent with increased nigral dopamine neuron activity and previous evidence showing that leptin amplifies striatal dopamine release, systemic leptin increases locomotor behavior. This increase in motor activity complements the well-established inhibitory effect of leptin on food intake and adds an additional dimension to the regulation of energy balance by this hormone.

Keywords

Dopamine neurons, energy homeostasis; leptin receptors; locomotor activity; obesity; GABA neurons; somatodendritic dopamine release; D1 dopamine receptors

Introduction

Midbrain dopamine neurons provide modulatory input to diverse brain regions, thereby playing pivotal roles in movement, motivation, and reward. Dopamine neurons in the substantia nigra pars compacta (SNc) provide dopamine to the dorsal striatum that invigorates movement, registers salience, and facilitates motor learning; loss of the nigrostriatal dopamine pathway results in the motor deficits of Parkinson's disease (Carli et al., 1985; Howe and Dombeck, 2016; da Silva et al., 2018). Axonal dopamine release in the striatum and somatodendritic dopamine release in the SNc both contribute to the regulation of motor output (Bergquist et al., 2003; Crocker, 1997; Zhou et al., 2009). Somatodendritic dopamine release modulates dopamine neuron activity via D2 dopamine autoreceptors (Beckstead et al., 2004; Gantz et al., 2013; Rice and Patel, 2015; Hikima et al., 2021, 2022), and also acts at pre- and postsynaptic D1 dopamine heteroreceptors that regulate GABA neuron excitability in the substantia nigra pars reticulata (SNr) (Miyazaki and Lacey, 1998; Radnikow and Misgeld, 1998; Trevitt et al., 2001; Zhou et al., 2009).

Increasing evidence indicates that metabolic hormones, including leptin, insulin, and ghrelin (Narayanan et al., 2010; Thompson and Borgland, 2013; Labouèbe et al. 2013; Murray et al., 2014; Stouffer et al. 2015; Ferrario et al., 2016; Geisler and Hayes, 2023; Patel et al. 2023), contribute to the regulation of dopaminergic circuits. The influence of leptin is perhaps the least well understood of these hormonal regulators, in part because of the complex interplay between its CNS and peripheral actions (Flier and Maratos-Flier, 2017). Leptin is synthetized by the obese gene (*ob*) primarily in white adipose tissue (Zhang et al. 1994), with circulating levels that are proportional to body-fat mass and which vary diurnally (mid-morning nadir, nocturnal peak in humans) (Ahrén et al., 2000; Pan and Myers, 2018; Obradovic et al., 2021). Leptin crosses the blood-brain barrier (Banks et al., 1996), informing the CNS about the status of body energy stores, with established actions in the hypothalamus via abundant leptin receptors (LepRs) (Considine et al., 1996; Elmquist et al., 1998; Figlewicz et al., 2003; Leinninger and Myers, 2008). The hypothalamus is far from its only site of action, however, as LepRs are expressed throughout the brain, including by midbrain dopamine neurons (Figlewicz et al., 2003; Hommel et al., 2006; Leshan et al., 2010; Patterson et al., 2011; Omrani et al., 2021), midbrain GABAergic neurons (Omrani et al., 2021), and striatal cholinergic interneurons (ChIs) (Mancini et al., 2022). Studies of leptin's influence on the incentive value of food as well as on motor behavior have focused primarily on dopamine neurons of the ventral tegmental area (VTA) (Fulton et al., 2000; Kiefer et al., 2005; Hommel et al., 2006; Figlewicz et al., 2006; Fulton et al., 2006; Roseberry et al., 2007; Fernandes et al., 2015; Murakami et al., 2018; Omrani et al., 2021). Indeed, when infused into the VTA, leptin inhibits VTA dopamine neuron activity and decreases food intake (Hommel et al., 2006; Murakami et al., 2018); however, these actions are indirect and involve leptin-dependent activation of inhibitory VTA GABA neurons (Omrani et al., 2021).

Possible actions of leptin on motor-related SNc dopamine neurons have been neglected. Given evidence for LepRb expression in SNc neurons (Figlewicz et al., 2003; Leinninger and Myers 2008; Leshan et al., 2010) and limited input from the hypothalamus to SNc (Watabe-Uchida et al., 2012; Brown et al., 2019), we hypothesized that leptin directly modulates SNc neuron activity. We tested this using whole-cell recording in SNc dopamine neurons in ex vivo midbrain slices and found that leptin indeed directly increases SNc dopamine neuron excitability and boosts somatodendritic release. We then examined the influence of leptin on SNr GABA neurons, the primary motor output neurons of rodent basal ganglia (Hikosaka, 2007; Grillner and Robertson, 2016). We found that leptin also increases SNr GABA neuron excitability, but indirectly through a dopamine-dependent local circuit. Overall, our data document non-hypothalamic targets of leptin that can influence physical activity and thus energy homeostasis.

Materials and Methods

Animals

All mouse handling was done in accordance with the recommendations of the National Institute of Health, using protocols that were approved by the NYU Grossman School of Medicine and the University of Michigan Animal Care and Use Committees. Male C57BL/6J mice (4-12 weeks of age) were used in all physiology studies and were purchased from Jackson Laboratory, then group-housed under controlled temperature and humidity conditions and maintained on a 12:12 h light cycle (lights on at 06:30) with food and water *ad libitum* at the NYU Animal Unit. All experiments were conducted between 9:30 and 15:30. Expression of LepRb in the SNc and SNr was assessed using male and female double homozygous $LepRb^{EGFP}$ mice expressing enhanced green fluorescence protein (EGFP) coupled to LepRb (Leshan et al., 2010). $LepRb^{EGFP}$ mice (a gift from Prof. Martin G. Myers Jr. at the University of Michigan), which are a cross between $Lepr^{cre/cre}$ and Gt(*ROSA*)*26-Sor*^{tm2Sho} mice (Leshan et al., 2010).

Immunohistochemistry

For immunohistochemical studies, $LepRb^{EGFP}$ and wild-type mice were deeply anesthetized using sodium pentobarbital injected intraperitoneally and perfused transcardially with 0.1 M PBS (154 mM NaCl in 10 mM phosphate buffer, pH 7.3) followed by 4% freshly prepared paraformaldehyde (PFA) (Sigma-Aldrich, Saint Louis, MO, USA) in PBS. Brains were removed, post-fixed for 12-15 h in PFA, then cryoprotected by successive immersion in solutions of graded 10-30% sucrose in PBS. Frozen midbrain sections (50 µm thickness) were cut using a Cryocut 1800 cryostat (Belair Instrument Company) and processed for immunohistochemistry. Sections were washed 3 x 15 min in PBS + 0.1% Triton-X 100, then for 1 h in 20% normal donkey serum in PBS + 0.3% Triton-X100, followed by primary antibody incubation for 18-24 h at room temperature on an orbital shaker. Sections were washed 3 x 15 min in PBS/0.3% Triton X, then incubated in secondary antibodies for 2 h. After final washes in PBS alone, sections for LepRb assessment were mounted onto slides, air-dried, dehydrated in graded alcohols, then Citrosolv

and coverslipped in Krystalon (EMD Chemicals). Sections for D1R localization were washed in PBS, then mounted and coverslipped in Vectashield (VectorLabs). Distribution of LepRb was indicated by expression of EGFP immunoreactivity (EGFP-ir) using a chicken anti-EGFP (GFP-1020, dilution 1:500, Aves Labs). Dopamine neurons in SNc were identified by immunohistochemical detection of hydroxylase (TH), the rate-limiting enzyme for dopamine synthesis, using a sheep polyclonal anti-TH antibody (ab113; dilution 1:1000; Abcam) to assess TH immunoreactivity (TH-ir). SNr GABA neurons were identified using a guinea pig polyclonal anti-parvalbumin (PV) antibody (195 004; dilution 1:400; Synaptic Systems GmbH). Dopamine D1 receptors (D1Rs) were detected with a rat monoclonal antibody (D2844; dilution 1:200; Sigma-Aldrich) that has been validated previously, including by the absence of immunostaining in sections from a D1R knockout mouse (Stojanovic et al., 2017). Secondary antibodies were donkey anti-sheep Cy5 (713-175-147), donkey anti-chicken Cy3 (703-165-155), donkey anti-guinea pig Cy2, (706-225-148) and donkey anti-rat Alexa 488 (712-545-153), all from Jackson ImmunoResearch Laboratories). Cy secondary antibodies were applied at 1:200 dilution; Alexa 488 secondary at 1:400 dilution.

Images of immunostained tissue were obtained either with a Nikon Eclipse C1 confocal microscope (Nikon) and processed with Photoshop (Adobe Systems Incorporated) or were obtained with an Andor BC43 benchtop spinning disk confocal microscope (Oxford Instruments) and processed using with Imaris 10.1.1 analysis software (Oxford Instruments). Any alterations in brightness and/or contrast were made on the entire image. The fraction of SNc TH-ir neurons showing robust EGFP-ir in serial sections through the SNc was quantified using Image J.

Ex vivo slice physiology

Patch-clamp recording of SNc dopamine and SNr GABA neurons was performed in midbrain slices, as described previously (Beckstead et al., 2004; Avshalumov et al., 2005; Lee et al., 2013; Hikima et al., 2021, 2022). Each mouse was deeply anesthetized with isoflurane (Henry Schein Inc.), decapitated, and the brain removed for slicing. Coronal or horizontal slices (250 µm thickness) were cut using a Leica VT1200S vibrating blade microtome (Leica Microsystems,) in oxygenated ice-cold cutting solution containing (in mM): 200 sucrose, 2.5 KCl, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 7 $MgCl_2$, 1 ascorbate, 3 pyruvate, 7 glucose, 0.5 $CaCl_2$; then transferred to a recovery chamber for 40 min at 35^{o}C in modified aCSF containing (in mM): 115 NaCl, 2.5 KCl, 25 $NaHCO_3$, 1.25 NaH_2PO_4, 1 $MgCl_2$, 0.5 $CaCl_2$, 25 glucose, 1 ascorbate, 0.4 myo-inositol, 3 pyruvate, equilibrated with 95% O_2/5% CO_2. Slices remained in this medium at room temperature until used. Recording was at 32^{o}C in aCSF containing (in mM): 124 NaCl, 3.7 KCl, 26 $NaHCO_3$, 2.4 $CaCl_2$, 1.3 $MgSO_4$, 1.3 KH_2PO_4, 10 glucose, and bovine serum albumin (BSA, 0.1 mg/mL), equilibrated with 95% O_2/5% CO_2 and superfused at 1.5 mL/min (*e.g.*, Mancini et al., 2022).

Neurons were visualized at 40x using an Olympus BX50WI microscope (Olympus America). Recording pipettes were fabricated from fire polished 2.0 mm o.d. borosilicate capillary tubing (Sutter Instrument Company) using a P-97 Flaming/Brown micropipette puller (Sutter Instrument

Company). The pipette solution for current-clamp recording in SNc dopamine neurons contained (in mM): 120 K-gluconate, 20 KCl, 10 HEPES, 2 $MgCl_2$, 10 EGTA, 3 Na_2-ATP, 0.2 Na_3-GTP, pH 7.3 with KOH; pipette resistance was 2-3.5 MΩ (Hikima et al., 2021). For current-clamp recording of SNr GABA neurons, the pipette solution contained (in mM): 129 K-gluconate, 11 KCl, 10 HEPES, 2 $MgCl_2$, 10 EGTA, 3 Na_2-ATP, 0.3 Na_3-GTP, pH 7.3 with KOH (Lee and Tepper, 2007; Lee et al., 2013); pipette resistance was 3-5 MΩ. The internal solutions used for the two neuronal populations were based on previously published pipette solutions for each cell type that we study routinely in the lab. Although the compositions differed slightly, net K^+ (140 mM) was the same for both. In some experiments, the pipette backfill solution also included a mouse monoclonal antibody directed against LepR clone B-3 (Ob-R (B-3), sc-8391; dilution 1:100; Santa Cruz Biotechnology) alone or together with a blocking peptide (SC-8391 P; dilution 1:100; Santa Cruz). In other experiments, the backfill included a control immunoglobulin (IgG) (ab176094; dilution 1:100; Abcam) (Hikima et al., 2021) or a TRPC3 channel antibody (ACC-016; dilution 1:100; Alomone Labs) (Zhou et al., 2009; Lee et al., 2013).

Somatodendritic dopamine release in the SNc was monitored using voltage-clamp recording to detect dopamine D2 autoreceptor-activated G-protein-coupled inward rectifier K^+ (GIRK) current (Lacey et al., 1987; Beckstead et al., 2004; Beckstead et al., 2007; Ford, 2014; Hikima et al., 2021). These D2-mediated inhibitory currents (D2ICs) serve as an index of dopamine release; in mouse SNc, the commonly employed method of fast-scan cyclic voltammetry could not be used because of interfering serotonin detection (John et al., 2006; Rice and Patel, 2015). The pipette solution for voltage-clamp recording of SNc dopamine neurons contained (in mM): 115 K-methylsulfate; 20 NaCl; 1.5 $MgCl_2$; 5 HEPES; 10 BAPTA; 3 Na_2-ATP; 0.3 Na_3-GTP, pH 7.3 with KOH (Ford et al., 2006; Hikima et al., 2021). Pharmacological isolation of evoked D2ICs was achieved by including ionotropic GABA and glutamate receptor antagonists in the superfusing aCSF. The antagonists used were (in µM): 100 picrotoxin, 0.3 CGP55845, 10 DNQX and 50 D-AP5 (Hikima et al., 2021, 2022). Neurons were held at -60 mV and D2ICs were evoked using pulse-train stimulation (5 pulses, 40 Hz, 0.015-0.05 mA) delivered at 240 s intervals by a bipolar stimulating electrode positioned 50-100 µm anterior to recorded cells in horizontal slices (Beckstead et al., 2004; Hikima et al., 2021, 2022).

Current- and voltage-clamp data were obtained using a Multiclamp 700B amplifier. The signals were filtered at 2 kHz and digitized at 10 kHz by a Digidata 1550B for subsequent acquisition using Clampex 10.7 software (Molecular Devices). Access resistance (R_a) was monitored during the recordings. Experiments in current-clamp mode with changes in R_a > 20% were excluded, as were voltage-clamp recordings from cells with R_a > 15 MΩ.

Dopamine neurons in the SNc were distinguished from GABA neurons in the SNr based on their respective electrophysiological characteristics (Grace and Onn, 1989), including spontaneous firing rate (compare **Figs. 1B** and **5B**), and action potential (AP) width of >1.2 ms for dopamine, but less for GABA neurons (Lee and Tepper, 2009). Dopamine neurons were identified by a prominent hyperpolarization-induced sag in membrane potential in response to hyperpolarizing current steps, which was largely absent in GABA neurons (compare **Figs. 2A** and **6A**).

Hyperpolarization in the presence of an exogenous D2R agonist, quinpirole (1 μM), in current-clamp recording or the presence of evoked D2ICs in voltage-clamp recording was also used to confirm dopamine neuron identity.

Open-field test

The open-field test was used to assess locomotor activity in 17 male mice, 5-10 weeks old. A within subject design was used, such that each mouse received vehicle and leptin; the order of administration was counterbalanced, and injections were separated by 1 week. Open-field testing was conducted in a clear, open field arena (28 x 28 cm) equipped with infrared source and detector strips, placed in a ventilated and sound attenuating cubicle with ceiling lights (Med Associates Inc). Testing was conducted during the early light phase (between 3 and 6 h after lights on) when leptin levels are low, before the later nocturnal rise (Ahrén, 2000). This time period also avoided the confounding factor of increased locomotor activity that occurs naturally during the dark phase. On the test day, mice were weighed and transferred to the behavioral room 20 min before testing. Each mouse was then placed in the center of the arena, and activity monitored for 15 min before intraperitoneal (i.p.) injection with vehicle solution (Tris-HCl 20 mM, pH 8.0) or with 1.5 mg/kg leptin in vehicle. The dose of leptin used was selected based on previous studies (Lu et al., 2006; Garza et al., 2012). Immediately after injection, the mouse was returned to the activity box and the locomotor activity tracked for 1 h. The total distance moved in 1 h was measured in 5 min bins using the activity monitor software SOF-812 (Med Associates Inc.). After each test session, the apparatus was cleaned with ethanol (70%) to eliminate the odor of previously tested animals.

Drugs and chemicals

Components of aCSF and pipette solutions were obtained from Sigma-Aldrich, as were picrotoxin, D-AP5, FFA and α-MPT. Recombinant mouse leptin (carrier free; catalogue number 498-OB) was purchased from R&D Systems (Minneapolis, MN, USA). CGP 55845, DNQX, SKF 83566, and 2-aminoethoxydiphenyl borate (2-APB) were from Tocris Bioscience. Water soluble drugs were prepared as aqueous stock solutions. Leptin was reconstituted in 20 mM Tris-HCl, pH 8.0, and stored as frozen aliquots at -80^{o}C. Stock solutions of 2-APB were prepared in ethanol; picrotoxin, CGP 55845, DNQX, and FFA stock solutions were in dimethyl sulfoxide (DMSO, Sigma-Aldrich). Final concentration of ethanol or DMSO was <0.1% in aCSF (Lee et al., 2013). Drugs were diluted in aCSF immediately before use; BSA was included as a carrier to help maintain solubility of leptin and to minimize adherence of leptin and other drugs to the tubing and recording chamber.

Experimental design and statistical analysis

Baseline records were obtained in aCSF alone or in the presence of drug vehicle. The duration of leptin application was 45-60 min for current-clamp recording SNc and SNr neuron firing rates, as well as voltage-clamp recording of D2ICs in SNc dopamine. A maximum effect was observed after 30 min in most cells, so that results at 30 min time point were used for comparisons of the

effect of leptin with time-matched controls in aCSF alone or in a drug. In a few cells, the response was transient and declined after 30 min, but in most recordings a sustained increase was observed that remained constant from that point until the end of recording. Drugs were applied for 10-15 min before leptin, except for α-MPT which was present throughout recovery and in the slice recording chamber for a total exposure time of at least 2 h before recording. Physiological recordings were analyzed using Clampfit 11 (Molecular Devices). Spontaneous activity was recorded and spikes counted for 10 s every 2 min, without current injection. Firing rate was calculated at the time of maximal effect of leptin alone, typically 10-15 min after initiation of superfusion, and in time-matched controls. Input resistance for both cell types was calculated by plotting the amplitudes of hyperpolarizing and depolarizing current steps against the resulting membrane voltage deflections from resting potential. For hyperpolarizing current steps, the voltage deflections were taken at the steady-state after the initial sag; for depolarizing steps, the value was measured just prior to action potential (AP) initiation. Other AP parameters were measured from spike discharge patterns in the absence and presence of leptin (*e.g.*, **Figs. 2A and 6A**). To assess the effect of leptin on somatodendritic dopamine release, leptin was applied after stable baseline data were obtained. Changes in D2IC amplitude were assessed by comparing the average of the last three pre-leptin recorded points with the average of three records at the usual time of maximal effect for SNc dopamine neurons (30 min).

Data are presented as means ± SEM. In the electrophysiology experiments, n equals the number of cells recorded from at least three different animals; for behavioral analyses, n equals the number of animals tested. Statistical analysis was performed in Prism 8 (GraphPad Software Inc., San Diego, CA, USA) and significance was calculated using paired (within cells) or unpaired Student's *t*-tests or using two-way ANOVA as appropriate. Results were considered to be significant when $p < 0.05$.

Results

Leptin enhances dopamine neuron excitability in the SNc

The presence of LepRs in SNc has been assessed previously, although with limited quantitation (Figlewicz et al., 2003; Leshan et al., 2010; de Vrind et al., 2021). Of six LepR isoforms that have been identified, only the long form, LepRb, contains the complete intracellular domain required to mediate LepR signaling (Lee et al., 1996). To evaluate LepR expression in SNc dopamine neurons, we used $LepRb^{EGFP}$ mice that express EGFP coupled to LepRb (Leshan et al., 2010) and evaluated colocalization of EGFP-ir and TH-ir (**Fig. 1A**). Our analysis showed that a majority of TH-ir somata showed robust expression of LepRb (EGFP-ir) (56.4 ± 2.5%, averaged for 3 mice (2 female, 1 male); from a total of 90 sections, with EGFP-ir in 783 of 1,382 TH-ir cells in the SNc), and lower levels of EGFP-ir in other cells that were not quantified. Notably, LepRb expression was detected in dopamine neuron dendrites, as well as in cell bodies (**Fig. 1A**).

We next tested whether LepRs on SNc dopamine neurons were functional, using current-clamp recording of SNc dopamine neurons in ex vivo midbrain slices from wild-type mice. Dopamine neurons in mouse SNc are tonically active, with firing rates of 1-5 Hz, and exhibit a voltage sag in response to hyperpolarizing current injections (Lee and Tepper, 2009; Guzman et al., 2009; Hikima et al., 2021). Acute application of 30 nM leptin (Mancini et al., 2022), a physiologically relevant concentration (Caro et al., 1996; Hommel et al., 2006), caused an increase in firing rate in 6 of 8 recorded neurons, typically beginning within 10 min (control, 1.03 ± 0.16 Hz; leptin, 2.61 ± 0.44 Hz; $t = 4.400$, df = 7, $p = 0.0032$, paired *t*-test, n = 8 neurons from 6 mice) (**Fig. 1B**). In two of the leptin-sensitive neurons, the increase was transient and firing rate declined after reaching a maximum; in the remaining cells the enhancement persisted throughout the recordings.

To assess a possible indirect effect of leptin on SNc dopamine neuron activity via glutamate or GABA input to these cells, we applied leptin (30 nM) in the presence of a cocktail of ionotropic receptor antagonists (100 µM picrotoxin for GABA-A receptors, 300 nM CGP 55845 for GABA-B receptors, 10 µM DNQX for AMPA receptor, and 50 µM D-AP5 for NMDA receptors). The leptin-induced increase in dopamine neuron firing rate occurred in the presence of these antagonists, consistent with a direct effect of this hormone on SNc dopamine neurons (**Fig. 1C**; control 0.97 ± 0.22 Hz; leptin 2.80 ± 0.47 Hz; $t = 4.526$, df = 6, $p = 0.0040$, paired *t*-test, n = 7 neurons from 5 mice).

We tested a possible direct effect further using single-cell application of a monoclonal antibody raised against residues 870-894 of the intracellular C-terminal of the LepR, with evidence for selectivity indicated by the absence of immunostaining in mouse neurons after conditional LepR KO (de Lartigue et al., 2014) and appropriate labeling of LepR protein in Western blots (Chang et al. 2021). When the LepR antibody was included in the intracellular solution (1:100 dilution), application of leptin (30 nM) no longer had a significant effect on neuron firing rate (**Fig. 1D**; control 0.80 ± 0.26 Hz; leptin, 1.10 ± 0.37 Hz; $t = 2.393$, df = 6, $p = 0.0538$, n = 7 neurons from 6 mice). As a control, we included a non-specific immunoglobulin (IgG) in the recording pipette at the same 1:100 dilution; the presence of IgG had no effect on leptin-induced enhancement of SNc dopamine neuron firing rate (**Fig. 1E**; control, 1.04 ± 0.25 Hz; leptin, 3.15 ± 0.57 Hz; $t = 5.135$, df = 8, $p = 0.0009$, paired *t*-test, n = 9 neurons from 6 mice). As a second control, we found that pre-adsorption of the LepR antibody by its immunogenic peptide preserved the excitatory action of leptin, supporting the specificity of the antibody effect (**Fig. 1F**; control, 0.66 ± 0.11 Hz; leptin 2.08 ± 0.50 Hz; $t = 2.843$, df = 5, $p = 0.0361$, paired *t*-test, n = 6 neurons from 4 mice).

Notably, the increase in spontaneous activity of SNc dopamine neurons with leptin (30 nM) was not accompanied by a significant change in whole-cell input resistance (control, 104.2 ± 17.4 MΩ; leptin, 104.5 ± 9.1 MΩ; $F_{1, 42} = 0.0002$, $p = 0.9879$, n = 4 neurons from 4 mice) or membrane potential (**Fig. 2A,B**); the input resistance was found to be nonlinear over the range of current injection amplitudes tested. We also found no difference in other AP characteristics examined including amplitude, width at 50% repolarization, or afterhyperpolarization amplitude (**Fig. 2C-**

H; n = 8 neurons from 6 mice). Given the difficulty in determining resting membrane potential in a spontaneously active cell, we also assessed the influence of leptin on membrane potential when APs were prevented by a Na^+ channel blocker, tetrodotoxin (1 μM). In this condition, there was still no change in membrane potential when leptin was applied (control, -54.3 ± 1.8 mV; leptin, -54.0 ± 2.8 mV; t = 0.2258, df = 5, p = 0.8303, n = 6 neurons from 3 mice). Collectively, our data demonstrate that leptin acts directly via LepRs on SNc dopamine neurons to increase neuronal excitability.

Leptin boosts somatodendritic dopamine release in SNc in a Ca^{2+}-dependent manner

In addition to axonal dopamine release in striatal and cortical target regions, midbrain dopamine neurons also exhibit somatodendritic dopamine release (Geffen et al., 1976; Beckstead et al., 2004; Gantz et al., 2013; Ford, 2014; Rice and Patel, 2015; Hikima et al., 2021, 2022). Somatodendritically released dopamine acts at D2 autoreceptors to inhibit dopamine neuron activity (Lacey et al., 1987; Beckstead et al., 2004; Beckstead et al., 2007; Ford, 2014; Hikima et al., 2021), thereby influencing the pattern of dopamine signaling in striatal target regions (Gerfen and Surmeier, 2011; Paladini and Roeper, 2014; Sulzer et al., 2016). We hypothesized that leptin-enhanced dopamine neuron excitability would result in increased somatodendritic dopamine release. Application of leptin (30 nM) caused an increase in D2IC amplitude compared to vehicle controls (**Fig. 3A**; vehicle, initial 32.8 ± 5.2 pA; final 32.7 ± 5.2 pA; t = 0.03382, df = 4, p = 0.9746, paired t-test, n = 5 neurons from 3 mice; leptin, initial 25.0 ± 3.2 pA, final 33.3 ± 5.0 pA; t = 3.401, df = 7, p = 0.0114, paired t-test, n = 8 neurons from 5 mice). The increase was progressive, beginning within a few minutes of leptin application and showing a significant increase over time for leptin *vs*. vehicle in these same neurons (**Fig. 3B**; $F_{10, 110}$ = 2.181, p = 0.0241, two-way ANOVA). Given that a change in D2IC amplitude might reflect either a change in somatodendritic dopamine release or in D2 receptor sensitivity, we tested the effect of leptin on D2ICs elicited by application of a D2 receptor agonist, quinpirole. Brief (15 s) superfusion of 250 nM quinpirole produces a D2IC of comparable amplitude to that seen with local stimulation (Hikima et al., 2021, 2022). The presence of leptin did not modify the amplitude of quinpirole-induced currents (**Fig. 3C**; control before leptin, 38.1 ± 4.3 pA; after 30 min in leptin, 37.0 ± 6.1 pA; t = 0.3022, df = 5, p = 0.7747, paired t-test, n = 6 neurons from 6 mice). This result indicates that the leptin-induced increase in evoked D2IC amplitude reflects an increase in somatodendritic dopamine release, and not a change in D2 receptor sensitivity or GIRK channel function.

Activation of LepRs recruits several intracellular pathways, including that of phosphoinositide 3-kinase (PI3K) (Xu et al., 2005; Plum et al., 2006), which, by activation of phospholipase C (PLC) (Rameh et al., 1998), generates inositol trisphosphate (IP_3). In turn, IP_3 receptors (IP_3Rs) promote Ca^{2+} release from intracellular stores. Previous studies have shown that IP_3R activation and increased intracellular Ca^{2+} boost somatodendritic dopamine release from SNc dopamine neurons (Patel et al., 2009; Yee et al., 2019), possibly acting via the sensitive Ca^{2+} sensor, synaptotagmin 7, to promote release (Hikima et al., 2022). We assessed a role for IP_3R

activation in leptin-enhanced somatodendritic dopamine release using an IP_3R antagonist, 2-APB (30 μM) (Patel et al., 2009; Maruyama et al., 1997). This antagonist alone had no consistent effect on evoked D2IC amplitude (**Fig. 4A**; initial, 20.4 ± 2.0 pA, final 22.7 ± 5.4 pA; $t = 0.5063$, df = 4, $p = 0.6393$, paired *t*-test, n = 5 neurons from 5 mice). Moreover, when applied before leptin, 2-APB prevented the increase in somatodendritic dopamine release (**Fig. 4A**; 2-APB, 18.2 ± 4.4 pA; 2-APB + leptin, 19.2 ± 5.6 pA; $t = 0.4864$, df = 4, $p = 0.6521$, paired *t*-test, n = 5 neurons from 3 mice). In these neurons, the amplitude of evoked D2ICs was stable over time in either 2-APB alone or when leptin was applied in the presence of 2-APB, implying IP_3R involvement in the amplification of dopamine release by leptin (**Fig. 2B**; $F_{10, 88} = 0.1740$, $p = 0.9977$, two-way ANOVA).

We returned to current-clamp recording to assess the effect of 2-APB on leptin-enhanced dopamine neuron firing rate. In the presence of 2-APB, leptin failed to elicit an increase in firing rate (**Fig. 4C**; 2-APB, 0.71 ± 0.17 Hz; 2-APB + leptin, 0.70 ± 0.21 Hz; $t = 0.1832$, df = 5, $p = 0.8618$, paired *t*-test, n = 6 neurons from 3 mice). One caveat for interpreting this result is that 2-APB can also interfere with Ca^{2+} entry via Ca^{2+}-permeable cation channels, including some transient receptor potential (TRP) channels (Li et al., 2006). To assess this possible confounding factor, we evaluated the effect of leptin on dopamine neuronal activity in the presence of a non-selective TRP channel blocker, flufenamic acid (FFA, 20 μM) (Lee et al., 2013). Application of FFA neither altered spontaneous activity nor prevented the expected increase in SNc dopamine neuron firing rate by leptin (**Fig. 4D**; FFA, 1.37 ± 0.55 Hz; FFA + leptin, 3.22 ± 0.55 Hz; $t = 10.73$, df = 6, $p < 0.0001$, paired *t*-test, n = 7 neurons from 4 mice). These data are consistent with an involvement of the PI3K-PLC-IP_3 pathway, but not TRP channels, in the effect of leptin on SNc dopamine neuron excitability.

Leptin increases the spontaneous firing rate of SNr GABA neurons

The SNr, a primary motor output nucleus of the basal ganglia (Zhou and Lee, 2011), lies adjacent to the SNc. Dopamine neuron somata in the SNc extend dendrites into the SNr and intermingle closely with SNr GABA projection neurons (González-Hernández and Rodríguez, 2000; Rice and Patel, 2015) (see also **Fig. 1A**). Previously published studies found little mRNA for LepRb in rat SNr, despite its ready detection in SNc (Elmquist et al., 1998). We therefore examined LepRb in SNr GABA neurons in midbrain sections from $LepR^{EGFP}$ mice using immunostaining for EGFP to identify LepRb-expressing cells and immunoreactivity to PV (PV-ir) to identify SNr GABA neurons (**Fig. 5A**). We chose PV because of its relative abundance among the SNr GABA neuron population (Zhou et al., 2009), particularly in dorsolateral SNr (Lee and Tepper, 2007b). Consistent with low mRNA expression in rat SNr, we found weak LepRb expression in SNr GABA neurons (**Fig. 5A**), intermingled with EGFP-ir processes identified as SNc dopamine neuron dendrites (representative of sections from n = 3 mice, 2 females, 1 male) (**Fig. 1A**).

We then examined the effect of LepR activation on SNr GABA neuron activity using current-clamp recording during leptin exposure. Leptin (30 nM) caused a marked increase in firing rate

(**Fig. 5B**; control, 4.01 ± 0.66 Hz; leptin, 15.17 ± 2.20 Hz; t = 5.090, df = 6, p = 0.0022, paired t-test, n = 7 neurons from 6 mice). To test whether enhanced SNr GABA neuron excitability reflected a direct action of leptin on these cells, we again applied LepR antibody intracellularly via the recording pipette to impair LepR signaling. In contrast to the efficacy of this antibody in preventing the effect of leptin on SNc dopamine neurons (**Fig. 1D**), it did not alter the excitatory effect of leptin on SNr GABA neurons (**Fig. 5C**; anti-LepR, 9.16 ± 2.34 Hz; anti-LepR + leptin 19.11 ± 2.73 Hz; t = 6.268, df = 7, p = 0.0004, paired t-test, n = 8 neurons from 4 mice). The lack of effect of the LepR antibody is consistent with the paucity of LepRs in SNr GABAergic neurons (**Fig. 5A**) and provides further support for a LepR-selective effect of the antibody in SNc dopamine neurons (**Fig. 1D**). We then tested non-specific IgG in the pipette solution as an additional control. In cells recorded with IgG, increases in firing rate with leptin were similar to those recorded seen with the usual backfill solution (**Fig. 5D**; IgG, 7.14 ± 1.38 Hz; IgG + leptin 20.88 ± 2.62 Hz; t = 8.085, df = 6, p < 0.0002, paired t-test, n = 7 neurons from 3 mice). These results imply an indirect effect of leptin on SNr GABA neurons. We therefore tested possible effects on glutamate or GABA input to these cells by recording SNr GABA neuron activity during leptin exposure in the presence of GABA and glutamate receptor antagonists, as we did for SNc dopamine neurons. Under these conditions, leptin still increased SNr GABA neuron firing rate (**Fig. 5E**; antagonists, 4.60 ± 1.40 Hz; antagonists + leptin 13.91 ± 2.20 Hz; t = 4.529, df = 5, p = 0.0062, paired t-test, n = 6 neurons from 4 mice) indicating that neither GABA nor ionotropic glutamate receptors were involved in the actions of leptin on SNr GABA neuron excitability. The increase in spontaneous activity of SNr neurons in the presence of leptin (30 nM) was not accompanied by a significant change in membrane input resistance (control, 154.0 ± 9.6 MΩ; Leptin, 149.0 ± 13.2 MΩ; $F_{1, 54}$ = 0.04836, p = 0.8268, n = 5 neurons from 3 mice) (**Fig. 6A,B**), or in membrane potential, action potential threshold, or most other parameters (**Fig. 6C-H**). The exception was that AP amplitude was slightly, but significantly lower in leptin (**Fig. 6F**; control, 69.0 ± 5.1 mV; leptin, 56.7 ± 6.6 mV, p = 0.0280; t = 2.882, df = 6, paired t-test, n = 7 neurons from 6 mice).

Leptin indirectly enhances the activity of SNr GABA neurons via local dopamine release

Given that leptin enhanced somatodendritic dopamine release (**Fig. 3**), we next investigated whether leptin-enhanced SNr GABA neuron firing rate might be dopamine-dependent. Dopamine can alter intrinsic neuronal excitability (Gerfen and Surmeier, 2011), including increasing the excitability of SNr neurons downstream from D1 dopamine receptor activation (**Fig. 7A**) (Zhou et al., 2009). Zhou and colleagues used single-cell reverse transcription PCR (scRT-PCR) to identify mRNA for D1 receptors in SNr neurons that also express mRNA for the GABA synthesizing enzyme, glutamic acid decarboxylase (GAD1) (Zhou et al., 2009). Here we used immunohistochemistry to examine the presence of D1 receptor protein in PV-ir neurons in the SNr (**Fig. 7B**). The specificity of the anti-PV antibody was indicated by the absence of staining after its pre-incubation with full length parvalbumin (not illustrated). In coronal sections of wild-type mouse SNr, PV-ir neuronal perikarya in SNr contain clusters of D1R-ir puncta (**Fig. 7B**; representative of sections from 3 mice). No puncta were observed when the anti-D1 receptor

antibody was omitted. Z-stacks through PV-ir perikarya containing D1R puncta documented that the puncta were located on GABAergic cell bodies. We tested the functional involvement of D1 receptors in the effect of leptin on SNr neurons using a D1 receptor antagonist, SKF 83566 (5 µM) (Zhou et al., 2009). These experiments were conducted in the presence of ionotropic glutamate and GABA receptor antagonists to eliminate possible confounding effects D1-receptor antagonism on input from these transmitters acting via ionotropic receptors in the SNr, *e.g.*, D1 receptors on striatonigral GABA terminals (Trevitt et al., 2001). Antagonism of D1 receptors by SKF 83566 eliminated the enhancing effect of leptin on SNr neuron firing rate (**Fig. 7C**; SKF 83566, 5.14 ± 1.79 Hz; SKF + leptin, 7.85 ± 2.76 Hz; $t = 1.938$, df = 6, $p = 0.1007$, paired *t*-test, n = 7 neurons from 3 mice). We then assessed dopamine dependence of the leptin effect by depleting midbrain dopamine stores using α-methyl-ρ-tyrosine (α-MPT), an inhibitor of TH-dependent dopamine synthesis. Slices were incubated in α-MPT (30 µM) (Mercuri et al., 1989) during slice recovery and throughout recording in the superfusion chamber. Under these conditions, the effect of leptin on SNr GABA neuron excitability was again lost (**Fig. 7D**; α-MPT, 4.71 ± 0.94 Hz; leptin, 5.90 ± 0.80 Hz; $t = 1.144$, df = 7, $p = 0.2904$, paired *t*-test, n = 8 neurons from 6 mice).

Previous studies have shown that D1 receptor activation triggers the opening of TRPC3 channels in SNr GABA neurons, increasing their excitability (Zhou et al., 2009). We therefore tested whether TRPC3 channels were involved in the dopamine-dependent effect of leptin on these cells. Given the absence of specific TRPC3 receptor antagonists, we instead applied, via the recoding pipette, a TRPC3 channel antibody (1:100 dilution) raised against residues 822-835 in the C-terminal region of the protein (Zhou et al., 2009; Lee et al., 2013). Zhou and colleagues (2009) showed previously that intracellular application of this TRPC3 antibody prevented the enhancing effect of D1 agonists on SNr GABA neuron excitability. Consistent with this mechanism, interference with TRPC3 channels also inhibited the leptin-driven increase of neuronal excitability (**Fig. 7E**; TRPC3 antibody, 8.64 ± 2.14 Hz; leptin, 11.50 ± 2.11 Hz; $t = 1.788$, df = 6, $p = 0.1240$, paired *t*-test, n = 7 neurons from 4 mice).

In vivo leptin administration increases locomotor activity

Dopamine neuron activation in SNc and the release of dopamine play a key role in invigorating motor behavior (Carli et al., 1985; Howe and Dombeck, 2016; da Silva et al., 2018), whereas activation of the SNr suppresses movement (Hikosaka et al., 1993; Grillner and Robertson, 2016). To assess the net effect of leptin on motor activity in wild-type mice, we conducted open-field testing to assess spontaneous locomotion after i.p. injection of vehicle or leptin (1.5 mg/kg) (Lu et al., 2006; Garza et al., 2012). We intentionally examined the influence of leptin in the early light phase, when endogenous leptin levels are low, before the sleep-cycle rise in leptin production (Ahrén, 2000). We found a significant increase in locomotor activity when mice were given leptin *vs*. vehicle (**Fig. 8A,B**) ($F_{1, 384} = 6.409$, $p = 0.0117$; two-way ANOVA; n = 17 mice). The greatest increase was seen 30-35 min after leptin administration (5 min time bins) (**Fig. 8B,C)**. When the total distances for mice given vehicle or leptin during this 10 min period were

compared, the distance after leptin was significantly greater than when the same mice received vehicle (leptin, 811.4 ± 47.7 cm; vehicle, 537.8 ± 31.6 cm; $t = 2.371$, df = 32, $p = 0.0239$; paired *t*-test, n = 17 mice) (**Fig. 8C**).

Discussion

Brain motor circuitry is influenced by neurohormonal regulators that mediate cross-talk between circuits governing energy homeostasis and physical activity (Ferrario et al., 2024). Leptin is a key player in these incompletely understood interactions (Murray et al., 2014; Ceccarini et al., 2015; Pan and Myers, 2018). We show that leptin increases the activity of motor-regulating basal ganglia neurons through direct activation of SNc dopamine neurons, as well as an indirect, dopamine-dependent activation of SNr GABA neurons. Notably, SNc dopamine-neuron activity influences motor-related axonal dopamine release in the striatum (Howe and Dombeck, 2016; da Silva et al., 2018), as well as via somatodendritic dopamine release (Crocker, 1997; Trevitt et al., 2001; Bergquist et al., 2003; Zhou et al., 2009). Previous work showed that leptin boosts striatal dopamine release by increasing striatal ChI excitability (Mancini et al., 2022) and consequent activation of nicotinic ACh receptors that drive axonal dopamine release (Rice and Cragg, 2004, Threlfell et al., 2012; Patel et al. 2013; Kramer at al., 2022). Leptin activation of SNc dopamine neurons provides a second site of action for leptin-induced increases in locomotor activity we observed in wild-type mice following systemic leptin administration.

Direct activation of SNc dopamine neurons by leptin

Previous studies have documented LepRbs on SNc dopamine neurons (Figlewicz et al., 2003; Leshan et al., 2010; de Vrind et al., 2021). The proportion of expressing neurons in these autoradiographic and immunohistochemical studies is variable, however, ranging from a small minority to a majority. Our data from $LepRb^{EGFP}$ mice (Leshan et al., 2010; Patterson et al., 2011) show robust expression in 56% of SNc dopamine neurons (**Fig. 1A**). Correspondingly, a leptin-induced increase in SNc dopamine neuron activity seen in most recorded cells supports LepR functionality. An excitatory action of leptin on SNc dopamine neurons is consistent with previously reported leptin-dependent activation of VTA GABA neurons (Omrani et al., 2021) and striatal ChIs (Mancini et al., 2022). Leptin also enhanced evoked somatodendritic dopamine release in a process involving IP_3Rs, known regulators of SNc dopamine release (Patel et al., 2009; Yee et al., 2019). Interestingly, studies in *ob/ob* mice lacking leptin synthesis (Zhang et al., 1994) suggest that leptin-deficiency compromises dopamine stores for both axonal and somatodendritic release (Fulton et al., 2006; Roseberry et al., 2007). Our results show that in *ad-libitum*-fed wild-type mice, leptin dynamically enhances dopamine neuron activity and somatodendritic release.

Dopamine-dependent SNc-SNr interactions underlie the effect of leptin on SNr GABA neurons

Our data document that leptin-enhanced somatodendritic dopamine release affects SNr GABA neuron activity. The SNr is a basal ganglia nucleus with inhibitory GABA output neurons that limit movement when activated and facilitate movement when inhibited. Accumulating evidence suggests that SNr GABA neurons do not act in a simple binary manner; rather, subsets of SNr neurons exhibit opposing activity patterns to exert motor control (Fan et al., 2012; Freeze et al., 2013; Barter et al., 2015; Partanen and Achim, 2022). We find that SNr GABA neurons have very low $LepRb^{EGFP}$ expression. Consistent with this, leptin-induced excitation of SNr GABA neuron activity was unaffected by intracellular application of a LepRb antibody that prevented a leptin-mediated action on SNc dopamine neurons. Given the rich innervation of SNr by SNc neuron dendrites (González-Hernández and Rodríguez, 2000; Rice and Patel, 2015; Crittenden et al., 2016) and the finding that dopamine regulates the activity of SNr GABA neurons (Zhou et al., 2009), we hypothesized that enhanced somatodendritic dopamine release might underlie the effect of leptin in SNr. Zhou and colleagues (2009) found that D1 receptor activation by exogenous agonists and endogenous dopamine promotes TRPC3 channel opening in SNr GABA neurons, thereby increasing neuronal activity via an ultra-short SNc-SNr dopamine pathway (Zhou et al., 2009). We confirmed a basic element of this circuit by demonstrating D1 dopamine receptors on SNr GABA neurons. We also provided the first evidence that increased SNc dopamine neuron activity regulates this pathway: the effect of leptin was lost with dopamine depletion, D1-receptor antagonism, or interference with TRPC3 channels. These data resolve the paradoxical excitatory effect of leptin on SNr GABA neurons, despite low $LepRb^{EGFP}$.

Leptin, dopamine, and locomotor activity

Leptin-deficient *ob/ob* mice are obese and have low motor activity; however, hypoactivity is not simply a consequence of elevated body weight, since systemic or intracerebroventricular (icv) leptin increases motor activity before a significant decrease in weight is seen (Pelleymounter et al., 1995, Ribeiro et al., 2011). Additionally, acute icv leptin increases motor activity in *ad libitum*-fed rats, with decreased feeding and weight loss after several days of administration (Choi et al., 2008). We extend these findings by showing that systemic leptin increases locomotor activity in *ad libitum*-fed wild-type mice. Notably, regulation of motor behavior by leptin is bidirectional: abrupt suppression of leptin availability in a tet-off *ob/ob* mice decreases spontaneous activity (Ribeiro et al., 2011), and genetic expression of a LepRb blocker decreases voluntary wheel-running (Matheny et al., 2009). Together, these findings implicate motor activity as a factor in leptin-dependent energy balance, independent of suppression of food intake (Choi et al., 2008).

Implicating dopamine in motor effects of leptin, amphetamine-enhanced locomotor activity is amplified by leptin in wild-type mice, and a blunted amphetamine response in leptin-deficient *ob/ob* mice is restored after systemic leptin replacement (Fulton et al., 2006). The present findings together with leptin-enhanced striatal dopamine release (Mancini et al., 2022) identify neuronal substrates for leptin-enhanced locomotion, as well as decreased motor activity during

caloric restriction that naturally decreases leptin levels (Ceccarini et al., 2015). Of course, sites and mechanisms of leptin's action in vivo extend beyond increasing excitability in the nigrostriatal dopamine system. For example, leptin administration leads to enhanced amphetamine-induced dopamine release in rat nucleus accumbens that reflects short-term and longer-term effects of leptin on dopamine synthesis, including upregulating TH activity, as well as increasing dopamine transporter (DAT) (Perry et al., 2010). We also see an increase in DAT activity with acute leptin exposure in ex vivo mouse striatal slices (Mancini et al., 2022). These findings point to additional targets through which leptin can influence motor activity, enriching its regulatory repertoire.

The effect of chemogenetic activation of LepR-expressing neurons in the VTA or SN on locomotion has also been examined using region-selective viral expression of an excitatory DREADD in LepR-cre mice (de Vrind et al., 2021). Activation of LepR neurons in the VTA had no effect on motor behavior in either fed or food-restricted mice; similarly, activation of SN LepR neurons was without effect in fed mice, although decreased activity was seen after food-restriction (de Vrind et al., 2021). Given that selective chemogenetic activation of VTA dopamine neurons increases locomotion (Boekhoudt et al., 2016), the lack of a motor effect of LepR neuron activation in VTA is consistent with the expected inhibition of VTA dopamine neurons by LepR-expressing VTA GABA neurons (Omrani et al., 2021). Interestingly, selective chemogenetic stimulation of SNc dopamine neurons produces a minimal increase in locomotion compared to that achieved by stimulation of VTA dopamine cells (Boekhoudt et al., 2016). At first glance, this is surprising, given the known motor activating role of SNc dopamine neurons (*e.g.*, Howe and Dombeck 2016). However, two factors are likely involved. First, chemogenetic studies involve continuous activation of target neurons instead of the patterned activity characteristic of these cells. Second, given evidence for dopamine-dependent activation of SNr GABA neurons (Zhou et al., 2009 and **Fig. 7**), limited locomotor effects with chemogenetic activation of SNc neurons would be a predicted consequence of unpatterned dopamine-neuron activation and concurrent dopamine-dependent excitation of inhibitory SNr motor output.

Leptin and motivated behavior

Leptin decreases VTA dopamine neuron firing rate (Hommel et al., 2006; Murakami et al., 2018) via activation of VTA GABAergic neurons (Omrani et al., 2021). Leptin-induced inhibition of VTA DA neurons underlies its influence on motivated behaviors (Fulton et al., 2000; Kiefer et al., 2005; Figlewicz et al., 2006; Fulton et al., 2006; Hommel et al., 2006; Fernandes et al., 2015). Thus, although food restriction is associated with an overall decrease in locomotor activity, a drop in leptin levels that signals starvation also increases exploratory behavior. Experimentally, this is seen in the food anticipatory response, which increases after food restriction or in *ob/ob* mice lacking leptin (Ribeiro et al., 2011). Moreover, motivation, reward, and motor activity intersect in models of anorexia in which circulating leptin levels are low (Ceccarini et al., 2015) and motor activity is driven by running-wheel exercise (Exner et al., 2000; Verhagen et al., 2011).

Under these conditions, motor hyperactivity is *suppressed* by systemic leptin (Exner et al., 2000) or intra-VTA leptin (Verhagen et al., 2011).

Thus, leptin contributes to motivated behaviors in complementary, state-dependent ways. Interactions between dopamine transmission and leptin make intuitive sense in that dopamine is a permissive signal for exploration, approach, and goal-directed behaviors (Palmiter, 2008; Mohebi et al., 2019), whereas leptin signals whether energy stores are sufficient or insufficient, resulting in the regulation of motor behaviors aimed at energy expenditure or food acquisition (Frederich et al., 1995; Friedman and Halaas, 1998). These dopamine-dependent actions complement the roles of other neuronal populations regulating long-term energy homeostasis and locomotor activity. For example, selective restoration of LepRs in hypothalamic arcuate neurons in obese *db/db* mice with global LepR deletion is sufficient to normalize blood glucose levels and improve physical activity, albeit over a period of several weeks (Coppari et al., 2005; Huo et al., 2009).

Conclusions and limitations

We have identified and characterized leptin as a modulator of basal ganglia neurons involved in movement. A limitation of our data is that most experiments were conducted using male mice only. However, given that our immunohistochemical evaluation of LepRb in SNc and SNr neurons showed LepRb in TH-ir neurons of both females and males, the basic findings are likely to be sex-independent. Overall, our results show that leptin influences the activity of two different nigral neuron populations, implicating this metabolic hormone in the regulation of motor output sculpted by the basal ganglia.

Figures and Figure Legends

Fig. 1. LepRbs in SNc dopamine neurons increase neuronal excitability. A, Confocal images of a coronal section of SNc with dual immunohistochemical staining for TH and for EGFP, which labels cells expressing LepRb (scale bar = 20 µm). Merged image shows co-localization of TH-ir and EGFP-ir in a large subpopulation of dopamine neurons. Inset in the merged image panel shows EGFP-ir in TH-ir expressing SNc dopamine neuron dendrites projecting into the SNr; imaging intensity was increased compared to the larger SNc image to show co-localization in these small processes. Images are representative of results from 90 sections from 3 mice (2 females, 1 male). **B,** Representative current-clamp records showing spontaneous action potentials (APs) in SNc dopamine neurons before (control) and after application of leptin (30 nM). Leptin increases AP firing rate (control, 1.03 ± 0.16 Hz; leptin, 2.61 ± 0.44 Hz; t = 4.400, df = 7, p = 0.0032, leptin *vs.* control, n = 8 neurons from 6 mice; paired *t*-test)**. C,** Current-clamp recording from SNc dopamine neurons in the presence of ionotropic glutamate and GABA receptor antagonists and after leptin (30 nM) exposure. Leptin continued to cause a significant increase in AP firing rate when glutamate and GABA inputs were blocked (control 0.97 ± 0.22 Hz; leptin 2.80 ± 0.47 Hz; t = 4.526, df = 6, p = 0.0040, n = 7 neurons from 5 mice; paired *t*-test). **D,** Current-clamp records of spontaneous AP activity in SNc dopamine neurons after intracellular infusion of LepR-antibody. The presence of the antibody did not alter firing rate but prevented the effect of leptin (30 nM) (control, 0.80 ± 0.26 Hz; leptin, 1.10 ± 0.37 Hz; t = 2.393, df = 6, 0.0538, n = 7 neurons from 6 mice; paired *t*-test). **E,** Current-clamp recording, before and after leptin application, with control IgG included in the intracellular solution. The effect of leptin (30 nM) was unaffected by IgG (control, 1.04 ± 0.25 Hz; leptin, 3.15 ± 0.57 Hz; t = 5.135, df = 8, p = 0.0009, n = 9 neurons from 6 mice; paired *t*-test). **F,** Current-clamp recording before and after leptin application with the LepR antibody and its blocking peptide in the intracellular solution. Average AP firing frequency increased significantly with leptin (30 nM) demonstrating that the inhibitory effect of LepR antibody was neutralized by the blocking peptide (control, 0.66 ± 0.11; leptin, 2.08 ± 0.50 Hz; t = 2.843, df = 5, p = 0.0361, n = 6 neurons from 4 mice, paired *t*-test). Data are means ± SEM of recorded cells (*p < 0.05, **p < 0.01, ***p < 0.001, n.s. = not significant, leptin *vs.* control).

Fig. 2. Membrane properties of SNc dopamine neurons are unchanged by leptin exposure. A, Representative SNc dopamine neuron response to hyperpolarizing and depolarizing current steps (100 pA current steps from -400 to 100 pA) recorded before and after leptin (30 nM) application. **B,** Current-voltage plot of SNc dopamine neurons before and after leptin exposure (n = 4 dopamine neurons from 4 mice; $F_{1, 42}$ = 0.0002, p = 0.9879). **C,** Overlay of action potentials (APs) obtained under control conditions and after leptin (30 nM) application in the same SNc DA neuron. **D-H,** AP properties of SNc DA neurons before (Con) and after leptin (Lep) (n = 8 neurons from 6 mice; paired *t*-test): AP threshold (t = 2.039, df = 7, p = 0.0808, Con vs. Lep) (D); AP width at 50% repolarization (t = 0.6974, df = 7, p = 0.5080) (E); AP amplitude (t = 2.131, df = 7, p = 0.0706) (F); after hyperpolarization (AHP) amplitude (t = 1.635,

df = 7, p = 0.1461) (G); and resting potential (t = 1.664, df = 7, p = 0.1400) (H). Data are means ± SEM of recorded cells (n.s. = not significant).

Fig. 3. Leptin enhances somatodendritic dopamine release in the SNc. A, Representative evoked D2ICs recorded in the presence of vehicle and after leptin (30 nM) application, with mean changes in amplitude of evoked D2ICs showing an increase with leptin, but not time matched vehicle controls (vehicle, initial 32.8 ± 5.2 pA, final 32.7 ± 5.2 pA; t = 0.03382, df = 4, p = 0.9746, paired t-test, n = 5 neurons from 3 mice; leptin, initial 25 ± 3.2 pA, final 33.3 ± 5.0 pA; t = 3.401, df = 7, p = 0.0114, paired t-test, n = 8 neurons from 5 mice). **B,** Time course of the change in D2IC amplitude in vehicle alone or leptin in the neurons in (A). An increase in D2IC amplitude over time was seen in the presence of leptin *vs*. control ($F_{10, 110}$ = 2.181, p = 0.0241, two-way ANOVA). **C,** Lack of leptin effect on quinpirole-induced D2ICs. Representative D2ICs elicited by superfusion of quinpirole (Quin; 15 s, 250 nM) in the absence (control) or presence of leptin (30 nM). Quinpirole-induced currents were unaffected by the presence of leptin (initial control, 38.1 ± 4.3 pA; final in leptin, 37.0 ± 6.1 pA; t = 0.3022, df = 5, p = 0.7747, paired t-test, n = 6 neurons from 6 mice). Black bar indicates duration of quinpirole superfusion. Data are means ± SEM of recorded cells (*p < 0.05, n.s. = not significant).

Fig. 4. Leptin enhancement of somatodendritic dopamine release requires Ca^{2+} from intracellular stores. A, Representative evoked D2ICs recorded before and after application of 2-APB (30 μM) and before and after leptin application (30 nM) in the presence of 2-APB. Quantification of D2IC amplitude shows that the effect of leptin is lost in the presence of 2-APB (2-APB plus leptin initial 18.2 ± 4.1 pA, final 19.2 ± 5.2 pA; t = 0.4864, df = 4, p = 0.65, paired t-test, n = 5 neurons from 3 mice). **B,** Time course of the change in D2IC amplitude in 2-APB alone (30 μM) or together with leptin (30 nM) in the neurons in (A). Leptin application did not change D2IC amplitude in the presence of 2-APB ($F_{10, 88}$ = 0.1740, p = 0.9977, two-way ANOVA). **C,** Current clamp recording showing the spontaneous activity of dopamine neurons in the presence of 2-APB (30 μM) and after leptin application (30 nM). Mean action potential (AP) firing frequencies show that the effect of leptin is lost when 2-APB was pre-incubated (2-APB, 0.71 ± 0.17 Hz; 2-APB + leptin, 0.70 ± 0.21 Hz; t = 0.1832, df = 5, p = 0.8618, paired t-test, n = 6 neurons from 3 mice). **D,** Representative current-clamp recordings illustrating AP firing frequency of SNc dopamine neurons in the presence of FFA (20 μM) and after leptin exposure (30 nM). The data show that FFA did not interfere with the leptin-induced increase of dopamine neuron activity (FFA, 1.37 ± 0.55 Hz; FFA + leptin, 3.22 ± 0.55 Hz; t = 10.73, df = 6, p < 0.0001, paired t-test, n = 7 neurons from 4 mice). Data are means ± SEM; n.s. = not significant, ***p < 0.001.

Fig. 5. Leptin indirectly increases the excitability of SNr GABA neurons. A) Immunohistochemical localization of EGFP-ir to indicate LepRs in SNr GABA neurons. Left panel shows neurons stained for parvalbumin (PV). Neurons in the middle panel exhibit weak EGFP-ir, showing low levels of LepRb on GABA neurons. Red arrows indicate GABA cells with EGFP-ir; white arrows point to dendrites of SNc dopamine neurons that show strong EGFP-ir.

The merged image (right) shows the co-localization of PV-ir and EGFP-ir (red arrows). Images are representative of results from 3 mice (2 females, 1 male). Scale bar = 20 µm. **B,** Spontaneous activity of SNr GABA neurons before (control) and after application of leptin (30 nM). Bar graphs summarize the action potential (AP) firing frequency increase after leptin exposure (control, 4.01 ± 0.66 Hz; leptin, 15.17 ± 2.20 Hz; $t = 5.090$, df = 6, $p = 0.0022$, paired *t*-test, n = 7 neurons from 6 mice). **C,** Representative current-clamp records from SNr GABA neurons obtained with LepR-Ab in the pipette, before and after leptin (30 nM) application. The presence of LepR-Ab did not alter the usual effect of leptin on these cells (anti-LepR, 9.16 ± 2.34 Hz; anti-LepR + leptin 19.11 ± 2.73 Hz; $t = 6.268$, df = 7, $p = 0.0004$, paired *t*-test, n = 8 neurons from 4 mice). **D,** Control current-clamp recording of a SNr GABA neuron with IgG in the intracellular solution. The application of leptin (30 nM) caused an increase in the AP firing frequency (IgG, 7.14 ± 1.38 Hz; IgG + leptin 20.88 ± 2.62 Hz; $t = 8.085$, df = 6, $p = 0.0002$, paired *t*-test, n = 7 neurons from 3 mice). **E,** Representative current-clamp records showing the firing frequency of a SNr GABA neuron before and after leptin (30 nM) application in the presence of DNQX (10 µM), AP5 (50 µM), CGP 55845 (0.3 µM), picrotoxin (100 µM). The presence of these receptor antagonists did not alter the increase in AP firing rate induced by leptin (antagonists, 4.60 ± 1.40 Hz; antagonists + leptin 13.91 ± 2.20 Hz; $t = 4.529$, df = 5, $p = 0.0062$, paired *t*-test, n = 6 neurons from 4 mice). All values are means ± SEM (***$p < 0.001$, **$p < 0.01$, leptin *vs.* control, Student's paired *t*-test).

Fig 6. Electrical membrane properties of GABA neurons in SNr before and after leptin exposure. A, Current-clamp record from SNr GABA neurons before and after leptin (30 nM) application following hyperpolarizing and depolarizing current steps (100 pA steps, -400 to +100 pA). **B,** Current-voltage plot of SNr GABA neurons before and after leptin exposure (n = 5 GABA neurons from 3 mice). **C,** Superimposed action potentials (APs) obtained under control conditions and after leptin (30 nM) application. **D-H,** AP properties of SNr GABA neurons before (Con) and after leptin (Lep) (n = 7 neurons from 6 mice; paired *t*-test, Con vs. Lep): AP threshold ($t = 2.055$, df = 6, $p = 0.0857$) (D); AP width at 50% repolarization ($t = 0.3664$, df = 6, $p = 0.7266$) (E); AP amplitude ($t = 2.882$, df = 6, $p = 0.0280$) (F); after hyperpolarization (AHP) amplitude ($t = 2.380$, df = 6, $p = 0.0548$) (G); and resting potential ($t = 0.6687$, df = 6, $p = 0.5286$) (H). Data are mean ± SEM (n = 7 SNr neurons from 6 mice; *$p < 0.05$, n.s. = not significant, Student's paired *t*-test).

Fig. 7. Dendritically released dopamine promotes the excitability of SNr GABA neurons via D1 receptors and TRPC3 channels. A, Diagram of the ultra-short SNc-SNr dopamine pathway regulating the firing frequency of the SNr GABA neurons (modified from Zhou et al., 2009). **B,** D1 receptor immunoreactive (D1R-ir) clusters on PV-ir neuronal perikarya in SNr. Coronal section showing TH-ir neurons in SNc (purple), PV-ir neurons (red) and D1R-ir (green). Arrows point to accumulation of D1R-ir puncta in the perikarya of PV-ir (presumed GABA) neurons within SNr (right panel scale bar = 50 µm; left panel scale bar = 20 µm). **C,** Representative current-clamp record from a SNr GABA neuron before and during application of leptin (30 nM) in the presence of a D1R antagonist SKF83566 (5 µM). Antagonism of D1Rs

prevented the effect of leptin on SNr neuron action potential (AP) firing rate (SKF 83566, 5.14 ± 1.79 Hz; SKF + leptin, 7.85 ± 2.76 Hz; $t = 1.938$, df = 6, $p = 0.1007$, paired *t*-test, n = 7 cells from 3 mice). **D,** GABA neuron AP activity before and after leptin exposure after pre-incubation in a dopamine synthesis inhibitor, α-methyl-ρ-tyrosine (α-MPT; 30 μM). The effect of leptin was absent after interference with dopamine availability (α-MPT, 4.71 ± 0.94 Hz; leptin, 5.90 ± 0.80 Hz; $t = 1.144$, df = 7, $p = 0.2904$, paired *t*-test, n = 8 neurons from 6 mice). **E,** Current-clamp records from GABA neurons before and after leptin (30 nM) recorded with TRPC3-Ab in the intracellular solution; the effect of leptin was lost with the TRPC-Ab in the pipette (TRPC3 Ab, 8.64 ± 2.14 Hz; leptin, 11.50 ± 2.11 Hz; $t = 1.788$, df = 6, $p = 0.1240$, paired *t*-test, n = 7 neurons from 4 mice). Data graphs are means ± SEM (n.s. = not significant).

Fig. 8. Leptin increases locomotor activity in the open-field test. A, Representative tracking of distance moved in the open-field test for a given mouse injected with vehicle or 1.5 mg/kg leptin. **B,** Time course of distance moved during open-field testing after i.p. injection of vehicle or leptin. Data are presented in 5-min time bins following injection at 0 min; arrow indicates time of injection. Two-way ANOVA (time x treatment) indicated a significant difference between vehicle and leptin ($F_{1, 384} = 6.409$; $^{\#}p = 0.0117$; n = 17 mice); Fisher's LSD *post hoc* test revealed a significant difference for the 35 min time bin ($p = 0.020$; n = 17) and near-significance for the 30 min bin ($p = 0.0755$; n = 17). **C,** Total distance moved over the combined 30-35 min time bins (10 min total) was significantly greater when the mice were given leptin *vs*. vehicle (leptin, 811.4 ± 47.7 cm; vehicle, 537.8 ± 31.6 cm; $t = 2.371$, df = 32, $p = 0.0239$; paired t-test, n = 17). Data are mean ± SEM; $^{*}p < 0.05$ leptin *vs*. vehicle.

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Figure 1

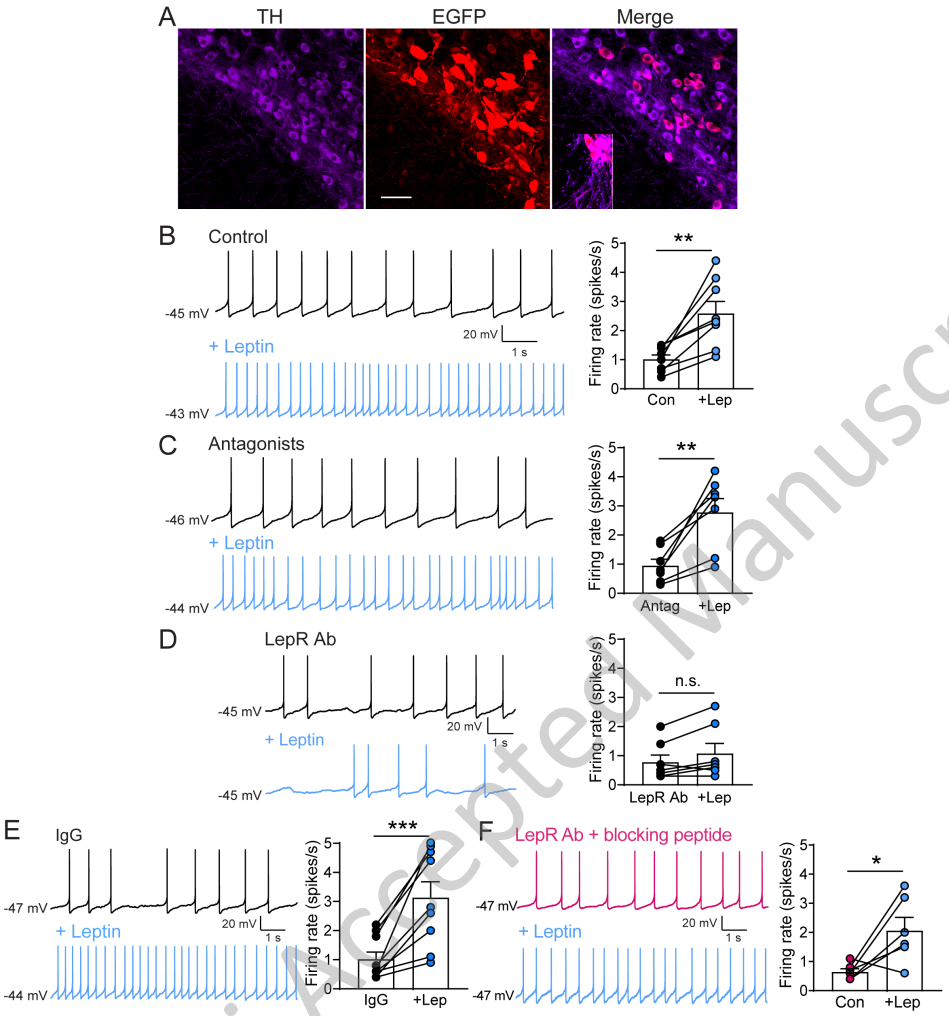
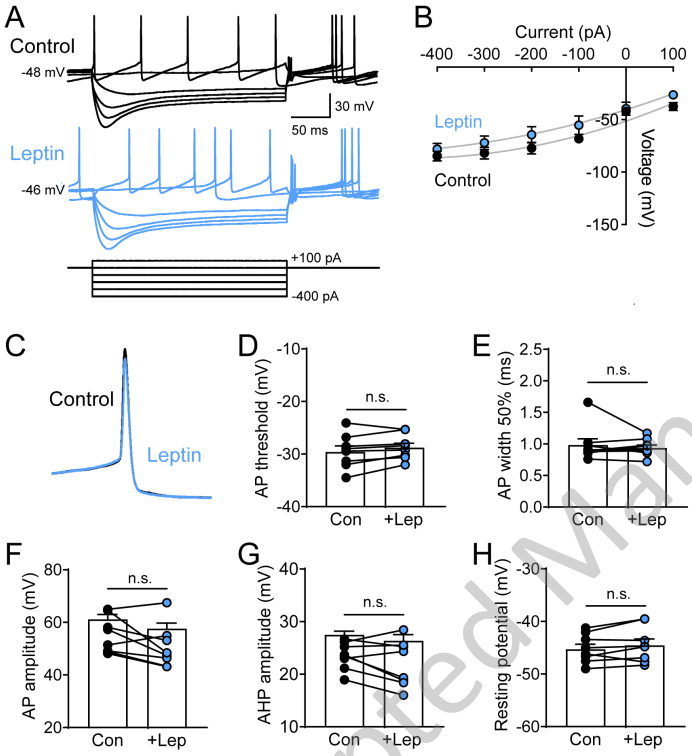


Figure 2



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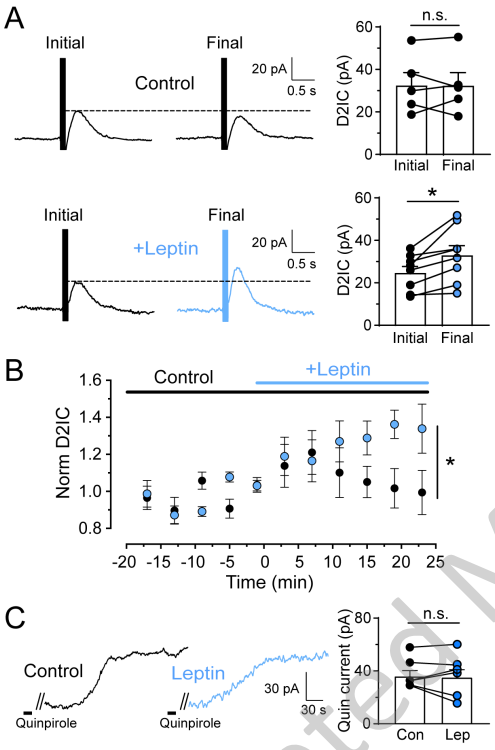

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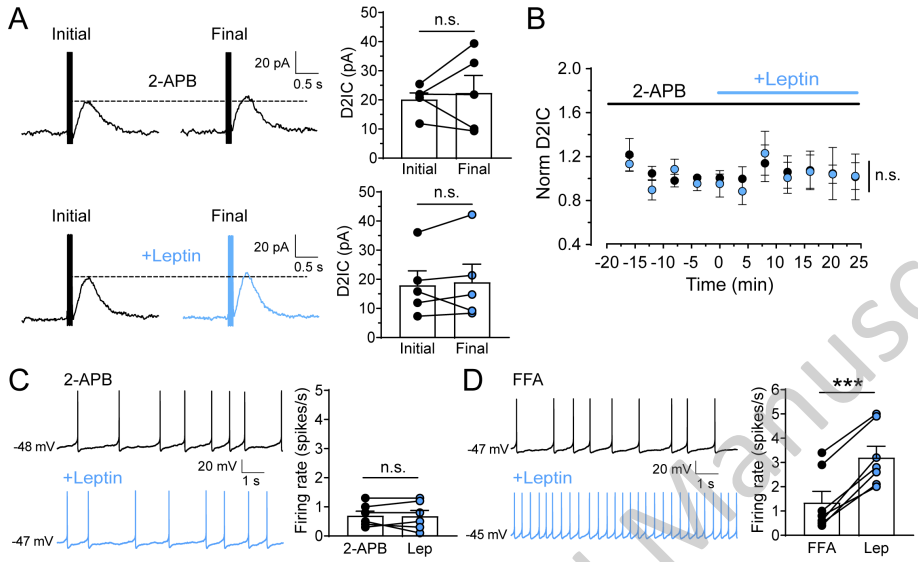
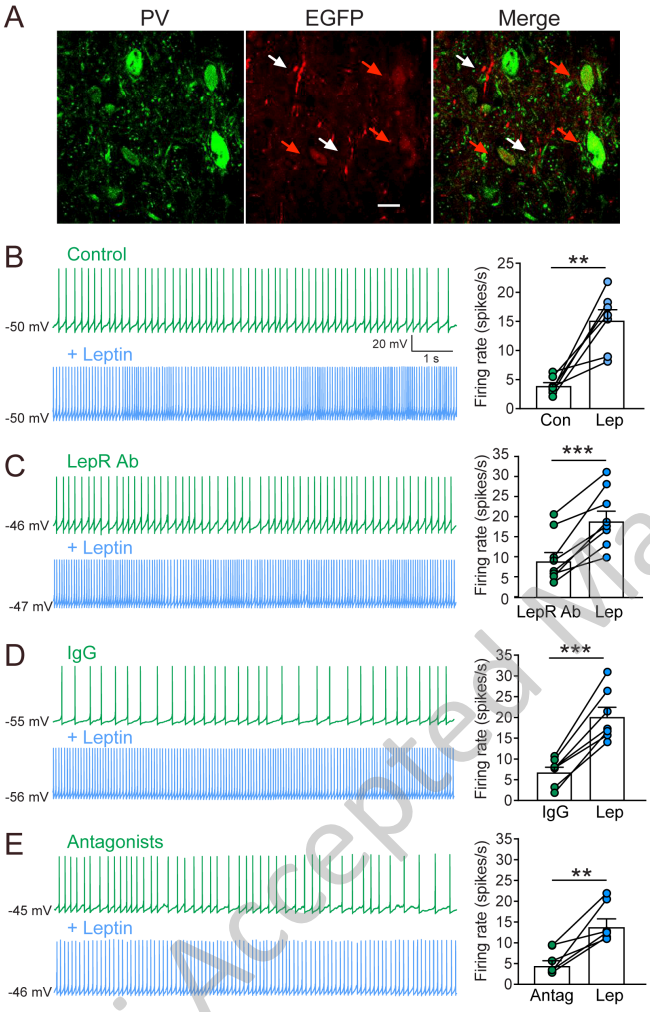


Figure 5



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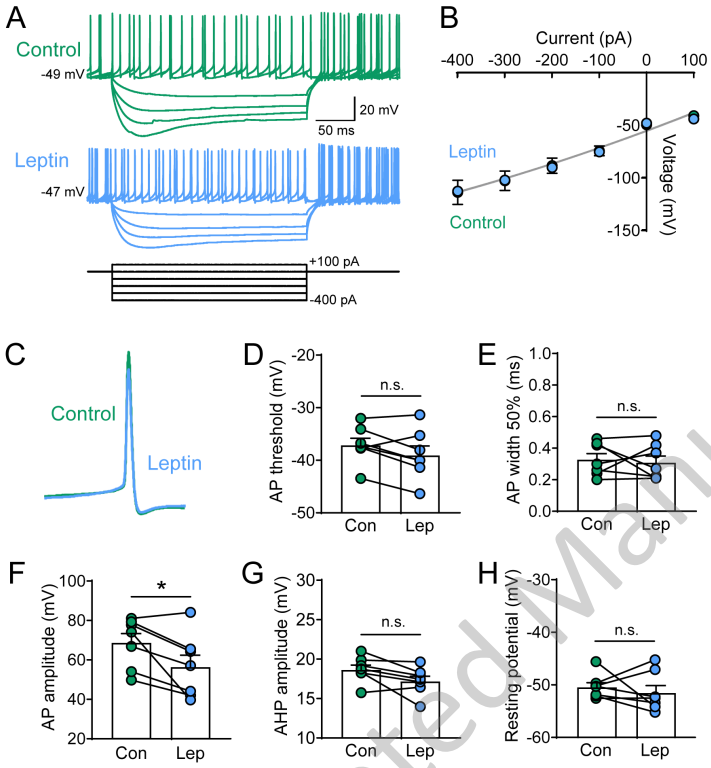

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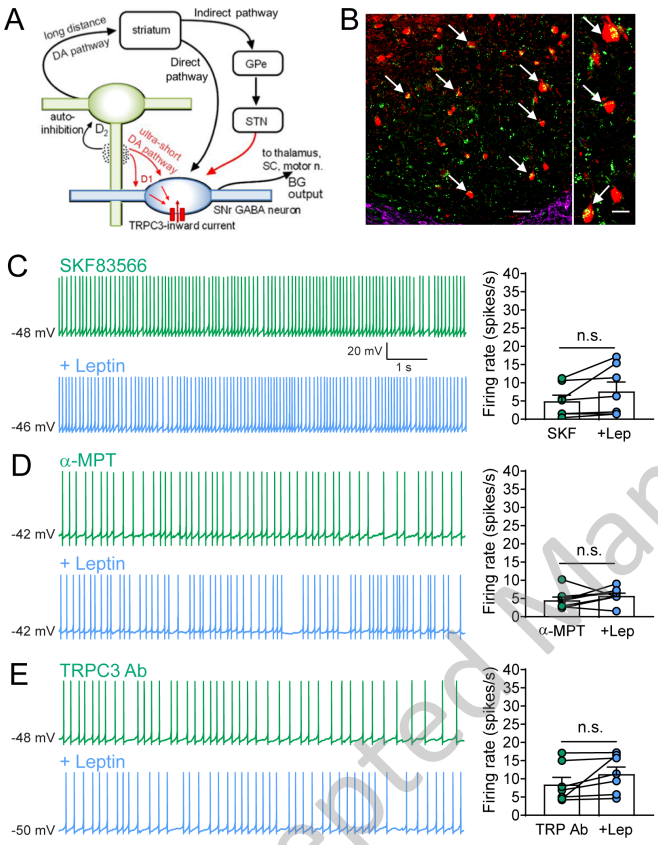

Figure 8