D1 and D2 antagonists reverse the effects of appetite suppressants on weight loss, food intake, locomotion and rebalance spiking inhibition in the rat NAc shell

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Abstract

Obesity is a worldwide health problem that has reached epidemic proportions. To ameliorate this problem one approach is the use of appetite suppressants. These compounds are frequently amphetamine congeners like diethylpropion (DEP), phentermine (PHEN) and bupropion (BUP) whose effects are mediated through serotonin, norepinephrine and dopaminergic pathways. The nucleus accumbens shell (NAc-shell) receives dopaminergic inputs and is involved in feeding and motor activity. However, little is known about how appetite suppressants modulate its activity. Therefore, we characterized behavioral and neuronal NAc-shell responses to short-term treatments of DEP, PHEN and BUP. These compounds caused a transient decrease in weight and food-intake while increasing locomotion, stereotypy and insomnia and evoked a large inhibitory imbalance in NAc-shell spiking activity that correlated with the onset of locomotion and stereotypy. Analysis of the local field potentials (LFPs) showed that all three drugs modulated beta, theta and delta oscillations. These oscillations do not reflect an aversive-malaise brain state as ascertained from taste aversion experiments, but tracked both the initial decrease in weight and food intake and the subsequent tolerance to these drugs. Importantly, the appetite suppressant-induced weight loss and locomotion were markedly reduced by intragastric (and intraNAc-shell) infusions of dopamine antagonists SCH23390 (D1-receptor) or raclopride (D2-receptor). Furthermore, both antagonists attenuated appetite suppressant-induced LFP’s oscillations and partially restored the imbalance in NAc-shell activity. These data revealed that appetite suppressant-induced behavioral and neuronal activity recorded in the NAc-shell depend on to various extents on dopaminergic activation thus point to an important role for D1/D2-like receptors (in the NAc-shell) in the mechanism of action for these anorexic compounds.

Key words: nucleus accumbens, appetite, amphetamine, anorexigenic, dopamine
**Introduction**

Obesity is a worldwide epidemic that predisposes individuals to a myriad of adverse health consequences (McPherson 2014). Although diet and exercise are the primary treatments for obesity, these activities are often supplemented by the use of appetite suppressants (Wilbert et al. 2011). Mild amphetamine analogues such as diethylpropion (DEP) and phentermine (PHEN) are among the most commonly used appetite suppressants (Hampp et al. 2013). Bupropion (BUP), an analogue of DEP, also produces weight loss (Gadde and Xiong 2007). The mechanisms by which these pro-drugs produce their anorexic effect are complex as they modulate the concentrations of serotonin, norepinephrine, and dopamine (Baumann et al. 2000) and evoke responses in various cortical and subcortical areas involved in feeding (Safta et al. 1976).

In humans the above named amphetamine congeners produce the sensation of fullness (serotonin), increase agitation, insomnia and energy expenditure (norepinephrine), as well as affect motivation and reward pathways (dopamine) (Moyers 2005; Offermeier and Potgieter 1972). To shed some light on their action we measured in rats how repeated treatments of these three anorexigenic compounds alter their feeding and motor behaviors and also how these behaviors are related to suppressant-evoked responses from the nucleus accumbens shell (NAc shell), an area that receives dopaminergic input, and that is involved in reward, feeding and locomotion (Carlezon and Thomas 2009; McGinty et al. 2013; Mogenson et al. 1980; Tellez et al. 2012).

Previous studies in rats found that intraperitoneal application of appetite suppressants increase dopamine levels in the NAc shell (Opacka-Juffry et al. 2014), increase locomotion and, for DEP, produce stereotypy (Reimer et al. 1995). The changes in locomotive behaviors have been attributed to the activation of dopamine D1-like and D2-like receptors (Janhunen et al. 2013). With regard to feeding, dopamine (DA) has been shown to play an important role (Costa et al. 2007; Rossi and Yin 2015). For example, alteration of dopamine levels (out of their
physiological range) such as a systemic increases or decreases of dopamine
impede feeding behavior (Szczypta et al. 1999). Furthermore, after ingestion of
food obese persons exhibit a decrease of DA release in their NAc shell compared
with that released by lean subjects (Wang et al. 2014). Moreover, compared to
their lean littermates, obese rats exhibit a decreased number of D2Rs in their
ventral striatum (Johnson and Kenny 2010). Likewise, morbidly obese persons
have fewer D2Rs in their ventral striatum than persons with normal BMIs (Wang et
al. 2009). Although these data point an important role of DA in feeding, it is
unknown to what extent the above mentioned anorectic drugs produce their
behavioral and neuronal effects via activation of DA receptors in the NAc shell.

In this study, we found that treatment of DEP, PHEN or BUP transiently decreased
food intake and increased weight loss, locomotion and stereotypy. Single-unit NAc
shell recordings showed that these appetite suppressants produced a net inhibitory
imbalance in the various neuron types that tracked onset of locomotion and,
stereotypy. Also changes in the local field potential (LFP) evoked by the appetite
suppressants tracked changes in weight loss and food intake (tolerance). A most
relevant finding was that inhibition of D1R or D2Rs both intragastrically and infused
in the NAc shell reversed to various extents both the DEP-induced behavioral and
electrophysiological responses. These data demonstrate that D1-like and D2-like
receptors are critically important factors in understanding how these anorectic
compounds induce weight loss.

Materials and Methods

Animals

A total of 147 male Sprague-Dawley rats ~250-300 g were used for all
experiments. Animals were housed individually and had ad libitum access to food
and water except when they were removed from their home cages for testing in an
operant box for multichannel recordings (see below) or in an open field arena for
locomotion and stereotypy studies. Room temperature was maintained at (21 ± 1
°C) on a 12/12 hour light-dark cycle (0600-1800 h). All procedures were approved by the CINVESTAV institutional animal care and use committee.

**Drugs and chemicals**

The appetite suppressants: diethylpropion hydrochloride (DEP) and phentermine hydrochloride (PHEN) were kindly provided by Productos Medix® (Mexico). Bupropion hydrochloride (BUP), R (+) – SCH-23390 hydrochloride (SCH) and S (-) – Raclopride (+) tartrate salt (RAC), Cholecystokinin-8 (CCK-8), and lithium chloride (LiCl) were obtained from Sigma Aldrich. Unless otherwise mentioned, these compounds were dissolved in physiological saline (0.9% NaCl) and administered intraperitoneally for the dose-response effect of DEP on feeding, locomotion, gastric emptiness and conditioned taste aversion experiments (Figs. 1, 2 and 6A). For all other experiments (Figs. 3-8) they were delivered intragastrically using a catheter (see below), in a volume of 1 mL/Kg. Sodium saccharin was obtained from Sigma Aldrich and was dissolved in water.

**Locomotion and stereotypy**

*Measurements of locomotion and stereotypy in an open field*

Locomotion was measured using an RV2 video processor (RV2, TDT systems, Alachua, USA -digital videotaped and real-time tracking). The RV2 video analyzer was placed in an open field arena (40L X 40W X 30H cm) containing a CCD camera (top view) with specialized software (VGAC Color Video Camera; RV map, TDT systems) to track the animal’s position in “x” and “y” coordinates. For stereotypy measurements we focused on head weavings since they were most readily measurable and quantifiable. We note that other stereotypic behaviors e.g., licking the walls and wood gnawing behavior, were also observed (mainly in the group DEP40, see Supplementary Video 1). We did not quantify other types of stereotypy since for the dose primarily used in most experiments (DEP20), head weavings were the most prominent stereotypy. For head weavings, three-color points (Red, Green and Blue) were glued to the animal and selected as targets for the nose, head and body, respectively and the same apparatus described above.
was used. In both cases, homemade MATLAB scripts calculated the locomotion (in cm) and stereotypy (Figs. 1C, D, 7B, 8C, 9C-D). Stereotypy was expressed as the occurrence of an angle more than 45° between the nose and head color mark targets (see supplementary video 1).

*Measurement of locomotion during multichannel recordings from the NAc shell*

During multichannel recordings (see below), the position of the rat's center of mass was tracked by using Ethovision XT10 (Noldus Information Technology, Netherlands) and reported as cm/min. Since this method only tracks one body point (the center of mass) stereotypy could not be measured during these recordings. In addition, only webcam videos where locomotion could be clearly decoded were included in the analysis (e.g., Figs. 4D, 5C, 6D, 7D and 10B). Onset of locomotion was computed by using a cumsum statistic (Gutierrez et al. 2006) to obtain the first bin after drug infusion where locomotion significantly increased above locomotion in the saline epoch.

**Behavioral procedures**

*Dose-response effect of DEP upon weight-loss and food intake (Fig. 1A-B)*

To determine whether DEP affects body weight and food intake in a dose-response manner, 23 lean male rats were assigned into four groups (Saline 0.9% (n=5), DEP10 (n=6), DEP20 (n=6) and DEP40 (n=6) mg/Kg) where they were provided with 100 g per day of standard rat chow (Purina Mexico) and *ad libitum* water. After seven days (to obtain a stable baseline- data not shown), rats received a daily intraperitoneal injection of one of the four treatments for fourteen consecutive days (days 1-14) that were followed by seven days (days 15-21) of recovery without treatment (withdrawal period). These behavioral experiments were carried out between 17:00 -18:00 hours (i.e., commencement of their active dark phase). Their body weight and 24 h food intake was measured daily 20 min before receiving an intraperitoneal injection of the corresponding treatment (see Figs. 1A and 1B).
Dose-response effect of DEP upon locomotor-activity and stereotypy in an open field arena (Fig. 1C-D)

In a different group of rats, we evaluated, in a dose-dependent manner, whether intraperitoneal injections of DEP affects locomotion and stereotypy. For this experiment, twelve rats were assigned to three groups (DEP10, DEP20 and DEP40 mg/kg; n = 4 per group). All experiments were conducted during the inactive light period. Locomotion and stereotypy (head weavings indicated by head oscillations) were measured for 90 minutes before (baseline) and after injection by the RV2 video processor described above. These experiments lasted eleven days of which, three days (S1-S3; Fig. 1C) were for habituation with only injection of saline. The next seven days (D1-D7) rats received daily injections of DEP followed by a day (S8) of saline injection. The ambulation and the stereotypic head movements were measured across the days as a delta (Δ) by subtracting the values of treatment by the values observed at baseline (see inset in Fig. 1C).

Effect of DEP20 on gastric emptiness (Fig. 2)

We assigned twenty-seven naïve rats into three equally sized groups. Briefly, animals were water and food deprived for 24 h, whence they received a single intraperitoneal injection of either saline, DEP20 or of 2 µg/kg CCK-8. Then 15 min after one of the injections animals received a 5 mL oral bolus of Ensure ®-chocolate flavor using an oral gavage needle, following the protocol described in (Conover et al. 1988). Finally, after 10 min animals were sacrificed and their stomachs were isolated with both sphincters occluded with silk threads. The wet stomach was weighed and inverted, then rinsed with water and placed in a heat oven at 45 °C for 24 h. The dry stomach was weighed and the difference on weight between wet and dry measurements was used as an indicator of gastric emptiness (Fig. 2).
Behavioral effect of DEP20 as unconditioned aversive stimuli (Fig. 6A)

Twelve naïve rats were randomly divided into two groups: LiCl (n=5) and DEP20 (n=7), following the protocol described in (Gutierrez et al. 2003). Briefly, animals were water deprived for 23.75 h and for three days, they had a daily period of 15-min access to water (Fig. 6A, W1-W3). On day 4 (ACQ, acquisition day), animals were allowed to drink a novel saccharin solution (0.1% w/v) for 15 min that served as conditioned stimuli, and 15 min later rats received a single intraperitoneal injection of either 0.4 M LiCl – 7.5 mL/kg- or DEP20 - 1 mL/Kg - that served as unconditioned aversive stimuli. On days 5 and 6, animals were given only water to allow recovery from gastric malaise. On the TEST day (day 7) rats were presented with 15 min (17:30-17:45 h) of 0.1% w/v saccharin followed by other 15 min of water (17:47–18:02 h). Following the TEST session, rats received three more extinction days (EXT1-3). Intake (mL) was measured at 0.5 mL resolution and displayed in Figure 6A.

Locomotion and stereotypy induced by DEP20, PHEN20 and BUP30 (Fig. 7B)

In the open field arena described above for a new cohort of rats (n=3 per group), locomotion and stereotypy (head weavings) were measured over seven days of repeated intragastric infusions of Saline, DEP20, PHEN20 and BUP30 (Fig. 7B). For the implantation of the intragastric catheter, we followed the protocol described by (Lukas and Moreton 1979; Ueno et al. 2012). Briefly, the hair on the mid-abdomen and dorsal neck areas was clipped and cleaned. To expose the stomach, a midline incision of 2-3 cm was made in the abdomen. With a 30-gauge needle we punctured the fundus and inserted 1 cm of the catheter into it (14 cm length and 0.76 mm diameter of silastic laboratory tubing; Dow Corning, USA, with one sterile rubber band braces glued at 1 cm from the tip). Then, we joined the catheter’s rubber band and the rats’ stomach using a non-absorbable silk suture (USP 6-0, ATRAMAT Mexico). We passed the opposite end of the catheter subcutaneously
until it exited the dorsal neck incision. Finally, the peritoneal cavity was carefully
stitched together using chromic catgut, whereas the abdominal and dorsal incision
was stitched together using a silk suture. After the animals were permitted to
recover, at least one-week from surgery before starting the treatment, they were
habituated to the open field for 3 days. In each experiment, a tube (30 cm length)
was connected to the catheter and to a syringe that was manually activated outside
of the behavioral box. The catheter patency was always confirmed at the end of all
experiments before perfusion of the animal.

*The effect of intragastric D1 (SCH23390) and D2 (raclopride) antagonists on
DEP20’s effect on weight loss, food intake, locomotion and stereotypy (Fig. 8)*

To understand the participation of D1Rs and D2Rs on DEP’s behavioral effects, we
tested and compared four groups of each comprised of three animals: saline,
DEP20, DEP20+RAC0.5 and DEP20+SCH1.5 where the numbers are in mg/Kg.
Briefly, rats were habituated to the open field arena for two days (data not shown).
Then each group received a daily intragastric infusion for seven days where their
locomotion and stereotypy were measured. We initially used this method because
intragastric infusions of appetite suppressants mimic how humans take these
compounds. As seen below, we also infused these antagonists directly into the
NAc shell. To determine whether DEP-induced neuronal modulation could be
reversed by i.g. infusion dopamine receptor antagonists, we first infused DEP20
followed 30 min later by one of the two antagonists (Fig. 8). The antagonist
concentrations were selected based on previous studies showing that RAC at a
systemic dose of 0.5 mg/Kg antagonized the locomotion effects induced by
amphetamine or methamphetamine (Broening et al. 2005; Janhunen et al. 2013;
Wright et al. 2013). Likewise, we initially tested using a high dose of SCH (3
mg/Kg), since this dose is used to prevent death from an overdose of
amphetamine (Derlet et al. 1990). However, over a seven days test period animals
generally did not tolerate this dose, and thus we could record NAc shell activity in
only one animal as seen in Figure 10. Consequently, we used SCH at 1.5 mg/Kg for the seven-day treatment period.

As a control we also tested if these DA antagonists by themselves produce any behavioral effects. This was accomplished using two additional control groups with each one receiving either only RAC0.5 or SCH1.5. Specifically, rats were introduced on the open field arena during 30 min as baseline (BL), followed by an intragastric injection of saline at 30 min and at 60 min (instead of DEP) they received an injection of either RAC0.5 or SCH1.5 (see Fig. 8C). Finally, at 90 min, they received a second infusion of saline.

**IntraNAc shell infusions of DA antagonists:**

*Surgery and intraNAc injection*

IntraNAc shell infusions of DA antagonists were performed to directly determine their effects on feeding and locomotion. Animals were implanted with an intragastric catheter, as described above and placed in a rat stereotaxic apparatus. For the bilateral intraNAc shell infusions two holes were drilled at: AP +1.4 mm; L ± 1 mm relative from Bregma. The tips of stainless steel guide cannula aimed at the NAc shell were bilaterally inserted at 6.5 mm DV from Bregma. Two screws served as anchors in the skull bone and the whole assembly was cemented to the skull with dental acrylic. A stylus was inserted into the cannula (to prevent clogging) and was removed before each injection. Rats were allowed to recover from surgery for at least seven days. Microinjections were given via a 30-gauge stainless steel injector 1.0 mm larger than the tip of the guide cannula, connected via a Teflon tubing to a 10-µL glass micro syringe (Hamilton 80366) that was attached to a microinfusion pump (KD scientific- KDS200 series). A total volume of 0.5 µL (0.33 µL/min) per hemisphere was infused daily across 7 days of treatment. The injector was left into the guide cannula one additional minute to allow complete effusion (Gutierrez et al. 2003).
Effect of D1 (SCH) and D2 (RAC) antagonists infused in the NAc shell upon DEP20’s effect on weight loss, food intake, locomotion and stereotypy (Fig. 9)

To investigate whether D1 and/or D2 receptors in the NAc shell mediates some (or all) of the pharmacological effects induced by intragastric DEP20, we used 18 animals randomly sorted into 6 groups of n = 3 (Fig. 9). After 2 days of habituation (data not shown), each animal was placed in the open field for 90 min and their locomotor activity was analyzed as described above using an RV2 video processor. After the initial 20 min baseline period, animals were briefly removed from the open field and were injected with 2.5 µg/0.5 µL of either SCH or RAC antagonists directly in the NAc shell (Baldo et al. 2002; van den Boss et al. 1988). After 30 minutes (Fig. 9C), all rats received the corresponding systemic infusion of either saline or DEP20 via the intragastric catheter. These behavioral experiments were carried out between 07:00 -19:00 hours (light phase). Their body weight and chow food intake was measured daily just before placement in the open field (Figure 9A-B).

Electrophysiology

Surgery

Surgical procedures for electrode implantation in the nucleus accumbens followed methods described previously (Tellez et al. 2012). Briefly, animals were anesthetized using an intraperitoneal injection of pentobarbital sodium (50 mg/Kg) and 0.1 ml atropine sulfate. A movable 4 x 4 microwire array comprised of formvar-coated tungsten wires (35 µm diameter) was unilaterally implanted in the NAc shell (centered from Bregma at coordinates: AP = 1.4, L = ±1, and DV = 7.5 mm). One stainless steel screw that was soldered to a silver wire (50 µm) at the surface of cerebellum served as ground.

Multichannel recordings from the NAc shell
All recordings began one-week after the post-surgery recovery period. They were performed in an operant behavioral box that was enclosed in a ventilated and sound-attenuating cubicle equipped with a webcam around 09:00 each day. Unless otherwise mentioned each recording session lasted 3 h and was divided into three 1 h epochs: baseline, infusion of saline and infusion of an appetite suppressant with or without a DA antagonist. Action potentials and LFPs were recorded using a Multichannel Acquisition Processor (Plexon, Dallas, TX). Only single neurons with action potentials with a signal-to-noise ratio larger than 3:1 were analyzed. Action potentials were identified online by means of voltage-time threshold windows and a three principal components contour template algorithm (Gutiérrez et al. 2010). Spikes were sorted using off-line sorter software (Plexon Dallas, TX) and the stability of waveform shape across the 3 h recording session was confirmed by plotting the average shape during baseline, saline and appetite suppressant epochs.

LFPs recorded from the NAc shell, were amplified 1000X, filtered 0.7–300 Hz and digitized at 1 kHz using a digital acquisition card (National Instruments, Austin, TX). For each LFP, the power spectral density (PSD) was estimated with the Welch’s method using a 2 s Hanning window with 50% overlap. Channels with exceptionally large noise levels, based on visual inspection, were excluded from analyses. From the remaining channels when an artifact event occurred (e.g., saturations and abrupt changes in voltage) the segments 100 ms before to 300 ms after the event were eliminated. The PSD of each LFP was averaged across all channels to generate a single PSD plot per experiment. We also report the average PSDs at delta (1-4 Hz), theta (6-9 Hz) and beta (15-30 Hz) bands (Halje et al. 2012). For comparison across experiments, PSDs were normalized to their z-score by subtracting their means and dividing them by the std’s of the baseline period. To allow time for the drugs to achieve a steady pharmacological state, the LFPs 5 min before and 10 min after the start of each epoch were not analyzed.
Weight loss, food intake, and locomotion for i.g. infusions of DEP, PHEN and BUP during multichannel recordings of the NAc shell activity

For these experiments we used 10 animals with electrode arrays implanted in their NAc shell (DEP20 n=4, PHEN20 n=4 and BUP30 n=2). Drug infusions were made via intragastric injections. The animal’s body weight and 24 h food intake were measured daily across 7 days 20 min before starting a recording session. Pilot experiments with BUP indicated that a dose of 15 mg/Kg was insufficient to induce decreases on weight, food intake, or changes in locomotion or NAc shell oscillations (data not shown). Consequently, based on rat studies showing that 30 mg/Kg of BUP reduced food intake (Janhunen et al. 2013), we used this dosage. For PHEN and DEP we used 20 mg/Kg based on previous studies and pilot experiments ((Roth and Rowland 1999), see Figure 1 for DEP20)).

It is important to note that in our pilot study (see Supplementary Video 1), despite having food available rats practically do not eat or drink anything during the initial 2-3 h after drug administration, where these drugs exert their maximal locomotor effects (see Fig. 7B). Given this information, we decided not to have food available during the multichannel recording sessions since rats will simply ignore it and also because the delivery of food pellets would interfere with our ability to accurately decode locomotion from the videos.

Modulation of NAc shell’s LFP oscillations induced by a gastric malaise agent (LiCl) (Fig. 6B-D)

To understand whether DEP20-induced LFP’s oscillations are related to any aversive-malaise brain state, we evaluated whether a prototypical gastric malaise agent, LiCl, was able to alter LFP’s oscillations in the same way as DEP20 did (Figure 4). We used 2 animals with a microarray of electrodes implanted in the NAc shell and with an intragastric catheter (see above). A total of 5 recording sessions were successfully performed (we let at least 2 days of rest between each recording
session to allow recovery from the sickness induced by previous injection of LiCl). To reduce animal distress we did not inject LiCl more than 3 times per animal. Each recording session contained four, 1 h, epochs: Baseline, Saline, LiCl (0.4 M) and DEP20, respectively. LFP’s oscillations and locomotion were analyzed and displayed in Figures 6B-D. From these experiments, we could only record a few single unit neurons (n = 12). Given this small sample size and because none of these neurons were significantly modulated by LiCl (data not shown) we could not make any conclusions from these data.

Intragastric NAc shell D1R and D2R antagonists’ modulation of NAc shell activity (Fig. 10)

To evaluate the effects of DA receptor antagonists upon modulating DEP20’s effects on NAc shell activity, we implanted 10 animals with multichannel electrodes in their NAc shell (DEP20+Rac0.5 n=2 rats; 81 neurons, and DEP20+SCH1.5 n=2 rats; 112 neurons, DEP20+SCH3, n=1; 34 neurons, RAC0.5 n=2; 93 neurons, and SCH n=3; 114 neurons). Experiments were performed in an operant chamber and each epoch was 30 min long. In order to increase the statistical power we pooled together the baseline and saline epochs (that were not significantly different, data not shown) and compared it against the firing rate on the DEP20 and antagonist epochs using a Kruskal-Wallis test. The normalized z-score peristimulus time histogram (PSTH) of significantly modulated neurons was obtained as were the normalized PSDs at beta, theta and delta bands (Figs. 10B and 10E). We tracked the animal’s locomotion, based on the center mass point method described above. Finally, to compare the global effect of DEP20 under chronic treatment of the above mentioned D1R or D2R antagonists, the population firing rate of all recorded neurons (modulated or not by DEP) were plotted aligning its response to time 0 min as the moment of DEP20 infusion, and normalizing their firing rate using the 30 min saline period as a baseline (Figs. 10C and F).

Histology
At the end of the experiments rats were injected with pentobarbital sodium (150 Kg/mg) and perfused with PBS, followed by 4% paraformaldehyde (PFH). Their brains were removed and placed in 10% sucrose/PFH (vol/vol) solution for 24 h followed by sequential increases in sucrose/PFH concentration until 30% at 72 h. To establish the placement of the electrodes brains were sectioned (50 µm) and stained with cresyl-violet.

**Data analyses**

All analyses were performed using matlab toolboxes and homemade custom scripts.

**Putative neuron-type classification**

Neurons were classified into putative cell-types according to four features: firing rate, coefficient of variation 2 (CV2), valley-to-amplitude ratio (VAR), and valley-to-peak width (V-P width) (see Tellez et. al. 2012). The firing rate was calculated as the number of spikes divided by the duration of baseline and/or saline periods (that were not significantly different; see Table 2). CV2 was calculated for each adjacent pair of inter-spike intervals (ISIs), and the average CV2_ISI was used in the assignment of neuron type. The two-ISI coefficient of variation was computed as CV2_ISI = |2(ISI2 - ISI1)/(ISI2 + ISI1)| (Holt et al. 1996). The VAR was calculated as the absolute value of the first valley in the waveform divided by the difference between its minimum value and the following maximum (Fig. 3A, inset, and (Yarom and Cohen 2011)). For computation of the V-P width (the time between the minimum value and the following maximum was calculated), see black waveform in Figure 3A, black waveform shape (pFSI). For each neuron recorded, these four features were computed and classified into 4 clusters by using a fuzzy cluster algorithm, and visualized by using principal component analysis (PCA) as seed followed by the fuzzy Sammon’s mapping plot as described in the Matlab Fuzzy Clustering & Data Analysis Toolbox.

**Hypnograms: the LFP’s brain state map**
For hypnograms (Fig. 3A; right panel) behavioral states were assigned using information obtained from the LFPs as outlined in (Gervasoni et al. 2004; Tellez et al. 2012). In brief, after elimination of segments with amplitude saturation, a sliding window Fourier-transform was applied to each LFP signal to calculate two spectral amplitude ratios (0.5–20/0.5–55 Hz and 0.5–4.5/0.5–9 Hz for ratio 1 and 2, respectively). PCA was then applied to these ratios obtained from all LFP channels, and the PCs were used as the overall ratios measure. These measures obtained for each second of data were further smoothed with a Hanning window (20 sec length). Finally, the two PCs of the spectral ratios were plotted against each other to construct a two-dimensional (2-D) state space (Fig. 3A right panel) where the density of points reflecting the relative abundance of the different brain states. REM sleep was not included in this analysis because the percentage of time the animals spent in REM was < 2% (unpublished observation). The final two-dimensional brain state map was selected and validated after visual inspection of animals behavior in the videotaped with three behavioral states (quiet wake (qW), slow wave sleep (SWS) and active wake (aW) (Tellez et al. 2012).

Active and Inactive neurons: after DEP, PHEN and BUP administration

Neuronal firing modulations such as those shown in Figures 3B and 7C were identified using a Kruskal-Wallis test for α < 0.05. Firing rates were pooled across baseline and saline epochs since differences between them were not significantly different (Table 2). Moreover, after DEP, PHEN and BUP infusions, because rats remained >80% of the time in the active awake state (Table 3), we compared the firing rates of the baseline-saline period, during the quiet wake times (computed from the hypnogram) against the firing rates during active awake (from the time interval 2:10 to 3 h; see Figs. 3B and 7C) after the infusion of the appetite suppressants.
Statistical analyses

Unless otherwise mentioned data is presented as mean ± SEM. Statistical differences between groups were assessed by a one-way ANOVA or repeated-measures ANOVA (RM ANOVA), followed by Fisher’s post-hoc analysis.

Results

Behavior

Effect of DEP upon weight-loss and appetite suppression

Preliminary experiments regarding the effects of DEP, PHEN and BUP on appetite suppression revealed that DEP gave the greatest response (see below and Figs. 1 and 7A) and therefore its effects were initially explored to determine an optimal concentration. Figure 1A shows a graph of the change in body weight across 14 days of daily intraperitoneal injections of: saline, 10, 20 and 40 mg/Kg of DEP (hereafter noted as DEP10, 20 and 40, respectively). This was followed by a seven-day withdrawal of treatment (to day 21). With saline injections (control) it is seen that the animals progressively gained weight until the trial ended on day 21. In contrast, increasing the DEP concentration caused an initial dose-dependent increase in weight loss whose effect decreased across the treatment period. Specifically, for DEP10 after day 7 the animals gained weight and from days 12 onward their weight approached that seen with saline injections. For DEP20, after the initial weight loss there was a recovery such that on day 12 their weight was the same as that at for day 1 (although it remained significantly below that of saline). After the withdrawal of treatment on day 15, the animals rapidly gained weight to that of animals treated with saline (day 21). For DEP40, there was a monotonic decrease in weight until day 7 that was maintained across the entire treatment period. Withdrawal of DEP40 also produced a weight gain, but the animal’s weight remained below that of saline. The overall mean (± SEM) change in body weight across the fourteen treatment days was 14.7 g ± 1.2, 4.9 g ± 3.6, -
4.9 g ± 2.8, -14 g ± 3.3, for saline, DEP10, 20, 40, respectively (main effect of dose: RM ANOVA $F_{(3, 19)} = 20.2, P < 0.0001$; days: $F_{(3, 13)} = 25.6, P < 0.0001$; and dose x days interaction: $F_{(39, 247)} = 13.5, P < 0.0001$). Post-hoc comparisons, relative to saline, indicated that DEP40 induced the greatest weight loss ($P < 0.0001$), followed by DEP20 ($P = 0.0001$), and DEP10 ($P = 0.038$). In summary, DEP induces a dose-dependent decrease in body weight.

In the same group of animals we also measured their food intake over 24 h (Fig. 1B). Over the 14 day treatment period, the percentage change of food intake from the day before treatment started was 4.1 ± 4.1% (mean ± SEM), -14.4 ± 5.2%, -15.1 ± 5.7% and -28.7 ± 4.8%, for saline, DEP10, 20 and 40, respectively (main effect of dose: RM ANOVA; $F_{(3, 19)} = 16.1, P <0.0001$; and days: $F_{(3, 13)} = 14.5, P <0.0001$ and significant interaction between dose and days: $F_{(39, 247)} = 3.59, P < 0.0001$). Although over the 21-day treatment and withdrawal periods the saline-injected animals exhibited a relatively constant food intake, the animals under treatment initially decreased food intake in a dose-dependent manner (reflected at day 2 for measurements made 24 h after) with DEP40 showing the greatest reduction (post-hoc, $P <0.0001$), followed by DEP20 ($P = 0.0007$) and DEP10 ($P = 0.0013$). Interestingly, DEP10 and 20 induced a similar suppression of appetite ($P = 0.78$, n.s.) in that after seven days rats treated with either dose exhibited a gradual recovery of food intake (Fig. 1B). For DEP40, after the large decrease in food intake on day 2, the animals increased their intake but to levels significantly below from that of the other treatments. Finally, seven days after drug withdrawal (Fig. 1B; day 21) the food intake of all DEP-treated groups achieved similar (or slightly higher) levels than for the saline group. These results show that over the treatment period the animals developed tolerance to the anorectic effects of DEP, but they consistently consumed 5-10% less chow food than saline group.

The effect of DEP on locomotion and stereotypy
Previous studies with rats reported that acute administration of DEP alters their locomotion and produces stereotypy (Reimer et al. 1995). Here we asked how a dose-dependent chronic treatment affects locomotion and stereotypic behavior (head weavings). In a new cohort of animals (n=12) we evaluated the effects of seven-day intraperitoneal injections of different doses of DEP on their locomotion (Fig. 1C). Relative to the initial 1.5 h baseline period, where the animals were free to move in an open field, during the three days of saline injections (S1-S3) they did not display marked changes in locomotion (Fig. 1C). Throughout the treatment period the increase in locomotion was DEP10 > DEP20 > DEP40. Moreover, throughout the treatment the responses to DEP10 and 20 remained elevated whereas the DEP40 responses showed a non-significant trend to decrease across days. Seven days of DEP treatment showed a main effect of dose RM ANOVA; D1-D7, $F_{(2, 9)} = 30.0, P = 0.0001$; and non-significant effect of days D1-D7: $F_{(2, 6)} = 1.48, P = 0.20$, with non-significant interaction $F_{(12, 54)} = 0.96, P = 0.49$). A Fisher’s post-hoc analysis showed that DEP10 induced more locomotion than DEP20 ($P = 0.008$), and in turn DEP20 more than DEP40 ($P < 0.002$). Moreover, one day after stopping treatment (S8), DEP’s effect approached baseline values. Thus, the recovery from locomotion is faster than for food intake and weight loss.

With respect to DEP-induced changes in stereotypy (Fig. 1D) three days of saline injections (S1-S3) were not significantly different from baseline. However, at days D1-2 the increase in stereotypy was DEP20 = DEP40 > DEP10 (RM ANOVA; D1-2, significant main effect of doses: $F_{(2, 9)} = 8.60, P = 0.008$, non-significant effect of days $F_{(2, 1)} = 0.15, P = 0.7$ and no interaction dose x days $F_{(2, 9)} = 1.21, P = 0.34$). However, with treatment the order changed because DEP10-induced stereotypy increased linearly to day 4 (For D1-4, no significant differences between doses: $F_{(2, 9)} = 0.92, P = 0.43$, but significant effect of days $F_{(2, 3)} = 4.42, P = 0.012$ and interaction dose x days, $F_{(6, 27)} = 3.71, P = 0.008$), whereas the others did not markedly change so at day 4 to day 7 the responses to all concentrations were not statistically different. These data show that seven treatment days of the three tested DEP concentrations induces stereotypy, but only DEP10 tends to sensitize
across repeated injections. After treatment, a single saline injection (S8, Fig. 1D), returned stereotypy to baseline levels. It follows that with respect to locomotion and stereotypy DEP does not produce long-term effects. In summary, since DEP20 provided significant, but not extreme changes in weight loss, food intake and motor effects relative to DEP10 or DEP40 in all subsequent experiments we chose to use this concentration.

A dose of DEP that suppresses food intake is not sufficient to delayed gastric emptiness

We also explored whether DEP20’s anorectic effect could be mediated by a peripheral vagal satiety pathway such as induced by CCK-8, a peptide which delays gastric emptiness (Chang et al. 2012). To test whether DEP20 can produce satiety by a similar mechanism, we performed a gastric emptiness assay using CCK-8 as a positive control (Fig. 2A). Briefly, after infusion of an oral Ensure gavage gastric emptiness was 5.01 ± 0.24 g, 5.18 ± 0.12 g and 6.18 ± 0.33 g for saline, DEP20 and CCK-8, respectively. Importantly, the differences were significant (one-way ANOVA, $F_{(2, 24)} = 6.67$, $P = 0.005$). A post-hoc analysis showed that a single intraperitoneal injection of CCK-8 delayed gastric emptiness relative to both saline ($P = 0.003$) and DEP20 group ($P = 0.008$), whereas DEP20 and saline were not significantly different ($P = 0.64$). Hence, unlike CCK-8 that delays gastric emptiness, DEP20 induced anorexia does not seem to be mediated via peripheral vagal satiety signals.

Electrophysiology

To relate the behavioral studies to neural recordings we recorded the responses to DEP20 in the NAc shell, a brain region involved in reward, feeding and motor activity (Brown et al. 2011; Kelley et al. 2005; Li et al. 2012). 154 well-isolated single neurons were recorded and were classified into putative medium Spiny Projection Neurons pSPNs ($n = 47$), putative Fast Spiking Interneuron’s pFSIs ($n =$
37), putative Choline Acetyl-Transferase interneurons pChATs (n = 36) and unidentified (n = 34) (see Table 1 for data regarding of firing rates and waveform shapes for each putative cell-type).

**DEP strongly modulates NAc shell neuronal activity**

Unless otherwise stated, all electrophysiological recordings followed the same protocol. That is, after 1 h baseline period where the animals were free to move, they received a daily intragastric infusion of saline (at 1 h) and DEP20 at 2 h. Recordings were usually terminated after 3 hours. Figure 3A shows a graph containing 154 classified neurons (pSPNs, pFSIs, pChATs and Us) that were recorded before and after the infusion of DEP20. Relative to the not significantly different baseline and saline epochs (Table 2), two types of modulatory responses were observed: those whose activity decreased (inhibited by the appetite suppressant; blue dots (68% (104/154)) and those that increased (activated by the appetite suppressant; red dots 9% (14/154; $\chi^2 (1) = 51.9, P < 0.001$). A representative activated response (below left panel) from a pSPN neuron and a representative inhibited response from a pChAT neuron (below right panel) are illustrated as well as their locations on the modulation map (Fig. 3A). As noted, most responses were inhibited and all neuronal types were equally affected ($\chi^2 (3) = 2.2, P = 0.53$, n.s., see Table 4). In contrast, for the activated responses, DEP20 significantly modulated more pSPNs than other cell-types ($\chi^2 (3) = 9.8, P = 0.019$; Table 4). We note that these activated pSPNs could be either D1+ or D2+ expressing cells, and that they might have different sensitivities to appetite suppressants (MacAskill et al. 2014). The remaining 36 neurons were unaffected.

Also seen in these two examples are that in the baseline and saline epochs the animals were either in a quiet awake (blue), active awake (green) or in slow wave sleep SWS (grey) states (Table 3). These three states were obtained from analysis of LFPs (Fig. 3A, right panel and Fig. 4A). In these experiments during the DEP20 epoch the animals were essentially in the active awake (aW) state (2-3h) the entire
epoch (green 50.6 min; Table 3). In this regard, after DEP20 infusion, the amount of SWS significantly decreased from 36.2 and 33.9 min (n.s) in the baseline and saline epochs, respectively to 0.7 min in the DEP20 epoch (Table 3; n=32 sessions; one-way ANOVA; main effect epochs, $F_{(2, 93)} = 113.7, P < 0.0001$).

With respect to changes in firing rates, except for the abrupt increases caused by the transitions between SWS and awake states (see pSPN neuron, Fig. 3A left panel below) there is not a marked change in spiking activity from baseline to saline (firing rates: pSPN; baseline = 0.57, saline= 1.1 Sp/s; pChAT; baseline = 9.1, saline 8.9 Sp/s). In contrast, about 5 min after the infusion of DEP20 there is a marked change in inhibition and/or activation that lasted more than 1 h (pSPN in 3.4 Sp/s after DEP20 and pChAT 3.1 Sp/s after DEP20; Fig. 3A). These changes are representative of the population responses (Table 2). The normalized individual and population activity (black traces) changes for 118 (104 inhibited and 14 activated) of the 154 neurons that significantly changed their firing rate in response to DEP20 are seen in Figure 3B. These data clearly show that NAc shell activity is strongly modulated by DEP20.

**DEP induces local field potential (LFP) oscillations in the NAc shell**

In addition to the recording single-unit activity we simultaneously recorded LFPs. Figure 4A shows 5 sec representative LFP recordings when the animals were in three distinct behavioral states. The quiet awake state (qW) is characterized by low amplitude and fast oscillations (blue traces), whereas during slow-wave sleep (SWS) they show their characteristic high amplitude low frequency delta (1-4 Hz) oscillations (grey traces). The DEP20 -induced active awake state (aW) is also characterized by high amplitude low frequency delta oscillations that over time become larger (green traces). Figure 4B shows a spectrogram (1-30 Hz) of the LFP’s over a single session that encompasses the baseline, saline and DEP20 epochs. It is readily seen that after DEP20 infusion the power at delta, beta and theta frequency bands greatly increased. Also shown is the animal’s locomotion
that increased upon DEP20 application. The panel below the hypnogram displays the animal’s behavior during the three epochs. Again, it clearly shows that in the DEP20 epoch the animal does not exhibit SWS and is primarily in the active awake state (Table 3). Figure 4C displays the average of 32 recording sessions of the normalized Power Spectral Density (PSD) of the LFPs for beta (15-30 Hz) theta (6-9 Hz) and delta (1-4 Hz) oscillations. Interestingly, there were no significant changes during the baseline or saline epochs; a result that could arise from an averaging of the quiet awake and slow wave sleep states that occur at different times across experiments. Nevertheless, after DEP20 infusion, large and rapid (~5 min) changes were revealed as a decreased z-score PSD at beta (Kruskal-Wallis; $H_{(2)} = 23800$, $P < 0.0001$) and theta ($H_{(2)} = 11122$, $P < 0.0001$) and an increase in delta oscillations ($H_{(2)} = 5090$, $P < 0.0001$; Table 5).

The mean locomotion responses (Fig. 4D) show that compared with the saline epoch (390 ± 12 cm/min), after DEP20 infusion there is a rapid (onset: 5.43 ± 0.44 min; n=22) increase in locomotion (1502 ± 20 cm/min) (Kruskal-Wallis; $H_{(1)} = 89.26$, $P < 0.0001$). In summary, these data show that DEP20 produces insomnia, increases locomotion, and alters oscillations in the NAc shell.

**DEP20-induced changes in NAc shell activity across treatment period**

Having shown that repeated intraperitoneal injections of DEP20 produce transient changes in weight loss and food intake (Fig. 1), we investigated if activity changes measured in the NAc shell would correlate with these behavioral changes. To achieve this, over 14 days, we gave daily intragastric infusions of saline and DEP20 while measuring the NAc shell activity. Animals given the intragastric treatment, like those given the intraperitoneal treatment, showed a transient increase in weight loss and decrease in food intake that adapted as the treatment progressed (Fig. 5A). To obtain a sufficient number of neurons for statistical analysis the treatment was divided into three periods (Fig. 5A): day 1, days 2-7 and days 8-14. The mean of the population firing rates and normalized firing rates for day 1 (purple), days 2-7 (orange) and days 8-14 (brown), over the baseline, saline
and DEP20 epochs are shown in Figure 5B and given in Table 6. Throughout the three epochs the greatest activity (baseline and evoked) occurred in days 2-7 and the least were in days 8-14. The normalized responses revealed no differences between the baseline and saline epochs and that the greatest inhibitory effect was during days 8-14 (Table 6).

Analysis of the LFP oscillations over these three periods shows dramatic changes in DEP20 responses (Fig. 5C, Table 6). Specifically, on day 1 there were statistically significant increases at beta, theta and delta oscillations. On days 2-7 all three oscillations decreased in magnitude and on days 8-14 the beta and theta oscillations became largely negative and the delta oscillations decreased to saline levels. Thus, the changes in weight loss and food intake are best correlated with increases in the oscillations on day 1 and the adaptation (tolerance) to the treatment is better correlated with the diminution of the amplitude of the oscillations over the succeeding treatment days.

DEP20 induces taste aversion to a novel tastant (similar to LiCl), but the NAc shell LFP’s oscillations induced by DEP20 are different than those evoked by LiCl.

In order to explore whether DEP20 has a potential to induce taste aversion, which may contribute to its anorectic effects, we performed a conditioned taste aversion experiment. We used DEP20 or LiCl (0.4 M), as unconditioned stimuli (US) and a novel tastant (saccharin) was the conditioned stimulus (CS). Figure 6A displays the 15 min intake of water and the CS across the experiment, note that during 3 days of baseline (W1-3), water intake gradually increased as rats habituate to drink its entire daily allotment of fluids within 15 min. On the acquisition day (ACQ) the animals were presented with a novel 0.1% saccharin solution in which they consumed less saccharin than water on W3 day. Surprisingly, in the TEST session, both DEP20 and LiCl groups rejected saccharin to the same extent (one-way ANOVA: $F_{(1, 10)} = 1.3, P = 0.28$), suggesting that they were able to associate a gastric distress induced by a single injection of DEP20 with the consumption of a novel tastant. This is reflected by the smaller intake of saccharin in the TEST
session compared to that in the ACQ day. Nevertheless, as measured by the faster extinction of DEP20 relative to the LiCl group the strength of the aversive memory induced by DEP20 was weaker in comparison to the one produced by LiCl, (RM ANOVA EXT1-3, main effect; $F_{(1, 10)} = 5.41, P =0.042$, across days $F_{(1, 2)}=30.91, P < 0.0001$). In summary, both DEP20 and LiCl reduced the consumption of a novel tastant.

In light of this result, we tested whether DEP20-induced LFP's oscillations could reflect any kind of aversive or gastric malaise induced brain state. Therefore, we recorded in LFPs the NAc shell while the same rat received saline, LiCl and finally DEP20 in the same session. Figure 6B shows the spectrogram of a representative experiment and Figure 6C shows the normalized PSD LFP's changes. It is seen that the injection of LiCl, despite the presence of a transit peak, showed a non-significant decrease in delta and theta oscillations but DEP20 significantly increased beta, theta and delta oscillations (Kruskal-Wallis; $H_{(2)} = 13124, P < 0.0001$, $H_{(2)} = 4146, P < 0.0001$, $H_{(2)} = 118, P < 0.0001$, respectively). The hypnogram is shown below panel 6B. Note that after DEP20, rats are primarily in the active awake state (green) with increased LFP's oscillations, whereas LiCl infusion prevents SWS (grey) by inducing gastric malaise but without increasing the LFP oscillations. The average locomotion (panel 6D) revealed that LiCl showed a "spike-patterned" locomotion profile that is likely caused by the distress, and not because LiCl increased exploratory locomotion as does DEP20. In summary, these data clearly shows that the LFP oscillations in the NAc shell evoked by LiCl, a prototypical gastric malaise agent, and DEP20 are significantly distinct.

Appetite suppressants phentermine (PHEN) and bupropion (BUP) also decrease food intake, increase locomotion, stereotypy and modulate NAc shell activity

In addition to DEP20, we tested two other commonly used appetite suppressants, PHEN20 and BUP30, in a new cohort of animals both to determine their effects and also to compare them to each other and to DEP20. Relative to saline
infusions, both PHEN20 and BUP30 caused a reduction in body weight, albeit to a lesser extent than DEP20 (Fig. 7A). Specifically, across seven days of treatment the change in body weight (mean ± SEM) was: saline (8.1 ± 1.2 g), DEP20 (-11.6 ± 2.3 g), PHEN20 (-0.6 ± 2.4 g) and BUP30 (5.7 ± 3.2 g), respectively. A RM ANOVA showed a significant effect between groups (main effect of treatment; F (3, 21) = 18.0, P < 0.0001), across 7 days (D2-D8; F (3, 6) = 13.7, P < 0.0001) and significant interaction between treatment and days (F (18, 126) = 3.7, P < 0.0001). Furthermore, PHEN20 and BUP30 also decreased food intake: saline (-0.7 ± 3.5%), DEP20 (-26.6 ± 5.5%), PHEN20 (-18.7 ± 3.3%) and BUP30 (-11.5 ± 4.4%) (main effect of treatment: RM ANOVA F (3, 21) = 9.2, P = 0.0004; effect across days F (3, 6) = 10.9, P < 0.0001; interaction between groups and days: F (18, 126) = 2.52, P = 0.0015). In comparison to saline, DEP20 (P < 0.0001) and PHEN20 (P = 0.002) significantly reduced food intake (post-hoc test), whereas BUP30 across days showed a non-significant trend (P = 0.064 n.s.), probably because it only significantly decreased food intake during days D5-D8 (Post-hoc; P = 0.04; Fig. 7A bottom panel).

We also measured during 30-minute epochs of baseline, saline and for 1.5 h after anorexic drug delivery how these three appetite suppressants affect locomotion (Fig. 7B, upper panel) and stereotypy (lower panel). In the initial 20 min of the baseline epoch, in all groups the animals normal arousal and accompanying locomotion gradually decreased and thus there was a small, but significant, decrease in locomotion between the baseline and saline epochs (RM ANOVA: main effect, F (3, 80) = 7.8, P < 0.0001). In the drug epoch the saline response remained consistently low whereas it greatly increased to DEP20, PHEN20 and BUP30. DEP20 induced a slightly greater locomotion (3925 ± 288 cm/min) than PHEN20 (3515 ± 199) and BUP30 (3325±233) with a significant increase of locomotion when compared with saline treated group (843±158) (main effect across treatment drug epoch: F (3, 78) = 90.02, P < 0.0001; effect across time: F (3, 17) = 15.5, P < 0.0001; interaction between treatment and time: F (51, 1326) = 5.69, P < 0.0001). The significant interaction indicates that BUP30-induced locomotion rapidly tends to return to baseline (also see Supplementary Video 1).
For stereotypy measurements, there was a small decrease from the baseline to the saline epochs (RM ANOVA: $F_{(3, 80)} = 3.4$, $P = 0.02$) that were followed by rapid increases after DEP20, PHEN20 and BUP30 infusions. DEP20 induced almost twice the number of head weavings (86 ± 7 counts/5 min) as PHEN20 (45 ± 5) or BUP30 (37 ±5). RM ANOVA showed a significant difference across treatment groups $F_{(3, 78)} = 75.0$, $P < 0.0001$ (main effect), effect across time: $F_{(3, 29)} = 61.3$, $P < 0.0001$ and interaction between treatment and time: $F_{(87, 2262)} = 16.1$, $P < 0.0001$.

Furthermore, post-hoc analysis revealed that DEP20 significantly induced more stereotypy than either PHEN20 ($P <0.0001$) or BUP30 ($P <0.0001$).

From some of the same animals shown in Figure 7A we recorded the activity from their NAc shell during the baseline, saline, PHEN20 (Fig. 7C left panel) or BUP30 (Fig. 7C right panel) epochs. As with DEP20, both these compounds evoked both decreases and increases in firing rate. PHEN20 was most similar to DEP20 in that it produced 64% (38/59) inhibited responses and only 5% activated (3/59; $\chi^2_{(1)} = 23$, $P < 0.0001$) (compared with 63% ($P = 0.91$ n.s.) and 11% for DEP20 ($P = 0.24$ n.s.; Table 4). With PHEN20, all cell-types were equally inhibited (Table 4; $\chi^2_{(3)} = 2.6$, $P = 0.44$), whereas only three neurons were activated (1 = pSPN and 2 = Unidentified, Table 4).

The application of BUP30 also induced a greater number of inhibited (44%, 48/110) than activated (15%, 16/110) responses ($\chi^2_{(1)} = 12.5$, $P < 0.0001$). The proportion of inhibited responses induced by BUP30 was not significantly different than for DEP20 (over 7 days of $\chi^2_{(1)} = 2.6$ $P = 0.1$ n.s.), or for PHEN20 ($\chi^2_{(1)} = 2.08$, $P = 0.14$). Activated responses were also not significantly different among drugs (BUP30 (15%), PHEN20 (5%) and DEP20 (11%); $\chi^2_{(2)} = 2.8$, $P = 0.23$).

BUP30 showed a non-significant trend to inhibit a greater percentage of pChAT neurons, than pSPN ($\chi^2_{(1)} = 3.4$, $P = 0.064$, see Table 4). In other words, over a 7 day treatment period all three compounds produced essentially the same effect.

Information about the average firing rate of activated and inhibited neurons by the three appetite suppressants are shown in Table 2.
With regard to the changes in LFPs evoked by PHEN20 and/or BUP30, we found both similarities and differences to those evoked by DEP20. Whereas over the entire 14 day treatment period DEP20 decreased beta and theta oscillations and increased delta oscillations, all three oscillations were increased by PHEN20 and BUP30 (over a 7 day period Figs. 5C, 7D and Tables 5 and 6). With regard to locomotion, we found that DEP20 infusions caused a greater increase in locomotion than either PHEN20 or BUP30 ($F_{(2, 48)} = 16.3, P < 0.0001$). Furthermore, the onset of locomotion induced by DEP20 (5.43 ± 0.44 min; $F_{(2, 49)} = 9.26, P = 0.0004$) was significantly shorter than for PHEN20 (19.1 ± 3.6; $P = 0.0003$) or BUP30 (16.7 ± 2.3 min; $P < 0.0001$), which did not differ ($P=0.28$).

In summary, repeated applications of these three appetite suppressants produce weight loss, decrease food intake increase locomotion, evoke a greater percentage of inhibited than activated NAc shell responses and evoke appetite suppressant-dependent changes in LFP’s oscillations in the NAc.

**DEP–induced locomotion, stereotypy, weight-loss and food intake are attenuated by intragastric infusion D1R and D2R antagonists**

Since the NAc shell receives dopaminergic input from various sources (Haber et al. 1985), we determined if the effects evoked by DEP20 would be modulated by intragastric infusion of either/or the D2R antagonist raclopride 0.5 mg/Kg (RAC0.5) and/or the D1R antagonist SCH23390 1.5 mg/Kg (SCH1.5). Figure 8A shows a plot of the change in body weight in naïve rats over seven days of daily treatment ($n = 3$, per group). As controls, we found that relative to saline, infusions of RAC0.5 (positive gain 18.4 ± 5.4 g) or SCH1.5 (11.8 ± 2.7 g) alone do not induce a significant weight loss although; intragastric infusions of DEP20 produce a large weight loss (-15.3 ± 3.1 g). This weight loss could be reversed by intragastric infusions of either DEP20+RAC0.5 (positive gain weight; 1.4 ± 1.9 g), or DEP20+SCH1.5 (-4.4 ± 5.4 g). RM ANOVA showed a significant main effect of group ($D2-D8; F_{(5, 12)} = 11.4, P = 0.0003$), and across days ($F_{(5, 6)} = 36.4, P$
<0.0001) and significant interaction between groups and days \( F_{(30, 72)} = 4.9, P < 0.0001 \). A post-hoc analysis showed in comparison to DEP20 both groups DEP20+RAC0.5 and DEP20+SCH1.5 (only for days 3-8) significantly attenuated the weight loss induced by DEP20 (All \( P \)'s < 0.05).

For these same animals the percentage change in 24 h food intake was also measured (Fig. 8B). Relative to day 1, the food intake over the treatment was: Saline \((4.9 \pm 9.8\%)\), RAC0.5 \((5.7 \pm 8.3\%)\), SCH1.5 \((2.54 \pm 5.7\%)\), DEP20 \((-28.7 \pm 3.8\%)\), DEP20+SCH1.5 \((-16.9 \pm 7.3\%)\) and DEP20+RAC0.5 \((-7.13 \pm 6.8\%)\) (RM ANOVA showed a significant main effect of group \( F_{(5, 12)} = 4.8, P = 0.011 \) and across days \( F_{(5, 6)} = 5, P = 0.0002 \) and significant interaction between groups and days \( F_{(30, 72)} = 2.9, P = 0.0001 \). The DEP20+SCH1.5 group tended to eat slightly more food than DEP20 group, although the differences were not significant \( (P = 0.21) \), whereas RAC0.5 significantly reversed food intake suppression induced by DEP20 \( (P = 0.032; \text{comparison DEP20 vs. DEP20+RAC0.5}) \). These data reveal that DEP’s effect on weight loss and food intake is markedly affected by inhibition of both D1 and D2 receptors. Below we showed that some of these effects are the result of inhibiting DA receptors in the NAc shell.

We also measured the animals changes in locomotion and stereotypy during a 2 h period (Fig. 8C each epoch being 30 min; baseline (BL), saline (Sal), DEP20 and antagonist). For locomotion, infusion of RAC0.5 \((812 \pm 137 \text{ cm / 5 min})\) or SCH1.5 \((1161 \pm 152 \text{ cm / 5 min})\) alone does not increase locomotion relative to saline \((1008 \pm 181 \text{ cm / 5 min})\). In all cases the animal’s locomotion decreased from the baseline (BL) to saline epoch and increased upon the infusion of DEP20 (Note that the responses to DEP20 are not identical, likely because they are affected by the previous treatment days). The DEP20-induced locomotion \((3957 \pm 293 \text{ cm / 5 min}; \text{RM ANOVA, from 1-120 min; main effect groups } F_{(5, 118)} = 45.1, P < 0.0001)\) was attenuated by subsequent administrations of either SCH1.5 \((3193 \pm 198 \text{ cm / 5 min}; P < 0.0001; \text{group DEP20+SCH1.5})\) or RAC0.5 \((2675 \pm 233 \text{ cm / 5 min}; P < \)
Locomotion was not significantly different between DEP20+RAC0.5 and DEP20+SCH1.5 ($P = 0.06$ n.s.).

Similar effects to those above were also found for stereotypy (Fig. 8D). Again, application of RAC0.5 or SCH1.5 alone does not evoke head weaving stereotypy (Fig. 8D). However, DEP20 evoked stereotypy ($97 \pm 7$ counts / 5 min; RM ANOVA, from 1-120 min; main effect groups $F_{(5, 118)} = 64.8$, $P < 0.0001$) was significantly reduced by either DEP20+RAC0.5 ($54 \pm 12$ counts / 5 min; Post-hoc $P = 0.0004$) or DEP20+SCH1.5 ($40 \pm 8$ counts / 5 min; $P < 0.0001$). Note that DEP20 –induced stereotypy remained at high levels for times longer than 1 h, whereas stereotypy declined within 30 min. in the presence of the antagonists. These results provide evidence for the involvement of DA receptors in the DEP20 -induced locomotion, stereotypy as well as on its anorectic effects.

**Blockade of D1R and D2R directly in the NAc shell diminishes the pharmacological effects of DEP20**

To directly address the question of whether DA receptors in the NAc shell mediate DEP’s behavioral effects we injected -either D1R (SCH) or D2R antagonist raclopride (RAC) - directly into the NAc shell, whereas above rats received intragastric infusion of DEP20. Figure 9A shows a plot of the change in body weight across seven days of daily treatment. Briefly, all groups received two injections: one directly in the NAc shell (Sal, SCH or RAC) and a second one intragastric (Sal or DEP20). A RM ANOVA analysis showed a significant main effect of group (D2-D8; $F_{(5, 12)} = 20.4$, $P < 0.0001$), and across days ($F_{(5, 6)} = 87.2$, $P < 0.0001$) and significant interaction between groups and days ($F_{(30, 72)} = 2.7$, $P = 0.0004$). A post-hoc analysis showed that relative to the control group Sal+Sal ($10 \pm 1.1$ g); neither the group RAC+Sal ($9.2 \pm 1.6$ g; $P = 0.74$) or SCH+Sal ($13.9 \pm 0.9$ g; $P = 0.13$) was significantly different from the control group, indicating that these DA antagonists in the NAc shell alone do not induce a significant weight loss. The weight loss produced by intragastric infusions of DEP20 was (Sal+DEP20; -6.4 ±
1.9 g; $P < 0.0001$) and it was significantly attenuated by intraNAc shell infusions of either SCH+DEP20 (positive gain weight; $2.1 \pm 2.5$ g; $P = 0.004$), or RAC+DEP20 (-0.8 ± 1.7 g; $P = 0.04$). Thus, D1 and D2 NAc shell receptors are clearly involved in DEP20’s induction of weight loss.

For the same animals, food intake was also measured (Fig. 9B). Specifically, a RM ANOVA, for days 2 to 8, showed a significant main effect of group ($F_{(5, 12)} = 7.5, P = 0.002$), a significant effect across days ($F_{(5, 6)} = 14.7, P < 0.0001$) and a significant interaction between groups and days ($F_{(30, 72)} = 3.2, P < 0.0001$). Overall, across seven days of treatment (D2-8), a post hoc analysis revealed that RAC+DEP20 showed a non-significant trend to consume more chow food relative to Sal+DEP20 group ($P = 0.075$), but largely reduced chow intake in Days 2-3 (one-way ANOVA: $F_{(1, 10)} = 10.61, P = 0.0086$; SAL+DEP20 Vs RAC+DEP20). Likewise, the SCH+DEP20 significantly ate more food than the Sal+DEP20 group ($P = 0.009$). No differences were found between SCH+DEP20 and RAC+DEP20 ($P = 0.28$). These data show that DEP20’s effects on food intake were partially, but significantly, reduced by NAc shell blockade of D1 and D2 receptors.

Locomotion and stereotypy were also measured under these conditions (Fig. 9C, see Methods). For locomotion, we found that relative to Sal+Sal (381 ± 79 cm / 5 min) NAc Shell infusion of RAC+Sal (404 ± 90 cm / 5 min) or SCH+Sal (347 ± 71 cm / 5 min) do not alter locomotion. In contrast, Sal+DEP20 induced a rapid onset (5 min) and robust locomotion (3303 ± 368 cm / 5 min; RM ANOVA; main effect groups $F_{(5, 118)} = 159.7, P < 0.0001$). For the RAC+DEP20 experiments the onset of locomotion induced by DEP20 was delayed by 15 min and the magnitude of locomotion was attenuated (2094 ± 199 cm / 5 min; $P < 0.0001$). Similar results were found for the SCH+DEP20 experiments, the onset of locomotion induced by DEP20 was further delayed by 35 min as well as its magnitude (881 ± 47 cm / 5 min; $P < 0.0001$).
Similar effects to those found for locomotion were replicated for head weaving stereotypy (Fig. 9D). As controls we found that RAC or SCH by themselves did not evoke head weaving stereotypy (Fig. 9D). Whereas DEP20 evokes a robust stereotypy (72 ± 2 counts / 5 min; RM ANOVA main effect of groups $F_{(5, 118)} = 212$, $P < 0.0001$) this effect was significantly diminished (but not eliminated) by either RAC+DEP20 (40 ± 2 counts / 5 min; Post-hoc $P < 0.0001$) or SCH+DEP20 (7 ± 1 counts / 5 min; $P < 0.0001$). Importantly, both groups RAC+DEP20 ($P < 0.0001$), but specially SCH+DEP20 ($P < 0.0001$) almost completely attenuated the DEP20-induced stereotypy. These results provide evidence for the involvement of NAc shell dopamine receptors in the ability of DEP20 to trigger and to maintain locomotion and stereotypy.

**DEP20-induced LFP oscillations and spiking inhibition in the NAc shell are mediated by D1R and D2R receptors**

Having shown that both i.g and intraNAc shell infusions affects behavior for technical reasons (to avoid drift in waveform stability induced by intraNAc microinjections) we only determined if intragastric infusion of the two tested DA antagonists would similarly affect DEP20 induced NAc shell activity, we recorded the electrophysiological responses over seven days of an i.g. infusion of RAC or SCH 30 min after the infusion of DEP20 (see below for controls). Figure 10A is a plot of the normalized color-coded PSTHs of all neurons (over 7 days) modulated by DEP20 during the Baseline (BL), Saline (Sal), DEP20 and RAC0.5 epochs. In the presence of RAC0.5 (DEP20+RAC0.5) the extreme bias towards the inhibition seen with DEP20 alone (only neurons recorded over 7 days of DEP20) were diminished since now only 30% (39/81) were inhibited and 48% (25/81) were activated ($\chi^2_{(1)} = 2.2$, $P = 0.13$, n.s.). The averaged normalized DEP20-induced z-score PSD for the beta, theta and delta oscillations over the treatment period are seen to decrease to baseline levels by RAC0.5 (Fig. 10B). Finally, RAC0.5 alone did not induce changes in the LFP’s oscillations at beta, theta and delta bands over
the 7 days of treatment (see Fig. 10B thinner traces), and neither induced locomotion during single-unit recordings (Fig. 10B bottom panel, pink trace).

RAC0.5 also attenuated and delayed the DEP20–induced increase in locomotion (Fig. 10B, bottom panel; cyan trace locomotion measured in the operant chamber; Kruskal-Wallis, $H_{(2)} = 53, P < 0.0001$) and delayed the onset of DEP20-induced locomotion by 11.8 ± 2.2 min, which was significantly slower to that normally evoked by DEP20 alone (6.2 ± 1.36 min; $F_{(1, 21)} = 5.05 P = 0.035$; over 7 days treatment).

Figure 10C shows the global population-firing rate of all 81 neurons recorded with DEP20+RAC0.5 (cyan). It is seen that the global inhibitory imbalance induced by DEP20 is largely abolished by repeated administrations of RAC0.5. Nevertheless, RAC0.5 alone modulated NAc shell single-unit activity although the percentage of neurons inhibited (n=29/93, 31%) was similar to the percentage of those activated (n= 26/93, 28%) ($P = 0.72$, n.s., data not shown). In summary, during the infusion of RAC0.5 alone the average population activity of all 93 neurons recorded showed a slight but transient activation that rapidly returned to baseline (< 4 min; see Fig. 10C, pink trace). It follows that RAC0.5 alone does not induce an inhibitory imbalance in the NAc shell population activity.

We also tested SCH1.5 alone and found that it modulated single-unit activity to about the same extent inducing nearly the same amount of inhibition (n = 34/114 neurons, 29.8%) as activation (n = 35/114 neurons, 30%; Data not shown). Accordingly, SCH1.5 alone does not produce any inhibitory imbalance at the population activity level (see Fig. 10F, black trace). In this regard, SCH1.5 did not restore the DEP20-induced inhibitory imbalance such that with SCH1.5 there were 63% (71/112) inhibited and 17.8% (20/112) activated ($\chi^2 = 20.8, P < 0.0001$; Fig. 10D) neurons. This result was unexpected given the effect of SCH1.5 on the behavioral data (Fig. 8, DEP20+SCH1.5). Consequently, we determined if SCH3 would restore the DEP20-induced imbalance. Indeed, under this condition 41% (14/34) were inactivated and 21% (7/34) activated (the proportions of inactive/active responses were not significantly different; $\chi^2 = 1.7, P = 0.18$ n.s.).
Thus, SCH3 effectively rebalanced the DEP20-induced imbalance (inhibited/activated) by decreasing the number of neurons with inhibited responses and slightly increasing the number of active responses.

With regard to the DEP20-induced beta, theta and delta oscillations over the treatment period, it is seen that the onset of DEP20-induced oscillations were markedly delayed by SCH1.5 and even more for SCH3 (Fig. 10E). As a control we found that SCH1.5 alone did not modulate beta, theta or delta LFP’s oscillations (Fig. 10E, thinnest traces). In addition, after the infusion of SCH1.5, the magnitude of the LFP’s PSD slightly changed (from 3.0 ± 0.08 in the DEP20 epoch to 2.59 ± 0.08 in the SCH epoch). Likewise, for SCH3, it also marginally changed from 1.18 ± 0.06 in the DEP20 epoch to 0.92 ± 0.07 in the SCH1.5 epoch. Nevertheless, SCH markedly attenuated DEP20-induced beta, theta and delta oscillations in a dose-dependent manner. Likewise, SCH decreased the locomotion in a dose dependent manner. It also delayed the onset of DEP20-induced locomotion from 6.2 ± 1.4 min to 11.5 ± 2.5 min (SCH1.5) and 24.9 ± 4.5 min (SCH3) ($F_{(2, 33)} = 11.64, P = 0.0001$, green; Fig. 10E bottom panel).

Finally, to determine at the population firing rate level whether SCH would reversed the inhibition induced by DEP20, we plotted in Figure 10F the population activity of all the recorded neurons (modulated or not by DEP20). It is seen that SCH reversed the strong inhibition evoked by DEP20 in a dose-dependent manner at the population NAc shell activity level.

Discussion

The most common recommendations for persons wanting to lose weight are dietary changes, exercise and, in many cases, the short term use of appetite suppressants. Many prescribed suppressants, like diethylpropion (DEP), phentermine (PHEN) and bupropion (BUP), are mild psychostimulants and, as such, increase wakefulness, increase psychomotor activity, cause changes in mood, and produce weight loss (Silverstone 1992). To cause all these changes
these compounds must affect many brain areas, in part, by increasing the concentration of neurotransmitters such as serotonin (to reduce craving), norepinephrine (to increase activity and metabolism) and dopamine (for reward and motor activity) (Rothman et al. 2002). Nevertheless, despite their extensive usage, their evoked behavioral changes and their corresponding neuronal correlates remain poorly understood. For this reason, in rats, we investigated behavioral and concurrently neural responses in the nucleus accumbens shell (NAc shell) to repeated intragastric treatments of DEP, PHEN and BUP.

In this study we found that all three anorexic compounds increased weight loss, decreased food intake and increased locomotion and stereotypy although the animals developed tolerance to these drugs with increasing treatments. Recordings of responses from the NAc shell revealed that these appetite suppressants evoked a global inhibitory imbalance involving all putative neuronal types that correlated with the onset of locomotion and stereotypy. Analysis of the LFPs revealed that these compounds initially increased delta, theta and beta oscillations, which correlated with the animal’s initial weight loss and decrease in food intake also with the subsequent tolerance developed during treatment. In addition, we identified roles for dopamine D1-like and D2-like receptors in the DEP20-induced effect on weight loss, food intake, locomotion, stereotypy, global inhibition of NAc shell spiking activity and changes in LFP oscillations. Thus, activation of D1-like and D2-like receptors by DEP (and presumably like other appetite suppressants (Janhunen et al. 2013)) are important factors in the ability of this class of anorectic compounds to induce weight loss and corresponding behavioral changes.

Comparison of the appetite suppressants:

Previous studies in humans revealed that at appropriate concentrations DEP, PHEN and BUP produce weight loss (Hendricks et al. 2009) with the order DEP >= PHEN > BUP (Cercato et al. 2009; Suplicy et al. 2014). Here, using rats, we found
that DEP20 was slightly more potent than PHEN20 with regard to weight loss (Fig. 7A), locomotion, and stereotypy (Fig. 7B) although for food intake it was equipotent (suppression). Nevertheless our data show that these three drugs are in fact more similar than different. For example, the efficacy of the drugs were not significantly different with respect to times in behavioral states (Table 3), putative cell-types of NAc shell neurons activated or inhibited (Tables 1, 4), changes in firing rates (Inhibited/Activated; Table 2), and initial effects on LFPs (Figs. 4, 5 and 7). Thus in the NAc shell these appetite suppressants evoke a similar neuronal signature. In this regard, similarities were expected since they are all mild congeners of amphetamine.

The effects of these appetite suppressants on weight loss and food intake are of clinical interest because after the initial decrease of weight and food intake the animals developed tolerance to them which resulted in increasing their food intake and re-gaining weight (Fig. 7A). These results parallel findings in humans taking these same appetite suppressants (Fernstrom and Choi 2008). Although, humans develop tolerance to the weight loss effects induced by appetite suppressants with a different time scale (in months, not weeks), they follow the same pattern as rats. For humans, after 5-6 months on treatment their weight loss plateaus. From this point their body weight is either maintained or is slowly increased (Cercato et al. 2009). Although the mechanisms explaining why appetite suppressants developed tolerance are largely unknown, we found that LFP oscillations in the NAc shell can be used as a biomarker to determine when tolerance to DEP20 develops.

Locomotion and stereotypy

Locomotion involves any type of forward non-repetitive movement whereas stereotypy behaviors are repetitive motor patterns, such as head oscillations, with no obvious function (Mason 1991). Although all three DEP concentrations tested increased locomotion (Fig. 1C and (Reimer et al. 1995)), DEP10 produced the
greatest increase in locomotion, a result that contrasts to the food intake studies, where the DEP40 had the greatest effect (Fig. 1A). DEP also induces stereotypy (Safta et al. 1976) and, as with locomotion, after stopping treatment for one day the stereotypic response returned to baseline, a result similar to that observed after chronic treatment with amphetamine (Borison et al. 1977). This is the first report showing that, like DEP and amphetamine (Safta et al. 1976), PHEN20 and BUP30 also induce head-weaving stereotypy (Fig. 7B), suggesting this behavior is a hallmark of this class of appetite suppressants. The rapid onset of locomotor activity suggests that DEP can be rapidly metabolized and cross the blood brain barrier (Carlsson and Johansson 1978; Cox and Maickel 1972).

**Behavioral mechanisms of appetite suppressants hypophagia**

In this study we used information that showed that amphetamine delayed the onset of eating and increased the inter-meal interval (Blundell et al. 1976). These data rule out an effect on satiety signals, but support the idea that amphetamine-induced anorexia evokes motor stimulatory effects that compete (interfere) with feeding (Wolgin 2000). In agreement with this idea, our data indicate that, unlike the anorexic effect of the short-term satiety peptide CCK-8, which delays gastric emptying, DEP20-induced anorexia is not mediated by a mechanism that involve delaying gastric emptiness (Fig. 2). This result is also consistent with our data showing that rats on appetite suppressants completely neglect chow food during the initial 2 h after injection (see Supplementary Video 1, see also (Ghosh and Parvathy 1976)). In other words, the data suggest that the greater the locomotion and stereotypy, the greater the food suppression (Fig. 7B).

Another mechanism of appetite-suppressants to induce anorexia is its potential to induce gastric malaise. It is known that amphetamine can induce taste aversion (CTA) (see Carey, 1973). Here we show that DEP20, and presumably like other appetite suppressants, can also induce taste aversion to a novel palatable tastant ((Fenu and Di Chiara, 2003)). We suggest that DEP20’s ability to induce taste aversion can partially contribute to its anorectic effects by decreasing the
consumption of novel foods and/or by reducing the probability of trying novel foods. However, DEP20’s anorectic effect induced on a highly familiar food cannot readily be attributed to taste aversion because it is much more difficult to acquire aversion to familiar foods (Garcia et al., 1974). Moreover, because the animals increased their food intake across injection days (Figure 1A), it is unlikely this can be attributed to conditioned taste aversion, which shows little tolerance (Garcia et al., 1974). Thus, not all anorectic effects of appetite suppressants can be accounted by its potential to induce gastric malaise. Additional evidence against the possibility that animals developed a strong taste aversion to chow is the finding that rats dramatically increased food intake the very first day after drug withdrawal (data not shown) and this overconsumption of chow (relative to saline group) still persisted even 7 days after drug withdrawal (see Fig. 1A; day 21).

In summary, the behavioral effects induced by these appetite suppressants reflect the strong interconnection among different brain networks controlling weight-loss, feeding, sleep, locomotion and stereotypy (Costa et al. 2007; Nicola 2010; Seiden et al. 1993; Tellez et al. 2012). As outlined below, dopamine plays important roles in relating all these behaviors (de Araujo et al. 2012; Dzirasa et al. 2006).

Neuronal modulation of the NAc shell single-unit activity

To determine possible neuronal correlates underling these behavioral effects we performed recordings in the NAc shell, a brain region that integrates inputs from limbic brain regions and transmits them to motor and feeding regions (Hajnal and Norgren 2004; Mogenson et al. 1980). For this reason appetite suppressants are likely acting in multiple brain regions and that the NAc shell is not the only area important for the action of these appetite suppressants (Shi et al. 2000). However, here we found that DA receptors in the NAc shell contribute, to most if not all, of the pharmacological effects induced by DEP20 (Fig. 9). Furthermore, the NAc shell is a critical region for amphetamine’s effects since direct infusions of large doses of amphetamine into the NAc shell can suppress food intake and stimulate
locomotion (Carr and White 1986; Kelley et al. 1989). In contrast, lower doses of amphetamine can promote food intake (Evans and Vaccarino 1990; 1986; Wise et al. 1989), suggesting that the level of dopamine stimulation in the NAc can have a bidirectional control upon food intake. In the same vein, it has been shown that pharmacological inactivation of the NAc shell using low doses of the GABAA agonist muscimol promotes feeding, although higher doses suppressed feeding, and induced locomotion (Kelley et al. 1989; Stratford and Kelley 1997).

This biphasic effect has been substantiated by the finding of a moderate inhibitory imbalance in the population NAc shell produced by rats eating hedonically positive foods (Krause et al. 2010; Tellez et al. 2012), whereas Krause et al. 2010 showed that electrical stimulation of the NAc shell stops feeding, uncovering that NAc activity can have a bidirectional control upon feeding. Here we found a large inhibitory unbalanced in the rat NAc activity obtained with appetite suppressants, which decreases food intake and increases locomotion and stereotypy. We are aware that these data add a degree of complexity to both the “gating feeding hypothesis” which states that inhibition in the NAc allows feeding behavior (Krause et al. 2010) and to the “NAc decreased (reward) / increased (aversion) activity hypothesis” (Carlezon and Thomas 2009). Thus, the strength of the inhibitory imbalance of the population NAc shell activity can correlate with multiple and sometimes conflicting behavioral outputs, such as food intake and appetite suppressant’s induced-anorexia.

An important result of this study was that all three appetite suppressants evoked a large inhibitory imbalance in the NAc shell that involved all putative cell types and that matched the onset and continuance of locomotion and stereotypy (Fig. 7 and Tables 1 and 4). These locomotor behaviors are incompatible both with food intake and sleep. These data also show that pSPNs are the neurons most likely to be activated by DEP20 (Table 4) and that BUP30 inhibited a greater percentage of pChAT neurons than the other appetite suppressants (Table 4); a finding consistent with its anti-nicotinic activity (Arias 2009). In summary, we have
identified a correlation between changes in NAc shell single unit activity and the onset of motor changes.

**LFP’s oscillations in the NAc shell**

Whereas appetite suppressants cause a rapid imbalance in the firing rate of NAc shell neurons that correlate with the onset of locomotion and stereotypy, changes in LFPs correlate with the longer-term behaviors involving food intake and the accompanying weight loss. This behavior is illustrated in experiments with DEP20 over 14 days (Fig. 5 and Table 6). However, for all the tested compounds one day after treatment beta, theta and delta oscillations were large and positive and that this change is associated with the largest decrease in food intake (Figure 5C and Table 6). However, on subsequent treatment days the magnitude of the three oscillations decreased and food intake increased. This is the first report regarding the measurement of the temporal changes evoked by appetite suppressants in the NAc shell and their relation to food intake. We are aware that these oscillations are also involved in motor and other behaviors (Jenkinson and Brown 2011) and that in rats cortical EEG’s studies that have shown that DEP evokes robust cortical delta oscillations (Safta et al. 1976). We hypothesize that amplification of delta oscillations during awake states, over those seen in SWS (Steriade et al. 2001) is related to conditions were DA levels are out of their physiological dynamic range (Costa et al. 2006; Lemaire et al. 2012) and under situations where energy consumption needs to be reduced (Dworak et al. 2011). Likewise, beta oscillations are normally high during tonic muscle rigidity in preparation to voluntary movement, but they are amplified in Parkinson Disease patients where DA levels are lower of its physiological range (Jenkinson and Brown 2011). It is possible that the decrease of DEP20- induced beta oscillations might be related to a DA alteration after chronic use, whereas theta oscillations induced by appetite suppressants might be related to locomotion and exploratory head weaving behaviors (Buzsaki 2005).
DEP20 induced LFP’s oscillations do not reflect and aversive-gastric malaise brain state

Fenu and Di Chiara (2003) reported that the D1 receptor antagonist, SCH39166, administered before amphetamine, either systematically or directly in the NAc shell, prevented the facilitation of conditioned taste aversion induced by amphetamine. They concluded that amphetamine facilitates taste aversion learning via D1 receptors of the NAc shell. This result highlights an important role of dopamine release in the NAc shell and the acquisition of taste aversion induced by amphetamine. Although the neuronal correlates of the aversive potential of appetite suppressants is beyond the scope of this manuscript, we provide evidence indicating that DEP20-induced LFP’s oscillations do not correlate with an aversive-gastric malaise induced brain state. This is because a prototypical gastric malaise agent LiCl did not alter NAc shell LFP’s oscillations (Fig. 6C). Indeed, no LiCl-induced LFP oscillations occurred even when LiCl induced insomnia, or distress movements (e.g., lying on the belly) even though it has been shown that LiCl can also increase dopamine release in the NAc shell (Ventura et al. 2007).

Dopamine receptors and DEP’s effect

The three appetite suppressants tested induce the release of norepinephrine, serotonin and dopamine (Opacka-Juffry et al. 2014). Here we show that their appetite suppressing and motor effects, as well as changes in NAc shell activity, can in part be accounted for by their ability to release dopamine. In this regard, amphetamine also causes DA to be released in the rat NAc (Daberkow et al. 2013) and also alters their feeding and motor behaviors (stereotypies)(Seiden et al. 1993). Indeed, a general dopaminergic tone appears to be necessary to express the full magnitude of locomotor activity and stereotypy induced by amphetamine (Sotnikova et al. 2005), DEP (Samanin and Garattini 1993), PHEN (Offermeier and Potgieter 1972) or BUP (Janhunen et al. 2013). What has not been tested is how these drugs affect the NAc shell activity and if DA antagonists could reverse their
effects. Here we showed that DEP’s effects on locomotion and stereotypy were attenuated by both systemic and intraNAc shell infusions of D1R and D2R antagonists (Fig. 8 and 9). In agreement with the proposed role of these receptors, we found that SCH23390 strongly delays the onset and magnitude of locomotion and stereotypy suggesting that activation of D1Rs are necessary for triggering these behaviors (Szczykpka et al. 1999). Our data also indicates that D1Rs can also be involved in food intake and weight loss. In agreement with D2Rs prominent role mediating feeding, energy homeostasis (Kim et al. 2010) and obesity (Johnson and Kenny 2010), we observed that D2R antagonist reversed the anorectic and weight loss effects induced by DEP and (when infused intragastrically) reverse the net inhibitory imbalance in the NAc shell produced by DEP. Nevertheless, D2Rs seem to also play an important role triggering locomotion and stereotypy. Our data favors the idea of a more cooperative action of both D1 and D2 like receptors -in the brain and in the NAc- mediating the pharmacological effects of this class of appetite suppressants.

In summary, these results demonstrate that appetite suppressants modulate weight loss, food intake, locomotion and stereotypy. Finally, in contrast to the belief that serotonin and norepinephrine are major contributors to the action of these drugs, we found that both their induced behavioral and electrophysiological changes were largely reversed by D1R and D2R antagonists, thus providing a role for these receptors in the anorexia induced by these appetite suppressants.

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Figure 1. The effects of diethylpropion (DEP) on weight loss, feeding, locomotion, and stereotypic-head movements. A, The change in body weight of control rats injected intraperitoneally daily with saline (○) from day 1 to day 14 compared with rats injected with DEP at (10 (■), 20 (▲) and 40 ( ◆) mg/Kg). These concentrations are hereafter named DEP10, 20 and 40, respectively. The grey shading depicts the change in body weight measured 20 min before each DEP injection. The break in the axis indicates where the treatment was stopped. The horizontal dotted line represents no weight change. B, The DEP-induced change in food intake for the same subjects shown in panel “A.” C, The effects of DEP10, 20 and 40 (relative to baseline) on the animal’s locomotion (in cm / 1.5 h). This protocol consisted of three days of saline treatment (S1-S3), seven days of DEP treatment (D1-D7) and one final day of saline treatment (S8). The inset displays the daily protocol. D, It shows the DEP–induced effect on stereotypic head movements (counts / 1.5 h). In this and subsequent figures the symbols represent mean ± SEM and the shading treatment times.

Figure 2. DEP20 at a dose that suppresses food intake does not delay gastric emptying. The plot shows the difference in weight between wet and dry stomach in g, as a measure of gastric emptiness (see Methods for details). Note that DEP20 did not differ from saline group, whereas CCK-8 delayed gastric emptiness. Data represents mean ± SEM. * indicates \( P < 0.05 \).

Figure 3. Intragastric infusions of DEP20 modulate NAc shell spiking activity. A, A modulation map for DEP20 responses was computed with 154 neurons using a fuzzy cluster algorithm to classify them into four putative cell-types: pSPN (medium spiny projection neuron) (+), pFSI (fast spiking interneuron) (○), pChAT (choline acetyltransferase interneurons) (●), unidentified, U (X) (see Table 1). For each cell-type a representative waveform is given as well as significantly inhibited (blue dots) and activated (red dots) responses. V-P is the valley to peak width and a waveform illustrating the amplitude. Upper right panel shows a brain state map (hypnogram) that was computed from the LFP’s in the NAc shell (see Fig. 4A) illustrating the three major behavioral states of an animal during the trial: active awake (aW), quiet awake (qW) and slow wave sleep (SWS) (Table 3). Each dot represents the principal component (PC’s) ratio between two power ranges for each second of LFP’s activity. Each dot falling into the gray ellipsoid corresponds to periods where the animals were in SWS, and the blue and green ellipsoids represent when the animals were in the qW and aW states, respectively. Left bottom panel: Excitatory changes from a representative pSPN during baseline (BL), saline (Sal) and after DEP20 infusion. During the transitions between SWS and qW states the firing rate transiently increased but dramatically increased after infusion of DEP20. The colors on the abscissa represent blue, grey and green for the qW SWS and aW states, respectively. Lower right panel. Inhibitory response of a representative pChAT neuron for the BL, Sal and
DEP20 epochs. **B**, Population responses of 118 (of 154) neurons recorded during the BL, Sal and DEP20 epochs. The activity of each neuron is normalized to its z-score value and is plotted in a color-coded population PSTH. It is seen that 68% (n = 104; black overlap line indicate their average population response) were inhibited by DEP20 and 9% (n = 14) were activated. The other neurons (23%) were unaffected. The horizontal dotted white line indicates the division between inhibited activated responses.

**Figure 4. DEP20 increases oscillations in the NAc shell.** **A**, Raw traces of local field potentials (LFPs) recorded while an animal was in the: quiet awake (blue; qW), slow wave sleep (grey; SWS) and active awake (green; aW) states, the latter shown 15 and 51 minutes after intragastric infusion of DEP20. **B**, Spectrogram of the LFPs taken from 1 to 30 Hz for the baseline, saline and DEP20 epochs (recorded on Day 1). The animal’s locomotor activity is shown as a thin white trace (scale on right). Note that after DEP20 rats are primarily in the active awake state (aW- green; Table 3). The delta power (1-4 Hz) increases during SWS (panel below) and exhibits larger amplitude after DEP20. The colored numbers (below) indicate the times from where the raw LFP’s traces were obtained (plotted in panel **A**). **C**, The normalized and smoothed power spectral densities (PSDs) at the beta (15-30), theta (6-9) and delta (1-4 Hz) frequency bands obtained for baseline, saline and DEP20 epochs across the entire 14 day treatment period. During the DEP20 epoch delta oscillations increased whereas beta and theta oscillations decreased. **D**, Locomotor activity measurements (cm/min) obtained during baseline and saline and DEP20 epochs. These data show that DEP20 clearly increases locomotion (also Table 6).

**Figure 5. Behavioral and neuronal changes of NAc shell activity across 14 days of DEP20 treatment.** **A**, Plots of weight loss and food intake over 14 day treatments of saline or DEP20. With intragastric saline the animal’s weight monotonically increased across days whereas for DEP20, after a decrease that lasted until day 9, the animals gained weight at the same rate as the saline treated animals. The DEP20 group transiently reduced food intake then it increased with increasing treatment days. Purple indicates day 1, orange days 2-7 and brown days 8-14. **B**, A plot of the neuronal population changes observed across repeated intragastric DEP20 infusions. The colors codes are the same as in A. Relative to treatment day 1, during days 2-7, DEP20 increased global firing rates during baseline and saline epochs, whereas after DEP20 for days 8-14 the activity decreased. For days 8-14 relative to baseline the activity is lower in all three epochs. The right panel shows the normalized activity in the baseline, saline and DEP20 epochs. The latter reveals a much larger inhibition during days 8-14. The inhibitory to activated responses are also presented. **C**, Changes in the LFP’s PSDs at beta, theta and delta frequencies across days. All DEP20-evoked oscillations were positive at day 1 but all decreased across days with the beta and delta oscillations becoming negative at days 8-14. The lowest panel depicts the locomotion evoked by DEP20 across days.
Figure 6. DEP20 induces taste aversion to a novel 0.1% saccharin solution, but DEP20-induced LFP’s oscillations are unrelated to an aversive-malaise brain state. A. Displays the daily 15 min intake (in mL) of water (W1-3) and sodium saccharin (ACQ, TEST and EXT1-3). The arrow, in ACQ day, indicates the time of injection of LiCl or DEP20, 15 min after consumption of the novel saccharin. The TEST session occurred 72 h after ACQ day, note that both groups rejected saccharin at similar levels, but during the EXT1-3 days, DEP20 extinguishes the taste aversion faster than the LiCl group (* P <0.05; RM ANOVA; EXT1-3). B, Spectrogram of the NAc shell LFP for the Baseline, Saline, LiCl (0.4M) and DEP20 epochs, and its corresponding hypnogram is displayed below. The white trace shows the animal’s locomotor activity (scale on right). C, The normalized power spectral densities (PSDs) at the beta (15-30), theta (6-9) and delta (1-4 Hz) frequency bands across the experiment. D, Average locomotor activity (cm/min) obtained during each epoch uncovers that LiCl upsets the animal, resulting in distress movements, but not stereotypy and exploratory locomotion.

Figure 7. The appetite suppressants phentermine (PHEN) and bupropion (BUP) also modulate appetite, locomotion and NAc shell activity. A, A graph showing the change in weight over a seven-day intragastric treatment (shaded) with saline, PHEN20, BUP30 and DEP20. Relative to saline, the three treatments produced a significant weight loss with DEP20 exhibiting the greatest loss. The weight loss with BUP30 was significant for only 2 days. In contrast to DEP20, during treatment PHEN20 and BUP30 treated animals began to recover their weight. Panel below: For the same animals as in “A” the change in 24 h food intake was unmodified with saline infusions, but transiently decreased with PHEN20 and DEP20. BUP30 gradually and slightly decreased food intake during treatment. B, The effect on locomotion of repeated infusions of PHEN20, BUP30 and DEP20 measured in an open-field arena. All groups displayed a gradual decrease in exploratory activity within 20 min from introduction into the open field (Baseline; BL). Intragastric infusions were then made at 30 min intervals with saline and at 60 min the corresponding appetite suppressant. Panel below: The quantification of head weavings (stereotypy) caused by these three appetite suppressants. Relative to saline, DEP20 induced the largest effect and PHEN20 and BUP30 the smallest. C, A color-coded PSTH showing the number of NAc shell neurons that were inhibited or activated by PHEN20 (left panel) and BUP30 (right panel). Each neuron was normalized to z-score values (see Methods). The black tracings are the mean PSTH of the inhibited and activated responses. The horizontal line separates these populations. D, The mean LFP’s normalized PSDs at beta, theta and delta frequencies during baseline, saline and PHEN20 and BUP30 epochs. Both compounds increased the LFP’s oscillations for the three frequency bands. The bottom most panels show that PHEN20 and BUP30 both increase locomotion, albeit with a delay.

Figure 8. Intragastric dopamine receptor antagonists D1R (SCH23390- SCH1.5) and D2R (Raclopride- RAC0.5) attenuate DEP20’s effect on weight loss, food intake,
locomotion and stereotypic-head movements. A, The change in body weight during seven days of treatment with daily intragastric infusions of saline (○), RAC0.5 (□), SCH1.5 (◇), DEP20 (▲), DEP20+RAC0.5 (■) and DEP20+SCH1.5 (♦) starting on day 1 and terminating on day 7 (shading). It is seen that the weight loss produced by DEP20 is reduced in the presence of either RAC0.5 or SCH 1.5. Note that neither D2 (□) nor D1 (◇) antagonists alone induced weight loss. B, A graph showing the change in 24 h food intake under the same conditions as in panel “A.” Infusion of DEP20+RAC0.5 or DEP20+SCH1.5 increased the food intake compared with that of DEP20 alone. C, The effect of these DA antagonists on locomotion in the same subjects and grouping as in “A” measured in an open-field arena. All groups displayed a gradual decay on exploratory activity within 20 min from introduction to the open field (Baseline; BL). Intragastric infusions were then made at 30 min intervals with saline, at 60 min DEP20 and at 90 min with and/or without RAC0.5 or SCH1.5. It is seen compared with DEP20 alone that repeated administrations of DEP20+RAC0.5 or DEP20+SCH1.5 decreased the magnitude and delayed the onset on locomotion. D, A graph showing the reduction in the stereotypy induced by DEP20+RAC0.5 or DEP20+SCH1.5.

Figure 9. IntraNAc shell infusion of either D1R (SCH) or D2R (RAC) antagonists attenuated the effect of intragastric infusion of DEP20's induced effects on weight loss, food intake, locomotion and stereotypic-head movements. A, The change in body weight (in g) across seven days of treatment: Note that all groups received two injections; one directly in the NAc shell (Sal, SCH or RAC) and a second one intragastric (Sal or DEP20). Thus, the group Sal+Sal received saline (intraNAc) + saline (intragastric), whereas the group Sal+DEP20 received saline (IntraNAc) + DEP20 (intragastric), and so on for all other groups. B, A graph showing the change over 7 days in Chow food intake per 24 h with the same protocol as in A. C, Displays the effect of SCH and RAC antagonists upon locomotion measured in the open-field arena, in the same subjects shown in “A”. At 20 min animals were briefly removed from the open field and received an intra-NAc infusion –note the interruption in the x-axis at 20-25 min. At 30 min all animals received the corresponding intragastric infusion. D, A graph plotting head weaving stereotypy under the same conditions as in C.

Figure 10. Intragastric dopamine D1R and D2R antagonists reverse the DEP20-induced inhibition/activation asymmetry in the rat’s NAc shell. A, A plot of the activity of 64 (of 81) NAc shell neurons that were significantly inhibited (30%) or activated (48%) by DEP20 across 7 days of chronic treatment of RAC0.5. Each of the four epochs was 30 min long. Note that compared to DEP20 alone (only using neurons recorded over 7 days from Fig. 3B) the percentages of neurons inhibited during the DEP20 epoch in the presence of chronic treatments of RAC0.5, decreased and the percentage activated increased. The black traces are the mean activity of the two respective categories. B, The grand average of 9 experiments showing the normalized PSD at delta frequency from the same experiments.
shown in upper panel over all days. It is seen that infusion of RAC0.5 greatly diminished the changes in all DEP20-induced oscillations. Note that infusion of RAC0.5 alone (thinner traces) did not change LFP’s oscillations. The panel below shows that the locomotion increases evoked by DEP20 are also decreased by RAC0.5 (cyan), whereas RAC0.5 alone did not increase locomotion (pink trace). C, A plot of the normalized global population responses of all neurons recorded (n=131) with DEP20 alone (green, same data as in Fig. 3, aligned to DEP injection time= 0 min) vs. all 81 neurons recorded with DEP20 with chronic treatment of RAC0.5 (cyan trace need over same treatment days, DEP20+RAC0.5). Note that after the infusion of RAC0.5 the population-firing rate returned to near baseline levels. Finally, the infusion of RAC0.5 alone did not produce any inhibitory imbalance (pink trace). The color lines below indicate the bins (1 min resolution) with significant increases (red) or decreases (blue) of population activity relative to saline firing rates (time interval = -30 to 0 min). D, Plots results showing repeated administration of two doses of D1R antagonist (SCH1.5 and SCH 3) infused after DEP20. The firing rate modulation of 91 out of 112 (and 21 out of 34) single neurons recorded in the NAc shell, during 30 min epochs of BL, Sal and DEP20, SCH1.5 and DEP20, SCH3 infusions, respectively. It is seen that under repeated administration SCH, DEP20 does not produce a robust spiking inhibition (SCH1.5 (63%) and SCH3 (41 %) compared with DEP20 alone (63%-82/131). Rather it increases its activation (SCH1.5 (18%) and SCH3 (21%) compared with DEP20 alone (11%-14/131)). E, Plots the LFP’s PSD (upper) and locomotor activity (below) induced by SCH1.5 (red trace) and 3 (blue trace), SCH1.5 alone (black trace). D1R antagonist attenuates in a dose dependent manner the DEP20-induced delta oscillations and delayed the onset and diminished the magnitude of locomotion evoked by DEP20. Note all oscillations are positive. The infusion of SCH1.5 alone (see thinnest lines) does not change LFP’s oscillations, values around 0 z-score. F, The average firing rate of all neurons recorded under repeated treatment of DEP20 followed by SCH at a dose of 1.5 (red trace) and 3 mg/Kg (blue trace), and SCH1.5 alone (black trace). Note that SCH23390 in a dose-dependent manner reversed the inhibition/activation imbalance induced by DEP20.
# Table 1. Characteristics of putative cell types of NAc Shell

<table>
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<tr>
<th>Experiment</th>
<th>Waveform Shape</th>
<th>Putative cell types</th>
<th>FR (Hz)</th>
<th>CV2</th>
<th>VAR</th>
<th>V-P width (ms)</th>
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<tbody>
<tr>
<td>DEP20</td>
<td><img src="image1" alt="waveform" /></td>
<td>pSPN (n=47)</td>
<td>3.18±0.37</td>
<td>1.01±0.01*</td>
<td>0.72±0.02</td>
<td>0.32±0.01</td>
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<td></td>
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<td>pFSI (37)</td>
<td>12.07±1.68*</td>
<td>0.81±0.01</td>
<td>0.64±0.03</td>
<td>0.30±0.01*</td>
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<td>pChAT (36)</td>
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<td>0.69±0.01</td>
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<td>Unidentified (34)</td>
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<td>pChAT (19)</td>
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<td>BUP30</td>
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<td>pFSI (20)</td>
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<td>pChAT (19)</td>
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<td></td>
<td>Unidentified (29)</td>
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<td>0.99±0.01</td>
<td>0.73±0.02</td>
<td>0.24±0.01</td>
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CV2, coefficient of variation 2; VAR, valley to amplitude ratio; V-P, valley to peak width. Values are mean±SEM. *P < 0.05, comparison across putative cell-types.
### Table 2. Firing rates as a function of drug

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<tr>
<th>Drug</th>
<th>Type of response</th>
<th>Firing rate (Sp/s) per 60 min epoch</th>
<th>Baseline ± SEM</th>
<th>Saline ± SEM</th>
<th>Drug ± SEM</th>
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<td>DEP20</td>
<td>days 1-14</td>
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<td>3.1 ± 0.01</td>
<td>3.1 ± 0.04</td>
<td>7.9 ± 0.18 *</td>
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<tr>
<td></td>
<td>Activated (n=14)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Inhibited (n=104)</td>
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<td>5.3 ± 0.01</td>
<td>5.6 ± 0.01</td>
<td>1.8 ± 0.04 *</td>
</tr>
<tr>
<td></td>
<td>days 1-7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Activated (n=14)</td>
<td></td>
<td>3.1 ± 0.01</td>
<td>3.1 ± 0.04</td>
<td>7.9 ± 0.18 *</td>
</tr>
<tr>
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<td>Inhibited (n=82)</td>
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<td>5.7 ± 0.01</td>
<td>6.0 ± 0.03</td>
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<td>PHEN20</td>
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<td>2.6 ± 0.02</td>
<td>6.2 ± 0.08 *</td>
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<td>Activated (n=3)</td>
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<td>Inhibited (n=38)</td>
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<td>3.9 ± 0.01</td>
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<td>Activated (n=16)</td>
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<td>3.6 ± 0.01</td>
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Values are mean ± SEM. * P < 0.05, comparison across epochs.
Table 3. *Time spent in each behavioral state across 60 min epochs*

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<td>Baseline</td>
<td>Saline</td>
<td>Drug</td>
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<td></td>
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<td>17.7 ± 1.9</td>
<td>18.9 ± 1.6</td>
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<td>0.7 ± 0.2 *</td>
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<td>aW</td>
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<td>13.9 ± 2.7</td>
<td>18.3 ± 2.2</td>
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<td>PHEN20</td>
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<td>BUP30</td>
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<tr>
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<td>aW</td>
<td>13.9 ± 2.7</td>
<td>18.3 ± 2.2</td>
<td>52.3 ± 2.3*</td>
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Values are mean ± SEM. * P < 0.05 significantly different from both baseline and saline epochs. # P < 0.05 significantly different from saline epoch. qW = quiet wake; SWS = Slow Wave Sleep; aW = active awake.
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<th>Drug</th>
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<th>pFSI</th>
<th>pChAT</th>
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<tr>
<td>DEP 20</td>
<td>days 1-14</td>
<td>Activated 14 (9%)</td>
<td>10/47 (21%)</td>
<td>1/37 (3%)</td>
<td>1/36 (3%)</td>
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<tr>
<td></td>
<td>Inhibited 104 (68%)</td>
<td>24/47 (51%)</td>
<td>24/37 (65%)</td>
<td>28/36 (78%)</td>
<td>28/34 (82%)</td>
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<tr>
<td></td>
<td>days 1-7</td>
<td>Activated 14 (11%)</td>
<td>10/41 (24%)</td>
<td>1/34 (3%)</td>
<td>1/27 (4%)</td>
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<td>Inhibited 82 (63%)</td>
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<td>21/34 (62%)</td>
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<td>23/29 (79%)</td>
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<td>PHEN 20</td>
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<td>Activated 3 (5%)</td>
<td>1/16 (6%)</td>
<td>0/9 (0%)</td>
<td>0/19 (0%)</td>
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<td></td>
<td>Inhibited 38 (64%)</td>
<td>11/16 (69%)</td>
<td>5/9 (55%)</td>
<td>16/19 (84%)</td>
<td>6/15 (40%)</td>
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<td>BUP 30</td>
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<td>4/20 (20%)</td>
<td>0/19 (0%)</td>
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<td>Inhibited 48 (44%)</td>
<td>13/42 (31%)</td>
<td>6/20 (30%)</td>
<td>14/19 (74%)</td>
<td>15/29 (52%)</td>
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Values are number of neurons per indicated population within each cell-type, with percentages given in parentheses. *P < 0.05 comparison between activated vs. inhibited and #P < 0.05, comparison across all four putative cell-types.
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<tr>
<th>Drug</th>
<th>Frequency band</th>
<th>Z-score PSD of NAc Shell’ LFP for epoch</th>
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<td>Baseline</td>
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<td>Drug</td>
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<td>0.069±0.004#</td>
<td>0.979±0.025 *</td>
</tr>
<tr>
<td>PHEN20</td>
<td>days1-7</td>
<td>Beta</td>
<td>0.002±0.006</td>
<td>0.106±0.007#</td>
<td>0.913±0.029 *</td>
</tr>
<tr>
<td></td>
<td>days1-7</td>
<td>Theta</td>
<td>0.030±0.004</td>
<td>0.051±0.005</td>
<td>0.863±0.007 *</td>
</tr>
<tr>
<td></td>
<td>days1-7</td>
<td>Delta</td>
<td>0.018±0.004</td>
<td>-0.085±0.006#</td>
<td>1.121±0.030 *</td>
</tr>
<tr>
<td>BUP30</td>
<td>days1-7</td>
<td>Beta</td>
<td>-0.024±0.005</td>
<td>0.002±0.006</td>
<td>0.290±0.031 *</td>
</tr>
<tr>
<td></td>
<td>days1-7</td>
<td>Theta</td>
<td>0.068±0.003</td>
<td>-0.143±0.004</td>
<td>0.098±0.007 *</td>
</tr>
<tr>
<td></td>
<td>days1-7</td>
<td>Delta</td>
<td>0.047±0.004</td>
<td>-0.257±0.004#</td>
<td>0.177±0.031 *</td>
</tr>
</tbody>
</table>

Values are mean±SEM. * P < 0.05, significantly different from both baseline and saline epochs. # P < 0.05 significantly different from baseline epoch.
**Table 6. Behavioral and neuronal changes on NAc shell activity after repeated use of appetite suppressants**

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Periods</th>
<th>Relative to day before treatment</th>
<th>Activity</th>
<th>Baseline</th>
<th>Saline</th>
<th>Drugs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Change in bodyweight (g)</td>
<td>24 h chow intake (%)</td>
<td>Firing rate (Hz)</td>
<td>z-score (PSD)</td>
<td>Distance (cm/min)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>All (n=22)</td>
<td>Inhibited (19)</td>
<td>Activated (3)</td>
<td>Beta (15-30Hz)</td>
<td>Theta (6-9 Hz)</td>
</tr>
<tr>
<td>PHEN20</td>
<td>Days 1</td>
<td>- 9.5 ± 2.5</td>
<td>- 45.4 ± 13.8</td>
<td>6.3 ± 0.01</td>
<td>3.1 ± 0.01</td>
<td>-0.017 ± 0.003</td>
</tr>
<tr>
<td>DEP20</td>
<td>Days 8-14</td>
<td>- 1.8 ± 2.1</td>
<td>- 9.6 ± 2.9</td>
<td>6.3 ± 0.01</td>
<td>3.1 ± 0.01</td>
<td>-0.017 ± 0.003</td>
</tr>
<tr>
<td></td>
<td>Days 2-7</td>
<td>- 12.3 ± 1.9</td>
<td>- 29.4 ± 4.2</td>
<td>6.3 ± 0.01</td>
<td>3.1 ± 0.01</td>
<td>-0.017 ± 0.003</td>
</tr>
<tr>
<td>BUP30</td>
<td>Days 2-7</td>
<td>- 2.2 ± 1.1</td>
<td>- 15.7 ± 1.8</td>
<td>6.3 ± 0.01</td>
<td>3.1 ± 0.01</td>
<td>-0.017 ± 0.003</td>
</tr>
<tr>
<td></td>
<td>Days 2-7</td>
<td>- 7.9 ± 4.1</td>
<td>- 12.8 ± 5.5</td>
<td>5.1 ± 0.02</td>
<td>5.4 ± 0.01</td>
<td>0.030 ± 0.009</td>
</tr>
</tbody>
</table>

Values are mean ± SEM. * P< 0.05, comparison across periods
Gastric emptiness (g)

- Saline (n=9)
- DEP20 (n=9)
- CCK-8 (n=9)

* n.s.
A) LFPs004
B) LFPs005
C) LFPs007

1) Quiet Wake (qW)
2) Slow Wave Sleep (SWS)
3) 15 min after DEP20
4) 51 min after DEP20

B) Baseline Saline DEP20

Frequency (Hz) vs. Time (min)

Delta Theta Beta

C) z-score (PSD)

(n=22 sessions)

D) Distance (cm/min) vs. Time (min)

(n=22 sessions)
**A**

Baseline Frequency (Hz)

<table>
<thead>
<tr>
<th></th>
<th>5</th>
<th>10</th>
<th>15</th>
<th>20</th>
<th>25</th>
<th>30</th>
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</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td></td>
<td></td>
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</tbody>
</table>

**C**

Baseline Saline LiCl(0.4M) DEP

<table>
<thead>
<tr>
<th>Frequency (Hz)</th>
<th>30</th>
<th>25</th>
<th>20</th>
<th>15</th>
<th>10</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beta</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Theta</td>
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<tr>
<td>Delta</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**D**

Distance cm/min

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distance</td>
<td>1800</td>
<td>1234</td>
<td>3456</td>
<td>7890</td>
<td>2345</td>
</tr>
</tbody>
</table>

**Intake (mL/15 min)**

- **WATER**
- **SACCHARIN**

**LiCl 0.4M (n=5)**

**DEP20(n=7)**