Glucagon-like Peptide-1 Receptor Signaling in the Lateral Parabrachial Nucleus Contributes to the Control of Food Intake and Motivation to Feed

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Glucagon-like peptide-1 receptor signaling in the lateral parabrachial nucleus contributes to the control of food intake and motivation to feed

Running Title: lPBN GLP-1R signaling contributes to food intake control

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Abstract

Central glucagon-like peptide-1 receptor (GLP-1R) activation reduces food intake and the motivation to work for food, but the neurons and circuits mediating these effects are not fully understood. While lateral parabrachial nucleus (lPBN) neurons are implicated in the control of food intake and reward, the specific role of GLP-1R expressing lPBN neurons is unexplored. Here, neuroanatomical tracing, immunohistochemical, and behavioral/pharmacological techniques are used to test the hypothesis that lPBN neurons contribute to the anorexic effect of central GLP-1R activation. Results indicate that GLP-1 producing neurons in the nucleus tractus solitarius project monosynaptically to the lPBN, providing a potential endogenous mechanism by which lPBN GLP-1R signaling may exert effects on food intake control. Pharmacological activation of GLP-1R in the lPBN reduced food intake, and conversely, antagonism of GLP-1R in the lPBN increased food intake. Additionally, lPBN GLP-1R activation reduced the motivation to work for food under a progressive ratio schedule of reinforcement. Taken together, these data establish the lPBN as a novel site of action for GLP-1R-mediated control of food intake and reward.

Introduction

Glucagon-like peptide-1 (GLP-1) is a neuropeptide involved in the control of food intake and glycemia that is primarily released from preproglucagon-expressing enteroendocrine L cells in the small intestine and in nucleus tractus solitarius (NTS) neurons of the caudal brainstem (Holst, 2007). Given the increasing attention paid to: (1) the GLP-1 system as a target for obesity treatment (Astrup *et al*, 2009), (2) the role of central nervous system (CNS) GLP-1 receptor (GLP-1R) signaling in mediating long-acting GLP-1R agonist effects (Hayes *et al*, 2010; Kanoski *et al*, 2011a), and (3) the anatomically distributed nature of the control of energy balance (Grill and Hayes, 2012), it is important to expand the analysis of GLP1-R expressing nuclei to identify the neurons and circuits that contribute to its energy balance effects. Although GLP-1Rs are widely expressed throughout the brain, studies have focused mainly on GLP-1R signaling in the hypothalamus (McMahon and Wellman, 1998; Schick *et al*, 2003) and NTS (Hayes *et al*, 2009; Hayes *et al*, 2011). Only recently have the functional contributions of GLP-1R-expressing nuclei in the ventral tegmental area (VTA) and the nucleus accumbens (NAc) to food intake and reward been addressed (Alhadeff *et al*, 2012; Dickson *et al*, 2012; Dossat *et al*, 2011; Mietlicki-Baase *et al*, 2013a). Here, we investigate the role of endogenous and exogenous GLP-1R signaling in the parabrachial nucleus (PBN) in the control of feeding and motivation to feed.

The pontine PBN is an important neural processing hub that receives input from and projects to neurons in a variety of brain nuclei considered key contributors to energy balance and reward control. Monosynaptic connections from the NTS (both the rostral and caudal divisions) conveying visceral afferent signals (including taste and gastrointestinally-

derived satiation signals) are processed by PBN neurons (Blessing, 1997; Cho *et al*, 2002; Norgren, 1978). PBN neurons in turn project to various nuclei critical for the control of energy balance including but not limited to nuclei of the hypothalamus (Blessing, 1997; Norgren, 1976), amygdala (Blessing, 1997; Norgren, 1976), VTA (Miller *et al*, 2011), and NAc (Li *et al*, 2012). The lateral subnuclei of the PBN (lPBN), which receive visceral afferent information from the caudal NTS, also receive descending projections from several nuclei that are implicated in food intake control, including nuclei of the amygdala, bed nucleus of the stria terminalis, and the hypothalamus (Blessing, 1997; Zhang *et al*, 2011a). Previous studies have demonstrated that a variety of neurochemicals including endocannabinoids (DiPatrizio and Simansky, 2008), opioids (Chaijale *et al*, 2013; Wilson *et al*, 2003), γ-aminobutyric acid (GABA) (De Oliveira *et al*, 2011; Wu *et al*, 2009), glutamate (Wu *et al*, 2012) and melanocortin (Skibicka and Grill, 2009) act in the lPBN to alter food intake and/or reward processing, highlighting the relevance of additional focus on the lPBN as a hindbrain region relevant to the control of energy balance. Despite the presence of GLP-1R mRNA (Merchenthaler *et al*, 1999) and terminals of GLP-1-immunopositive fibers (Rinaman, 2010) in the PBN, a direct analysis of lPBN GLP-1R signaling in the control of feeding behavior remains unexplored.

The studies described here combine neuroanatomical tracing, immunohistochemical, and behavioral/pharmacological techniques to study the neural connections between NTS GLP-1-producing neurons and the lPBN, as well as the effects of lPBN GLP-1R signaling on feeding and motivation to work for food. Collectively, the results obtained indicate that GLP-1R signaling in the lPBN is physiologically relevant for the

control of food intake and motivation to work for food, putatively through monosynaptic projections from NTS GLP-1-producing neurons.

Materials and Methods

Subjects and Drugs

Adult male Sprague-Dawley rats (250-300g upon arrival; Charles River Laboratories, Wilmington, MA) were individually housed in hanging metal cages on a 12h light/12h dark cycle and had *ad libitum* access to standard pelleted chow (Purina Rodent Chow, 5001) and water except when otherwise noted. All procedures conformed to and received approval from the institutional standards of the University of Pennsylvania animal care and use committee.

The long acting GLP-1R agonist exendin-4 (American Peptide Co., Sunnyvale, CA) and GLP-1R antagonist exendin-(9-39) (Bachem Americas, Torrence, CA) were dissolved in artificial cerebrospinal fluid (aCSF). The monosynaptic retrograde tracer Fluorogold (Fluorochrome, LLC; Denver, CO) was diluted to 2% in distilled water.

Surgery

Rats received intramuscular ketamine (90mg/kg; Butler Animal Health Supply, Dublin, OH), xylazine (2.7mg/kg; Anased, Shenandoah, IA) and acepromazine (0.64mg/kg; Bitler Animal Health Supply) anesthesia and subcutaneous analgesia (2.0mg/kg Metacam; Boehringer Ingelheim Vetmedica, St. Joseph, MO) for all surgeries.

Unilateral 26-gauge guide cannulae (Plastics One; Roanoke, VA) were stereotaxically implanted in the lPBN or the cerebral aqueduct according to the following coordinates.

lPBN guide cannulae were positioned ±2.0mm lateral from midline, 0.6mm anterior to lambda, and 5.7mm ventral from skull surface using a 20° angle (negative slope in anterior to posterior direction) with the injector aimed 2.0mm below the end of the guide cannula. Cannula placements were histologically confirmed postmortem. A representative image of the injection site is depicted in **Figure 1C**. Animals with injection sites that were not within the lPBN were excluded from analyses. On this basis, two animals were excluded from Experiment 4, three animals were excluded from Experiment 5, and one animal was excluded from Experiment 8. Aqueduct guide cannulae were positioned anterior to the PBN, ±2.0mm medial from midline, 8.2mm caudal anterior from bregma, and 3.85mm ventral from skull using a 20° angle (negative slope in the lateral to medial direction). Cannula placements were functionally confirmed via measurement of the sympathoadrenal-mediated glycemic response to 5-thio-D-glucose (210µg/2µL in artificial cerebrospinal fluid, aCSF) injected into the aqueduct as previously described (Ritter *et al*, 1981). A post-injection increase in blood glucose level of at least 100% from baseline was necessary for subject inclusion.

Experimental Procedures

Experiment 1: lPBN Fluorogold tracing and NTS GLP-1 immunohistochemistry (IHC)

Using a protocol similar to one previously established in our laboratory (Alhadeff *et al*, 2012), rats (n=5) were lightly anesthetized and received a unilateral 300nl injection of 2% (w/v) Fluorogold (FG; Fluorochrome, LLC; Denver, CO) via an automated syringe pump (PHD Ultra; Harvard Apparatus; Holliston, MA) directed to the lPBN. Four days later, rats were deeply anesthetized and transcardially perfused with 0.1M pH 7.4 PBS (Boston

Bioproducts; Ashland, MA), followed by 4% paraformaldehyde (PFA; Boston Bioproducts; Ashland, MA). Brains were removed and postfixed in 4% PFA for 4h and subsequently stored in 20% sucrose in 0.1M PBS at 4°C overnight. Coronal sections (30µm) were cut from the caudal hindbrain using a cryostat (Leica 3050S; Leica Corp., Deerfield, IL) and collected serially in glass jars. Brain sections were stored at 4°C overnight until the start of IHC.

IHC for GLP-1 was conducted according to modified previous procedures (Alhadeff *et al*, 2012; Zhang *et al*, 2011b). Briefly, sections were treated consecutively with 1% H_20_2 in precooled methanol, 0.3% glycine, and blocking solution (5% normal donkey serum in 0.1M PBS with 2.5% TritonX); then incubated with the GLP-1 primary antibody [GLP-1-(7-37) antiserum; Bachem Americas, Torrance, CA] at a 1:2000 concentration in blocking solution for 18h at room temperature. Sections were then washed and incubated with the secondary antibody (Dylight 549; Jackson ImmunoResearch Laboratories, West Grove, PA) at a 1:500 concentration in blocking solution for 2h. Detection of FG autofluorescence was observed under a special filter (C-FL UV-2A; Nikon Instruments, Melville, NY) on a fluorescence microscope (Nikon 80i; Nikon Instruments, Melville, NY).

Brain sections were mounted on slides and coverslipped with Fluorogel (Electron Microscopy Sciences; Hatfield, PA). Using fluorescence microscopy (Nikon 80i; NIS Elements AR 3.0) at 10X and 20X magnification, neurons expressing FG and GLP-1 immunofluorescence were quantified from coronal sections (≥8 sections per brain) of the caudal brainstem between -14.8mm and -14.1mm from bregma, according to the stereotaxic atlas of Paxinos and Watson (Paxinos, 2005).

Experiment 2: Parenchymal dose selection: evaluation of food intake effects of GLP-1R agonist and antagonist delivery to the cerebral aqueduct

To ensure that doses used in subsequent lPBN experiments were subthreshold for effect when delivered into the cerebroventricular system, rats (n=9) maintained *ad libitum* on high-fat diet (HFD; 45% kcal/fat, Research Diets, New Brunswick, NJ) for 5 days and habituated to experimental procedures received a 200nl unilateral injection of aCSF, exendin-4 (0.025 or 0.05μg), or exendin-(9-39) (10 or 20μg) in the aqueduct in a within-subjects, counterbalanced experimental design immediately before the onset of the dark cycle. The effects of aqueduct-delivered exendin-4 and exendin-9 on HFD (vs. chow) intake were examined given that the drug effects are more pronounced with HFD when delivered to some central nuclei (see following experiments and (Alhadeff *et al*, 2012)). HFD intake was measured at 1h, 3h, 6h, and 24h accounting for spillage. Body weight and water intake was measured 24h post-injection. At least 48h elapsed in between drug injection conditions.

Experiment 3: Effects of lPBN GLP-1R activation on chow intake

Rats (n=15) were housed in a custom made, automated feedometer, which consists of hanging wire cages with a small access hole to a food cup resting on an electronic scale. The associated software (LabView) records the weight of the food cup every 10s. Following habituation to the cages and powdered standard chow for 5 days, rats received a 100nl unilateral injection of aCSF or exendin-4 (0.025 or 0.05μg) in a within-subjects, counterbalanced experimental design immediately before the onset of the dark cycle. These exendin-4 doses were determined to be subthreshold for a reduction in food intake

when delivered to the aqueduct cannula positioned just rostral from the PBN (*Experiment 2*). Automated food measurements were made for the 24h post-injection; body weight and water intake were manually recorded 24h post-injection. At least 48h elapsed in between drug injection conditions.

Experiment 4: Pica effects of lPBN GLP-1R activation

To determine whether reductions in feeding resulting from lPBN GLP-1R activation might be attributable to nausea/malaise, pica (the ingestion of nonnutritive substances) was assessed as previously reported (Alhadeff *et al*, 2012; Kanoski *et al*, 2012) in rats (n=8) maintained *ad libitum* on standard chow and habituated to *ad libitum* access to kaolin pellets (aluminum silicate; Research Diets; New Brunswick, NJ) for 4 days. For testing, rats received a 100nl unilateral lPBN injection of aCSF or exendin-4 (0.05μg) in a within-subjects, counterbalanced experimental design immediately before onset of the dark cycle. Food intake, body weight, and kaolin intake were measured 24h post-injection, accounting for spillage. At least 48h elapsed in between drug injection conditions.

Experiment 5: Effects of lPBN GLP-1R activation on high-fat diet intake

Rats (n=11) maintained on *ad libitum* HFD for 5 days received a 100nl unilateral injection of aCSF or exendin-4 (0.025 or 0.05μg) in a within-subjects, counterbalanced experimental design immediately before the onset of the dark cycle. HFD intake was measured manually at 1h, 3h, 6h, and 24h accounting for spillage. Body weight and water intake were measured 24h post-injection. At least 48h elapsed in between drug injection conditions.

Experiment 6: Effects of lPBN GLP-1R antagonist on standard chow or high-fat diet intake

Rats maintained *ad libitum* on standard chow (n=10) or on HFD (n=11) for at least 5 days received a 200nl unilateral injection of aCSF or exendin-(9-39) (20µg) in a within-subjects, counterbalanced experimental design immediately before the onset of the dark cycle. This dose was determined to be subthreshold for an effect on food intake when delivered to the aqueduct (*Experiment 2*). Standard chow or HFD intake was measured manually at 1h, 3h, 6h, and 24h accounting for spillage. Body weight and water intake were measured 24h post-injection. At least 48h elapsed in between drug injection conditions.

Experiment 7: Effects of lPBN GLP-1R signaling on progressive ratio (PR) operant responding for high-fat chocolate flavored pellets

Rats (n=15) maintained *ad libitum* on standard chow were habituated to high-fat (35%), chocolate-flavored pellets (Bio-Serv; Frenchtown, NJ) in their home cage and trained as previously described (Mietlicki-Baase *et al*, 2013b) to press a lever for these pellets at an FR5 schedule of reinforcement (5 lever presses required to receive 1 pellet). For all training sessions, the right lever was the active lever and an inactive left lever served as a control for non-conditioned increases or decreases in operant responding.

Rats were given two tests in a within-subjects design using a PR schedule of reinforcement, and they received one FR5 session on the day between tests. A 100nl unilateral lPBN injection of 0.025µg of exendin-4 or aCSF was delivered 4h prior to each PR test session in a within-subjects, counterbalanced experimental design. The 4h latency between injection and PR test was chosen to be in the middle of the 3-6h time point where

ad-libitum food intake is reduced by lPBN exendin-4 administration (*Experiments 3 and 5*). Animals were returned to their home cage for the 4h between injection and test session. During the PR test, the effort required to obtain each pellet increased exponentially throughout the session as previously described (Kanoski *et al*, 2013; Mietlicki-Baase *et al*, 2013b), using the formula $F(i)=5e^{0.2i}-5$, where F(i) is the number of lever presses required to obtain the next pellet at i=pellet number. The PR session ended when a 20min period elapsed without the rat earning a pellet.

Experiment 8: Effects of lPBN GLP-1R stimulation and blockade on activity

To determine whether alterations in feeding and food motivation resulting from lPBN GLP-1R activation might be attributable to changes in activity, rats [two groups, n=11 for exendin-4, n=10 for exendin-(9-39)] maintained *ad libitum* on standard chow were habituated to a plexiglass chamber (74cm long, 57.4cm wide, 24.7cm wall height) for 30min each for 3 consecutive days. For testing, rats received a 100nl unilateral lPBN injection of aCSF or exendin-4 (0.025µg), or a 200nl unilateral lPBN injection of aCSF or exendin-(9-39) (20µg) in a within-subjects counterbalanced experimental design, 4h prior to video recording (timing of injection designed to mimic that of *Experiment 7* – lPBN exendin-4 effects on progressive ratio responding). Rats were then placed in the Plexiglass chamber and recorded with a camera for 30min. At least 48h elapsed between drug injection conditions. Videos were analyzed using ANY-Maze software (Stoelting Co., Wood Dale, IL) for total distance traveled and "time active". To calculate time spent active, the data were analyzed using two different criteria were used for time inactive: 1s and 2s (e.g. the 1s criterion requires the animal to be completely still for 1s to be considered not active).

Statistical Analyses

Data for each experiment were analyzed separately using Statistica (version 7; StatSoft Inc., Tulsa, OK) and expressed as mean ± SEM. For all behavioral experiments, repeated measures ANOVA and *post hoc* Neumann-Keuls comparisons were made. Alpha levels were set to $\alpha=0.05$ for all analyses.

Results

Experiment 1: GLP-1-producing neurons in the NTS project monosynaptically to the lPBN

Analysis of caudal NTS brain sections showed an average of 16.6 ± 1.5 GLP-1-producing neurons and 104.8 ± 9.9 FG-expressing neurons (e.g. neurons that project directly from NTS to lPBN) per 30μm coronal brain section. Overall, 71.3% of FG-expressing neurons in the NTS projected ipsilaterally the FG-injected lPBN, consistent with a classic anatomical study showing that the majority of NTS projections to PBN are ipsilateral (Norgren, 1978). Quantification of the colocalization of GLP-1 and FG immunoreactivity showed that 23.2 ± 4.2% of ipsilateral NTS GLP-1-producing neurons and 10.6 ± 3.0% of contralateral NTS GLP-1 producing neurons project monosynaptically to the lPBN (**Figure 1B)**. A representative image of a double-labeled NTS GLP-1 producing neurons following lPBN-injected fluorogold is shown in **Figure 1A.**

Experiment 2: Parenchymal dose selection: evaluation of food intake effects of GLP-1R agonist and antagonist delivery to the cerebral aqueduct

Cerebral aqueduct delivery of exendin-4 at either dose did not significantly affect cumulative HFD intake at 1h [$F(2,16)=0.62$], 3h [$F(2,16)=0.25$], 6h [$F(2,16)=.1.59$], or 24h

[F(2,16)=1.17] compared to vehicle treatment (**Figure 2A**). Similarly, exendin-(9-39) delivered to the aqueduct did not significantly affect cumulative HFD intake at 1h [F(2,16)=3.54], 3h [F(2,16)=0.85], 6h [F(2,16)=2.27], or 24h. [F(2,16)=0.31] (**Figure 2B**).

Experiment 3: lPBN GLP-1R activation reduces standard chow intake

lPBN GLP-1R activation significantly reduced standard chow intake. There was a significant main effect of lPBN exendin-4 (0.025μg and 0.05μg) on cumulative food intake at 6h [F(2,28)=4.74, p<.05] and 24h [F(2,28)=4.51, p<.05], and on non-cumulative food intake from 3-6h [F(2,28)=4.11, p<.05]. Post-hoc comparisons showed that a significant reduction in cumulative food intake at 6h and 24h (**Figure 3A**) and non-cumulative food intake (**Figure 3B**) from 3-6h was observed with each dose of exendin-4 compared to vehicle treatment. There was a non-significant trend for lPBN exendin-4 effect on 24h change in body weight [F(2,28)=3.18, p=.057] (**Figure 3C**) and a significant main effect on 24h water intake [F(2,24)=6.41, p<.01] (**Figure 3D**).

Experiment 4: lPBN GLP-1R activation does not cause pica

lPBN exendin-4 (0.05μg in aCSF) delivery did not affect 24h kaolin intake compared to vehicle treatment [F(1,8)=1.42] (**Figure 3E**).

Experiment 5: lPBN GLP-1R activation reduces high-fat diet intake

lPBN GLP-1R activation significantly reduced high-fat diet (HFD) intake. There was a significant main effect of lPBN exendin-4 (0.025μg and 0.05μg) on cumulative HFD intake at 6h [F(2,20)=7.34, p<.01] and 24h [F(2,20)=20.89, p<.01] along with a significant main effect on non-cumulative HFD intake from 3-6h [F(2,20)=29.31, p<.01] and 6-24h

[F(2,20)=18.41, p<.01]. Post-hoc comparisons showed that each dose of exendin-4 significantly reduced cumulative food intake at 6h and 24h (**Figure 4A**), and non-cumulative food intake from 3-6h (**Figure 4B**), compared to vehicle treatment. There was a significant main effect of lPBN exendin-4 (reduction) on 24h change in body weight [F(2,20)=7.53, p<.01] (**Figure 4C**) and 24h water intake [F(2,20)=8.52, p<.01] (**Figure 4D**).

Experiment 6: lPBN GLP-1R antagonism increases the intake of standard chow and high-fat diet

lPBN GLP-1R antagonism significantly increased cumulative chow and HFD intake at 6h post-injection [F(1,9)=11.27, p<.01; F(1,10)=10.17, p<.01, respectively] (**Figure 5A, 5C**), and cumulative chow intake at 24h post-injection [F(1,9)=8.85, p<.05]. There was no change in 24h body weight in animals maintained on chow or HFD [F(1,9)=3.44; F(1,10)=0.37, respectively] (**Figure 5B, 5D**).

Experiment 7: lPBN GLP-1R activation reduces progressive ratio operant responding for high-fat, chocolate-flavored pellets

lPBN GLP-1R activation significantly reduced the number of pellets earned [F(1,14)=16.08, p<.01] (**Figure 6A**), as well as the number of active lever presses [F(1,14)=15.42, p<.01] (**Figure 6B**), compared to vehicle treatment in *ad libitum* fed rats. The number of presses on the inactive control lever was not influenced by lPBN exendin-4 delivery [F(1,14)=0.56] (**Figure 6A**).

Experiment 8: lPBN GLP-1R stimulation or blockade has minimal effects on overall activity

lPBN GLP-1R stimulation did not affect total distance traveled in the activity test [F(1,10)=2.18] (**Figure 7A**). lPBN GLP-1R stimulation also did not affect the total time active when the "1s" analysis of inactivity was assigned to the data [F(1,10)=0.79] (**Figure 7B**), but produced a significant reduction in activity when the "2s" analysis of inactivity was assessed [F(1,10)=5.75, p=.038) (**Figure 7C**). lPBN GLP-1R blockade did not affect total distance traveled in the activity test [F(1,10)=0.97] (**Figure 7D**). Likewise, lPBN GLP-1R blockade had no effect on total time active using the "1s" analysis [F(1,9)=1.57] (**Figure 7E**) or "2s" analysis [F(1,9)=0.79] (**Figure 7F**).

Discussion

Accumulating evidence supports a role for central GLP-1R signaling in the control of food intake that involves contributions from several anatomically distributed GLP-1R expressing nuclei including subnuclei of the hypothalamus [e.g. paraventricular nucleus (PVH) (McMahon *et al*, 1998), lateral (LH) and medial (VMH and DMH) nuclei (Schick *et al*, 2003)], the NTS (Hayes *et al*, 2009; Hayes *et al*, 2011), and most recently, the reward-associated VTA and NAc (Alhadeff *et al*, 2012; Dickson *et al*, 2012; Dossat *et al*, 2011; Mietlicki-Baase *et al*, 2013a). Evidence presented here establishes a role for GLP-1R signaling in neurons of the lPBN. Our results show that GLP-1-expressing neurons of the NTS project directly to the lPBN, and suggest that these projections are physiologically relevant for the control of food intake as antagonism of GLP-1R in the lPBN increases both standard chow and high-fat diet intake. When lPBN GLP-1R are activated by parenchymal agonist delivery, food intake and body weight are reduced, as is the motivation to work for palatable food.

Previous research shows dense projections from the caudal NTS to the PBN (Rinaman, 2010), as well as GLP-1R mRNA and GLP-1 immunoreactivity in the PBN (Merchenthaler *et al*, 1999; Rinaman, 2010). Those studies, however, left unresolved whether PBN neurons receive monosynaptic projections from NTS GLP-1-producing neurons to the PBN. Using a strategy similar to Larsen et al. (Larsen *et al*, 1997) and Alhadeff et al. (Alhadeff *et al*, 2012), neuroanatomical tracing combined with immunohistochemistry for GLP-1 showed that approximately 23% of ipsilateral and 10% of contralateral GLP-1-producing NTS neurons project directly to the lPBN. Although post-mortem analysis of PBN tissue shows that the 300nl injection spanned the majority of the PBN and was centered at the lPBN, we cannot completely exclude the possibility that (1) not all of the axon terminals in the PBN took up the Fluorogold tracer or (2) some of the injected FG could have spread outside of the lateral subnucleus of the PBN. Despite these minor methodological limitations, these neuroanatomical tracing data, together with the current pharmacological results showing that lPBN-directed GLP-1R antagonism increases food intake, provide evidence for an endogenous contribution of lPBN GLP-1R signaling to the control of food intake.

An important topic for further research is to determine which physiological signals activate these NTS-to-PBN GLP-1 neurons. One relevant class of signals to pursue is satiation signals arising from the gut in response to the ingestion of food, such as gastric distension and the release of cholecystokinin (CCK). Gastric distension activates NTS GLP-1 producing neurons (Vrang *et al*, 2003), and has also been shown to stimulate PBN neural activity through the visceral afferent pathway (Baird *et al*, 2001; Karimnamazi *et al*, 2002). Additionally, CCK, which is released from the intestine following the intake of fat and/or

protein (Ritter, 2004), stimulates NTS GLP-1 producing neurons (Hisadome *et al*, 2011). Thus, it is possible that GLP-1 is released in the lPBN as a consequence of activation of NTS GLP-1 neurons following post-prandial satiation signaling; this speculation requires direct investigation.

Although GLP-1 is released from enteroendocrine L cells in the intestine in addition to neurons in the NTS, GLP-1 is rapidly degraded by the enzyme dipeptidyl peptidase-IV (DPP-IV) so that its half-life is only 1-2 minutes (Holst, 2007). However, there are two long-acting, DPP-IV-resistant GLP-1R agonists that are FDA approved for the treatment of type-2 diabetes mellitus: exendin-4 (Byetta®, the GLP-1R agonist used in the current studies) and liraglutide (Victoza®). Following peripheral administration, these long-acting GLP-1R agonists can enter the brain (Goke *et al*, 1995) and activate central GLP-1R to reduce food intake (Kanoski *et al*, 2011b). Thus, it is possible that peripheral GLP-1R agonists may reach and act in the lPBN to reduce food intake and motivation. This untested idea has clinical implications for the development of therapeutics for the treatment of obesity.

Exendin-4 delivered to the lPBN, at doses subthreshold for effect when delivered ventricularly, significantly reduced cumulative chow (*Experiment 3*) and HFD (*Experiment 5*) intake at 6h and 24h. For both food types, non-cumulative effects of exendin-4 were strongest in the 3-6h time bin. GLP-1R antagonist delivery increased food intake with a latency of feeding effect similar to that seen with the agonist, providing a complementary time course for effects. It is interesting to note that several hours elapse between the agonist or antagonist injection and the time that the effects on intake are observed. A cautious explanation for this observation is that lPBN GLP-1R activation involves longer-

term changes in gene transcription and protein synthesis to elicit its energy balance effects, a notion that has been discussed previously in more detail (Grill *et al*, 2012; Hayes, 2012; Hayes *et al*, 2010). It is also likely that lPBN GLP-1R signaling not only results in cellular changes in the lPBN itself, but also engages other brain nuclei to exert effects on feeding and food reward, a process that may take hours to manifest. Elucidating the brain nuclei, projection pathways and neurotransmitter systems that are involved in the mediation of lPBN GLP-1R signaling induced changes in food intake is therefore a topic that warrants further research.

Recent evidence supports the idea that central GLP-1R signaling is involved in motivational aspects of food intake (Alhadeff *et al*, 2012; Dickson *et al*, 2012; Dossat *et al*, 2011; Mietlicki-Baase *et al*, 2013a). Here, we investigated whether lPBN GLP-1R signaling contributes to food reward. An interpretation of the current findings is that lPBN GLP-1R activation reduces food intake, at least in part, by reducing the motivation to work for food. Indeed, lPBN GLP-1R activation significantly reduced active lever presses and the number of food reinforcers obtained on a progressive ratio schedule of reinforcement where the effort required to obtain a reinforcer exponentially increased with each successive reinforcer earned. Given the direct anatomical connections between the PBN and other energy-balance relevant and reward-related brain nuclei, it is not surprising that lPBN GLP-1R activation contributes to the control of food intake and motivation to work for food. For example, there are monosynaptic connections from the PBN to the VTA (Miller *et al*, 2011) and the NAc (Brog *et al*, 1993; Li *et al*, 2012), brain nuclei of the mesolimbic pathway that are involved in modulating dopamine neurotransmission and food motivation. Indeed, recent evidence suggests that the PBN may provide synaptic information about gustation to

dopaminergic neurons in the VTA (Overton *et al*, 2014). Thus, lPBN GLP-1R-expressing neurons may be engaging these structures to exert effects on the motivation to feed. Future studies should determine the precise anatomical output(s) of lPBN GLP-1R-expressing neurons.

The complementary effects of lPBN GLP1-R agonist and antagonist delivery on feeding is consistent with an interpretation of a role for endogenous lPBN GLP-1R signaling in the control of food intake. However, there are a few potential alternate explanations for our findings that lPBN GLP-1R activation reduces food intake and the motivation to feed. Given that systemically administered GLP-1R agonists are associated with nausea in some humans (Buse *et al*, 2004; Buse *et al*, 2009) and can produce a conditioned taste avoidance in rodents (Kinzig *et al*, 2002), we wanted to examine the possibility that lPBN GLP-1R activation may be eliciting malaise which could contribute to the suppression of food intake following direct exendin-4 administration. Thus, pica, an experimental rodent model used to measure nausea/malaise (Andrews and Horn, 2006), was directly examined. Pica involves the consumption of nonnutritive substances (e.g. kaolin clay) in response to nausea-inducing agents (Andrews *et al*, 2006) and this model of nausea is especially useful in rodent species that lack the physiology to vomit. The finding that lPBN GLP-1R activation by exendin-4 does not induce pica indicates that the reduction in food intake by lPBN GLP-1R activation is not likely explained by nausea/malaise. This finding is interesting given that there are populations of neurons in the lPBN, especially in the external lateral subregion, that are activated by noxious stimuli (e.g. lipopolysaccharide or cisplatin) (De Jonghe and Horn, 2009; Elmquist *et al*, 1996; Gaykema *et al*, 2009). However, there is a dissociation between the food intake effects and malaise effects of central GLP-1R

signaling in some regions of the brain (Alhadeff *et al*, 2012; Kanoski *et al*, 2012; Kinzig *et al*, 2002), i.e., it is established that GLP-1R signaling in certain brain nuclei can cause reduction in food intake without signs of malaise, and vice versa. Based on the current data, it appears that the GLP-1 system in the lPBN is affecting food intake independent of nausea/malaise.

It is also possible that drug-induced effects on activity may underlie reductions in food intake and progressive ratio responding. Although, lPBN exendin-4 significantly reduced lever pressing in a progressive ratio test, the exendin-4 treated rats pressed the lever over 100 times indicating that they were able to execute and sustain operant responding. Nevertheless, *Experiment 8* was included to directly test whether lPBN GLP-1R agonism or antagonism affects activity. We found a small but significant reduction in activity by lPBN exendin-4 in one of the parameters measured in this experiment, presenting a potential confound in interpreting the progressive ratio results. Overall, however, data analyzed using several parameters align to suggest that there is no compelling evidence that lPBN GLP-1R activation or blockade affects activity; suggesting that the changes in food intake and motivation to obtain food observed in the current experiments are not likely explained by general changes in activity. While we favor reduced feeding motivation as an explanation for the reduction in food intake and operant responding by lPBN exendin-4, we acknowledge that GLP-1R stimulation may reduce several types of motivated behaviors (e.g. drug taking, sexual activity, etc.), including the motivation to feed. This is an interesting idea that is not directly addressed in these experiments but should be tested in future studies.

Collectively, results of a range of behavioral/pharmacological studies are consistent and show for the first time that lPBN GLP-1R signaling is physiologically relevant to the control of food intake and motivation to work for palatable food. That NTS GLP-1-producing neurons project monosynaptically to the lPBN provides an endogenous mechanism by which lPBN GLP-1R signaling may contribute to the control of food intake. These results may have broader implications for the development of future GLP-1-based pharmacological treatments for obesity and overconsumption of energy dense/highly-palatable foods.

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Figure Legends

Figure 1. Colocalizion of caudal NTS GLP-1 producing neurons and lPBN-injected Fluorogold. **A**, representative 20X magnification image of a coronal NTS section; red immunofluorescence represents Fluorogold-expressing neurons and green immunofluorescence represents GLP-1-expressing neurons. **B**, quantification of neurons in the caudal NTS showed that 23.2 ± 4.2% of ipsilateral NTS GLP-1 neurons and 10.6 ±3.0% of contralateral NTS GLP-1 neurons project monosynaptically to the lPBN (means ± SEM). **C**, representative image of lPBN injection site (white arrow). CB, cerebellum; lPBN, lateral parabrachial nucleus; scp, superior cerebellar peduncle; mPBN, medial parabrachial nucleus; 4V, 4th ventricle.

Figure 2. GLP-1R agonist (**A**) or antagonist (**B**) injected into the aqueduct just rostral from the level of the PBN had no effect on cumulative food intake (means ± SEM).

Figure 3. lPBN GLP-1R activation by exendin-4 reduced standard chow and water intake. **A**, cumulative chow intake; **B**, noncumulative chow intake; **C**, 24h change in body weight; **D**, 24h water intake; **E**, 24h kaolin intake (means ± SEM, *p<0.05).

Figure 4. lPBN GLP-1R activation by exendin-4 reduced high fat diet intake, body weight, and water intake. **A**, cumulative high fat diet intake; **B**, noncumulative high fat diet intake; **C**, 24h change in body weight; **D**, 24h water intake (means ± SEM, *p<0.05, **p<0.01).

Figure 5. lPBN GLP-1R antagonism by exendin-(9-39) increased standard chow and high fat diet intake. **A**, cumulative chow intake; **B**, 24h change in body weight for animals

maintained on chow; **C**, cumulative HFD intake; **D**, 24h change in body weight for animals maintained on HFD (means ± SEM, *p<.05, **p<0.01).

Figure 6. lPBN GLP-1R activation by exendin-4 reduced operant lever responding under a progressive ratio schedule of reinforcement for a high-fat chocolate-flavored reinforcer. **A**, number of active and inactive lever presses; **B**, number of reinforcers earned (means ± SEM, **p<0.01).

Figure 7. lPBN GLP-1R activation by exendin-4 had minimal effect on activity parameters: **A**, total distance traveled; **B**, total time active, with animal still for >1s to be considered inactive; **C**, total time active, with animal still for >2s to be considered inactive. lPBN GLP-1R blockade by exendin-(9-39) had no effect on activity: **A**, total distance traveled; **B**, total time active, with animal still for >1s to be considered inactive; **C**, total time active, with animal still for >2s to be considered inactive (means ± SEM, *p<.05).

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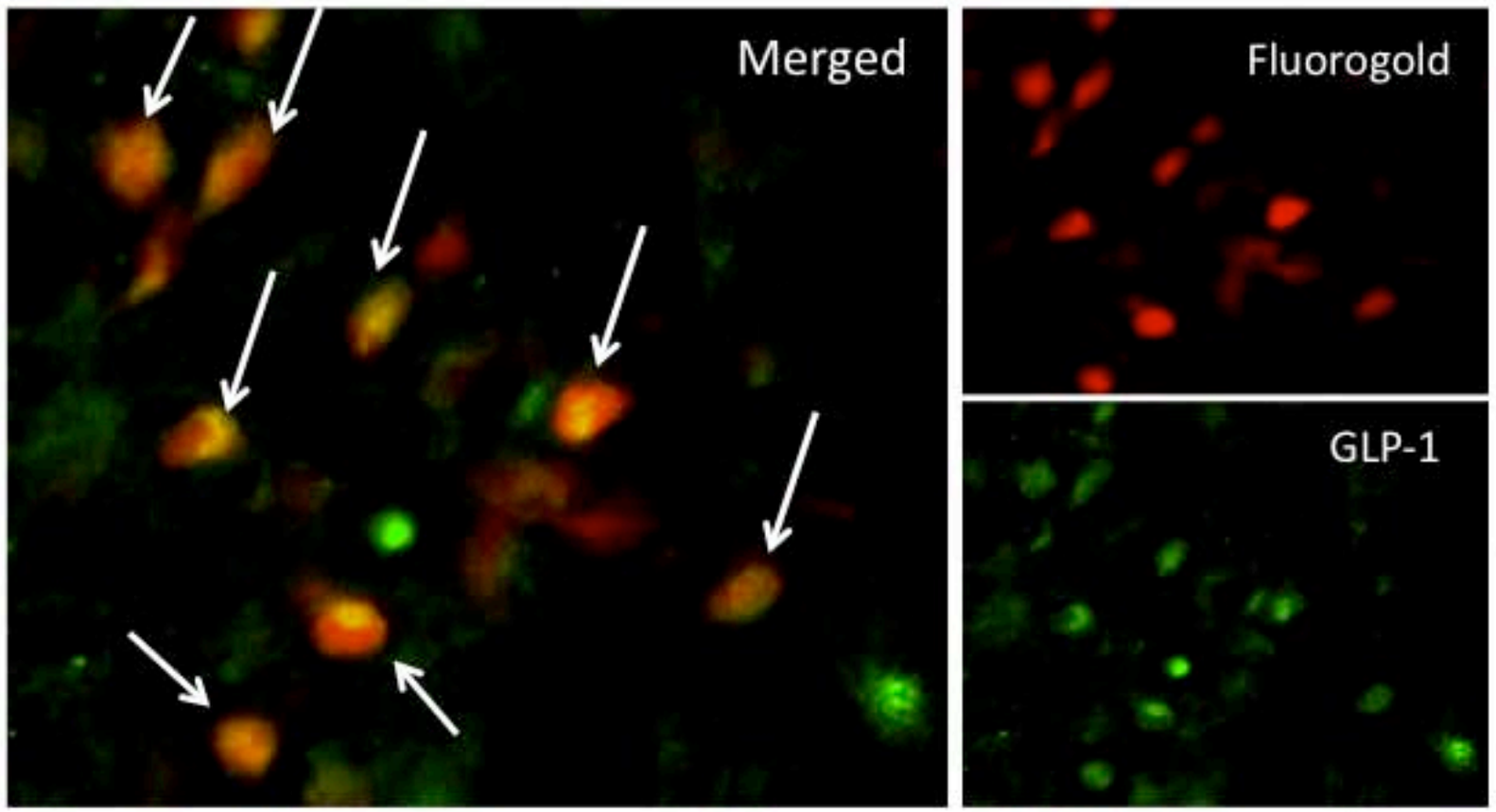
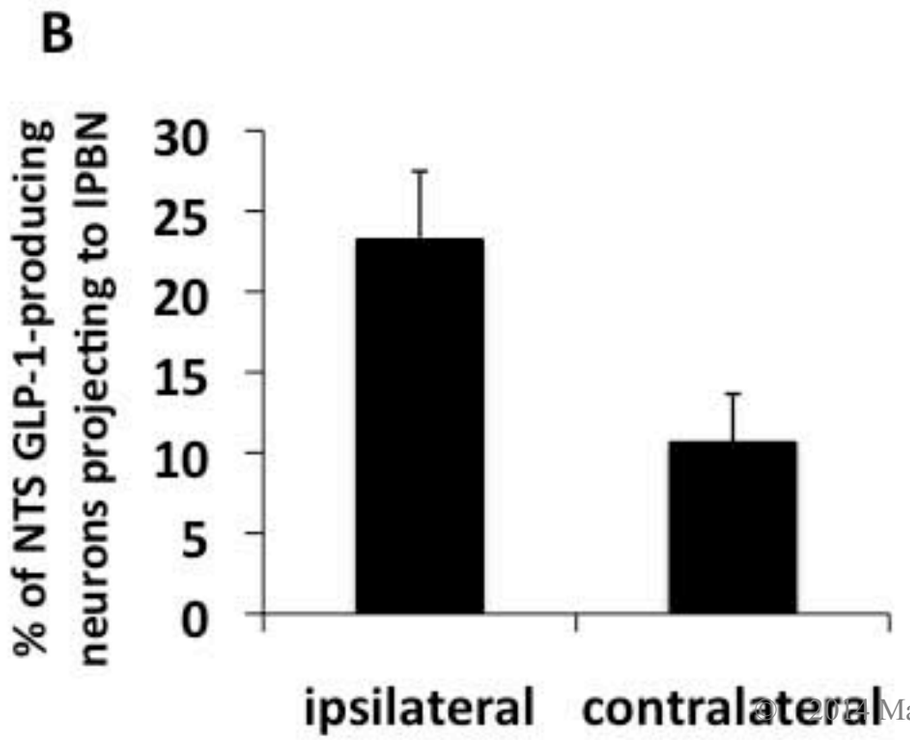
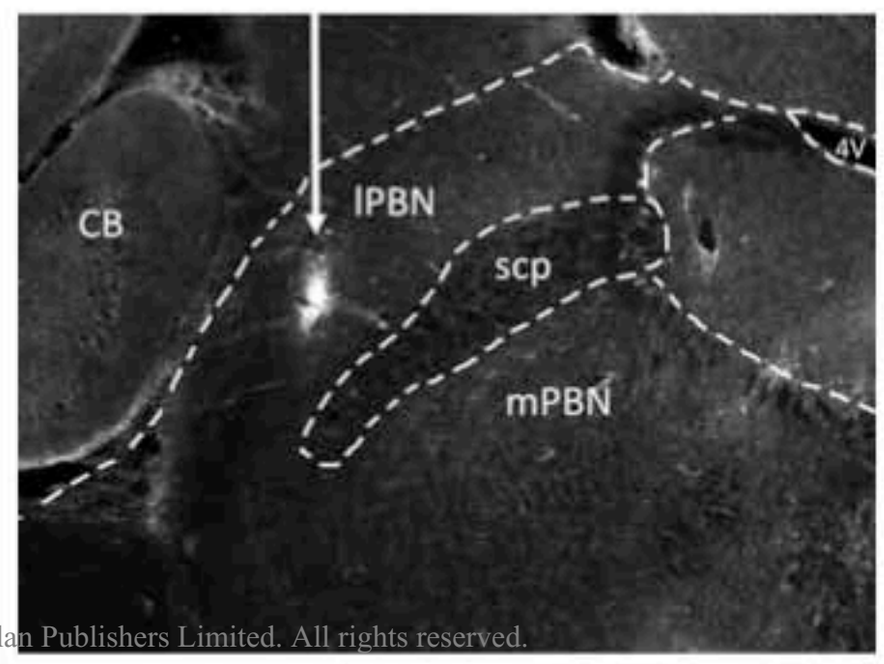
Figure 1







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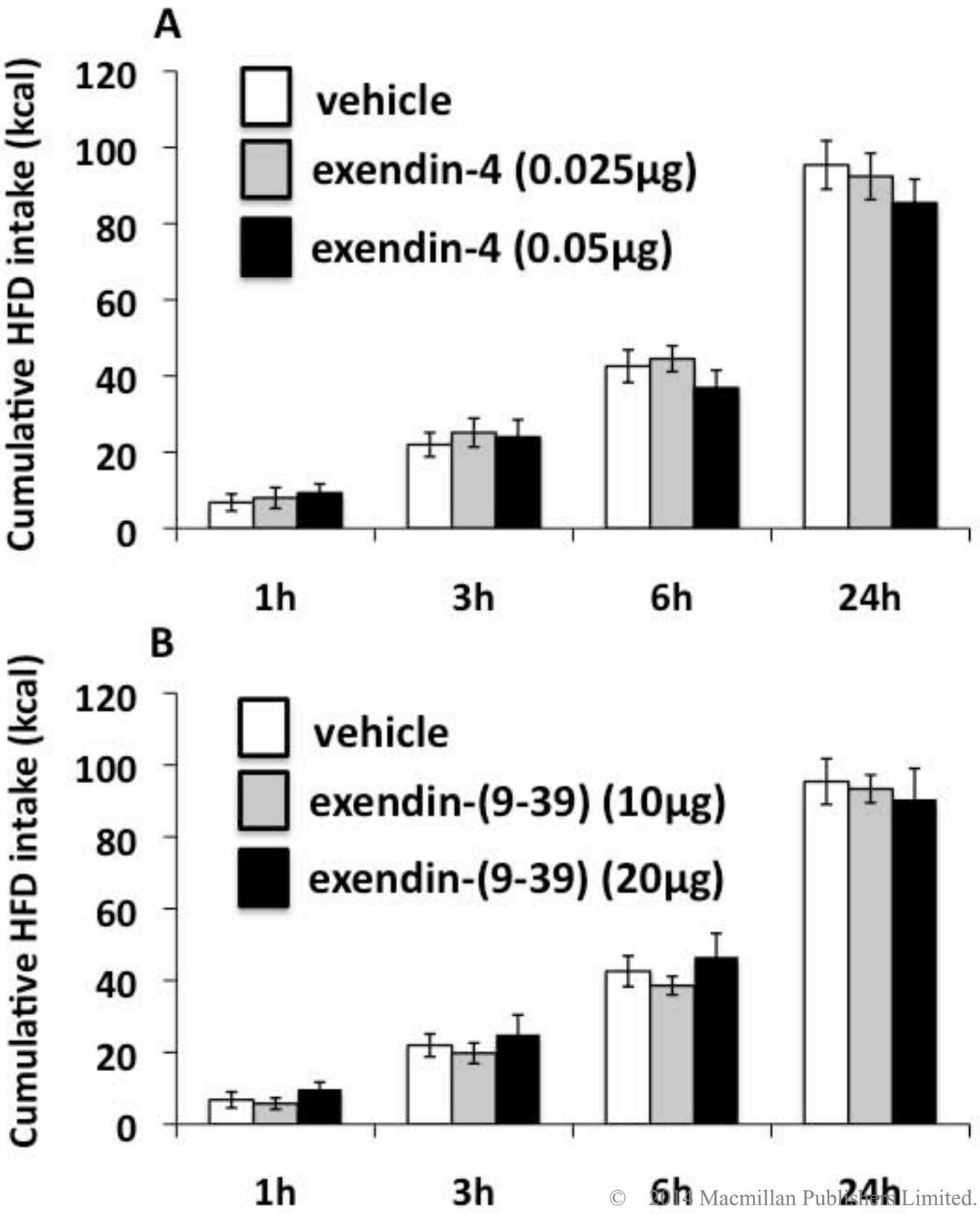

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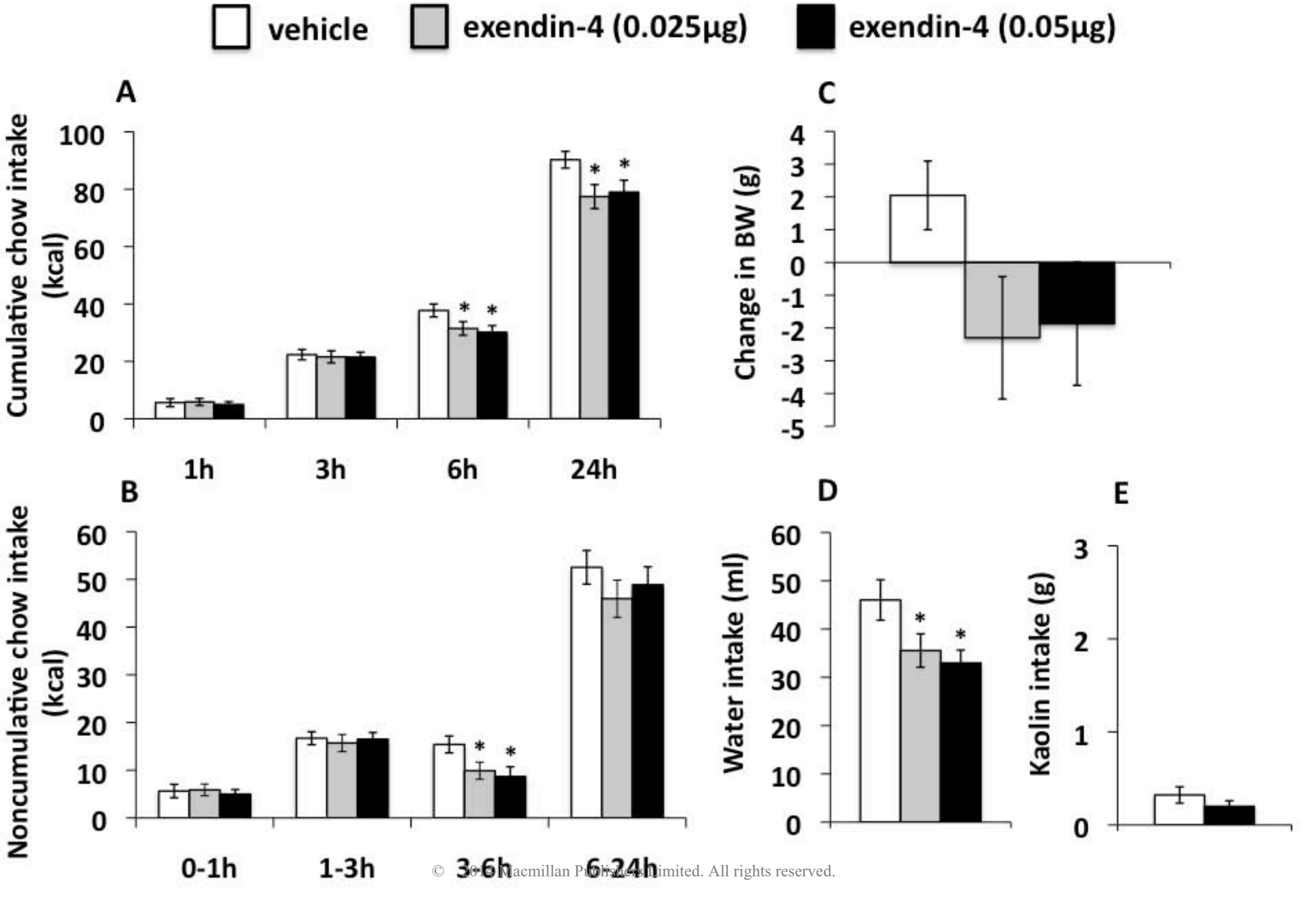

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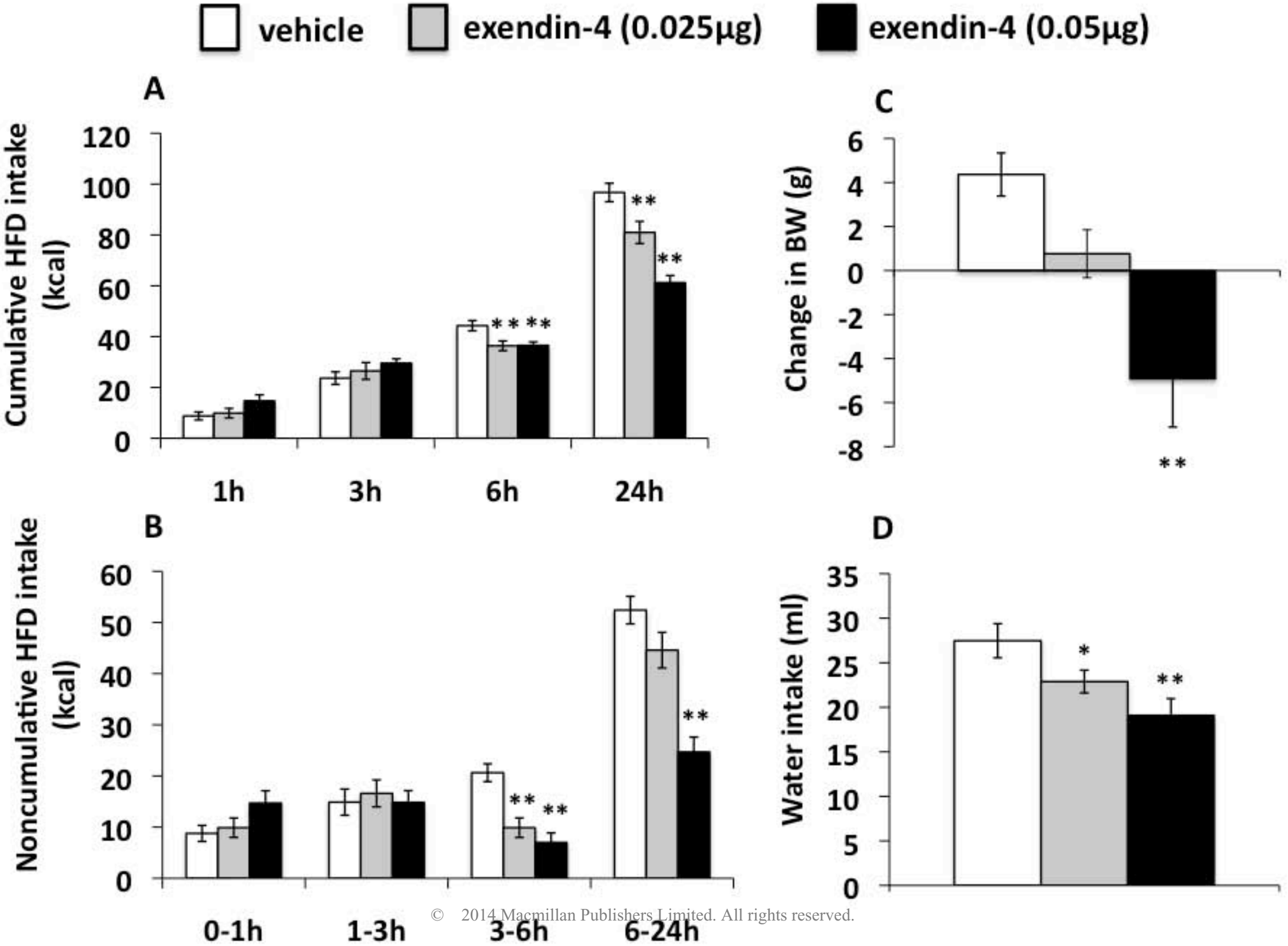

Figure 5

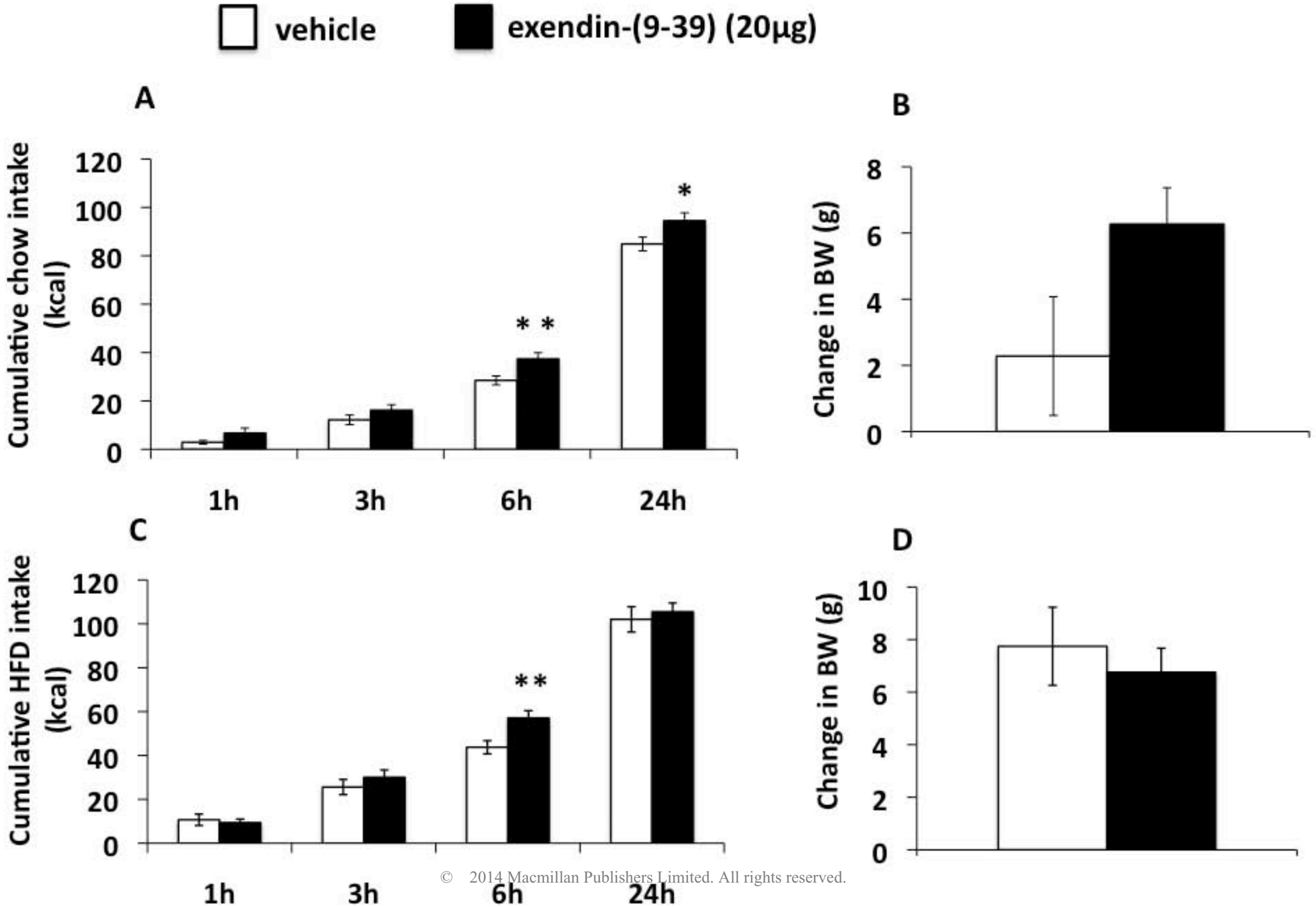

Figure 6

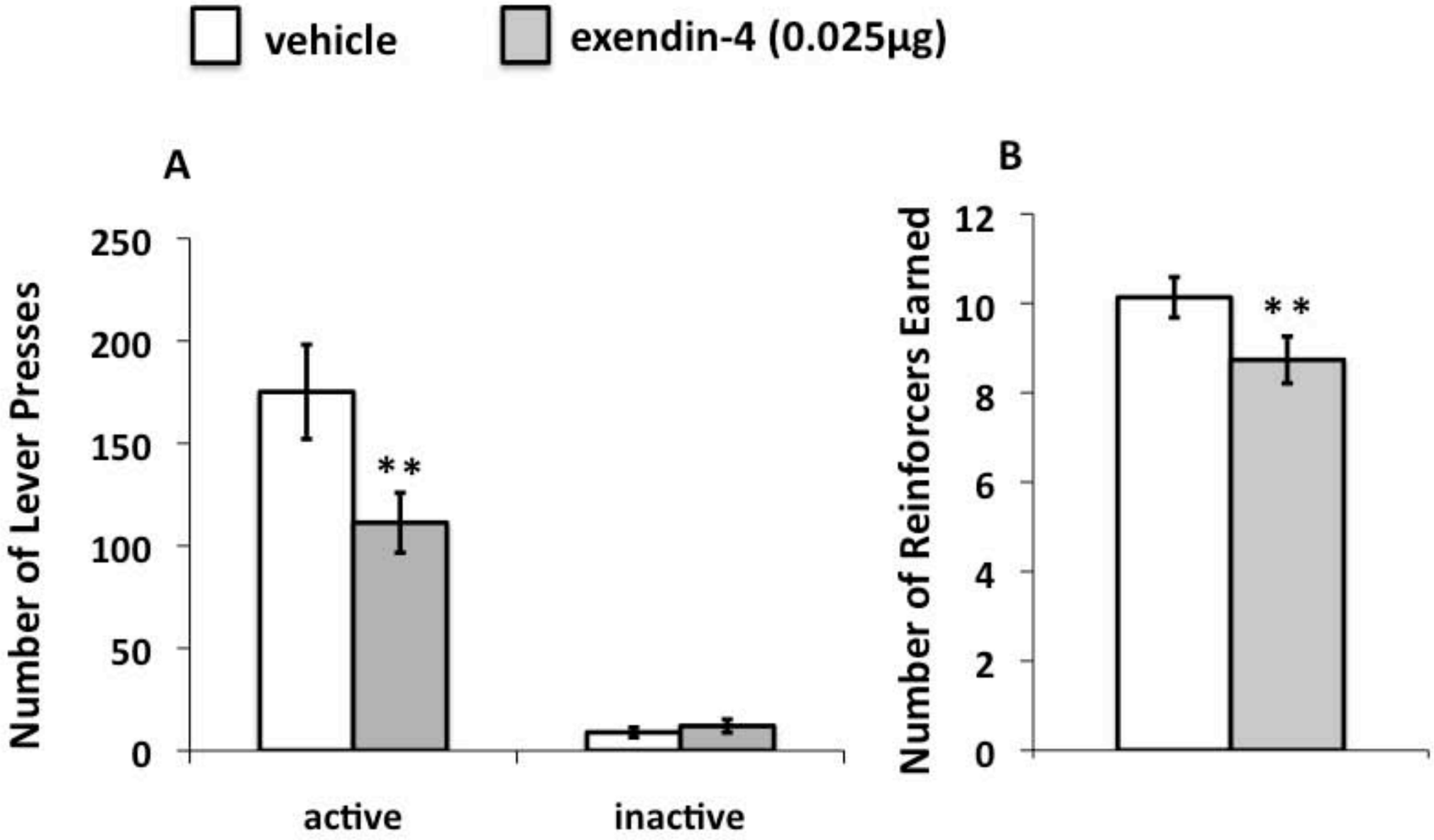

Figure 7