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# GLP-1 is both anxiogenic and antidepressant; divergent effects of acute and chronic GLP-1 on emotionality

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ABSTRACT

Glucagon-like peptide 1 (GLP-1), produced in the intestine and hindbrain, is known for its glucoregulatory and appetite suppressing effects. GLP-1 agonists are in clinical use for treatment of type 2 diabetes and obesity. GLP-1, however, may also affect brain areas associated with emotionality regulation. Here we aimed to characterize acute and chronic impact of GLP-1 on anxiety and depression-like behavior. Rats were subjected to anxiety and depression behavior tests following acute or chronic intracerebroventricular or intra-dorsal raphe (DR) application of GLP-1 receptor agonists. Serotonin or serotonin-related genes were also measured in the amygdala, DR and the hippocampus. We demonstrate that both GLP-1 and its long lasting analog, Exendin-4, induce anxiety-like behavior in three rodent tests of this behavior: black and white box, elevated plus maze and open field test when acutely administered intraperitoneally, into the lateral ventricle, or directly into the DR. Acute central GLP-1 receptor stimulation also altered serotonin signaling in the amygdala. In contrast, chronic central administration of Exendin-4 did not alter anxiety-like behavior but significantly reduced depression-like behavior in the forced swim test. Importantly, this positive effect of Exendin-4 was not due to significant body weight loss and reduced food intake, since rats pair-fed to Exendin-4 rats did not show altered mood. Collectively we show a striking impact of central GLP-1 on emotionality and the amygdala serotonin signaling that is divergent under acute versus chronic GLP-1 activation conditions. We also find a novel role for the DR GLP-1 receptors in regulation of behavior. These results may have direct relevance to the clinic, and indicate that Exendin-4 may be especially useful for obese patients manifesting with comorbid depression.



## 1. Introduction

Obesity is a major global health concern and significant efforts are now underway to find an effective pharmacotherapy to aid weight-reduction and metabolic health improvement. Since body weight and food intake are under the tight control of the central nervous system (CNS) (Berthoud, 2002) it is likely that effective anti-obesity therapeutics will target the CNS. With targeting the CNS, potential psychiatric side effects of any treatment should be closely examined, especially since many of the substances known to control body weight have also been shown to impact mood and emotionality. Recently withdrawn anti-obesity treatment, rimonabant, was revealed to induce significant psychiatric side effects, namely anxiety and depression, after the drug was already approved (Moreira et al., 2009). Animal models confirm this negative impact on mood (Navarro et al., 1997). Therefore, a very careful investigation of mood impact of any potential new treatment is warranted.

GLP-1 is well recognized for its role in energy balance regulation (Holst, 2007). It is produced in the intestinal L-cells and also in neurons primarily in the nucleus of the solitary tract (NTS) of the hindbrain (Merchenthaler et al., 1999). In addition microglia have recently been indicated as a potential CNS source of GLP-1 secretion (Kappe et al., 2012). Plasma GLP-1 levels, and also the activity of GLP-1 producing neurons increase in response to a meal (Parker et al., 2010). Analogs of GLP-1, including Exendin-4 (EX4), are approved for clinical use in patients with type 2 diabetes due to their glucoregulatory effects, effects thought to be

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primarily mediated by peripheral GLP-1 receptors (GLP-1R). GLP-1R activation also reduces food intake and body weight, effects largely mediated by the central GLP-1R (Trapp and Richards, 2013; Turton et al., 1996). Another GLP-1 analog, liraglutide, was just recently approved by the FDA for use in clinical populations of obese patients due to its weight-loss effect. Importantly, GLP-1 analogs were clearly shown to rapidly cross the blood brain barrier (Kastin and Akerstrom, 2003). GLP-1 is postprandially secreted with another peptide, glucagon-like peptide-2 (GLP-2) which has been shown to have similar effects on food intake in addition to effects in the gastrointestinal tract (Brubaker et al., 1997; Thulesen, 2004). Moreover GLP-2 receptors are expressed in many areas associated with depression such as the hippocampus and cerebral cortex, and has previously been shown to display anti-depressant like effects (Iwai et al., 2009; Lovshin et al., 2004; Nelson et al., 2007; Tang-Christensen et al., 2000). However, much less in known about its role in anxiety-like behavior.

GLP-1Rs are also found in areas not classically associated with energy balance regulation but rather regulation of mood and emotionality including the amygdala, the dorsal raphe (DR) and the hippocampus (Merchenthaler et al., 1999). The role of GLP-1R in the DR is entirely unexplored. Furthermore, little is known about the effects of GLP-1 on mood, and the few studies that are available produced conflicting results. Some preclinical studies found that CNS-directed GLP-1 injections in rodents may increase anxiety (Gulec et al., 2010; Kinzig et al., 2003), others show no changes in anxiety-like behavior (Krass et al., 2012, 2015), and recent studies even found an anxiolytic effect (Sharma et al., 2015a,b,c; Komsuoglu Celikyurt et al., 2014). While in one clinical study no anxiogenic properties were observed after intravenous administration of GLP-1 (Strawn et al., 2008). Another study in diabetic patients, receiving continuous 6-month treatment with a GLP-1 analog, did not find elevated anxiety and depression scores in quality of life tests, on the contrary scores were significantly reduced and the well-being scores were superior to those of the control group (Grant et al., 2011). Nonetheless studies on diabetic patients, while clinically relevant, make it difficult to decouple the potential beneficial effect of the treatment on mood from mood improvement associated with improvement of diabetic symptoms. Thus, further studies are still needed to understand the conflicting results of GLP-1 treatment on mood.

Here we aimed to understand both acute and chronic impact of central GLP-1 and its clinically utilized analog, EX4, on anxiety and depression-like behaviors. We used several complementary rat models of anxiety-like behavior: the elevated plus maze (EPM), the open field, and the black and white chamber test in combination with central and intraparenchymal DR-directed GLP-1 microinjections. We further evaluated whether both acute and chronic GLP-1 alters depression—like behavior in a forced swim test (FST) (Porsolt et al., 1977). Lastly, because of the important role of serotonin in mood, along with the pattern of results obtained with GLP-1 reminiscent of the effect seen with selective serotonin reuptake inhibitors (SSRIs), we evaluated the impact of central GLP-1R activation on serotonin turnover and expression of serotonin-related genes in key emotionality brain regions: the amygdala, the DR and the hippocampus.

## 2. Materials and methods

### 2.1. Animals

Adult male Sprague-Dawley rats, weighing 200–250 g (Charles River, Germany) were housed in individual plastic cages under 12/12 h dark/light cycle, at 20 °C and 50% humidity. Standard chow (Harlan Tekland; Norfolk, England) and water were available ad libitum unless otherwise indicated. The University of Gothenburg Institutional Animal Care and Use Committee guidelines approved all experiments (ethical permission 195-13).

### 2.2. Drugs

GLP-1 (7-36), Exendin-4 (EX4; GLP-1R agonist), GLP-2 and Angiotensin II were purchased from Tocris (Bristol, UK). All substances were dissolved in artificial cerebrospinal fluid (aCSF), vehicle for central injections, and stored as aliquots in −20 °C.

### 2.3. Brain cannulation

For behavioral testing, all rats were implanted with a guide cannula (26 gauge cannula; Plastics One, Roanoke, VA) allowing brain drug injections targeting the lateral ventricle (LV) or DR under ketamine/xylazine anesthesia. The cannulas were positioned and attached to the skull with dental acrylic and jeweler's screws and closed with an obturator (Dickson et al., 2012). The following coordinates were used for the LV: ±1.6 from the midline, 0.9 mm posterior to bregma, and 2.0 mm ventral to skull, with injector aimed 4.0 mm ventral to the skull; and for the DR guide cannula: 7.7 mm posterior to bregma, and 4.8 mm ventral from the surface of the skull, with injector aimed 6.8 mm ventral to skull. The LV placement was verified with the angiotensin II drinking test. Briefly, angiotensin II was injected at a dose of 20 ng in 2 μl of aCSF and water intake was measured immediately. Rats that drank at least 5 ml of water in 30 min were considered to have a correct cannula placement. The microinjection site for the DR guide cannula was verified post mortem by microinjection of India ink at the same microinjection volume (0.3 μl) used throughout the study.

### 2.4. Behavioral testing

Three complementary tests were used to examine anxiety-like behavior. Although all three tests represent approach–avoidance anxiety testing procedures, it is important to emphasize that the pharmacology and the neurobiology behind the response in each test may differ, thus the use of this battery-style approach for assessing novel pharmacological agents is proposed to offer more accurate conclusions on their anxiety impact. All behavioral tests were carried out during the light cycle, avoiding only the two hours after the lights were turned on and the two-hour period preceding the dark onset. All tissue collection was performed during the same hours of the light cycle as behavioral testing.

### 2.5. Elevated plus maze (EPM)

The EPM, originally described by Pellow and colleagues (Pellow et al., 1985), is a well-established rodent test used to characterize anxiety-like behavior. The open arms of the maze provide an anxiogenic environment, and less time spent exploring the open arms is indicative of higher levels of anxiety-like behavior. The EPM apparatus (Med-Associates, Georgia, VT, USA) was made of black acrylic plastic and consisted of two open arms, 10 × 50 cm, and two closed arms 10 × 50 × 40 cm and a central square platform. Each rat was placed on the central platform and allowed to move freely for 5 min. The amount of time spent on each arm was detected by measuring beam breaks of infrared lasers located at the beginning of each arm and recorded with MED-SYST-8A PCI operating and software package, provided by Med-Associates. The EPM was cleaned with a damp tissue after each rat. All behavioral tests were carried out during the light cycle as described above; this was done to minimize the potential hunger-suppressing effect of GLP-1R (ad libitum

fed rats eat little during the light cycle, thus both treatment rats and control rats should have low levels of hunger).

### 2.6. Open field test

Rats were placed in a brightly lit arena (68 × 68 × 35 cm, Kungsbacka mät-och reglerteknik AB, Fjärås, Sweden or 43 × 43 × 31 cm, Med-Associates, Georgia, VT, USA) and allowed to freely explore for 60 min during the open field test. The open field chambers were equipped with a grid of photocells for detecting movement. Photocells were coupled to a computer-based system that registers the animals' movements in the box (Ericson et al., 1991) or time spent in the center or periphery of the box. The brightly lit exposed arena is a highly anxiogenic environment and anxiety levels were assessed by the amount of activity of the rat in the more anxiety-provoking central compartment.

### 2.7. Black and white test

The boxes (Med-Associates, Georgia, VT, USA) consisted of two connected compartments with distinct visual qualities, a dark side (0 lux) and a light side with a light source (174 lux). The key measure in this test is a change in willingness of a subject to explore the light side during a 30 min test session. The light compartment is believed to be more aversive to the rat than the dark compartment (Crawley, 1981). Anxiolytic drug administration increases the amount of time spent in the light compartment.

### 2.8. Forced swim test (FST)

The FST was originally developed by Porsolt and colleagues (Porsolt et al., 1977) to screen for anti-depressive effect of pharmacotherapeutics. Here we used the modified version of the FST shown to provide a greater reliability for detection of depressant or antidepressant-like effects for compounds that affect the serotonergic system (Slattery and Cryan, 2012). Briefly all rats were allowed to swim individually in an acrylic cylinder (height: 58 cm, diameter: 21 cm) filled with fresh water before each trial (25 °C and 30 cm deep). A 15 min pre-swim was performed 24 h prior to test session. On the test day, animals were treated with either vehicle or drug and allowed to swim for a period of 5 min; the amount of time the rats spent floating (immobility), actively swimming or climbing was recorded with a video camera and later scored by an experimenter blinded to the treatment conditions. Immobility was defined as an absence of any movement other than those necessary to keep the head and nose above the surface of the water and was considered an index of depressive behavior.

#### 2.8.1. PICA test

To determine whether anxiety response to GLP-1R activation is associated with nausea, the PICA response was measured (consumption of non-nutritive substances that mimics emesis in species not capable of the emetic response). Rats were allowed to sample kaolin for at least 3 days before the Ex4 or GLP-1 injection to avoid association of kaolin with injections. Kaolin intake was measured at 1 and 24 h after injection. The doses of Ex4 (0.05 μg) and GLP-1 (2 μg) were chosen based on their earlier shown ability to induce anxiety-like behavior when injected into the DR.

### 2.9. RNA isolation and mRNA expression

DR and amygdala gene expression levels were measured after acute or chronic (daily, for 10 days) LV injection of EX4 (0.2 μg) or vehicle (aCSF). The following serotonin production, receptor, degradation or uptake-related genes were examined: *Tph2*, *Htr1a*, *Htr2a, Htr2c, Htr3a, Htr5a, Maob* and *SERT*. These genes were chosen based on their previously shown importance for anxiety or depression-like behaviors as discussed in detail in the discussion section. Ninety minutes after EX4 or aCSF injection (for the acute study) or after the last behavioral test of the chronic study the brains were rapidly removed and DR or amygdala was dissected using a brain matrix based on the areas position in a rat brain atlas. The tissues were then frozen in liquid nitrogen and stored at −80 °C. Gene expression was determined using TaqMan RT-PCR. For details see supplementary information.

### 2.10. Serotonin turnover

For the acute effect of EX4 on serotonin turnover thirty minutes after the LV injection of EX4 or vehicle, rats were lightly sedated using isoflurane and decapitated. For chronic effect of EX4 the brains were dissected 24 h after the last EX4 injection. Brains were rapidly removed and the amygdala or the hippocampus was dissected using a brain matrix. Dissected tissue was frozen on dry ice and stored in −80 °C. Tissue concentrations of serotonin and its metabolite (5-HIAA) were determined via high performance liquid chromatography (Hansson et al., 2014). For details see supplementary information.

## 3. Experimental design

### 3.1. Effect of acute intraperitoneal, central or intra-DR GLP-1, EX4 and GLP-2 administration on anxiety-like behavior

To assess the impact of peripheral acute EX4 injection (clinically relevant compound and clinically relevant route of application) on anxiety-like behavior two doses of EX4 (0.1 or 1.0 μg/kg) or saline were injected intraperitoneally. The EPM and the open field testing were performed 20 or 25 min after EX4 injection, respectively. To assess the impact of central GLP-1, EX4 and GLP-2 on anxiety-like behavior, separate groups of rats received LV injections of GLP-1 (10 μg), EX4 (0.15 μg), GLP-2 (10 μg) or vehicle (1 μl of aCSF). Injections were delivered 20 min prior to each anxiety test. Doses were chosen based on their previously reported effectiveness in reduction of food intake or food reward (Dickson et al., 2012; Tang-Christensen et al., 2000; Turton et al., 1996). To assess the impact of GLP-1, EX4 and GLP-2 in the DR on anxiety-like behavior, separate groups of rats received DR-directed microinjections of GLP-1 (2 μg), EX4 (0.05 μg), GLP-2 (2 μg) or 0.3 μl of aCSF 20 min prior to each anxiety test. These doses were chosen to be below LV threshold for effect to avoid potential confounding effect of potential drug leakage into the cerebral aqueduct located just above the DR. For this, and below described designs, all rats were naive to the tests performed except for the intraperitoneal injection experiment, which utilized a counterbalanced (Latin square) design (all rats received each drug condition separated by at least 48 h). To make sure that the repeated exposure to the EPM or the open field test did not change behavior during the test, we assessed the effect of multiple exposure to the test statistically, by comparing the performance of vehicle-injected rats on each test day, and did not find any significant effect of exposure # for either EPM or open field (ANOVA on amount of time spent in the center of open field, $F_{(2,13)} = 1.01$, $p = 0.39$; EPM open arm time: $F_{(2,13)} = 0.47$, $p = 0.64$). During the EPM test six rats were taken out from the central experiment and five rats from the IP experiment due to open arm entry sensor malfunction (no data recorded).

### 3.2. Anti-depressive effect of acute central GLP-1R activation

To assess the effect of GLP-1R stimulation on depression-like behavior, EX4 (0.2 μg) or vehicle (1 μl) was injected into the LV.

Effects of acute treatment were evaluated after one single injection of EX4. The FST test was performed for each rat individually 1 h after drug administration.

### 3.3. Impact of chronic central GLP-1R activation on anxiety and depression-like behavior

Rats were injected with EX4 (0.2 μg) or vehicle (1 μl of aCSF) once a day, intra-LV (for a total of 9 days of injections). A third group of rats was included in order to determine whether the emotionality effect of chronic GLP-1R activation, previously shown to reduce food intake and body weight, was not simply due to body weight or body fat loss. These rats were pair-fed daily to the amount of chow eaten by the EX4-treated rats. The body weight and food intake of each rat was measured daily. The FST was performed on day 9 of injections and the EPM was performed on day 10. Both tests were performed during the light cycle 24 h after the last central injection to separate the chronic and acute effect of the treatment. One rat was excluded from the chronic treatment FST test since he was taken out of the water before completion of the test.

### 3.4. Statistics

All statistical analysis was performed in Graph Pad Prism software (GraphPad Software, Inc., GraphPad, San Diego, CA). Statistical significance was analyzed using Student's *t* test or ANOVA followed by Holm–Sidak's multiple comparison test. Kruskal–Wallis test was used when unequal variances were discovered. All data are presented as mean ± standard error of the mean (de Morentin et al., 2014).

## 4. Results

### 4.1. Acute peripheral administration of EX4 increases anxiety-like behavior

Acute intraperitoneal injection of EX4 dose-dependently increased anxiety-like behavior in EPM test as suggested by the reduced % time spent in the open arm of the maze produced by the 1 μg/kg dose of EX4 (Fig. 1A ; repeated measures ANOVA; $F_{(2,20)} = 4.31$, $p < 0.05$) and reduced number of entries to the open arms (Fig. 1B; repeated measures ANOVA; $F_{(2,20)} = 4.93$, $p < 0.05$). EX4 at the 1 μg/kg dose also potently reduced the amount of time spent in the center of the open field arena (Fig. 1C; repeated measures ANOVA; $F_{(2,30)} = 9.25$, $p < 0.001$).

### 4.2. Acute central administration of GLP-1 and EX4 increases anxiety-like behavior

Acute LV injection of either one of the GLP-1R agonists, GLP-1 or EX4, increased anxiety-like behavior in the black and white test, during which the rats injected with the GLP-1 agonists chose to spend significantly less time in the bright compartment (Fig. 2A ; ANOVA; $F_{(2,26)} = 5.95$, $p < 0.01$) of the black and white box without altering (ANOVA; $F_{(2,26)} = 0.95$, p = 0.4) overall physical activity (Fig. 2B). Elevated plus maze data indicate a similar trend for increased anxiety after central EX4 treatment, however due to high variability the results do not reach statistical significance; Kruskal–Wallis test due to unequal variances ($p = 0.27$ for % time in open arm; Fig. 2C, and $p = 0.14$ for% open arm entries; Fig. 2D). Since the amount of time spent in the open arms of the EPM was relatively low for the vehicle-treated rats it is possible the effect size of EX4 may have been larger or more consistent if the baseline was higher. However in the experiment where rats were peripherally injected with EX4 the amount of time spent in the open arm was nearly five times higher by the vehicle-treated rats, yet the effect size of EX4 in this test was also very small (albeit significant). GLP-1 significantly reduced activity in the center of the open field arena, a behavior associated with increased anxiety in rodents (Fig. 2E) without changing the activity in the periphery of the arena (Fig. 2F). EX4 also significantly reduced activity in the center of the open field arena ($F_{(2,20)} = 3.58$, $p < 0.05$), a behavior associated with increased anxiety in rodents (Fig. 2G) but also changed the activity in the periphery of the arena ($F_{(2,20)} = 5.04$, $p < 0.05$; Fig. 2H).

### 4.3. The effect of acute central GLP-1R activation on depression-like behavior

No statistically significant differences were found between rats acutely treated with EX4, or GLP-1 and vehicle treated rats on the amount of time spent swimming, floating or climbing during the FST test (Fig. 3).

### 4.4. Impact of acute central GLP-1R stimulation on serotonin turnover and serotonin receptor expression

The serotonin turnover, calculated as a ratio of 5-HIAA to serotonin was significantly increased by acute central EX4 treatment in the amygdala (Fig. 4A) but not the hippocampus (Fig. 4B). These data suggest an impact of central GLP-1R activation on serotonin signaling in the amygdala. Central injection of EX4 also significantly increased the mRNA expression of two serotonin receptors, 5-$HTR_{2A}$ and 5-$HTR_{2C}$, in the amygdala (Fig. 4C) but did not alter gene expression for *Tph2*, *5HT*$_{1A}$ and *SERT* in the DR (Fig. 4D).

### 4.5. Acute DR administration of GLP-1 and EX4 on anxiety-like behavior

Based on the observed impact of GLP-1R activation on serotonergic turnover, we hypothesized that GLP-1 may be exerting its anxiogenic effects by acting directly on the DR nucleus. To test this, rats were microinjected with GLP-1, EX4 or aCSF directly into the DR. Similar to the results obtained with the LV injection, rats injected with GLP-1 or EX4 into the DR spent significantly less time in the white chamber of the black and white box (ANOVA; $F_{(2,33)} = 7.44$, $p < 0.005$) without altering their general motor activity ($F_{(2,33)} = 1.39$, $p = 0.26$) compared to the vehicle treated group (Fig. 5A and B). Rats microinjected with EX4 or GLP-1 into the DR also spent three times less time in the open arms of the maze (ANOVA; $F_{(2,23)} = 4.00$, $p < 0.05$) compared to vehicle treated controls (Fig. 5C), they were also less likely to enter the open arm (ANOVA; $F_{(2,23)} = 3.61$, $p < 0.05$) (Fig. 5D). However they did not display signs of malaise as measured by the PICA response ($F_{(2,32)} = 0.51$, $p = 0.6$ and $F_{(2,32)} = 0.44$, $p = 0.6$ for 1 and 24 h respectively) (Fig. 5E). PICA test outcome, together with the lack of effect on general motor activity of these treatments, strongly suggest a lack of illness or malaise coupled to the anxiety-like behavior induced by DR-targeted GLP-1R activation.

### 4.6. Acute LV and DR GLP-2 administration impact on anxiety

GLP-2 reduced anxiety-like behavior in the open field test, as demonstrated by increased activity in the center of the open field arena after LV treatment (Fig. S1A). However GLP-2 produced no changes in anxiety-like behavior in the EPM or black and white test (Fig. S1C–E). Microinjection of GLP-2 into the DR did not significantly change anxiety-like behavior, measured by the EPM test (Fig. S1F).

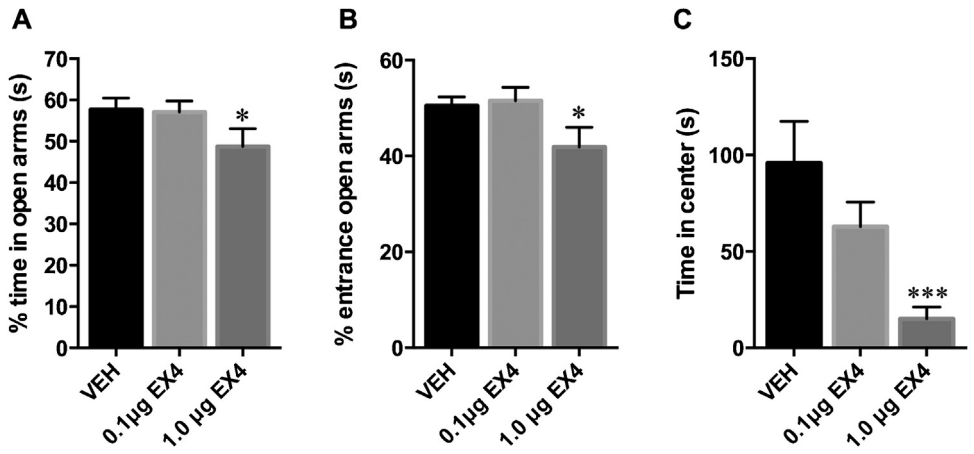


**Fig. 1.** Peripheral acute injection of GLP-1 analogue, EX4, increases anxiety-like behavior. EX4 reduced the amount of time spent in the open arm of the elevated plus maze (A) and reduced the % of open arm entries, $n = 11$ (B). Peripheral EX4 also potently reduced the amount of time spent in the central region of the open field, $n = 16$ (C). Data are expressed as mean ±SEM. $^{*}p < 0.05$, $^{***}p < 0.0005$.

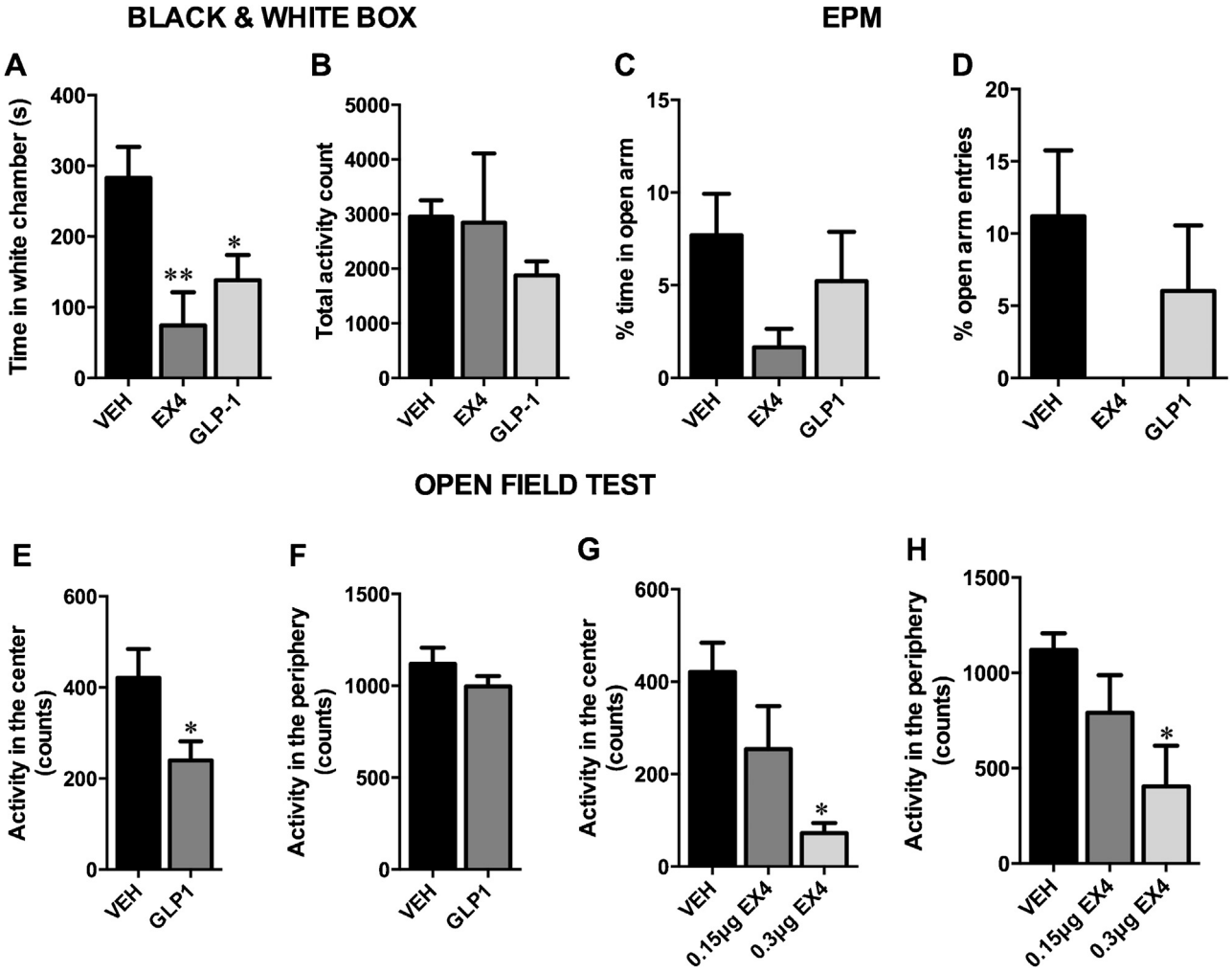


**Fig. 2.** Central acute GLP-1R activation increases anxiety-like behavior. GLP-1 or EX4 potently reduce the amount of time spent in the white chamber of the black and white box (A) without altering overall physical activity (B). $n = 7$–14 per each treatment group. Elevated plus maze data indicate a similar trend for increased anxiety after central EX4 treatment, however due to high variability the results do not reach statistical significance;% time in open arm (C) and % open arm entries (D). $n = 5$–11 per each treatment group. Central GLP-1 injections also reduced activity in the central region of the open field (E, $p < 0.05$), without changing the activity in the peripheral region (F). $n = 11$–14. EX4 also significantly reduced activity in the center of the open field arena, a behavior associated with increased anxiety in rodents (Fig. 2G) but also changed the activity in the periphery of the arena (Fig. 2H). $n = 3$–14. Data are expressed as mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.005$.

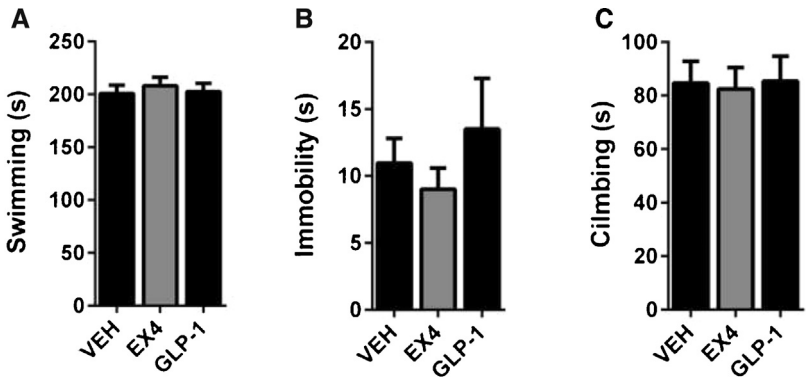


**Fig. 3.** Acute GLP-1R activation does not change depression-like behavior. No statistically significant differences were found between rats acutely treated with EX4, or GLP-1 and vehicle treated rats on the amount of time spent swimming (A), floating (B) or climbing (C) during the forced swim test. Data are expressed as mean ± SEM. $n = 12–21$ per each treatment group.

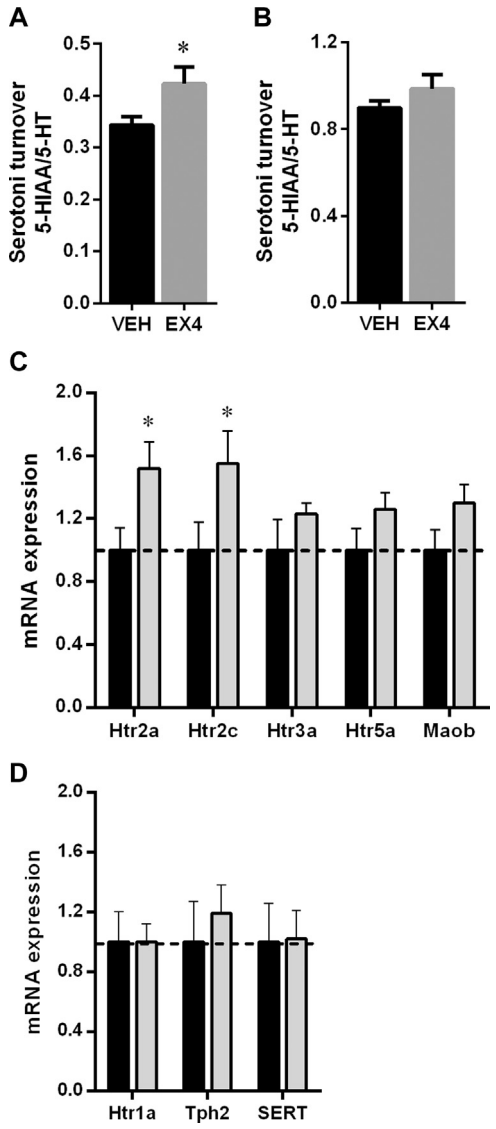


**Fig. 4.** Acute GLP-1R activation alters serotonin turnover and serotonin receptor expression in the amygdala. Acute injections of EX4 into the lateral ventricle increase 5-HIAA/5-HT turnover in the amygdala (A) but not the hippocampus (B). This treatment also increases the expression of amygdala serotonin receptors previously associated with anxiety behavior control: $5HT_{2A}$ and $5HT_{2C}$ (C). On the other hand the same treatment does not change the expression of serotonin neuron autoreceptors or serotonin synthesizing enzyme in the dorsal raphe (D). Data are expressed as mean ± SEM. n = 7-8 per each treatment group. $^*p < 0.05$.

### *4.7. Chronic central GLP-1R activation reduces depression-like behavior without affecting anxiety*

In contrast to the acute effects of EX4, rats chronically treated with EX4 displayed reduced depression-like behavior as evidenced by a reduced amount of time spent on the passive behavior (immobility) ($F_{(2,32)} = 5.487$, $p < 0.01$; Fig. 6B) and increased active behavior (Swimming) (ANOVA; $F_{(2,32)} = 9.244$, $p < 0.001$; Fig. 6A) during the FST. Since the amount of time spent on passive behavior was relatively low in the vehicle-treated group it is possible that the effect size of EX4 may have been larger in rats displaying higher amount of passive behavior. In the chronic treatment experiment an additional group of rats was tested, a group that had their body weight matched to that of the EX4-treated rats in order to discern whether the positive effect of EX4 on mood was connected to the body weight loss and reduced food intake displayed by the EX4-treated rats. Food was given once daily to pair-fed subjects. Importantly, body weight loss, matched to the loss measured in EX4-treated rats, did not change FST behavior leading to the conclusion that the positive effects of EX4 on mood in the FST were not due to the weight-loss effects of EX4. Anxiety-like behavior, measured in the EPM test, was not altered by chronic treatment with EX4 (Fig. 6D and E). Thus, chronic injections of EX4 were neither anxiogenic nor anxiolytic.

As expected based on the well known weight-reducing effect of the central GLP-1R activation, rats chronically treated with EX4 significantly reduced their body weight (Fig. 6F). Two-way repeated measures ANOVA indicated a significant effect of treatment ($F_{(8,256)} = 6.017$, $p < 0.001$) and day ($F_{(2,32)} = 8.437$, $p < 0.0001$). The reduction in body weight was consistent with reduced chow intake (compared to the vehicle-treated rats) ($F_{(2,32)} = 5.49$, $p < 0.001$; Fig. 6G). After two days the animals stopped losing additional weight and began to gradually return to their starting weight. Pair-fed group displayed a slightly larger weight-loss compared to EX4-treated group, a similar effect was indicated by a previous study where body weight loss was measured during nine days of peripheral EX4 treatment (Yang et al., 2014). Animals pair-fed to EX4 treated rats lost a similar amount of weight to the EX4 treated rats indicating that body-weight loss after EX4 is primarily due to reduced calorie intake rather than increased energy expenditure.

Restriction of food intake to the amount voluntarily eaten by EX4-treated rats resulted in a significant increase in the expression of genes encoding 5-$HTR_{1A}$ ($F_{(2,30)} = 6.42$, $p < 0.005$), 5-$HTR_{2A}$ ($F_{(2,30)} = 8.26$, $p < 0.005$), 5-$HTR_{2C}$ ($F_{(2,30)} = 10.45$, $p < 0.0005$), and 5-$HTR_{3A}$ ($F_{(2,30)} = 12.53$, $p < 0.0001$) but not Tph2 ($F_{(2,30)} = 1.83$, $p = 0.18$) in the amygdala (Fig. 6H). While EX4 treatment did not change the expression of any genes measured when compared to

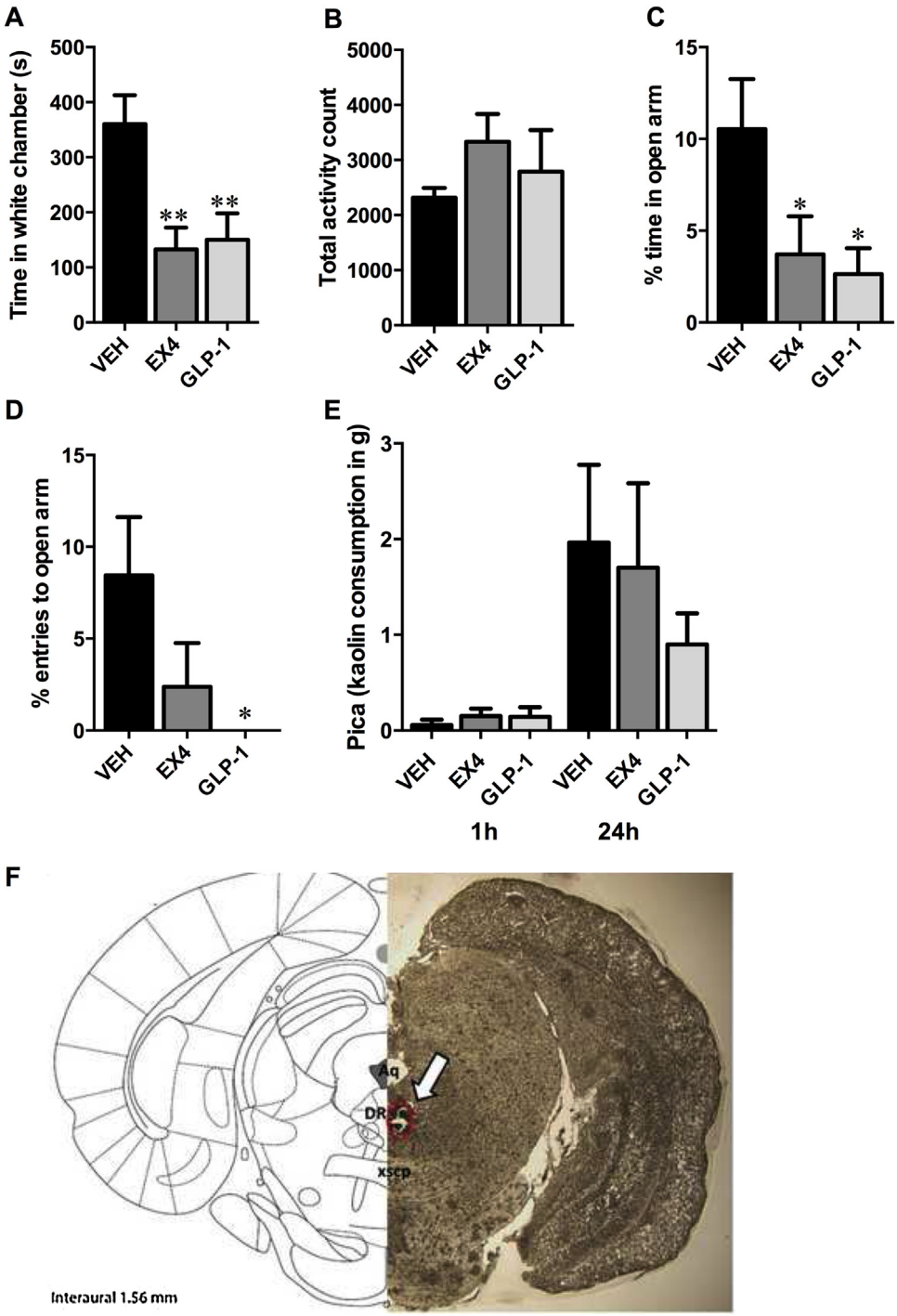


**Fig. 5.** Dorsal raphe GLP-1R activation is sufficient to increase anxiety-like behavior. GLP-1 or EX4 potently reduce the amount of time spent in the white chamber without altering general motor activity in the black and white box (A-B). $n = 9–15$ per each treatment group. Also elevated plus maze data suggest increased anxiety-like behavior since the rats treated with EX4 or GLP-1 spent three times less time in the open arms of the maze compared to vehicle treated controls (C), they are also less likely to enter the open arm (D). However they do not display signs of malaise as measured in the PICA response (E). Notably the doses of agonists used did not produce a significant effect on anxiety-like behavior when applied to the ventricle, indicating the raphe as an important neural substrate for the anxiety effect of GLP-1. $n = 7–10$ per each treatment group. Data are expressed as mean ± SEM. Photomicrograph of a 40 μm coronal section of rat brain illustrating the injection site and schematic representation of the dorsal raphe according to the rat brain atlas (F). $^{*}p < 0.05$, $^{**}p < 0.005$.

ad libitum-fed controls, it did alter gene expression compared to pair-fed rats (arguably a more appropriate control since it takes into account the changes in the brain induced by reduced body weight in EX4-treated rats). Thus, EX4 attenuated the increased expression of serotonin receptor genes which was induced by restricted feeding, as shown by the reduced expression compared to pair-fed rats. Serotonin turnover was not elevated after chronic EX4 treatment (Fig. 6I).

## 5. Discussion

Using three tests of anxiety-like behavior, we show that acute peripheral or central GLP-1R activation is anxiogenic and identify

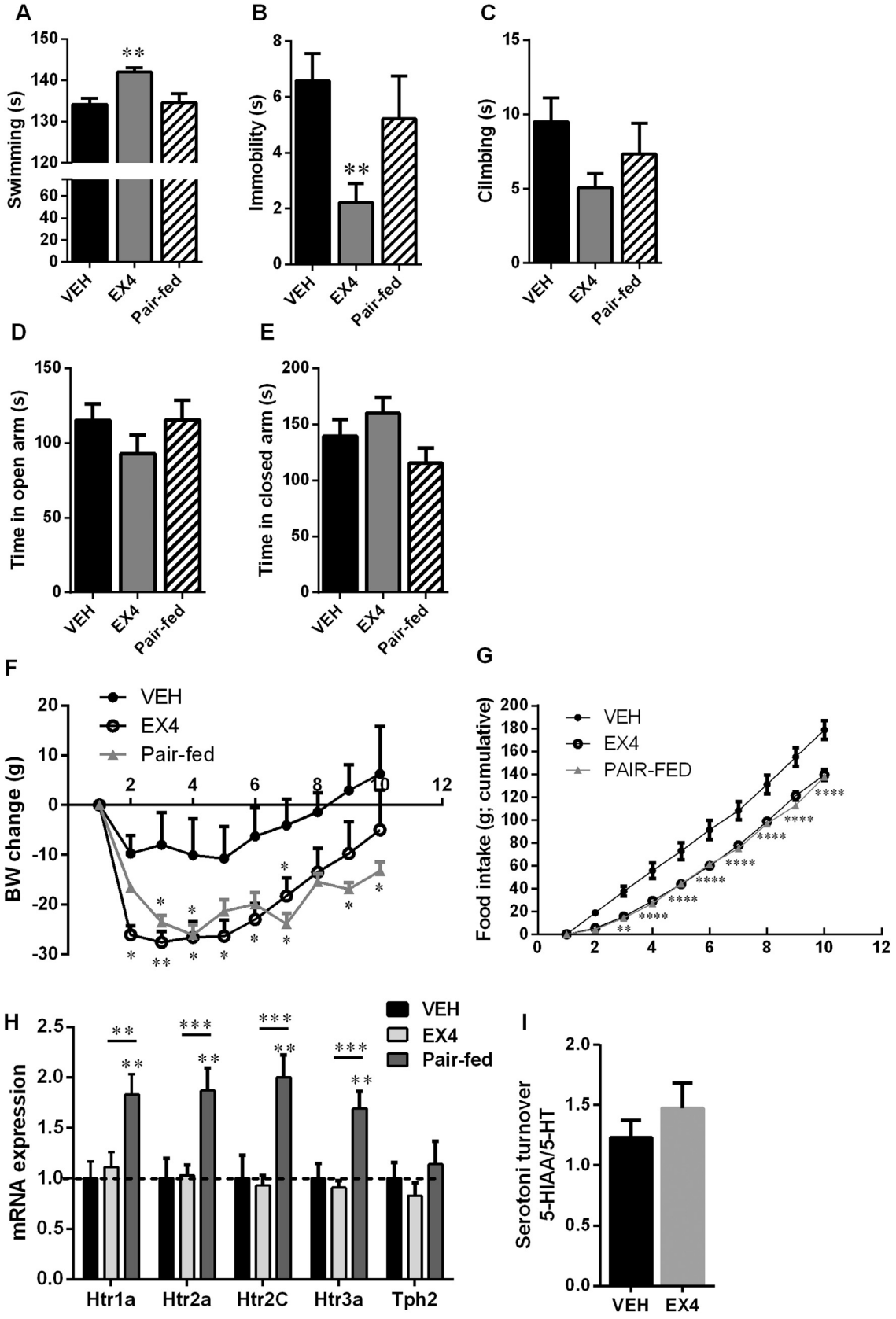


**Fig. 6.** Chronic GLP-1R activation reduces depression-like behavior. In contrast to the acute effects of EX4, rats chronically treated with EX4 displayed reduced depression-like behavior as evidenced by an increased amount of time spent on active behavior—swimming (A) and a reduced amount of time spent on passive behavior—immobility (B) in the forced swim test. Rats pair-fed to the amount of food eaten by EX4 rats did not show any significant changes in behavior. Climbing behavior was not significantly altered by any of the treatments (C). Chronic EX4 treatment or pair feeding did not change anxiety-like behavior (D-E). Rats chronically treated with EX4 reduced their body weight (F). The reduction in body weight was consistent with reduced chow intake in EX4 treated rats compared to the vehicle treated animals (G). Restriction of food intake to the amount voluntarily eaten by EX4-treated rats resulted in a significant increase in the expression of genes encoding 5-$HTR_{1A}$, 5-$HTR_{2A}$, 5-$HTR_{2C}$, and 5-$HTR_{3A}$ in the amygdala, this effect was attenuated by chronic EX4 treatment (H). Chronic EX4 treatment did not significantly increase serotonin turnover in the amygdala (I). Data are expressed as mean ± SEM. $n = 8$–14 per each treatment group. $^{*}p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.0005$, $^{****}p < 0.00005$.

the DR as a neural substrate for this effect. Increased anxiety can result from elevated serotonergic neurotransmission in the amygdala; therefore serotonin turnover and gene expression of several serotonergic receptors were analyzed in this area. Central acute GLP-1R activation resulted in increased serotonin turnover and increased expression of several serotonin receptors in the amygdala. In contrast to the acute effects of central GLP-1R activation, chronic daily central treatment with the GLP-1 analog, EX4, did not alter anxiety-like behavior but instead led to reduced depression-like behavior in the FST. Chronic GLP-1R activation, unlike the acute treatment, did not increase serotonin turnover or serotonin receptor expression in the amygdala.

The presence of GLP-1R in the DR suggests a potential role for GLP-1 in emotional processes (Merchenthaler et al., 1999). Serotonin-producing neurons are localized in the raphe nuclei, which send projections throughout the brain. Accordingly, the amygdala receives serotonergic innervation from the DR nucleus (Vertes, 1991) while the hippocampus receives serotonergic innervation from the median raphe (McQuade and Sharp, 1997). Guided by our results indicating an increased anxiety-like behavior after central GLP-1R agonist application and increased serotonin turnover in the amygdala, but not the hippocampus, we hypothesized that the anxiogenic effects of GLP-1 may originate from direct DR GLP-1R activation. The results of the DR-directed GLP-1 and EX4 microinjections confirm this idea and show that DR GLP-1R activation alone is sufficient to increase anxiety-like behavior. The preproglucagon neurons situated in the hindbrain's solitary tract may be a primary source of the endogenous ligand to the raphe as GLP-1-producing neurons from this area were recently shown to innervate the brainstem raphe nuclei (Llewellyn-Smith et al., 2013).

While selective serotonin reuptake inhibitors (SSRIs) are used to effectively treat a spectrum of anxiety disorders, some patients may paradoxically experience an increase in anxiety during the initial stages of SSRI treatment, an effect that eventually subsides after chronic treatment. Acute SSRI administration increases 5-HT concentrations in the amygdala and hippocampus (Bosker et al., 2001; Hervas et al., 2000) and can enhance amygdala activity in healthy humans (Del-Ben et al., 2005; McKie et al., 2005) and exacerbate the expression of anxiety (Grillon et al., 2007). The early onset anxiety that subsides with chronic exposure and improvement of depression symptoms, but only after chronic exposure seen here with GLP-1R activation, resembles the action of the SSRIs. SSRIs, or other agents that increase serotonin signaling, can elicit anxiety, both in rodents and humans, during the first few days of treatment, and weeks before anxiolytic and anti-depressant effects emerge (Nutt et al., 1999; Pettersson et al., 2015; Sinclair et al., 2009; Wise et al., 1972).

$5HT_{1A}R$ is an autoreceptor in the DR, known to inhibit DR serotonin neurons (Asan et al., 2013). In the current study, however, the expression of this receptor or the enzyme responsible for serotonin synthesis is not altered in the DR. In contrast, in the amygdala acute EX4 injection increased the expression of several serotonin receptors ($5HT_{2A}$ and $5HT_{2C}$) in addition to increasing serotonin turnover. The acute anxiogenic effect of SSRI may involve $5HT_{2C}$Rs in the basolateral amygdala (Vicente and Zangrossi, 2012). Wealth of evidence from both animal and clinical studies suggests an anxiogenic role of $5HT_{2C}$. Overexpression of $5HT_{2C}R$ in limbic areas results in increased anxiety (Kimura et al., 2009), and conversely $5HT_{2C}R$ knock-out mice display reduced anxiety-like behavior (Heisler et al., 2007). Nevertheless amygdala serotonin receptor expression alone may not be entirely predictive of increased anxiety, as all serotonin receptors measured were strikingly increased in the amygdala of rats pair-fed to EX4 rats, compared to their ad libitum-fed controls, yet the pair-fed rats did not show elevated levels of anxiety or depression-like behavior. The lack of changes in anxiety or depression by restricted feeding (pair fed to the EX4 group) is curious as it may indicate that reduced intake or body weight can be dissociated from the positive effects of EX4 on mood, and also that reduced intake itself does not produce beneficial or negative changes in mood. It is also possible that the lack of anxiety or depression-like behavior in pair-fed animals is due to the opposing effects of the different 5HTRs that were overexpressed in these animals. While $5HT_{2A}R$ and $5HT_{2C}R$ are associated with increased anxiety, $5HT_{3A}R$ stimulation decreases anxiety behavior within the amygdala (Bhatnagar et al., 2004; Harrell and Allan, 2003; Kelley et al., 2003; Vicente and Zangrossi, 2012; Zangrossi and Graeff, 1994). Therefore increased expression of these receptors together may not lead to any behavioral differences regarding anxiety or depression. Furthermore, chronic food restriction per se has previously been shown to increase depression and anxiety-like behavior in rats as shown in FST and EPM (Jahng et al., 2007). However, animals in the cited experiment were food restricted for a longer period of time, therefore the effects on 5HT expression may precede an anxious phenotype, which may have been present after further food deprivation. In contrast, other studies have shown that caloric restriction instead reduces anxiety and depression (Lutter et al., 2008). This would indicate that the anorexic effects of EX4 may be sufficient to exert the anti-depression effects demonstrated in this study. However, in the current study pair-fed animals did not display these same characteristics after chronic treatment indicating that the effects on mood shown here are not due to food restriction alone. In contrast to these acute effects of EX4 on amygdala serotonin receptors, chronic treatment with EX4 did not change the expression of any serotonin receptors measured when compared to ad libitum fed rats, or potently reduce the expression of all serotonin receptors measured when compared to the pair-fed control group.

The serotonergic hypothesis of depression is still debated, especially considering the discrepancy between the immediate increase in serotonin produced by SSRIs and a much delayed relief in depression symptoms along with the fact that nearly a third of patients do not get symptom relief from SSRI treatment (Rossetti et al., 2014). Anti-depression therapies have been suggested to increase neurogenesis (Anacker et al., 2011; Mendez-David et al., 2013; Santarelli et al., 2003). Interestingly several lines of evidence suggest that EX4 is also neurotrophic and neuroprotective (Holscher, 2014; Li et al., 2009). Thus, it is possible that the anti-depressant effect of GLP-1R activation is linked to its action to reduce neurodegeneration and increase neurogenesis, though this idea is still rather speculative and requires further research. The divergent role of EX4 on anxiety and depression-like behaviors is also reminiscent of one previously observed with genetic overexpression of brain derived neurotrophic factor (BDNF) (Govindarajan et al., 2006).

In addition to serotonin, the GLP-1 system has also been shown to interact with dopamine (Anderberg et al., 2014; Egecioglu et al., 2013; Richard et al., 2015; Sorensen et al., 2015). Dopamine has been implicated in depression (Dunlop and Nemeroff, 2007). Optogenetically-driven inhibition of VTA dopamine neurons has been shown to induce depression-like behavior (Tye et al., 2013). Furthermore this phenotype can be reversed by photoactivation of VTA dopamine neurons projecting to the ventral striatum (Tye et al., 2013). Interestingly, pretreatment with the GLP-1R agonist, EX4, was previously shown to attenuate striatal cocaine-induced dopamine release in mice (Sorensen et al., 2015). In addition EX4 has also been shown to have an anti-psychotic-like effect after pretreatment with the non-selective dopamine receptor agonist-apomorphine (Dixit et al., 2013), and to decrease locomotor activity induced by administration of amphetamine, a drug which acts to increase central dopamine levels (Erreger et al., 2012). These studies suggest the ability of EX4 to decrease central dopamine levels; depression may be associated with reduced dopaminergic

transmission, indicating that the effects of GLP-1 and its agonists, shown here, are unlikely to be mediated through actions on the dopaminergic pathway. However, all GLP-1 studies discussed above examined acute perturbations of the GLP-1 system, thus the impact of chronic manipulations, as done here, on the dopaminergic striatal transmission remains unknown and could be of interest for the role of the GLP-1 system in modulating depression-like behavior. The dopaminergic pathway has also been implicated in anxiety-like behavior (Bananej et al., 2012; de la Mora et al., 2010, 2012; Mohammadi et al., 2015). For instance apomorphine may have anxiolytic properties through its actions on the amygdala (Zarrindast et al., 2011). In addition D1 and D2 antagonists have been shown to increase anxiety by acting in this area (Zarrindast et al., 2011). Central acute GLP-1R activation has previously been shown to elevate dopamine turnover in the amygdala and the anorexic effects of GLP-1 may be partly mediated through actions on D2 receptors within this area (Anderberg et al., 2014). As GLP-1 has been shown to elevate dopamine activity in the amygdala, this would instead imply a reduction in anxiety-like behavior based on previous literature, thus dopaminergic signaling, at least in the amygdala, remains an unlikely candidate mediator of the acute anxiogenic effect of GLP-1 or its analogs.

Our results are strengthened by the fact that treatment with the endogenous ligand, GLP-1, as well as the clinically utilized stable analog, EX4, resulted in elevated anxiety. Interestingly, however, GLP-2, another peptide produced from the same precursor gene as GLP-1 (Tang-Christensen et al., 2000), did not increase anxiety. In fact LV-injected GLP-2 tended to reduce anxiety-like behavior. Thus, central GLP-1 and GLP-2, despite the similarities in their production and release, may have different roles in the regulation of anxiety. This may be surprising as many of the central effects of GLP-1, for example reduction of food intake, body weight, gastrointestinal motility and regulation of blood glucose are, to some extent, mimicked by GLP-2 (Guan, 2014). However, this is not the first time these two co-secreted peptides were reported to have divergent effects, previous studies have shown that when released in the intestine these peptides have opposing impact on intestinal lipid packaging (Hein et al., 2013).

The current study, as well as several others (Gulec et al., 2010; Kinzig et al., 2003; Moller et al., 2002), finds a clear anxiogenic effect of central acute GLP-1R stimulation. In contrast to the anxiogenic effects of acute GLP-1 and EX4 treatment reported here, peripheral treatment with another GLP-1 agonist, liraglutide, or the DPP-IV inhibitor sitagliptin, was very recently shown to decrease anxiety-like behavior measured in the EPM task (Sharma et al., 2015a,b,c). Similarly a whole-body knockout of the GLP-1R results in either non-altered anxiety-like behavior or an increased anxiety-like behavior in the EPM test in mice (Harasta et al, 2015; MacLusky et al., 2000). Though it is important to note that other parameters, like the body-weight of the null mice which is the same or even lower than that of control mice, are inconsistent with the well documented role of GLP-1R in body weight regulation (MacLusky et al., 2000; Seeley et al., 2000). The divergent results in the earlier mentioned pharmacological studies compared to the current study can be due to the different routes of drug administration (peripheral vs. central), different GLP-1 analogs/enhancers used (liraglutide or sitagliptin vs. EX4 or GLP-1), different rat strains (Wistar vs. Sprague Dawley), or different testing time point in relation to drug injections (4 h vs. 20 min). While any of these differences could have contributed to the contrasting results, the strain differences could have been of particular importance since it was previously reported that Wistar Kyoto rats and Sprague Dawley rats have different FST responses at baseline but also, importantly, respond differently to noradrenergic or serotonergic antidepressants in the FST test (Lopez-Rubalcava and Lucki, 2000). Furthermore of the four doses tested only one dose of liraglutide or sitagliptin increased EPM open arm entry, indicating that this effect may not be robust (Sharma et al., 2015a,b,c). In order to eliminate the route of administration as a potential source of varied effect of GLP-1 analogs on anxiety-like behavior, in the current study we also tested the impact of peripherally injected EX4 on anxiety-like behavior and found that in line with our central-injection results, peripherally injected EX4 resulted in anxiety-like behavior in both the EPM and open field tests. An anxiolytic effect of chronic EX4 administration was also indicated by another study, performed in diabetic mice (Komsuoglu Celikyurt et al., 2014). These differences may be due to different species (mice vs. rats) or the disease state of the animals during testing (streptozotocin-induced diabetic mice vs. healthy rats), the latter idea is especially compelling since diabetes alone seems to induce severe anxiety in the mice that is partially attenuated by EX4 (Komsuoglu Celikyurt et al., 2014). Additionally, relieving diabetes alone, in other words improved glucoregulation produced by peripheral treatment with EX4, may be a major contributing factor to the reduced anxiety in these mice. In addition, recently, another group reported no effects on anxiety-like behavior measured in the black and white test after acute or chronic subcutaneous administration of the GLP-1 agonists EX4 or liraglutide in mice (Krass et al., 2012, 2015).

In line with the results of the current central administration focused study, three recent studies report a clear anti-depressive impact of peripheral EX4 or liraglutide (Sharma et al., 2015a; Isacson et al., 2011; Komsuoglu Celikyurt et al., 2014). Sharma and colleagues show that chronic liraglutide treatment reverses depressive behavior associated with long-term antipsychotic treatment in female rats (Sharma et al., 2015a), providing additional evidence of the potential anti-depressive effects of chronic GLP-1 agonist treatment. They also show that in control rats four days of peripheral liraglutide injections was sufficient to reveal a reduced immobility behavior in the FST test. In contrast to the differential effects on anxiety reported by Komsuoglu Celikyurt and colleagues (Komsuoglu Celikyurt et al., 2014), compared to this study, a profound effect of EX4 on anti-depressive behavior was reported in the diabetes-induced mice, further supporting our results of the anti-depressive effects of EX4 and implicating that these effects may be present both in healthy and diabetic subjects. Similarly our results are in line with another previous study in which peripherally administered EX4 was shown to decrease immobility in the FST after chronic, but not acute treatment (Isacson et al., 2011). These studies collectively also indicate that the antidepressant effects of GLP-1 agonist treatment are present after peripheral administration, in addition to central administration as in the current study, which implicates that peripherally injected GLP-1 agonists can affect emotionality behavior. Thus these effects may also be present in patients receiving peripherally administered GLP-1 analogs. One recent study did not find any effects of 35-day peripheral treatment with either EX4 or liraglutide in rats or mice on FST (Krass et al., 2015). However significant methodological differences exist between this study and all other studies discussed in this paragraph. Namely in the study by Krass et al. (2015), rats received the pre-test 35 days before the FST test, and, importantly, the FST test lasted 10 min for rats and 6 min for mice, and only behavior from the last 6 min for rats and last 4 min of the test for mice was analyzed. Finally, in (Krass et al., 2015) both rats and mice were also subjected to an open-field test immediately before the FST test. These differences are substantial and may have contributed to the lack of effect in the study by Krass and colleagues.

Acute GLP-1R stimulation led to increased anxiety-like behavior while chronic stimulation of these receptors led to reduced depression-like behavior. It is possible that the differential effects displayed after chronic treatment with the agonist may indicate potential desensitization at the central GLP-1R, however previous studies administering GLP-1 or its analogs chronically demon-

strated potent effects of GLP-1, with respect to body weight, food intake and glucose homeostasis throughout a 7 or 14 day treatment period (Baggio et al., 2004; Davis et al., 1998). Desensitization studies conducted in cells have previously shown a concentration-dependent decrease in specific binding of the GLP-1R; however this did not result in functional desensitization (Davis et al., 1998). In addition in vivo studies did not indicate the presence of receptor desensitization after chronic GLP-1R stimulation regarding its effects on food intake (Davis et al., 1998) supporting the idea that the effects on mood in this study are not due to differences in receptor sensitivity caused by chronic stimulation.

The higher prevalence of depression and other mood disorders in obese patients and negative effects on mood of previously available anti-obesity treatments highlight the need for experimental studies on the impact on mood of newly emerging obesity treatments. Here we show that GLP-1R activation, a recently approved anti-obesity treatment, induces anxiety immediately after the commencement of the treatment, this anxiety subsides with chronic treatment and is in fact replaced by positive effects on mood manifested by reduced depression-like behavior. These results may have direct relevance to the clinic, and indicate that EX4 may be especially useful for those obese patients manifesting with comorbid depression. They also may warrant vigilance on the part of treating physicians during the initial phase of the treatment where, similarly to what has been previously observed with SSRIs, an elevation in anxiety behavior may be observed.

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The authors declare no conflict of interest. The funders had no role in the study.

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The funders had no involvement in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the article for publication.

## Contributors

All authors have approved the final article.

All authors made substantial contributions to all of the following:

(1) The conception and design of the study, or acquisition of data, or analysis and interpretation of data.
(2) Drafting the article or revising it critically for important intellectual content.
(3) Final approval of the version to be submitted.

K.P.S conceived and designed the study, drafted and approved the manuscript

R.H.A., J.E.R., C.H, F.B, H.N participated in acquisition of the data, revising the manuscript and approving the final version.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.psyneuen.2015.11.021.

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