Activation of glucagon-like peptide-1 receptors reduces the acquisition of aggression-like behaviors in male mice

Jesper Vestlund, Qian Zhang, Olesya T. Shevchouk, Daniel Hovey, Lundström Sebastian, Lars Westberg and Elisabet Jerlhag

INTRODUCTION

Aggression is a complex social behavior, which is provoked in the defense of limited resources including food and mates. Recent advances show that the gut-brain hormone ghrelin modulates aggressive behaviors. As the gut-brain hormone glucagon-like peptide-1 (GLP-1) reduces food intake and sexual behaviors its potential role in aggressive behaviors is likely. Therefore, we investigated a tentative link between GLP-1 and aggressive behaviors by combining preclinical and human genetic-association studies. The influence of acute or repeated injections of a GLP-1 receptor (GLP-1R) agonist, exendin-4 (Ex4), on aggressive behaviors was assessed in male mice exposed to the resident-intruder paradigm. Besides, possible mechanisms participating in the ability of Ex4 to reduce aggressive behaviors were evaluated. Associations of polymorphisms in GLP-1R genes and overt aggression in males of the CATSS cohort were assessed. In male mice, repeated, but not acute, Ex4 treatment dose-dependently reduced aggressive behaviors. Neurochemical and western blot studies further revealed that putative serotonergic and noradrenergic signaling in nucleus accumbens, specifically the shell compartment, may participate in the interaction between Ex4 and aggression. As high-fat diet (HFD) impairs the responsiveness to GLP-1 on various behaviors the possibility that HFD blunts the ability of Ex4 to reduce aggressive behaviors was explored. Indeed, the levels of aggression was similar in vehicle and Ex4 treated mice consuming HFD. In humans, there were no associations between polymorphisms of the GLP-1R genes and overt aggression. Overall, GLP-1 signaling suppresses acquisition of aggressive behaviors via central neurotransmission and additional studies exploring this link are warranted.

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Another gut-brain hormone is glucagon-like peptide-1 (GLP-1), which is well-known for its ability to suppress food intake, appetite, or body weight and to modulate glucose homeostasis (for review see [35]). These physiological properties have contributed towards the approval of GLP-1 receptor (GLP-1R) agonists for treatment of diabetes type 2 [36] and obesity [37]. Besides metabolic homeostasis, GLP-1R agonists like exendin-4 (Ex4) attenuate reward from addictive drugs and palatable food in male rodents [38–42]. More recent studies reveal that GLP-1R regulates social behaviors as Ex4 decrease sexual behaviors in male mice [43, 44]. Aggression is another social behavior driven by the defense of food and mates, consummatory behaviors regulated by GLP-1 signaling (for review see refs. [1, 45]). Together with the findings that obesity and aggression share overlapping genes [46] and are co-morbid in epidemiological studies [47, 48], indicate that obesity and aggression share common risk factors; plausibly GLP-1 signaling. Indeed, the potential role of GLP-1R for aggression is unknown.

To explore this knowledge gap, we herein investigated the link between GLP-1R activation and aggressive behaviors by combining preclinical experiments and a human genetic-association study. We investigated whether acute or repeated treatment with Ex4 decreases the expression and acquisition of aggressive behavior in male mice in the resident-intruder paradigm. Peripheral and central mechanisms contributing towards the ability of Ex4 to reduce aggressive behaviors were then evaluated by means of various molecular biology and biochemical methods. First, the ex vivo levels of monoamines in aggression-related brain areas and the plasma concentrations of corticosterone and testosterone were measured in male mice treated with Ex4 or vehicle during the resident intruder test. Besides, the levels of noradrenalin, and serotonin-related proteins and the counts of FOS immunoreactivity, a marker of neuronal activity, was determined.

Exposure to high-fat diets (HFD) reduces preference for palatable foods, depression-like behaviors and drug reward, a decline plausibly involving altered dopaminergic neurotransmission [49–51]. Besides, in male mice HFD enhances aggression [52] and impairs the responsiveness to GLP-1 on various behaviors [53–55]. Additional pilot resident intruder experiments therefore assessed the possibility that HFD (i.e., peanut butter) blunts the ability of Ex4 to reduce aggressive behaviors. Additionally, the protein levels of noradrenalin, and serotonin-related proteins of these mice were assessed. Finally, we assessed the association between polymorphisms in GLP-1R genes and self-reported overt aggression in young men from the Child and Adolescent Twin Study in Sweden (CATSS).

MATERIAL AND METHODS

Herein a combination of preclinical experiments and a human genetic study are used to study the hypothesis that activation of GLP-1R suppresses aggression behaviors. Indeed, the resident-intruder model is a valid model to study aggressive behaviors directed towards others (overt aggression) in male mice [8]. As genetic vulnerability contributes to the expression of overt aggression in humans (for review see ref. [56]), the identification of genes associated with this behavior can be useful when studying mechanisms underlying aggression.

Preclinical study

**Animals.** To establish a robust aggressive behavior in the resident mouse (C57Bl/6 N), an intruder mouse of a smaller, submissive strain 129/SvEv was used [33]. The animals and their situation were identical to our previous study [8].

The conducted experiments were approved by the Swedish Ethical Committee on Animal Research in Gothenburg (Ethical number: 3348/20; 96/15). Males were studied as they show a robust response in the resident intruder test, whereas laboratory female mice in general display no or low aggression in this test and as lactating or gestational female mice with higher aggression most likely involve other neurobiological substrates [8–10, 14].

**Drugs and peanut butter.** Two different doses of Ex4 (1.2 µg/kg or 2.4 µg/kg; Tocris Bioscience, Abingdon, United Kingdom) dissolved in vehicle (0.9% NaCl) were injected intraperitoneal (IP) 10 min prior to the test [39]. The selected doses were used as they do not alter locomotor behavior in an open field test per se, whereas the higher one blocks the alcohol-induced locomotor stimulation in male mice [39]. Peanut butter (Green Choice; Coop, Gothenburg, Sweden) was used.

**Resident intruder paradigm.** The resident intruder paradigm was used in order to evaluate the effect of acute or repeated administration of Ex4 on aggressive behaviors, and to explore the possible influence of peanut butter on such behavioral outcome. The resident intruder paradigm is a valid model of overt aggression, where a male mouse defends his territory against a specific intruder [8, 14]. It was conducted as described in previous studies [8, 33, 57, 58]. Importantly, all mice (resident and intruder) had full access to regular Chow throughout the experimental setup, except during the daily resident intruder encounter (maximum 10 min per day). The mice were not food restricted as we aim to explore the effect of Ex4 during regular physiological conditions. Besides, after food restriction access to food during the resident intruder test decrease aggressive behaviors [59], a possible confounding factor eliminated in the present design.

**Experimental designs.** The designs of the test differ as they either investigate the effect of acute or repeated Ex4 treatment on aggressive behavior.

**Experiment 1** (Fig. 1, Panel A) was designed to study the effect of an acute injection of Ex4 on aggressive behavior in mice with an established aggression. Therefore, only mice displaying aggressive behavior on day 6 was included in the statistical analysis. Therefore, two non-aggressive mice were excluded from the analysis. After six initial training days the resident mice were divided into two future treatment groups, where the mice had similar attack latency during day 1–6. Thereafter, the mice received either an acute Ex4 (2.4 µg/kg, IP) or vehicle injection on the final test day (day 7).

In contrast to experiment 1, low aggression was not an exclusion criterion for experiment 2–5 as these evaluate the possibility that repeated Ex4 treatment during aggression acquisition dose-dependently reduces aggressive behaviors in male mice. As lower innate level of aggressive-like behavior might confound the obtained data, vehicle controls are included and treatment were either randomized (experiment 2), stratified based on acquired aggression level (experiment 1) or innate aggression level (experiment 3,4,5). In experiment 2 (Supplementary Fig. 1, Panel A), a pilot test, the resident mice were randomized to future treatment prior to training (day 0). The resident mice were then injected with either Ex4 (2.4 µg/kg, IP) or vehicle on each training (day 1–6) and on the test day (day 7).

In experiment 3 (Fig. 2, Panel A), the resident mice were untreated during the first training day (day 1), and were thereafter stratified to future treatment groups with similar baseline attack latency. The resident mice were injected with either Ex4 (2.4 µg/kg, IP) or vehicle on each training (day 2–5) and on the test day (day 6). Thereafter the mice were euthanized, brains and brains (measurements of monoamine levels in aggression-related areas) as well as plasma (determination of corticosterone and testosterone concentrations) were collected. In this experiment, two mice were excluded due to repeated jumping out of the cage or to violent behavior already at day 1.

**Experiment 4** (Fig. 3) was conducted to explore if a low dose of Ex4, as the high dose, reduces aggressive behaviors. Therefore, the resident mice were untreated during the first training day (day 1), and were thereafter stratified to future treatment groups with similar baseline attack latency. The resident mice were injected with either Ex4 (1.2 µg/kg, IP) or vehicle on each training (day 2–5) and on the test day (day 6). After the test, the mice were euthanatized and brains (determination of noradrenalin/serotonin-related proteins or FOS immunoreactivity) were collected. One mouse was excluded as its body weight was lower than the body weight of the submissive mice.

As HFD impairs the responsiveness to GLP-1 on various behaviors the possibility that HFD blunts the ability of Ex4 to reduce aggressive behaviors was explored in experiment 5 (Fig. 4). The resident mice had free access to HFD (i.e., peanut butter) and Chow in their home cage prior to the resident intruder test, allowing acclimatization to HFD. The mice were untreated during the first training day (day 1), and were thereafter stratified to future treatment groups with similar baseline attack latency. The resident mice were injected with Ex4 (2.4 µg/kg, IP) or vehicle on each training (day 2–5) and on the test day (day 6). Each day, the pre-weighed peanut butter was introduced to the
mice after resident intruder test removed and weighed prior to the following test (24-h intake). The mice had access to HFD, but not chow, during the daily encounter (maximum 10 min per day). Neither the dominant or submissive mice appeared to eat HFD during the resident intruder test as evident by visual observation. In fact, access to food during test in combination with food restriction decrease aggression behavior in the resident intruder test [59], the visual inspection in combination with free access to food indicate that the mice were not hungry when performing the test. After the test day, the mice were euthanatized and brains (measurement of serotonin/noradrenalin-related proteins) were collected.
Ex vivo biochemical analyses. After experiment termination, tissues were collected and placed on dry ice and frozen at −80 °C until analysis as described before [33]. Ex vivo measurements (HPLC-EC, ELISA, western blot, or immunohistochemistry) were conducted as they allowed determination of downstream mechanisms responsible for the ability of Ex4 to reduce aggressive behavior. Besides, the effect of HFD exposure on the expression of GLP-1R was examined by means of reverse transcription and real-time PCR.

An established HPLC-EC system [33, 60] was used to measure monoamines in important brain areas (Table 1) from mice treated with Ex4 (2.4 μg/kg). The plasma levels of corticosterone or testosterone, important for aggression (for reviews see refs. [1, 32]), were measured in these mice with a specific Enzo ELISA (AH diagnostics, Stockton, Sweden) as described previously [33].

Two separate western blot studies were conducted from brains collected from mice of the resident intruder test i) treated with Ex4 (1.2 μg/kg) or vehicle and eating chow or ii) treated with Ex4 (2.4 μg/kg) or vehicle and exposed to HFD during test. Western blot of serotonin or noradrenergic proteins in NAC, specifically the core and shell compartments, were conducted as the neurochemical data reveal that these mediate the Ex4 reduced aggression. Specifically, for NAc shell both the ventral and medial subregions were selected. Moreover, dorsal raphe was explored as this area provides the main serotoninergic projections to NAc. Detailed protocol described in Table 2. After being blocked with 5% skim milk for 1 h at room temperature, the membrane was sequentially incubated with primary/secondary antibodies according to Table 3. β-actin was used as a loading control.

The relative density of the target protein band was normalized to the density of the β-actin band to represent the relative expression of the target protein.

The number of FOS positive cells were counted in brain slices from mice treated with Ex4 (1.2 μg/kg; n = 3) or vehicle (n = 3–4) from the resident intruder experiment (Fig. 3A) as described in detail elsewhere [61]. NAC, specifically the core and shell regions, was assessed as neurotransmitter and protein content in this area was associated with aggressive behaviors. As for the Western Blot studies, the ventral rather than medial subregion was selected. To highlight the possibility that Ex4 alters neuronal activation in dorsal raphe responsible for the main serotonin to NAC, the number of FOS positive cells was counted in this area. Besides, additional areas important for aggression was analyzed (Table 2). A series of 2 pictures per brain region of each section was obtained by a Nikon light microscope (Nikon Eclipse 90i, UK) and was described previously [33].

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The expression of GLP-1R was explored in NAC and dorsal raphe after 7 days of access to either chow (n = 18) or the combination of HFD and chow (n = 10) in the home cage to male mice. After termination, the NAC and dorsal raphe were collected by punching. Due to optimizing the detection of GLP-1R, regions from three mice were pooled to one sample (n = 6 per group).

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Statistical analysis of the human genetic study. A linear mixed effect model in the MIXED procedure of SAS 9.3 (SAS Institute, Inc, Cary, NC, USA), was used. The analysis includes scores from all genotyped individual. This model adjusts for (i) the dependent nature of twin observations and (ii) dependence of individuals from the same family. Two separate variance–covariance matrices was modeled (monozygotic and dizygotic twins), as monozygotic and dizygotic twins share 100% and 50% of their genome respectively. Correlations between individuals in groups (1) and (2) were calculated with an R-side random effects with an unstructured variance–covariance matrix. The Bonferroni correction was used to control for multiple testing, where a primary analysis of 5 SNPs yielded a corrected alpha of 0.01.
Fig. 2  Repeated exendin-4 treatment suppressed the acquisition of aggressive behaviors in male mice. A schematic illustration of the resident intruder experiment (Panel A) with repeated exendin-4 (Ex4) treatment. At day 1, the mice were trained in the resident intruder paradigm and the time to first attack was scored. This allows stratification (#; with similar attack score between future treatment groups) into Ex4 (2.4 µg/kg, IP, N = 7) or vehicle (Veh, IP, n = 7) injections throughout training (day 2–5) and at the test day (day 6). During the training, the latency to attack was recorded (Panel B), and aggressive behaviors (attack, threat,Panel C) and non-aggressive behaviors (social and non-social, Panel D) were scored for 10 min at the test day. Directly after the test the mice were euthanized, and the brains and plasma from each mouse were collected for ex-vivo analyses (Panel E). A in these mice the baseline latency to attack was similar between future treatment groups (t(12) = 0.04, P = 0.9687). A repeated treatment with Ex4 during training increased the latency to attack (treatment F(1,12) = 8.10, P = 0.0148, time F(3,36) = 4.09, P = 0.0134, interaction F(3,36) = 2.11, P = 0.1163, n = 7 per group). Post-hoc analysis reveal that this difference is evident at day 5 (P = 0.0023). B This is further evident as the area under the curve for attack latency during training is higher in Ex4 treated mice compared to those treated with vehicle (t(12) = −2.20, P = 0.0455). At test day, Ex4 reduced aggressive behaviors (Panel C), and enhanced social behaviors (Panel D). Compared to vehicle, Ex4 (C) did not affect the attack duration (t(12) = 1.71, P = 0.1131), but (D) reduced attack frequency (t(12) = −2.64, P = 0.0218) and (E) increased attack latency (t(12) = 2.49, P = 0.0286). Ex4 treated mice had no alterations in threat (F) duration (t(12) = 0.15, P = 0.8824), (G) frequency (t(12) = 0.98, P = 0.3448), (H) latency, (t(12) = 0.50, P = 0.6291) compared to vehicle-treated mice. Ex4 did not alter (I) social behavior duration (t(12) = 1.75, P = 0.1065), but (J) increased social behavior frequency (t(12) = 2.89, P = 0.0136) and (K) tended to reduce the social behavior latency (t(12) = 2.07, P = 0.0607). Ex4 did not affect (L) non-social behavior duration (t(12) = 0.33, P = 0.7482). In an attempt to define underlying mechanisms, the levels of monoaminergic neurotransmission in various aggression-related areas and the plasma levels of testosterone or corticosterone were investigated in these mice (Panel E). Compared to vehicle, repeated Ex4 injections to mice in the resident intruder test decreased (M) noradrenalin (NA) (t(12) = 2.78, P = 0.0168), (N) serotonin (5-HT) (t(12) = 2.91, P = 0.0132) and (O) 5-HIAA (t(12) = 2.83, P = 0.0152) levels in nucleus accumbens (NAc). Contrarily, there were no differences between treatments when it comes to (P) 5-HT turnover (t(12) = 0.53, P = 0.6027), (Q) dopamine (DA) (t(12) = 0.66, P = 0.5236), (R) DOPAC (t(12) = 0.14, P = 0.8898), (S) 3-MT (t(12) = 0.72, P = 0.4849) levels or (T) DA turnover (t(12) = 1.36, P = 0.1977) in NAc.In these mice, Ex4 (n = 6, one sample excluded due to contamination) does not alter the levels of (U) corticosterone or (V) testosterone in plasma compared to vehicle (n = 7). The plasma concentrations of (U) corticosterone (t(11) = 1.52, P = 0.1571) and (V) testosterone (t(11) = 0.35, P = 0.7300) did not differ between mice treated with Ex4 (n = 6, one sample contaminated following handling) or vehicle (n = 7) from these experiments. Data are presented as mean ± SEM; significant data are illustrated by *P<0.05, **P<0.01.

RESULTS
Acute Ex4 did not alter expression of aggressive behavior in male mice
In mice assigned to either vehicle or Ex4 treatment at the test day after aggression training (Fig. 1, Panel A) displayed a similar baseline attack latency during this period (Panel B; day 1–6). At test day, acute administration of Ex4 (2.4 µg/kg) did neither alter aggressive behaviors (Panel C, Fig. 1A–F) or non-aggressive (Panel D; Fig. 1G–J) behaviors compared to vehicle.

Repeated treatment of Ex4 reduced the acquisition, but not expressing, of aggressive behaviors in male mice
As acute treatment did not alter aggressive behaviors, the effect of repeated Ex4 on aggressive behaviors was explored. Mice were treated during training (day 1–6), and on the test day (day 7) (Supplementary Fig. 1, Panel A). Compared to vehicle, repeated treatment of Ex4 (2.4 µg/kg) increased the latency to attack in these mice (Supplementary Fig. 1, Panel B). On the test day, there were no differences in aggressive behaviors (Supplementary Fig. 1, Panel C) or non-aggressive behaviors (Supplementary Fig. 1, Panel D) between Ex4 and vehicle treatment. These findings suggest that Ex4 reduced acquisition of aggression, but not the expression of aggression. Therefore, a different design was used onwards where the effect on repeated Ex4 injection on the acquisition of aggression was explored (Figs. 2 and 3, Panel A).

Repeated treatment of Ex4 dose-dependently reduced the acquisition of aggressive behaviors in male mice
Subsequent experiments investigate the possibility that repeated Ex4 treatment dose-dependently (2.4 or 1.2 µg/kg) reduces the acquisition of aggressive behaviors.

Firstly, in mice with similar baseline aggression, repeated Ex4 (2.4 µg/kg) injection during training increased the latency to attack, a difference specifically evident at day 5 (Fig. 2A). This is further evident as the AUC for attack latency was higher in Ex4 treated mice compared to vehicle mice (Fig. 2B). At test day Ex4 did not alter attack duration (Fig. 2C), but reduced attack frequency (Fig. 2D) and increased attack latency (Fig. 2E). Despite a robust effect on attack behaviors, Ex4 did not alter threat duration, frequency or latency (Fig. 2F–H). When it comes to non-aggressive behaviors (Panel D), Ex4 did not affect social behavior duration (Fig. 2I), whereas it increased social behavior frequency (Fig. 2J), without altering the social behavior latency (Fig. 2K), or non-social behavior duration (Fig. 2L). In an attempt to define underlying mechanisms, the levels of monoaminergic neurotransmission in various aggression-related areas and the plasma levels of testosterone or corticosterone were investigated in these mice. Repeated Ex4 treatment did not alter the levels of monoamines, their metabolites or turnover in VTA, prefrontal cortex, hypothalamus, hippocampus or amygdala (Supplementary Table 3), but altered neurotransmission in NAc (Fig. 2, Panel E). Specifically, repeated Ex4 injections decreased noradrenaline, serotonin and 5-HIAA levels (Fig. 2M–O), without altering serotonin turnover, the levels of dopamine, DOPAC, 3-MT or dopamine turnover (Fig. 2P–T). Correlational analyses were conducted in an attempt to correlate Ex4-induced alterations in neurotransmission in NAc with the behavioral outcomes (Table 5). Noradrenaline levels correlated with attack latency and social behavior frequency. 5-HT levels correlated with attack latency and social behavior frequency, whereas 5-HIAA levels correlated with attack duration, attack frequency, attack latency, social behavior frequency and social behavior latency. Moreover, Ex4 did not alter the levels of corticosterone or (Fig. 2U, V) testosterone in plasma.

As demonstrated in Fig. 3 (Panel B), a low dose of Ex4 (1.2 µg/kg) during training did not alter the latency to attack (Fig. 3A) or the AUC (Fig. 3B). On the test day, a low dose of Ex4 decreased aggressive behaviors (Panel C). Ex4 did not alter attack duration, frequency or latency (Fig. 3C, D), but decreased threat duration and frequency (Fig. 3F, G) and increased threat latency (Fig. 3H). Besides, the scores of social behavior duration, social behavior frequency, social behavior latency or non-social behavior duration do not differ between treatment groups (Fig. 3I–L; Panel D).

Western blot studies (Fig. 3, Panel B; n = 3 per group) were conducted on these mice to further indicate that Ex4-reduced aggression involves serotoninergic and noradrenergic neurotransmission in NAc, specifically the core or shell compartment. Additionally, raphe was studied as it sends the main serotoninergic projection to NAc. These studies revealed that a low dose of Ex4 enhanced the TH2 protein levels of raphé (P < 0.05, Fig. 3L–N). There were no observed differences in any investigated protein protein between treatments in NAc core (Fig. 3O, P). In NAc shell, the protein levels of DBH (P < 0.05), NET (P < 0.01), or SERT...
(P < 0.01) (Fig. 3Q, R) were lower in Ex4 compared to vehicle-treated mice. No differences were observed when it comes to GLP-1R or 5HT1B.

Immunohistochemical detection of neuronal activity in dorsal raphe (Panel F) reveal that the low dose of Ex4 did not alter the number of cells positive for FOS in this area (Fig. 3S, T, P = 0.1878). As Ex4 alters neurotransmission in NAc shell, we aimed to study the possibility that treatment alters neuronal activity by means of immunohistochemical studies (Fig. 3, Panel F). Similar to the western blot findings, the low dose of Ex4 did not alter the
**Fig. 3** Repeated treatment with a low dose of exendin-4 reduces aggressive behaviors in male mice. The outline of the resident intruder experiment with a low dose of exendin-4 (Ex4) is illustrated in Panel A. At day 1, the mice were trained in the resident intruder paradigm and the time to first attack was scored. This allows stratification (ψ; with similar attack score between future treatment groups) into Ex4 (1.2 µg/kg, IP, n = 8) or vehicle (veh, IP, n = 7) injections throughout training (day 2–5) and at the test day (day 6). During training latency to attack was recorded (Panel B), and aggressive behaviors (attack, threat, Panel C) and non-aggressive behaviors (social and non-social, Panel D) were scored for 10 min at the test day. Directly after the test, the mice were euthanized, and the brains from each mouse were collected for ex-vivo analyses (Panel E, F). A in these mice the baseline latency to attack was similar between future treatment groups (t(13) = 0.20, P = 0.8476). A low dose of Ex4 did not alter the latency to attack (treatment F(1,13) = 0.31, P = 0.5850, time F(3,39) = 1.64, P = 0.1959, interaction F(3,39) = 0.96, P = 0.4221; n = 8, Ex4, n = 7, veh), possibly implying that higher doses of Ex4 are required to influence attack latency during the training. B This is further evident as the area under the curve was similar between vehicle and Ex4 treated mice (t(13) = 0.30, P = 0.7704). At test day, a low dose of Ex4 affected some aggressive (Panel C), but not any non-aggressive (Panel D) behaviors. Ex4 (ψ) did notalter attack duration (t(13) = 0.75, P = 0.4646), or (B) attack frequency (t(13) = 1.48, P = 0.1619), but (A) tended to enhance the or attack latency (t(13) = 2.02, P = 0.0650). In addition, Ex4 (ψ) reduced attack duration (t(13) = 3.15, P = 0.0077), (H) suppressed threat frequency (t(13) = 3.94, P = 0.0017) and (H) enhanced threat latency (t(13) = 4.24, P = 0.0010). The low dose of Ex4 did not alter (I) social behavior duration (t(13) = 0.002, P = 0.9982), (J) social behavior frequency (t(13) = 0.65, P = 0.5281), (K) social behavior latency (t(13) = 0.59, P = 0.5639) or (L) non-social behavior duration (t(13) = 1.21, P = 0.2471). Western blot (Panel E) show (M) the expression of tryptophan hydroxylase (TPH2) of raphe from mice of the above resident intruder test, (N) and the TPH2 levels are higher after a low dose of Ex4 (n = 3) compared to vehicle (n = 3). Western blots show the expression of dopamine-b-hydroxylase (DBH), noradrenaline reuptake transporter (NET), serotonin reuptake transporter (SERT), glucagon-like peptide-1 receptor (GLP1R), serotonin-1B-receptor (5HT1B) of (O) nucleus accumbens (NAc) core, (P) where no differences are evident between treatments, or (Q) NAc shell. R Compared to vehicle, a low dose of Ex4 reduces the protein levels of DBH, NET, SERT, without altering the levels of GLP1R, or SHT1B in NAc shell. β-actin was always used as a loading control. The relative density of the target protein band was normalized to the density of the β-actin band to represent the relative expression of the target protein. FOS immunoreactivity (Panel F) of one representative picture of (S) dorsal raphe and (U) NAc core and shell, where the FOS positive cells were quantified. Compared to vehicle (n = 4, and 2–4 slices from each animal) the low dose of Ex4 (n = 3, and 2–4 slices from each animal) did not alter the number of cells positive for FOS immunoreactivity in (T) dorsal raphe (ψ) NAc core (ψ) NAc shell, (V) lateral ventricle (LV), anterior commissure (AC). Data are presented as mean ± SEM; significant data are illustrated by *P < 0.05, **P < 0.01, ***P < 0.001.

**DISCUSSION**

Herein we report that repeated, but not acute, administration of Ex4 dose-dependently reduces the acquisition, but not expression, of aggression-like behaviors in male mice in the resident intruder test. As evident from neurochemical and western blot studies, this reduction correlates with neurotransmission of serotonin as well as noradrenaline in NAc, specifically the shell compartment. Besides, intake of an HFD blunts the ability of Ex4 to decrease aggression. Although this interaction is evident in male mice, no association between overt aggression in young men and polymorphisms in the GLP-1R genes was found.

As shown in various experiment herein, repeated Ex4 treatment during training increases the latency to attack, and thereafter suppresses attack behaviors and as a compensation increases social behaviors at test day. A similar effect is obtained by a lower dose of Ex4, where lower threat behaviors and tendency to increase attack latency is evident in Ex4 treated mice compared to those treated with vehicle. It may therefore be suggested that the anti-aggressive effects of Ex4 are more pronounced during acquisition rather than during full expression of the behavior. Supportively, an acute injection of Ex4 does not influence the expression of aggressive behaviors in mice already trained to be aggressive. This is further supported as the ability of Ex4 to reduce aggression disappears after a prolonged treatment schedule. The possibility that GLP-1 influences the acquisition but not expression should be determined in detail in upcoming studies. It should also be considered that tolerance to Ex4 may influence this outcome. Indeed, tolerance may be associated with a desensitization of the GLP-1R to Ex4, which already has been demonstrated in cell cultures [70]. The findings that Ex4 causes tolerance to other reward-related behaviors [71], an effect not evident by GLP1R agonists like dulaglutide [72], raises the need for additional aggression studies with dulaglutide.

As evident from the neurochemical and western blot studies, alterations in serotonergic and noradrenergic neurotransmission in NAc appear important for the ability of Ex4 to reduce aggression. Specifically, the NAc of chow-consuming mice with low expression of noradrenaline, serotonin and 5-HIAA and in consistency lower protein levels of DBH, NET, and SERT after high and low dose of Ex4 treatment respectively. A behavioural-neurotransmission correlation is
evident as both serotonin and 5-HIAA correlates to attack latency. However, the interpretation of these data is limited as a small number of animals were used in these western blot studies. Given the preliminary outcome and putatively contrasting outcome of the present data, additional experiments designed to define the exact circuits and mechanisms involved are thus warranted. The western blot findings imply that the shell compartment of NAc, rather than any other studied areas was associated with the Ex4 reduced aggression. Given that monoaminergic signaling in NAc is central for the reward of attack...
null
like aggression. One of these tentative mechanisms could be a
other domains may in
reduced depressive state as repeated Ex4 decreases immobility in
no treatment effect on these hormones. Another possibility is a
aggression \[84
and corticosterone, which previously have been associated with
systemically, peripheral signals may participate in the ability of Ex4
processes and subsequently aggression. That is unlikely since
Another tentative explanation is that Ex4 alter explorative
\[89
factor. Moreover, as the outcome of GLP-1R activation on anxiety-

In our pilot experiment with HFD exposure the aggression levels
continuous access to an HFD (peanut butter) blunts the ability of
Ex4 to reduce aggression in male mice. On this note, HFD impairs
the response to Ex4 when it comes to feeding or vagal afferent
activation \[52–55\]. It should however be taken into consideration
that the diet per se causes less aggression although this appears
less likely as HFD rather enhances aggression in rodents \[52\]. Nonetheless, this cannot be ruled out as a non-HFD (chow) control
condition was not included in this experiment. The possibility that
vehicle-treated mice eat HFD rather than engage in fighting \[59\] is
another tentative explanation to the blunted Ex4 reponse after
HFD. However, this is unlikely as neither vehicle or Ex4-treated
mice eat HFD during any of the resident-intruder daily encounter.
Neither did Ex4 treatment alter food intake outside the test,
indicating that food-restriction does not influence the obtained
data. The pilot nature of this study raises the concern that the
interpretation of these experiments should be cautious and
additional experiments should explore this interaction in detail.
The western blot data further reveal that the protein levels of SERT
in NAc shell returns to baseline in mice consuming HFD and
treated with Ex4 compared to those consuming chow. This implies
that serotonin in NAc shell may participate in the interaction
between GLP-1, aggression and HFD. It should also be noted that
the protein levels of TPH2 of raphe are elevated and DBH in NAc
are lower in Ex4 mice in both chow and HFD consuming mice.
Moreover, the NET levels are low versus high after Ex4 in mice
are lower in Ex4 mice in both chow and HFD consuming mice.

### Table 3. Selected proteins, antibodies, dilutions.

| Target protein | Primary antibody (COMPANY, City, Country) | Dilution/ incubation | Secondary antibody (COMPANY, City, Country) | Dilution/ incubation |
|----------------|------------------------------------------|----------------------|---------------------------------------------|----------------------|
| TPH2           | Rabbit anti-TPH2 LS-C346253, Lifespan Bio, Seattle, WA, USA | 1:500 in 5% skim milk/ overnight | Scanlater Eu-labeled Goat Anti- Rabbit IgG R8029 Molecular Devices, Sunnyvale, CA, USA | 1:5000 in 5% skim milk/ RT 1 h |
| DBH            | Rabbit anti-DBH b209487, Abcam, Cambridge, UK | 1:500 in 5% skim milk/ overnight | Scanlater Eu-labeled Goat Anti-Rabbit IgG R8029 Molecular Devices, Sunnyvale, CA, USA | 1:5000 in 5% skim milk/ RT 1 h |
| NET            | Rabbit anti-NET LS-C408311, Lifespan Bio, Seattle, WA, USA | 1:1000 in 5% skim milk/ overnight | Scanlater Eu-labeled Goat Anti-Rabbit IgG R8029 Molecular Devices, Sunnyvale, CA, USA | 1:5000 in 5% skim milk/ RT 1 h |
| SERT           | Rabbit anti-SERT ab9726, Sigma Aldrich, Saint Louis, MO, USA | 1:500 in 5% skim milk/ overnight | Scanlater Eu-labeled Goat Anti-Rabbit IgG R8029 Molecular Devices, Sunnyvale, CA, USA | 1:5000 in 5% skim milk/ RT 1 h |
| SHT1B          | Rabbit anti-SHT1B SAB4501471, Sigma Aldrich, Saint Louis, MO, USA | 1:500 in 5% skim milk/ overnight | Scanlater Eu-labeled Goat Anti-Rabbit IgG R8029 Molecular Devices, Sunnyvale, CA, USA | 1:5000 in 5% skim milk/ RT 1 h |
| GLP1R          | Rabbit anti-GLP1R PAS–70645, Invitrogen, Waltham, MA, USA | 1:500 in 5% skim milk/ overnight | Scanlater Eu-labeled Goat Anti-Rabbit IgG R8029 Molecular Devices, Sunnyvale, CA, USA | 1:5000 in 5% skim milk/ RT 1 h |
| β-actin        | Mouse anti-β-actin #3700, Cell Signaling, Danvers, MA, USA | 1:1000 in 5% skim milk/ overnight | Scanlater Eu-labeled Goat Anti-Mouse IgG R8028 Molecular Devices, Sunnyvale, CA, USA | 1:5000 in 5% skim milk/ RT 1 h |

Description of the selected proteins as well as primary/secondary antibody, dilutions and incubation times used. Tyrosine Hydroxylase Isoforms 2 (TPH2), Dopamine 1 (DBH), Norepinephrine Transporter (NET), Serotonin Transporter (SERT), 5-hydroxytryptamine receptor 1B (SHT1B), Glucagon-like peptide 1 receptor (GLP1R).

### Table 4. Coordinate(s) for the selected brain regions sliced for the immunohistochemical staining.

| Brain region                | Coordinate(s) |
|-----------------------------|----------------|
| Nucleus accumbens           | +1.8 and +0.9 |
| Paraventricular thalamus    | −0.2           |
| Bed nucleus of the stria terminalis | −0.2          |
| Medial preoptic area        | +0.2 and −0.2 |

Coordinates are presented in mm from bregma.

Although reward processing might influence the obtained data, other domains may influence the outcome of a complex behavior like aggression. One of these tentative mechanisms could be a suppressed locomotor activity which is unlikely as the selected Ex4 doses do not alter locomotor activity per se in the open field \[39\]. Another tentative explanation is that Ex4 alter explorative processes and subsequently aggression. That is unlikely since the resident-intruder test is performed in the home-cage of the resident mice where exploration is low. As all drugs were injected systemically, peripheral signals may participate in the ability of Ex4 to reduce aggressive behaviors. These may include testosterone and corticosterone, which previously have been associated with aggression \[84–87\]. However, this appears less likely as there were no treatment effect on these hormones. Another possibility is a reduced depressive state as repeated Ex4 decreases immobility in the forced-swim test \[88, 89\]. Anxiety is another confounding factor. Moreover, as the outcome of GLP-1R activation on anxiety-like behavior are inconsistent and depend on contextual factors \[89–94\] the possible influence on such behavior is complex. Although, upcoming studies should evaluate the complex association between anxiety state, aggression, contextual factors, and GLP-1 further.

In our pilot experiment with HFD exposure the aggression levels is similar between vehicle and Ex4-treated mice, indicating that
Inter-batch variation may also influence the data but appropriate stratification and vehicle controls are included to minimize this confounder. Therefore, strict comparisons between experiments is difficult. Herein, we show that a low, but not high dose of Ex4 attenuates threat behavior. These differences in behaviors may be linked to dose-dependent effects of Ex4 treatment, but may also be attributed to inter-batch variation in aggression levels. The current experiments were performed in male mice and as aggressive behavior is an innate sexually dimorphic behavior most commonly studied in males (for review see ref. [97]). However, the lack of inclusion of females should be considered as a major limitation with the present study. Notably, in contrast to C57BL6 female mice lactating or gestational females, female hamsters, female California or Swiss webster mice or female rats display aggression in the female intruder test [49–51]. Supportively, Ex4 does not alter the intake of foods considered rewarding (HFD) [95, 96], thus including mice with no aggression on day 1, may confound the other findings. Hence, upcoming studies should investigate the role of Ex4 on aggression in such female species and strains as this would further contribute to the understanding of the neurobiological underpinnings of female aggression compared to intermale aggression.

Here, we found no associations between polymorphisms in the GLP-1R genes and overt aggression in young men of the CATSS cohort, indicating that genetic alterations of GLP-1R does not influence the expression of aggression. However, several limitations such as small sample size and that only young males with Caucasian descent were included may have contributed to the negative outcome. Contrarily, a link between a studied GLP1R gene polymorphisms is associated with AUD, increased alcohol intravenous intake and higher BOLD response in the right globus pallidus in response to notification of high monetary reward [68]. Thus, we conclude that genetic variation of GLP-1R genes does not contribute to vulnerability to engage in overt aggression, which is supported by the preclinical data where Ex4 does not alter the expression in mice with an established behavior. However, as we further report that Ex4 attenuates the acquisition of aggression in male mice, the role of these GLP-1R polymorphisms in the acquisition of aggressive behaviors should be studied in men.

The pharmacological studies of male mice show that GLP-1R activation inhibits the acquisition of aggressive-like behaviors. It might be suggested that these findings may have a clinical application as the GLP-1R agonist, liraglutide, decreases aggressive behaviors in a man with autism-spectrum disorder [102]. On the other hand, GLP-1R may be a less suitable target to treat aggression in humans as GLP-1R activation did not alter expression of aggression in male mice with an established application as the GLP-1R agonist, liraglutide, decreases aggres-

Table 5. Correlational analyses between levels of noradrenaline, serotonin or 5-HIAA in nucleus accumbens (NAc) and behaviors in male mice from the resident intruder test which were injected repeatedly with exendin-4 or vehicle.

| Noradrenaline in NAc (pmol/mg) | Serotonin in NAc (pmol/mg) | 5-HIAA in NAc (pmol/mg) |
|-------------------------------|----------------------------|------------------------|
| Attack duration (s)           |                            |                        |
| 0.2205 (−0.3512−0.6724)      | 0.4488                     | 0.2932 (−0.2811−0.7129) |
| 0.0343*                       |                            |                        |
| Attack frequency              |                            |                        |
| 0.4209 (−0.1412−0.7778)      | 0.1340                     | 0.3555 (−0.2157−0.7455) |
| 0.0343*                       |                            |                        |
| Attack latency (sec)          | −0.7434 (−0.9136−0.3515)   | 0.0023**               |
| −0.6237 (−0.8673−0.1393)     | 0.0171*                    | −0.5461 (−0.8348−0.0219) |
| 0.0433*                       |                            |                        |
| Threat duration (s)           | 0.02718 (−0.5108−0.5498)   | 0.9265                 |
| 0.1416 (−0.4206−0.6252)      | 0.6291                     | 0.2559 (−0.3178−0.6925) |
| 0.3772                        |                            |                        |
| Threat frequency              | −0.0058 (−0.5348−0.5264)   | 0.9842                 |
| 0.1243 (−0.435−0.6144)       | 0.6720                     | 0.0222 (−0.5144−0.5463) |
| 0.9400                        |                            |                        |
| Threat latency (s)            | −0.3664 (−0.751−0.2038)    | 0.1975                 |
| −0.4223 (−0.7785−0.1395)     | 0.1325                     | −0.0299 (−0.5517−0.5087) |
| 0.9191                        |                            |                        |
| Social behavior duration (s)  | −0.2057 (−0.6638−0.3647)   | 0.4806                 |
| −0.2775 (−0.7043−0.2968)     | 0.3369                     | −0.1246 (−0.6145−0.4347) |
| 0.6714                        |                            |                        |
| Social behavior frequency     | −0.5559 (−0.839−0.0359)    | 0.0390*               |
| −0.5626 (−0.8419−0.0457)     | 0.0362*                    | −0.7681 (−0.9227−0.401) |
| 0.0013**                      |                            |                        |
| Social behavior latency (s)   | 0.3023 (−0.2718−0.7178)    | 0.2934                 |
| 0.256 (−0.3174−0.6927)       | 0.3763                     | 0.5505 (0.0281−0.8367) |
| 0.0414*                       |                            |                        |
| Non-social behavior duration (s) | −0.07643 (−0.5834−0.4733) | 0.7951               |
| −0.165 (−0.6396−0.4006)      | 0.5729                     | −0.5001 (−0.8145−0.0416) |
| 0.0686                        |                            |                        |

Data are presented with Pearson r together with the 95% confidence interval (CI) and the corresponding P-value for each correlational analysis. P < 0.05 is considered as statistically significant; *P < 0.05; **P < 0.01.
behavior. On this note, the results from the genetic study indicate that GLP-1R may have limited importance for the regulation of the expression of aggression in men. Despite this, additional studies are warranted to gain more mechanistic insight into the anti-aggressive properties of GLP-1R activation.

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AUTHOR CONTRIBUTIONS
EJ designed experiment; JV, OTS, QZ performed the pre-clinical experiments and analyzed pre-clinical data; LS provided human genetical data; DH and LW analyzed human genetical data; EJ, JV wrote the first draft of the paper. All authors contributed to and approved the final manuscript.

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JV is currently employed by AstraZeneca, but his experimental contribution was done before this. This does not alter the authors’ adherence to any of the journal’s policies on sharing data and materials. The remaining authors declare no competing interests.

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Correspondence and requests for materials should be addressed to Elisabet Jerlhag.

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