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Insights into the central pathways involved in the emetic and behavioural responses to exendin-4 in the ferret

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ABSTRACT

Background: GLP-1 receptor agonists are utilised for the treatment of Type-2 diabetes but can be associated with undesirable effects of nausea and vomiting.
Objectives: To investigate the role of GLP-1 receptors in mechanisms of emesis, behaviours indicative of nausea (BIN) and food intake in the ferret.
Results: Exendin-4 (10 and 30 nmol, i.c.v.) induced emesis, inhibited food intake, and increased the frequency of BIN. Increases in c-Fos in the brainstem, midbrain and forebrain occurred in animals exhibiting emesis; no activation of the brainstem occurred in animals not vomiting. Exendin-4 (10 nmol, i.c.v.) when preceded by i.c.v. saline (15 μl), was not emetic but induced BIN and inhibited food intake; exendin (9–39) (100 nmol) reduced BIN only. c-Fos showed that consistent with the absence of emesis in saline/exendin-4 treated animals there was no increase in c-Fos in the brainstem, but it increased in midbrain and forebrain nuclei. Excepting the amygdala, exendin (9–39) was without efffect on the increases in c-Fos. Analysis of c-Fos data showed a positive linear relationship between midbrain and forebrain areas irrespective of the occurrence of emesis induced by exendin-4. In contrast, brainstem and midbrain c-Fos levels were positively correlated, but only in animals with emesis.
Conclusions: The brainstem is critical for exendin-4-induced emesis but suppression of food intake and BIN involves more rostral brain sites. Exendin-4-induced BIN and c-Fos activation of the amygdala are sensitive to exendin (9–39), whereas the suppression of food intake is not implicating separate control mechanisms for emesis and BIN.

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1. Introduction

Peptide hormones released from the digestive tract can act on local target tissues including smooth muscle, interstitial cells of Cajal (ICC), epithelial cells and neurones (e.g. enteric nervous system, vagal afferents) (Blair et al., 2014; Brookes et al., 2013; Furness, 2012; Williams et al., 2016). The action on vagal afferent terminals either directly via receptors or secondary to an action of another process (e.g. disruption of motility, secretion from enterochromaffin cells) can also modulate the central nervous system (Brookes et al., 2013; Dockray, 2014; Sanger et al., 2013). Peptide hormones may also act on the brain within the circumventricular organs located in the third (e.g. organum vasculosum lamina terminae, subfornical organ (OVLT), bed nucleus of the stria terminalis (BNST)) or fourth ventricles (area postrema; AP), as the blood brain barrier (BBB) is relatively permeable in these regions (Borison, 1989; Johnson and Gross, 1993; Kash et al., 2015). Peptides can also act on the brain if transported across the BBB, or if the BBB becomes permeabilised by disease (e.g. local inflammation, cerebral oedema) (Banks, 2006). Substances in the cerebrospinal fluid CSF can influence brain function and autonomic outflow via access to the circumventricular organs where the CSF-brain barrier is similarly relatively permeable (Leslie, 1986).

There has been an increasing interest in the central effects of gastrointestinal peptides because of their involvement in the control of food intake and in the sensations of hunger and satiety. Glucagon like peptide (GLP-1) is of particular interest because of its ability to regulate blood glucose in Type 2 diabetes, decrease appetite, reduce food intake and

Abbreviations: AP, area postrema; Arc, arcuate nucleus; BBB, blood brain barrier; BIN, behaviours indicative of "nausea"; BNST, bed nucleus of the stria terminalis; CC, cingulate cortex; CeA, central nucleus of amygdala; CSF, cerebrospinal fluid; DHM, dorsal medial nucleus of hypothalamus; Ex-4, exendin-4; Ex-(9–39), exendin-(9–39); GLP-1, glucagon-like peptide-1; ICC, interstitial cells of Cajal; i.c.v., intracerebroventricular; NTS, nucleus tractus solitarius; OVLT, organum vasculosum lamina terminae; PVN, paraventricular nucleus of hypothalamus; SFO, subfornical organ; SON, supraoptic nucleus; VMH, ventral medial nucleus of hypothalamus.

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cause weight loss (Buse et al., 2013; Meier, 2012; Secher et al., 2014; van Bloemendaal et al., 2014). Research and clinical utility are enhanced by the increasing availability of non-peptide, small molecule GLP-1 receptor agonists (e.g. exendin-4). In addition, the availability of selective GLP-1 receptor antagonists, such as exendin (9–39) (Goke et al., 1993; Kanoski et al., 2011), has facilitated identification of the sites at which GLP-1 receptor agonists act. GLP-1 receptors have been implicated in emesis induced by the cytotoxic agent cisplatin in *Suncus murinus* (Chan et al., 2013; Chan et al., 2014) and cisplatin-induced kaolin consumption in the rat (De Jonghe et al., 2016) which is argued to be an indicator of nausea in species such as the rat which lack an emetic reflex (Andrews and Sanger, 2014; Horn et al., 2013).

As part of a study investigating the site(s) at which GLP-1 receptor agonists act to induce both their desirable effect of reduced food intake, and their undesirable adverse effects of nausea, vomiting and hypertension, we have investigated the effect of both peripheral and central administration of the selective GLP-1 receptor agonist, exendin-4, in *Suncus murinus* (Chan et al., 2013) and the peripheral administration exendin-4 in the ferret (Lu et al., 2014). *Suncus murinus* and ferrets are used for these studies as in contrast to rodents they possess an emetic reflex (Horn et al., 2013). In *Suncus murinus*, exendin-4 given into the ventricles of the brain induced emesis more reliably than when given peripherally and the emetic effect was blocked by exendin (9–39), given as a pre-treatment via the same route (Chan et al., 2013; Chan et al., 2014). Moreover, exendin-4-induced emesis and associated behavioural changes were associated with increases of c-Fos levels in the AP, nucleus tractus solitarius (NTS), central nucleus of the amygdala (CeA), and hypothalamus (Chan et al., 2013).

The ferret has been widely used for mechanistic studies of emesis and has demonstrated translation to humans in the area of anti-emesis (Percie du Sert and Andrews, 2014). The ferret is also suitable for investigations of digestive tract and cardiovascular effects of pharmacological agents using implanted telemetry (e.g. Lu et al., 2014; Percie du Sert et al., 2009) and additionally the ferret has a behavioural repertoire that is arguably better characterised for studying "behaviours indicative of nausea" (BIN) (see Method for details and references). Studies of GLP-1 receptors and emesis initially performed in *Suncus murinus* were therefore extended to investigate i.c.v. exendin-4 and exendin (9–39) the ferret in which GLP-1 receptor immunoreactivity is known to be widely distributed in the brain (Lu et al., 2014). This study aimed firstly to characterise the emetic and behavioural effects of i.c.v. exendin-4 to identify a dose that could be used in subsequent studies using implanted telemetry. Secondly, c-Fos expression in the brain was quantified at doses of exendin-4 inducing emesis to provide insights into the nuclei likely to be involved in emesis. Having identified a dose of exendin-4 inducing emesis we then aimed to investigate the effects of exendin-(9–39) or saline vehicle given 15 min before exendin-4 (cf. Suncus protocol, (Chan et al., 2013; Chan et al., 2014)). The results provide insights into the central sites at which GLP-1 receptors are implicated in the genesis of the side effects of GLP-1 receptor agonists but the unexpected findings from the study with exendin (9–39) raise issues about reproducibility and technical issues of i.c.v. administration that have wider implications for studies of emesis.

2. Materials and methods

2.1. Animals

Castrated adult male fitch ferrets (1.51 ± 0.08 kg; $n = 37$) were obtained from Southland Ferrets (Invercargill, New Zealand). They were housed individually in observation cages (0.5 m × 0.5 m × 0.5 m) in a temperature-controlled room at 24 ± 1 °C under artificial lighting, with lights on between 06:00 to 18:00 h, and with a relative humidity of 50 ± 5%. Food (TriPro super premium chicken meal formula dog food, American Nutrition, USA) and water were available ad libitum except when indicated. All experiments were conducted under licence from the Government of the Hong Kong SAR and the Animal Experimentation Ethics Committee, The Chinese University of Hong Kong.

2.2. Cannulation of the third ventricle

Animals were fasted overnight with free access to water. They were injected with buprenorphine (0.05 mg/kg, s.c. Temgesic®), and anaesthesia was induced by ketamine (20 mg/kg i.m.; Alfasan, Holland) to enable intubation (2/0 tube) to permit anaesthesia with 1.5% isoflurane (Halocarbon Products Corporation, USA) in oxygen (Narkomed 2C, Drager, USA). Rectal temperature was monitored and maintained at 37 °C (UCI#390 Analogue moist heating pad, Rebirth Medical & Design, Inc., Taiwan). The level of anaesthesia was monitored during surgery by the absence of the limb withdrawal reflex. Ferrets were then placed into a stereotaxic frame equipped with custom-made ear-bars and mouthpieces (David Kopf Instruments, Tujunga, USA). The temporalis muscles were exposed via a skin incision and displaced to expose the skull. A hole was drilled in the skull: coordinates for the third ventricle: 17.3 mm anterior to lambda and at the midline. Coordinates were derived from studies in our laboratory (Rudd et al., 2006) with guide-cannula placement confirmed (dye injection and histology) and literature on the topographical anatomy of the ferret brain and skull (Lawes and Andrews, 1987). A 30-gauge cannula was then inserted into the hole in the skull, 8 mm below the surface of the dura. Two screws were fixed into the skull and dental cement was applied to secure the screws and guide cannula in place. The muscle layer was closed with a 2/0 curved cutting needle and the skin was closed with a 2/0 straight cutting needle. The wound was treated with Opsite® (Smith and Nephew, UK) silicone dressing. Guide-cannula placement was confirmed by dye injection post mortem on completion of the study.

2.3. Assessment of the effect of intracerebroventricular exendin-4 and exendin (9–39) on behaviour, food intake, and c-Fos expression in the brain

Prior to experimentation, all ferrets ($n = 37$) had free access to food and water. To determine if the effect of exendin-4 on behaviour including emesis (see below for definitions) was dose-related, animals were randomised to receive saline (15 μl, i.c.v.), or exendin-4 (3–30 nmol, i.c.v. in 15 μl saline) and additionally were randomised to one of four injection times (09.00, 10.00, 14.00 or 16.00). Doses of exendin-4 given i.c.v. were initially based upon studies in *Suncus murinus* (Chan et al., 2013; Chan et al., 2014), but also included a 10 times higher dose (30 nmol) to take into account the larger brain size and ventricular volume of the ferret. Spontaneous food and water consumption during the 60 min observation period were also measured. 60 min post exendin-4 administration, the animals that had received either saline (15 μl), exendin-4 at 10 nmol or 30 nmol i.c.v. (i.e. the groups with an emetic response) were deeply anaesthetized with pentobarbitone (40 mg/kg, i.p.) (Dorminal®, Alfasan, Woerden, Holland) and perfused via the heart with ice-cold saline (120 ml) followed by 4% paraformaldehyde in phosphate-buffered saline (PBS; 200 ml). A 60-min time period was chosen primarily as c-Fos activity is reported to peak at 60–90 min following challenges (Hoffman et al., 1993) and we also considered (based upon comparable studies in *Suncus murinus* (Chan et al., 2011)) that any emetic response to i.c.v. exendin-4 would occur within minutes of injection. Brains were removed and post-fixed in 4% paraformaldehyde overnight at 4 °C and placed in an aluminium foil container filled with O.C.T. compound (Tissue-Tek, Sakura, USA). The preparations were frozen in isopentane pre-cooled liquid nitrogen for c-Fos immunohistochemistry (see below).

Following identification of a dose of i.c.v. exendin-4 (10 nmol) that induced emesis in 75% of the animals and that produced the highest level of behaviours indicative of nausea (see below and Results Section 3.1), we then investigated the effect of exendin-(9–39) on the responses in another group of animals using a dose of exendin (9–39) (100 nmol), 10× > than the dose of exendin-4 (10 nmol); the same

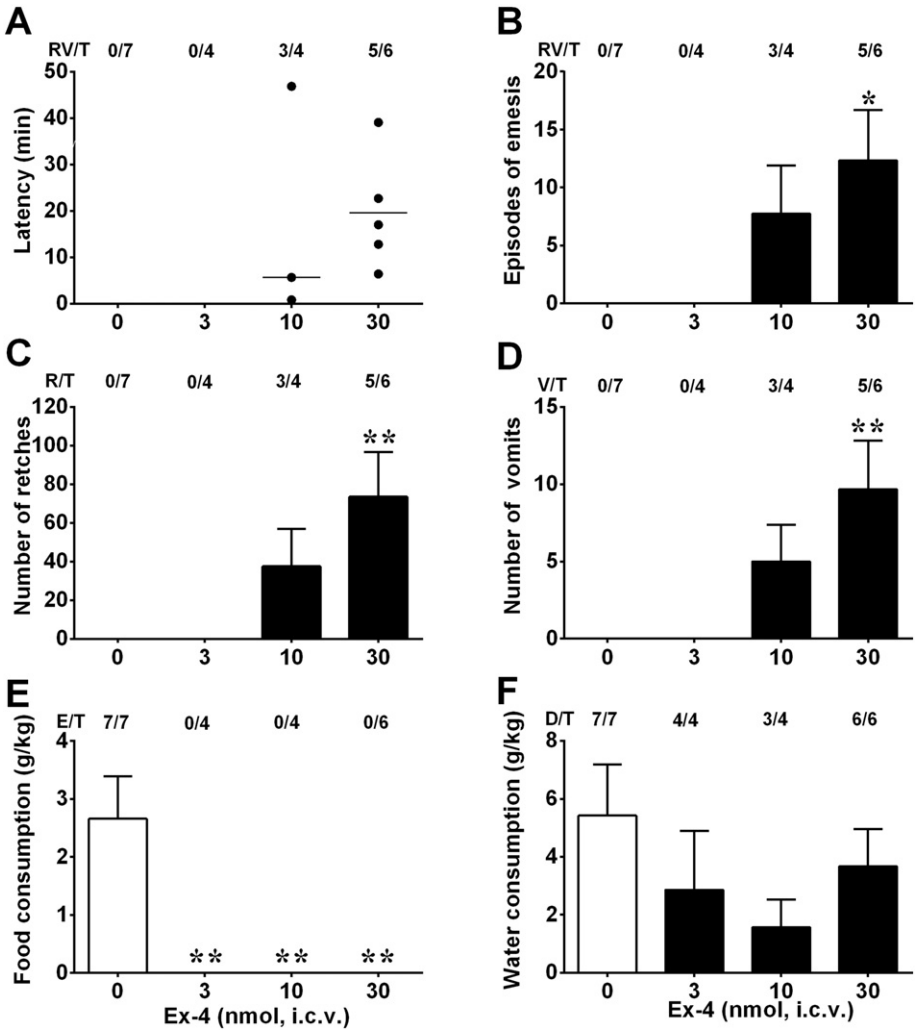


Fig. 1. Effect of an i.c.v. administration of exendin-4 (Ex-4) (3–30 nmol) or saline (15 μl) on emesis, food and water intake in the ferret. (A) The latency to the first emetic episode, (B) the number of episodes, (B) retches, (C) vomits, (E) food intake, and (F) water intake are shown. Episode, and retching and vomiting data represents the mean ± s.e.m. of 4–7 animals; latency data are shown as individual responses, with lines indicating the median response. The number of animals retching (R) and/or vomiting (V), Eating (E), and Drinking (D) out of the number of animals tested (RV/T, R/T, V/T, E/T, and D/T) is also shown. Significant differences compared to the saline control group are indicated as $^{*}P < 0.05$, $^{**}P < 0.01$ (One-way ANOVA followed by Bonferroni test).

dose ratio used in ferrets and *Suncus murinus* to demonstrate involvement of GLP-1 receptors in glucose homeostasis (Chan et al., 2011; Lu et al., 2014). Animals were randomised to the experimental groups and exendin (9–39) (100 nmol, i.c.v.), or saline (15 μl, i.c.v.) was administered 15 min before exendin-4 (10 nmol, i.c.v.) and food intake, water consumption and behaviour were monitored as described above. Following observation animals were deeply anesthetize and brains were fixed for processing of c-Fos activity as described above.

2.4. c-Fos immunohistochemistry

The methodology for c-Fos immunohistochemistry followed that previously used in studies of pathways implicated in emesis in the ferret (Billig et al., 2001; Boissonade and Davison, 1996; Boissonade et al., 1994; Zaman et al., 2000). Frozen tissues were sectioned at 40 μm in the coronal plane using a freezing microtome and incubated at room temperature for 1 h in 0.01% H_2O_2. The free floating sections were blocked with 1.5% normal goat serum containing 0.3% Triton X-100 in PBS (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, USA) for 1 h. Sections were then incubated with rabbit anti-c-Fos antibody (1:10,000, Ab5, Oncogene Research Products, Cambridge, USA), without washing for 48 h, at 4 °C. The sections were subsequently washed and incubated with secondary goat-anti-rabbit antibody (1:200; Vector Laboratories) for 1 h, followed by Vectastain avidin–biotin complex reagent for 1 h (1:100; Vectastain Elite ABC kit, Vector Laboratories, Burlingame, USA). c-Fos expression was visualised using a commercially available peroxidase substrate (Vector® VIP kit, Vector Laboratories, Burlingame, USA). The number of c-Fos-like-immunoreactive cells was counted manually using a Zeiss Axioskop 2 plus microscope (Carl Zeiss Inc. Thornwood, USA) equipped with a Zeiss Axiocam 2 camera. The person analysing the c-Fos immunohistochemistry was unaware of the treatments given. Neurones were considered c-Fos positive if they contained a visible nuclear dark purple c-Fos immunoreactive product. The number of positive neurones was counted in pre-defined brain regions of interest (see below), consistent with previous methodology in ferrets (Billig et al., 2001) and *Suncus murinus* (Chan et al., 2013). To quantify c-Fos expression in the brain, three sections from each region of interest were analysed. Nuclei were identified from ferret brain topographical anatomical guides (Kroenke et al., 2014; Lawes and Andrews, 1998), publications utilising c-Fos in the ferret (Dampney et al., 2003; Zaman et al., 2000), and a ferret brain stereotaxic atlas constructed using Nissl stained sections (Rudd laboratory, unpublished). The rostro-

caudal coordinates (measured from the obex) of sections in which c-Fos positive neurones were counted were +16.44 mm, +16.56 mm, +16.68 mm, for the arcuate nucleus (Arc), ventral medial nucleus of hypothalamus (VMH), and dorsal medial nucleus of hypothalamus (DMH) respectively; +16.8 mm, +16.92 mm. +17.04 mm for the central nucleus of the amygdala (CeA), paraventricular nucleus of the hypothalamus (PVN), and the supraoptic nucleus (SON), respectively; +17.32 mm, +17.44 mm, +17.56 mm, for the bed nucleus of the stria terminalis (BNST), cingulate cortex (CC); −0.12 mm, +0 mm, and +0.12 mm for the AP and NTS. For the areas, a grid of 450 × 600 μm^2 was superimposed on the centre of each brain region and the number of c-Fos positive cells on both sides of each section was manually counted at 20 times magnification; counts from 3 sections were averaged per animal. In addition to analysis of c-Fos counts from individual brain nuclei, the total activity in brain stem (AP + NTS), mid-brain (Arc + VMH + DMH + PVN + SON + BNST) and forebrain (CeA + CC) was also compared between treatments.

2.5. Behavioural definitions

Emesis was characterised as rhythmic abdominal contractions that were either associated with oral expulsion of solid or liquid material from the gastrointestinal tract (i.e. vomiting), or not associated with passage of material (i.e. retching). An episode of retching and/or vomiting was considered separate when the animal changed its location in the observation cage, or when the interval between retches and/or vomits exceeded 5 s (Rudd et al., 1994). Behaviours quantified were: *scratching* (scratching the floor using forelimbs); *backing* (backward walking); *burrowing* (digging with forelimbs into the bedding and moving forward with the head under the bedding); *licking* (tongue protrusion and movement) *walking* (a period of spontaneous locomotor activity lasting at least 1 s); *rearing* (standing up on hind legs); *grooming* (using mouth to clean its body); *scratching body* (using its hind legs to scratch body); *rolling* (extending body and rolling on the floor); *head shaking* (shaking only its head); *wet dog shaking* [(Zaman et al., 2000) for definition]. Following the administration of exendin-4, many of the individual behaviours increased, but when all the individual measures were collated the variability was high. However, a more detailed analysis revealed that increases were contributed primarily by changes in episodes of floor scratching, backward walking and burrowing. Burrowing, backing and licking have been quantified in ferrets and given cytotoxic drugs, radiation or morphine-6-glucuronide and have been included as components of a "nausea" score (Bermudez et al., 1988; Hawthorn and Cunningham, 1990; King and Landauer, 1990; Miner and Sanger, 1986; Sharkey et al., 2007). Therefore, we separated burrowing, backing, and licking from the other behaviours and added the scores to those for floor scratching (a behaviour probably related to burrowing; digging in bedding chips has been used as an index of an aversive response in rats, (Iida et al., 2003)). The clusters of behaviours were dose-related and classified collectively as BIN. An overall indication of the activity level was derived by summing scores for other behaviours (walking, rearing, grooming, body scratching, rolling, head shake, and wet dog shake). Behaviours considered to be indicative of reduced activity were: *laying down* (laying on the back or with the stomach pressed on the floor and immobile) or *curling up* (adopting a normal sleeping posture). The total time during which these two behaviours were observed was used to calculate the % overall time spent exhibiting each behaviour. Analysis of patterns of behaviour rather than individual behaviours have been used in *Suncus murinus*, and this approach reduces inter-individual animal behavioural variability (Horn et al., 2011).

2.6. Statistical analysis

Statistical analyses were performed using GraphPad Prism version 5 (GraphPad Prism version 5.0, Inc., California, USA). Differences between the behaviour, food consumption, and the number of c-Fos positive cells were assessed using one-way ANOVA followed by a Bonferroni's multiple comparison tests or by using a paired Student's *t*-test, as appropriate. All data are expressed as mean ± s.e.m. Differences were considered significant when $P < 0.05$.

2.7. Drug formulation

Exendin-4 and exendin (9–39) amide (American Peptide Company, Sunnyvale, CA) were dissolved in sterile saline (0.9% w/v), which was used as the vehicle for control studies.

3. Results

3.1. The emetic potential of centrally administered exendin-4 and its ability to modify behaviour

The i.c.v. administration of saline did not induce either retching or vomiting in any of the animals tested. Exendin-4 at 3 nmol, i.c.v., failed to induce either retching or vomiting in 4 animals studied, but at 10 nmol, i.c.v., 3 out of 4 animals had an emetic response (37.5 ± 19.4 retches and 5.0 ± 2.4 vomits), although the latency was variable (0.82 to 46.8 min; Fig. 1A–D). Exendin-4 at 30 nmol, i.c.v., induced emesis in 5 out 6 ferrets (73.5 ± 23.1 retches and 9.7 ± 3.1 vomits; Fig. 1A–D) but as with the lower dose the latency was variable (ranging from 6.4 to 39.1 min). Exendin-4 at all doses (3, 10, 30 nmol, i.c.v.) abolished food consumption when compared with the saline treated group

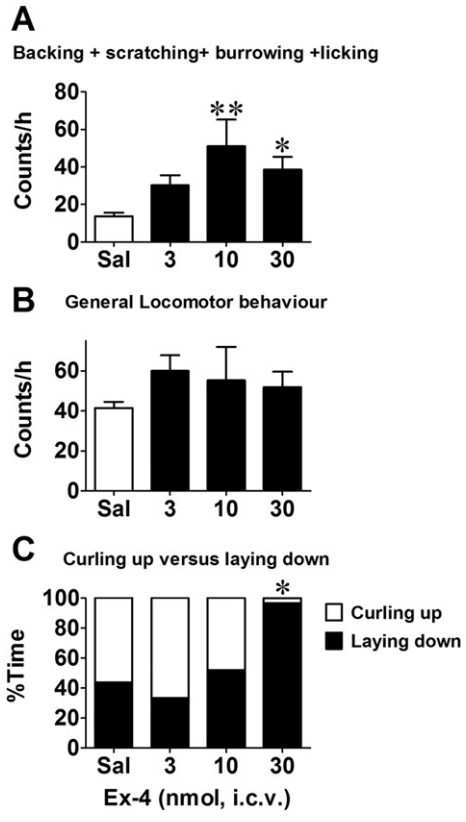
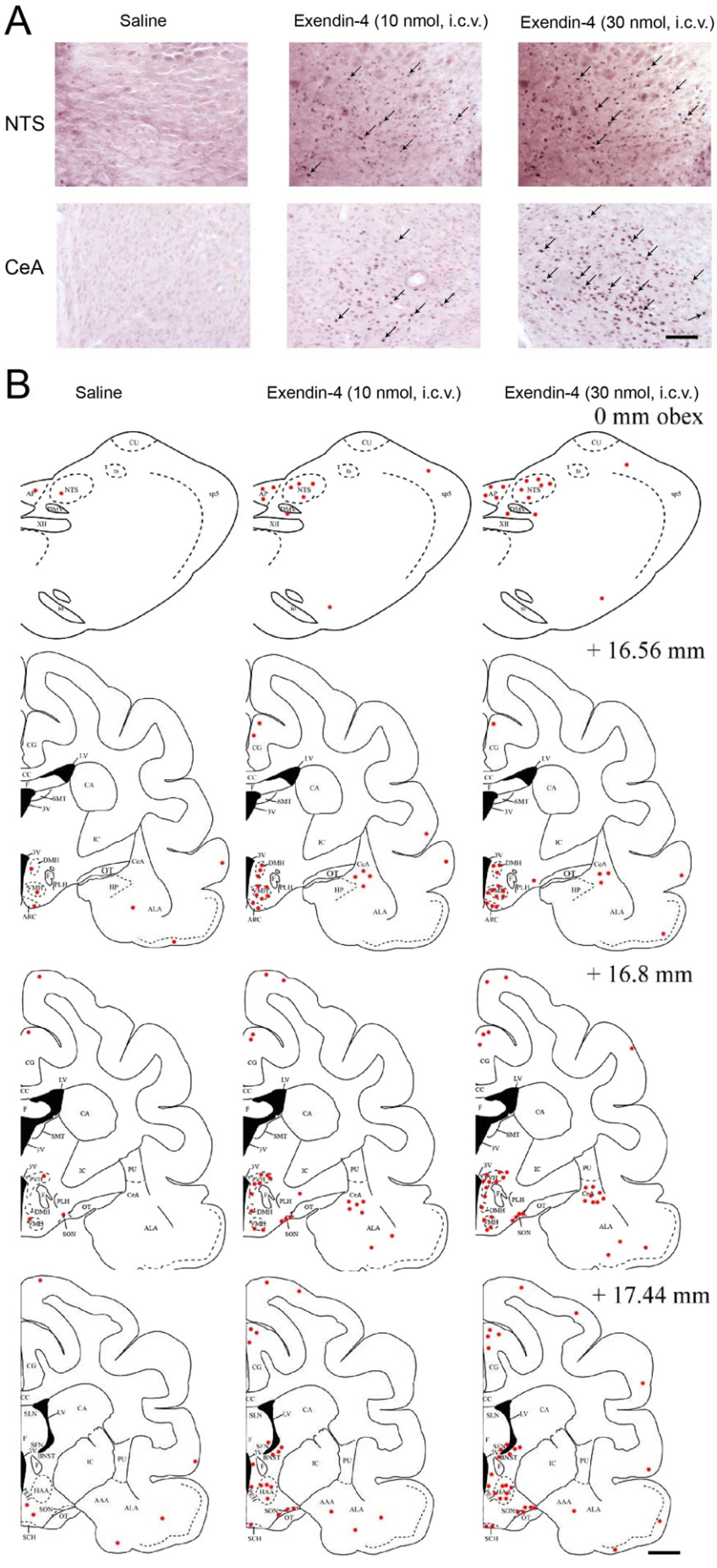


Fig. 2. Effects of drug administration on ferret behaviours. The effect of saline (sal; single dose, 15 μl) and increasing doses of exendin-4 (Ex-4; 3, 10, 30 nmol) given i.c.v. on (A) backing + burrowing + licking + scratching (BIN: behaviours indicative of nausea; sum of backing + scratching + burrowing + licking-see Methods for definitions), (B) general locomotor activity, and (C) proportion of time spent curling up versus laying down. Data represents the mean ± s.e.m. of 4–7 animals. Significant differences compared to the Sal group are indicated as $^{*}P < 0.05$, $^{**}P < 0.01$ (One-way ANOVA followed by Bonferroni test).


A
Saline
Exendin-4 (10 nmol, i.c.v.)
Exendin-4 (30 nmol, i.c.v.)
NTS
CeA
B
Saline
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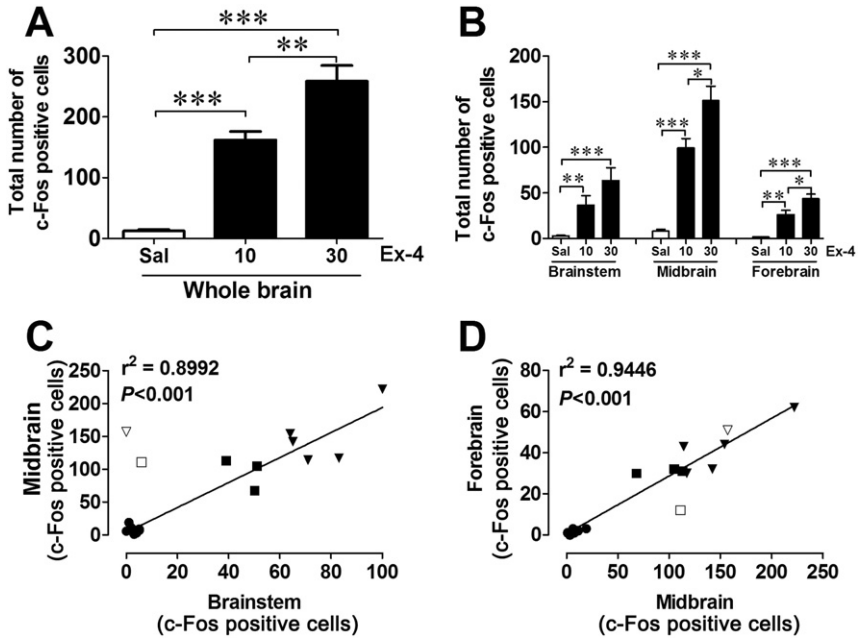


Fig. 4. Total c-Fos immunoreactivity in whole brain, brainstem, midbrain, and forebrain (see Methods for definitions) after i.c.v. administration of saline (sal; 15 μl) or exendin-4 (Ex-4; 10 nmol, 30 nmol). Exendin-4 (Ex-4; 10 nmol and 30 nmol, i.c.v.) increased total c-Fos immunoreactivity in (A) whole brain, (B) brainstem, midbrain, and forebrain. The correlation of total c-Fos immunoreactivity for (C) brainstem vs. midbrain, and (D) midbrain vs. forebrain were analysed by linear regression; black circles represent saline group, black squares represent exendinx-4 (10 nmol) group with emesis, open square represent exendin-4 (10 nmol) without emesis, black triangles represent exendin-4 (30 nmol) group with emesis, open triangle represents exendin-4 without emesis. Sal = saline, Ex-4 = exendin-4. Data represents the mean ± s.e.m. of 4–7 animals. The linear regression analysis fit excluded those animals not exhibiting emesis following exendin-4 treatment. Significant differences between treatment groups in panels A and B are indicated as $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (One-way ANOVA followed by Bonferroni test).

($P < 0.01$; $n = 4$–7; Fig. 1E) and this effect occurred irrespective of the induction of emesis. Conversely, exendin-4 did not significantly affect water intake at any of the doses (3, 10, 30 nmol) studied (Fig. 1F).

Ferrets treated with saline exhibited a low level of spontaneous behaviour during the 60 min observation time post injection; the lowest dose of exendin-4 (3 nmol, i.c.v.) did not increase the level of individual counts of backing, burrowing, licking or scratching, but this cluster of behaviours (BIN) was increased significantly ($P < 0.05$) with exendin-4 at 10 and 30 nmol, i.c.v. (Fig. 2A), with the largest effect at 10 nmol. None of the doses of exendin-4 affected the general locomotor behaviour (Fig. 2B). However, the percentage of time lying down was increased significantly by exendin-4 at 30 nmol, i.c.v., with an associated decrease in the percentage of time curled up ($P < 0.05$; Fig. 2C).

3.2. Effect of i.c.v. exendin-4 on brain c-Fos immunoreactivity

Representative photomicrographs of c-Fos expression within the hindbrain and forebrain are shown in Fig. 3. In saline treated animals, the number of c-Fos positive cells was low (Figs. 3, and 5). Exendin-4 (10, 30 nmol, i.c.v.; doses at which emesis and BIN increased) increased the number of c-Fos positive neurones in each major brain region (brain stem, midbrain and forebrain) with the response being larger for 30 nmol compared to 10 nmol in the brain as a whole (Fig. 4A), and for both the midbrain and forebrain (Fig. 4B). Taking the data together for individual animals for saline ($n = 7$), exendin-4 at 10 nmol ($n = 4$) and 30 nmol ($n = 6$) for the major brain regions a positive linear correlation was shown between the level of activation in the brainstem and the midbrain ($r^2 = 0.8992$, $P < 0.001$, $n = 15$; excluding two animals without an emetic response and no brainstem c-Fos activity above saline levels–see below; Fig. 4C) and the midbrain and the forebrain ($r^2 = 0.9446$, $P < 0.001$, $n = 17$; Fig. 4D).

Examination of the 10 individual brain areas revealed a significant increase ($P < 0.05$; Fig. 5) in c-Fos by exendin-4 (10, 30 nmol, i.c.v.). The mean number of c-Fos positive neurones was significantly greater for the 30 nmol dose compared with the 10 nmol dose only in the paraventricular nucleus of the hypothalamus (PVN) and the CeA (Fig. 5). Examination of the rank order of the percentage increase in the mean number of c-Fos positive neurones (compared to saline) following the highest dose, 30 nmol, i.c.v., in animals with an emetic response revealed the greatest increases in the AP (3570%) > CeA (3080%) > PVN (2500%) > bed nucleus of stria terminalis (BNST) (2570%) > supraoptic nucleus (SON) (1630%) > arcuate nucleus (Arc) (1440%) > NTS (1209%) > cingulate cortex (CC) (972%).

In both the 10 nmol and 30 nmol groups, one animal did not exhibit emesis, although food intake was inhibited and both animals showed behavioural changes (BIN, see below). The c-Fos data from these two animals showed no increase in c-Fos positive cells in the AP and NTS compared to the number of c-Fos positive cells in saline treated animals; this is in marked contrast to the significant increases in c-Fos in both the AP and NTS in all eight animals of the

Fig. 3. Representative photomicrographs and schematic representation illustrating c-Fos expression in the ferret brain after i.c.v. administration of saline (no emesis) and exendin-4 (10 nmol, 30 nmol) in animals that retched and/or vomited. (A) Representative photomicrographs illustrating c-Fos expression (violet nuclear label) in the caudal brainstem (NTS) and forebrain area (CeA). Arrows point out some of the many activated c-Fos positive cells. Scale bar = 100 μm. (B) A schematic representation of coronal sections depicting the distribution of c-Fos immunoreactivity in the ferret. Scale bar = 1 mm. One red dot represents ~5 c-Fos positive cells.

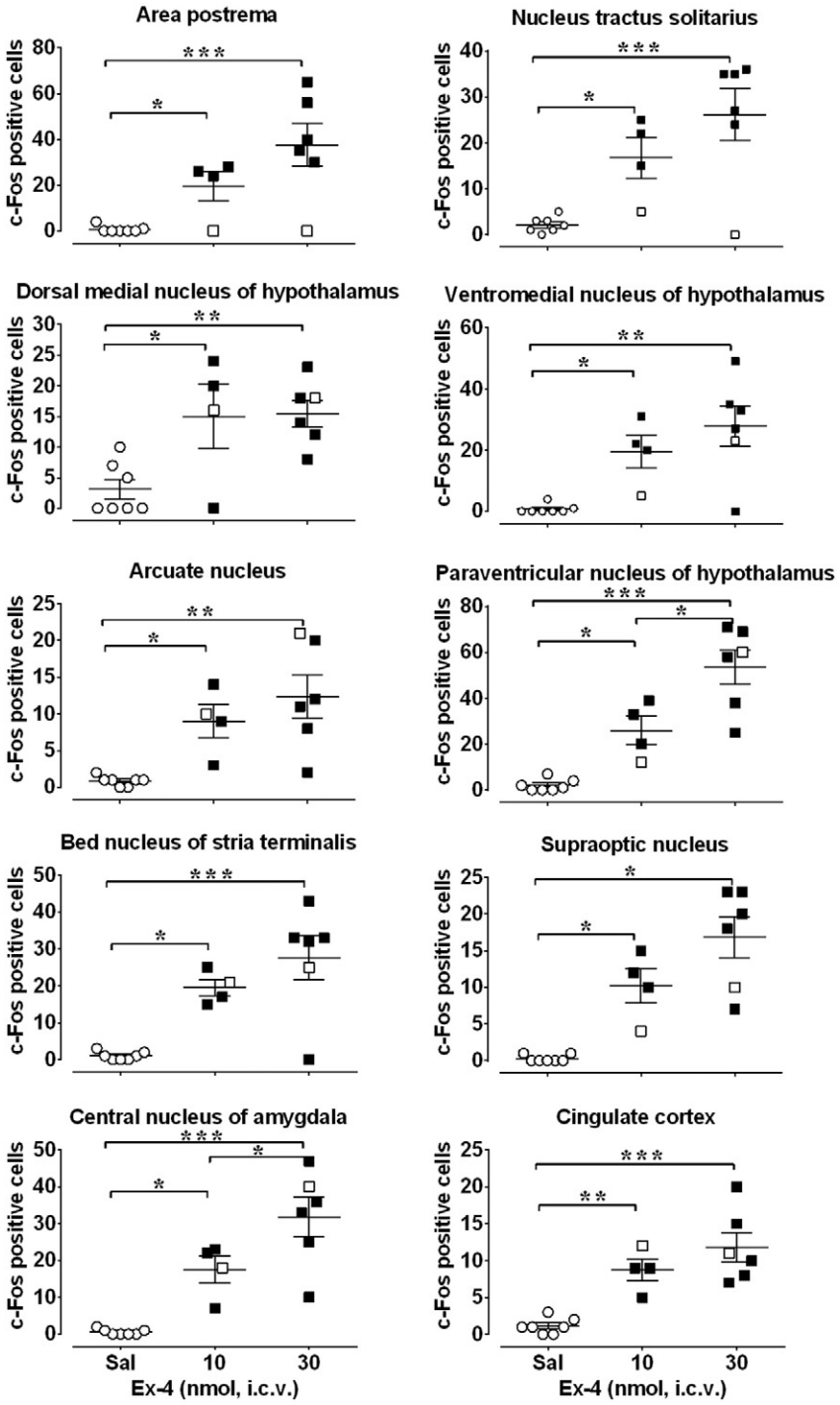


Fig. 5. Action of an i.c.v. administration of exendin-4 to induce c-Fos expression in the brain. Exendin-4 (Ex-4; 10 nmol and 30 nmol, i.c.v.) increased c-Fos expressions in the area postrema, nucleus tractus solitarius, dorsal medial nucleus of hypothalamus, ventromedial nucleus of hypothalamus, arcuate nucleus, paraventricular nucleus of hypothalamus, bed nucleus of stria terminalis, supraoptic nucleus of hypothalamus, central nucleus of amygdala, and cingulate cortex. Data represents the mean ± s.e.m. of 4–7 animals. Open-square symbols represent ferrets that had no emesis and filled symbols show ferrets exhibiting emesis. Significant differences between treatment groups are indicated as $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (One-way ANOVA followed by Bonferroni test).

animals with an emetic response given exendin-4 (3 at 10 nmol, 5 at 30 nmol) (Fig. 5). In contrast to the lack of effect of exendin-4 in the brainstem of the animal lacking an emetic response, the 10 nmol dose of exendin-4 evoked an increase in the number of c-Fos positive neurones of the same magnitude as in animals with an emetic response in five (DMH, VMH, Arc, BNST, CeA) of the eight brain regions rostral to the brainstem. At a higher dose of 30 nmol, all eight rostral (DMH, VMH, Arc, PVN, BNST, SON, CeA and CC) structures showed c-Fos increases of a similar magnitude in animals with or without an emetic response (Fig. 5).

3.3. Effect of vehicle (saline 15 μl) or exendin (9–39; 100 nmol) on exendin-4 (10 nmol)-induced changes of behaviour

A double i.c.v. administration of saline (saline/saline) was used as a control for the effect of exendin (9–39) on the profile of action of

exendin-4 (10 nmol). Animals ($n = 4$) treated with exendin-4 (10 nmol) preceded by saline (15 μl) had no emesis but food intake was abolished in all animals (reduction in food intake in saline/saline treated animals was 2.85 ± 0.47 g/kg vs saline/exendin-4 treated animals 0 ± 0 g/kg; $P < 0.01$). Exendin-4 (10 nmol) preceded by saline also significantly ($P < 0.01$) increased the total counts of BIN, but had no effect on either general locomotor behaviour, or the percentage of time curled up vs laying down (Fig. 6).

Administration of exendin (9–39) (100 nmol) with saline (15 μl) did not induce emesis, had no significant effect on BIN, locomotor behaviour, or the percentage time curled up vs laying down when compared to saline/saline (Fig. 6). In addition, exendin (9–39) was without effect on spontaneous food intake over a 60 min period post injection (saline/saline 2.85 ± 0.47 g/kg vs saline/exendin (9–39) 4.04 ± 1.15 g/kg, $n = 4$). Exendin (9–39) (100 nmol) given 15 min before exendin-4 (10 nmol) significantly reduced BIN in comparison to saline/exendin-4 alone to values comparable to those in animals treated with saline/saline or exendin (9–39) and saline (Fig. 6). In all four animals given exendin (9–39) before exendin-4, there was no food intake in the 60 min observation period, as was also the case in animals given saline and exendin-4 (see above).

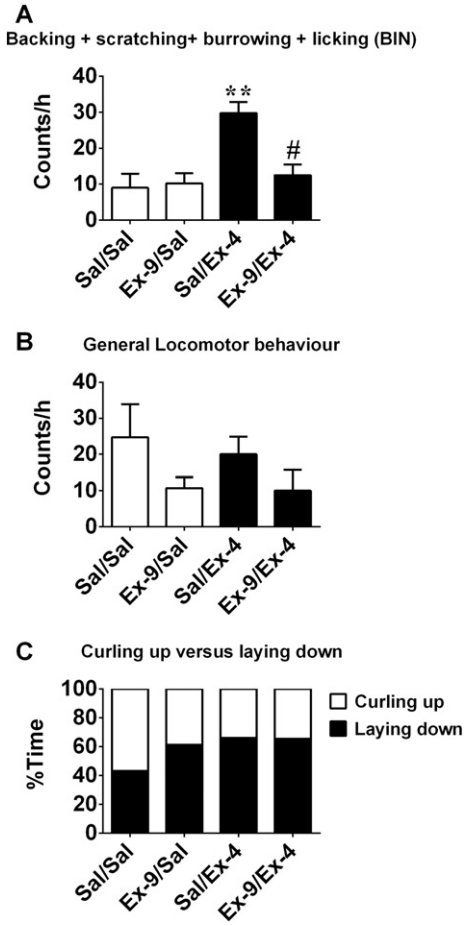


Fig. 6. Effects of i.c.v. saline, exendin-4 and exendin (9–39) alone and in combination on ferret behaviours. Panel A: Behaviours indicative of nausea (BIN: sum of backing + scratching + burrowing + licking-see Methods for definitions); Panel B: general locomotor activity; Panel C: proportion of time spent curling up versus laying down. For each parameter the effect of a double dose of saline (15 μl ×2, saline/saline), exendin (9–39) (Ex-9, 100 nmol/saline 15 μl), exendin-4 (saline, 15 μl/Ex-4, 10 nmol) and exendin (9–39) with exendin-4 (Ex-9100 nmol/Ex-4, 10 nmol) given i.c.v. is shown. Data represents the mean ± s.e.m. of 4 animals. Significant differences between Sal/Sal and Sal/Ex-4 groups were indicated as $^{**}P < 0.01$; significant differences between Ex-9/Sal and Ex-9/Ex-4 groups were indicated as $^{\#}P < 0.05$.

3.4. Effect of vehicle (15 μl) or exendin (9–39) (100 nmol) on exendin-4 (10 nmol)-induced changes of c-Fos in the brain

I.c.v. injection of saline/saline, or saline/exendin (9–39; 100 nmol), had a minimal effect on the number of c-Fos positive cells in all ten brain nuclei studied. Consistent with a failure to induce emesis, exendin-4 (10 nmol, i.c.v.) preceded by saline (sal/exendin-4) also failed to increase c-Fos counts in the AP and NTS, but significant increases of c-Fos counts occurred in all rostral brain regions (Fig. 7, Fig. 8, Fig. 9), except the arcuate nucleus (Arc) (Fig. 8).

Administration of exendin (9–39; 100 nmol) before exendin-4 (10 nmol) had little effect on the c-Fos response compared to saline/exendin-4 alone when the total c-Fos activity in the major brain regions was analysed (Fig. 9). However, analysis of individual nuclei revealed that in the CeA, the number of c-Fos positive cells was not significantly different from either saline/saline, or exendin (9–39)/saline groups, and was zero in 3 out of 4 animals (Fig. 8).

3.5. Comparison of behavioural response to exendin-4 (10 nmol) with or without preceding saline (15 μl) administration

The dose of exedin-4 used in the above studies investigating the effect of exendin (9–39) was selected from the dose-response study reported in Section 3.1 (Fig. 1) and performed under identical conditions; doses were also extrapolated from our previous studies on glucose homeostasis (Chan et al., 2011; Lu et al., 2014). The data reported here can only be interpreted in the light of the behavioural and c-Fos data from the initial dose-response study (Results Section 3.1) which is used here for comparison. Both studies were conducted using animals from the same batch animals in both studies were randomised to the study treatment groups with the two studies performed in close (within one month) succession. There were no overt differences in environmental conditions, food or housing between the animals used in the two studies. It should be noted that statistical comparison between the two studies is not possible as the animals studies were undertaken one month apart and randomisation to the sub-groups was not contemporaneous so we limit ourselves to presenting the data with qualitative comparisons.

A comparison of the total counts of BIN, general locomotor behaviour and the percentage time curled up vs laying down and food intake (saline 2.85 ± 0.47 g/kg vs saline/saline 2.66 ± 0.73 g/kg; $n = 4$–7) showed similar values between animals receiving one or two 15 μl i.c.v. injections of saline. However, when exendin-4 (10 nmol) was preceded by saline (15 μl; $n = 4$) in comparison to the same dose of exendin-4 alone (10 nmol; n = 4), there were fewer episodes (41% fewer of clustered backing + burrowing + licking + scratching scores) of BIN, and a reduced (63% fewer) level of clustered general locomotor behaviour, but no difference in the percentage of time spent curled up vs laying down percentage in present study. In both protocols exendin-4 (10 nmol) abolished food consumption ($n = 4$ in each study) compared to that measured over a 60 min period following either a single or double i.c.v. injection of saline. Whilst both retching (37.5 ± 19.4) and vomiting (5.0 ± 2.4) was evoked in three out of four animals in the original exendin-4 (10 nmol) study, none of the four animals had emesis when the exendin-4 (10 nmol) was preceded by saline.

3.6. Comparison of the c-Fos response to exendin-4 (10 nmol), with or without a preceding saline (15 μl) administration

In the second study, i.c.v. exendin-4 (10 nmol) preceded by saline increased the number of c-Fos positive neurones in seven of eight midbrain and forebrain nuclei but was without effect on the number of c-Fos positive neurones in either the AP or NTS. This contrasts with the initial dose-response study using the same dose of exedin-4 (10 nmol) but without a preceding saline injection in which there was increased

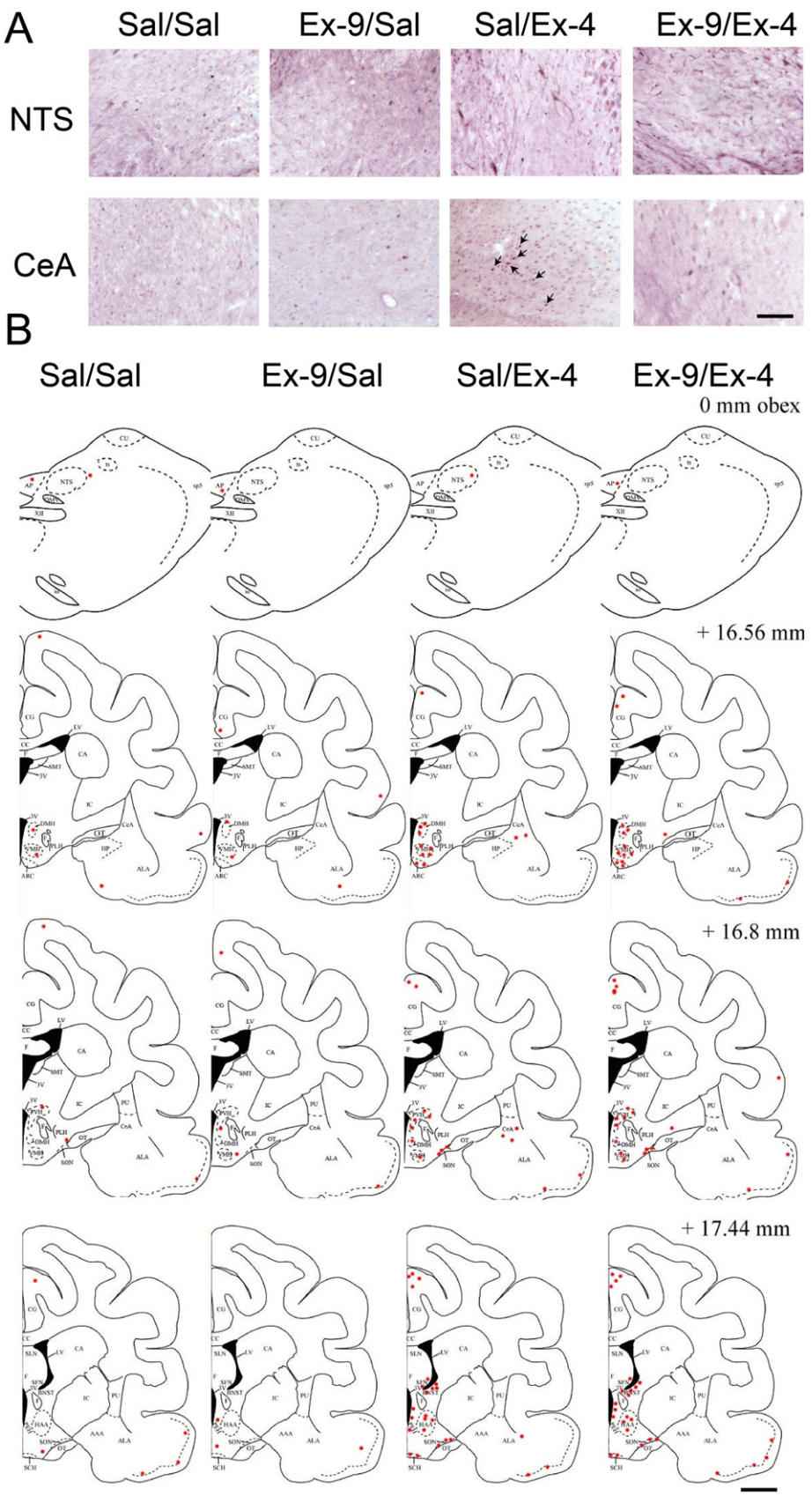


Fig. 7. Representative photomicrographs and schematic representation illustrating c-Fos expression in the ferret brain after i.c.v. administration of saline (15 μl × 2, Sal/Sal), exendin (9–39) (Ex-9, 100 nmol/Sal, 15 μl), exendin-4 (Sal, 15 μl/Ex-4, 10 nmol) and exendin (9–39) with exendin-4 (Ex-9100 nmol/Ex-4, 10 nmol) given i.c.v. is shown. (A) Representative photomicrographs illustrating c-Fos expression (violet nuclear label) in the caudal brainstem (NTS) and forebrain area (CeA). Arrows point to representative c-Fos positive cells. Scale bar = 100 μm. (B) A schematic representation of coronal sections depicting the distribution of c-Fos immunoreactivity in the ferret. Scale bar = 1 mm. One red dot represents ~5 c-Fos positive cells.

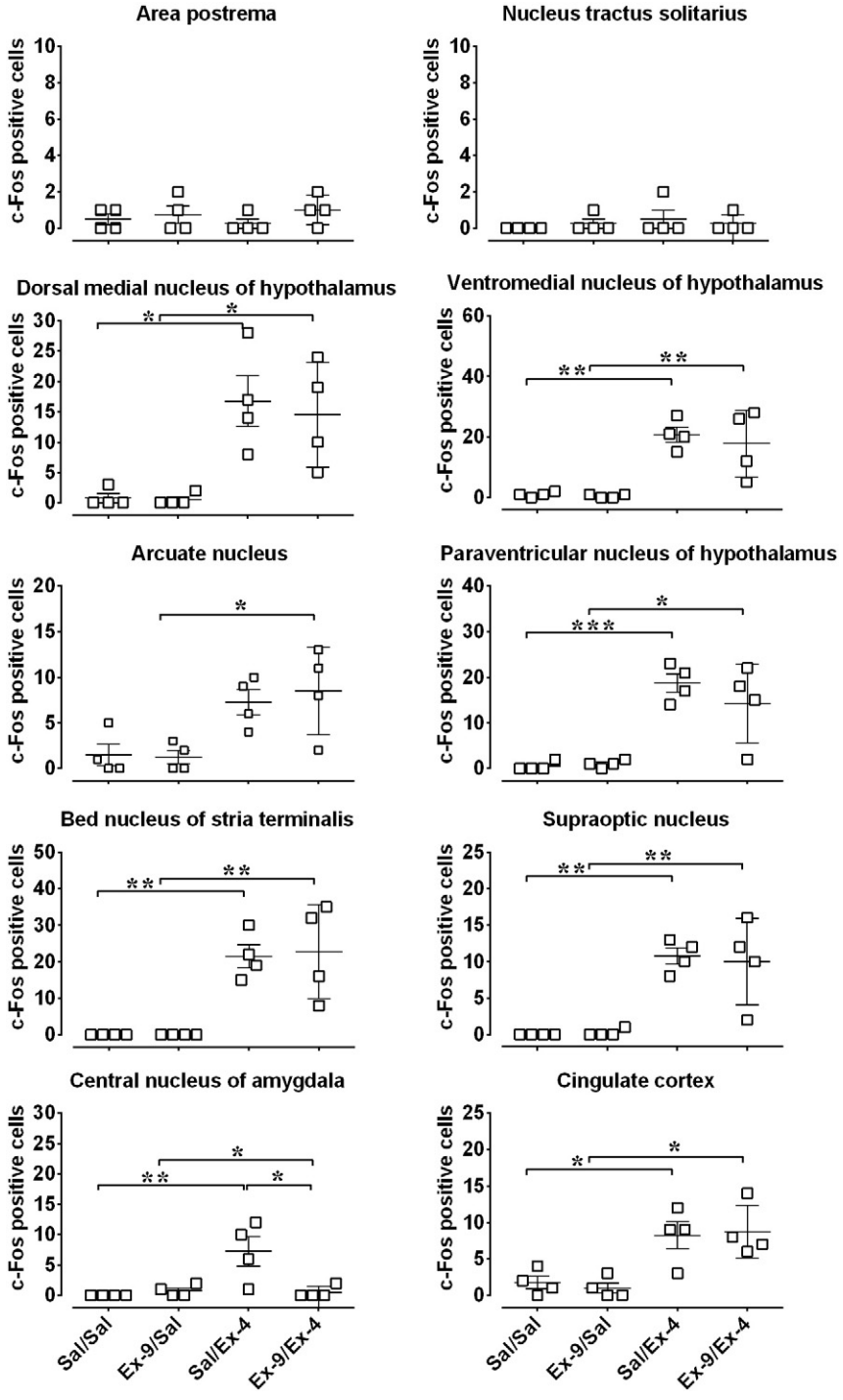


Fig. 8. Effect of exendin (9–39; 100 nmol) on exendin-4-(10 nmol) induced c-Fos expression in the ferret brain. Sal/Sal = saline 15 μl × 2, Ex-4 = exendin-4, Ex-9 = exendin (9–39); Data represents the mean ± s.e.m. of 4 animals with individual data points shown. Significant differences between groups are indicated as $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (One-way ANOVA followed by Bonferroni test).

activity in all ten nuclei studied including the AP and NTS. The striking difference between the two studies can be seen by comparing total c-Fos counts for brainstem, midbrain and forebrain nuclei in the two studies (Fig. 10A). Further analysis shows that the total c-Fos counts for midbrain and forebrain regions from the second exendin-4 study (no emesis) are clustered with those from the initial exendin-4 dose response study (emesis) (Fig. 10B). In addition, with regards to the brainstem, the counts from the present study cluster with the counts from two animals in the previous dose response study in which no emesis was seen and the cluster of points from all the animals with emesis (both studies) cluster away from the points of animals with emesis (Fig. 10C).

4. Discussion

The first part of the study showed that doses of exendin-4 (10 and 30 nmol) with the potential to induce emesis also increased behaviours which have been argued to be indicative of nausea in the ferret

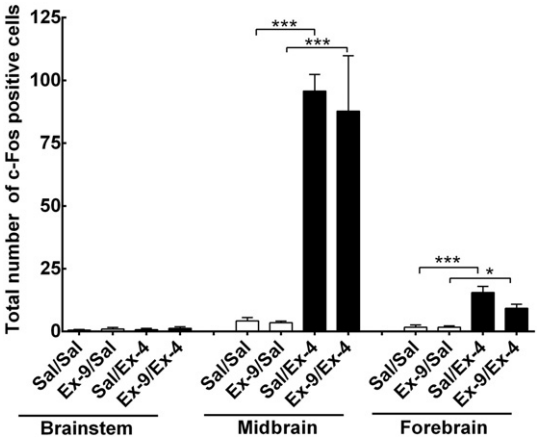


Fig. 9. The total number of c-Fos positive cells in all brainstem (NTS + AP), midbrain (DMH + VMH + Arc + PVN + BNST + SON) and forebrain regions (CeA + CC) in animals given i.c.v. injections of saline (15 μl ×2, saline/saline), exendin (9–39) (Ex-9, 100 nmol/saline 15 μl), exendin-4 (saline, 15 μl/Ex-4, 10 nmol) and exendin (9–39) with exendin-4 (Ex-9100 nmol/Ex-4, 10 nmol). Note that no animals in these groups had emesis, behavioural data from the same animals is plotted in Fig. 6 and food intake was suppressed by Sal/Ex-4 and in the Ex-9/Ex-4 group (see text for details). Values are plotted as mean ± s.e.m., $n = 4$ animals with data derived from the sum of c-Fos counts from 3 sections per nucleus, per animal (see Methods for details). $^{*}P < 0.05$, $^{***}P < 0.001$ (One-way ANOVA followed by Bonferroni test).

(Bermudez et al., 1988; Gonsalves et al., 1996; Hawthorn and Cunningham, 1990; King and Landauer, 1990; Lau et al., 2005a; Lau et al., 2005b). Although it is impossible to know what the animal is actually experiencing (for discussion of the issues see Andrews and Sanger, 2014), the argument that such behaviours may be specifically related to "nausea" or "visceral malaise" is strengthened by the lack of change in a suite of other behaviours that are indicative of overall locomotor activity. The initial dose-response study shows that the dose of exendin-4 required to reduce food intake (3 nmol) is lower than that required to induce emesis and BIN (10, 30 nmol) an observation consistent with the view that inhibition of food intake may be an indication of emetic potential when given at a higher dose (see Stern et al., 2011 p215, for discussion). This study has also shown a characteristic pattern of brain c-Fos activation induced by i.c.v. exendin-4 (10 nmol and 30 nmol) including brainstem nuclei previously implicated in emesis (AP and NTS) and more rostral nuclei implicated in endocrine, autonomic and behavioural responses to a range of substances with emetic potential. However, when we attempted to investigate if all aspects of the response to i.c.v. exendin-4 in the ferret were sensitive to the selective GLP-1 receptor antagonist, exendin (9–39) (Goke et al., 1993; Kanoski et al., 2011), the emetic response to a dose of exendin-4 (10 nmol) that had previously been effective in 75% of animals in inducing emesis (Results, Section 3.1) was no longer effective, although all other aspects of the response (abolition of food intake, induction of BIN, increase in c-Fos in supramedullary regions) were preserved. The possible reasons for the difference in response between the two studies which with the exception of saline administration were otherwise technically identical are discussed below and include a commentary of the significance of the overall results for understanding the emetic effects of exendin-4 and GLP-1 receptors.

4.1. The area postrema and nucleus tractus solitarius are critical sites for the induction of emesis by i.c.v. exendin-4

Exendin-4 at 10 and 30 nmol, i.c.v., induced emesis, but in both dose groups a single animal failed to have emesis yet food intake was abolished, and BIN increased (Fig. 1). The c-Fos activation pattern in the two animals not exhibiting emesis showed no increased c-Fos count in either the AP or NTS, but the level of activity in six midbrain and two forebrain nuclei was comparable to animals in which there was an emetic response (see Fig. 10B and C). In the present study, the four animals (saline/exendin-4, 10 nmol) without an emetic response also showed no increased activity in either the AP or NTS, but the c-Fos responses were identical in pattern of c-Fos activation and the magnitude of the total c-Fos response did not differ in the midbrain or forebrain between the two studies (Fig. 10A). Taking the pattern of c-Fos data from the six animals without emesis (four from the second saline/exendin-4 study, and two from the exendin-4 dose response study) and the eight animals with emesis (initial study, three with 10 nmol and five with 30 nmol) indicates a mechanism for central exendin-4 induction of emesis reliant on activation of the AP and NTS; there appears to be no relationship between the presence or absence of emesis and c-Fos activity in any of the other eight nuclei studied. An additional conclusion is that activation of midbrain or forebrain nuclei is not critical for induction of emesis, a conclusion consistent with the induction of emesis and fictive emesis in decerebrate animals and working-heart perfused brainstem studies (Horn et al., 2013; Smith et al., 2002). Furthermore, although the connections between the NTS and the midbrain are well established ((Stern et al., 2011) for review), the activation of midbrain structures in animals following i.c.v. exendin-4 is not critically dependent upon activation of the NTS (or AP). It also argues against descending pathways from the midbrain (closer to 3rd ventricle) leading to NTS activation. However, we are unable to exclude the possibility that when the brainstem is activated by i.c.v. exendin-4, it provides an additional drive to the midbrain although there is no difference in magnitude of c-Fos activation under the two conditions (Fig. 10A).

4.2. Exendin-4 suppression of food intake and increase in behaviours indicative of nausea can occur in the absence of emesis and brainstem c-Fos activity

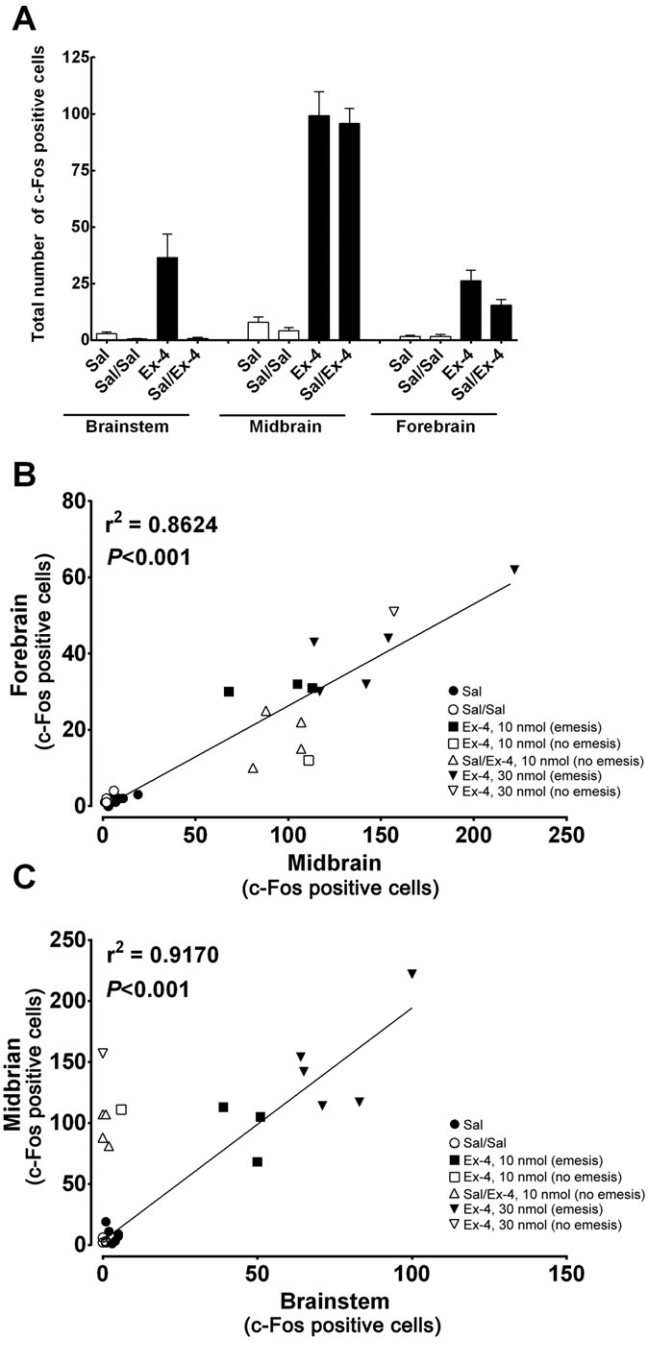
The reduction in food intake and increased BIN can occur in the absence of emesis and an increase in brainstem c-Fos activity. This observation is consistent with the view that suppression of food intake and increase in BIN are not critically dependent upon an action of exendin-4 on the brainstem with subsequent activation of midbrain or forebrain structures and hence both effects can be produced by a primary effect of exendin-4 on sites accessible from the third ventricle. The level of c-Fos activity in the midbrain and forebrain in animals without emesis but with suppressed food intake and increased BIN does not differ (Figs. 4 and 10), supporting the view that even in animals with emesis the brainstem may not be the primary site at which exendin-4 acts to produce these effects. Exendin-4 could act on regions in the third ventricle where the BBB/CSFB barrier is relatively permeable (SFO, OVLT, median eminence, sub-commissural organ), or by transport across the CSFB barrier to nuclei subjacent to the third ventricle. Identification of the exact site requires local microinjection studies but GLP-1 receptors are widely distributed in the brain, including several structures with c-Fos activity increased by exendin-4 (Gu et al., 2013; Lu et al., 2014; Merchenthaler et al., 1999).

4.3. The GLP-1 receptor antagonist, exendin (9–39), appears to have a differential effect on food intake suppression, BIN and c-Fos in the forebrain

Caution must be exercised over interpretation of the studies with the GLP-1 receptor antagonist, exendin (9–39), as only a relatively small number of animals were studied ($n = 4$), and because only a single dose was used (100 nmol) although this was ten times the dose of exendin-4 (10 nmol), and the pharmacological profile of exendin (9–39) is incompletely characterised in the ferret (Lu et al., 2014).

We found that an increase in BIN induced by i.c.v. exendin-4 (10 nmol) in the absence of an emetic response was significantly reduced by exendin (9–39), without affecting the other behavioural

measurements including the suppressed food intake. Similar studies in *Suncus murinus* have shown that exendin (9–39) blocked the exendin-4-induced emesis, but not the inhibition of food intake (Chan et al., 2013). However, in *Suncus murinus*, exendin (9–39) blocked c-Fos activation by exendin-4 in the brainstem and hypothalamus, and reduced c-Fos activation in the CeA. In the present study in the ferret, the exendin-4 induced c-Fos activity in CeA was the *only* brain region where exendin (9–39) had an effect producing a reduction of 89%. It appears that with the possible exception of the CeA, BIN are not reflected by c-Fos activity in other limbic regions or the hypothalamus in the ferret. Certainly, exendin-4 does not produce complex behavioural changes in *Suncus murinus* that could be considered equal to the repertoire of BIN recorded in ferrets (Rudd, unpublished data). This raises the question of the differential role of the CeA between species and the control of motor outputs and possibly the gating or expression of information perceived as "nausea". An alternative explanation is that whilst the pathway(s) by which exendin-4 induces BIN are antagonised by exendin (9–39) in ferrets, the pathways leading to c-Fos activation and the reduction in food intake involve exendin-4 acting on a non-classical GLP-1 receptor. The involvement of the CeA in "nausea" induced by GLP-1 receptor agonists is further supported by rodent studies, showing that injection of GLP-1 into this brain area induced CTA when paired with a flavour and CTA induced by lithium chloride was reduced by injection of a GLP-1 receptor antagonist, des His^1 Glu^9 exendin-4 (Kinzig et al., 2003). The CeA was also activated in a previous ferret study following peripherally administered lithium chloride and cholecystokinin evoked behavioural signs of "nausea" (Billig et al., 2001). However, again, the cluster of exendin-4-induced BIN we report here in ferrets is not seen in rodents; this may again explain why c-Fos expression could be antagonised by the GLP-1 receptor antagonist. Further studies of GLP-1 receptor antagonists are required using wider range of doses but if the i.c.v. route is used the technical issues relating to the saline control injection reported here will need to be resolved.



4.4. The emetic, food intake, BIN and forebrain c-Fos effects of exendin-4 (10 nmol, i.c.v.) with and without preceding i.c.v. saline administration

Exendin-4 (10 nmol) completely abolished food intake in all animals in both studies reported here and there was an increase in BIN, but when animals received i.c.v. saline (15 μl) 15 min prior to exendin-4, no emesis occurred. This observation suggests that emetic and food intake effects of exendin-4 are not critically linked and taken together with the c-Fos observations indicate that suppression of food intake can occur without activation of either the AP or NTS. Although baseline BIN was not different between the studies, and increased in both studies in response to exendin-4, the magnitude of the increase was smaller in the group that also received i.c.v. saline. The baseline level of general locomotor behaviour was lower (63%) in the saline/saline treated animals, but the percentage time curled up vs laying down was not. It is also notable that although the level of c-Fos activity in the midbrain was very similar between the exendin-4 alone and the saline exendin-4 groups, the activity in the forebrain was lower in the saline + exendin-4 group (Fig. 10A).

How can the difference between the two studies with exendin-4 which differ in the administration of i.c.v. saline be explained? It is

Fig. 10. Panel A. The total number of c-Fos positive cells in all brainstem (NTS + AP), midbrain (DMH + VMH + Arc + PVN + BNST + SON) and forebrain regions (CeA + CC) studied in animals given i.c.v. injections of saline (Sal,15 μl), saline/saline (Sal/Sal, 15 μl × 2), exendin-4 (Ex-4, 10 nmol) or saline followed by exendin-4 (Sal 15 μl/Ex-4, 10 nmol). The data from the Sal/Sal and Sal/Ex-4 groups is replotted from Fig. 9 and the data for Sal and Ex-4 (10 nmol) groups replotted from Fig. 4, with both studies using identical methodology (see text for details). Note the marked difference between Ex-4 (10 nmol) and Sal/Ex-4 (10 nmol) in the brainstem; three of four animals had emesis in response to Ex-4 (10 nmol) whereas no animal given Sal/Ex-4 (10 nmol) had emesis. Data are mean ± s.e.m. ($n = 4$ in each group). Note that statistical comparisons between the studies are not valid as animals were not randomised to all study groups at the same time. **Panel B**. Correlation between midbrain and forebrain total c-Fos counts combining data from Fig. 4C and D and Fig. 10A. Filled circles (●): data from animals given Sal; Open circles (○): data from animals given Sal/Sal; Filled squares (■): data from animals given i.c.v. Ex-4, 10 nmol with emesis; Open square(□): data from animals given i.c.v. Ex-4, 10 nmol without emesis; Open triangles (△): Data from animals given Sal/Ex-4 (15 μl, 10 nmol) without emesis. Filled inverted triangle (▼): Data from animals given i.c.v. Ex-4, 30 nmol with emesis; Open inverted triangle (▽): Data from animals given i.c.v. Ex-4, 30 nmol without emesis. **Panel C**. Correlation (excluding Ex-4 animals without emesis) between brainstem and midbrain total c-Fos counts using all the c-Fos data from the present study. Symbols as panel B. Note that in contrast to panel B where data from animals with and without emesis all cluster along the same line in panel C, data from animals without emesis in both Ex-4 10 nmol studies reported study cluster together but away from all other points and illustrating that whilst the brainstem is relatively inactive in the animals given i.c.v. Ex-4 when emesis does not occur, the midbrain level of activity is comparable to animals with emesis.

possible that the emetic response incidence (75%; 3 out of 4 animals) to exendin-4 (10 nmol) in the first dose-response study was not representative of the overall sensitivity of the ferret population. However, it seems unlikely (but theoretically possible) that by chance from the same initial batch of animals we selected one group of four animals, three of which were responsive to 10 nmol and another four none of which had an emetic response to the same dose. Even at a dose of 30 nmol emesis does not occur in all animals (83%; 5 out of 6) indicating inter-animal variability in the emetic sensitivity to exendin-4.

The lack of reproducibility in the studies performed one month apart with animals from the same initial batch could be due to a "technical or methodological error" such as in drug preparation, but this is not supported by the consistent changes c-Fos changes in the supramedullary structures in response to 10 nmol exendin-4 nor by the reduction of food intake or increased BIN. Administration of saline requires animal handling and restraint, so it is possible that this may contribute to the baseline difference in general locomotor behaviour although the animals were adapted to the procedure by frequent handling. The similarity of baseline c-Fos levels in brainstem, midbrain and forebrain in animals with one saline injection (control for dose-response study) vs two saline injections (control for exendin-(9–39) study) indicates that saline itself is not having a gross effect on the brain. If our hypothesis that emesis requires exendin-4 to act on the brainstem is correct (and assuming no major inter group sensitivity differences) then it suggests that the saline injection is preventing the subsequent injection of exendin-4 accessing the brain stem possibly because of limited flow through the narrow aqueduct of Sylvius. An alternative and perhaps more plausible explanation is that the saline reduces the rate of access of the subsequent exendin-4 injection to the brainstem so that the concentration drops below the threshold required for activation of the AP and emesis. The dose–response study (Results Section 3.1) showed that 3 nmol exendin-4 did not induce emesis or BIN, but did inhibit food intake. An observation from a cat study (Borison, 1959) is relevant as when adrenaline or apomorphine were given into the lateral ventricle emesis and a range of behavioural responses occurred but when the injection was confined to the fourth ventricle only emesis occurred.

Whilst we have only been able to speculate on the differences between the two studies and caution is essential in interpretation of the data, particularly in making statistically robust conclusions because of the relatively small number of animals involved, we argue that the data does allow some conclusions to be drawn about the central effects of exendin-4 and GLP-1 receptors.

5. Conclusion

This paper reports data from a study with a partially unexpected and unpredictable outcome which may be due to a methodological issue related to a double i.c.v. injection necessitated by the experimental design although other explanations are possible (see above). Irrespective of the explanation for the variability in the emetic response to exendin-4 the data provides insight into the sites and mechanisms by which i.c.v. exendin-4 causes emesis, BIN and suppression of food intake. The behavioural observations combined with c-Fos provide evidence that whilst the brainstem (NTS/AP) is critical for induction of emesis by exendin-4, suppression of food intake and induction of BIN can be induced by an action at brain sites accessible from the third ventricle alone. Exendin-4-induced BIN in the ferret and c-Fos activation of the CeA are sensitive to exendin (9–39), whereas the suppression of food intake is not at the same dose of antagonist implicating different mechanisms in mediation of these actions of a GLP-1 receptor agonist.

Conflict of interest

The authors have declared that no conflict of interest exists.

Acknowledgments

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References

Andrews, P.L.R., Sanger, G.J., 2014. Nausea and the quest for the perfect anti-emetic. Eur. J. Pharm. Biopharm. 722, 108–121.
Banks, W.A., 2006. The blood-brain barrier as a regulatory interface in the gut-brain axes. Physiol. Behav. 89, 472–476.
Bermudez, J., Boyle, E.A., Miner, W.D., Sanger, G.J., 1988. The anti-emetic potential of the 5-hydroxytryptamine3 receptor antagonist BRL 43694. Br. J. Cancer 58, 644–650.
Billig, I., Yates, B.J., Rinaman, L., 2001. Plasma hormone levels and central c-Fos expression in ferrets after systemic administration of cholecystokinin. Am. J. Phys. Regul. Integr. Comp. Phys. 281, R1243–R1255.
Blair, P.J., Rhee, P.L., Sanders, K.M., Ward, S.M., 2014. The significance of interstitial cells in neurogastroenterology. J. Neurogastroenterol. Motil. 20, 294–317.
Boissonade, F.M., Davison, J.S., 1996. Effect of vagal and splanchnic nerve section on Fos expression in ferret brain stem after emetic stimuli. Am. J. Phys. 271, R228–R236.
Boissonade, F.M., Sharkey, K.A., Davison, J.S., 1994. Fos expression in ferret dorsal vagal complex after peripheral emetic stimuli. Am. J. Phys. 266, R1118–R1126.
Borison, H.L., 1959. Effect of ablation of medullary emetic chemoreceptor trigger zone on vomiting responses to cerebral intraventricular injection of adrenaline, apomorphine and pilocarpine in the cat. J. Physiol. 147, 172–177.
Borison, H.L., 1989. Area postrema: chemoreceptor circumventricular organ of the medulla oblongata. Prog. Neurobiol. 32, 351–390.
Brookes, S.J., Spencer, N.J., Costa, M., Zagorodnyuk, V.P., 2013. Extrinsic primary afferent signalling in the gut. Nat. Rev. Gastroenterol. Hepatol. 10, 286–296.
Buse, J.B., Nauck, M., Forst, T., Sheu, W.H., Shenouda, S.K., Heilmann, C.R., Hoogwerf, B.J., Gao, A., Boardman, M.K., Fineman, M., Porter, L., Schernthaner, G., 2013. Exenatide once weekly versus liraglutide once daily in patients with type 2 diabetes (DURATION-6): a randomised, open-label study. Lancet 381, 117–124.
Chan, S.W., Lin, G., Yew, D.T.W., Rudd, J.A., 2011. A physiological role of glucagon-like peptide-1 receptors in the central nervous system of *Suncus murinus* (house musk shrew). Eur. J. Pharmacol. 668, 340–346.
Chan, S.W., Lin, G., Yew, D.T.W., Yeung, C.K., Rudd, J.A., 2013. Separation of emetic and anorexic responses of exendin-4, a GLP-1 receptor agonist in *Suncus murinus* (house musk shrew). Neuropharmacology 70, 141–147.
Chan, S.W., Lu, Z., Lin, G., Yew, D.T.W., Yeung, C.K., Rudd, J.A., 2014. The differential antiemetic properties of GLP-1 receptor antagonist, exendin (9–39) in *Suncus murinus* (house musk shrew). Neuropharmacology 83, 71–78.
Dampney, R.A., Polson, J.W., Potts, P.D., Hirooka, Y., Horiuchi, J., 2003. Functional organization of brain pathways subserving the baroreceptor reflex: studies in conscious animals using immediate early gene expression. Cell. Mol. Neurobiol. 23, 597–616.
De Jonghe, B.C., Holland, R.A., Olivos, D.R., Rupprecht, L.E., Kanoski, S.E., Hayes, M.R., 2016. Hindbrain GLP-1 receptor mediation of cisplatin-induced anorexia and nausea. Physiol. Behav. 153, 109–114.
Dockray, G.J., 2014. Gastrointestinal hormones and the dialogue between gut and brain. J. Physiol. 592, 2927–2941.
Furness, J.B., 2012. The enteric nervous system and neurogastroenterology. Nat. Rev. Gastroenterol. Hepatol. 9, 286–294.
Goke, R., Wagner, B., Fehmann, H.C., Goke, B., 1993. Glucose-dependency of the insulin stimulatory effect of glucagon-like peptide-1 (7–36) amide on the rat pancreas. Res. Exp. Med. (Berl) 193, 97–103.
Gonsalves, S., Watson, J., Ashton, C., 1996. Broad spectrum antiemetic effects of CP-122,721, a tachykinin NK_1 receptor antagonist, in ferrets. Eur. J. Pharmacol. 305, 181–185.
Gu, G., Roland, B., Tomaselli, K., Dolman, C.S., Lowe, C., Heilig, J.S., 2013. Glucagon-like peptide-1 in the rat brain: distribution of expression and functional implication. J. Comp. Neurol. 521, 2235–2261.
Hawthorn, J., Cunningham, D., 1990. Dexamethasone can potentiate the anti-emetic action of a $5HT_3$ receptor antagonist on cyclophosphamide induced vomiting in the ferret. Br. J. Cancer 61, 56–60.
Hoffman, G.E., Smith, M.S., Verbalis, J.G., 1993. c-Fos and related immediate early gene products as markers of activity in neuroendocrine systems. Front. Neuroendocrinol. 14, 173–213.
Horn, C.C., Henry, S., Meyers, K., Magnusson, M.S., 2011. Behavioral patterns associated with chemotherapy-induced emesis: a potential signature for nausea in musk shrews. Front. Neurosci. 5, 88.
Horn, C.C., Kimball, B.A., Wang, H., Kaus, J., Dienel, S., Nagy, A., Gathright, G.R., Yates, B.J., Andrews, P.L.R., 2013. Why can't rodents vomit? A comparative behavioral, anatomical, and physiological study. PLoS One 8, e60537.
Iida, T., Moriyama, T., Kobata, K., Morita, A., Murayama, N., Hashizume, S., Fushiki, T., Yazawa, S., Watanabe, T., Tominaga, M., 2003. TRPV1 activation and induction of nociceptive response by a non-pungent capsaicin-like compound, capsiate. Neuropharmacology 44, 958–967.
Johnson, A.K., Gross, P.M., 1993. Sensory circumventricular organs and brain homeostatic pathways. FASEB J. 7, 678–686.
Kanoski, S.E., Fortin, S.M., Arnold, M., Grill, H.J., Hayes, M.R., 2011. Peripheral and central GLP-1 receptor populations mediate the anorectic effects of peripherally administered GLP-1 receptor agonists, liraglutide and exendin-4. Endocrinology 152, 3103–3112.

Kash, T.L., Pleil, K.E., Marcinkiewcz, C.A., Lowery-Gionta, E.G., Crowley, N., Mazzone, C., Sugam, J., Hardaway, J.A., McElligott, Z.A., 2015. Neuropeptide regulation of signaling and behavior in the BNST. Mol. Cell 38, 1–13.

King, G.L., Landauer, M.R., 1990. Effects of zacopride and BMY25801 (batanopride) on radiation-induced emesis and locomotor behavior in the ferret. J. Pharmacol. Exp. Ther. 253, 1026–1033.

Kinzig, K.P., D'Alessio, D.A., Herman, J.P., Sakai, R.R., Vahl, T.P., Figueiredo, H.F., Murphy, E.K., Seeley, R.J., 2003. CNS glucagon-like peptide-1 receptors mediate endocrine and anxiety responses to interoceptive and psychogenic stressors. J. Neurosci. 23, 6163–6170.

Kroenke, C.D., Mills, B.D., Olavarria, J.F., Neil, J.J., 2014. Neuroanatomy of the Ferret Brain with Focus on the Cerebral Cortex, Biology and Diseases of the Ferret. John Wiley & Sons, Inc., pp. 69–80.

Lau, A.H., Kan, K.K., Lai, H.W., Ngan, M.P., Rudd, J.A., Wai, M.K., Yew, D.T.W., 2005a. Action of ondansetron and CP-99,994 to modify behavior and antagonize cisplatin-induced emesis in the ferret. Eur. J. Pharmacol. 506, 241–247.

Lau, A.H., Ngan, M.P., Rudd, J.A., Yew, D.T.W., 2005b. Differential action of domperidone to modify emesis and behaviour induced by apomorphine in the ferret. Eur. J. Pharmacol. 516, 247–252.

Lawes, I.N., Andrews, P.L.R., 1987. Variation of the ferret skull (*Mustela putorius furo* L.) in relation to stereotaxic landmarks. J. Anat. 154, 157–171.

Lawes, I.N.C., Andrews, P.L.R., 1998. Neuroanatomy of the ferret brain. The Biology and Diseases of the Ferrets, 2nd edition Williams & Wilkins, Co., Baltimore, pp. 71–102.

Leslie, R.A., 1986. Comparative aspects of the area postrema: fine-structural considerations help to determine its function. Cell. Mol. Neurobiol. 6, 95–120.

Lu, Z., Percie Du Sert, N., Chan, S., Yeung, C.K., Lin, G., Yew, D.T.W., Andrews, P.L.R., Rudd, J.A., 2014. Differential hypoglycaemic, anorectic, autonomic and emetic effects of the glucagon-like peptide receptor agonist, exendin-4, in the conscious telemetered ferret. J. Transl. Med. 12, 327.

Meier, J.J., 2012. GLP-1 receptor agonists for individualized treatment of type 2 diabetes mellitus. Nat. Rev. Endocrinol. 8, 728–742.

Merchenthaler, I., Lane, M., Shughrue, P., 1999. Distribution of pre-pro-glucagon and glucagon-like peptide-1 receptor messenger RNAs in the rat central nervous system. J. Comp. Neurol. 403, 261–280.

Miner, W.D., Sanger, G.J., 1986. Inhibition of cisplatin-induced vomiting by selective 5-hydroxytryptamine M-receptor antagonism. Br. J. Pharmacol. 88, 497–499.

Percie du Sert, N., Chu, K.M., Wai, M.K., Rudd, J.A., Andrews, P.L.R., 2009. Reduced normogastric electrical activity associated with emesis: a telemetric study in ferrets. World J. Gastroenterol. 15, 6034–6043.

Percie du Sert, N., Andrews, P.L.R., 2014. The Ferret in Nausea and Vomiting Research: Lessons in Translation of Basic Science to the Clinic, Biology and Diseases of the Ferret. John Wiley & Sons, Inc., pp. 735–778.

Rudd, J.A., Jordan, C.C., Naylor, R.J., 1994. Profiles of emetic action of cisplatin in the ferret: a potential model of acute and delayed emesis. Eur. J. Pharmacol. 262, R1–R2.

Rudd, J.A., Ngan, M.P., Wai, M.K., King, A.G., Witherington, J., Andrews, P.L.R., Sanger, G.J., 2006. Anti-emetic activity of ghrelin in ferrets exposed to the cytotoxic anti-cancer agent cisplatin. Neurosci. Lett. 392, 79–83.

Sanger, G.J., Broad, J., Andrews, P.L.R., 2013. The relationship between gastric motility and nausea: gastric prokinetic agents as treatments. Eur. J. Pharmacol. 715, 10–14.

Secher, A., Jelsing, J., Baquero, A.F., Hecksher-Sorensen, J., Cowley, M.A., Dalboge, L.S., Hansen, G., Grove, K.L., Pyke, C., Raun, K., Schaffer, L., Tang-Christensen, M., Verma, S., Witgen, B.M., Vrang, N., Bjerre Knudsen, L., 2014. The arcuate nucleus mediates GLP-1 receptor agonist liraglutide-dependent weight loss. J. Clin. Invest. 124, 4473–4488.

Sharkey, K.A., Cristino, L., Oland, L.D., Van Sickle, M.D., Starowicz, K., Pittman, Q.J., Guglielmotti, V., Davison, J.S., Di Marzo, V., 2007. Arvanil, anandamide and N-arachidonoyl-dopamine (NADA) inhibit emesis through cannabinoid CB1 and vanilloid TRPV1 receptors in the ferret. Eur. J. Neurosci. 25, 2773–2782.

Smith, J.E., Paton, J.F., Andrews, P.L.R., 2002. An arterially perfused decerebrate preparation of *Suncus murinus* (house musk shrew) for the study of emesis and swallowing. Exp. Physiol. 87, 563–574.

Stern, R.M., Andrews, P.L.R., Koch, K.L., 2011. Nausea: Mechanisms and Management. Oxford University Press, Inc., New York.

van Bloemendaal, L., Ten Kulve, J.S., la Fleur, S.E., Ijzerman, R.G., Diamant, M., 2014. Effects of glucagon-like peptide 1 on appetite and body weight: focus on the CNS. J. Endocrinol. 221, T1–16.

Williams, E.K., Chang, R.B., Strochlic, D.E., Umans, B.D., Lowell, B.B., Liberles, S.D., 2016. Sensory neurons that detect stretch and nutrients in the digestive system. Cell 166, 209–221.

Zaman, S., Woods, A.J., Watson, J.W., Reynolds, D.J., Andrews, P.L.R., 2000. The effect of the NK_1 receptor antagonist CP-99,994 on emesis and c-fos protein induction by loperamide in the ferret. Neuropharmacology 39, 316–323.