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A glucagon-like peptide-1 receptor agonist reduces intracranial pressure in a rat model of hydrocephalus

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Overline:

One sentence summary:

GLP-1R agonists show promise as a therapeutic agent to lower intracranial pressure in rodents.

Abstract

Current therapies for reducing raised intracranial pressure (ICP) under conditions such as idiopathic intracranial hypertension or hydrocephalus have limited efficacy and tolerability. Thus, there is a pressing need to identify alternative drugs. Glucagon-like peptide-1 receptor (GLP-1R) agonists are used to treat diabetes and promote weight loss but have also been shown to affect fluid homeostasis in the kidney. Here, we investigated whether exendin-4, a GLP-1R agonist, is able to modulate cerebrospinal fluid (CSF) secretion at the choroid plexus and subsequently reduce ICP in rats. We used tissue sections and cell cultures to demonstrate expression of GLP-1R in the choroid plexus and its activation by exendin-4, an effect blocked by the GLP-1R antagonist exendin 9-39. Acute treatment with exendin-4 reduced Na^+ K^+ ATPase activity, a key regulator of CSF secretion, in cell cultures. Finally, we demonstrated that administration of exendin-4 to female rats with raised ICP (hydrocephalic) resulted in a GLP-1R-mediated reduction in ICP. These findings suggest that GLP-1R agonists can reduce ICP in rodents. Repurposing existing GLP-1R agonist drugs may be a useful therapeutic strategy for treating raised ICP.

Introduction

Elevated intracranial pressure (ICP) is caused by alterations in the volume of either cerebral blood, cerebrospinal fluid (CSF) or brain tissue. CSF volume is tightly regulated and depends on the balance between CSF secretion, which is modulated predominantly by the choroid plexus, and drainage through the arachnoid granulations and lymphatic (*1*). Reducing CSF volume, by either CSF drainage or decreasing CSF secretion is used therapeutically to lower ICP (*2, 3*) in conditions characterized by raised ICP such as idiopathic intracranial hypertension and hydrocephalus.

In the choroid plexus, CSF is secreted by the choroid plexus epithelial (CPe) cells, and is driven by net movement of sodium ions (Na^+) from the blood into the cerebral ventricles. This creates an osmotic gradient, which drives water transport into the cerebral ventricles. There are numerous ion channels involved in this process, but the apical Na^+ K^+ ATPase that pumps Na^+ into the ventricles is the most important of these channels and represents the rate limiting step (*4, 5*). Specific inhibition of the Na^+ K^+ ATPase with ouabain, reduces CSF secretion by 70-80% (*6*). As such, the CPe cells function akin to inverted renal proximal tubule epithelial cells with an analogous mechanism of fluid transport (*7, 8*).

The incretin glucagon-like peptide-1 (GLP-1), is a gut peptide secreted by the distal small intestine in response to food intake (*9*). GLP-1 stimulates glucose-dependent insulin secretion and inhibits glucagon release, lowering blood glucose (*10*). In addition, GLP-1 is synthesized in neurons of the nucleus tractus solitarius, which project to the hypothalamus (*11*) and promote satiety and weight loss (*12-14*). GLP-1 signals through the GLP-1 receptor (GLP-1R), a class-B G protein-coupled receptor expressed in selected cell types within the central nervous system including the hypothalamus, hippocampus, olfactory cortex, circumventricular organs, hindbrain and choroid plexus (*15-17*).

GLP-1 also has effects on renal proximal tubule Na^+ secretion, reducing Na^+ reabsorption and increasing diuresis (*18*). Here, GLP-1R activation stimulates the conversion

of adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP) by adenylate cyclase. cAMP activates protein kinase A (PKA), which inhibits the Na^+ H^+ exchanger, thereby preventing Na^+ reabsorption into the bloodstream (*18*). The diuretic actions of incretins have led to investigation of their use as antihypertensive agents (*19*). Similar to its activity in the kidney, we hypothesize that GLP-1 also modulates Na^+ transport and subsequently fluid movement at the choroid plexus. We propose that GLP-1R activation may inhibit the basal Na^+ H^+ exchanger through cAMP-dependent PKA activation, thus impeding the Na^+ K^+ ATPase-dependent secretion of CSF. Stabilized GLP-1 mimetics are widely used to treat diabetes and obesity, and therefore could be repurposed for treating raised ICP.

In the present study, we used tissue sections and CPe cell cultures to assess the localization and distribution of the GLP-1R in rat and human choroid plexus and determined the effects of GLP-1R stimulation on CSF secretion. Furthermore, we conducted in vivo studies to evaluate the effects of GLP-1R agonists on ICP in a hydrocephalus rat model with raised ICP.

Results

GLP-1R expression in human choroid plexus tissue

Immunohistochemical analysis using haematoxylin and eosin staining confirmed that human donor tissue comprised the choroid plexus, demonstrating choroid plexus morphology including a cuboidal CPe cell monolayer resting on a basement membrane, the underlying interstitial tissue and capillary vessels (**Fig. 1A)**. *GLP-1R* mRNA expression in five human choroid plexus samples was compared to known commercially available GLP-1R-positive tissues (pooled samples; see methods for source details). Human pancreas had the highest expression of *GLP-1R* mRNA, with heart and ovary having the least. Human choroid plexus

showed *GLP-1R* mRNA expression (**Fig. 1B**). To determine the localization of the receptor protein, paraffin embedded human choroid plexus sections were immunostained with a specific monoclonal antibody to human GLP-1R previously validated in human and monkey tissue (*20, 21*). Based on the morphology of the choroid plexus, GLP-1R positive staining was detected in CPe cells (**Fig. 1C-F**). Together, these studies demonstrate that the human choroid plexus expresses GLP-1R mRNA and protein.

Exendin-4 treatment of whole rat choroid plexus in vitro

Given the lack of validated antibodies against rodent GLP-1R, we instead incubated whole rat choroid plexus in vitro with a fluorescently tagged GLP-1R agonist, exendin-4 (FLEX), to demonstrate the presence of the receptor in the choroid plexus. After 15 minutes of 1μM FLEX incubation, only a few CPe cells were positive for FLEX within the cytoplasm (**Fig. 2A**). However this increased by 30 minutes (**Fig. 2A**). In both cases, GLP-1R appeared to localize predominantly in the cytoplasm, consistent with agonist-induced receptor internalization and trafficking, most likely via endosomes (*22*). The GLP-1R antagonist exendin 9-39 (1μM) reduced the number of FLEX-positive cells within the choroid plexus (**Fig. 2A**), suggesting specific binding of the FLEX ligand to GLP-1R.

Next, we determined *Glp-1r* mRNA expression in whole rat choroid plexus tissue after incubation with 100nM exendin-4. Incubation of the rat choroid plexus with exendin-4 for 3 hours showed an increase in *Glp-1r* mRNA compared to artificial CSF (3.21 ± 0.70 fold, $P<0.01$), with a return to baseline at 6 hours (0.78 ± 0.12 fold) (**Fig 2B**). There was also a small but detectable increase in Na^+ K^+ *atpase* mRNA expression after 3 hours of exendin-4 treatment compared to incubation with artificial aCSF (1.82 ± 0.28 fold; $P<0.05$), which again returned to baseline at 6 hours (0.97 ± 0.21 fold) (**Fig. 2C**). The expression of other channels

and transporters involved in CSF secretion, including the water channel aquaporin 1 (*Aqp1*) and the Na^+ H^+ exchanger (*Nhe1*), were not altered after exendin-4 treatment (**Fig. 2D, E**).

Exendin-4 treatment increases cAMP and reduces Na^+ K^+ ATPase activity

To explore further the effects of exendin-4 on the choroid plexus, monolayers of rat CPe cells were grown in culture. These CPe cells were characterized using antibodies against specific identity markers and were shown to be similar to their in vivo counterparts (**Fig. S1A**), including the expression of *Glp-1r* mRNA (**Fig. S1B**). To determine the effect of exendin-4 on GLP-1R signaling, cAMP generation was assessed using two enzyme immunoassay systems. Treatment of CPe cells with exendin-4 increased cAMP compared to control (2.14 ± 0.61 fold, $P<0.01$) (**Fig. 3A**) in a concentration-dependent manner, and this could be inhibited by exendin 9-39 (**Fig. 3B**). Forskolin, an adenylate cyclase activator, was used as a positive control to maximally stimulate cAMP production (**Fig 3A-B**) (5.30 ± 0.74 fold compared to control).

The role of GLP-1R signaling in CSF secretion was assessed in rat CPe cell cultures by measuring Na^+ K^+ ATPase activity (proposed as a marker of CSF secretion from the choroid plexus) (*6*). Exendin-4 treatment reduced Na^+ K^+ ATPase specific phosphate production compared to control (39.3 ± 9.4%, $P<0.05$) (**Fig. 3C**). In addition, inhibition of PKA with PKI-16-22-amide (PKI) abolished the exendin-4-induced reduction in Na^+ K^+ ATPase activity (95.4 ± 17.6%, $P<0.05$) (**Fig. 3C**).

Exendin-4 treatment reduces ICP in conscious rats

To establish whether exendin-4 was able to modulate ICP in vivo, healthy female adult rats were implanted with an ICP monitor (Day 0) before receiving daily subcutaneous (SC) injections of either saline or 20 µg/kg exendin-4 for 5 days (day 2-6). ICP was measured before and after the SC injection on days 2, 4 and 6 (**Fig. 4A**). Examples of the ICP traces are shown

in **Fig. 4B**. On the first day of treatment (day 2), exendin-4 significantly reduced ICP 10 minutes after the SC injection; by 30 minutes ICP was 65.2 ± 6.6% of baseline compared to 91.0 ± 3.9% of baseline in saline-treated rats (P<0.01) (**Fig. 4C**). A similar drop in ICP was observed on day 4 (50.4 ± 6.9% of baseline; P<0.001) and day 6 (54.5 ± 8.2% of baseline; P<0.001), 30 minutes after exendin-4 administration (**Fig. 4D-E**).

In addition to reducing ICP immediately after treatment, exendin-4 had a cumulative effect on reducing ICP. Exendin-4 caused a significant reduction in ICP measured pre-dose on day 2 (baseline, 100%) to day 4 (79.3 ± 7.3%; P<0.05) and day 6 (72.5 ± 5.6%; P<0.01) (**Fig. 4F**), which was not observed in saline-treated rats (day 2, baseline 100%; day 4, 95.5 ± 13.6%; day 6, 105.3 ± 12.5%; **Fig. 4G**).

As there is evidence that weight loss can alter ICP (*23*), weights were monitored over the treatment period. Whilst both saline- and exendin-4-treated rats lost weight during treatment (P<0.05), there was no significant difference between the groups at any time point (**Fig. 4H**). In the saline group, weight change correlated with alterations in ICP (r=0.710, P=0.032), although no relationship was detected for the exendin-4 treatment group (r=-0.300, P=0.552) (**Fig. 4I**).

The effect of SC administration of 20 µg/kg exendin-4 on blood and CSF pH and CSF electrolytes was analyzed 60 minutes post-treatment. Exendin-4 maintained normal blood pH (7.35 ± 0.01; **Fig. 4J**), however, it caused a reduction in CSF pH (7.41 ± 0.03; P<0.05) compared to saline (blood pH 7.35 ± 0.03, CSF pH 7.61 ± 0.07) (**Fig. 4K**). CSF Na^+ concentration remained unaltered (saline, 150.3 ± 0.9; exendin-4, 150.3 ± 0.6) (**Fig. 4L**), whereas the concentration of Cl^- ions in the CSF was reduced in the exendin-4 group (117 ± 0.5 mmol/L; P<0.05) compared to the saline group (123.8 ± 0.9 mmol/L) (**Fig. 4M)**. Exendin-4 treatment also increased the concentration of Ca^{2+} ions in the CSF (1.09 ± 0.01, P>0.05) compared to saline (1.03 ± 0.02) (**Fig. 4N**).

Exendin-4 acts via GLP-1R in the brain to reduce ICP in rats

To assess whether the reduction in ICP was specific to the brain, exendin-4 was injected into the lateral ventricle through an intracerebroventricular (ICV) cannula in anesthetized rats. ICV delivery of exendin-4 reduced ICP over time, which was significantly different from baseline at 15 minutes (68.9 ± 6.4%, $P<0.05$). ICV delivery of saline also reduced ICP over time (technical effect due to the ICV cannula itself reducing ICP), and this was significantly different from baseline at 50 minutes (74.5 ± 7.9%, $P<0.05$). Over the 60 minutes of ICP measurement, ICV delivery of exendin-4 significantly reduced the area-under-the-curve (AUC) of ICP compared to saline delivered via the same route (3852 ± 397 versus 4974 ± 262 AUC, $P<0.05$) (**Fig. 4O**). To determine if the effects of exendin-4 on ICP were mediated by the GLP-1R, the antagonist exendin 9-39 was continuously infused (4 µg/hour) into the lateral ventricle for 2 days prior to SC administration of either saline or 20 µg/kg exendin-4. SC injection of exendin-4 (ICV saline + SC exendin-4) lowered ICP ($P<0.0001$) compared to a SC injection of saline with ICV delivery of exendin 9-39 (ICV exendin 9-39 + SC saline; **Fig. 4P**). Central ICV exendin 9-39 infusion decreased the ICP-lowering effect of SC exendin-4 at 5 minutes (ICV exendin 9-39 + SC exendin-4 96.7 ± 13.7% vs ICV saline + SC exendin-4 65.7 ± 8.3%, $P<0.001$) (**Fig. 4P)**. These data suggest that exendin-4 in part exerts its effects on ICP via the GLP-1R signaling pathway in the brain.

Exendin-4 reduces ICP in a dose-dependent manner and the effects last for 24 hours

Rats were treated subcutaneously with 1, 3 and 5 µg/kg exendin-4 to determine whether exendin-4 reduces ICP at lower concentrations. At 60 minutes 1, 3 and 5 µg/kg exendin-4 significantly reduced ICP to 79.0 ± 7.0% of baseline ($P<0.05$), 69.9 ± 8.8% of baseline ($P<0.0001$) and 60.6 ± 3.6% of baseline ($P<0.0001$), respectively, compared to saline (97.2 ±

2.5% of baseline) (**Fig. 5A-B**). Five µg/kg exendin-4 showed the greatest reduction in ICP and the effect was still present 3 hours after the treatment (P<0.001). Conversely, in 1 and 3µg/kg exendin-4 groups ICP had returned to baseline by 3 hours **(Fig. 5C**).

Alterations in mRNA and protein expression of GLP-1R and molecules involved in CSF secretion were assessed in the choroid plexus of rats 3 hours after treatment with 1, 3 and 5 µg/kg exendin-4. *Glp-1R* and Na^+ K^+ *atpase* mRNA expression was not altered by exendin-4 treatment (**Fig. 5D-E**). Conversely, there was a 2-fold increase in the amount of *Aqp1* mRNA in the 5 µg/kg exendin-4 treatment group (P<0.05) (**Fig. 5F**), and a 4-fold increase in the amount of *Nhe1* mRNA expression in the 1 µg/kg exendin-4 treatment group (P<0.05) (**Fig. 5G**). Although no significant changes were observed in Na^+ K^+ *atpase* mRNA expression, there was a small increase in Na^+ K^+ ATPase protein in the 5 µg/kg exendin-4 treatment group (2.16 ± 0.22 AU, P<0.05) (**Fig 5H-I**). Two bands were observed for the water channel aquaporin 1 (AQP1), representing the glycosylated (top band) and non-glycosylated (bottom band) forms of AQP1 (**Fig. 5H**). The total amount of AQP1 protein was slightly reduced by 1 and 3 µg/kg exendin-4 treatment but not with the higher 5 µg/kg exendin-4 dose (1 µg/kg, 1.96 ± 0.17 AU, P<0.05, 3 µg/kg, 1.75 ± 0.08 AU, P<0.01, 5 µg/kg, 2.75 ± 0.30 AU) (**Fig. 5J**). The ratio of glycosylated AQP1 to non-glycosylated AQP1 was increased after 1 and 3 µg/kg exendin-4 treatment but not after 5 µg/kg exendin-4 treatment (1µg/kg , 0.97 ± 0.06 AU, P<0.05, 3 µg/kg, 1.08 ±0.06 AU, P<0.01, 5 µg/kg, 0.81 ± 0.08 AU) (**Fig. 5K**). Glycosylation is important for intracellular trafficking and protein stability, making proteins more resistant to proteolysis (*24*), therefore these data suggest that exendin-4 may lower AQP1 through enhanced degradation of the non-glycosylated AQP1.

The effect of 5 µg/kg exendin-4 was monitored for 24 hours in healthy rats to determine its duration of action. A single SC injection of 5 µg/kg exendin-4 maintained lower ICP compared to saline over 24 hours and returned to the pre-dose ICP baseline at 24 hours (1 hour, 60.2 ±

3.5%, P<0.0001, 3 hours, 71.3 ± 3.7%, P<0.001, 6 hours, 70.3 ± 4.0%, P<0.0001, 12 hours, 88.9 ± 16.6%, P<0.01, 24 hours, 100.3 ± 14.3%, P<0.01) (**Fig. 6A**). Effects on weight and food and water intake were also noted in relation to changes in ICP over 24 hours. Although exendin-4 caused a greater reduction in weight at 3 and 6 hours (**Fig. 6B**), there were no differences between food or water intake at any time point between exendin-4-treated and saline-treated rats (**Fig. 6C-D**). *Glp-1R, Na^+ K^+ atpase* and *Nhe1* mRNA expression did not change over the 24 hour period (**Fig. 6E-F,H**). As shown previously, 5 µg/kg exendin-4 increased *Aqp1* mRNA expression at 3 hours compared to saline, although this was not observed at any other time point (**Fig. 6G**). There were also no significant changes in the amount of Na^+ K^+ ATPase or AQP1 protein over the 24 hour time period (**Fig. 6I-L**).

Exendin-4 treatment reduces ICP in a rodent model of raised ICP

To determine the efficacy of exendin-4 to reduce ICP under conditions of raised ICP, a well-characterized kaolin model of hydrocephalus in rats was used. Kaolin, an aluminium silicate, acts as an irritant, inducing an inflammatory response with concomitant deposition of collagen and dense fibrosis in areas of the subarachnoid space close to the injection site, which leads to raised ICP (*25, 26*). Kaolin was injected into the cisterna magna, leading to development of hydrocephalus, before implantation of the ICP monitor. ICP was recorded before and after a SC injection of either saline or 20 µg/kg exendin-4 (**Fig. 7A**). The injection of kaolin significantly increased baseline ICP (11.1 ± 1.3 mmHg; P<0.0001) compared to that of normal rats (5.5 ± 0.4 mmHg) (**Fig. 7B**). Exendin-4 treatment significantly reduced ICP almost immediately after the SC injection, and at 30 minutes was 62.6 ± 5.1% of baseline (P<0.0001) compared to 105.0 ± 4.6% of baseline in saline-treated rats (**Fig. 7C**). Eight rats in the kaolin group had baseline ICP values of greater than 10 mmHg and had an average baseline ICP of 13.7 ± 0.7mmHg. In these rats (ICP >10mmHg), the ICP values at 30 minutes were 56.6 ±

5.7% of baseline (n=4) in the exendin-4 treatment group compared to 106.7 ± 8.6% of baseline (n=4) in the saline treatment group (**Fig. 7C**). In the rodents with elevated ICP, the ICP waveform was very unstable, with the appearance of B-waves characteristic of pathologically elevated ICP and a reduction in brain compliance (*27*). These were abolished in rats receiving exendin-4 but not saline (**Fig. 7D**).

Discussion

The aim of the present study was to establish whether GLP-1 had a role in modulating CSF secretion and ICP. We were able to demonstrate that the GLP-1R agonist exendin-4 was able to reduce ICP in conscious healthy female rats and in a rat model of raised ICP. In addition, our results suggest that the ICP-lowering properties of exendin-4 may occur through reduced CSF secretion at the choroid plexus, implied by the reduction in Na^+ K^+ ATPase activity in CPe cells. Furthermore, our data suggest that exendin-4 modulates CSF production in vitro through the GLP-1R/cAMP/PKA signaling pathway.

Alvarez et al. (*15*) first described the presence of the GLP-1R in the rat ependyma and choroid plexus by *in situ* hybridisation, but did not characterize the cellular localization of this receptor. Our studies corroborate these findings and demonstrate further that *GLP-1R* mRNA and protein are present in both rat and human choroid plexus. We localized the GLP-1R protein in tissue sections of the human choroid plexus to the CPe cells using a monoclonal antibody, and showed the presence of the receptor in the rat choroid plexus using fluorescently tagged exendin-4. We note that no specific antibody exists for mouse/rat tissue so rodent tissue was not examined for GLP-1R protein expression. In any case, our studies are in keeping with others showing localization of the GLP-1R in monkey kidney and human GLP-1R transfected cells (*20, 21*). G-protein coupled receptors undergo internalization, trafficking and recycling/degradation following agonist stimulation (*28*). We speculate that such dynamics

may allow the GLP-1R to be stimulated from both sides of the choroid plexus (**Fig. S2A**). Although GLP-1R mRNA and protein expression were in general low, it has recently been shown that activation of the receptor requires femto- to picomolar concentrations of GLP-1R, so even faced with low abundance, signaling would be expected in the presence of exendin-4 (*29*).

We successfully cultured monolayers of rat CPe cells, which we used as an in vitro cell culture model of the rat choroid plexus to assess CSF secretion. The Na^+ K^+ ATPase is localized to the apical surface and is the driving force for transporting Na^+ ions from the choroid plexus into the CSF against its concentration gradient. Many studies have demonstrated that modulation of Na^+ K^+ ATPase expression or activity directly correlates with CSF secretion (*6, 30-33*). We were able to show that exendin-4 reduces Na^+ K^+ ATPase activity, suggesting reduced CSF secretion at the choroid plexus. Previous studies have shown similar effects of exendin-4 on Na^+ K^+ ATPase activity in the renal system (*34*). In kidney proximal tubule epithelial cells and pancreatic beta cells, GLP-1 modulates Na^+ concentration through increased cAMP and PKA activation (*18, 35*). Using two different techniques, exendin-4 was seen to induce a concentration-dependent rise in cAMP in the choroid plexus, which was inhibited by the GLP-1R antagonist, exendin 9-39. Furthermore, a PKA inhibitor blocked the effects of exendin-4 on Na^+ K^+ ATPase activity, although we acknowledge that such approaches can be non-specific and further studies using specific knockout animals are required. Altogether, these data indicate that the cAMP/PKA-dependent pathway may be involved in the GLP-1R-mediated reduction in CSF secretion at the choroid plexus. In the kidney, GLP-1R agonist treatment increases diuresis through phosphorylation of the Na^+ H^+ exchanger (*18, 36*). There are PKA phosphorylation sites present on both the Na^+ H^+ exchanger and the Na^+ K^+ ATPase (*37*), therefore, in the choroid plexus, phosphorylation of either the Na^+ H^+ exchanger or the Na^+ K^+ ATPase may result in inhibition of Na^+ transport across the cells

and thus CSF production (**Fig. S2B-C**). In the choroid plexus, the Na^+ K^+ ATPase can also be phosphorylated by PKC (*37*). Interestingly, GLP-1R is able to signal through the PKC pathway in pancreatic beta cells (*29, 38, 39*). Therefore, the GLP-1R/PKC signaling pathway may also have a role in reducing CSF secretion and warrants further investigation.

The key finding of this study is that subcutaneous exendin-4 treatment is able to reduce ICP in vivo in normal rats and rats with raised ICP. In addition, the effect on ICP of a single administration of exendin-4 lasted for 24 hours and cumulative dosing reduced the pre-dose ICP. This suggests that exendin-4 may be able to maintain low ICP over a long period. This is an important advance, as there are very limited specific therapeutic options to clinically reduce and maintain low ICP under conditions of raised ICP. The main therapeutic agent for managing chronic raised ICP is acetazolamide, a carbonic anhydrase inhibitor. However, in idiopathic intracranial hypertension, acetazolamide is associated with limited efficacy and poor tolerability (48% withdrawal) (*2*), and is contraindicated for use in premature infants with post-haemorrhagic hydrocephalus (*40*). On the other hand, treatment with incretin mimetics is generally well tolerated, with the main side effects being transient nausea, constipation and diarrhea, and these drugs do not induce hypoglycemia (*41*). In patients taking the GLP-1R agonist liraglutide, drug withdrawal due to side effects was only 5.4% in the cohort receiving the highest dose (3mg; *12*).

There are, however, a number of limitations to the present study. To determine the central actions of exendin-4 on ICP, we had to deliver exendin-4 directly into the brain's ventricular system. The injection itself may have a direct effect on ICP and could mask any changes in ICP relating to the treatment. To try to minimize these effects, we implanted an ICV cannula 2 days prior to the injection. Nonetheless, as it was not possible to completely seal the system, ICP showed a slight decrease in saline-treated rats. However, we were still able to establish a significant reduction in ICP with exendin-4 treatment. The study design was also

limited by the lack of blinding during the intervention, although the data were analyzed by different individuals with the same outcome. ICP was monitored continuously via automated software thus removing measurement bias. It will be of interest to study in the future, prolonged dosing in a rat model of hydrocephalus. However, this will require considerable technical optimization, given that ICP is notoriously difficult to measure in such models where recordings are typically only accurate immediately before euthanasia (*42*, *43*).

GLP-1R agonists also have peripheral actions that have the potential to indirectly affect ICP. Whilst incretin mimetics have been shown to acutely increase heart rate and blood pressure (*44*), hypertension would be expected to cause the opposite effect to that seen here due to increased choroid plexus permeability and fluid secretion (*45*, *46*). Indeed, our data imply that the effect of exendin-4 on ICP dynamics is through central mechanisms, since ICV infusion of exendin 9-39 partially inhibited the action of SC exendin-4. Exendin 9-39 may not have fully inhibited the actions of exendin-4, since the inhibitor was infused into the ventricle rather than being given as a bolus injection. However, it is also possible that the effects of exendin-4 are not fully mediated by GLP-1R and this requires further investigation. Previous studies have also demonstrated only moderate effects on attenuating exendin-4 induced food intake suppression at early time points following ICV bolus of exendin 9-39 (*47*). Nevertheless, the central actions of exendin-4 are further supported by the fact that exendin-4 lowered CSF pH whereas blood pH remained unchanged, which is supported by other studies showing that GLP-1 does not affect blood pH (*19*). It remains unclear how the subcutaneous administration of the GLP-1R agonist exendin-4 exerts its central effects on the choroid plexus. Following subcutaneous administration, circulating exendin-4 may cross the fenestrated capillaries in the choroid plexus and stimulate the GLP-1R on the basolateral side of the CPe cells. Otherwise, it is possible that exendin-4 crossed the blood brain barrier (*48*, *49*) or entered the CSF via the circumventricular organs, where it is able to stimulate the receptors on the apical surface of the

CPe cells. Indeed, liraglutide readily crosses into the hypothalamic arcuate nucleus (*50*), and in vivo imaging studies in rodents using fluorescently-tagged ghrelin show passage of the gut peptide to the same region *via* fenestrated capillaries of the median eminence (*51*). Lastly, exendin-4 may stimulate vagal afferents that project to the nucleus tractus solitarius (*11*). This may lead to secretion of GLP-1 through a widespread network of fibres projecting to the third ventricle allowing GLP-1 to enter the CSF (**Fig. S2A**).

In summary, exendin-4 reduces Na^+ K^+ ATPase activity at the choroid plexus, implying a reduction in CSF secretion, and lowers ICP in conscious rats with and without elevated ICP. This work demonstrates that GLP-1R agonists may provide an alternative treatment for raised ICP in conditions such as idiopathic intracranial hypertension and hydrocephalus, and warrants further clinical investigation in humans.

Materials and Methods

Study design

The main aim of this study was to explore the potential of exendin-4, a GLP-1R agonist, to modulate CSF secretion and subsequently reduce ICP. Three experimental studies were performed: (i) in vitro analysis of the GLP-1R and downstream signaling pathway in human and rat choroid plexus, GLP-1R expression was determined through mRNA analysis, immunostaining of human choroid plexus tissue sections and fluorescently tagged exendin-4 binding to rat choroid plexus explants. The downsteam signaling pathway was assessed in rat CPe cell culture by measuring cAMP generation and Na^+ K^+ ATPase activity. In vivo studies to determine the efficacy of exendin-4 to reduce ICP were conducted in (ii) healthy rats and (iii) in a pathological model of raised ICP, a rat model of hydrocephalus. The sample size (n=4-9 per experimental group) for the in vivo studies was based on the resources equation as the effects size was unknown. Exact numbers for each experiment are included below and in the figure legends. The investigators were not blinded when conducting or evaluating the experiments and the rats were randomly assigned to the treatment and control groups.

Human tissue

Human choroid plexus samples were obtained from the Parkinson's UK Brain Bank at Imperial College London, under the ethical approval of the Wales Research Ethics Committee (Ref. No.

08/MRE09/31+5). Informed consent was obtained for the use of post mortem tissue for research. Samples were stored in RNALater at -80^{0}C before being processed for qPCR following the protocol stated in the supplementary methods. Pooled human pancreas (540023), heart (540011) and ovary (540071) RNA was purchased from Agilent Technologies. Fresh choroid plexus samples were fixed in 4% formaldehyde before embedding in paraffin wax.

Experimental animals

For the in vitro work, 150-200g female Sprague-Dawley rats (Charles River) were used at the University of Birmingham in accordance with the Animals and Scientific Procedures Act 1986, licensed by the UK Home Office and approved by the University of Birmingham Ethics Committee. For the in vivo studies, which were conducted in Rigshospitalet-Glostrup, 150-250g female Sprague-Dawley rats (Taconic) were housed in groups of 4, kept under a 12 hour light/dark cycle with free access to food and water. All experimental procedures were approved by the Danish Animal Experiments Inspectorate (license number 2014-15-0201-00256 and 2012-15-2934-00283). After treatments and surgical procedures, the rats were monitored daily for any adverse effects. Female rats were used to ensure the results were relevant to conditions such as idiopathic intracranial hypertension.

Daily subcutaneous injection of exendin-4 in normal conscious rats. On day 0, the epidural ICP probe was implanted and the animal allowed to recover. On day 2, 4 and 6, for the ICP recordings the rats were sedated with midazolam (2.5 mg/kg subcutaneous injection) in an infusion cage (Instech Laboratories), which had a swirl lever arm to ensure unhindered movement. A stable baseline ICP reading was recorded for around 30 minutes before the rats received a SC injection of either saline (n=9) or 20µg/kg exendin-4 (n=9). ICP was recorded

for a further 60 minutes after which the rat was returned to its normal cage. The daily SC injections of saline or exendin-4 were performed at similar times of the day for each rat.

ICV injection of exendin-4 in anesthetized rats. To determine whether the effects of exendin-4 on ICP were due to central activity the rats were fitted with an ICV cannula at the same time as the epidural ICP probe implantation and the rat allowed to recover. Subsequent ICP recordings during exendin-4 treatment were done under anaesthesia. A stable baseline ICP reading was recorded for around 30 minutes before the following treatments were then administered ICV in a counterbalance design: (1) 1µl saline (n=8) and (2) 0.3µg/1µl exendin-4 (n=6). ICP was recorded for a further 60 minutes after which the rat was allowed to recover. Injection treatments were separated by 2-3 days.

Continuous ICV infusion of exendin 9-39 with SC injection of exendin-4 in conscious rats. To determine whether the effects of exendin-4 on ICP are through the GLP-1R, rats were fitted with an osmotic pump attached to an ICV cannula containing either saline or exendin 9-39 at the same time as the epidural ICP probe implantation. On day 2 the rats were sedated, a stable baseline recorded before a SC injection of either saline or 20µg/kg exendin-4. ICP was then recorded for a further 60 minutes. The rats were therefore assigned to 3 treatment groups: (1) Saline filled osmotic pump with SC injection of exendin-4 (ICV saline + SC exendin-4; n=6); (2) exendin 9-39 filled osmotic pump with SC injection of saline (ICV exendin 9-39 + SC saline; n=5); and (3), exendin 9-39 filled osmotic pump with SC injection of exendin-4 (ICV exendin 9-39 + SC exendin-4; n=6).

Exendin-4 dose response and time course experiment. Rats underwent the same procedure as outlined in experiment 1. For the dosing experiment the rats were given either 1 (n=6), 3

(n=6) or 5µg/kg exendin-4 (n=6) and ICP recorded for 3 hours. For the time course experiment rats were given either saline (n=18 for ICP data but only 4 were used for choroid plexus analysis) and ICP recorded for 24 hours, or 5µg/kg exendin-4 and the ICP recorded for 6 (n=6), 12 (n=6) and 24 hours (n=12 for ICP data but only 6 were used for choroid plexus analysis). After each time point the rats were killed with an overdose of euthatol and transcardially perfused with ice cold PBS. The choroid plexus was then dissected, frozen immediately and stored at -80ºC for qPCR and western blot analysis (described in detail in the supplementary methods).

SC injection of exendin-4 in conscious hydrocephalic rats. We used the kaolin model of hydrocephalus as our model of raised ICP. On day 0 the rats received an injection of kaolin to induce hydrocephalus and the rat allowed to recover. On day 6-8 the rats were fitted with an epidural ICP probe and the rat was then allowed to recover in the infusion cages still connected to the transducer so that the ICP could be continuously measured overnight to establish raised ICP. The following morning, after establishing the baseline ICP reading was stable, the rats received a SC injection of either saline (n=6, n=4 >10mmHg) or 20µg/kg exendin-4 (n=6; n=4 >10mmHg). ICP was then recorded for a further 60 minutes.

Statistical analysis

Values are represented as mean and standard error of the mean (SEM). The majority of the data was analyzed using GraphPad Prism software, however, the time course experiment with 5µg/kg exendin-4 was analyzed using SPSS due to missing data points. For the ELISA cAMP analysis, the non-parametric Kruskall-Wallis test was used, and was followed by Mann-Whitney test (two-tailed) with the appropriate adjustment for multiple comparisons (Bonferroni). T-test or One-way ANOVA (followed by a post hoc Tukey test) was used for the

comparison of qPCR, western blot and Na^+ K^+ ATPase activity. Two-way ANOVA with Sidak's multiple comparison test was used for the comparison of ICP between two groups over a period of time. Values were considered statistically significant when P values were *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. Individual level data are included in table S1.

Supplementary Materials

Supplementary Materials and Methods

Fig. S1. Characterisation of primary rat choroid plexus epithelial cells *in vivo* and *in vitro.*

Fig. S2. Suggested route for GLP-1 action at the choroid plexus

Table S1. Individual level data corresponding to the different figures.

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Author contributions

A.S. was responsible for the study concept. H.B., A.G., D.J.H. and A.S. conceived and designed the experiments; H.B. conducted the following in vitro experiments: immunohistochemistry, Na^+ K^+ ATPase activity assay, cAMP assay, rat qPCR and western blot, and FLEX analysis); C.W. performed human qPCR and cAMP assays; A.G. M.U. and J.M contributed to the immunohistochemistry data; H.B., M.U. J.M. and S.H. performed the ICP recordings; H.B. and M.U. analyzed the data; H.B., M.U., A.G., D.J.H., R.J and A.S co-wrote the manuscript and all authors reviewed the final version.

Competing interests

A.S. holds patent # PCT/GB2015/052453 related to this work entitled "elevated intracranial pressure treatment". R.J. has given lectures for Pfizer, Berlin-Chemie, Norspan, Merck and Autonomic Technologies and has been a member of the advisory boards of Autonomic Technologies, Medotech and ElectroCore.

Figure legends

Fig. 1. GLP-1R expression in post-mortem human choroid plexus tissue in vitro. (A) Representative image of haematoxylin and eosin staining of human choroid plexus tissue section demonstrating classic choroid plexus morphology. **(B)** The histogram shows *GLP-1R* mRNA expression in human pancreas (n=1), heart (n=1), ovary (n=1) and choroid plexus (n=5). (**C**-**D**) Representative images of GLP-1R staining of paraffin-embedded human choroid plexus counterstained with haemotoxylin. Sections were incubated without primary antibody (**C**) and with the human GLP-1R antibody MAb 3F52 (**D**). (**E**-**F**) High magnification of the boxed regions shown in C and D respectively. Scale bars, 100µm, BV – blood vessel and CPe – choroid plexus epithelial cell.

Fig. 2. Expression of GLP-1R after treatment with exendin-4 in rat choroid plexus in vitro. (**A**) Representative images of rat choroid plexus after treatment with artificial CSF (aCSF) as control or fluorescently labelled exendin-4 (FLEX) in the presence or absence of the GLP-1R antagonist exendin 9-39. DAPI (blue) was used as a nuclear marker; scale bar, 50µm (insert, 25µm). **(B-E)** The histograms represent the fold change in mRNA expression of *Glp-1r* (**B**), *Na^+ K^+ atpase* (**C**), *Aqp1* (**D**) and *Nhe1* (**E**) (aCSF n=6; 3hr n=7, 6hr n=7) *P<0.05, **P<0.01; ANOVA with Tukey's multiple comparisons test.

Fig. 3. Effect of exendin-4 treatment on cAMP and Na^+ K^+ ATPase activity in CPe cells. (A-B) The histograms represent the amount of cAMP generated after incubation with control, exendin-4 with and without 1µM exendin 9-39 and forskolin (positive control) using two different methods of cAMP detection (A - control n=8, exendin-4 n=8, Forskolin n=5, B -

control n=5, 1nM n=5, 10nM n=6 and 100nM exendin-4 n=5; with 1µM exendin 9-39 n=6, n=5 and n=5 respectively). (**C**) Na^+ K^+ ATPase activity was measured by determining the concentration of inorganic phosphate generated by the hydrolysis of ATP that was sensitive to ouabain (Na^+ K^+ ATPase inhibitor) (control n=13, exendin-4 n=7; PKI n=8; exendin-4 + PKI n=8). *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001, NS no significance. (A) Kruskal-Wallis followed by Mann-Whitney tests (Bonferroni correction); (B-C) ANOVA with Tukey's multiple comparisons test. Protein kinase A inhibitor, PKI.

Fig. 4. Effect of exendin-4 on ICP in healthy conscious rats. (A) Overview of the experimental design in normal rats. Rats were fitted with an epidural ICP probe and allowed to recover. Treatment was given daily for 5 days and ICP was recorded on days 2, 4 and 6, before and after the rats received a subcutaneous (SC) injection of either saline (n=9) or 20µg/kg exendin-4 (n=9). **(B)** Example ICP traces of saline (*blue*) and exendin-4 (*red*) treatment. Spikes in the trace represent when the animal was moving (*) and accurate recording of ICP was confirmed by the response to jugular vein compression. **(C-E)** Line graphs showing the percentage of baseline ICP after SC injection of either saline or exendin-4 on day 2 **(C)**, day 4 **(D)** and day 6 **(E)**. **(F-G)** Histograms showing the pre-dose and 60 minutes post treatment ICP values (% of baseline on day 2) on days 2, 4 and 6 for exendin-4 (**F**) and saline (**G**). (**H**) Line graph of the % change in weight from day 2 (start of treatment) showing that both saline and exendin-4 treated rats lost weight but there was no significant difference between the groups on day 4 or 6. (**I**) Scatter plot of weight change (g) vs ICP change (mmHg) in the saline (*blue* n=4) and exendin-4 (*red* n=5) groups. (**J-N**) Histograms showing blood pH (**J**) and CSF pH (**K**), and the concentration of Na^+ (**L**) Cl^- (**M**) and Ca^{2+} (**N**) in the CSF, 60 minutes after a SC injection of either saline or 20µg/kg exendin-4. **(O)** ICP was measured before and after a 1µl intracerebroventricular (ICV) injection of either saline (n=8) or 0.3µg exendin-4 (n=6). (**P**)

Exendin 9-39 was continually infused (4µg/µl/hr) into the lateral ventricle (ICV) and ICP was measured before and after a SC injection of either 20µg/kg exendin-4 (ICV exendin 9-39 + SC exendin-4, n=6) or saline (ICV exendin 9-39 + SC saline, n=5) and compared to continuous saline infusion (ICV Saline + SC exendin-4, n=6). *P<0.05, **P<0.01, ***P<0.001; (C-H, O-P) Two way ANOVA with Sidak's multiple comparison test; (J-N) T-test (two-tailed).

Fig. 5. Effects of different doses of exendin-4 on ICP, mRNA and protein expression in healthy conscious rats. (A-B) Dose-response of exendin-4's effects on ICP following SC administration of 1 (n=6), 3 (n=6), 5 (n=23) and 20 µg/kg (n=9) exendin-4 compared to saline (n=18) at 30 and 60 minutes. **(C)** Line graph showing the percentage of baseline ICP after treatment with 1, 3 or 5µg/kg exendin-4 measured over 3 hours. **(D-G)** The histograms show *Glp-1R* **(D)**, *Na^+ K^+ atpase* **(E)**, *Aqp1* **(F)** and *Nhe1* **(G)** mRNA expression in the rat choroid plexus after saline treatment (n=4) or treatment with 1 (n=5), 3 (n=6), 5 µg/kg (n=6) exendin-4. **(H)** Representative western blots and **(I-K)** semi-quantitative protein analysis for **(I)** Na^+ K^+ ATPase (112kDa) and **(J)** total AQP1, either non-glycosylated (NG, 29kDa) or glycosylated (G, 35kDa); β-actin (42kDa) loading control. **(K)** Histogram shows the ratio of G to NG AQP1. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. (B-C) Two-way ANOVA with Sidak's multiple comparison test; (D-G and I-K) ANOVA with Tukey's multiple comparison test.

Fig. 6. Effects of Exendin-4 time course on ICP, mRNA and protein expression in healthy conscious rats. (A) Line graph showing the percentage of baseline ICP after a single SC injection of saline (n=18) or 5µg/kg exendin-4 (n=24) measured over 24 hours. **(B-D)** Histograms showing weight loss (B), water intake (C) and food intake (D) in rats treated with saline (n=4) or 5µg/kg exendin-4 at 3 (n=6), 6 (n=6) and 24 hours (n=6). **(E-H)** Histograms representing *Glp-1r* **(E)**, *Na^+ K^+ atpase* **(F)**, *Aqp1* **(G)** and *Nhe1* **(H)** mRNA expression in the

rat choroid plexus after treatment with saline (n=4) and 5µg/kg exendin-4 at 3 (n=6), 6 (n=5) and 24 hours (n=5). **(I)** Representative western blots and **(J-L)** semi-quantitative protein analysis for **(J)** Na^+ K^+ ATPase (112kDa) and **(K)** total AQP1 either nonglycosylated (NG, 29kDa) or glycosylated (G, 35kDa); β-actin (42kDa) loading control. **(L)** The histogram shows the ratio of G to NG AQP1. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001; (A-D) Two-way ANOVA with Sidak's multiple comparison test; (E-H and J-L) ANOVA with Tukey's multiple comparison test.

Fig. 7. Effect of exendin-4 on ICP in a rat model of raised ICP (hydrocephalic). (A) Overview of the experimental plan. Kaolin was injected into the cisterna magna to induce hydrocephalus. On Day 6 the ICP monitor was implanted under anaesthesia and ICP was recorded overnight to allow the ICP to normalize after implantation. On Day 7, the rats were given a SC injection of either saline (n=6) or 20µg/kg Exendin-4 (n=6), and ICP was recorded for a further 60 minutes. **(B)** Dot plot showing the individual baseline ICP values (mmHg) for the normal rats and rats injected with kaolin. The kaolin group had significantly higher baseline ICP values compared to the normal group, with 8/12 rats having an ICP value of >10mmHg. **(C)** Line graph showing the percentage of baseline ICP after treatment with either saline (dark blue, n=6) or exendin-4 (dark red, n=6). The groups could also be further divided into those with ICP >10mmHg in the saline group (light blue, n=4) and exendin-4 group (light red, n=4). **(D)** Example ICP trace in a hydrocephalic rat before and after treatment with exendin-4. Before treatment the rat exhibited pathological ICP B-waves (*b*), which were abolished following treatment with exendin-4. **** P<0.0001; (B) T-test (two tailed); (C) Two-way ANOVA with Sidak's multiple comparison test.