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

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**Supplementary Information**

**Supplementary Materials and Methods**

## Reagents

Exendin-4 (GLP-1R agonist), exendin- 9-39 (GLP-1R antagonist), ouabain (specific $Na^+$ $K^+$ ATPase inhibitor), 3-Isobutyl-1-methylxanthine (IBMX; phosphodiesterase inhibitor) and Forskolin (adenylate cyclase activator) were purchased from Sigma-Aldrich. For the in vivo studies exendin 9-39 was purchased from Cohesion Biosciences (CCP1199) and Abcam (ab141101). Fluorescently tagged exendin-4 (FLEX) was purchased from AnaSpec (AS-63899). The myristoylated PKA inhibitor (PKI)–(14-22)-amide was purchased from Merck Chemicals. Mouse monoclonal antibody against human GLP-1R protein was purchased from the Developmental Studies Hybridoma Bank (Iowa; Mab 3F52, deposited by Knudsen, L.B.). Primary antibodies for choroid plexus epithelial (CPe) cell characterization included antibodies against transthyretin (TTR; sheep, ab9015, Abcam), $Na^+$ $K^+$ ATPase (rabbit, ab76020, Abcam; mouse, 05-369, Millipore), zona occludens-1 (ZO-1; rabbit, 61-7300, Life Technologies), aquaporin 1 (AQP1; rabbit, AB3065, Abcam; rabbit, AB2219, Millipore) and β-actin (mouse, A5441, Sigma Aldrich). For immunohistochemistry, the Alexa Fluor® labelled secondary antibodies were purchased from Life Technologies and for western blot, HRP-conjugated secondary antibodies were bought from Cell Signaling Technology. Cell culture reagents were from Life Technologies or Sigma-Aldrich and unless specified all other chemicals were purchased from Sigma-Aldrich. For surgical procedures the midazolam was purchased from B. Braun and the fluanisone and fentanylcitrate from the Danish pharmacy supply.

## In vitro experiments

**Whole Choroid plexus.** The choroid plexus from the lateral ventricles were dissected and placed in artificial CSF (aCSF; 118mM NaCl, 22mM $NaHCO_3$, 1.45mM $K_2HPO_4$, 1mM $MgSO_4$, 1mM $CaCl_2$ and 10mM glucose). To ~~evaluate the effects of exendin-4 on~~demonstrate the presence of the GLP-1R ~~localisation within the cell~~in the choroid plexus, whole choroid plexus was incubated with aCSF containing (a) 1µM FLEX for 15 and 30 minutes or (b) 1µM exendin 9-39 for 10 minutes followed by 1µM FLEX for 30 minutes. ~~100nM Exendin 9-39 for 15 minutes followed by 100nM exendin-4 for 30 minutes or 100nM exendin-4 only for 15 and 30 minutes~~. Whole choroid plexus was then fixed and visualized under a Zeiss LSM 510 UV-confocal microscope (Carl Zeiss)~~stained following the protocol described below~~. To determine the effects of exendin-4 on GLP-1R mRNA expression the choroid plexus was incubated with aCSF containing 100nM exendin-4 for 3 and 6 hours, immediately frozen in liquid nitrogen and stored at -80$^0$C.

**Primary CPe cell culture.** Choroid plexus tissue from lateral and fourth ventricles were dissected and incubated with 0.25% trypsin solution for 2.5 hours at 4°C followed by 30 minutes at 37°C. Trypsin digestion was stopped by the addition of newborn calf serum and the cell suspension was centrifuged at 20g for 10 minutes. Cells were resuspended in DMEM/F12 supplemented with 10% FBS, 1% penicillin/streptomycin, 4mM L-glutamine, 200ng/ml hydrocortisone, 5ng/ml sodium selenite and 10ng/ml EGF. 20µM cytosine arabinoside was used for the first 4 days in culture to limit the growth of fibroblasts (*53*). Initially the cells were seeded onto a laminin coated 6 well plate and allowed to grow for 2 days before being transferred to laminin coated 96 well plates or 12 well inserts (Greiner Bio-One Ltd). On day 4 the media was replaced with DMEM/F12 supplemented with 10% FBS and 1% penicillin/streptomycin and changed every 2-3 days. After reaching confluency, CPe cells were serum deprived for 3 days prior to the beginning of the studies (between days 10-14).

**Immunofluorescent staining.** For staining of rat brain tissue sections, rats were euthanized with rising $CO_2$ and immediately perfused transcardially with 10mM PBS, pH 7.4 (PBS) followed by 4% paraformaldehyde (PFA; Alfa Aesar) in PBS. Brains were postfixed overnight at $4^0$C, cryoprotected by sequential immersion in 10%, 20% and 30% sucrose in PBS at $4^0$C, embedded in OCT (Fisher Scientific), and 15-µm-thick coronal sections cut on a cryostat (Bright Instruments), mounted on charged microscope slides and stored at $-20^0$C until use. Sections were first washed in PBST (PBS containing 0.3% Tween20), blocked in PBST containing 2% bovine serum albumin (BSA) and 15% normal goat serum (NGS) for 20 minutes at room temperature, and then incubated with the primary antibody (PBST with 2% BSA) at 4°C overnight. After washing in PBST, sections were incubated for 1 hour at room temperature in the dark with the appropriate Alexa Fluor 488 labelled secondary antibody diluted in PBST containing 2% BSA and 1.5% NGS. Finally, sections were washed in PBST before mounting in Vectashield containing the nuclear stain DAPI (Vector Laboratories).

For fluorescence labelling of CPe cells, samples were first fixed in PBS containing 2% PFA and 2% glucose for 20 minutes at room temperature, washed in PBS and then permeabilized with methanol for 6 minutes at room temperature. The cells were stained using the same technique described above except that PBST was substituted with PBS.

Stained cells and sections were viewed under a Zeiss LSM 510 UV-confocal microscope (Carl Zeiss) and multiple Z-stack images were taken.

**Immunoperoxidase staining.** For staining of paraffin-embedded human choroid plexus, the sections were dewaxed and dehydrated to distilled water. Sections were treated for 30 minutes in Tris-EDTA buffer (pH 9.0) at 95ºC in a waterbath for antigen retrieval. The sections were then cooled in PBST before incubation with 1% $H_2O_2$ (70% methanol in PBS) for 30 minutes

to inhibit endogenous peroxidase. Sections were washed in PBST, blocked in PBST containing 2% BSA and 15% normal serum for 1 hour at room temperature, and then incubated in primary antibody solution at 4°C overnight. Sections were again washed in PBST before incubation in biotinylated secondary antibody solution (Vector Laboratories) for 30 minutes at room temperature. Sections were washed in PBST and then incubated for 30 minutes at room temperature in Avidin/Biotin Complex (ABC; Vectastain Elite ABC kit, Vector Laboratories) following the manufacturer's instructions. After rinsing in PBST, sections were treated with 3'3 diaminobenzidine (DAB) substrate (Vector Laboratories), washed in distilled water, counterstained with haematoxylin, washed in running water before dehydration, cleared in xylene and mounted in Vectamount medium (Vector Laboratories).

**Quantitative polymerase chain reaction (qPCR).** For qPCR studies the choroid plexus was dissected, immediately frozen in liquid nitrogen and stored at -80$^0$C. Primary cultures of CPe cells were grown on 12 well inserts until confluency. Total RNA was extracted using the GenElute mammalian total RNA extraction kit and carried out according to the manufacturer's instructions. RNA was reverse transcribed to complementary DNA (cDNA) using a high capacity reverse transcription kit (Life Technologies) or iScript cDNA synthesis kit (Biorad) according to the manufacturer's protocol. Taqman Gene Expression Assays (Life Technologies) were used to assess the expression of GLP-1R (assay number Rn00562406_m1 and Hs00157705_m1), $Na^+$ $K^+$ ATPase (assay number Rn01533986_m1), AQP1 (assay number Rn00562834_m1) and NHE1 (assay number Rn00561924_m1). The 18S ribosomal subunit was used as an endogenous reference (4319413E) and samples were run in triplicate. The cycle number at which the particular sample crossed that threshold (Ct) was used to determine the levels of gene expression and ΔCt was calculated as the difference between the Ct (gene of interest) and the Ct (endogenous reference).

**Western blot.** The choroid plexus was dissected, immediately frozen in liquid nitrogen and stored at -80$^0$C. Tissues were homogenised in ice cold RIPA lysis buffer and centrifuged at 13,000g to remove cell debris. Tissue lysates (10µg protein) were separated on a 4-12% tris-glycine gel. The proteins were transferred onto a polyvinylidene difluoride membrane and subsequently blocked with 5% skimmed milk powder in TBST (TBS pH 7.4 with 0.5% Tween20) for 1 hour at room temperature before incubation with the primary antibody diluted in milk/TBST overnight at 4$^0$C. After washing in TBST the membranes were incubated with HRP-conjugated secondary antibody diluted in milk/TBST for 1 hour at room temperature. The bands were detected using ECL reagents (Amersham) and developed onto film.

**cAMP assay.** The effect of exendin-4 on the downstream GLP-1R signaling pathway was assessed by measuring the levels of cAMP in CPe cells using two different techniques. The first assay was the Amersham cAMP Biotrak Enzyme immunoassay System (RPN 225, GE Healthcare Life Sciences). CPe cells were grown on a 96 well plate (described previously) and, on the day of the experiment, incubated in aCSF supplemented with 1mM IBMX containing; aCSF only (n=8), 100nM exendin-4 (n=8) or 100nM Forskolin (positive control; n=5) for 30 minutes at 37$^0$C. The cells were subsequently lysed and cAMP detected according to the manufacturer's instructions. The second assay was the LANCE® (Lanthanide chelate excite) cAMP 384 kit (PerkinElmer). CPe cells were grown in flasks and then trypsinized to form a single cell suspension. The cells were incubated in stimulation buffer (PBS with 5.5mM glucose, 0.1% BSA and 0.5mM IBMX) containing; 1nM (n=5), 10nM (n=6) and 100nM exendin-4 (n=5), with and without 1µM exendin 9-39 (n=6, n=5 and n=5 respectively), and forskolin (n=6) as a positive control. cAMP was then detected according to the manufacturer's instructions.

**$Na^+$ $K^+$ ATPase activity assay.** The effect of exendin-4 on $Na^+$ $K^+$ ATPase activity in the choroid plexus was evaluated by the colorimetric measurement of phosphate released from ATP hydrolysis with the use of a phosphate assay kit (ab65622, Abcam); with $Na^+$ $K^+$ ATPase activity being defined as the portion of phosphate produced that is sensitive to ouabain. CPe cells were incubated with aCSF for 1 hour at $37^0$C before incubation in aCSF containing; 100nM exendin-4 (n=7), 5µM PKI-16-22-amide (n=8), 100nM exendin-4 + 5µM PKI-16-22-amide (n=8); in the presence and absence of 1mM ouabain for 30 minutes at $37^0$C. The cells were then lysed on ice and spun at 13,000g to remove cell debris. Phosphate was measured as per the manufacturer's instructions. Briefly the reaction mix was added to the samples and incubated at room temperature for 60 minutes before the plate was read at 690nm. $Na^+$ $K^+$ ATPase activity was calculated as the difference between the amount of phosphate produced in the presence and absence of ouabain for each treatment.

## In vivo experiments

**Epidural ICP probe implantation.** Implantation of an epidural ICP probe and its validation were recently published as a methodological work that contains all technical and surgical detail (*54*). The rats were anaesthetized (2.7ml/kg subcutaneous injection containing 1.25mg/ml midazolam, 2.5mg/ml fluanisone and 0.079mg/ml fentanylcitrate), placed in a stereotactic frame (David Kopf Instruments) and a 2cm-midline incision was performed on top of the skull and the bone was exposed by retracting the skin and soft tissue. A dental drill was used to make 4 burr holes in the skull; one large hole was carefully drilled to expose the dura mater enabling placement of the epidural ICP probe (C313G-3UP, PlasticsOne), with the cannula cut to be level with the base of the pedestal. The other 3 smaller holes were used to fit anchoring screws to the skull. The epidural pressure bolt and the anchoring screws were placed and aligned with

the interior surface of the skull and secured using dental resin-cement (Clearfil SA Cement, RH Dental). The epidural ICP probe and the transducer (DTX-Plus™, Argon Medical Devices) were then connected by a polyethylene tube filled with sterile water, ensuring the absence of air bubbles. The pressure signal was visualized and recorded using Perisoft v.2.5.5 (Perimed). Correct ICP signal was confirmed by the transient elevation of ICP after jugular vein compression. When the ICP recording procedure was completed the epidural pressure cannula was closed with a bite proof cap (303DCFTX2, PlasticsOne) and the rat allowed to recover.

There was one modification with the epidural ICP probe implantation in the hydrocephalic rats; before the epidural pressure bolt was placed on the dura, a small hole (1mm in diameter) was made with forceps in the dura.

**Intracerebroventricular (ICV) injection**

During the epidural ICP probe surgery, animals receiving ICV treatments also had an ICV cannula implanted at the same time. An additional burr hole was made 0.8mm posterior and 1.6mm lateral to Bregma. The cannula was inserted into the left lateral ventricle, fixed with dental resin-cement and closed by a cap with a dummy cannula to maintain patency. For the ICV injection rats were anesthetized and connected to the transducer to measure ICP. Once a stable baseline had been established a 5µl Hamilton syringe connected via tubing to an internal cannula was used for the ICV injection.

**Osmotic pump implantation**

Osmotic pumps (model 1003D, Alzet, Durect Corporation, California, USA) were prepared under sterile conditions and primed in sterile saline overnight at 37°C to allow prompt delivery after implantation. The ICV cannula (brain infusion kit 1, Alzet) was set to 4mm and attached to the pump via 5cm catheter tubing containing saline. The osmotic pump was filled with either

saline or 4mg/ml exendin 9-39, thus the infusion rate was 4µg/µl/hr (around 100µg per day). The fluid in the pump and the fluid in the catheter tubing were separated by an air bubble to delay the start of exendin 9-39 until implantation.

For implantation of the osmotic pump, the head of anaesthetized rat was fixed in a stereotactic frame, the dorsal skull was exposed and a burr hole sited in the parietal bone 0.8mm posterior and 1.6mm lateral to Bregma. The ICV cannula was inserted into the left lateral ventricle and fixed in place with glue to 2 stabilising screws (PlasticsOne), and the osmotic pump was implanted subcutaneously in the neck region. The epidural ICP probe was then implanted as above.

**Induction of hydrocephalus**

We used the kaolin model of hydrocephalus as our model of raised ICP. The rats were anaesthetized and the head fixed in a stereotactic frame with the neck flexed down in a 90 degree angle to horizontal and secured in this position. A mark was made on the skin above the dorsal atlanto-occipital membrane between the skull and the first cervical spinosus. The percutaneous injection was performed using an insulin syringe with a 30 gauge needle, which was slowly advanced in a vertical direction until there was a loss of resistance and 80 µL of sterile kaolin suspension (0.250 mg/mL in Ringer's lactate solution - 1.4mM $Ca^{2+}$, 4mM $K^{+}$, 130mM $Na^{+}$, 109mM $Cl^{-}$, 28mM lactate) injected gradually (8.5 µL/s). Following the injection the neck was extended, the head released from the stereotactic frame and the animal allowed to recover.

**Blood and electrolyte Measurements.** Blood and CSF pH and electrolytes were measured immediately using an ABL80 FLEX blood gas analyzer (Radiometer Medical ApS).

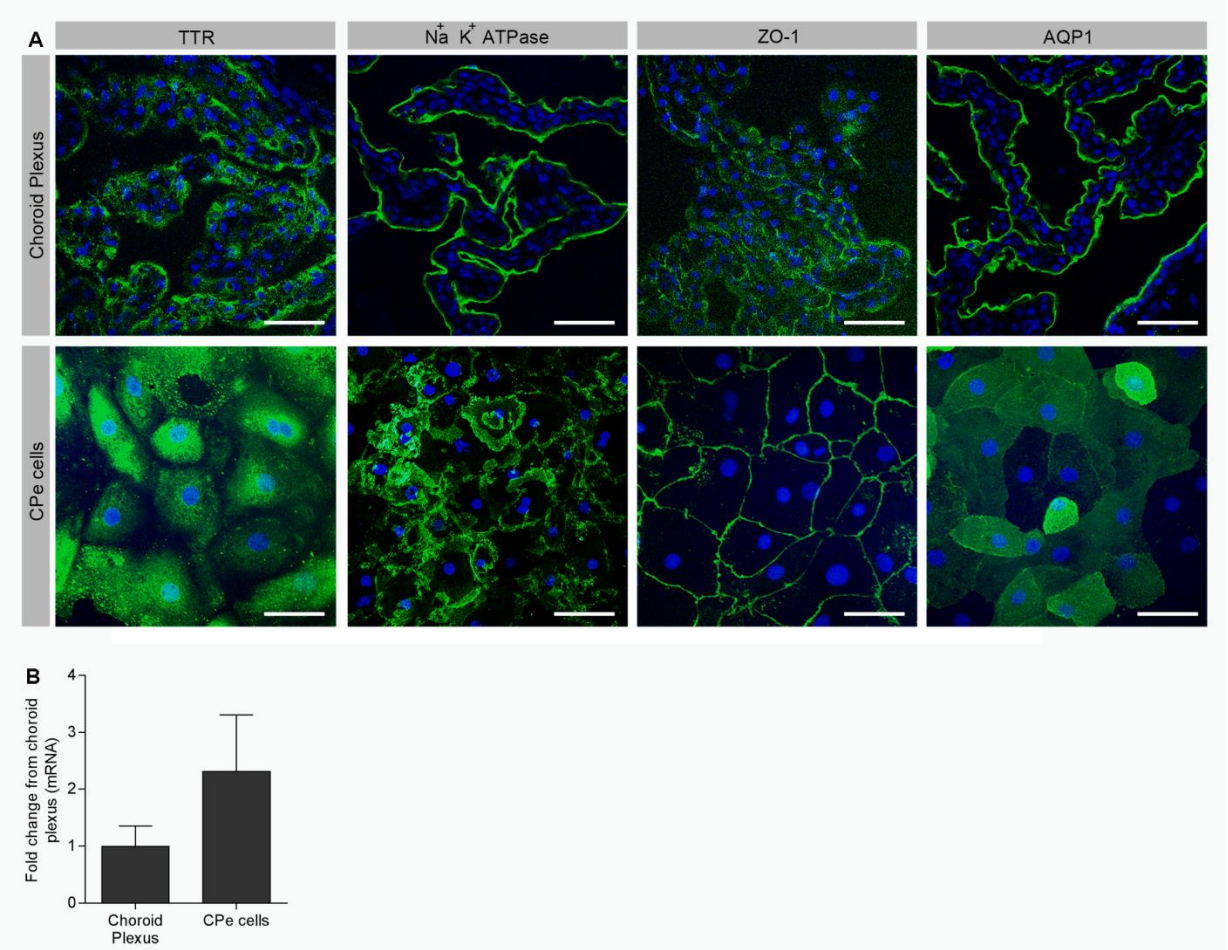


**Fig. S1. Characterisation of primary rat choroid plexus epithelial (CPe) cells in vivo and in vitro. (A)** The identity of CPe cells in culture was determined by immunohistochemistry using antibodies against: (1) transthyretin (TTR), a CPe cell marker; (2) $Na^+$ $K^+$ ATPase, ion pump involved in actively moving $Na^+$ out of the CPe cells and into the CSF, (3) zona occludens-1 (ZO-1), a tight junction protein; and (4) aquaporin 1 (AQP1), the most prominent water channel in the choroid plexus. TTR staining (*green*) was observed in the cytoplasm of the CPe cells; ZO-1 (green) was localised at the interface between the cells indicating the presence of tight junctions; $Na^+$ $K^+$ ATPase and AQP1 *(green*) were present on the apical surface of the epithelial cells indicating the polarisation of the cells in vitro and in vivo. **(B)** The histogram represents the fold change in *Glp-1r* mRNA from whole choroid plexus + SEM (choroid plexus n=3; CPe cells n=3), demonstrating *Glp-1r* mRNA is present in both the CPe cells and whole choroid plexus. DAPI (blue) was used as a nuclear marker, scale bar - 50µm.

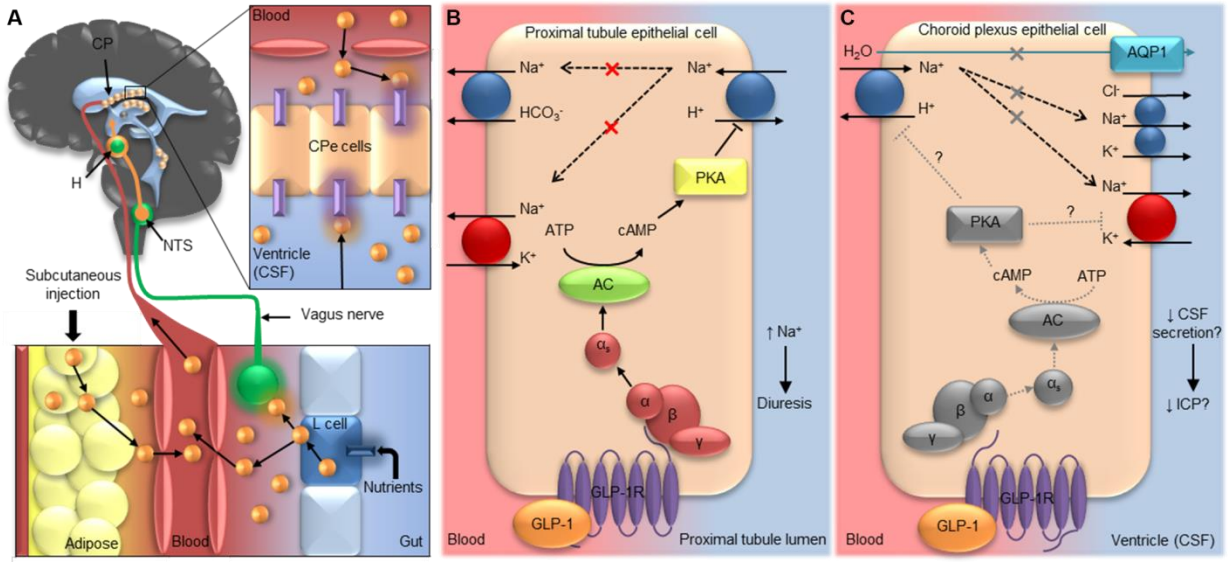


**Fig. S2. Suggested route for GLP-1 action at the choroid plexus. (A)** Under physiological conditions, GLP-1 is secreted by L cells in response to nutrients in the gut and then enters the bloodstream or activates the vagus nerve. Therapeutic administration of GLP-1 mimetics is via a subcutaneous injection. Once in the bloodstream, GLP-1 could bind to GLP-1 receptors (GLP-1Rs) on the basal surface of the choroid plexus epithelial (CPe) cells or could cross the blood brain barrier and enter the CSF. Alternatively, the vagus nerve could stimulate GLP-1 production at the nucleus tractus solitarius (NTS), which has fibres projecting to the hypothalamus (H) adjacent to the CSF. This allows GLP-1 secretion into the CSF, from where it can bind with GLP-1Rs on the apical surface of the CPe cells. **(B)** In the kidney proximal tubule cells, the binding of GLP-1 to its receptor stimulates the conversion of adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP) by adenylate cyclase (AC) through $G\alpha_s$ protein subunit. cAMP activates protein kinase A (PKA), which phosphorylates the $Na^+$ $H^+$ exchanger resulting in its inhibition, thus preventing $Na^+$ reabsorption. **(C)** We hypothesize that activation of GLP-1R on choroid plexus epithelial cells stimulates AC, which converts ATP to cAMP. cAMP subsequently activates PKA which could phosphorylate either the $Na^+$ $H^+$ exchanger or the $Na^+$ $K^+$ ATPase, reducing $Na^+$ transport from blood into the CSF. This would decrease CSF production and potentially reduce ICP**.**