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

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1 **Supplementary Information**

2

3

4 **Supplementary Materials and Methods**

5 **Reagents**

6 | Exendin-4 (GLP-1R agonist), exendin_9-39 (GLP-1R antagonist), ouabain (specific Na⁺ K⁺
7 | ATPase inhibitor), 3-Isobutyl-1-methylxanthine (IBMX; phosphodiesterase inhibitor) and
8 | Forskolin (adenylate cyclase activator) were purchased from Sigma-Aldrich. For the in vivo
9 | studies exendin 9-39 was purchased from Cohesion Biosciences (CCP1199) and Abcam
10 | (ab141101). Fluorescently tagged exendin-4 (FLEX) was purchased from AnaSpec (AS-
11 | 63899). The myristoylated PKA inhibitor (PKI)–(14-22)-amide was purchased from Merck
12 | Chemicals. Mouse monoclonal antibody against human GLP-1R protein was purchased from
13 | the Developmental Studies Hybridoma Bank (Iowa; Mab 3F52, deposited by Knudsen, L.B.).
14 | Primary antibodies for choroid plexus epithelial (CPE) cell characterization included antibodies
15 | against transthyretin (TTR; sheep, ab9015, Abcam), Na⁺ K⁺ ATPase (rabbit, ab76020, Abcam;
16 | mouse, 05-369, Millipore), zona occludens-1 (ZO-1; rabbit, 61-7300, Life Technologies),
17 | aquaporin 1 (AQP1; rabbit, AB3065, Abcam; rabbit, AB2219, Millipore) and β-actin (mouse,
18 | A5441, Sigma Aldrich). For immunohistochemistry, the Alexa Fluor[®] labelled secondary
19 | antibodies were purchased from Life Technologies and for western blot, HRP-conjugated
20 | secondary antibodies were bought from Cell Signaling Technology. Cell culture reagents were
21 | from Life Technologies or Sigma-Aldrich and unless specified all other chemicals were
22 | purchased from Sigma-Aldrich. For surgical procedures the midazolam was purchased from B.
23 | Braun and the fluanisone and fentanylcitrate from the Danish pharmacy supply.

24

25 **In vitro experiments**

26 **Whole Choroid plexus.** The choroid plexus from the lateral ventricles were dissected and
27 placed in artificial CSF (aCSF; 118mM NaCl, 22mM NaHCO₃, 1.45mM K₂HPO₄, 1mM
28 MgSO₄, 1mM CaCl₂ and 10mM glucose). To ~~evaluate the effects of exendin-4 on~~demonstrate
29 the presence of the GLP-1R ~~localisation within the cell in the choroid plexus~~, whole choroid
30 plexus was incubated with aCSF containing (a) 1µM FLEX for 15 and 30 minutes or (b) 1µM
31 exendin 9-39 for 10 minutes followed by 1µM FLEX for 30 minutes. 100nM Exendin 9-39 for
32 15 minutes followed by 100nM exendin-4 for 30 minutes or 100nM exendin-4 only for 15 and
33 30 minutes. Whole choroid plexus was then fixed and visualized under a Zeiss LSM 510 UV-
34 confocal microscope (Carl Zeiss)~~stained following the protocol described below~~. To determine
35 the effects of exendin-4 on GLP-1R mRNA expression the choroid plexus was incubated with
36 aCSF containing 100nM exendin-4 for 3 and 6 hours, immediately frozen in liquid nitrogen
37 and stored at -80°C.

38
39 **Primary CPe cell culture.** Choroid plexus tissue from lateral and fourth ventricles were
40 dissected and incubated with 0.25% trypsin solution for 2.5 hours at 4°C followed by 30
41 minutes at 37°C. Trypsin digestion was stopped by the addition of newborn calf serum and the
42 cell suspension was centrifuged at 20g for 10 minutes. Cells were resuspended in DMEM/F12
43 supplemented with 10% FBS, 1% penicillin/streptomycin, 4mM L-glutamine, 200ng/ml
44 hydrocortisone, 5ng/ml sodium selenite and 10ng/ml EGF. 20µM cytosine arabinoside was
45 used for the first 4 days in culture to limit the growth of fibroblasts (53). Initially the cells were
46 seeded onto a laminin coated 6 well plate and allowed to grow for 2 days before being
47 transferred to laminin coated 96 well plates or 12 well inserts (Greiner Bio-One Ltd). On day 4
48 the media was replaced with DMEM/F12 supplemented with 10% FBS and 1%
49 penicillin/streptomycin and changed every 2-3 days. After reaching confluency, CPe cells were
50 serum deprived for 3 days prior to the beginning of the studies (between days 10-14).

51

52 **Immunofluorescent staining.** For staining of rat brain tissue sections, rats were euthanized
53 with rising CO₂ and immediately perfused transcardially with 10mM PBS, pH 7.4 (PBS)
54 followed by 4% paraformaldehyde (PFA; Alfa Aesar) in PBS. Brains were postfixed overnight
55 at 4°C, cryoprotected by sequential immersion in 10%, 20% and 30% sucrose in PBS at 4°C,
56 embedded in OCT (Fisher Scientific), and 15-µm-thick coronal sections cut on a cryostat
57 (Bright Instruments), mounted on charged microscope slides and stored at -20°C until use.
58 Sections were first washed in PBST (PBS containing 0.3% Tween20), blocked in PBST
59 containing 2% bovine serum albumin (BSA) and 15% normal goat serum (NGS) for 20
60 minutes at room temperature, and then incubated with the primary antibody (PBST with 2%
61 BSA) at 4°C overnight. After washing in PBST, sections were incubated for 1 hour at room
62 temperature in the dark with the appropriate Alexa Fluor 488 labelled secondary antibody
63 diluted in PBST containing 2% BSA and 1.5% NGS. Finally, sections were washed in PBST
64 before mounting in Vectashield containing the nuclear stain DAPI (Vector Laboratories).

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

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

71

72 **Immunoperoxidase staining.** For staining of paraffin-embedded human choroid plexus, the
73 sections were dewaxed and dehydrated to distilled water. Sections were treated for 30 minutes
74 in Tris-EDTA buffer (pH 9.0) at 95°C in a waterbath for antigen retrieval. The sections were
75 then cooled in PBST before incubation with 1% H₂O₂ (70% methanol in PBS) for 30 minutes

76 to inhibit endogenous peroxidase. Sections were washed in PBST, blocked in PBST containing
77 2% BSA and 15% normal serum for 1 hour at room temperature, and then incubated in primary
78 antibody solution at 4°C overnight. Sections were again washed in PBST before incubation in
79 biotinylated secondary antibody solution (Vector Laboratories) for 30 minutes at room
80 temperature. Sections were washed in PBST and then incubated for 30 minutes at room
81 temperature in Avidin/Biotin Complex (ABC; Vectastain Elite ABC kit, Vector Laboratories)
82 following the manufacturer's instructions. After rinsing in PBST, sections were treated with
83 3'3 diaminobenzidine (DAB) substrate (Vector Laboratories), washed in distilled water,
84 counterstained with haematoxylin, washed in running water before dehydration, cleared in
85 xylene and mounted in Vectamount medium (Vector Laboratories).

86

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

101
102 **Western blot.** The choroid plexus was dissected, immediately frozen in liquid nitrogen and
103 stored at -80°C. Tissues were homogenised in ice cold RIPA lysis buffer and centrifuged at
104 13,000g to remove cell debris. Tissue lysates (10µg protein) were separated on a 4-12% tris-
105 glycine gel. The proteins were transferred onto a polyvinylidene difluoride membrane and
106 subsequently blocked with 5% skimmed milk powder in TBST (TBS pH 7.4 with 0.5%
107 Tween20) for 1 hour at room temperature before incubation with the primary antibody diluted
108 in milk/TBST overnight at 4°C. After washing in TBST the membranes were incubated with
109 HRP-conjugated secondary antibody diluted in milk/TBST for 1 hour at room temperature. The
110 bands were detected using ECL reagents (Amersham) and developed onto film.

111
112 **cAMP assay.** The effect of exendin-4 on the downstream GLP-1R signaling pathway was
113 assessed by measuring the levels of cAMP in CPe cells using two different techniques. The
114 first assay was the Amersham cAMP Biotrak Enzyme immunoassay System (RPN 225, GE
115 Healthcare Life Sciences). CPe cells were grown on a 96 well plate (described previously) and,
116 on the day of the experiment, incubated in aCSF supplemented with 1mM IBMX containing;
117 aCSF only (n=8), 100nM exendin-4 (n=8) or 100nM Forskolin (positive control; n=5) for 30
118 minutes at 37°C. The cells were subsequently lysed and cAMP detected according to the
119 manufacturer's instructions. The second assay was the LANCE® (Lanthanide chelate excite)
120 cAMP 384 kit (PerkinElmer). CPe cells were grown in flasks and then trypsinized to form a
121 single cell suspension. The cells were incubated in stimulation buffer (PBS with 5.5mM
122 glucose, 0.1% BSA and 0.5mM IBMX) containing; 1nM (n=5), 10nM (n=6) and 100nM
123 exendin-4 (n=5), with and without 1µM exendin 9-39 (n=6, n=5 and n=5 respectively), and
124 forskolin (n=6) as a positive control. cAMP was then detected according to the manufacturer's
125 instructions.

126

127 **Na⁺ K⁺ ATPase activity assay.** The effect of exendin-4 on Na⁺ K⁺ ATPase activity in the
128 choroid plexus was evaluated by the colorimetric measurement of phosphate released from
129 ATP hydrolysis with the use of a phosphate assay kit (ab65622, Abcam); with Na⁺ K⁺ ATPase
130 activity being defined as the portion of phosphate produced that is sensitive to ouabain. CPe
131 cells were incubated with aCSF for 1 hour at 37⁰C before incubation in aCSF containing;
132 100nM exendin-4 (n=7), 5μM PKI-16-22-amide (n=8), 100nM exendin-4 + 5μM PKI-16-22-
133 amide (n=8); in the presence and absence of 1mM ouabain for 30 minutes at 37⁰C. The cells
134 were then lysed on ice and spun at 13,000g to remove cell debris. Phosphate was measured as
135 per the manufacturer's instructions. Briefly the reaction mix was added to the samples and
136 incubated at room temperature for 60 minutes before the plate was read at 690nm. Na⁺ K⁺
137 ATPase activity was calculated as the difference between the amount of phosphate produced in
138 the presence and absence of ouabain for each treatment.

139

140 **In vivo experiments**

141 **Epidural ICP probe implantation.** Implantation of an epidural ICP probe and its validation
142 were recently published as a methodological work that contains all technical and surgical detail
143 (54). The rats were anaesthetized (2.7ml/kg subcutaneous injection containing 1.25mg/ml
144 midazolam, 2.5mg/ml fluanisone and 0.079mg/ml fentanylcitrate), placed in a stereotactic
145 frame (David Kopf Instruments) and a 2cm-midline incision was performed on top of the skull
146 and the bone was exposed by retracting the skin and soft tissue. A dental drill was used to make
147 4 burr holes in the skull; one large hole was carefully drilled to expose the dura mater enabling
148 placement of the epidural ICP probe (C313G-3UP, PlasticsOne), with the cannula cut to be
149 level with the base of the pedestal. The other 3 smaller holes were used to fit anchoring screws
150 to the skull. The epidural pressure bolt and the anchoring screws were placed and aligned with

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

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

161

162 **Intracerebroventricular (ICV) injection**

163 During the epidural ICP probe surgery, animals receiving ICV treatments also had an ICV
164 cannula implanted at the same time. An additional burr hole was made 0.8mm posterior and
165 1.6mm lateral to Bregma. The cannula was inserted into the left lateral ventricle, fixed with
166 dental resin-cement and closed by a cap with a dummy cannula to maintain patency. For the
167 ICV injection rats were anesthetized and connected to the transducer to measure ICP. Once a
168 stable baseline had been established a 5µl Hamilton syringe connected via tubing to an internal
169 cannula was used for the ICV injection.

170

171 **Osmotic pump implantation**

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

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

179 For implantation of the osmotic pump, the head of anaesthetized rat was fixed in a
180 stereotactic frame, the dorsal skull was exposed and a burr hole sited in the parietal bone
181 0.8mm posterior and 1.6mm lateral to Bregma. The ICV cannula was inserted into the left
182 lateral ventricle and fixed in place with glue to 2 stabilising screws (PlasticsOne), and the
183 osmotic pump was implanted subcutaneously in the neck region. The epidural ICP probe was
184 then implanted as above.

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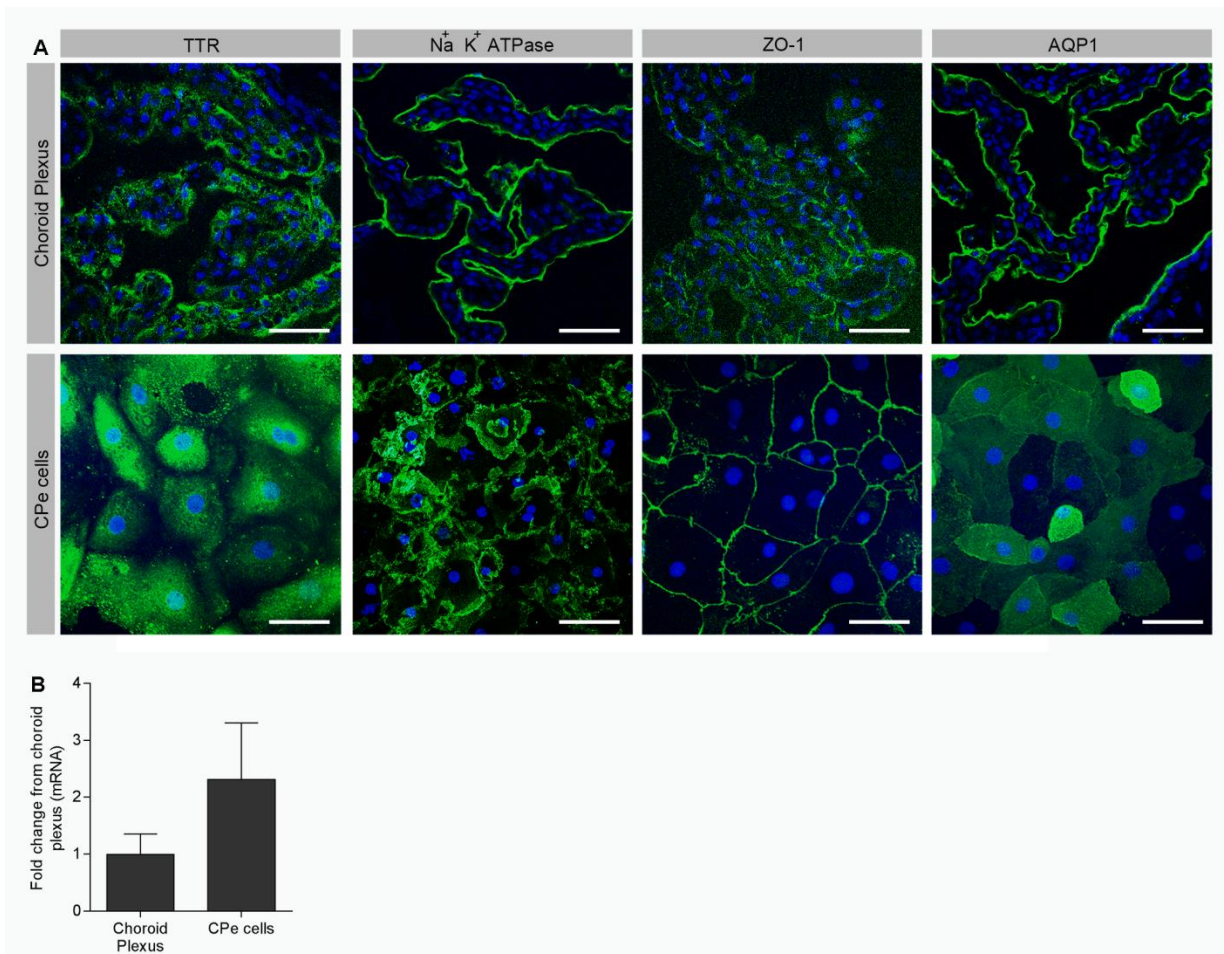
186 **Induction of hydrocephalus**

187 We used the kaolin model of hydrocephalus as our model of raised ICP. The rats were
188 anaesthetized and the head fixed in a stereotactic frame with the neck flexed down in a 90
189 degree angle to horizontal and secured in this position. A mark was made on the skin above the
190 dorsal atlanto-occipital membrane between the skull and the first cervical spinosus. The
191 percutaneous injection was performed using an insulin syringe with a 30 gauge needle, which
192 was slowly advanced in a vertical direction until there was a loss of resistance and 80 µL of
193 sterile kaolin suspension (0.250 mg/mL in Ringer's lactate solution - 1.4mM Ca²⁺, 4mM K⁺,
194 130mM Na⁺, 109mM Cl⁻, 28mM lactate) injected gradually (8.5 µL/s). Following the injection
195 the neck was extended, the head released from the stereotactic frame and the animal allowed to
196 recover.

197

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

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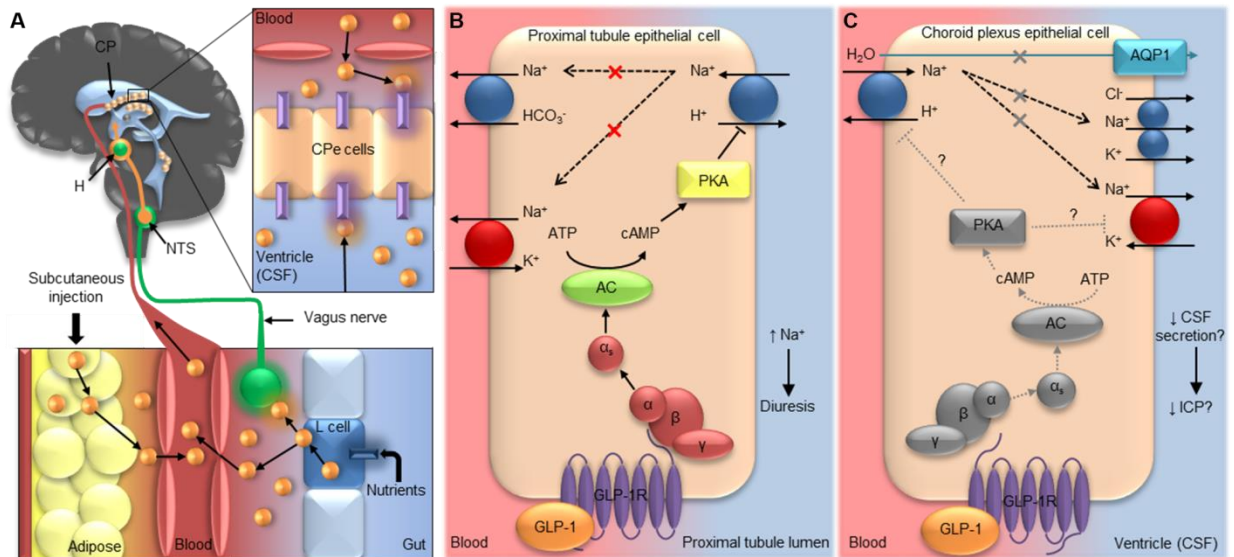
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Fig. S1. Characterisation of primary rat choroid plexus epithelial (CPE) cells in vivo and in vitro. (A) The identity of CPe cells in culture was determined by immunohistochemistry using antibodies against: (1) transthyretin (TTR), a CPe cell marker; (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.



214

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