

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

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

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25 **Overline:**

26

27 **One sentence summary:**

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

29

30 **Abstract**

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

45

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47

48

49 **Introduction**

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

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

65 The incretin glucagon-like peptide-1 (GLP-1), is a gut peptide secreted by the distal
66 small intestine in response to food intake (9). GLP-1 stimulates glucose-dependent insulin
67 secretion and inhibits glucagon release, lowering blood glucose (10). In addition, GLP-1 is
68 synthesized in neurons of the nucleus tractus solitarius, which project to the hypothalamus (11)
69 and promote satiety and weight loss (12-14). GLP-1 signals through the GLP-1 receptor (GLP-
70 1R), a class-B G protein-coupled receptor expressed in selected cell types within the central
71 nervous system including the hypothalamus, hippocampus, olfactory cortex, circumventricular
72 organs, hindbrain and choroid plexus (15-17).

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

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

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

89

90 **Results**

91

92 *GLP-1R expression in human choroid plexus tissue*

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

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

106

107 *Exendin-4 treatment of whole rat choroid plexus in vitro*

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

117 Next, we determined *Glp-1r* mRNA expression in whole rat choroid plexus tissue after
118 incubation with 100nM exendin-4. Incubation of the rat choroid plexus with exendin-4 for 3
119 hours showed an increase in *Glp-1r* mRNA compared to artificial CSF (3.21 ± 0.70 fold,
120 $P < 0.01$), with a return to baseline at 6 hours (0.78 ± 0.12 fold) (**Fig 2B**). There was also a
121 small but detectable increase in *Na⁺ K⁺ atpase* mRNA expression after 3 hours of exendin-4
122 treatment compared to incubation with artificial aCSF (1.82 ± 0.28 fold; $P < 0.05$), which again
123 returned to baseline at 6 hours (0.97 ± 0.21 fold) (**Fig. 2C**). The expression of other channels

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

126

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

128 To explore further the effects of exendin-4 on the choroid plexus, monolayers of rat CPe cells
129 were grown in culture. These CPe cells were characterized using antibodies against specific
130 identity markers and were shown to be similar to their in vivo counterparts (**Fig. S1A**),
131 including the expression of *Glp-1r* mRNA (**Fig. S1B**). To determine the effect of exendin-4 on
132 GLP-1R signaling, cAMP generation was assessed using two enzyme immunoassay systems.
133 Treatment of CPe cells with exendin-4 increased cAMP compared to control (2.14 ± 0.61 fold,
134 $P < 0.01$) (**Fig. 3A**) in a concentration-dependent manner, and this could be inhibited by exendin
135 9-39 (**Fig. 3B**). Forskolin, an adenylate cyclase activator, was used as a positive control to
136 maximally stimulate cAMP production (**Fig 3A-B**) (5.30 ± 0.74 fold compared to control).

137 The role of GLP-1R signaling in CSF secretion was assessed in rat CPe cell cultures by
138 measuring Na⁺ K⁺ ATPase activity (proposed as a marker of CSF secretion from the choroid
139 plexus) (6). Exendin-4 treatment reduced Na⁺ K⁺ ATPase specific phosphate production
140 compared to control ($39.3 \pm 9.4\%$, $P < 0.05$) (**Fig. 3C**). In addition, inhibition of PKA with PKI-
141 16-22-amide (PKI) abolished the exendin-4-induced reduction in Na⁺ K⁺ ATPase activity (95.4
142 $\pm 17.6\%$, $P < 0.05$) (**Fig. 3C**).

143

144 *Exendin-4 treatment reduces ICP in conscious rats*

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

149 in **Fig. 4B**. On the first day of treatment (day 2), exendin-4 significantly reduced ICP 10
150 minutes after the SC injection; by 30 minutes ICP was $65.2 \pm 6.6\%$ of baseline compared to
151 $91.0 \pm 3.9\%$ of baseline in saline-treated rats ($P < 0.01$) (**Fig. 4C**). A similar drop in ICP was
152 observed on day 4 ($50.4 \pm 6.9\%$ of baseline; $P < 0.001$) and day 6 ($54.5 \pm 8.2\%$ of baseline;
153 $P < 0.001$), 30 minutes after exendin-4 administration (**Fig. 4D-E**).

154 In addition to reducing ICP immediately after treatment, exendin-4 had a cumulative
155 effect on reducing ICP. Exendin-4 caused a significant reduction in ICP measured pre-dose on
156 day 2 (baseline, 100%) to day 4 ($79.3 \pm 7.3\%$; $P < 0.05$) and day 6 ($72.5 \pm 5.6\%$; $P < 0.01$) (**Fig.**
157 **4F**), which was not observed in saline-treated rats (day 2, baseline 100%; day 4, $95.5 \pm 13.6\%$;
158 day 6, $105.3 \pm 12.5\%$; **Fig. 4G**).

159 As there is evidence that weight loss can alter ICP (23), weights were monitored over
160 the treatment period. Whilst both saline- and exendin-4-treated rats lost weight during
161 treatment ($P < 0.05$), there was no significant difference between the groups at any time point
162 (**Fig. 4H**). In the saline group, weight change correlated with alterations in ICP ($r = 0.710$,
163 $P = 0.032$), although no relationship was detected for the exendin-4 treatment group ($r = -0.300$,
164 $P = 0.552$) (**Fig. 4I**).

165 The effect of SC administration of $20 \mu\text{g/kg}$ exendin-4 on blood and CSF pH and CSF
166 electrolytes was analyzed 60 minutes post-treatment. Exendin-4 maintained normal blood pH
167 (7.35 ± 0.01 ; **Fig. 4J**), however, it caused a reduction in CSF pH (7.41 ± 0.03 ; $P < 0.05$)
168 compared to saline (blood pH 7.35 ± 0.03 , CSF pH 7.61 ± 0.07) (**Fig. 4K**). CSF Na^+
169 concentration remained unaltered (saline, 150.3 ± 0.9 ; exendin-4, 150.3 ± 0.6) (**Fig. 4L**),
170 whereas the concentration of Cl^- ions in the CSF was reduced in the exendin-4 group (117 ± 0.5
171 mmol/L ; $P < 0.05$) compared to the saline group ($123.8 \pm 0.9 \text{ mmol/L}$) (**Fig. 4M**). Exendin-4
172 treatment also increased the concentration of Ca^{2+} ions in the CSF (1.09 ± 0.01 , $P > 0.05$)
173 compared to saline (1.03 ± 0.02) (**Fig. 4N**).

174

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

176 To assess whether the reduction in ICP was specific to the brain, exendin-4 was injected into
177 the lateral ventricle through an intracerebroventricular (ICV) cannula in anesthetized rats. ICV
178 delivery of exendin-4 reduced ICP over time, which was significantly different from baseline at
179 15 minutes ($68.9 \pm 6.4\%$, $P < 0.05$). ICV delivery of saline also reduced ICP over time
180 (technical effect due to the ICV cannula itself reducing ICP), and this was significantly
181 different from baseline at 50 minutes ($74.5 \pm 7.9\%$, $P < 0.05$). Over the 60 minutes of ICP
182 measurement, ICV delivery of exendin-4 significantly reduced the area-under-the-curve (AUC)
183 of ICP compared to saline delivered via the same route (3852 ± 397 versus 4974 ± 262 AUC,
184 $P < 0.05$) (**Fig. 4O**). To determine if the effects of exendin-4 on ICP were mediated by the GLP-
185 1R, the antagonist exendin 9-39 was continuously infused ($4 \mu\text{g}/\text{hour}$) into the lateral ventricle
186 for 2 days prior to SC administration of either saline or $20 \mu\text{g}/\text{kg}$ exendin-4. SC injection of
187 exendin-4 (ICV saline + SC exendin-4) lowered ICP ($P < 0.0001$) compared to a SC injection of
188 saline with ICV delivery of exendin 9-39 (ICV exendin 9-39 + SC saline; **Fig. 4P**). **Central**
189 **ICV exendin 9-39 infusion decreased the ICP-lowering effect of SC exendin-4 at 5 minutes**
190 **(ICV exendin 9-39 + SC exendin-4 $96.7 \pm 13.7\%$ vs ICV saline + SC exendin-4 $65.7 \pm 8.3\%$,**
191 **$P < 0.001$) (**Fig. 4P**). These data suggest that exendin-4 in part exerts its effects on ICP via the
192 GLP-1R signaling pathway in the brain.**

193

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

195 Rats were treated subcutaneously with 1, 3 and $5 \mu\text{g}/\text{kg}$ exendin-4 to determine whether
196 exendin-4 reduces ICP at lower concentrations. At 60 minutes 1, 3 and $5 \mu\text{g}/\text{kg}$ exendin-4
197 significantly reduced ICP to $79.0 \pm 7.0\%$ of baseline ($P < 0.05$), $69.9 \pm 8.8\%$ of baseline
198 ($P < 0.0001$) and $60.6 \pm 3.6\%$ of baseline ($P < 0.0001$), respectively, compared to saline ($97.2 \pm$

199 2.5% of baseline) (**Fig. 5A-B**). Five $\mu\text{g}/\text{kg}$ exendin-4 showed the greatest reduction in ICP and
200 the effect was still present 3 hours after the treatment ($P<0.001$). Conversely, in 1 and $3\mu\text{g}/\text{kg}$
201 exendin-4 groups ICP had returned to baseline by 3 hours (**Fig. 5C**).

202 Alterations in mRNA and protein expression of GLP-1R and molecules involved in CSF
203 secretion were assessed in the choroid plexus of rats 3 hours after treatment with 1, 3 and 5
204 $\mu\text{g}/\text{kg}$ exendin-4. *Glp-1R* and $\text{Na}^+ \text{K}^+ \text{atpase}$ mRNA expression was not altered by exendin-4
205 treatment (**Fig. 5D-E**). Conversely, there was a 2-fold increase in the amount of *Aqp1* mRNA
206 in the 5 $\mu\text{g}/\text{kg}$ exendin-4 treatment group ($P<0.05$) (**Fig. 5F**), and a 4-fold increase in the
207 amount of *Nhe1* mRNA expression in the 1 $\mu\text{g}/\text{kg}$ exendin-4 treatment group ($P<0.05$) (**Fig.**
208 **5G**). Although no significant changes were observed in $\text{Na}^+ \text{K}^+ \text{atpase}$ mRNA expression,
209 there was a small increase in $\text{Na}^+ \text{K}^+$ ATPase protein in the 5 $\mu\text{g}/\text{kg}$ exendin-4 treatment group
210 (2.16 ± 0.22 AU, $P<0.05$) (**Fig 5H-I**). Two bands were observed for the water channel
211 aquaporin 1 (AQP1), representing the glycosylated (top band) and non-glycosylated (bottom
212 band) forms of AQP1 (**Fig. 5H**). The total amount of AQP1 protein was slightly reduced by 1
213 and 3 $\mu\text{g}/\text{kg}$ exendin-4 treatment but not with the higher 5 $\mu\text{g}/\text{kg}$ exendin-4 dose (1 $\mu\text{g}/\text{kg}$, 1.96
214 ± 0.17 AU, $P<0.05$, 3 $\mu\text{g}/\text{kg}$, 1.75 ± 0.08 AU, $P<0.01$, 5 $\mu\text{g}/\text{kg}$, 2.75 ± 0.30 AU) (**Fig. 5J**). The
215 ratio of glycosylated AQP1 to non-glycosylated AQP1 was increased after 1 and 3 $\mu\text{g}/\text{kg}$
216 exendin-4 treatment but not after 5 $\mu\text{g}/\text{kg}$ exendin-4 treatment (1 $\mu\text{g}/\text{kg}$, 0.97 ± 0.06 AU,
217 $P<0.05$, 3 $\mu\text{g}/\text{kg}$, 1.08 ± 0.06 AU, $P<0.01$, 5 $\mu\text{g}/\text{kg}$, 0.81 ± 0.08 AU) (**Fig. 5K**). Glycosylation is
218 important for intracellular trafficking and protein stability, making proteins more resistant to
219 proteolysis (24), therefore these data suggest that exendin-4 may lower AQP1 through
220 enhanced degradation of the non-glycosylated AQP1.

221 The effect of 5 $\mu\text{g}/\text{kg}$ exendin-4 was monitored for 24 hours in healthy rats to determine its
222 duration of action. A single SC injection of 5 $\mu\text{g}/\text{kg}$ exendin-4 maintained lower ICP compared
223 to saline over 24 hours and returned to the pre-dose ICP baseline at 24 hours (1 hour, $60.2 \pm$

224 3.5%, $P < 0.0001$, 3 hours, $71.3 \pm 3.7\%$, $P < 0.001$, 6 hours, $70.3 \pm 4.0\%$, $P < 0.0001$, 12 hours,
225 $88.9 \pm 16.6\%$, $P < 0.01$, 24 hours, $100.3 \pm 14.3\%$, $P < 0.01$) (**Fig. 6A**). Effects on weight and food
226 and water intake were also noted in relation to changes in ICP over 24 hours. Although
227 exendin-4 caused a greater reduction in weight at 3 and 6 hours (**Fig. 6B**), there were no
228 differences between food or water intake at any time point between exendin-4-treated and
229 saline-treated rats (**Fig. 6C-D**). *Glp-1R*, $Na^+ K^+ atpase$ and *Nhe1* mRNA expression did not
230 change over the 24 hour period (**Fig. 6E-F,H**). As shown previously, 5 $\mu\text{g}/\text{kg}$ exendin-4
231 increased *Aqp1* mRNA expression at 3 hours compared to saline, although this was not
232 observed at any other time point (**Fig. 6G**). There were also no significant changes in the
233 amount of $Na^+ K^+$ ATPase or AQP1 protein over the 24 hour time period (**Fig. 6I-L**).

234

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

236 To determine the efficacy of exendin-4 to reduce ICP under conditions of raised ICP, a well-
237 characterized kaolin model of hydrocephalus in rats was used. Kaolin, an aluminium silicate,
238 acts as an irritant, inducing an inflammatory response with concomitant deposition of collagen
239 and dense fibrosis in areas of the subarachnoid space close to the injection site, which leads to
240 raised ICP (25, 26). Kaolin was injected into the cisterna magna, leading to development of
241 hydrocephalus, before implantation of the ICP monitor. ICP was recorded before and after a
242 SC injection of either saline or 20 $\mu\text{g}/\text{kg}$ exendin-4 (**Fig. 7A**). The injection of kaolin
243 significantly increased baseline ICP (11.1 ± 1.3 mmHg; $P < 0.0001$) compared to that of normal
244 rats (5.5 ± 0.4 mmHg) (**Fig. 7B**). Exendin-4 treatment significantly reduced ICP almost
245 immediately after the SC injection, and at 30 minutes was $62.6 \pm 5.1\%$ of baseline ($P < 0.0001$)
246 compared to $105.0 \pm 4.6\%$ of baseline in saline-treated rats (**Fig. 7C**). Eight rats in the kaolin
247 group had baseline ICP values of greater than 10 mmHg and had an average baseline ICP of
248 13.7 ± 0.7 mmHg. In these rats (ICP > 10 mmHg), the ICP values at 30 minutes were $56.6 \pm$

249 5.7% of baseline (n=4) in the exendin-4 treatment group compared to $106.7 \pm 8.6\%$ of baseline
250 (n=4) in the saline treatment group (**Fig. 7C**). In the rodents with elevated ICP, the ICP
251 waveform was very unstable, with the appearance of B-waves characteristic of pathologically
252 elevated ICP and a reduction in brain compliance (27). These were abolished in rats receiving
253 exendin-4 but not saline (**Fig. 7D**).

254

255 **Discussion**

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

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

274 may allow the GLP-1R to be stimulated from both sides of the choroid plexus (**Fig. S2A**).
275 Although GLP-1R mRNA and protein expression were in general low, it has recently been
276 shown that activation of the receptor requires femto- to picomolar concentrations of GLP-1R,
277 so even faced with low abundance, signaling would be expected in the presence of exendin-4
278 (29).

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

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

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

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

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

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

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

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

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376 **Materials and Methods**

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378 **Study design**

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

392

393 **Human tissue**

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

396 08/MRE09/31+5). Informed consent was obtained for the use of post mortem tissue for
397 research. Samples were stored in RNALater at -80°C before being processed for qPCR
398 following the protocol stated in the supplementary methods. Pooled human pancreas (540023),
399 heart (540011) and ovary (540071) RNA was purchased from Agilent Technologies. Fresh
400 choroid plexus samples were fixed in 4% formaldehyde before embedding in paraffin wax.

401

402 **Experimental animals**

403 For the in vitro work, 150-200g female Sprague-Dawley rats (Charles River) were used at the
404 University of Birmingham in accordance with the Animals and Scientific Procedures Act 1986,
405 licensed by the UK Home Office and approved by the University of Birmingham Ethics
406 Committee. For the in vivo studies, which were conducted in Rigshospitalet-Glostrup, 150-
407 250g female Sprague-Dawley rats (Taconic) were housed in groups of 4, kept under a 12 hour
408 light/dark cycle with free access to food and water. All experimental procedures were approved
409 by the Danish Animal Experiments Inspectorate (license number 2014-15-0201-00256 and
410 2012-15-2934-00283). After treatments and surgical procedures, the rats were monitored daily
411 for any adverse effects. Female rats were used to ensure the results were relevant to conditions
412 such as idiopathic intracranial hypertension.

413

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

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

422

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

431

432 **Continuous ICV infusion of exendin 9-39 with SC injection of exendin-4 in conscious rats.**

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

442

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

445 (n=6) or 5µg/kg exendin-4 (n=6) and ICP recorded for 3 hours. For the time course experiment
446 rats were given either saline (n=18 for ICP data but only 4 were used for choroid plexus
447 analysis) and ICP recorded for 24 hours, or 5µg/kg exendin-4 and the ICP recorded for 6 (n=6),
448 12 (n=6) and 24 hours (n=12 for ICP data but only 6 were used for choroid plexus analysis).
449 After each time point the rats were killed with an overdose of euthatol and transcardially
450 perfused with ice cold PBS. The choroid plexus was then dissected, frozen immediately and
451 stored at -80°C for qPCR and western blot analysis (described in detail in the supplementary
452 methods).

453
454 **SC injection of exendin-4 in conscious hydrocephalic rats.** We used the kaolin model of
455 hydrocephalus as our model of raised ICP. On day 0 the rats received an injection of kaolin to
456 induce hydrocephalus and the rat allowed to recover. On day 6-8 the rats were fitted with an
457 epidural ICP probe and the rat was then allowed to recover in the infusion cages still connected
458 to the transducer so that the ICP could be continuously measured overnight to establish raised
459 ICP. The following morning, after establishing the baseline ICP reading was stable, the rats
460 received a SC injection of either saline (n=6, n=4 >10mmHg) or 20µg/kg exendin-4 (n=6; n=4
461 >10mmHg). ICP was then recorded for a further 60 minutes.

462
463 **Statistical analysis**

464 Values are represented as mean and standard error of the mean (SEM). The majority of the data
465 was analyzed using GraphPad Prism software, however, the time course experiment with
466 5µg/kg exendin-4 was analyzed using SPSS due to missing data points. For the ELISA cAMP
467 analysis, the non-parametric Kruskal-Wallis test was used, and was followed by Mann-
468 Whitney test (two-tailed) with the appropriate adjustment for multiple comparisons
469 (Bonferroni). T-test or One-way ANOVA (followed by a post hoc Tukey test) was used for the

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

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477 **Supplementary Materials**

478 **Supplementary Materials and Methods**

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

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

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

482

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653 **Author contributions**

654 A.S. was responsible for the study concept. H.B., A.G., D.J.H. and A.S. conceived and
655 designed the experiments; H.B. conducted the following in vitro experiments:
656 immunohistochemistry, Na⁺ K⁺ ATPase activity assay, cAMP assay, rat qPCR and western
657 blot, and FLEX analysis); C.W. performed human qPCR and cAMP assays; A.G. M.U. and
658 J.M contributed to the immunohistochemistry data; H.B., M.U. J.M. and S.H. performed the
659 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-
660 wrote the manuscript and all authors reviewed the final version.

661

662 **Competing interests**

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

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672 **Figure legends**

673

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

683

684 **Fig. 2. Expression of GLP-1R after treatment with exendin-4 in rat choroid plexus in**
685 **vitro.** (A) Representative images of rat choroid plexus after treatment with artificial CSF
686 (aCSF) as control or fluorescently labelled exendin-4 (FLEX) in the presence or absence of the
687 GLP-1R antagonist exendin 9-39. DAPI (blue) was used as a nuclear marker; scale bar, 50µm
688 (insert, 25µm). (B-E) The histograms represent the fold change in mRNA expression of *Glp-*
689 *1r* (B), *Na⁺ K⁺ atpase* (C), *Aqp1* (D) and *Nhe1* (E) (aCSF n=6; 3hr n=7, 6hr n=7) *P<0.05,
690 **P<0.01; ANOVA with Tukey's multiple comparisons test.

691

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

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

703
704 **Fig. 4. Effect of exendin-4 on ICP in healthy conscious rats.** (A) Overview of the
705 experimental design in normal rats. Rats were fitted with an epidural ICP probe and allowed to
706 recover. Treatment was given daily for 5 days and ICP was recorded on days 2, 4 and 6, before
707 and after the rats received a subcutaneous (SC) injection of either saline (n=9) or 20µg/kg
708 exendin-4 (n=9). (B) Example ICP traces of saline (*blue*) and exendin-4 (*red*) treatment. Spikes
709 in the trace represent when the animal was moving (*) and accurate recording of ICP was
710 confirmed by the response to jugular vein compression. (C-E) Line graphs showing the
711 percentage of baseline ICP after SC injection of either saline or exendin-4 on day 2 (C), day 4
712 (D) and day 6 (E). (F-G) Histograms showing the pre-dose and 60 minutes post treatment ICP
713 values (% of baseline on day 2) on days 2, 4 and 6 for exendin-4 (F) and saline (G). (H) Line
714 graph of the % change in weight from day 2 (start of treatment) showing that both saline and
715 exendin-4 treated rats lost weight but there was no significant difference between the groups on
716 day 4 or 6. (I) Scatter plot of weight change (g) vs ICP change (mmHg) in the saline (*blue* n=4)
717 and exendin-4 (*red* n=5) groups. (J-N) Histograms showing blood pH (J) and CSF pH (K), and
718 the concentration of Na^+ (L) Cl^- (M) and Ca^{2+} (N) in the CSF, 60 minutes after a SC injection
719 of either saline or 20µg/kg exendin-4. (O) ICP was measured before and after a 1µl
720 intracerebroventricular (ICV) injection of either saline (n=8) or 0.3µg exendin-4 (n=6). (P)

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

726
727 **Fig. 5. Effects of different doses of exendin-4 on ICP, mRNA and protein expression in**
728 **healthy conscious rats. (A-B)** Dose-response of exendin-4's effects on ICP following SC
729 administration of 1 (n=6), 3 (n=6), 5 (n=23) and 20 µg/kg (n=9) exendin-4 compared to saline
730 (n=18) at 30 and 60 minutes. (C) Line graph showing the percentage of baseline ICP after
731 treatment with 1, 3 or 5µg/kg exendin-4 measured over 3 hours. (D-G) The histograms show
732 *Glp-1R* (D), *Na⁺ K⁺ atpase* (E), *Aqp1* (F) and *Nhe1* (G) mRNA expression in the rat choroid
733 plexus after saline treatment (n=4) or treatment with 1 (n=5), 3 (n=6), 5 µg/kg (n=6) exendin-4.
734 (H) Representative western blots and (I-K) semi-quantitative protein analysis for (I) *Na⁺ K⁺*
735 *ATPase* (112kDa) and (J) total AQP1, either non-glycosylated (NG, 29kDa) or glycosylated
736 (G, 35kDa); β-actin (42kDa) loading control. (K) Histogram shows the ratio of G to NG AQP1.
737 *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. (B-C) Two-way ANOVA with Sidak's
738 multiple comparison test; (D-G and I-K) ANOVA with Tukey's multiple comparison test.

739
740 **Fig. 6. Effects of Exendin-4 time course on ICP, mRNA and protein expression in healthy**
741 **conscious rats. (A)** Line graph showing the percentage of baseline ICP after a single SC
742 injection of saline (n=18) or 5µg/kg exendin-4 (n=24) measured over 24 hours. (B-D)
743 Histograms showing weight loss (B), water intake (C) and food intake (D) in rats treated with
744 saline (n=4) or 5µg/kg exendin-4 at 3 (n=6), 6 (n=6) and 24 hours (n=6). (E-H) Histograms
745 representing *Glp-1r* (E), *Na⁺ K⁺ atpase* (F), *Aqp1* (G) and *Nhe1* (H) mRNA expression in the

746 rat choroid plexus after treatment with saline (n=4) and 5µg/kg exendin-4 at 3 (n=6), 6 (n=5)
747 and 24 hours (n=5). **(I)** Representative western blots and **(J-L)** semi-quantitative protein
748 analysis for **(J)** Na⁺ K⁺ ATPase (112kDa) and **(K)** total AQP1 either nonglycosylated (NG,
749 29kDa) or glycosylated (G, 35kDa); β-actin (42kDa) loading control. **(L)** The histogram shows
750 the ratio of G to NG AQP1. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001; (A-D) Two-way
751 ANOVA with Sidak's multiple comparison test; (E-H and J-L) ANOVA with Tukey's multiple
752 comparison test.

753
754 **Fig. 7. Effect of exendin-4 on ICP in a rat model of raised ICP (hydrocephalic).** **(A)**
755 Overview of the experimental plan. Kaolin was injected into the cisterna magna to induce
756 hydrocephalus. On Day 6 the ICP monitor was implanted under anaesthesia and ICP was
757 recorded overnight to allow the ICP to normalize after implantation. On Day 7, the rats were
758 given a SC injection of either saline (n=6) or 20µg/kg Exendin-4 (n=6), and ICP was recorded
759 for a further 60 minutes. **(B)** Dot plot showing the individual baseline ICP values (mmHg) for
760 the normal rats and rats injected with kaolin. The kaolin group had significantly higher baseline
761 ICP values compared to the normal group, with 8/12 rats having an ICP value of >10mmHg.
762 **(C)** Line graph showing the percentage of baseline ICP after treatment with either saline (dark
763 blue, n=6) or exendin-4 (dark red, n=6). The groups could also be further divided into those
764 with ICP >10mmHg in the saline group (light blue, n=4) and exendin-4 group (light red, n=4).
765 **(D)** Example ICP trace in a hydrocephalic rat before and after treatment with exendin-4. Before
766 treatment the rat exhibited pathological ICP B-waves (*b*), which were abolished following
767 treatment with exendin-4. **** P<0.0001; (B) T-test (two tailed); (C) Two-way ANOVA with
768 Sidak's multiple comparison test.