Neuroprotective exendin-4 enhances hypothermia therapy in a model of hypoxic-ischaemic encephalopathy

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Hypoxic-ischaemic encephalopathy remains a global health burden. Despite medical advances and treatment with therapeutic hypothermia, over 50% of cooled infants are not protected and still develop lifelong neurodisabilities, including cerebral palsy. Furthermore, hypothermia is not used in preterm cases or low resource settings. Alternatives or adjunct therapies are urgently needed. Exendin-4 is a drug used to treat type 2 diabetes mellitus that has also demonstrated neuroprotective properties, and is currently being tested in clinical trials for Alzheimer’s and Parkinson’s diseases. Therefore, we hypothesized a neuroprotective effect for exendin-4 in neonatal neurodisorders, particularly in the treatment of neonatal hypoxic-ischaemic encephalopathy. Initially, we confirmed that the glucagon like peptide 1 receptor (GLP1R) was expressed in the human neonatal brain and in murine neurons at postnatal Day 7 (human equivalent late preterm) and postnatal Day 10 (term). Using a well characterized mouse model of neonatal hypoxic-ischaemic brain injury, we investigated the potential neuroprotective effect of exendin-4 in both postnatal Day 7 and 10 mice. An optimal exendin-4 treatment dosing regimen was identified, where four high doses (0.5 μg/g) starting at 0 h, then at 12 h, 24 h and 36 h after postnatal Day 7 hypoxic-ischaemic insult resulted in significant brain neuroprotection. Furthermore, neuroprotection was sustained even when treatment using exendin-4 was delayed by 2 h post hypoxic-ischaemic brain injury. This protective effect was observed in various histopathological markers: tissue infarction, cell death, astrogliosis, microglial and endothelial activation. Blood glucose levels were not altered by high dose exendin-4 administration when compared to controls. Exendin-4 administration did not result in adverse organ histopathology (haematoxylin and eosin) or inflammation (CD68). Despite initial reduced weight gain, animals restored weight gain following end of treatment. Overall high dose exendin-4 administration was well tolerated. To mimic the clinical scenario, postnatal Day 10 mice underwent exendin-4 and therapeutic hypothermia treatment, either alone or in combination, and brain tissue loss was assessed after 1 week. Exendin-4 treatment resulted in significant neuroprotection alone, and enhanced the cerebroprotective effect of therapeutic hypothermia. In summary, the safety and tolerance of high dose exendin-4 administrations, combined with its neuroprotective effect alone or in conjunction with clinically relevant hypothermia make the repurposing of exendin-4 for the treatment of neonatal hypoxic-ischaemic encephalopathy particularly promising.

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Introduction

Hypoxic-ischaemic encephalopathy (HIE) is a serious complication of labour caused by reduced blood flow and oxygen supply to the neonatal brain. This can result in mortality for the infant or significant and lasting brain damage. HIE is a global problem with an estimated incidence of 1.5 per 1000 live births. Fifteen to 20% of HIE neonates die during the postnatal period, and an additional 25% develop irreversible and lifelong mental and physical disabilities including cerebral palsy (Shankaran, 2012). In 2010, HIE was associated with 2.4% of the total Global Burden of Disease (Lee et al., 2013).

The known temporal sequence of events in the brain following hypoxia-ischaemia in rodent models has defined a therapeutic window of opportunity of up to ~8 h based on a time course of secondary energy failure and other measures of secondary brain injury (Blumberg et al., 1997; Gilland et al., 1998a, b). Early interventions have shown efficacy when initiated early and within this window (Nijboer et al., 2011). Reducing the body temperature (hypothermia) of human neonates within 6 h of hypoxia-ischaemia onset and with duration of 72 h is the only clinically approved treatment (Edwards et al., 2010). Hypothermia is thought to protect neurons by reducing cerebral metabolic rate (Rosomoff and Holaday, 1954) and concentrations of glutamate and nitric oxide (Thoresen et al., 1997), inhibiting cerebral energy failure, preserving high-energy phosphates (Thoresen et al., 1995) and preventing inflammatory cascades (Inamasu et al., 2000). However, while hypothermia therapy is very promising, up to 55% of treated neonates cannot be saved (Gluckman et al., 2005). Therefore, there is a need to develop therapies that are either more effective than hypothermia, or can be used in combination to enhance its therapeutic efficacy.

Exendin-4 (also known as exenatide) is a small peptide drug approved by the Food and Drug Administration (FDA) in 2005 and European Medicines Agency (EMA) in 2006 for the treatment of type 2 diabetes mellitus. It is an analogue of the human glucagon-like peptide-1 (GLP-1) gut hormone peptide that plays a role in regulating blood sugar levels by enhancing insulin production from the pancreas. While GLP-1 has a half-life of ~1.5 min (Deacon et al., 1996), exendin-4 can reach 60–90 min (Nielsen et al., 2004), making it of therapeutic value, and is administered twice daily after meals. The positive neurological effects of exendin-4 were first recognized by improvements in neuropathic aspects in type 2 diabetes mellitus patients under treatment (Grant et al., 2011). Exendin-4 also efficiently crosses the blood–brain barrier (Kastin and Akerstrom, 2003; Zanotto et al., 2017) and its cellular receptor (GLP1R) is found throughout the brain (Wei and Mojsov, 1995). Although there is no definitive unifying mechanism, in vitro and in vivo studies have suggested that exendin-4 has neuroprotective, neurotrophic (Perry et al., 2002), neurogenic (Bertilsson et al., 2008), anti-inflammatory (Teramoto et al., 2011), anti-apoptotic (Tews et al., 2009) and mitoprotective (Fan et al., 2010; Kang et al., 2015) properties. These findings, together with the excellent safety profile of exendin-4 in patients with type 2 diabetes mellitus, has led to recent and ongoing clinical trials examining the neuroprotective properties of exendin-4 in patients with Parkinson’s (NCT01174810; NCT01971242; Aviles-Olmos et al., 2013a; Athauda et al., 2017) and Alzheimer’s disease (NCT01255163). Recently, the Parkinson’s disease trial has reported that patients on exendin-4 show a statistically significant improvement in clinical motor and cognitive measures compared to control group. This effect persisted 12 weeks after ending the exendin-4 treatment (Athauda et al., 2017).

Given the need to develop effective treatments for neonatal HIE, the encouraging animal and clinical studies supporting the neuroprotective properties of exendin-4 make it an attractive therapeutic option. Little is known regarding the use of exendin-4 in perinatal animals, and there are no neonatal clinical studies. Therefore, we conducted a preclinical assessment of exendin-4 using an established mouse model of neonatal hypoxia-ischaemia causing widespread cerebral damage (Carlsson et al., 2012; Rocha-Ferreira et al., 2015). In this study, we confirmed the presence of GLP1R in the perinatal human and murine brains. We demonstrated significant dose-dependent neuroprotective and anti-inflammatory effect of exendin-4 treatment that is dose-dependent and the effects of which can be maintained even when administration is delayed post-hypoxia-ischaemia. Furthermore, we conducted a toxicity study to examine the safety of high dose repeat
administration of exendin-4 in perinatal mice and demonstrate safety and tolerance to the drug. Finally, we established its ability to be used synergistically with therapeutic hypothermia that enhances neuroprotection and ameliorates brain damage.

Materials and methods

Study approval

All UK mice experiments were approved by the Ethics Committee of the University College London and were carried out by licensed personnel (PPL, PCC436823) in concordance with the UK Home Office Guidelines [Animals (Scientific procedures) Act, 1986]. All mice experiments undertaken in Sweden conform to the Swedish Board of Agriculture and were approved by the Gothenburg Animal Ethics Committee (61-2014 and 01-2016). CD1 mice (Charles River) were bred in house with a 12-h light/dark cycle and had free access to food and water. Breeding cages contained igloo housing with free access to an exercise plate. Once weekly, enrichment food in the form of dry nuts and fruits was sprinkled onto the cages to allow foraging. All animal experiments included male and female littermates randomly allocated to the different experimental groups to reduce bias and followed the ARRIVE guidelines (Kilkenny et al., 2011). For the human post-mortem preterm study, brain tissue samples were collected from Perinatal Pathology autopsy services at Hammersmith Hospital and St Thomas’s Hospital, London, UK. Parental consent was received in accordance to the guidelines of the National Health Services (NHS), UK. Study ethics were obtained from the Hammersmith and Queen Charlotte’s and Chelsea Research Ethics Committee, NHS Research Ethics Services, UK [Post-mortem magnetic resonance imaging study on developing brain (supported by the Medical Research Council, UK); ethics number 07/H0707/139].

Blood analysis

Postnatal Day 7 (P7) mice underwent four high doses of exendin-4 (0.5 μg/g per dose, Enzo) via intraperitoneal administration (5 μg/g, 12 h apart). Naïve or saline-injected mice acted as controls (n = 6 per group). Twelve hours after the last injection, blood samples taken via cardiac puncture and collected in EDTA-coated tube. The analysis was performed by MRC Harwell Clinical Pathology laboratory (Mary Lyon Centre, UK). Different parameters were obtained: total white blood cells, neutrophils, lymphocytes, monocytes, eosinophils and basophils counts, haematocrit, platelets, red blood cells, haemoglobin and mean corpuscular volume. Blood glucose levels (mmol/l) were measured using a blood glucose monitor (CodeFree, SD Biosensor) in naïve controls and mice following a single exendin-4 high dose administration (0.5 μg/g). Blood samples were collected via cardiac puncture at 0.5 h, 1 h, 2 h, and 4 h post-exendin-4 injection.

Cyclic AMP assay

Brains of P7 naïve mice were collected at 2 h, 4 h, 8 h and 12 h following a single intraperitoneal injection of exendin-4 (0.5 μg/g). Saline-treated controls were collected 2 h post-injection (n = 4 per group). All samples were processed using cAMP direct immunoassay kit as per manufacturer’s instructions (Abcam).

Hypoxia-ischaemia surgery

Human brain maturation equivalent late preterm (P7) and term (P10) (Semple et al., 2013) CD1 littermate mice were anaesthetized with isoflurane (5% induction, 1.5% maintenance). The left common carotid artery was permanently ligated. Following 1 h recovery, pups were placed in a hypoxic chamber (8% O2, 36°C) for 30 min (P7) or 20 min (P10).

Hypothermia treatment

Within 10 min post-hypoxia-ischaemia, P10 mice underwent a single high dose of either exendin-4 (0.5 μg/g) or saline and were placed in individual compartments within a hypothermia (33°C) or normothermia (36°C) chamber for 5 h. One probe in each chamber monitored environmental temperature and one animal in each chamber was randomly selected to be used as a temperature-monitoring sentinel to measure core temperature using a rectal probe (T21, 0.41 mm diameter; Physitemp Instruments). The use of a rectal probe is in agreement with previous hypothermia studies in mice and rats demonstrating a good correlation between core body temperature measured this way and brain temperature in small rodent pups (Thoresen et al., 1996b). The alternative use of telemetry is promising but more expensive and technically challenging. Room temperature was also recorded. Computer Software Daisy lab 10.0 (Physitemp Instruments) was used to monitor the temperature, and values from each probe were recorded every 5 min (Supplementary Fig. 1). It was decided prior to the experiment that sentinel mice (n = 8 normothermia, n = 8 hypothermia) were excluded from the final analysis, as restraint-associated stress from the rectal probe has shown a neuroprotective effect (Thoresen et al., 1996a).

Exendin-4 treatment

Different dose regimens of exendin-4 were used to establish optimum treatment in the P7 hypoxia-ischaemia injury model, with initiation within the therapeutic window. Animals were randomized to: (i) saline (n = 14); (ii) one high dose exendin-4 (0.5 μg/g) administered immediately after hypoxia-ischaemia (n = 14); (iii) four high doses of exendin-4 administered every 12 h, starting immediately after hypoxia-ischaemia (n = 14); (iv) four low doses of exendin-4 (0.05 μg/g) administered every 12 h, starting immediately after hypoxia-ischaemia (n = 14); and (vi) four doses of exendin-4 administered every 12 h, starting with a 2 h delay after hypoxia-ischaemia (n = 14) (Fig. 1A). In the P10 combined exendin-4 (0.5 μg/g) and hypothermia treatment, animal groups consisted of control normothermia + saline (NT SAL, n = 24), single treatments normothermia + exendin-4 (NT EX4, n = 25) and hypothermia + saline (HT SAL, n = 25), and combined treatment hypothermia + exendin-4 (HT EX4, n = 25) (Fig. 1B).

Tissue preparation

Highest level of widespread neuronal caspase-3 expression within the acute injury phase occurs 48 h post-hypoxia-
ischaemia (Johnston et al., 2011). Therefore, this time point was chosen for early evaluation of neuropathological markers in the P7 study. P7 mice were perfused with 4% paraformaldehyde (PFA) 48 h post-hypoxia-ischaemia, brains transferred to 30% sucrose and snap frozen. P10 mice were perfused in the same manner 7 days after hypoxia-ischaemia, a time point when the secondary phase of brain injury is completed (Gilland et al., 1998a). Coronal brain sections (40-μm thickness) starting from the point of fusion of the corpus callosum were collected onto TBSAF wells. Every 10th section, in a total of five sections (400 μm apart) per brain underwent histochemistry, immunohistochemistry or immunofluorescence. Whole postmortem preterm neonatal brains were fixed in 4% formalin for 7 weeks before anatomical positions from the frontal, occipital, and parietal lobes were selected and processed using a Leica tissue processor. The blocks were sectioned at 6-μm thickness using a microtome, mounted onto SuperFrost™ Plus slides and allowed to dry for 4 days.

**Histochemistry**

P7 naïve, saline- and high dose exendin-4-treated mice (no hypoxia-ischaemia, n = 6 per group) were culled 12 h after the last exendin-4 injection (four doses 12 h apart). Different organs: brain, heart, spleen, liver, lung, pancreas and kidney were stained with haematoxylin and eosin for histopathological assessment.

**Immunohistochemistry**

Brain sections were treated as previously described (Rocha-Ferreira et al., 2015). In brief, slides were fixed in 4% PFA, blocked with 5% goat serum (Sigma-Aldrich) for 30 min and incubated overnight with MAP2 (1:1000, Sigma), alpham (1:5000, Serotec), CD68 (1:2000, Biorad), GFAP (1:6000, Dako) or ICAM1 (1:3000, Pharmingen) antibodies. Sections were then incubated with appropriate biotinylated secondary antibody (anti-rat or anti-rabbit; 1:100) for 1 h at room temperature, followed by 1 h incubation with ABC (1:100; Vector) solution and visualized with 3,3’diaminobenzidine (DAB)/hydrogen peroxide (Sigma). The reaction was stopped and washed twice in bidistilled water. Slides were dehydrated by consecutive immersion in increasing concentrations of ethanol, isopropanol and xylene, then covered using DEPEX. Postmortem neonatal sections were stained as previously shown (Carlsson et al., 2011). Slides underwent routine deparaffinization, followed by 15 min 1% hydrogen peroxide/PBS-Tween incubation before 15 min block with 5% goat serum. Overnight incubation with GLP1R (1:100, Novus Bio) was followed by 2 h room temperature incubation with biotinylated goat anti-rabbit secondary antibody (1:1000). Sections were incubated for 1 h with ABC (1:20) and visualized with DAB/hydrogen peroxide. The reaction was stopped, and sections covered as described above.

**TUNEL staining**

Murine brain sections were incubated in 3% hydrogen peroxide/methanol (15 min) followed by 2 h incubation at 37°C with terminal deoxynucleotransferase and deoxyuridine triphosphate solution (Roche). The reaction was stopped by incubation in terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL) stop solution for 10 min. Slides were incubated in ABC solution (1 h, room temperature) and visualized using DAB enhanced with cobalt nickel in the presence of hydrogen.
peroxide. The reaction was stopped, and sections covered as
described above.

**Immunofluorescence and scanning confocal microscopy**

Naïve P7, P10 and adult 10 week CD1 mice brain sections
\((n = 3 \text{ per group})\) were stained by simultaneous overnight
incubation with rabbit anti-GLP1R (1:50, Novus Bio) and mouse
anti-NeuN (1:1000, Millipore), mouse anti-GFAP (1:2000,
Millipore) or rat anti-CD68 (1:2000, Bio-Rad) antibodies.
Representative images were captured using a Zeiss LSM710
confocal microscope and Zen software (Carl Zeiss AG). Post-
mortem neonatal sections were simultaneously incubated with
confocal microscope and Zen software (Carl Zeiss AG). Post-
mortem neonatal sections were simultaneously incubated with
GLP1R (1:100) and NeuN (1:500) antibodies. Sections were
acted with fluorochrome-conjugated secondary antibody
(1:1000, Alexa Fluor® 488 and 546) and mounted with a
Fluoromount-G

\[\text{TM}\] mounting medium (ThermoFisher Scientific).
Representative images were captured using a Leica SP5 confocal
microscope equipped with LAS-AF software. All images were
processed using Imaris v.9.1 (Bitplane AG). Because of known
difficulties associated with the GLP1R antibodies (Drucker,
2013), negative GLP1R antibody stained sections were also
used for all developmental time points and in both murine and
human tissue (Fig. 2 and Supplementary Fig. 2).

**Quantitative polymerase chain reaction**

The distribution of GLP1R gene expression was assessed in
different brain regions: olfactory bulb, cortex, hypothalamus,
thalamus, hippocampus, cerebellum, pons and medulla. Naïve
brains were extracted at P7, P10 and 10 weeks, and RNA was
isolated using RNeasy® Mini kit (Qiagen) and first-strand
cDNA was generated using High-Capacity cDNA Reverse
Transcription kit (Applied Biosystems). Quantitative RT-PCR
was carried out using a StepOnePlus (Applied Biosystems)
with the SsoAdvanced™ Universal SYBR® Green PCR Core
Reagents Supermix (Bio-Rad). Primers were designed to detect
mouse Glp1r: forward 5'-AGACCGTTGACAAATTGAGA-3';
reverse 5'-TGGCGCTTCCGTGAGG-3' and Gapdh housekeeping

\[\text{gene: forward 5'-GTTGTCCTCTGCGACTTCA-3'; reverse 5'-}
\text{GGTGGTCCAGGTTTCTTA-3'. Data from StepOne™}
software v2.3 were calibrated to Gapdh and the relative quan-
tification of gene expression was performed using the comparativa
CT method.

**Data analysis**

Infarct area was measured using ImageJ software (NIH). The
intact Nissl+ areas of the isocortex, pyriform cortex, hippocam-
pus, striatum, thalamus and external capsule brain regions were
delineated bilaterally and the ipsilateral regions were subtracted
from the contralateral regions and expressed as percentage
tissue loss. This method assumes that the contralateral hemi-
sphere represents maximal intact area (100%); however, there
are instances where because of potential symmetrical differences
between hemispheres, the intact ipsilateral hemisphere is larger
in area size and the resulting measurement shows a negative
value. TUNEL+ cells were quantified under ×20 magnification
in three separate fields per assessed brain region. Microglial
activation was assessed using semi-quantitative score as previ-
ously described (Rocha-Ferreira et al., 2015), with a scale of:
0 (no activation, ramified microglia) to 4 (widespread amoeboid
microglia), GFAP, ICAM1 and CD68 immunoreactivity was
measured by quantitative thresholding image analysis using
Image Pro Premier software (Media Cybernetics) as previously
described (Rocha-Ferreira et al., 2016). In brief, three non-
overlapping RGB images from the assessed brain regions were
captured using a live video camera (Nikon, DS-Fi1) mounted
onto a Nikon eclipse E600 microscope at ×40 magnification for
both GFAP and ICAM1 stainings. Similarly, 30 non-
overlapping RBG images from brain, heart, spleen, liver, lung,
pancreas and kidney were captured for CD68 measurements.
The threshold setting was kept constant for all acquired images.
MAP-2 staining was used for quantification of volumetric

tissue loss from five brain levels, starting from level 1 (L1,
fusion of corpus callosum) and continuing until level 5 (L5, late
hippocampus), as well as total volumetric tissue loss (Stridh
et al., 2013). Macroscopic score of tissue loss was performed
using a scale comprising of 0, no visible injury; 1, 25%; 2,
50%; and 3, 75% hemispheric injury/loss.

**Statistical analysis**

Experimental cohorts consisted of 14–25 mice, based on power
calculations as described by Dupont and Plummer (1990). The
levels of significance are 5% with 80% power as a minimum.
The noise and effect size were estimated through our previous
experience and publications using this mouse model (Wang
et al., 2010; Carlsson et al., 2011) and calculations were
performed using publicly available software, PS: Power
vanderbilt.edu/wiki/Main/PowerSampleSize) (Dupont and
Plummer, 1998). Data were analysed using the GraphPad
Prism v6.0. All assessments were performed blindly. Average ±
standard error of the mean (SEM) was recorded for all data
(Supplementary Table 1) and was first analysed with the Kolmogorov–Smirnov normality test. As the data
did not follow Gaussian distribution, the Kruskal–Wallis
non-parametric test was applied, followed by Dunn’s test
(Supplementary Table 2). *P < 0.05, **P < 0.01, ***P < 0.001 and
****P < 0.0001.

**Data availability**

All raw data are available from the corresponding author on
request.

**Results**

**GLP1R is expressed in the human and murine neonatal brain**

Firstly, we sought to confirm the presence of GLP1R both in
the human and murine brains. Confocal imaging using anti-
bodies against GLP1R and neural cell-specific markers showed
GLP1R co-localization predominantly in neurons (NeuN) in
each assessed developmental ages: adult (10 weeks), P10 and P7
(Fig. 2A). At 10 weeks of age, co-localization of GLP1R was
Figure 2 GLP1R expression in the human preterm and murine brain across different developmental ages.

(A) Immunofluorescence and scanning confocal microscopy studies demonstrating GLP1R is expressed in neurons (NeuN) in the mouse brain at 10 weeks (adult), P10 and P7, (B) with only small colocalization with astrocytes (GFAP) at 10 weeks of age, and (C) no co-localization with microglia (CD68) cells in any of the different developmental ages. The first row of micrographs (A–C) show negative staining for GLP1R antibody. (D) Immunofluorescence studies of post-mortem human preterm brain tissue shows co-localization of GLP1R expression in neurons (NeuN) in the frontal lobe and hippocampus Ammon’s horn, with the first row of micrographs for each human brain region containing negative staining for GLP1R antibody. A median filter was applied to the images to reduce noise. (E) Relative quantification of GLP1R expression by quantitative PCR in different brain regions at 10 weeks, (F) P10 and (G) P7 (n = 6 per age group). CBL = cerebellum; CTX = cortex; HIP = hippocampus; HPT = hypothalamus; MDL = medulla; OB = olfactory bulbs; THL = thalamus. Scale bar = 20 μm in C, 10 μm in D.
observed mostly in neurons but also in astrocytes (GFAP). This astrocyte co-localization was not observed at P10 and P7 (Fig. 2B). No microglia (CD68) co-localization with GLP1R was observed in any of the different developmental ages (Fig. 2C). Post-mortem neonatal sections showed GLP1R expression in the frontal lobe and hippocampus Ammon’s horn. Confocal imaging confirmed specific co-localization with neurons (NeuN) (Fig. 2D). Quantitative PCR analysis using primers against Glp1r and control Gapdh mRNA for normalization of data (Supplementary Fig. 2) revealed GLP1R expression across the different brain regions examined in adult (10 weeks) and neonatal (P10 and P7) mice. Glp1r mRNA was particularly present in the cortex, cerebellum and olfactory bulb of adult (Fig. 2E), P10 (Fig. 2F) and P7 mice (Fig. 2G), and also in the hippocampus of P10 and P7 mice.

**Exendin-4 reduces brain infarction in a dose- and time-dependent manner**

To assess the efficacy of exendin-4 brain protection against neonatal hypoxia-ischaemia, tissue infarction was measured 48 h post-insult using different dosing and concentration regimens, and timing of intervention. Saline-treated hypoxia-ischaemia littermates served as controls (Fig. 3A). The Nissl measurement as a percentage of tissue loss showed overall profound and consistent injury in the ipsilateral hemisphere of saline-treated hypoxia-ischaemia littermates served as controls (Fig. 3 A). Evaluation of cell death as quantification of TUNEL + cells was a reduced weight gain in comparison to saline controls, which reached significance at the 36 h (P = 0.0212) and 48 h (P = 0.0148) time points post-hypoxia-ischaemia. Both immediate and 2-h delay four high dose exendin-4 treatments resulted in initial non-significant weight loss when compared to baseline weight, with visible signs of recovery 48 h post-hypoxia-ischaemia. In the exendin-4 (four doses group) mice weighed significantly less than the low-dose group at 12 h (P = 0.0150) and 24 h (P = 0.0096) time points, and significantly less than saline-injected mice at 24 h (P = 0.0074), 36 h (P = 0.0315) and 48 h (P = 0.0269) post-hypoxia-ischaemia (Fig. 3D). There was no exendin-4-mediated modulation of weight in the 2-h-delayed four-dose treatment (2H) and low-dose-treated groups. To assess how quickly exendin-4 was crossing the blood–brain barrier and potentially initiating a cellular response in the CNS, we measured alterations in cyclic AMP (cAMP) expression, a known second messenger of GLP1R signalling. Naïve animals were given one high dose of exendin-4 and cAMP was measured in the brain at 2h, 4h, 8h and 12h. Significantly higher total brain cAMP was measured at 2h (P = 0.0021) and 4h (P = 0.0281) when compared to saline-injected controls at 2h (Supplementary Fig. 4).

**Exendin-4 prevents cell death and neuroinflammation**

Evaluation of cell death as quantification of TUNEL + cells 48 h after late preterm hypoxia-ischaemia demonstrated substantial overall cell loss in the control saline-treated group (310.5 ± 62.9) (Fig. 4A). Four administrations of high dose exendin-4 started either immediately post-hypoxia-ischaemia or 2 h later significantly reduced the overall number of TUNEL + cells (P = 0.0002 and P < 0.0001, respectively) (Fig. 4B). Furthermore, this protective effect was significant across all individually assessed brain regions: isocortex (P < 0.0001) and striatum (P < 0.0001) (Fig. 4C), pyriform cortex (P = 0.0001, SAL versus EX4, P < 0.0001) (Fig. 4D), external capsule (SAL versus EX4, P < 0.0001, SAL versus 2H, P < 0.0001) (Fig. 4E), hippocampus (SAL versus EX4, P = 0.0253, SAL versus 2H, P = 0.0037) (Fig. 4F), striatum (SAL versus EX4, P < 0.0001, SAL versus 2H, P < 0.0001) (Fig. 4G) and thalamus (SAL versus EX4, P < 0.0001, SAL versus 2H, P = 0.0010) (Fig. 4H). Microglial activation (alphaM, emβ2) was significantly reduced in both the four administrations of exendin-4 (P = 0.0006) and 2 h delayed intervention cohorts (P < 0.0001) compared to the saline-treated controls (Fig. 5A and Supplementary Fig. 5). This effect was observed throughout all assessed brain regions:
isocortex (SAL versus EX4, P = 0.0001, SAL versus 2H, P = 0.0024), pyriform cortex (SAL versus EX4, P = 0.0002, SAL versus 2H, P = 0.0011), hippocampus (SAL versus EX4, P < 0.0001, SAL versus 2H, P = 0.0005), striatum (SAL versus EX4, P < 0.0001, SAL versus 2H, P = 0.0001), thalamus (SAL versus EX4, P < 0.0001, SAL versus 2H, P < 0.0001) and external capsule (SAL versus EX4, P < 0.0001, SAL versus 2H, P = 0.0006). Astrogliosis (GFAP) was substantially suppressed in both the four administrations of exendin-4 (P = 0.0038) and 2-h delayed intervention cohorts (P = 0.0155) compared to the saline-treated controls (Fig. 5B and Supplementary Fig. 5). Regional analysis showed both immediate and 2-h delayed four-dose regimen treatments had significantly less GFAP in the isocortex (P = 0.0115 and P = 0.0416), hippocampus (P = 0.0006 and P = 0.0011), striatum (P = 0.0279 and P = 0.0412) and external capsule (P = 0.0004 and P = 0.0005, respectively). The pyriform cortex of EX4 mice had significantly less astrogliosis (P = 0.0551) than SAL-treated hypoxic-ischaemic mice. Immediate administration of the four doses of exendin-4 showed the highest inhibition of endothelial activation (ICAM1) (P = 0.0050) (Fig. 5C and Supplementary Fig. 5). This effect was present in most assessed brain regions: isocortex (P < 0.0001), hippocampus (P = 0.0443), striatum

Figure 3 Evaluation of optimal exendin-4 dose and time treatment regimen in the P7 late preterm model. (A) Representative whole brain micrographs of the different treatment groups: saline (n = 14, eight male and six females); one high dose exendin-4 (n = 14, eight male and six female), four high doses exendin-4 (n = 14, seven male and seven female) and four low-doses exendin-4 (n = 14, seven male and seven female) started immediately after hypoxia-ischaemia, and four high doses exendin-4 initiated 2 h after hypoxia-ischaemia (n = 14, seven male and seven female). (B) Effect on ipsilateral hemispheric tissue loss of different dose regimen started immediately after hypoxia-ischaemia and (C) delayed start of exendin-4 treatment. (D) Weight gain over a 48 h period following immediate exendin-4 administration at the different doses and (E) different time. Data presented as individual animals ± SEM and analysed using Kruskal-Wallis Dunn’s test. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. Scale bar = 2 mm. EX-4 = exendin-4; HI = hypoxia-ischaemia.
(P = 0.0136), thalamus (P = 0.0171) and external capsule (P = 0.0057). Four doses of exendin-4 administration with a 2-h delay inhibited the overall increase in ICAM1 (P = 0.0356), which was also observed in the isocortex (P = 0.0004) and external capsule (P = 0.0300).

Repeated high doses of exendin-4 is non-toxic in neonatal mice

The therapeutically optimal exendin-4 dose (0.5 μg/g) given every 12 h over a 48-h period in this study are substantially higher than those used clinically in diabetic patients. Therefore, we conducted an examination in naïve (no hypoxia-ischaemia) P7 mice for any toxicity or adverse effects. High dose exendin-4 did not alter blood glucose levels when compared to saline-treated or naïve controls (0 h, 0.5 h, 1 h, 2 h and 4 h time points) (Fig. 6A). The exendin-4 four high dose regimen in naïve animals resulted in reduced weight gain at 24 h (naïve versus EX4, P = 0.0552, SAL versus EX4, P = 0.0558) and 36 h (naïve versus EX4, P = 0.0558, SAL versus EX4, P = 0.05598 time points) (third and fourth injections), with partial recovery to baseline weight measurement (0 h time point) by 48 h after final injection (Fig. 6B).

As exendin-4 has been shown to modulate peripheral immune cells (Yanay et al., 2015; He et al., 2017), blood tests were conducted after completion of the four high dosing regimen. Counts of various blood cell populations and biochemistry: total white blood cells (Fig. 6C), neutrophils (Fig. 6D), lymphocytes (Fig. 6E), monocytes (Fig. 6F), eosinophils (Fig. 6G), basophils (Fig. 6H), haematocrit (Fig. 6I), platelets (Fig. 6J), red blood cells (Fig. 6K), haemoglobin (Fig. 6L) and mean corpuscular volume (Fig. 6M) in mice treated with the high dose exendin-4 regimen were all normal with no significant differences to naïve unadministered mice and saline-injected mice. Given the systemic administration of exendin-4, in addition to the brain we also harvested the heart, spleen, liver, lung, pancreas and kidney for analysis. Haematoxylin and eosin staining did not reveal any obvious fibrosis or abnormalities in cellular or tissue architecture in mice receiving the high dose exendin-4.
Figure 5 Exendin-4 suppresses glial cell activation 48 h after hypoxia-ischaemia. Overall and regional assessment of microglial activation (alphaM, OM1β2) through semi-quantitative score (A), and quantitative immunoreactivity analysis of astrocytes (GFAP) (B) and endothelial cells (ICAM1) (C). Data presented as individual animals ± SEM and analysed using Kruskal-Wallis Dunn’s test. *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001.
High dose exendin-4 enhances hypothermia neuroprotection

Following hypoxia-ischaemia, animals were randomly allocated into four groups: (i) normothermia and a single saline injection; (ii) normothermia and a single high dose exendin-4; (iii) hypothermia and a single saline injection; and (iv) hypothermia and a single high dose exendin-4 (Fig. 8A). The sex of each animal is shown in Supplementary Table 3. Macroscopic visual scoring of brain injury 7 days post-hypoxia-ischaemia demonstrated a more pronounced loss of tissue in the normothermia + saline (NT SAL) control group when compared to normothermia + exendin-4 (NT EX4, $P = 0.0003$) and hypothermia + saline (HT SAL, $P = 0.0171$), as well as the combined treatment of hypothermia + exendin-4 (HT EX4, $P < 0.0001$) groups (Fig. 8B). Additionally, combined hypothermia + exendin-4 showed significantly less macroscopic tissue loss when compared to hypothermia treatment + saline (HT SAL, $P = 0.0532$). Exendin-4 single high dose either alone or in combination with hypothermia did not affect weight gain (Fig. 8C). To more accurately assess tissue loss, volume infarction was measured. Normothermia mice demonstrated substantial total volume tissue loss (11.6 ± 1.2 mm$^3$). Exendin-4
Figure 7 histopathological organ assessment. (A) Representative micrographs of haematoxylin and eosin stained brain, including hippocampus (hip) level and visceral organs: heart, liver, pancreas, spleen, lung and kidney showing no abnormal histopathology in naïve, saline (SAL) or exendin-4 (EX4) alone treatments. (B) Quantitative immunoreactivity threshold measurements of macrophages (CD68) in the brain, (C) heart, (D) spleen, (E) liver, (F) lung, (G) pancreas and (H) kidney for the different groups (n = 6 per group). Data presented as individual animals ± SEM and analysed using Kruskal-Wallis Dunn’s test. *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001.
single high dose (3.9 ± 1.4 mm$^3$, $P = 0.0003$), hypothermia alone (5.2 ± 1.2 mm$^3$, $P = 0.0115$) and combined hypothermia and exendin-4 (1.1 ± 0.6 mm$^3$, $P < 0.0001$) treatments substantially reduced the infarction volume (Fig. 8D). Analysis of volume loss across different brain levels showed that in level 1, NT EX4 and HT EX4 animals were significantly protected ($P = 0.0067$ and $P < 0.0001$). All three treatments resulted in significant protection when assessing levels 2–5: level 2 NT EX4 ($P = 0.0067$), HT SAL ($P = 0.0527$), HT EX4 ($P < 0.001$); level 5 NT EX4 ($P = 0.0006$), HT SAL ($P = 0.0206$), HT EX4 ($P < 0.0001$) (Fig. 8E). Additionally, HT EX4 animals had substantially less volume loss than HT SAL when assessing levels 1 ($P = 0.0093$) and 2 ($P = 0.0541$).

Discussion

There is an enormous unmet clinical need for effective interventions against neonatal HIE. We have conducted a
preclinical study of the therapeutic efficacy and safety of exendin-4 in a perinatal mouse model of HIE. Here we establish for the first time that exendin-4 treatment after neonatal HIE is highly neuroprotective. Furthermore, we demonstrate that exendin-4 can be used in synergy with hypothermia, the current clinical standard of care for HIE, to enhance its therapeutic efficacy.

In our late preterm results, intraperitoneal administration of 0.5 μg/g exendin-4 as a four-dose 12 h interval regimen started immediately after hypoxia-ischaemia is significantly neuroprotective. Delaying the start of this exendin-4 treatment regimen by 2 h also significantly protects the immature brain with no significant difference in efficacy when compared to immediate administration post-hypoxia-ischaemia. The significant therapeutic efficacy was also measured in other readouts of neuropathology including a reduction of TUNEL+ cell death and microglia/macrophage, astrocytes and endothelial cells activation markers remained low in contrast to untreated hypoxia-ischaemia groups. Our 2 h delayed exendin-4 regimen results are of particular clinical relevance and may signify a potentially extended window of opportunity in which therapeutic exendin-4 could be administered. In the adult murine stroke model using transient middle cerebral artery occlusion, Teramoto et al. (2011) were able to retain reduced infarct volume only up to 1 h delayed administration of high dose exendin-4.

This study shows that exendin-4 alone or in combination with therapeutic hypothermia significantly protects the neonatal brain against term HIE. Hypothermia is the only standard treatment of care for term HIE in developed countries and is not used in preterm infants or in developing countries. The latter has a high association with infection, where hypothermia may have limited or even a detrimental effect (Robertson et al., 2008; Osredkar et al., 2014). Furthermore, hypothermia is only partially protective, and alternative or adjunct therapies are needed. Because of the variability in the efficacy of hypothermia in the neonatal rodent model of HIE, we administered a single high dose of exendin-4 in combination with hypothermia to allow clear dissection of a potential synergistic adjunct role between both treatments. Exendin-4 administration provided neuroprotection against tissue loss in the P10 term mouse comparable to the P7 single-dose results, demonstrating that the same dose is applicable for both developmental ages. Hypothermia alone also proved significantly neuroprotective; however, the addition of a single dose of exendin-4 treatment enhanced hypothermia protection both in the macroscopic injury score and regional infarct volume assessments.

Exendin-4 is an FDA and EMA approved drug used clinically for the treatment of type 2 diabetes mellitus (Furman, 2012). Epidemiological studies have established comorbidity links between type 2 diabetes mellitus and several adult onset neurodegenerative and cerebrovascular disorders, where patients with type 2 diabetes mellitus have increased risk of Alzheimer’s disease (Peila et al., 2002), Parkinson’s disease (Santiago and Potashkin, 2013) and stroke (Putaala et al., 2011). This suggests shared disease mechanisms, and inflammation, aberrant insulin and insulin-like growth factor 1 (IGF1) signalling and mitochondria dysfunction have shown to contribute to pathogenesis of these conditions (Kruyt et al., 2010; Donath and Shoelson, 2011; Clark et al., 2012; Aviles-Olmos et al., 2013b). Most neonatal studies rely on extensive research of adult experimental models, and despite biological differences, inflammation (Hagberg et al., 2015) and mitochondrial dysfunction (Hagberg et al., 2014) are also major hallmarks of HIE. There are other reasonable links between hypoxia-ischaemia and the aforementioned adult disorders. Preclinical rat studies have shown neonatal hypoxia-ischaemia as a precursor to diabetes, metabolic syndrome and stroke (Mcpherson et al., 2009), and IGF1 treatment offers neuroprotection after hypoxia-ischaemia (Brywe et al., 2005). Therefore, exendin-4-mediated prevention of mitochondrial damage and stimulation of mitochondria biogenesis (Fan et al., 2010), anti-inflammatory properties and sustained neuroprotection beyond the period of intervention (Athauda et al., 2017) make it an attractive therapeutic approach in the treatment of HIE. In fact, exendin-4 has already been used in neonatal animals to prevent adolescent and adult disorders without adverse effects. Transient neonatal preconditioning with exendin-4 in lamb (Gatford et al., 2013) and rat (Stoffers et al., 2003) intrauterine growth restriction models reduced both visceral fat accumulation, a risk factor for type 2 diabetes mellitus, and oxidative stress (Raab et al., 2009). Neonatal exendin-4 protected against juvenile and adult rat myocardial ischaemic injury and preconditioned mitochondria (Brown et al., 2010). Its administration in neonatal wild-type C57BL/6 mice prevented increased adult body weight and fat mass, and increased energy levels via GLP1R activation (Rozo et al., 2017).

The optimal exendin-4 dose concentration used in this study (0.5 μg/g) is significantly higher than in the treatment of type 2 diabetes mellitus (0.1 μg/kg) and in the ongoing Alzheimer’s and Parkinson’s disease trials. The doses used in this study are based on the findings by Teramoto et al. (2011), which required the same dose concentration or higher (10, 50 μg/100μl per mouse) to exert protection against transient middle cerebral artery occlusion (Teramoto et al., 2011). We observed that reducing the dose concentration by 10-fold to 0.05 μg/g resulted in abolition of the exendin-4 neuroprotective effect in the HIE model. This high dose requirement may reflect the acute and rapid nature of the injury. Hypoglycaemia is a serious risk for infants suffering from HIE, and blood glucose is continuously monitored in neonatal intensive care units (Tam et al., 2012). Therefore, we monitored blood glucose at different time points after high dose exendin-4 injection, and the known glucose lowering effects of exendin-4 was not observed when compared to saline-treated/basal level controls. This differs from many studies where exendin-4 was associated with substantial reduction in blood glucose levels. However, many of these preclinical exendin-4 studies use diabetic models, which have altered baseline glucose (Young et al., 1999). Clinically, exendin-4 is given to
patients with type 2 diabetes mellitus as an adjunct to metfor- 
min, sulfonylurea or basal insulin (Inzucchi et al., 2015), and it is the exendin-4 addition to metformin and/or sulfo-
ylurea that enables type 2 diabetes mellitus patients to 
achieve glycaemic control via improvement of β-cell func-
tion (Kendall et al., 2005). These results are compatible 
with exendin-4’s positive effect on glucose homeostasis. 
Moreover, exendin-4 administration in healthy individuals 
reduces fasting glucose levels without reaching hypogly-
caeic levels (Edwards et al., 2001). Our assessment of 
any adverse effects to this transient systemically adminis-
tered high dose exendin-4 regimen did not reveal any toxic-
ity through blood, macrophage-inflammatory or 
histological analyses. Our results indicate that the high 
dose regimen used in this study is safe and well-tolerated 
in mice. These findings are supported by a pharmacology 
and toxicity review of exenatide (Byetta®) reporting that 
doses of 450 times the clinical dose in normal glycaemic 
monkeys produces no hypoglycaemia, neurological signs or path-
ology (https://www.accessdata.fda.gov/drugsatfda__
docs/nda/2009/021919s000pharmr.pdf). Chronic toxicity 
studies in monkeys administered very high doses of up to 
1000 µg/kg over a 28-day period resulted in no mortality. 
Any risks are further mitigated as we have advocated high 
doses administered once or every 12 h over a limited 48 h 
period (for optimal therapy) when the pathological cascade 
associated with HIE is most significant. The lack of com-
mercially available kits to measure exendin-4 makes com-
parison of plasma levels in this study with those in humans 
difficult. However, the same review shows that a 200 µg/kg 
subcutaneous administration in mice leads to a C_{max} value 
of 318.507 pg/ml compared to a human that had received 
10 µg/subject and a C_{max} of 251 pg/ml.

In our study, high dose exendin-4 in post-hypoxic-ischaem-
ic injury or naïve mice resulted in an initial weight drop, 
with a return to baseline level (0 h time-point equivalent) 
48 h after start. However, a 10-fold lower dose of exendin-
4 (0.05 µg/g) given four times had no effect on body weight 
when compared to controls, suggesting that this dose might be too low for exendin-4 to sufficiently activate GLP1R in 
the brain of neonates. Interestingly, P10 mice treated with 
one high dose exendin-4 used in conjunction with hypo-
thermia showed no weight change in comparison to con-
trols. This could signify that hypothermia may have a modulating effect on exendin-4-mediated activation of 
GLP1R, although not enough to diminish its neuroprotective 
effects in the HIE model.

Exendin-4 was administered intraperitoneally and several 
studies have shown that it can readily cross the blood–brain 
barrier (Kastin and Akerstrom, 2003; Zanotto et al., 2017) 
and interact directly with GLP1R in neural cells. Our measurements of cAMP (a second messenger of 
GLP1R) in the brain at regular time intervals following a 
single intraperitoneal administration of exendin-4 to naïve 
P7 mice showed significantly elevated levels at the earliest 
2 h time point. This suggests that exendin-4 rapidly crosses 
the blood–brain barrier and initiates a pharmacological 
response. GLP1R is expressed in the human (Wei and 
Mojsov, 1995), rat (Göke et al., 1995) and mouse (Hamilton and Hölscher, 2009) brain. Similarly, we have shown ubiquitous expression of GLP1R throughout the 
naïve mouse brain irrespective of developmental age (P7, 
P10 and 10 weeks) and confirm for the first time in the 
human preterm neonatal brain. In the neonatal period (P7 
and P10) GLP1R seemed to predominantly co-localize with 
neuronal cells, whereas GLP1R in adult mice also showed 
co-localization with astroglia. We believe that this is the 
first study to confirm GLP1R expression in the neonatal 
mouse brain, and its main co-localization with neurons at 
this developmental stage.

The precise mechanisms of action of exendin-4 neuropro-
tection are still not fully understood. Several studies have 
demonstrated a multitude of neuroprotective actions, with 
exendin-4-mediated PI3K surge in different areas of the 
CNS, leading to increase in phosphorylation of AKT via 
PI3K signalling pathway. This mechanism of action is thought 
to increase the anti- versus pro-apoptotic bcl-2 family protein balance (Brywe et al., 2005; Athauda and 
Foltyne, 2016), attenuate neuroinflammation and stabilize 
the blood–brain barrier post-transient middle cerebral 
artery occlusion in non- (Chen et al., 2016) and diabetic 
mice (Li et al., 2016). Intracellular cAMP levels are also 
raised in exendin-4-treated post-transient middle cerebral 
artery occlusion as a result of increased GLP1R expression 
(Teramoto et al., 2011; Kim et al., 2017). Exendin-4 ad-
ministration also increases islet-brain 1, partially inhibits 
JNK and attenuates downstream COX-2 and prostaglandin 
E2 after transient middle cerebral artery occlusion (Kim 
et al., 2017). Exendin-4 treatment has resulted in mitochon-
dria biogenesis (Fan et al., 2010; Kang et al., 2015), 
protection from reactive oxygen species (Teramoto et al., 
2011; Li et al., 2013), inflammatory inhibition (Teramoto 
et al., 2011), neurogenesis (Bertilsson et al., 2008), neuro-
trophic effects (Perry et al., 2002) and cell survival (Teramoto et al., 2011; Candeias et al., 2017). These neu-
roprotective effects are of extreme importance in offsetting 
the mechanisms of HIE injury.

The current study was designed to assess exendin-4 drug 
preclinical safety and efficacy as a standalone therapy, its 
capacity to augment therapeutic hypothermia neuroprotec-
tion, and not specifically the exendin-4 mechanisms of 
action. However, aspects of our study suggest that exen-
din-4 may act to maintain neuronal viability and modulate 
neuroinflammation. Microglia, astroglia and endothelial 
cells are a source of inflammatory response and reactive 
oxidant species after HIE (Dietrich, 2002; Hagberg et al., 
2015; Rocha-Ferreira et al., 2016). In our study, four high 
dose treatments of exendin-4 started either immediately or 
2 h post-hypoxia-ischaemia significantly reduced prolifera-
tion of microglia and its morphological change into phagocytic amoeboid microglia. GFAP expression in astro-
cyes also remained low and there was reduced ICAM1 
immunoreactivity. ICAM1 upregulation is associated with 
diapedesis, transendothelial migration and further
recruitment of peripheral inflammatory cells (Dietrich, 2002). In neonatal hypoxia-ischaemia, ICAM1 is also associated with alterations of the brain microvasculature and breakdown of the blood–brain barrier (Lai et al., 2017). Our ICAM1 results showed increased ICAM1 immunoreactivity, particularly in the areas surrounding the tissue infarction observed in the hypoxia-ischaemia group, an effect not observed in the exendin-4-treated groups. This suggests that exendin-4 treatment prevents breakdown of the blood–brain barrier. This is in agreement with a study by Zanotto et al. (2017) where exendin-4 treatment reversed blood–brain barrier permeability and reduction of blood–brain barrier-specific proteins in diabetes mellitus rats. The suggested beneficial effect of reduced activation of these cells occurs in parallel with significantly reduced tissue infarction assessed through Nissl staining and cell death (TUNEL assay) in exendin-4-treated mice. The neuroprotective effect of exendin-4 alone seemed moderately long-lasting, as seen in the P10 single dose treatment part of the study. Our studies showed predominant neuronal expression of GLP1R. Therefore, it cannot be excluded that the reduced glial cell activation may be secondary to reduced neurodegeneration. Additionally, high dose exendin-4 administration resulted in significantly higher levels of brain cAMP, suggesting involvement of the cAMP signalling pathway, also proposed in other studies (Teramoto et al., 2011; Kim et al., 2017). Interestingly, metformin, another type 2 diabetes mellitus medication with diverse pharmacological activities, also ameliorates brain infarction in neonatal hypoxic-ischaemic rats (Fang et al., 2017).

This preclinical study in a mouse model of acute perinatal HIE is an essential first step to potentially advance the use of exendin-4 for clinical benefit. Using severe hypoxic-ischaemic injury in both the late preterm and term models has shown exendin-4’s efficacy in both ages. Preterm treatment with exendin-4 alone is highly attractive as therapeutic hypothermia is not common practise at this age, and the successful 2-h delayed start of exendin-4 treatment offers a larger therapeutic window of opportunity within which to administer the drug. Exendin-4 term treatment combined with therapeutic hypothermia has provided enhanced protection as seen in the brain macroscopic score and regional volume measurement. Other proof of concept studies of exendin-4 have already been translated into early clinical trials for neurological diseases such as Alzheimer’s disease (NCT01255163) and, in particular, Parkinson’s disease for which initial reports are highly promising (NCT01174810; NCT01971242; Aviles-Olmos et al., 2013a; Athauda et al., 2017). This study and these early clinical trials support the continued investigation of exendin-4 for clinical translation as a treatment for HIE.

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Competing interests

The authors declare no conflict of interests regarding the research, authorship, and/or publication of this article.

Supplementary material

Supplementary material is available at Brain online.

References


