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## Incretin mimetics as pharmacological tools to elucidate and as a new drug strategy to treat traumatic brain injury

Nigel H. Greig<sup>\*1</sup>, David Tweedie<sup>1</sup>, Lital Rachmany<sup>2</sup>, Yazhou Li<sup>1</sup>, Vardit Rubovitch<sup>2</sup>, Shaul Schreiber<sup>3</sup>, Yung-Hsiao Chiang<sup>4,5</sup>, Barry J. Hoffer<sup>6</sup>, Jonathan Miller<sup>6</sup>, Debomoy K. Lahiri<sup>7</sup>, Kumar Sambamurti<sup>8</sup>, Robert E. Becker<sup>1,9</sup>, and Chaim G. Pick<sup>2</sup>

<sup>1</sup> Drug Design & Development Section, Translational Gerontology Branch, Intramural Research Program, National Institute on Aging, National Institutes of Health, Baltimore, MD 21224, USA

<sup>2</sup> Department of Anatomy & Anthropology, Sackler School of Medicine and Sagol School of Neuroscience, Tel-Aviv University, Tel-Aviv, Israel.

<sup>3</sup> Department of Psychiatry, Tel Aviv Sourasky Medical Center and Sackler Faculty of Medicine, Tel-Aviv University, Tel-Aviv, Israel.

<sup>4</sup> Department of Neurosurgery, Taipei Medical University Hospital, Taipei City 110, Taiwan (R.O.C.)

<sup>5</sup> Ph.D. Program for Neural Regenerative Medicine, Graduate Institute of Neural Regenerative Medicine, Taipei Medical University, Taipei City 110, Taiwan (R.O.C.)

<sup>6</sup> Department of Neurosurgery, Case Western Reserve University School of Medicine, Cleveland, OH 44106, USA

<sup>7</sup> Department of Psychiatry, Indiana University School of Medicine Indianapolis, IN 46202, USA

<sup>8</sup> Department of Neuroscience, Medical University of South Carolina, Charleston, SC 29425, USA

<sup>9</sup> Aristeia Translational Medicine, Park City, UT 84098, USA.

### Abstract

Traumatic brain injury (TBI), either as an isolated injury or in conjunction with other injuries, is an increasingly common occurring event. An estimated 1.7 million injuries occur within the US each year and 10 million people are affected annually worldwide. Indeed, some one-third (30.5%) of all injury-related deaths in the U.S. are associated with TBI, which will soon outstrip many common diseases as the major cause of death and disability. Associated with a high morbidity and mortality, and no specific therapeutic treatment, TBI has become a pressing public health and medical problem. The highest incidence of TBI occurs among young adults (15 to 24 years age) as well as in the elderly (75 years and older) who are particularly vulnerable as injury, often associated with falls, carries an increased mortality and worse functional outcome following lower

\* Corresponding author: Nigel H. Greig <[Greign@grc.nia.nih.gov](mailto:Greign@grc.nia.nih.gov)>.

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initial injury severity. Added to this, a new and growing form of TBI, blast injury, associated with the detonation of improvised explosive devices in the war theaters of Iraq and Afghanistan, are inflicting a wave of unique casualties of immediate impact to both military personnel and civilians, for which long-term consequences remain unknown and may potentially be catastrophic. The neuropathology underpinning head injury is becoming increasingly better understood. Depending on severity, TBI induces immediate neuropathological effects that for the mildest form may be transient but with increasing severity cause cumulative neural damage and degeneration. Even with mild TBI, which represents the majority of cases, a broad spectrum of neurological deficits, including cognitive impairments, can manifest that may significantly influence quality of life. In addition, TBI can act as a conduit to longer-term neurodegenerative disorders. Prior studies of glucagon-like peptide-1 (GLP-1) and long-acting GLP-1 receptor agonists have demonstrated neurotrophic/neuroprotective activities across a broad spectrum of cellular and animal models of chronic neurodegenerative (Alzheimer's and Parkinson's diseases) and acute cerebrovascular (stroke) disorders. In line with the commonality in mechanisms underpinning these disorders as well as TBI, the current article reviews this literature and recent studies assessing GLP-1 receptor agonists as a potential treatment strategy for mild to moderate TBI.

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## Introduction: Traumatic brain injury

Traumatic brain injury (TBI) is a significant cause of disability and death worldwide; but particularly in industrialized countries. Beyond any ensuing physical disabilities and the signature cognitive deficits, predominantly in attention, learning and memory, and higher-order executive functions, TBI is a major conduit for the development of chronic neurodegenerative disorders, especially Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS) and chronic traumatic encephalopathy (CTE) [1]. Within the US alone it is estimated that at least 1.7 million people suffer a TBI event annually, which results in some 235,000 hospitalizations and an excess of 50,000 deaths. Indeed, at least 5.3 million Americans are currently living with a long-term disability associated with a TBI incident [2]. In this regard, the elderly are particularly vulnerable to TBI. Often associated with falls in this increasingly large segment of the population, it carries a higher mortality and worse functional outcome following lower initial injury severity compared to younger adults [3]. Worldwide, the incidence of TBI is approximately 0.5% per year.

TBI is also a much too common occurrence among military forces serving in modern combat operations. Military operations ongoing in Iraq and Afghanistan (Operation Enduring Freedom for the war in Afghanistan, and Operation Iraqi Freedom together with Operation New Dawn - for military operations in Iraq after August 2010) have spanned more than a decade from their initiation in 2001 and have involved more than 2.4 million U.S. and coalition personnel. Bomb explosions, primarily caused by improvised explosive devices, have become ever more frequent in these war theaters, impacting military personnel and increasingly civilians as a consequence of urban terrorist attacks and sectarian violence. Estimates range from approximately 15% [4] to 19.5–22.8% of all returning deployed US troops [5] suffering a blast exposure TBI, with the total number of such injuries estimated as high as 320,000 [4,6]. The vast majority of TBIs experienced by military personnel have been classified as mild injuries, based upon clinical severity [7]. However, many involved

likely have repeated injury, where cumulative long-term effects could have particularly serious implications. Emerging evidence suggests possible dose- and frequency-dependent associations between TBI and a risk of neurodegenerative disease; however, a threshold for clinical manifestation remains to be determined and more than likely could be affected by genetic predisposition and environmental factors [8,9].

Some 10% of all concussive TBIs result in significant cognitive and emotional dysfunction, requiring around-the-clock care. The more prevalent mild injuries frequently result in significant long-term effects on an individual's health. TBI can cause meaningful deficiencies across a broad range of brain functions, but mild and moderate TBIs characteristically induce headaches, impairments in sleep, memory, attention and cognitive processes, as well as stress, and affective disorders [10]. Such clinical problems may persist long after the injury occurrence or even permanently. There is a range of similarities and differences in relation to blast exposure TBI, where symptoms include headache, nausea, vomiting, dizziness/balance problems, fatigue, sleep disturbances, together with concentration and cognitive impairments [7,11]. Such symptoms are reported in more than 70% of cases, occur in the absence of overt histological or standard clinical radiological evidence of damage, and are often accompanied by ringing in the ears and sensitivity to light and/or sound. Clearly acutely disabling, particularly in a battlefield environment, such symptoms may resolve or persist for years following initial injury, degrading the subjects' quality of life and placing an enormous burden on military, veteran and civilian health care systems. Although there are symptomatic treatments to address some of the above-described aspects of mild TBI, the development of effective pharmacological treatments to protect against the injury-induced secondary neurodegeneration that primarily contributes to and largely underpins cognitive dysfunction has been slow and only relatively recently is being evaluated under true battlefield conditions [7].

During recent years, advances in our knowledge of molecular mechanisms that regulate the health and survival of neurons together with an understanding of key pathways induced by TBI that lead to neuronal dysfunction and death [12-14] are being applied to the development of experimental drugs with features potentially beneficial for mild TBI treatment – whether concussive or blast related. Primary brain injury is induced by the immediate insult to the head, likely as a consequence of mechanical forces inducing shearing and compression of neuronal and vascular tissue at the time of impact, and rotational head acceleration. A cascade of pathological events may then follow that leads to further secondary brain injury that takes place from minutes to days following the trauma [15]. Most of the damage apparent in mild injury patients derives from the secondary events of the trauma, which includes brain edema, ischemia, inflammatory responses, free radical generation, elevated excitatory neurotransmitters (e.g., glutamate excitotoxicity), loss/disruption of synaptic connections or altered synaptic physiology and DNA damage [12-16]. This leads to neuronal dysfunction, as manifested by changes in long-term potentiation [17] and dendritic and synaptic loss (personal communication: Dr. Ronald F. Mervis, Center for Aging and Brain Repair, University of South Florida College of Medicine, Tampa, Florida 33612, USA), and when cellular damage is sufficiently profound the pro-apoptotic protein p53 will initiate the process of apoptosis [18-25] – that may then exacerbate inflammatory processes and instigate the development of a self-propagating adverse cycle of events [26-32]. Clinical and

experimental research indicates that the hippocampus is particularly vulnerable to secondary damage, underpinning the manifestations of deficits in the hippocampal-dependent functions of learning and memory [33-37]. Such ensuing secondary injury may be amenable to intervention and is worsened by secondary physiological insults. A clear conundrum is that the resulting impairments render the TBI victim less capable of avoiding further potential head injury, thereby increasing the likelihood of repetitive mild TBI to which the brain is yet more vulnerable. Specific risk factors for poor outcome after TBI have been established, some of which recognized at the time of injury, are age, gender, mechanism of injury and presenting signs, whereas others, including hyperglycemia, hypoxia and hypotension, are potential areas for medical intervention [38]. Although prompt and specialist neurocritical care is associated with improved outcome [7,39], to date, no successful drug treatments have been approved for improving patient outcome [38].

### Animal models of TBI

Across the clinical spectrum of all TBIs there is significant heterogeneity and thus numerous TBI models have been developed that mimic, to a lesser or greater degree, select aspects of human injury [40]. The classification of human TBI is conventionally based on the presenting symptoms and level of consciousness, routinely using the Glasgow Coma Scale (GCS). Albeit patients may have a similar GCS score, they may have remarkably different radiological appearances (such as contusions, skull fractures, blood-brain barrier disruption, hematoma, axonal injury, etc. – or, indeed, none of these), reiterating that TBI is not a single disorder. Mimicking all aspects of TBI in a single animal model is clearly not feasible, and thus for translational purposes when assessing treatment strategies the use of multiple models would appear prudent. Recent comprehensive reviews of TBI animal models are Marklund & Hillered [40] and Xiong et al [41].

In humans, the form of concussive mild TBI that can occur in traffic accidents, sports injuries and falls can, to a varying degree, be mirrored in a classic weight drop rodent model [42]. In this closed head model, injury is diffuse and occurs throughout the brain, both ipsi- and contralateral to the side of impact. In some TBIs a focal lesion ensues, with the most common in human TBI being a cortical contusion in which destruction of brain tissue with micro hemorrhages can occur [40]. Such contusions frequently arise in frontal and temporal regions, although a contre-coup contusion may occasionally occur within a brain region opposite to initial injury, and such contusions can noticeably expand during early days post-TBI. A widely used model in this context of moderate TBI involves fluid percussion injury where damage is inflicted by a pendulum striking a piston associated with a fluid reservoir in contact with the brain surface via a craniotomy. This generates a fluid pressure pulse to the brain that, depending on craniotomy placement (whether lateral or midline) can result in a local focal cortical contusion and diffuse subcortical neuronal injury to the hippocampus and thalamus [43]. This model can additionally reproduce intracranial hemorrhage, brain swelling and progressive grey matter damage that are classical features of human moderate TBI [43]. A further commonly used more severe contusion TBI model that mimics focal human TBI is the controlled cortical impact model, which generates extensive cortical tissue loss, together with hippocampal and thalamic damage [44]. Additionally, in recent years, interest has grown in the development of blast TBI models, and several have been

established in rodents [45-49]. In general, these models of blast injury involve either the use of a compression-driven shock tube to simulate blast effects in a confined laboratory setting or the exposure of animals to a controlled detonation, providing a 'real world' model of an improvised explosive device [49].

Potential limitations to currently available animal models are that although there are significant similarities in the neurophysiology between non-human mammals, particularly rodents, and human brain, there are clear differences as well in brain size, complexity and white to grey matter ratio. Whether or not such dissimilarities translate to differences in behavior, neurochemistry and drug responses after TBI is unknown, and thus the importance of assessing more than a single animal model for translational drug studies is reiterated.

### **The counterbalance between neuronal cell death and survival as a target for TBI therapy**

The most widely studied type of programmed cell death in the nervous system, apoptosis [18,24,28,50], is a process regulated by specific cysteine proteases - caspases. Numerous triggers of neuronal apoptosis exist, such as oxidative stress, cell surface receptor engagement (e.g., activation of glutamate receptors or TNF- $\alpha$  receptor engagement), trophic factor insufficiency, DNA damage and accumulation of damaged proteins – all of which have been reported following TBI [51,52]. Several families of proteins and specific biochemical signal-transduction pathways regulate cell death. Cell death signaling can involve plasma membrane death receptors, mitochondrial death proteins, proteases, kinases, and transcription factors. Players in the cell death and cell survival orchestra include the Fas receptor, Bcl-2 and Bax (and their homologues), cytochrome c, caspases, and extracellular signal-regulated protein kinases [18,24,28,50,53]. Other forms require gene activation, RNA synthesis, and protein synthesis. Irrespective of this, many share biochemical cascades that regulate ensuing cell death processes and, thereby, provide targets for intervention.

Counterbalancing this, biochemical cascades can be triggered that activate specific proteins associated with cell survival. For example, numerous growth factors and G-protein coupled receptors activate PI3 kinase, a well-known upstream regulator of cell survival that activates Akt. This has the capacity to phosphorylate a wide variety of substrate proteins, including specific death cascade proteins, such as Bad and caspase 9, to inhibit their ability to induce cell death [54]. It is apparent that Akt promotes cellular survival via a series of distinct pathways that involve the Forkhead family of transcription factors, GSK-3 $\beta$ ,  $\beta$ -catenin, eIF2B, c-Jun, CREB, Bad, IKK, p53, and JIPs [55]. As apoptosis and opposing cellular survival pathways are finely tuned both within neurons and glial cells and are activated in neurodegenerative diseases and TBI, pharmacological approaches to up or down regulate them provide the opportunity to utilize them as 'pharmacological tools' to elucidate the relevance of specific biochemical cascades in TBI and neurodegenerative disorders as well as to test investigational drugs for treatment.

It is, additionally, clear that following any form of brain injury, particularly TBI, new neurons can be generated from stem cells within the subgranular layer of the dentate gyrus and the subventricular zone throughout life [56-61] and some of them are capable of migrating into the granule cell layer of the dentate gyrus [57]. There, they develop granule cell morphology and neuronal markers [62], connect to their target area [63] and become

functionally integrated into local circuitry [64], similar to mature cells [65-67]. During TBI, however, there is a loss of neural precursor cells from these neurogenic areas with an accompanying reduction in newly generated neurons [58]. This is associated with learning impairments [68] and an increase in new glial cells [58]. Neural stem cells are exquisitely sensitive to trauma and inflammatory cytokines [69,70], which are known to be elevated in TBI, and to impact their differentiation and survival [58]. The neurogenesis/regenerative process provides an additional intervention strategy to potentially maximize neural stem cell survival and optimize differentiation towards neurons versus glial cells.

### **Incretin mimetics as a mechanism-based mild TBI treatment strategy to promote neuronal survival**

Counterbalancing apoptotic pathways leading to cell death are biochemical cascades that promote cell survival [50,53]. To this end, we and others have been assessing cell survival/neuroprotection consequent to G-protein coupled receptor (GPCR) activation, focusing on the glucagon-like peptide-1 (GLP-1) receptor (R) that is of clinical relevance to type 2 diabetes mellitus (T2DM) and neurodegenerative disorders [71-79]. The incretin GLP-1 (Figure 1) is an endogenous 30-amino acid insulinotropic peptide that controls blood glucose levels via activation of the GLP-1R on pancreatic  $\beta$ -cells [80,81]. GLP-1 derives from the post-translational modification of proglucagon and is secreted from enteroendocrine L cells present throughout the small and large intestine where its basal secretion is rapidly and substantially elevated in response to food intake [81-84]. Interestingly, such cells display a molecular profile that largely overlaps with other gut endocrine cell types and co-express multiple peptide hormones that, depending on their post-translational modification by activities such as prohormone convertase 1/3, can generate a diversity of essential physiological peptides along the length of the gastrointestinal tract [85]. GLP-1 has an array of physiological actions, with its insulinotropic ones being the most notable and well studied [Figure 2]. These are exerted through a distinct GPCR, the GLP-1R that is highly expressed on pancreatic islet  $\beta$  cells. The insulinotropic actions of GLP-1 include glucose-dependent stimulation of insulin secretion and inhibition of glucagon secretion [82]. GLP-1 additionally confers glucose sensitivity to glucose-resistant  $\beta$  cells and acts as a trophic agent, inducing pancreatic  $\beta$ -cell proliferation and neogenesis, as well as inhibiting  $\beta$ -cell apoptosis [80-82]. It is hence a key regulator of  $\beta$ -cell mass that, with its other physiological actions, spurred the development of GLP-1R agonists for treatment of T2DM [80-84]. The biochemical cascades and cellular repertoire responsible for effective GLP-1R signaling within the  $\beta$ -cell to promote GLP-1 mediated trophic actions have been an area of intense study and recently, together with a detailed analysis of the gut-brain GLP-1 axis, have been comprehensively reviewed by Campbell & Drucker [82]. As the insulinotropic actions of GLP-1 and agonists are glucose-dependent, advantageously and unlike other current agents for diabetes treatment, their pharmacological actions are not associated with a high risk of hypoglycemia [80,83,84]. As illustrated in Figure 2, the GLP-1R is also widely expressed in nonislet cells where its activation additionally exerts indirect metabolic actions. Hence there is considerable interest in identifying extrapancreatic actions of GLP-1R activation [86]. GLP-1 is additionally generated within the central nervous system, chiefly in the caudal part of the nucleus of the solitary tract within the

brainstem [87-91], from where it can diffuse within brain to induce assorted metabolic, vascular and neuroprotective actions (Figure 2).

Like most endogenous peptides, however, GLP-1 is short-lived in the circulation following its release [92-94]. It is rapidly degraded by the effective protease dipeptidyl peptidase IV (DPP-IV), rendering it inert at the GLP-1R, and then cleared renally. Elegant studies by Eng and colleagues [95,96] resulted in the discovery and characterization of a naturally occurring DPP-IV resistant GLP-1R agonist, exendin-4 (Ex-4), present within the saliva of a large and heavy-bodied venomous lizard (Gila monster, *Heloderma suspectum*) that reaches up to 1.25 feet in length and is native to the western and southern foot hills of Arizona through to the Mexican state of Sonora. Purely synthetic forms of Ex-4 as well as more recent GLP-1 and Ex-4 analogues have been generated (Figure 1). These possess substantially longer half-lives than GLP-1, and exenatide (synthetic Ex-4 as a twice daily (*Byetta*) or once weekly (*Bydurion*) formulation), liraglutide (*Victoza*, once daily) and Lixisenatide (*Lyxumia*, once daily but not yet approved within the US) are now in clinical use as agents for T2DM [80-84].

Although chiefly localized to pancreatic islets [81,82], GLP-1R expression also occurs on neurons throughout the brain and nervous system (primarily localized to dendrites [92]), which GLP-1 and analogues can readily enter after systemic administration [93-96]. Expanding upon our prior interest of GLP-1 and Ex-4 in T2DM, their insulinotropic and antiapoptotic actions [93,94,97,98], the structure-activity relation of their amino acid sequence [99-101], and their translation to clinic [102,75], we hypothesized that GLP-1R stimulation in brain would provide neurotrophic/protective activity as (i) GLP-1 has trophic action on  $\beta$ -cells [103-108], and (ii) the GLP-1R is coupled to the cAMP second messenger pathway, increases in which are well documented to be associated with neuroprotection [109,110] - a function very different from its prior known role in brain in the regulation of food intake and satiety [111,112]. We hence characterized the action of GLP-1 analogs on neuronal cells both in cell culture and animal studies [113-122] demonstrating potent neurotrophic and neuroprotective actions that have been widely confirmed [75-79,123-127].

Our cell culture studies with both immortal PC12 and rat primary hippocampal cells established GLP-1R expression and activation stimulated adenylyl cyclase to elevate intracellular cAMP in a manner similar to pancreatic  $\beta$ -cells [113]. GLP-1 and analogs induced differentiation in PC12 cells in a manner similar to nerve growth factor (NGF), inducing dendrite extension that was reversed by co-incubation with the selective GLP-1R antagonist (Ex 9-39 (Figure 1)). Across a variety of neuronal cell types, co-incubation of neurons with GLP-1 and/or Ex-4 enhanced the robustness of their neuronal phenotype, as exemplified by cholinergic cells expressing elevated levels of cholinergic markers (e.g., choline acetyltransferase) [122] and dopaminergic neurons heightened tyrosine hydroxylase activity [118]. GLP-1 analogs enhanced NGF initiated differentiation and rescued degenerating cells from NGF-mediated withdrawal in the absence of cellular dysfunction or toxicity [113]. This indicates that this incretin can provide trophic support in the absence of growth factors, which has been confirmed in other neuronal cell types [122]. The binding affinity of GLP-1 for receptors on hippocampal neurons ( $EC_{50}$  value of 14nM – a concentration apparently achievable [120]) is in the range of its binding to pancreatic  $\beta$ -cells

[99,100], and GLP-1 analogs provided complete protection against apoptotic cell death induced by glutamate, amyloid- $\beta$  ( $A\beta_{1-42}$ ), hypoxia and a variety of oxidative stressors (e.g.,  $Fe^{2+}$ ,  $H_2O_2$ , 6-hydroxydopamine) [113-115,118,120-122].

Our translational studies and the elegant work of others across numerous independent laboratories have demonstrated that GLP-1R agonists are effective in animal models of (i) AD: reducing levels of amyloid precursor protein (APP),  $A\beta$  and tau hyperphosphorylation, augmenting long-term potentiation (LTP), inducing synapogenesis, augmenting neurogenesis, suppressing neuroinflammation, reversing brain insulin resistance and improving memory performance [114,115,118,128-140]. (ii) Peripheral neuropathy: promoting neurite outgrowth and inhibiting neuron dysfunction and loss [117,141-144]. (iii) Stroke: reducing stroke volume and neuroinflammation, and augmenting neuronal survival and neurogenesis [118,145-149]. (iv) PD: augmenting dopaminergic cell survival, reducing neuroinflammation, increasing neurogenesis and improving motor coordination [118,150-152], which has recently translated to motor and cognitive improvements in moderately affected PD patients in an open label clinical trial [153]. (v) ALS: promoting motor neuron survival [122,154,155], (vi) Huntington's disease [119] and (vii) across a number of excitotoxic lesions models [114,155]. In defining cellular pathways mediating these neurotrophic/protective actions, thus far our studies implicate participation of PI3-kinase and ERK/MAPK dependent pathways, with additional involvement of protein kinase-A (PKA) signaling [72,121] to divert signaling away from apoptosis towards cell survival. In light of the numerous commonalities existing across these disorders and their occurrence in TBI (neuronal cell death, glutamate excitotoxicity, neuroinflammation, synaptic loss, etc.) we hypothesized that GLP-1R agonists would be useful to mitigate TBI on two complementary levels: (i) as neuroprotective/neurotrophic agents, and (ii) as anti-hyperglycemic agents – since TBI-induced hyperglycemia is associated with increased mortality and morbidity in humans with moderate to severe injury [38,157-159].

### Traumatic brain injury and GLP-1 receptor agonists

Our studies have focused on three complementary rodent models. In one, experimental concussive (weight drop) mild TBI was induced in anesthetized mice using the closed scalp, head trauma device described previously [21,22,25,36,37,42] in which a defined weight (up to 30 g) was dropped through a metal tube of fixed length (80 cm). The opening of the tube was positioned directly over the cushioned animal's head just anterior to the right ear, and the animal held in a manner such that the force of impact to the skull generated antero-lateral movements without any rotational movements, analogous to that occurring during closed head injury in a car accident. This induced a diffuse injury within the brain in which apoptotic neurons appear throughout the cerebral cortex and hippocampus both ipsi- and contralateral to the injury site [21,22]. Pro- and anti-apoptotic markers (epitomized by p53 and Bcl2, respectively) are up regulated by as little as a 5 g insult when evaluated at 72 h [21,22], with degeneration of neurons and their processes becoming apparent at insults of 15 g within cortical regions and 25 to 30 g in hippocampus and dentate gyrus, as evaluated by silver staining with verification of apoptosis by TUNEL staining [21,22] as well as by Fluoro-Jade B and NeuN immunohistochemistry [25]. Likewise, markers associated with mitochondrial dysfunction and failure, cytochrome-c and Bax, together with markers of



inflammation, TNF- $\alpha$ , are found to be elevated within 8 to 72 hours [24,25,160]. Interestingly, when evaluated with a broad neurological battery 1 or 24 hours post-injury, it is not possible to differentiate mild TBI from control (uninjured) mice. However, with the use of selected behavioral paradigms initiated as early as 72 hours (forced swim/Porsolt test [24]) or at 7 and 30 days for learning and memory tests (such as Y-maze and novel object recognition paradigms [161-164]) mild TBI and uninjured animal can readily be differentiated, and such cognitive deficits in spatial and non-spatial tasks have been shown to persist for as long as 90 days in the absence of gross neurological sequelae [42], as occurs in the human condition [1].

The use of the very same mouse strain in a 'real life' blast injury mild TBI model [49] allows one to not only assess the potential of experimental treatment strategies but, importantly, to also compare between deficits and associated mechanisms induced by blast and concussive mild TBI. Our blast mild TBI model involves a controlled explosion (500 g TNT) detonated 4 and 7 m from anesthetized mice to expose them to a blast pressure of 5.5 and 2.5 PSI, respectively, in line with human exposure to an improvised explosive device. Similar to our concussive (weight drop) mild TBI model, a diffuse neuronal injury occurs, with axonal and myelin abnormalities, and the development of persistent select cognitive deficits in the absence of alterations in basic neurological assessment or brain gross pathology [49]. Interestingly, whereas both forms of mild TBI are distinctly different regarding the mechanism of trauma induction (blast vs. concussive injury), there are striking similarities in the cognitive and affective status of exposed animals and, similar to humans, the examined indices of cognition and anxiety-like behavior in mice subjected to both forms of TBI were largely alike [165]. Contrasting with these similar mouse behavioral outcomes following either concussive or blast mild TBI, we observed marked differences in gene expression profiles by RNA extracted from intact TBI hippocampal tissue. Although there was a degree of overlap in genes co-regulated by the two distinctly different mechanisms of injury, notably, there were a larger number of genes uniquely expressed in an injury specific manner. Consequently, molecular pathways, particularly associated with ribosomal proteins, the electron transport chain, oxidative stress, inflammation and neurogenesis, displayed highly similar patterns of injury-dependent regulation. However, while these data suggest the involvement of common cascades initiated in response to different types of head injury, there was a divergence in individual gene expression within these pathways, suggesting that the two types of injury (blast vs. concussive mild TBI) are different at the molecular level [165]. Interestingly, pathways associated with AD displayed a markedly different form of regulation depending on the type of TBI [165]. Furthermore, particularly in the blast mild TBI model, changes were evident in other pathways associated with neurological disorders, including the 'Parkin Pathway' involving genes associated with PD, as well as genes found to be reduced in major depressive disorders and schizophrenia [165]. This is in line with multiple studies linking TBI to long-term neuropsychiatric/ neurodegenerative disorders as recently reviewed by Walker & Tesco [166].

Remarkably, administration of a clinically translatable dose of Ex-4 to mice with either form of mild TBI (blast or concussive), whether administered prior to or shortly following insult, fully ameliorated the cognitive deficits [161,162] (Figure 3). Importantly, the mild TBI-induced gene expression changes, including genes associated with AD, were largely

prevented by Ex-4 both at the pathway analysis level as well as at the individual gene level, as illustrated in Figure 4 for mice exposed to concussive (weight drop) mild TBI. As described, blast mild TBI primarily impacted the very same gene pathways as shown changed for concussive mild TBI, albeit that in large part different individual genes were up and down regulated by blast mild TBI within these pathways [165]. Our studies indicate that Ex-4 likewise ameliorated the majority of these at individual and pathway levels. Hence, taken together, these data suggest a strong beneficial action of Ex-4 in managing secondary events induced by these two very different forms of mild TBI.

In light of the fact that no single animal TBI model perfectly mimics the human condition [40], additional studies to verify the activity of Ex-4 in moderate TBI were undertaken – specifically, in rat following fluid percussion injury. As the most commonly used rodent model of moderate TBI, fluid percussion injury replicates many of the key cognitive and histological changes evident in human head injury [167,168]. Of the two variations of the model developed, central (i.e., midline) fluid percussion injury was induced in anesthetized animals that produces both focal and diffuse pathology. This generates both primary (a local contusion, edema, and hemorrhage at the gray/white matter interface) and secondary damage (gliosis, inflammation, axonal injury, and delayed cell death) [166,167] and, similar to human TBI, this model adversely impacts hippocampal function and produces long-term cognitive and memory deficits that can last for weeks or months after injury [169,170]. Administration of Ex-4 post injury at a dose comparable to the above described mild TBI mouse studies dramatically mitigated fluid percussion injury-induced cognitive impairments, as assessed in the classical Morris water maze paradigm [171].

Interesting and further supportive evidence for GLP-1R activation as a therapeutic strategy for TBI derives from the elegant studies of Heile and Brinker [172,173], who, together with colleagues, have developed an approach in which encapsulated human mesenchymal stem cells generate and secrete steady-state levels of GLP-1. The implantation of these cells in the lateral ventricle of rats subjected to controlled cortical impact-induced moderate TBI significantly attenuated hippocampal neuronal cell loss as well as cortical glial and neuronal cyto-skeletal abnormalities [172].

## Conclusions

In summary activation of the GLP-1R mediated incretin pathway has been demonstrated to be both neurotrophic and neuroprotective across a broad number of cellular and animal neurological models, and to provide functional and behavioral benefits across a wide range of pathophysiological paradigms. These have been seen in independent laboratories, and verified across different animal models of the same disease in the fields of AD, PD, stroke, peripheral neuropathy and, more recently, ALS and TBI (71-79). In large part, commonalities in fundamental biochemical cascades appear to be triggered across diverse acute and chronic neurological disorders that cause neuronal cell populations associated with defined disease to become dysfunctional and undergo apoptosis. The GLP-1 signaling pathway can clearly counter-balance these and, as has been successfully undertaken for GLP-1R activation in pancreatic  $\beta$ -cells, these pathways are now being mapped for the GLP-1R on neurons [72,120]. Not only does the GLP-1R appear to be present across a

number of neuronal cell types (e.g., motor neurons, neural stem cells, etc.) that underlies its diverse actions to provide neurotrophic/protective actions, augment long-term potentiation and synaptic plasticity, and favorably impact neurogenesis, but its appearance on other cells types, for example on multiple immune cell subpopulations [174-176], and glial cells [177] accounts for other positive actions as well, for example suppression of proinflammatory cytokines and reduced microglial activation within the brain. Most likely it is the combination of these actions, rather than any specific and separate one, that supports the current promise of GLP-1R activation as a therapeutic strategy across neurological disorders, including TBI. Maximizing the translational potential of the strategy is clearly crucial, although three GLP-1R agonists are approved and well tolerated in T2DM [80-84] and promising clinical studies in PD [153] support repositioning for neurological disorders. Careful and hypothesis-driven preclinical and clinical studies are warranted [178] to define the optimal neurological use of this promising drug class.

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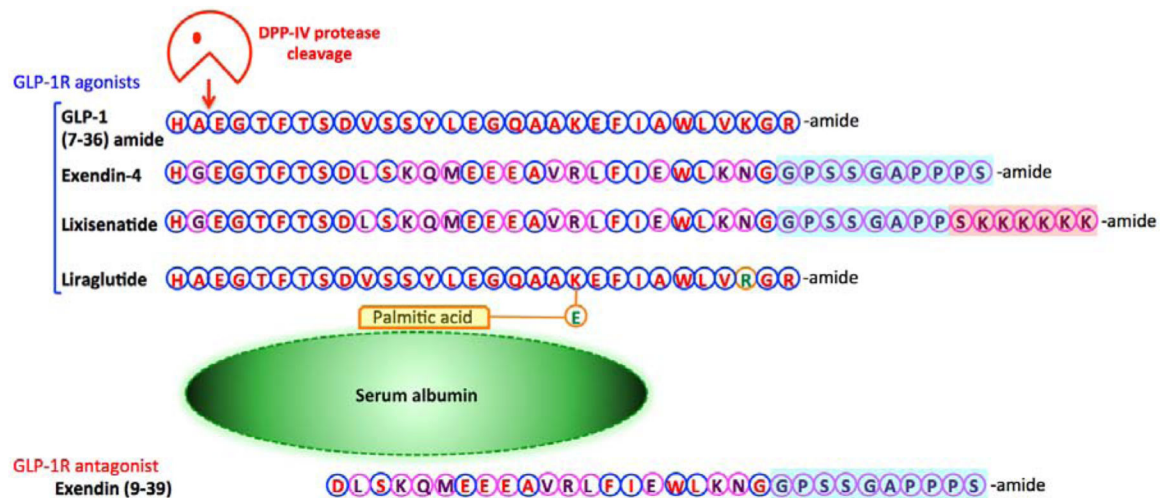


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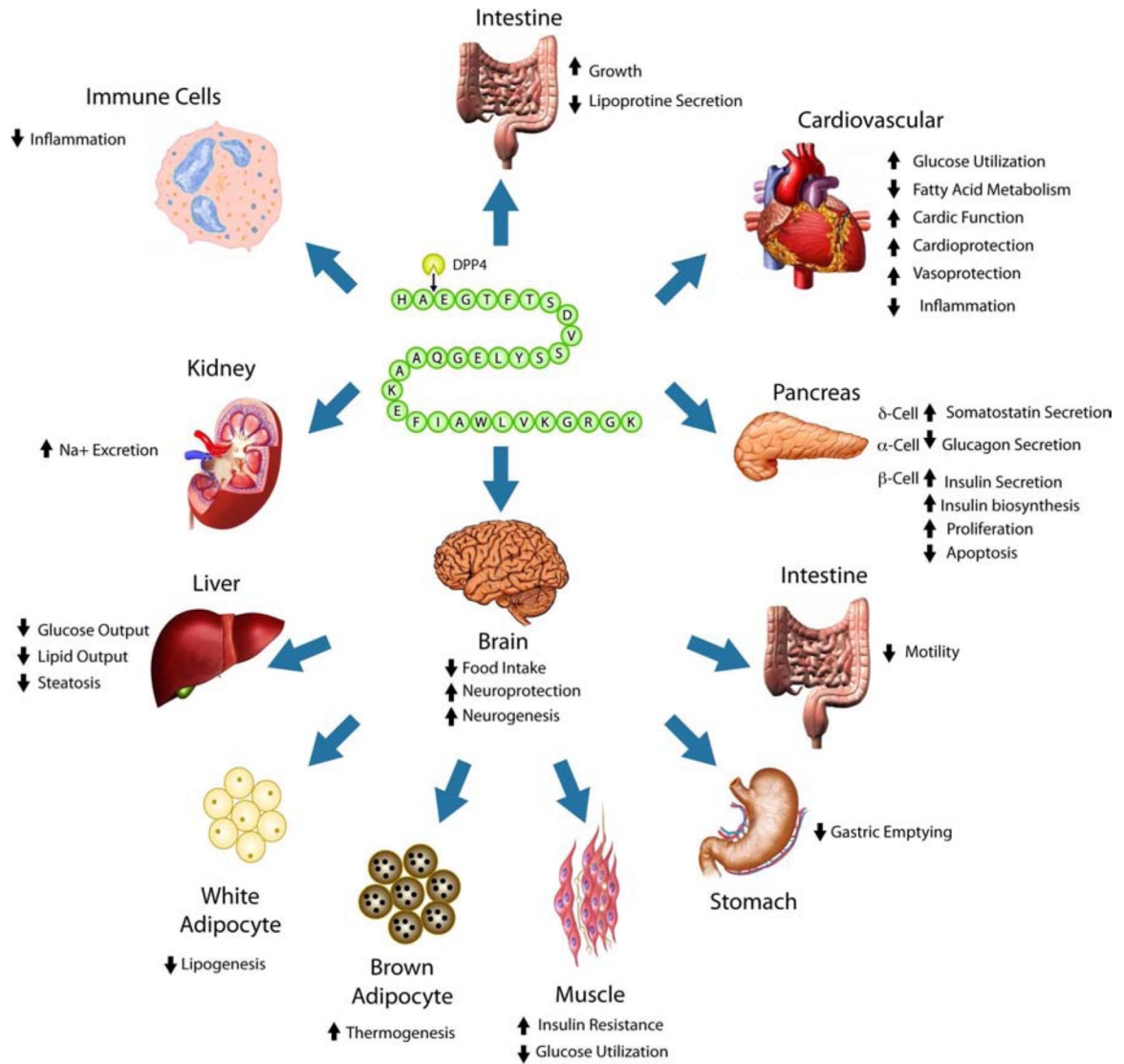
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**Figure 1.**

Amino acid sequence of GLP-1 and that of the long-acting GLP-1 analogs, exendin-4 (Ex-4), lixisenatide and liraglutide. Ex-4 is known clinically as Byetta and Bydureon for subcutaneous (s.c.) twice a day and extended release (once weekly) dosing, respectively. Lixisenatide, known clinically by its trade name Lyxumia, and liraglutide (Victoza) are administered s.c. once daily. Amino acid homology (blue circles) and differences (fuchsia circles), in comparison to GLP-1, are highlighted. The peptidase cleavage of GLP-1 by DPP-IV is noted. Of relevance Ex-4 and lixisenatide are close analogues that differ in their tail region. GLP-1 and liraglutide are likewise close analogues, with the latter possessing a C-16 fatty acid (palmitic acid) with a glutamic acid spacer attached to the lysine residue at position 26, permitting its binding to albumin to augment its half-life. By contrast, exendin (9-39) is a widely used pharmacological tool that is an antagonist at the GLP-1R.

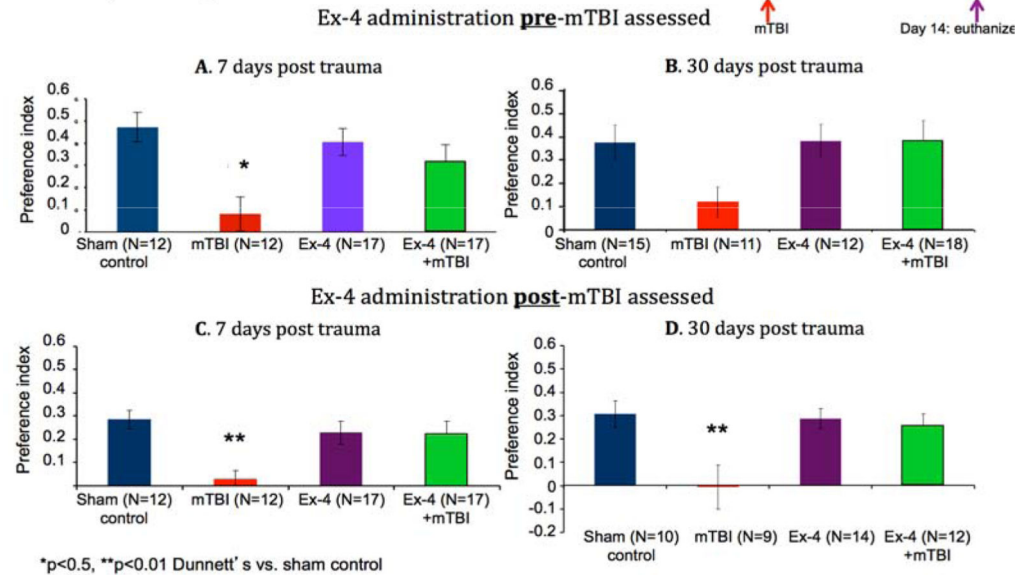


**Figure 2.**

Direct (upper) and indirect (lower) pharmacological actions of GLP-1R agonists. (Upper) GLP-1 agonists act directly by stimulating the GLP-1R to induce multiple coordinated actions at the level of pancreatic islet cells, within the heart, gastrointestinal tract, on subsets of immune cells and within the brain. (Lower) Within the brain, that GLP-1R agonists appear to freely enter, activation of select GLP-1R signaling pathways instigates biological actions within the gastrointestinal tract, liver and adipose tissue. Importantly, the activation of GLP-1R signaling cascades within the central and peripheral nervous systems appear to underpin the benefits of GLP-1R agonists described in preclinical animal models of AD, PD, stroke, ALS, Huntington's disease and, more recently TBI (adapted from Campbell and Drucker [82] and Salcedo et al., [75]).

### Weight drop (30 g) concussive mTBI

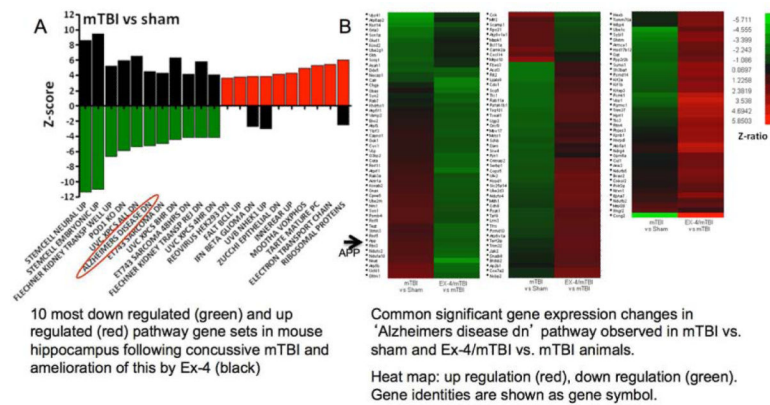
#### Novel object recognition



**Figure 3.**

Ex-4 Post-treatment protects against concussive mild TBI (mTBI)-induced cognitive loss, as assessed by novel object recognition at 7 day and 30 day testing post mTBI in two separate groups of mice (a similar protection was provided in a blast-TBI model that closely mimics military personnel trauma). Concussive (30 g weight drop) mTBI induces an impairment in visual memory (red column), as assessed by the novel object recognition paradigm, that was fully ameliorated when Ex-4 was administered (green column) pre-trauma (**A** (7 days), **B** (30 days)) and post-trauma (**C** (7 days), **C** (30 days)). Mice undergoing cognitive testing initiated on day 7 were euthanized on day 14, and their hippocampus was subjected to gene expression analyses.

The mouse Ex-4 dose was 3.5 pM/kg/min (subcutaneously administered as a steady-state dose), which is 21 ug/kg/day and equivalent to a dose of 1.7 ug/kg/day in a human following normalization of body surface area between mouse and human. This dose compares favorably to once weekly exenatide LAR: 2 mg/week that provides a 60 kg human subject 4.8 ug/kg/day. Significantly impaired behavior compared to sham (uninjured) controls: Fisher's LSD post hoc, \*p<0.05, \*\*p<0.01]. Performance of mice was quantitatively assessed 7 and 30 days following mTBI as a preference index, calculated as (time near the new object–time near the old object)/(time near the new object+time near the old object). Values are mean±SEM, [adapted from 161].



**Figure 4.**

Gene array analyses of the impact of concussive (30 g weight drop) mTBI (versus uninjured controls) on the hippocampus of animals derived from Figure 3, and the ameliorative effects of Ex-4 administration. **(A)** Pathway analysis: the effects of mild TBI (mTBI) vs. sham on the 10 most down-regulated (green) and up-regulated (red) pathway gene sets in mouse hippocampus are shown (pathway Z-scores are presented). The effects of treatment of mTBI with Ex-4 are shown in the **black** bars. In these black bars Ex-4 treatment of mTBI induced a change in gene sets relative to the mTBI group. Where no black bar is present, Ex-4 had no effect on gene sets relative to mTBI. In large part, treatment with Ex-4 prevented the down-regulation of the 10 most affected pathways associated with mTBI, while Ex-4 treatment had beneficial effects upon three of 10 up-regulated pathways.

**(B)** Selecting the 'Alzheimers disease dn' pathway as a representative of those whose changes induced by mTBI were ameliorated by Ex-4, the pathway is opened to reveal the individual genes that comprise it (whose gene identities are shown as their gene symbol), presented as a classic heat map: up-regulation (red) and down-regulation (green). Remarkably, across the majority of individual genes mTBI-induced up-regulation is countered by Ex-4 mitigation. APP up-regulation by mTBI is noted, in line with TBI providing a conduit towards AD, which is mitigated by Ex-4. The scale for expression level is shown on the upper right, and the group comparisons are shown at the bottom of each section on the heat map. Adapted from Tweedie et al., [162].