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Review article

Interactions of Glutamate and Gamma Amino Butyric Acid with the insulin-like growth factor system in traumatic brain injury (TBI) and/or cardiovascular accidents (CVA or stroke): A systematic review

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ABSTRACT

The brain maintains homeostasis of neural excitation in part through the receptor-mediated signaling of Glutamate (Glu) and Gamma Amino Butyric Acid (GABA), but localized injuries cause cellular release of excess Glu leading to neurotoxicity. The literature strongly supports the role of Insulin-like growth factor-1 (IGF-1) in adult brain neuroprotection and repair, and research supporting the existence of molecular interactions between Glu, GABA, and IGF-1 *in vitro* and in normal animals raises the question of whether and/or how the Glu/GABA system interacts with IGF-1 post-injury. This systematic review was undertaken to explore works addressing this question among adults with a history of traumatic brain injury (TBI) and/or cerebrovascular accident (CVA; stroke). The literature was searched for human and animal studies and only four animal papers met inclusion criteria. The SYRCLE criteria was used to evaluate risk of bias; results varied between categories and papers. All the included studies, one on TBI and three on stroke, supported the molecular relationship between the excitatory and IGF-1 systems; two studies provided direct, detailed molecular evidence. The results point to the importance of research on the role of this protective system in pathological brain injury; a hypothetical proposal for future studies is presented.

1. Introduction

Research literature supports the concept that Glutamate (Glu) and Gamma (ɣ) Amino Butyric Acid (GABA) play important roles in the homeostatic control of excitation and inhibition of neural responses in the normal brain (e.g., see review by Guerriero et al., 2015). However, when the brain is injured, excessive glutamate is released into the extracellular space. Downstream pathologic events include further neural tissue and cell damage (excitotoxicity). The overall goal of this systematic review is to examine the current research literature on potential attempts of the brain to repair or counteract the excitotoxic damage caused by traumatic brain injury (TBI) and/or cardiovascular accident (CVA; stroke) by activation of neuroprotective factors. Specifically, we focus on the potential links between Glu, GABA and Insulin-like Growth Factors (IGFs). As overviewed below, IGF-1 is a leading candidate for potential clinical applications based on the extant literature supporting its neuroprotective and neuro-regenerative functions in the mature brain. Further, there is strong support for the existence of regulatory cellular and molecular links between IGF and the Glu/GABA systems from research in cultured cells and explants and in normal and long-lived animals. Recent data on animals and human longevity implicates a molecular link between the excitation and IGF-1 systems in the successful aging process (Zullo et al., 2019). Establishing that these tri-molecular interactions function in disease states such as TBI and stroke may inform the molecular pathways underlying disease progression, the procedures for planned and ongoing trials (e.g., see review by Hayes et al., 2021), and potentially increase the number of key targets for further investigation.

There are two types of CVA: 1) an ischemic event occurs when blood supply or other particles block the blood supply to part of the brain; and, 2) a hemorrhagic stroke occurs when a blood vessel bursts in the brain

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(https://www.cdc.gov). Ischemic stroke is the most common form of CVA, accounting for approximately 71% of all strokes globally (Campbell et al., 2019; Feng et al., 2020; The GBD 2016 Lifetime Risk of Stroke Collaborators, 2018). The animal models of ischemia-reperfusion used in the studies reviewed here are most closely related to ischemic stroke in humans. Mechanistically, ischemia is part of the complex series of events following TBI when contusions disrupt or lead to occlusion of the normal cerebrovascular circulation to affected cerebral tissues (Kowalsky et al., 2017). A TBI is defined as a "disruption in the normal function of the brain that can be caused by a bump, blow, or jolt to the head, or penetrating brain injury" (https://www.cdc.gov). TBI and CVA were selected for this review as based on the robust literature on the relevant cellular and molecular events in these disease states, as well as the interests of this laboratory.

1.1. The brain excitation system and excitotoxicity

The imbalance between the Glu and GABA systems following TBI and/or stroke has been well documented (Imbrosci and Mittman, 2011). For a comprehensive review of homeostatic and post-TBI mechanisms of Glu and GABA action, including description of receptor classes and their roles. As a brief overview, Glu is normally stored within vesicles in the pre-synaptic neuron until a de-polarizing current leads to its controlled release into the synapse, thereby positioning it for the activation of signaling receptors in post-synaptic cells. Excess Glu in the synapse is taken up by its transporter protein (the excitatory amino acid transporter2 (EAAT2)/glutamate transporter-1 (GLT-1)) and shuttled to neighboring astrocytes for metabolic conversion to glutamine, which is then transported back to pre-synaptic neurons to serve as precursor for Glu synthesis. In addition to GL-1, the glutamate transporter known as GAST (and its human counterpart the excitatory amino acid transporter-1 (EAAT1) is considered a second major glutamate transported in the brain (Pajarillo et al., 2019).

Following cell injury or death, Glu is released into the extracellular space in excessive quantities leading to increased excitation signaling. In several pathologic processes, including ischemic injury and severe TBI, the transporter protein EEAT2 that normally carries Glu to astrocytes for metabolic processing is downregulated (Guerriero et al., 2015). This might be expected to dampen the normal clearance mechanism and contribute to pathology.

Glutamine also serves as a precursor for GABA in GABAergic neurons, where it is first converted to Glu and then rapidly to GABA by the action of glutamate decarboxylase. Like Glu, GABA is normally stored in pre-synaptic neurons and following its release into the synapse it activates GABA receptors or it is bound by its transporter protein, GAT-1, and shuttled back to the presynaptic neuron. The GAT-3 transporter protein also helps to regulate extracellular GABA concentrations and GABA tonic and phasic inhibition (Kersante et al., 2013; Melone et al., 2014). Extant evidence indicates that inhibitory GABA signaling is reduced relatively fast following lesion induction (Imbrosci and Mittman, 2011) and only slowly returns to a subnormal level at two months post-lesion.

1.2. The insulin-like growth factors (IGFs)

As noted above, the Insulin-like Growth Factor (IGF) system has been strongly implicated in repair of the central nervous system (CNS) (Anderson et al., 2019; Mangiola et al., 2015 (review); Santi et al., 2018; Trejo et al., 2007). IGF-1 is the main signaling ligand in this growth factor system, and therefore the major focus of this short introduction. The more limited but important work on the IGF-binding proteins in response to injury will also be addressed in the Results section within the context of their interactions with the Glu and/or GABA systems. For more detailed information about the IGF molecular system, including the IGF-2 ligand, signaling receptors and binding proteins, please see Dyer et al. (2016); Lewitt & Boyd (2019) and Pardo et al., 2019).

The evidence for the role of IGF in TBI includes the finding that increases in IGF-1 mRNA and protein are consistent responses to brain injury; indeed, the IGF-1 protein increases in or near contusion sites (Madathil et al., 2010; Sandberg, Nordqvist et al., 1997; Santi et al., 2018; Walter et al., 1997). Increased IGF-1 localizes to reactive microglial cells, astrocytes, and neurons within one to three days post-injury (Fernandez and Torres-Aleman, 2012; Santi et al., 2018; Walter et al., 1997). Further evidence implicating IGF-1 in reparative functions comes from studies of the systemic and/or intracerebral administration of IGF-1 to animal models of TBI. For example, Santi et al. (2018) showed that serum IGF-1 specifically infiltrates brain sites injured by controlled cortical impact, and that chronic subcutaneous administration of IGF-1 leads to a slow but significant recovery of sensorimotor functions, as observed at 4 weeks of treatment. An important observation of this study was that even though IGF-1 naturally increased at injury sites via de novo synthesis, cellular translocations and/or infiltration from serum, this increase was insufficient to restore sensorimotor functions, which required additional exogenous IGF-1. Similarly, Carlson & Saatman (2018) reported that chronic intracerebroventricular infusion of IGF-1 for 7 days following TBI induction increased IGF-1 in the cortex and hippocampus of the injured mice, generated increased number of immature neurons in the hippocampus (neurogenesis), and resulted in improved motor and cognitive functions. Madathil et al. (2013) showed an increase in hypertrophic astrocytes and associated IGF-1 by 72 h post-trauma and greater neuron survival at 10 days after injury. These changes were accompanied by improved motor and cognitive responses.

Increases in endogenous IGF-1 have also been reported following hypoxic-ischemic brain injury in animal models of ischemic stroke (Belharz et al., 1998; Schwab et al., 1997) and exogenous IGF administration has been linked to its accumulation at peri-infarct sites, as well as to reparative functions that include increased blood vessel density and blood flow, neuronal cell preservation, increased neurogenesis, and, transcriptional down-regulation of inflammatory mediators in the affected site or ischemic hemisphere (Guan et al., 1992; Selvamani et al., 2012; Serhan et al., 2020; Zhu et al., 2009). Further, multiple clinical connections between IGF-1 and ischemic stroke have been observed in humans and recently summarized by Hayes et al. (2021). The results of the large Framingham cohort study are noteworthy, as the results showed a significant inverse correlation between levels of IGF-1 in the circulation and stroke incidence. However, it is not known if or how much of the effect of IGF on stroke incidence risk is direct.

1.3. Molecular interactions of the excitation and IGF systems In Vitro

Studies of cerebellar granule neurons led to the notion that GABA receptor B can transactivate the IGF-1 receptor to enhance cell survival (Tu et al., 2010). In other words, the activated $GABA_B$ receptor (a G protein metabolomic receptor) can "hijack" the IGF-1 receptor's intracellular machinery to augment GABA's direct signaling of pro-survival effects. .

Detailed molecular analysis using creative molecular tools to isolate, stabilize, and activate the $GABA_B$ receptor enabled investigators to isolate the receptor with its associated molecular complexes and to study the exact sequence of events underlying the transactivation events (Lin et al., 2012). The results showed that the two receptors ($GABA_B$ and IGF-1) and their associated cytoplasmic proteins formed a multimolecular complex. A critical step initiating transactivation involved the activation of Focal adhesion kinase (FAK) by the $GABA_B$ receptor, which helped to bridge the two receptor complexes and to initiate the activation of IGF signaling intermediates, including Akt protein kinase (Figure 1). Akt is believed to be a key determinant of the downstream functions of IGF in neuroprotection.

Transactivation activities are important, not only because they link different signaling pathways, but also because they bypass the need for the ligands to bind their extracellular receptors and any associated

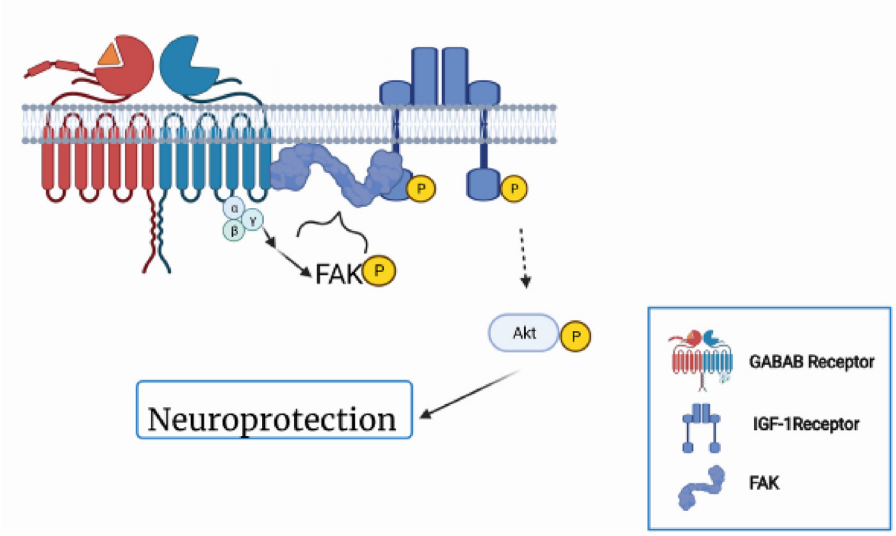


Figure 1. Rendition of the Transactivation of the $GABA_B$ Receptor. The diagram presents a simplified version of the Transactivation of theIGF-1 Receptor by the $GABA_B$ Receptor, based on published work of Tu et al. (2010). Activation of the $GABA_B$ receptor by the GABA ligand leads to the activation of FAK (focal adhesion kinase), which is believed to form a molecular bridge linking the $GABA_B$ and IGF-1 receptors and their relevant intracellular pathway proteins. This complex formation is involved in the intracellular activation of the IGF-1 receptor and downstream AKT protein leading to neuroprotection. Figure created with Biorender.com.

controls. In the case of IGF-1, these activities might circumvent extracellular regulatory controls imposed by the IGF-binding proteins. The metabotropic glutamate receptor (mGlu2) has also been implicated in the transactivation of the IGF-1R pathway through the intermediate action of Focal adhesion kinase (FAK) (Hu et al., 2019). In this case transactivation of IGF intermediates channels the signaling cascade to the extracellular signal regulated kinase ½ (ERK ½) pathway.

The transactivation experiments summarized above relied on cellular responses in culture, mostly of fetal or neonatal origin, and/or isolated molecular complexes, but they open interesting questions of their relevance to adult *in vivo* systems (e.g., how these processes might be integrated in time and place following TBI). These questions and issues notwithstanding, the results of adult animal studies are consistent with, and generally support, the *in vitro* findings. For example, in a rat model of ischemia/reperfusion, activation of $GABA_A$ and $GABA_B$ receptors via infusion of the corresponding receptor agonists mucinol and baclofen led to neuroprotection of hippocampal CA-1 pyramidal cells (Xu et al., 2008). This protection was reduced when two intermediates in the IGF signaling pathway were inhibited (PI-3 Kinase activity and downstream Akt phosphorylation). This study supports the concept that GABA signaling stimulates the effect of IGF-1 on neuroprotection. Another example includes the link between glutamate and IGF-1 signaling, as supported by early studies in the cerebellum (Castro-Alamancos and Torres-Aleman, 1993). These investigators showed that injections of glutamate into the cortex and deep cerebellar nuclei of normal rat brains resulted in release of GABA and that co-administration of glutamate and IGF-1 (but not pre-glutamate administration of IGF-1) could inhibit this effect. Again, these experiments suggested interrelationships between the Glu and IGF-1 signaling systems even though precise mechanisms of action, including possible transactivations were not explored. Taken together, studies to date indicate that the Glu/GABA and IGF systems are functionally related under some circumstances, even though the direction of the effects (i.e., maintenance of neurotoxicity or neuroprotection) might depend on brain and cell localization, event timing, and/or pathophysiological status.

2. Method

We followed PRISMA guidelines for systematic reviews (Moher et al., 2009). In order to answer the question posed for the systematic review, we initially searched for publications in both the human and animal literature. Given the lack of relevant publications regarding these conditions among humans, we present findings from animal models of TBI and stroke.

2.1. Systematic review protocol

The present review was conducted using the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) method (Moher et al., 2009). This included a rigorous assessment of bias, with a thorough search strategy. Key questions (KQ) investigated the regulatory molecular interactions between the excitatory (e.g., glutamate, GABA) and IGF systems post TBI and/or CVA. A protocol for our review of animal studies is registered in PROSPERO International prospective register of systematic reviews under registration number CRD42021227570.

2.2. Study selection criteria

Eligibility Criteria were defined according to the PI(E)CO(TS) framework (Moher et al., 2009):

Population – Animals that receive a defined injury known to initiate a TBI or CVA-like condition.

Intervention/Exposure – Modification of IGF, GABA, and/or glutamate function(s) as defined in each study.

Comparator - No specific comparison group required for inclusion.

Outcome - Molecular interactions between glutamate or GABA and insulin-like growth factors among animal models of TBI and/or CVA.

Timing/Setting - Restrictions were not based on timing, setting, or study design.

Only studies including original data published in a peer-reviewed journal were included.

2.3. Literature search strategies

Databases were searched since inception using controlled subject heading vocabulary and key words for TBI or stroke, combined with controlled subject heading vocabulary and key words for IGF and GABA, or IGF and glutamate (see Table 1). Final searches included the combination of IGF terms with GABA and/or glutamate terms and TBI and/or stroke terms (Table 1, item 24) and excluded immature subjects (Table 1, item 25). Searches were limited to English. References were mined for additional studies. The final literature searches of OVID Medline, EMBASE, Web of Science Core Collection, Cochrane Library, and Google Scholar were conducted on January 7, 2021 Complete references were

Table 1. OVID medline search strategy.

1 stroke/or stroke, lacunar/or cerebral infarction/or infarction, anterior cerebral artery/or infarction, middle cerebral artery/or infarction, posterior cerebral artery/or brain infarction/or cerebrovascular disorders/or Brain Ischemia/or Brain stem infarctions/or cerebral hemorrhage/or basal ganglia haemorrhage/or Intracranial Hemorrhages/or Subarachnoid Hemorrhage/or Basal ganglia cerebrovascular disease/
2 (stroke* or hemorrhage* or haemorrhage* or ischemia* or ischaemia* or "basal ganglia cerebrovascular disease" or "basal ganglia cerebrovascular diseases" or post-stroke*).tw,kw.
3 ((cerebral or brain or cerebrovascular or "brain stem" or "basal ganglia" or intracranial or subarachnoid) adj1 (infarction* or ischaemia* or ischemia* or disorder* or hemorrhage* or haemorrhage*)).tw,kw.
4 1 or 2 or 3 *(Combination of all terms to search for stroke)*
5 exp Brain Injuries/or exp Brain Edema/or exp Cerebrovascular Trauma/or exp Craniocerebral Trauma/or exp Coma/or exp Glasgow Outcome Scale/or exp Glasgow Coma Scale/
6 ((brain* or capitis or cerebr* or crani* or hemispher* or intercran* or intracran* or inter-cran* or intra-cran* or skull*) adj4 (contusion* or damag* or fractur* or injur* or trauma* or wound*)).tw,kw.
7 ((brain or crani* or cerebr* or head or intercran* or intracran* or inter-cran* or intra-cran*) adj4 (bleed* or haematoma* or haemorrhag* or hematoma* or hemorrhag* or pressure)).tw,kw.
8 ("Glasgow coma scale" or "Glasgow coma score" or "Glasgow outcome scale" or "Glasgow outcome score" or "Glasgow coma scales" or "Glasgow coma scores" or "Glasgow outcome scales" or "Glasgow outcome scores").tw,kw.
9 ((brain or cerebral or intracranial) adj3 (edema or oedema or swell*)).tw,kw.
10 ("diffuse axonal injury" or "diffuse axonal injuries" or mtbi or whiplash).tw,kw.
11 ("fluid percussion injury" or "controlled cortical impact" or "weight drop injury" or "fluid percussion injuries" or "controlled cortical impacts" or "weight drop injuries").tw,kw.
12 5 or 6 or 7 or 8 or 9 or 10 or 11 *(Combined search terms for TBI)*
13 exp Receptor, IGF Type 1/or exp Receptor, IGF Type 2/
14 ("insulin-like growth factor" or "insulin-like growth factors" or "insulin like growth factor" or "insulin like growth factors" or igf).tw,kw.
15 13 or 14 *(Combined search terms for IGF)*
16 exp gamma-Aminobutyric Acid/or exp receptors, gaba/or exp GABA Agents/or exp GABA Plasma Membrane Transport Proteins/or exp GABAergic Neurons/or exp GABA Uptake Inhibitors/
17 ("gamma-aminobutyric acid" or "gamma aminobutyric acid" or gaba or gaba-a or gaba-b or "gaba a" or "gaba b" or "gaba receptor a" or "gaba receptor-a" or "gaba-receptor a" or "gaba receptor b" or "gaba receptor-b" or "gaba-receptor b" or gabapentin or gabaergic or gabitril or tiagabine).tw,kw.
18 16 or 17 *(Combined search terms for GABA)*
19 exp Glutamates/
20 ("glutamic acid" or "glutamic acids" or glutamate or glutamates or "excitatory amino acid" or "excitatory amino acids").tw,kw.
21 19 or 20 *(Combined search terms for glutamate)*
22 4 or 12
23 18 or 21
24 15 and 22 and 23 *(Final search strategy for inclusions:[combined IGF terms]and [combined stroke or TBI terms] and [combined GABA or Glu terms]*
25 24 not ((exp child/or exp Adolescent/or exp Infant/) not exp Adult/)
26 limit 25 to english language

Note: Database: OVIDMEDLINE (R) ALL 1946 to January 06, 2021.

exported into EndNote X9, duplicates were removed, and then imported into Covidence review software (https://www.covidence.org).

2.4. Data extraction

The PRISMA literature flow diagram is shown in Figure 2. For the screening phase, two reviewers (TM and TK) selected relevant abstracts and disagreements in the selection process were resolved by KSY with consensus from the reviewers. The final papers elected in the eligibility phase were selected by two reviewers (TM and HW) and disagreements resolved by LAB with consensus from the reviewers.

Data from included articles were abstracted into an evidence table (Table 2) by the lead author with final group consensus. Extracted data from each article included a description of the injury model, sample, experimental question and approach, outcome collection measures, and outcome results.

2.5. Risk of bias

Studies included in this review were independently evaluated for study design and risk of bias (RoB) by two reviewers (ASH, KSY), and was followed by critical discussions (ASH and TM) with consensus. Included studies were assessed for RoB using the SYRCLE's quality assessment tool for animal studies. The SYRCLES tool groups items for bias by domain: selection, performance, detection, attrition, reporting, and other bias. To inform the study design appraisal, studies were classified using the Taxonomy of Study Design Tool. Each of the RoB domains, if applicable, was rated as low, high, or unclear. Ratings were based only on information reported in the published study.

2.6. Data analysis

Variability of study designs and outcome measurement precluded a meta-analytic approach to synthesis. Results were not quantitatively synthesized because only 4 studies met inclusion criteria and there were diverse measurement approaches to assess outcomes relevant to the review KQs. Therefore, a descriptive synthesis approach was used.

3. Results

The SYRCLE evaluations are presented in Table 2 and discussed in the next section (DISCUSSION).

Please see Table 3 for a summary of the methods and results of each individual study, including the injury model used, the species weight/age and number of animals, the experimental question, approach, outcome measures and results.

3.1. Study by Sandberg Nordqvist et al. (1997)

This work examined the transcriptional changes of the IGF-1 and IGF binding proteins 2 and 4 (IGFBP-2 and IGFBP-4) genes in a rat model of mild TBI induced by controlled concussion. It was shown that IGF-1, IGFBP-4, and IGFBP-2 mRNAs were induced in the cortical and hippocampal tissue adjacent to the contusion site at 2 days following injury. The increase in binding protein mRNAs extended along the ipsilateral cortex. To examine the potential role of glutamate in the activation of these responses, the investigators used inhibitors targeted at the two types of ionotropic glutamate receptors: a non-competitive antagonist of the N-methyl-D-aspartate (NMDA) receptor (MK-801) and an antagonist of the α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptor (CNQX). These inhibitors or the saline controls were injected i.p. into the animals at 30 min prior to and two days following contusion. The MK-801 inhibitor was effective in blocking IGF-1 and IGFBP-4 mRNA increases close to the contusion site and also the IGFBP-2 mRNA increases at the cortex temporal to the contusion site (but not close to the contusion site). CNQX was also able to block IGF-1, but not the binding proteins, suggesting that glutamate can signal through either receptor to regulate IGF-1 mRNA levels, but the induction of the binding proteins occurs only through the NMDA receptor. Overall, the paper provided evidence for a molecular link between glutamate signaling and increased transcriptional activation of various members of the IGF family in an animal model of mild TBI. The authors suggested that the combination of glutamate receptor inhibition and IGF-1 treatment might increase neuronal survival.

The other three studies evaluated in this systematic review were based on studies on animal models of ischemia (impaired blood flow to the brain).

3.2. Study of Garcia-Galloway et al. (2003)

The study by Garcia-Galloway et al. (2003), explored whether insensitivity to the pro-survival actions of IGF-1 is a feature of excess glutamate signaling. Initially, this possibility was examined in cultured cerebellar granule cells treated with cytotoxic doses of glutamate (100–500 μM). This treatment promoted cell death and inhibited IGF-1 signaling.

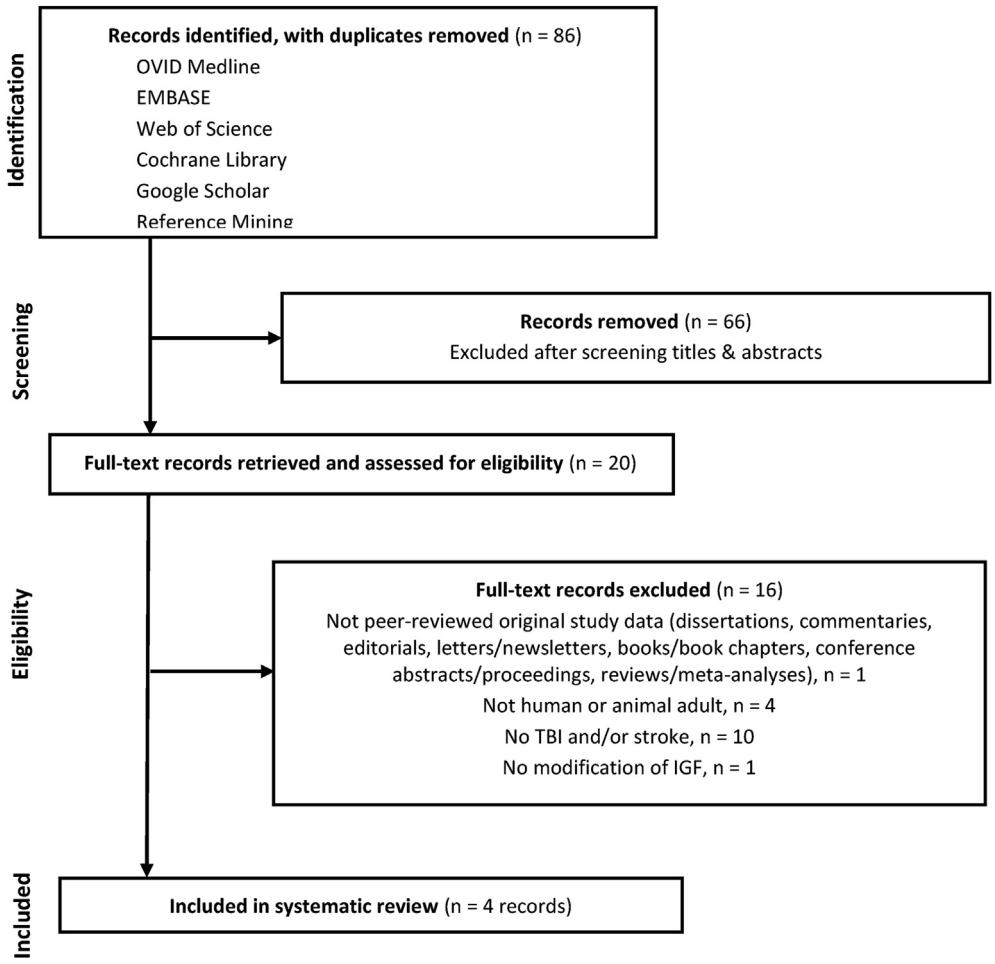


Figure 2. PRISMA literature flow diagram.

Then, the specific step(s) involved in IGF-1 inhibition were explored. Normally, the IGF-ligand binds to and phosphorylates its receptor (IGF-RI), which initiates a cascade of events starting with the activation (phosphorylation) of the cytoplasmic Insulin Receptor Substrate (IRS) protein. In the present study, excess glutamate was found to inhibit the activation of the IRS protein (insulin-receptor substrate), through a pathway that involved the activation of protein kinase C (PKC). PKC phosphorylated the IRS protein at an unusual serine site, leading to its inactivation. PKC activity and the neurotoxic effects of glutamate could be inhibited by the PKC inhibitor, Ro320432. This inhibitor was then tested *in vivo* using an animal model of ischemia-reperfusion, and it was shown that it effectively increased the activation of IGF-1 signaling intermediates following intracerebral ventricle (icv) infusion of IGF-1. It is of note that this was done in the presence of an inhibitor of glutamate reuptake (L-trans-pyrrolidine-2,4,- dicarboxylic acid or PDCA) to maintain excess levels of glutamate). The PKC inhibitor also reduced the infarct size by 50% one week following injury. It was not explicitly stated if PDCA was administered in the latter experiment, but we assume that the authors repeated the methods of the previous *in vivo* experiments. Overall, the study supported the effect of cytotoxic levels of glutamate on the inhibition of IGF-1 pro-survival pathways following ischemia-reperfusion. The authors suggested that the inhibition of IGF-1 signaling by glutamate may be an additional route contributing to excitotoxic neuronal damage.

3.3. Study of Hwang et al. (2004)

The work of Hwang et al. (2004) examined the spatial and temporal alterations in endogenous IGF-1 protein in the hippocampus after exposure to transient hypoxia in a Mongolian gerbil model. The study indicated that IGF-1 protein increased in areas of the hippocampus, such that there was an elevation in IGF-1 protein in the CA-1[a] area. One day following ischemic injury, IGF-1 and GAD67 protein (the enzyme that produces GABA) were both detected by immunostaining in the stratum pyramidale area (but higher magnifications and/or co-localizations in specific cells were not shown). The elevation of IGF-1 in the hippocampal CA-1 area persisted up to at least 4 days post-ischemia reperfusion, at which time IGF-1 protein was associated with microglia and astrocytes. This was evidenced by immunostaining with antibodies to IGF-1 and the respective cell marker proteins (OX-42 for microglia and glial fibrillary acidic protein [GFAP] for astrocytes). A more transient elevation was seen after injury in the CA 2/3 and Dentate Gyrus areas of the hippocampus, where levels decreased after 4 days. This study showed that ischemia-reperfusion substantially increased IGF protein in various areas of the hippocampus, at least over the short term. The authors proposed that increases in IGF-1 in astrocytes and microglial cells are associated with mechanisms that compensate for neuronal cell death in animal models of ischemia-reperfusion.

3.4. Study of Lu et al. (2020)

The final included study by Lu et al. (2020) investigated the role of neuron-derived 17-β estradiol (E2) on the injured ischemic brain. To this end, the authors established and validated a mouse model with a brain-specific neuron deletion of the aromatase gene. The latter gene encodes the structure for the aromatase enzyme involved in synthesis of E2. Global cerebral ischemia in control mice (intact aromatase activity and normal E2 levels) resulted in multiple changes in the hippocampus including

Footnote[a]: CA area = Cornus Ammonis area 1 in the hippocampus.

Table 2. Study of design and risk of bias of included studies (N = 4).

Author	Study Design	Selection Bias			Performance Bias		Detection Bias		Attrition Bias	Reporting Bias	Other Bias
		Random Group Allocation	Groups Similar at Baseline	Blinded Group Allocation	Random Housing	Blinded Interventions	Random Outcome Assessment	Blinded Outcome Assessment	Reporting of Drop-outs	Selective Outcome Reporting	
Garcia-Galloway et al. (2003) (68)	Non-randomized controlled experiments	●	○	●	●	●	●	◐*	◐	○	◐
Hwang et al. (2004) (51)	Non-randomized controlled experiments	●	○	●	●	●	●	●	◐	○	◐
Lu et al. (2020) (38)	Non-randomized controlled experiments	●	○	●	●	●	◐*	◐*	◐*	○	○
Sandberg Nordqvist et al. (1997) (29)	Non-randomized controlled experiments	●	◐	●	●	●	●	●	◐	○	◐

Note. Risk of bias: ○ = low; ●= high; ◐ = unclear; * additional explanatory notes included.

* Notes: Blinded outcome assessment in the Garcia-Galloway et al. (2003) study is reported as unclear, but it is noteworthy that the authors indicated that the outcome of the key *in vivo* assessment of infarct size was blinded. This would have earned them a low-risk score, but it was not clear whether other assessments were likewise blinded. Detection bias and attrition bias were reported as unclear for the study of Lu et al. (2020) received unclear score, even though Random outcome assessment was reported by these authors for some of the outcomes. However, they did not report on randomization of all of the outcomes, and they did not explain how they randomized selected outcomes. This group also reported that the outcome assessor for the behavioral tests was blinded, but it was not clear if other tests were also blinded. Incomplete outcomes of the global cerebral ischemia were reported but not clear if all other outcomes were complete (i.e., if any other animals were lost during the experimentation).

Neuronal damage; astrocyte activation; and induction of Brain Derived Neurotropic Factor (BDNF), IGF-1, and the glutamate transporter GLT-1. These changes were accompanied by impaired hippocampal cognition. On the other hand, global cerebral ischemia in the knockout mice showed that deletion of E2 resulted in enhanced neuronal depletion, reduced astrogliosis, reduced BDNF, IGF-1 and GLT-1, and greater cognitive impairments. These findings pointed to a neuroprotective effect of E2. Part of this protective mechanism was on the effect of E2 on the inhibition of FGF-2 signaling, which normally compromises reactive astrogliosis and the production of BDNF, IGF-1, and GLT-1.

4. Discussion

Overall, findings suggest that research on interrelationships between the excitatory and IGF-1 systems in TBI and/or stroke continues to be nascent. This notwithstanding, the information provided in the four extracted papers, taken together with the background data provide interesting directions for future studies, which are presented below as a hypothetical model. With this in mind, we first discuss the results of the SYRCLE risk of bias evaluations, as well as important questions raised by the extracted papers.

4.1. SYRCLE risk of bias

As noted, the quality of each study was informed by the risk of bias evaluations recommended by the SYRCLE's quality assessment for animal studies. In summary, all 4 studies had threats to selection, performance, and detection biases due to the experiments being non-randomized and non-blinded. The study authors did not clearly report drop-outs resulting in threats to attrition bias. However, reviewers did not note threats to reporting bias; all studies had adequate selective outcome reporting. It is recognized that: (1) most of the studies evaluated here (with exception of the study of Lu et al., 2020) were carried out before the SYRCLE quality assessment guidelines for animal studies was published in 2009; (2) it is more difficult for investigators of experimental animal studies to comply with all the risk of bias requirements than it might be for researchers of pre-clinical intervention studies. This is likely because many experimental studies are more limited in animal numbers, and more exhaustive in the number of interventional manipulations and their outcome analysis (e.g., examination of various inhibitors or phosphorylation activation events to pinpoint the precise effect on a signaling pathway and examination of outcomes by various technical methods). These caveats notwithstanding, the SYRCLE evaluations are presented in the spirit of contributing to the continuing awareness of the importance of these elements to the overall quality of animal studies.

4.2. Future questions related to each study

The study of Sandberg Nordqvist et al. (1997) pointed to the key relationship between glutamate signaling and IGF-1 transcriptional regulation and raised several important question(s), including: (1) is the IGF-1 mRNA translated into protein and what are the cell types involved in IGF production; (2) is the action of glutamate on IGF signaling direct, or are there intermediate effectors or cellular activities involved 3) what are the functions of the two binding proteins in the cortex and hippocampus and are they co-localized with IGF proteins? Or with distinct matrix components? These are relevant questions since IGF-BPs can be inhibitory to IGF-1 or synergistic with this ligand; alternatively, the binding proteins can even act independently of IGF (Burgdorf et al., 2017; Dyer et al., 2016; Fernandez and Torres-Aleman, 2012; Lewitt and Boyd, 2019). The paper of Garcia-Galloway et al. (2003) provided information on the role of excess levels of glutamate on IGF-1 signaling and at the same time opened the question of the effects of different levels of glutamate.

The studies of Hwang et al. (2004) and of Lu et al. (2020) each showed a circumstantial relationship between Glutamate or GABA and IGF-1. In the former study, the relationship was suggested by the localization of two proteins (IGF-1 and GAD67) in the same hippocampal tissue compartment. However, co-immunolocalization of the two

Table 3. Data extraction.

Article	Injury model	Species, weight/ age, and n	Experimental Question and Study aim	Experimental Approach	Outcome Measures	Results
Sandberg Nordqvist et al. (1997)	Mild TBI model (contusion) was established by stereostactically guided weight drop injury according to published methods.	Nineteen male Sprague-Dawley rats (240–260 g). The number of rats per experimental or control groups was not explicitly stated.	Is the regulation of IGF-1 and its binding proteins 2 and 4 after cerebral contusion dependent on glutamate signaling? "We studied the influence of glutamate transmission on IGF-1, IGF-BP2 and IGF-BP4 mRNA levels after traumatic brain injury by treating animals with MK-801,CNQX, or saline" (p. 456)	Injected rats with specific inhibitors of different types of glutamate receptors (the inhibitors included dizocilpine maleate [MK-801], an antagonist of the N-methyl-D-Aspartate (NMDA) receptors; and cyano-7-nitroquinoxaline-2,3-dione [CNQX], an antagonist of the α amino-3-hydroxy-5-methyl-4 isoxazole propionate-kainate (AMPA/kainite) receptors). Injections were done 30 min prior to contusion and then twice a day for 2 days.	Quantitative in-situ hybridization was used to measure levels of the IGF and IGF-BPs mRNAs around the brain contusion site aided by computer-assisted image analysis. Images were assessed 2 days post-contusion following treatment of the rats with either one of the inhibitors or without inhibitor (only saline).	IGF-1 mRNA was undetectable in controls but strongly induced in cortical and hippocampal tissue adjacent to the contusion site at 2 days post-injury. Treatment with either one of the glutamate receptor inhibitors completely blocked the IGF-1 mRNA induction. IGF-BP2 and IGFBP4 mRNAs were modestly expressed in controls and BP-2 increased in the cortex and hippocampus post-injury, but only the most distal increase in cortex mRNA was inhibited by MK-801 (but not CNQX). BP-4 increased markedly in the cortex close to the contusion site and was inhibited only by MK-801.
Garcia-Galloway et al. (2003)	Ischemic injury model induced by occlusion of the medial cerebral artery and reperfusion 90 min later according to published methods.	The experiments included four groups of Wistar rats (250–300 g) with at least five animals per treatment.	Is a loss of sensitivity to the pro-survival actions of IGF-1 a common feature of damaged neurons resulting from excitotoxic glutamate signaling? "We now have explored whether loss of sensitivity to the prosurvival actions of IGF-1 is a common feature of damaged neurons. For this purpose, we analyzed excitotoxic neuronal damage due to excess glutamate signaling…." (p. 1028).	The initial studies were carried out on cell cultures and showed that excess glutamate inhibited IGF. The *in vitro* studies included the use of a variety of specific inhibitors to examine how/at which point in the signaling cascades excess glutamate inactivated IGF signaling. The inhibitor able to block the *in vitro* pathway by which glutamate blocked IGF-1 action (Ro324032: a protein kinase C inhibitor) (or the saline vehicle) was then injected into normal rats or those with an ischemic brain lesion.	Western blots were used to assess changes in phosphorylated IGF intermediates by quantitative image analysis both *in vitro* and *in vivo* studies. The animal experiments also included assessment of the size of the infarct after inhibition of the glutamate effect on IGF signaling, as compared to saline injected controls a week after ischemic injury.	The authors established that excitotoxic levels of glutamate act through protein kinase C to phosphorylate the IGF intermediate (IRS) in an alternative site (serine vs the usual tyrosine), which results in IGF insensitivity. The finding that inhibition of the glutamate pathway results in reduction of the ischemic contusion site indirectly points to the contribution of (uninhibited) IGF to the increased size of the injury/ contusion area.
Hwang et al. (2004)	Ischemia was induced by occlusion of both common carotid arteries by use of non-traumatic aneurism clips for 5 min followed by blood flow reperfusion.	Male Mongolian gerbils weighing 55–70 g were used (animal numbers not provided except for sham-operated animals, n = 10)	What are the spatial and temporal alterations in endogenous IGF-1 in the hippocampus after exposure to transient forebrain ischemia? "…in the present study, we examined the spatial and temporal alterations of endogenous IGF-1 immunoreactivity in the hippocampus after exposure to transient forebrain ischemia in the Mongolian gerbil." (p.150).	Creation of ischemia-reperfusion model and sham operated control animals followed by examination of changes in IGF immunoreactivities in subareas of the hippocampal brain region.	Immunohistochemistry and Western blot analysis at 12 h, 1 day, and 4 days after ischemia-reperfusion.	IGF, as assessed by immunohistochemistry, increases in the CA1, CA2/3 and Dentate Gyrus (DG) areas of the hippocampus after transient ischemia. Temporal patterns of expression differ, such that IGF remains elevated in the CA1 after 4 days post-ischemia, while it decreases in the CA2/3 and DG. In the CA-1 IGF and GAD67 are co-localized in the stratum pyramidale. At 4 days post ischemia, IGF colocalizes with markers of microglia (stratum pyramidale) and with markers for astrocytes in the S. oriens and radiatum.

(*continued on next page*)

proteins was missing and would be warranted. The question of the potential effect of IGF-1 on GAD67 synthesis is also interesting and relevant. The study of Lu et al. (2020) demonstrated the co-regulation of IGF-1 and the glutamate transporter protein-1 (GLT-1) downstream of E2, suggesting that both proteins might participate in estrogen-regulated pro-survival activities. However, the nature of the specific molecular interactions, if any, between BDNF, IGF-1 and GLT-1 remained unexplored in this study. It will be interesting for future studies to evaluate these potential interactions, e.g., does E2 directly activate GLT-1 transcription or is this mechanism mediated by BDNF or IGF-1?

Table 3 (*continued*)

Article	Injury model	Species, weight/ age, and n	Experimental Question and Study aim	Experimental Approach	Outcome Measures	Results
Lu et al. (2020)	Global cerebral ischemia was induced by 2- vessel occlusion of the two carotid arteries for 20 min according to published methods.	Age-matched 3 to 5- month-old mice. N reported as at least 3 per group.	What is the role of neuron-derived 17-β estradiol in the hippocampus in global cerebral ischemia (as assessed using the knockout mouse for aromatase)? "In the current study, we sought to use the FBN-ARO-KO mice to determine the role of neuron-derived E2 in the injured ischemic brain". (p. 7536)	Aromatase is a critical enzyme involved in synthesis of 17-β estradiol (E2). Aromatase knockout mice and its floxed control were created to assess the role of E2 in the hippocampus following ischemia. In addition, intracerebroventricular injections of FGF-receptor blocking antibodies were used to test the effect of E2 on FGF2 activities in ischemic animals. Replenishment of E2 to knockout mice was used to establish the reversibility of the effects of gene deletion.	Biochemical outcome measures included Western blotting with image analysis of band intensities; immunohistochemical analysis; isolation of astrocytes, ELISA of homogenates; and RNA sequencing analysis. Behavioral outcomes included the Barnes Maze test, the Novel Object Recognition test, and the Open field test. Outcomes were measured at 3, 7, and 14 days following cerebral ischemia reperfusion.	Global cerebral ischemia (control mice) resulted in changes in the hippocampal CA1 area that included neuronal damage; astrocyte activation; induction of BDNF, IGF-1 and GLT-1; and impaired hippocampal dependent cognition. E2 depletion was associated with increased FGF-2 signaling; enhanced neuronal damage, reduced astroglyosis, reduced IGF-1, BDNF and GLT-1 and reduced cognitive impairments. The effects of E2 were mediated by FGF-2, which is a suppressor of astrocyte activation.

Abbreviations: 17-β estradiol (E2); Brain derived neurotrophic factor (BDNF); Fibroblast growth factor (FGF); Glutamate decarboxylase 67 kDa(GAD 67); Glutamate Transporter-1 (GLT-1); Insulin-like growth factor (IGF); Insulin Receptor Substrate (IRS); IGF binding protein 2 and 4 (IGF-BP2 and IGF-BP4); messenger RNA (mRNA). FBN-ARO-KO = Forebrain specific aromatase knockout.

4.3. Hypothetical picture

If supplemented by knowledge from related research (discussed in the Introduction), a hypothetical picture of events in the Glu/GABA and IGF systems following brain injury can be assembled from the results summarized in this review. Of note, the hypothetical schema presented below assumes similarities between critical TBI and CVA events, which would require further evaluation.

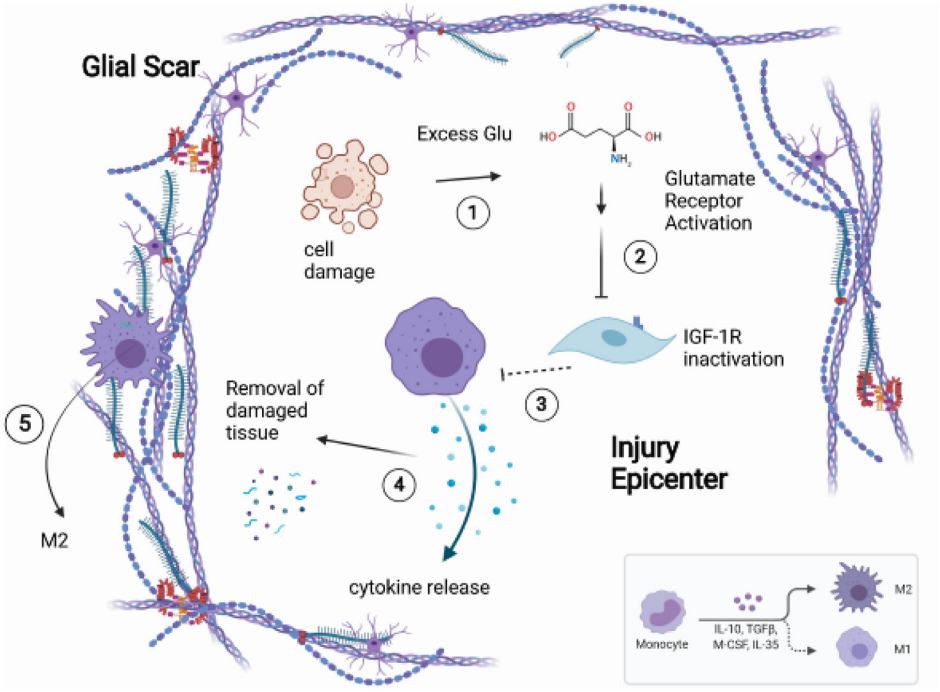
The proposed hypothesis/model, diagramed in Figure 3 A and B, pictures an initial event that includes rupture of surrounding blood vessel integrity and the infiltration of inflammatory/immune cells including macrophages. Additionally, other inflammatory cells such as brain-derived microglia are recruited to the lesion (e.g., O'Donnell et al., 2002). These inflammatory cells and related mediators and proteolytic enzymes are instrumental and necessary to clear matrix and cellular debris from the area. Excess glutamate released by injured and dead cells (and possibly other wound "alarmins" released by cell injury and death), as well as additional signaling proteins (e.g., FGF-2 as suggested by Lu et al. (2020)) help to tone down the production of active IGF-1 (e.g., Garcia-Galloway et al., 2003), which would otherwise be expected to antagonize the inflammatory cascade (Serhan et al., 2020) and compromise debridement of the lesion site. While these events are taking place, a glial scar forms around the central injury and physically contains the damage to the injury site, thereby protecting surrounding surviving tissue and associated neurons from the toxic environment of the wound epicenter (Raposo and Schwartz, 2014). The glial scar is formed by a complex matrix that includes glycosaminoglycans (hyaluronan, chondroitin-and heparan sulfate) collagens I and IV and other matrix elements (Anik et al., 2011). The eventual breakdown of scar components would enable the matrix to be transformed into one supporting axonal sprouting and tissue regeneration (Mutoji et al., 2021). The literature suggests that the glial scar breakdown is mediated at least in part through phenotypic channeling of infiltrating monocytes to a reparative Th2 phenotype, via binding to matrix CSPGs (Dzyubenko et al., 2018; Schecter et al., 2011), and/or a hyaluronan-based complex (Lauer, M.E. et al., 2013). IGF-1 signaling also affects the transition of monocytes to the Th2 phenotype (Kooijman and Coopers, 2004). Mechanical signals, partly derived from cellular and matrix changes in the post-injury phase are also likely to be contributory to the initiation of repair signaling (Hemphill et al., 2015).

4.4. Future research

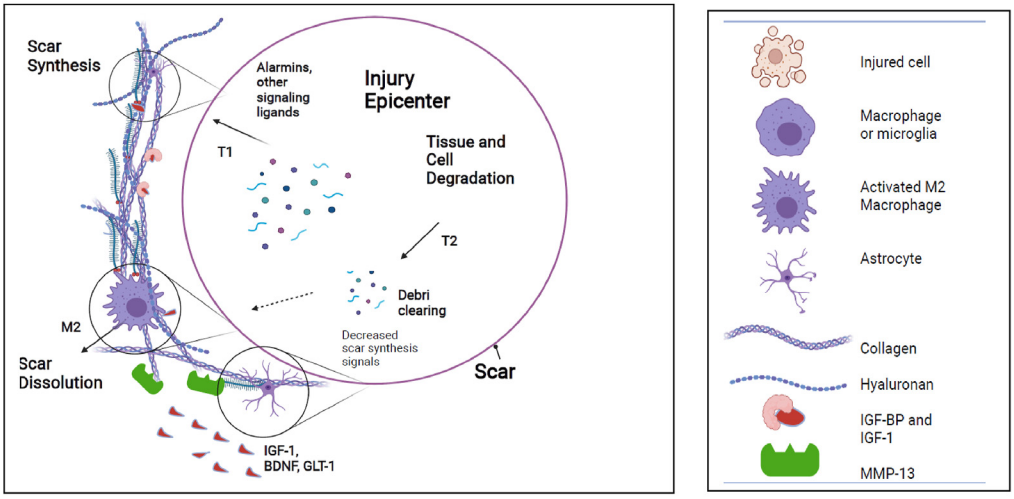
In summary, several research questions of importance to the understanding of disease pathogenesis arise from the above model: (1) do degradation products and/or related alarmins released from cells in the injury epicenter diffuse to the glial scar and convey molecular information that maintains the glial matrix for as long as it is needed? A related question is whether the concentration of these catabolic products decrease with time as the injured epicenter heals; (2) does IGF-1 bind to and/or interact with the epicenter structures or glial matrix components (and/or associated binding proteins) to form an immobile reservoir that limits its access the cellular receptors, but that is available for future action following release ?(e.g., such as occurs in cartilage, Bhakta et al., 2000); (3) at what point in the post-injury cascade does IGF signaling occur and how is this controlled (e.g., by lowered glutamate and/or other alarmins or FGF-2, and/or by GABA signaling?; and (4) do IGF-BPs, particularly IGF-BP5 play a role in the controlled release and/or activation of IGF-1? Walter et al. (1997) reported that IGFBP-5 is regulated at the injury margins during the transition from acute to chronic stage; hence it would be important to determine if IGF-1 is co-regulated with this BP.

While it is proposed here that additional work on animal models of TBI and CVA would help to inform trials for clinical modalities in humans, it is worth noting that clinical trials for TBI have frequently failed, in part due to the complexity of the pathology and to the absence of biomarkers that are firmly related to spatiotemporal events taking place in the brain (Loane and Faden, 2010). An example of promising ventures would be the use of proton magnetic resonance spectroscopy studies such as those initiated by Harris et al. (2012) to investigate the spatiotemporal presentation of various neurochemicals in animal models of TBI (such as Glu and GABA) and/or timed micro dialysis of released substances at different anatomical sites; such studies would provide relevant insight particularly if coupled to serum studies that might later provide an effective bridge for assessing markers in human serum.

Others have suggested various approaches to develop clinical trials for the human diseases, for example the use of IGF-1, its analogues, short tripeptides derived from the parent molecule and/or microRNAs to modulate IGF activity (Hayes et al., 2021; De Diego et al., 2006; Hayes et al., 2021; Ikeda et al., 1995; Sara et al., 1989; Saura et al., 1999; Selvamani et al., 2012; Vaaga et al., 2014). As noted above, the timing of



A: Hypothesis-Injury Response



B: Hypothesis-Transition to Reparative Response

Figure 3. Panel A: Hypothesis-Injury Response. Notes: The injury epicenter is surrounded by a glial scar. 1. In the injury site, cells are damaged and dying. They release excess levels of Glutamate (Glu) into the extracellular space, which then induce hyperactivation of surrounding cells with Glu receptors. 2. In cells with IGF-1 receptors, excess Glu signals block IGF receptor activation. 3. The inhibitory signaling effect of IGF on the inflammatory response is blocked (e.g., the secretion of cytokines by microglial cells and/or the transformation of monocytes into M1 type macrophages that produce inflammatory cytokines). 4. The inflammatory response is important for clearing the injured cell and matrix debri from the epicenter. 5. The glial scar prevents spreading of the injury. Unknown signals, likely including Chondroitin Sulfate proteoglycans lead to the binding of monocytes to the glial scar, which are then transformed to the M2 reparative type in the presence of IGF-1. Created with Biorender.com. Panel B: Hypothesis-Transition to Reparative Response. Notes: Time 1 (T1). During the initial post-injury phase, signals from the injured site and surrounding vasculature stimulate glial scar synthesis by activated astrocytes. The scar helps to contain toxic and deleterious materials to the lesion epicenter and spares healthy surrounding tissue. T2. As cell lysis diminishes and the debri from the injury is cleared (e.g., by M1 macrophages and microglia), concentrations of Glu decrease to non-toxic levels and the inhibition of IGF-1 synthesis in the epicenter and surrounding scar tissue is diminished. IGF-1 is deposited in larger quantities by cells in the glial scar matrix and accumulates in the matrix by binding to several IGF-BPs including IGFBP-4 and IGFBP-5. Under the influence of incompletely understood signals, monocytes bind to the chondroitin-sulfate containing matrix and transform to the M2 restorative phenotype in the presence of IGF-1. Release of MMP-13 from M2 cells then starts to dissolve the matrix and release entrapped growth factors, which subsequently contribute to re-building healthy matrix. Decrease in FGF-2 and other inhibitory signals lead to the synthesis of IGF-1, BDNF, and GLT-1 by astrocytes, thereby contributing to the reparative response. Created with Biorender.com.

IGF-1 therapies is likely to be of critical importance and should be informed by future experimental studies of the interrelationships between the excitotoxic system and IGF activity. In conclusion, the extant literature on the interactions of the excitation and IGF systems in normal animals points to a promising target for more detailed investigations in TBI and stroke models, focused on careful monitoring of time and spatial events as well as their peripheral manifestations.

5. Disclaimers

The views, opinions and/or findings contained in this article are those of the author(s) and should not be construed as an official Department of Defense or Veterans Affairs position, policy, or decision unless so designated by other documentation.

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