Multifunctional Drugs for Head Injury

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Summary: Traumatic brain injury (TBI) remains one of the leading causes of mortality and morbidity worldwide in individuals under the age of 45 years, and, despite extensive efforts to develop neuroprotective therapies, there has been no successful outcome in any trial of neuroprotection to date. In addition to recognizing that many TBI clinical trials have not been optimally designed to detect potential efficacy, the failures can be attributed largely to the fact that most of the therapies investigated have been targeted toward an individual injury factor. The contemporary view of TBI is that of a very heterogenous type of injury, one that varies widely in etiology, clinical presentation, severity, and pathophysiology. The mechanisms involved in neuronal cell death after TBI involve an interaction of acute and delayed anatomic, molecular, biochemical, and physiological events that are both complex and multifaceted. Accordingly, neuropharmacotherapies need to be targeted at the multiple injury factors that contribute to the secondary injury cascade, and, in so doing, maximize the likelihood of a successful outcome. This review focuses on a number of such multifunctional compounds that have shown considerable success in experimental studies and that show maximum promise for success in clinical trials. **Key Words:** Neurotrauma, statins, progesterone, erythropoietin, cyclosporin, toll-like receptors, magnesium, dexanabinol, bradykinin, substance P, minocycline, thyrotropin releasing hormone.

INTRODUCTION

Traumatic brain injury (TBI) is the leading cause of mortality and morbidity in industrialized countries in people under the age of 45 years of age, with the incidence of death reported as 20 to 30 per 100,000.[1] Motor vehicle accidents account for the majority of fatal head injuries, whereas falls increasingly account for nonfatal head injuries.[2,3] The social and economic cost of TBI to the community is substantial, given that those who survive TBI are often left with permanent neurological deficits that adversely affect their quality of life and sometimes require long-term rehabilitation.

Traumatic brain injury is caused by both primary and secondary injury mechanisms. Primary injury encompasses the mechanical forces at the time of the injury, which result in direct mechanical damage to neurons, axons, glia, and blood vessels through shearing, tearing, and stretching. The resultant injury includes diffuse axonal injury, hemorrhage, contusions, and laceration, with immediate clinical effects.[4] Primary injury is irreversible, and devices such as seat belts, airbags, and helmets have been introduced in an effort to prevent its occurrence.

Secondary injury is the sequence of cellular, neurochemical, and metabolic alterations initiated by the primary insult that continue to develop over time.[5] For example, the primary shearing forces that are applied to neurons at the time of the traumatic event cause massive ion fluxes across the neuronal membranes, widespread depolarization, and rapid release of neurotransmitters from affected cells. Subsequently, a host of biochemical events generate large amounts of toxic and proinflammatory molecules such as nitric oxide, prostaglandins, free radicals, and inflammatory cytokines, which lead to a breakdown of the blood–brain barrier and the development of edema. The associated increase in intracranial pressure (ICP) may then cause local hypoxia and ischemia, and herniation with subsequent neuronal cell death *via* necrosis and apoptosis. Typical secondary injury cascades such as this are thought to be associated with the development of many of the neurological deficits observed after TBI.[5] More important, the fact that these cascades manifest over minutes to days following the initial trauma suggests that there is a therapeutic window for treatment to prevent, attenuate, or at least delay the resultant neurological deficits.

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A number of experimental TBI models have been developed to study the mechanisms following trauma and to develop potential therapeutic interventions. In these preclinical studies, numerous pharmacological treatments have been identified, with at least 20 compounds and therapeutic interventions being the subject of more than 50 clinical trials over the last three decades.[6–8] Despite the encouraging preclinical results, none of these investigations have resulted in any consistent improvement in outcome in the clinical situation.

Aside from the potential shortcomings of the drugs themselves, a number of flaws have been recognized in how translations of experimental data have been attempted. For example, despite the recognition that metabolic and biochemical changes occur many hours after the initial trauma, most experimental studies use either pretreatment or very early (<30 min) treatment protocols; such early intervention is not always possible in clinical TBI. Drug dosing schedules also often differ between the preclinical and the clinical trials, with the latter frequently using lower doses (to avoid potential toxicity) or more frequent dosages (e.g., continuous infusions) that have not been supported by the preclinical data. Clinical trials also include a wide range of injury severities in the test group, whereas preclinical screening generally uses a very well-defined, highly controlled animal model of preselected severity. Moreover, the use of young healthy animals of a single sex results in a high injury reproducibility that is not apparent in clinical trials. Secondary insults such as CNS hypoxia and systemic injuries are also generally avoided in these animal TBI models but are commonplace in clinical TBI, especially in victims of motor vehicle accidents.

All of these individual differences have contributed to the failure to translate a successful pharmacological intervention to date,[7,8] and to the resultant reticence in the pharmacological industry to venture into acute brain injury trials. Nonetheless, many of these shortcomings can be easily addressed with appropriate preclinical screening and clinical trial design. In contrast, the complexity and heterogeneity of clinical TBI, and in particular the multifactorial nature of the secondary injury process, constitute one of the most significant hurdles to trial success.[8,9]

Given the multifactorial nature of the secondary injury process, it is unlikely that targeting a single factor will result in a significant improvement in outcome. Conversely, simultaneously targeting several injury factors may be the most likely therapeutic approach to improve outcome. A number of interventional pharmacologies have been developed that reportedly have multifunctional effects on secondary injury. The present review focuses on some of these multifactorial therapies, after first summarizing some of the major secondary injury processes identified in TBI.

SECONDARY INJURY

Excitotoxicity

Excitotoxicity is widely recognized as an important process in secondary damage and cell death following acute brain injury.[5] It is produced by excessive release of the excitatory amino acids such as glutamate and aspartate that stimulate a number of receptors, most notably the *N*-methyl-D-aspartate (NMDA) receptor complex that seems to play the most prominent role.[10] The overstimulation of the NMDA receptor[11] causes an ionic imbalance to occur, with Na^+ influx and K^+ efflux leading to further depolarization, thus overcoming the Mg^{2+} blockade of the NMDA receptor.[12] Exacerbating this situation is the reduction in glutamate reuptake associated with presynaptic ionic imbalances, thus further increasing the synaptic amino acid concentration. The high quantities of glutamate binding to the NMDA receptor promote substantial Ca^{2+} influx, resulting in increased intracellular Ca^{2+} concentration and the activation of a large number of calcium-dependent enzymes, including proteases, lipases, and endonucleases. Their activation leads to neuronal destruction.

Given the detrimental effects of excessive excitatory amino acid release, it is not surprising that numerous clinical trials have assessed the efficacy of NMDA antagonists on patient outcome following acute brain injury. Nine trials of compounds that attenuate excitotoxicity were identified,[13] none of which had a beneficial effect on outcome. One reason for the lack of efficacy may be the fact that the antagonists have usually been administered after the peak concentration of glutamate following the TBI has passed.[14] Alternatively, the non-NMDA glutamate receptors may also play a critical role in post-traumatic glutamate excitotoxicity.[15] Thus, glutamate toxicity is more complex than originally thought and may in fact be mediated by a number of different receptors.

Mitochondria

The mitochondrion has been shown to be a key participant in TBI-induced neuropathology,[16] and its dysfunction has serious implications for outcome following head injury. Indeed, compared with patients with marginal mitochondrial impairment, who demonstrate a good outcome, TBI patients with profound mitochondrial impairment have a poor prognosis.[17] Following injury, this organelle has been shown to undergo a mitochondrial permeability transition,[18] with the permeabilization of the inner mitochondrial membrane being associated with excessive calcium accumulation.[19,20] Mitochondrial permeability transition results in the release of cytochrome *c*, which is integrally involved in apoptotic cell death, as well as in uncoupling and inhibition of oxidative phosphorylation and the generation of mitochondrial reactive oxygen species (ROS).[20,21] This propensity of

mitochondria to undergo permeability transition has also been implicated in the selective vulnerability of different brain regions to an ischemic insult.[22] Interventions that attempt to block the mitochondrial permeability transition are thus designed to inhibit the destructive cellular cascades that follow such permeability. Continued calcium accumulation in mitochondria also results in a loss of matrix components, impairment of mitochondrial function, and swelling of the organelle leading to outer membrane rupture.[23] Such mitochondrial dysfunction would also lead to energy depletion, free radical release, and further cell death pathway activation.[24,25]

Oxidative stress

Oxidative stress can be defined as damage inflicted *via* processes involving production of ROS and their detrimental reactions with proteins, lipids, and deoxyribonucleic acid (DNA).[26] The ROS are highly reactive molecules that contain an unpaired electron in the outermost orbit, increasing their potential for chemical reactivity.[27] As normal by-products of oxidative metabolism, these ROS, or free radicals, are constantly produced, but their concentration is usually tightly controlled by endogenous antioxidant mechanisms. Traumatic brain injury, however, dramatically increases their production,[28] and the result is oxidative stress.

Brain tissue is extremely vulnerable to oxidative damage because of its high rate of oxidative metabolic activity, production of reactive oxygen metabolites, relatively low antioxidant capacity, low repair mechanism activity, nonreplicating nature of its neuronal cells, and the high ratio of membrane surface to cytoplasm.[29] The ROS can be generated *via* arachidonic acid cascade activity, catecholamine oxidation, mitochondrial leak, oxidation of extravasated hemoglobin, or neutrophils.[30] They initiate tissue damage through complex mechanisms, including excitotoxicity, metabolic failure, and disturbance of intracellular calcium homeostasis.[31]

Oxidative damage also frequently involves lipid peroxidation of neuronal, glial, and vascular cell membranes and of myelin, resulting in the decomposition of polyunsaturated fatty acids in lipid membranes, disruption of ionic gradients, and, if severe enough, membrane lysis.[30] Oxidative damage is tightly linked to other pathological mechanisms, such as Ca^{2+} overload, mitochondrial cytochrome *c* release, caspase activation, and apoptosis.[28] Administration of antioxidants has been shown to be effective in experimental models of TBI.[30] On their own, however, they have not shown efficacy in clinical trials of TBI.

Inflammation

Inflammation is considered critically important in TBI,[32] with inflammatory processes and chemokine signaling now considered major components of the secondary injury cascade and offering attractive potential targets for pharmaceutical neuroprotection.[33] Classically, the brain is considered as being shielded from the immune system by the blood–brain barrier, giving it an immuno-privileged position within the body. Instead, the CNS has its own, dedicated immune system mediated by glial cells, whose function is to engage in inflammatory processes that serve to defend the CNS from pathogens, as well as aiding in its recovery from insult and injury. However, an excessive activation of these mechanisms may result in a vicious cycle of severe, chronic neuroinflammation that may have deleterious effects, promoting or propagating neurodegeneration.[34]

Glial cell–mediated inflammation represents one mechanism by which inflammation can occur within the CNS, and it is probably associated with a number of chronic inflammatory conditions. However, a second pathway by which inflammation may occur in the CNS is *via* compromised blood–brain barrier function. The blood–brain barrier normally serves to shield the CNS from the peripheral immune system. After acute insults to the brain, however, blood–brain barrier function may become compromised for a period of time, allowing for the entry of immune cells from the circulation.[35,36] Transmigration of leukocytes after disruption of the blood–brain barrier may result in the activation of glial cells in the CNS. Both the infiltrating peripheral immune cells and activated glial cells then increase the production of cytokines, thus promoting neuroinflammation.[32] In addition, this transient alteration in blood–brain barrier function has been shown to contribute to the vasogenic component of cerebral edema after TBI.[37] Because compromised blood–brain barrier function may facilitate the acute inflammatory response after TBI, it may well serve as a target for anti-inflammatory drug development, insofar as agents capable of restoring or maintaining the integrity of the blood–brain barrier could help ameliorate the acute phase of CNS inflammation.[38]

Edema

One feature of CNS inflammation is cerebral edema, which can manifest either locally or diffusely throughout the brain. It is broadly defined as a volumetric increase in brain tissue volume due to an abnormal accumulation of fluid.[39] Although single types of edema rarely exist in practice,[39] cerebral edema is generally classified primarily as either vasogenic or cytotoxic, depending on the underlying mechanisms associated with the edema formation.

Specifically, vasogenic edema results from increased blood–brain barrier permeability, which causes a disruption to the balance between the oncotic and hydrostatic pressures that govern movement of fluid between blood plasma and brain interstitial fluid. The compromised barrier allows solutes such as protein exudates to escape from the cerebral vasculature and enter the interstitium

of brain parenchyma, resulting in a net movement of water down its pressure gradient and a subsequent gain of interstitial fluid.[40,41] The limited lymphatic system in the brain greatly impairs resorption of exudate from the extracellular space, and, accordingly, vasogenic edema then spreads throughout the extracellular space. This mechanism of movement explains why edema is seen primarily in the structurally ordered cerebral white matter, rather than in the more densely organized gray matter.[39,41]

In contrast, cytotoxic edema is characterized by intracellular swelling of neuronal, glial, and endothelial cells in the absence of any measurable breakdown of the blood–brain barrier.[41] Cytotoxic edema primarily occurs in the gray matter and is commonly associated with ischemia and energy depletion, in which failure of the ATPase pumps result in intracellular accumulation of sodium.[41] Glutamate-mediated excitotoxicity may also contribute, because, in addition to calcium accumulation, it also causes intracellular accumulation of sodium.[42] Water then follows by osmosis, thereby increasing intracellular fluid volumes and resulting in a concurrent reduction in extracellular space.[43]

Serious consequences of cerebral edema include raised ICP, reduced cerebral blood flow (CBF), reduced tissue oxygenation, CSF displacement, and deformation and herniation of brain tissue, all of which contribute substantially to increased morbidity and mortality following TBI.[44] Increased ICP has in fact been singled out as one injury factor that closely predicts outcome following severe brain injury,[45,46] and its elimination has been proposed as a highly neuroprotective measure, beyond its role only as a life-saving procedure to prevent cerebral herniation.[47] Despite the serious consequences associated with edema formation, there is currently no effective pharmacological treatment in clinical practice. Treatments to date (which include mannitol, hypertonic saline, glucocorticoids, hypothermia, and barbiturates) have had either limited success or have been completely ineffective.[48]

ATTENUATING SECONDARY INJURY

Statins

The 3-hydroxy-3-methyglutaryl coenzyme A (HMG-CoA) reductase inhibitors, or statins, have been recently attracting considerable attention as a potential neuroprotective therapy in acute brain injury, given the experimental evidence that has been accumulating, particularly in preclinical studies of stroke. The statins were originally developed as lipid-lowering drugs for use in reducing mortality associated with coronary artery and cerebral vascular diseases.[49,50] It was not long, however, before a number of properties other than those affecting cholesterol metabolism were observed.

At the level of the vasculature, statins have been shown to increase endothelium-derived nitric oxide production,[51] reduce vascular inflammation,[52] and reduce the volume of hematoma in hemorrhagic stroke.[53] There are also reports that statins decrease platelet aggregation and thrombosis.[54] At the level of the neurons, statins are thought to protect cortical neurons from NMDA-induced excitotoxic death as a result of changes in cell cholesterol homeostasis,[55] although others have reported that some statins exert specific antiexcitotoxic effects independent of cholesterol changes.[56] This cholesterol-independent neuroprotection against glutamate excitotoxicity may be mediated *via* activation of tumor necrosis factor receptor 2 (TNF-R2) signaling pathways.[57] Significant improvements in neurological outcome after stroke have also been reported, partly due to the ability of statins to enhance neurogenesis, synaptogenesis, and angiogenesis.[58]

Similar effects have also been noted for TBI: statins have been shown to reduce the secondary vascular injury, thrombosis, and lesion volume after TBI.[59] In addition, a neuroprotective effect on neurons in the CA3 region has been described after TBI, resulting in significant improvement of spatial learning at later timepoints.[60] The delay in cognitive improvement that was described in these studies suggest that statins may influence apoptosis, a possibility supported by the later observation that the ratio of Bax/Bcl-2 is significantly reduced in simvastatin-treated animals, favoring an antiapoptotic state.[61] Similar beneficial effects on locomotor outcome have also been described in spinal cord injury (SCI), with the authors attributing the neuroprotection to effects on endothelial dysfunction.[62]

Clearly, statins have multifactorial effects that contribute to their neuroprotective and neurorestorative status. Some are mediated at the level of the microvasculature, such as their ability to increase nitric oxide bioavailability, which regulates cerebral perfusion and improves endothelial function, and their ability to stabilize atherosclerotic plaques; others are independent of this system, including the ability of statins to serve as antioxidants, inhibitors of inflammatory responses, immunomodulatory agents, and regulators of progenitor cells.[63] Notably, recent reports suggest that, when combined with a phosphodiesterase 4 inhibitor, statins provide neuroprotection and promote neurorepair in demyelinating disease.[64] It is no surprise that statins are the subject of a number of ongoing clinical trials including stroke, subarachnoid hemorrhage, intracerebral hemorrhage, Alzheimer's disease, and multiple sclerosis—although not yet TBI.

Progesterone

A growing body of evidence indicates that progesterone, which can also be synthesized de novo by glial cells in the CNS, influences several brain functions *via* steroidal (genomic), neuroactive (nongenomic), and neuroste-

roidal actions.[65] Progesterone and its three reduced metabolites modulate neuronal excitability by interacting with the inhibitory $GABA_A$ receptors, as well as by modulating other neurotransmitter receptors, including serotonin, glycine, nicotinic acetylcholine, and kainate receptors.[65] The modulatory effects of progesterone are believed to involve anesthetic,[66] anxiolytic,[67] analgesic, and anticonvulsant properties.[68] Effects on sleep patterns, memory,[65] and depression[68] have also been reported.

Extensive neuroprotective and neurotrophic effects of progesterone have been demonstrated both *in vitro* and *in vivo* in a number of CNS injury models, including SCI,[69] stroke,[70,71] neurodegeneration,[72] and TBI.[73] In an *in vitro* model of SCI, progesterone was shown to protect against glutamate toxicity,[74] possibly by modulation of inhibitory ($GABA_A$) and excitatory (excitatory amino acids) neurotransmitter receptors.[75] After incomplete paraplegic SCI, rats treated with progesterone showed less tissue and white matter damage at the epicenter of the injury and evidenced a better functional activity as assessed using the Basso–Beattie–Bresnahan locomotor rating scale.[76] The restoration in functional outcome may be related to the ability of the hormone to restore choline acetyltransferase immunoreactivity and enhanced GAP-43 mRNA expression, as well as to increase mRNA for neuronal Na,K-ATPase.[77] Similar improvements in functional outcome have also been demonstrated in experimental stroke, in which progesterone administration reduced infarct volume.[71] These observations are consistent with the fact that progesterone reduces neuronal cell loss in the hippocampus when administered after global ischemia.[78]

In the peripheral nervous system, progesterone has been shown to modulate myelin protein synthesis,[79] possibly by stimulating the synthesis of specific myelin proteins or lipids. The increase in myelin basic protein has since been associated with an increased rate of remyelination of axons in both central and peripheral nerve preparations, suggesting potential neuroprotective effects in multiple sclerosis.[80]

In TBI, Roof et al.[81] were the first to note that females performed better than males in the Morris water maze after injury, and suggested that female hormones may play a protective role. This was subsequently confirmed when progesterone-treated male rats were less impaired on the Morris water maze task than vehicle-treated animals,[82] and the authors attributed this protective effect to progesterone. This effect of progesterone on posttraumatic performance in the Morris water maze was subsequently confirmed in studies using an air-driven, cortical contusion model of TBI in rats[83] and the positive effects of progesterone on cognitive outcome were linked to a reduction in neuronal cell loss.[84]

Although the mechanisms by which functional outcomes are improved after TBI are unknown, progesterone has the ability to reduce membrane lipid peroxidation after TBI,[85] indicative of an effect on oxidative stress. This effect on oxidative stress has been confirmed in tissue culture as well as in an *in vitro* stretch model of TBI. In both cases, progesterone reduced oxidative stress as reflected by 2-thiobarbituric acid, cytochrome oxidase, or manganese superoxide dismutase levels.[86] Attenuation of excitatory amino acid responses by progesterone has also been demonstrated,[87] with recent studies suggesting that NMDA mediated calcium influx is mediated by antagonizing σ_1 receptors, a distinct action that precedes the delayed activation by progesterone of the Src-ERK signaling pathway.[88] Progesterone has also been shown to inhibit cell death, particularly caspase-3 activation and subsequent apoptosis.[89] This inhibition of apoptosis may, in part, account for the reduced axonal damage observed in white matter after TBI following treatment with progesterone.[90]

The fact that progesterone reduces inflammation after TBI[91] may also contribute to the widely observed beneficial effects of the hormone on edema. Administration of progesterone after brain injury attenuated edema in both female and male animals,[92,93] irrespective of estrogen. Moreover, the reduction of edema was significant even when progesterone treatment was delayed for up to 24 h after injury.[94] These observations have since been confirmed in the bilateral medial frontal cortex injury model of trauma,[95] as well as in ischemic injury.[70]

The underlying mechanisms by which progesterone may reduce edema have not been fully elucidated, although several possible mechanisms have been proposed.[73] These include inhibition of active ion uptake through Na^+,K^+-ATPase, inhibition of vessel growth associated with leaky blood–brain barrier function after TBI, modulation of levels of vasopressin, inhibition of neurogenic inflammation,[96] and, finally, action as a free radical scavenger mediating lipid peroxidation.

Finally, systemic benefits of progesterone administration on soft tissue injury may also play a role. Subcutaneous progesterone administration following trauma-induced hemorrhage has been shown to ameliorate the proinflammatory response and reduce hepatocellular injury in ovariectomized female rats.[97] Related studies demonstrated that progesterone attenuated the cardiovascular depression and significantly improved cardiac output and heart performance and increased circulating blood volume.[98] These combined systemic effects of progesterone may be particularly beneficial in TBI victims with multisystem trauma, a common occurrence in motor vehicle accidents but frequently not accounted for in clinical trails.

The multifactorial nature of progesterone has seen it incorporated into several clinical trials investigating dis-

orders of the CNS, including TBI. The recently completed phase II ProTECT trial[99] confirmed the safety of progesterone administration in TBI patients, and demonstrated that those patients randomized to progesterone had a reduced 30-day mortality and were more likely to have a moderate to good outcome. This was later confirmed in a subsequent trial that demonstrated both reduced mortality and improved outcome, although the improvement was not related to any statistically significant effect on ICP.[100] The positive outcomes in both of these trials suggest that progesterone is highly worthy of a larger, multicenter trial.

Erythropoietin

The hormone erythropoietin (EPO) is the key hematopoietic growth factor in the human body, used extensively for the treatment of anemia, although recent evidence suggests that it may also have broad neuroprotective properties in the CNS following injury. EPO-derived peptides comprising approximately 7 to 25 amino acids are highly protective in experimental models of multiple sclerosis, acute stroke, and acute spinal cord and brain injury, as well as arthritis.[101]

Specifically, EPO-derived peptides have been shown to ameliorate the extent of concussive brain injury, the immune damage in experimental autoimmune encephalomyelitis, and the toxicity of kainite.[102] In a cryogenic model of cortical brain injury,[103] EPO administration significantly reduced vasogenic brain edema, attenuated blood–brain barrier breakdown, reduced lesion volume, and ameliorated motor dysfunction. Similarly, following traumatically induced contusion injury, EPO administration increased the neuronal density in the CA1 and CA3 region of the hippocampus, and significantly reduced the total contusion volume when administered within 6 h of injury.[104] Unfortunately, no functional outcome measures were reported. Nonetheless, in acute sciatic nerve crush injury, EPO treatment resulted in consistent functional neuroprotection, particularly noteworthy in that it was detectable even when EPO was administered up to 1 week after injury.[105]

In addition to providing neuroprotection by decreasing lesion volume and cell loss, EPO reportedly also facilitates neurorestoration by enhancing neurogenesis, subsequently improving sensorimotor and spatial learning function.[106] It has also been described as an anti-inflammatory agent that can protect against tissue damage in subjects having diverse forms of neural and non-neural organ system injury through downregulation of the inflammatory autoimmune components.[107] Indeed, the natural occurrence of EPO within the body, its ability to cross the blood–brain barrier, and the evidence that it is a neuroprotective agent that promotes neuronal regeneration has prompted suggestions that it offers considerable promise as a therapeutic agent for central nerve repair.[108] This view is supported by the observation that EPO receptor null in the nervous system aggravates sensorimotor deficits and impairs cortical neurogenesis, calcium accumulation, and amyloid precursor protein accumulation (axonal injury) after TBI.[109] Several clinical trials of EPO in CNS pathology are underway, including studies in TBI, stroke, subarachnoid hemorrhage, and hypoxic–ischemic encephalopathy, among others.

Minocycline

Minocycline is an antibiotic that, in contrast to related compounds such as tetracycline, is an effective antioxidant with free radical scavenging potency similar to that of vitamin E.[110] Minocycline has been reported to be an effective pharmacological agent for reducing tissue injury and neurological deficits after experimental TBI, most likely through a caspase-1-dependent mechanism.[111] Early studies have largely focused on its efficacy in experimental SCI, where it has been shown to significantly reduce the gross lesion size in the spinal cord and to promote superior behavioral recovery.[112] Although the mechanisms of this neuroprotection in SCI are unclear, the compound has been shown to significantly lower cytosolic cytochrome *c* at the epicenter, supporting inhibitory effects on apoptosis.[113] Such inhibition was associated with enhanced long-term, hindlimb locomotion relative to that of controls. Minocycline has also been shown to inhibit oligodendrocyte death after SCI, and to improve functional recovery,[114] perhaps by decreasing myelin damage.

Studies examining effects of minocycline in TBI suggest that long-term improvements in neurologic dysfunction may be mediated by the ability of minocycline to inhibit microglial activation, in addition to its antiapoptotic properties.[115] This view is not universally accepted, however. Bye et al.[116] have shown that minocycline decreases lesion volume and transiently improves neurological outcome. Given the time-course associated with the functional improvement, the authors suggest that the early beneficial effect is unlikely to be related to antiapoptotic mechanisms, because the density of apoptotic cells is not affected at early time-points. However, protection by minocycline is associated with a selective anti-inflammatory response in which microglial activation and interleukin-1β (IL-1β)expression are reduced, and neutrophil infiltration and expression of multiple cytokines are not affected. This may contribute to the neuroprotective effects observed. This is consistent with reports that repeated, daily, post-insult treatment with minocycline abolishes neuroinflammation and potentially provides neuroprotection to white matter for up to 1 week after hypoxia–ischemia in a rodent preterm model.[117] Nonetheless, different steroids, cyclosporin A, and FK506 all inhibit microglial activation and none have demonstrated significant neuroprotective effects in clinical trials. Al-

though it is not currently the subject of a clinical trial in TBI, minocycline is being examined in clinical trials of stroke, Huntington's disease, Parkinson's disease, and SCI.

Kinin antagonists

Kinins are a group of peptide mediators that have, among other effects, a proinflammatory action. Some of their inflammatory effects are mediated *via* the vasculature, where they promote inflammation by causing vasodilation and increased vascular permeability. There are two distinct families of kinins, the bradykinins and the tachykinins. The bradykinins are formed from the cleavage of the plasma globulin kininogen by plasma and tissue proteases known as kallikreins. The active peptides formed by this proteolytic cleavage are bradykinin and kallidin (lysyl bradykinin).

These kinins produce their effects through bradykinin receptors, with two subtypes of the receptor having been identified, B_1 and B_2. B_1 receptors are normally only expressed in very low levels, but are induced in damaged tissues by cytokines such as IL-1β. There has been significant interest in developing nonpeptide antagonists of the bradykinin receptor, given the potential ability for such agents to have anti-inflammatory and analgesic actions.[118] The potential role for these agents in managing neurological disorders that exhibit an inflammatory component has also been mooted,[119] although there is also some evidence to suggest that bradykinin may have anti-inflammatory and neuroprotective effects in the CNS by modulating microglial function.[120] Bradykinin receptor antagonists have been shown to improve neurological outcome following TBI,[121] in part by attenuating the increase in ICP.[122]

The tachykinins, which include substance P and neurokinin A, are released from nerves in the active form and produce their effects *via* tachykinin receptors. Substance P is an abundant neurotransmitter, being associated with both the peripheral and the central nervous systems. In the periphery, it is the predominant neurotransmitter found in nociceptive nerves. The release of substance P, and other neuropeptides, from sensory nerves is thought to play a significant role in the neural component of inflammation (i.e., neurogenic inflammation),[123] which encompasses increased vascular permeability and vasodilation. Indeed, it was found that mice that lack the receptor for substance P (tachykinin NK_1 receptor) fail to exhibit normal inflammatory responses. There is also growing evidence that substance P may play a significant role in the CNS, both from a physiological and a pathological angle. Its role in emesis has been clearly established clinically, with NK_1 antagonists having very potent antiemetic actions. There is also good evidence that NK_1 antagonists have antidepressant effects.[124] Substance P may also be an important mediator of neuroinflammation in the CNS. Nessler et al.[125] have shown that NK_1 antagonists can suppress autoimmune encephalomyelitis.

In TBI, there is accumulating evidence that NK_1 antagonists can ameliorate the inflammation associated with acute injury to the brain, particularly through maintaining the integrity of the blood–brain barrier.[37,126] When administered after TBI, substance P antagonists reduce blood–brain barrier permeability, reduce edema, decrease axonal injury, enhance neuronal survival, and improve both motor and cognitive outcome.[127] As with bradykinin, there is significant interest in developing nonpeptide antagonists of substance P for use in treating TBI.[128]

Toll-like receptor agonists

Toll-like receptors (TLRs) are a key element in the innate immune response, directly involved in detecting pathogen invasion or tissue damage, and in initiating a response to the challenge. Toll-like receptors recognize distinct components (pathogen-associated molecular patterns [PAMPs]), and activate intracellular signaling pathways that induce the expression of inflammatory genes.[129] Unlike antigen receptors, TLRs are encoded in the DNA and are expressed on the surface of antigen-presenting cells, dendritic cells, and macrophages. Activation of these receptors triggers the production of the main proinflammatory cytokines, TNF-α and IL-1β, as well as the release of other mediators, such as histamine and prostaglandins.

There is considerable interest in developing novel pharmaceutical agents that target the TLRs, in order to try and control neuroinflammation. The immunostimulatory properties of TLRs are being examined for their ability to generate tumor-specific immune responses directed against cerebral tumors, and the immunomodulatory properties are being investigated for their ability to suppress the acute inflammatory responses associated with ischemic and traumatic insults. A third component of TLR signaling has also begun to emerge, and this pathway exerts a direct neuroprotective effect. As a result, there is a growing interest in TLRs as potential novel targets for the treatment of acute CNS injury.[130]

Recently, Van Noort et al.[131] described the use of TLR3 agonists for the treatment of neurodegenerative disorders. There is evidence that TLR3-mediated responses can be distinct from the responses mediated by other members of the TLR family, being linked to a different signaling pathway. In response to most PAMPs, TLRs typically activate the NF-κB-mediated pathway, leading to the production of TNF-α, IL-lβ, IL-6, and nitric oxide. This typical response is designed to start the proinflammatory responses and eventually the adaptive immune responses. It has been shown, however, that TLR3 signaling in human mast cells not only fails to trigger TNF-α or IL-lβ, but instead inhibits degranula-

tion of these cells and inhibits their attachment to the extracellular matrix.

The present authors[131] have shown that activation of TLR3 on human astrocytes and fibroblasts results in a repair response consisting of enhanced production of a variety of anti-inflammatory, antifibrotic, proangiogenic, chemotactic, and neuroprotective mediators that—together—support regenerative responses. In addition, they have shown that stathmin and stathmin-like proteins can act as activators for TLR3 or can activate TLR3-mediated signaling. The stathmin-activated TLR3-mediated response of astrocytes includes production of a range of neuroprotective, anti-inflammatory, angiogenic and chemotactic mediators that support and promote regeneration.

In summary, TLR3 stimulation reinforces the natural innate immune mechanism of neuroprotection.[132] To date, few studies have examined the potential of toll-like receptor agonists in TBI.

Dexanabinol

Dexanabinol (also known as HU-211) is a nonpsychotropic analog of tetrahydrocannabinol that has a number of neuroprotective properties that have been demonstrated in a variety of experimental models of CNS pathologies,[133] including severe closed head injury, hypoxemia–ischemia, neurotoxin exposure, and nerve crush injury.[134–136] It is thought that the compound, which does not bind to the cannabinoid receptor, has a number of neuroprotective effects, including acting as an NMDA receptor antagonist,[137] a free radical scavenger and antioxidant,[138] and an inhibitor of cytokine TNF-α.[139]

Specifically, the dexanabinol molecule readily crosses the blood–brain barrier and weakly blocks NMDA receptors by interacting with a site close to, but distinct from, that of uncompetitive NMDA antagonists.[137] Accordingly, it is able to provide the therapeutic benefits of uncompetitive NMDA-receptor antagonists without the adverse psychotropic effects associated with this class of compounds.[134] By blocking the NMDA receptor, it attenuates calcium influx and thus reduces the likelihood of calcium-triggered autodestruction. This reduction in calcium entry would also contribute to the antioxidant properties of the compound, although it would not completely account for them, because the antioxidant potential of dexanabinol is more pronounced than that of MK-801.[133,138] What contributes to this additional antioxidant effect is unclear, but it is likely to involve direct scavenging of free radicals or the ability to increase endogenous antioxidant ability. Whatever the antioxidant mechanism, it is known that dexanabinol protects cultured neurons from the toxic effects of ROS.[140] Finally, its ability to inhibit TNF-α synthesis and other inflammatory cytokines confirms its anti-inflammatory potential, which has been demonstrated both *in vitro* and *in vivo*.[29] This anti-inflammatory effect would contribute to the observed attenuation of blood–brain barrier permeability after injury, with a consequent reduction in edema formation.

Given properties that attenuate at least three of the major known secondary injury factors in TBI, it is not surprising that dexanabinol was one of the first multifactorial drugs entered into clinical trials. Phase II clinical trials of dexanabinol indicated that it was safe and well tolerated by TBI patients, with few adverse consequences. It is worth noting that ICP in this study was better controlled in dexanabinol-treated patients than in the control group,[141] supporting a pronounced effect on edema development. Unfortunately, improvements in the control of ICP or quality of life were not observed in the larger phase III trial, and subgroup analysis did not support any differential treatment effects. Although dexanabinol was conclusively demonstrated to be safe, it was not efficacious in the treatment of TBI.[142]

Magnesium

The role of magnesium in acute brain injury and its potential as a neuroprotective therapy have been reviewed in detail elsewhere.[143–145] Briefly, brain magnesium decline is a ubiquitous feature of TBI and is associated with the development of motor and cognitive deficits. Experimentally, parenteral administration of magnesium up to 12 h post trauma restores brain magnesium homeostasis and profoundly improves both motor and cognitive outcome. Although the mechanism of action is unclear, magnesium has been shown to attenuate a variety of secondary injury factors, including brain edema, cerebral vasospasms, glutamate excitotoxicity, calcium-mediated events, lipid peroxidation, mitochondrial permeability transition, and apoptosis (reviewed by the present authors in 2002[143]). It is thus a truly multifactorial pharmacological intervention with a proven safety record in previous clinical studies.

Protective effects of magnesium administration on functional outcome have also been reported in acoustic trauma[146] and traumatic cortical lesions,[147] although results in hypoxia–ischemia have been mixed.[148–150] The fact that preinjury treatment is protective in this condition but postinjury treatment is less effective suggests that intracellular energy depletion may attenuate the neuroprotective effects of magnesium administration, perhaps by restricting the action of the ion to extracellular secondary injury factors. For example, at the intracellular level, magnesium has been shown to reduce apoptosis by both decreasing the expression of apoptosis-inducing p53-related factors[151] and by decreasing caspase-3 expression[149]; however, this effect on apoptosis is reduced in the presence of ATP depletion.[148,149] Similarly, magnesium decreases mitochondrial ultrastructural damage and improves respiratory function after TBI,[152] but may

exacerbate the rate of ATP depletion and acidosis in hypoxia–ischemia.[150] On the other hand, extracellular events such as the ability of extracellular magnesium to block the glutamate NMDA channel or sodium influx[153,154] would be independent of the intracellular ATP concentration. Thus, the efficacy of magnesium as a neuroprotective agent may depend somewhat on the energy status of the condition under study.

Injury-dependent declines in serum ionized magnesium have been noted in clinical TBI[155] and in aneurysmal subarachnoid hemorrhage.[156] Specifically in TBI, patients with a presenting serum magnesium of <1.3 mEq/L were 2.37 times more likely to have a poor outcome.[157] In terms of CSF magnesium, patients with initial high CSF magnesium were 7.63 times more likely to have a poor outcome, and those with severe head injury had elevated CSF magnesium during the entire observation period.[158] Notably, elevated CSF magnesium correlated with depressed serum magnesium only in patients with poor outcome.[157] In terms of magnesium treatment, a single bolus of magnesium is sufficient to improve neurological outcome in experimental studies—except in the presence of cerebral hemorrhage, in which case outcome was worsened by magnesium treatment.[159]

Nonetheless, the importance of restoring low serum magnesium levels to normal levels was highlighted in a study examining the neurological events associated with low serum magnesium in patients with advanced atherosclerosis.[160] Low serum magnesium levels were associated with a 3.29-fold increased risk of adverse neurological events, including stroke. It is still unclear, however, exactly how much of the serum magnesium enters the brain once the blood–brain barrier has closed. Hypermagnesemia produced only marginal increases in total and ionized CSF magnesium, suggesting that regulation of CSF magnesium is largely maintained following acute brain injury and limits its brain bioavailability.[161]

A recently completed clinical trial of magnesium in TBI[162] demonstrated that magnesium treatment did not improve outcome, and in fact may be harmful in some cases. Although some problematic issues with trial design and interpretation have been identified,[163] the results were nonetheless consistent with the negative preclinical studies. Specifically, the clinical trial demonstrated that continuous infusion of magnesium for 5 days was not superior to a single bolus on admission (the untreated control group had their serum magnesium normalized on admission), that high doses of magnesium were not superior to low doses of magnesium consistent with the established inverted-U dose–response curve, and that magnesium administration may, in some cases, be harmful (e.g., hemorrhage). All of these negative observations have been previously demonstrated in experimental studies.[159,164]

Other clinical trials that adopted treatment dosages consistent with those used in preclinical studies, and that did not administer magnesium to the control group, have shown that magnesium does have a neuroprotective effect in specific circumstances. For example, a prespecified interaction analysis of the neutral Intravenous Magnesium Efficacy in Stroke (IMAGES) trial revealed significant benefit from magnesium in patients with noncortical stroke.[165] Improvements in neonatal outcomes that are of potential clinical significance have been demonstrated following magnesium administration to mothers prior to preterm birth.[166] Infusion of magnesium in patients with subarachnoid hemorrhage has been shown to reduce the occurrence of delayed ischemia caused by cerebrovascular spasm.[167] Finally, magnesium administration has been shown to be particularly useful in preserving short-term memory and cortical control over brainstem functions after cardiac surgery.[168]

Clearly, the issue of magnesium neuroprotection in brain injury has not been resolved. Further trials investigating magnesium as a neuroprotective agent are currently in progress for stroke, subarachnoid hemorrhage, and the prevention of cerebral palsy. The only ongoing trial in TBI is directed at pediatric head injury.

Cyclosporin

Another potentially multifactorial compound that has been proposed as a multifactorial neuroprotective agent is cyclosporin-A (CyA). CyA is a short polypeptide that putatively exerts neuroprotective and neurotrophic effects in TBI, sciatic nerve injury, focal and global ischemia (for review, see Kaminska et al.[169]). Recent studies have demonstrated that, in addition to the well-described improvement in motor outcome observed with CyA administration after TBI,[170] the compound also improves cognitive performance.[171] This effect was dose-dependent and was correlated to an improvement in brain oxygen consumption after trauma. The preserved brain oxygen consumption after TBI was thought to reflect improved mitochondrial function, which is consistent with the ability of CyA to inhibit the mitochondrial permeability transition,[19,172,173] and in so doing, prevent mitochondrial swelling and improve energy recovery.[174] Such effects would also inhibit calcium accumulation, apoptosis and block free radical production.[175,176] Although CyA also inhibits calcineurin,[169] it is thought that the effects on mitochondrial activity are pivotal to its neuroprotective action.[177,178] Indeed, compared with FK506, a related immunosuppressant that inhibits calcineurin as effectively as CyA does,[169,179] CyA was superior in protecting against ischemic damage[177–180] and hypoglycemic brain injury,[181] presumably because of the superior ability of CyA to inhibit the mitochondrial permeability transition.[180] In addition to the suggestion that CyA completely inhibits excitotoxin-induced neuronal

cell death,[182] there is also a dose-dependent inhibition of amyloid precursor protein accumulation[183] and of traumatic axonal injury after TBI,[184] which may account for the reduced number of disconnected and dysfunctional axons following impact acceleration TBI.[185–188]

In clinical studies, phase II trials of CyA in TBI[189] have shown in the ascending dose study that patients with TBI demonstrated more rapid clearance and a wider distribution of CyA metabolites than other populations, thus complicating accurate dose determination for further trials. Nonetheless, even with the doses currently used, CyA administration in the early phase after TBI results in significantly higher extracellular fluid glucose and pyruvate, as well as a significant increase in mean arterial pressure and cerebral perfusion pressure.[190] The latter hemodynamic effects might contribute to the neuroprotective effect of CyA.

Thyrotropin releasing hormone

Thyrotropin releasing hormone (TRH) and its analogs have been promoted as potential multifactorial neuroprotectants in neurotrauma over a number of years.[191] Early experimental studies demonstrating that TRH and the early TRH analogs, such as CG3703 and YM-14673, improve motor outcome[192] attributed some of this improvement to physiological and metabolic actions of the compounds, including improving magnesium status[193] or bioenergetic state,[193] and promoting recovery in cerebral blood flow following brain trauma.[194] However, physiological actions of these compounds, including autonomic, analeptic, and endocrine effects, were considered undesirable from a clinical point of view. Aside from their potential use in recovery from disturbances of consciousness,[195] they were accordingly not vigorously pursued as neuroprotectants.

Alternative TRH analogs were designed to largely eliminate the undesirable physiological actions while preserving the neuroprotective effects,[196] and in so doing, the compounds have become even more of a multifactorial drug, now with nootropic actions, but without the adverse side effects. As a class of compounds, these more recent analogs have been shown to reduce cell death, protect against glutamate toxicity and β-amyloid-induced injury, reduce lesion volume, and produce highly significant improvements in motor and cognitive function in both *in vitro* and *in vivo* models of neurotrauma.[197] They have also been shown to significantly downregulate expression of mRNAs for cell cycle proteins, aquaporins, cathepsins, and calpain and to upregulate expression of brain-derived neurotrophic factor, hypoxia-inducible factor, and several heat-shock proteins.[197] The effect on aquaporins may explain the observed beneficial effects on ICP that have been reported, although this may also be attributed to the ability of the compound to reduce free radical reactions.[198]

CONCLUSIONS

The brain is the most complex organ in the body, and significant gaps still exist in our understanding of normal brain function and how it is affected by acute injury. Unlike other organ systems, we cannot readily relate cellular function to the function of the organ as a whole, and although neural pathways and the connections neurons make are a key aspect of normal brain function, we still have not resolved how structure, neurochemistry, and function are related. It is, therefore, not surprising that we have yet to understand how changes in structure and neurochemistry after acute injury translate into functional deficits, or how to prevent these functional deficits.

Nonetheless, we do understand that acute brain injury is a heterogenous type of injury, made up of immediate and delayed anatomic, molecular, biochemical, and physiological events that are both complex and multifaceted. So complex are these interactions that the concept of a simple magic bullet is no longer accepted by the research community, and the focus has now turned to interventions that can modulate a number of independent injury factors simultaneously. Although this seems an ambitious goal, several such interventions have already been identified from experimental studies and await clinical trial. Some of these have been summarized in the current review. However, translation efforts over the past few decades have not been successful, and a growing reticence to pursue clinical TBI studies is becoming apparent in the pharmaceutical industry.

In clinical trial design, it must be realized that TBI is a very heterogenous type of injury that varies widely in its etiology, clinical presentation, severity, and pathophysiology. Just as experimental models have been finely tuned and optimized to detect neuroprotective effects, so too must clinical trials be given every opportunity to detect an efficacious intervention. Only then can we have the opportunity to realize the potential of these multifactorial pharmacotherapies.

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