Alterations in Cholinergic Pathways and Therapeutic Strategies Targeting Cholinergic System after Traumatic Brain Injury

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Abstract

Traumatic brain injury (TBI) results in varying degrees of disability in a significant number of persons annually. The mechanisms of cognitive dysfunction after TBI have been explored in both animal models and human clinical studies for decades. Dopaminergic, serotonergic, and noradrenergic dysfunction has been described in many previous reports. In addition, cholinergic dysfunction has also been a familiar topic among TBI researchers for many years. Although pharmacological agents that modulate cholinergic neurotransmission have been used with varying degrees of success in previous studies, improving their function and maximizing cognitive recovery is an ongoing process. In this article, we review the previous findings on the biological mechanism of cholinergic dysfunction after TBI. In addition, we describe studies that use both older agents and newly developed agents as candidates for targeting cholinergic neurotransmission in future studies.

Key words: acetylcholine; cholinergic; nicotinic; traumatic brain injury

Introduction

TRAUMATIC BRAIN INJURY (TBI) is a common cause of death and disability worldwide. Among the survivors of TBI in the United States, 70,000 to 90,000 have substantial long-term loss of cognitive function that results in a lifetime of disability.¹ Neuropsychological tests that assess various aspects of behavior such as social function, cognitive abilities, and psychiatric symptoms at 10–20 years after TBI show significant behavioral impairment at such chronic time points.² Specifically, there are problems in memory, attention, and information processing among many others. Much has been discovered about the biological mechanisms of injury, which includes oxidative stress, inflammation, neurotransmitter dysfunction, and mitochondrial dysfunction. Development of pharmacological treatment for persons with TBI, however, has been difficult because of the wide heterogeneity of disease and mechanisms of injury.³

Acetylcholine (ACh) is a neurotransmitter that is composed of an ester of acetic acid and choline, and it is effective in regulating plasticity and arousal among many other functions. Cholinergic neurotransmission is a crucial factor in regulation of cognitive function, specifically in learning and memory,⁴ as well as attention.⁵ Both the septo-hippocampal cholinergic and the nucleus

basalis-neocortical cholinergic pathways are important components of the neural circuitry of cognition. In the septo-hippocampal pathway, neurons of the medial septum and the diagonal band of Broca innervate the hippocampus via the fimbria–fornix bundle and the supracallosal striae. The nucleus basalis consists of a collection of magnacellular cholinergic neurons in the basal forebrain that provide diffuse, predominantly ipsilateral, projections to most of the cerebral cortex. Cholinergic inputs to the medial prefrontal cortex of rats mediate attentional processing,⁶ and cholinergic inputs to hippocampus regulate memory consolidation.⁷

In neurodegenerative diseases, such as Alzheimer's disease, loss of cholinergic functions is believed to be an important contributor to cognitive deficits. Similarly, TBI induces dysregulation of the cholinergic system, and this is believed to be one of the significant underlying causes of impairment of cognitive functions. Because of ACh's major role in numerous cognitive processes, the relationship between alterations in cholinergic neurotransmission and cognitive deficits after TBI has been investigated in many studies. The role of cholinergic dysfunction after TBI leading to long-term deficits in learning and memory have been described previously.^{8,9}

As reviewed previously,^{9,10} the cholinergic system exhibits an acute surge in activity leading to massive release of ACh immediately after TBI.¹¹ Early studies documented acute cholinergic

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excess in cerebrospinal fluid (CSF) after TBI in humans.^{12,13} At later times, however, there is a persistent reduction in cholinergic function.^{14,15} In agreement with this, several studies have shown that TBI causes direct injury to cholinergic projections. Cholinergic neuronal loss is found in several areas of the forebrain such as the medial septal nucleus and nucleus of the diagonal band of Broca, which have major projections to the hippocampus.^{16,17} Human postmortem studies after TBI reported loss of ACh neurons in the nucleus basalis of Meynert, reflecting a general deficit in the cholinergic neurotransmission,¹⁸ and functional imaging of the brain after TBI suggested long-term cholinergic deficits.¹⁹ Overall, the cholinergic system undergoes drastic change throughout the days to months after TBI. Therapeutically attenuating the acute injury to the cholinergic system and enhancing its function chronically has been the challenge of TBI researchers.

In this review, we will outline the alterations in various components of the cholinergic system after TBI in both animal models and human clinical studies. Pharmacological agents targeting ACh neurotransmission have been shown in many studies to attenuate cognitive deficits in both neurodegenerative diseases as well as acute injury. For example, nicotinic agonists have shown promise in Alzheimer's disease.^{20–22} Similarly, acetylcholinesterase (AChE) inhibitors have been shown to be beneficial in Alzheimer's disease.^{23,24} The therapeutic effects of cholinergic agents on cognitive function were also evidenced in studies of TBI^{25–29} as well as stroke.^{30,31}

The importance of cholinergic signaling in TBI has been explored by a fair number of studies and reviews. An updated review is currently warranted, however, given the introduction of new pharmacological agents that are receptor specific and recent clinical trials. By reviewing and reinterpreting the studies that used pharmacological agents in the setting of TBI, both the cholinergic pathobiology of TBI as well as future strategies for targeting specific aspects of cholinergic deficit after TBI will be explored.

Muscarinic Receptors

Muscarinic acetylcholine receptors (mAChR) are G-protein coupled receptors that are important for neurogenesis,³² survival of newborn neurons,³³ and long-term potentiation (Fig. 1).^{34,35} These receptors are categorized to five subtypes (M₁–M₅). The M₁, M₃, and M₅ subtypes mediate excitatory function, whereas M₂ and M₄ subtypes mediate inhibitory function. Of particular interest is the M₂ autoreceptor, which is found presynaptically and inhibits ACh release. By activation of this receptor, ACh signaling is modulated by presynaptic feedback inhibition.

Alterations in mAChRs after TBI

Although human postmortem study of inferior temporal gyrus tissue showed no alteration in muscarinic receptor binding,³⁶ many animal studies showed reduction of mAChR at an early time point after injury ranging from hours to days. Newborn piglets subjected to fluid percussion injury (FPI) had reduced mAChR determined by autoradiography at 6 h after injury.³⁷ At as early as 2 h after FPI in rats, there was decreased affinity of muscarinic receptor to positron emission tomography (PET) radiotracer in the hippocampus.³⁸ An autoradiography study in rats also showed early decrease in mAChR affinity in the hippocampus and brainstem at 1 h.³⁹ Studies using the controlled cortical impact (CCI) model of injury, however, showed a decrease in muscarinic receptors at slightly later time points: the earliest time of mAChR reduction was at 24 h, but no significant decrease was found at 2 h.⁴⁰ Similarly, no decrease

was detected between the 1–24 h interval, and earliest mAChR deficit was found at 3 days by radioligand binding.⁴¹ This difference in timeline of mAChR downregulation is possibly because of the differences in the nature of injury: FPI induces more diffuse mechanical injury compared with CCI.

Although both types of injury produce diffuse axonal injury and cortical contusion injury, CCI's more focal distribution of energy may lead to delayed deficits in the pericontusional regions. In contrast, direct injury to a wider volume of tissue by FPI may lead to earlier changes in mAChR at pericontusional areas. mAChR, however, may also show compensatory increases at later time points, because an increased number of binding sites of mAChR was reported in the hippocampus and neocortex at 15 days after injury.⁴²

Most of these studies used PET tracer or radioactive ligand non-specific to muscarinic receptor subtypes, but several studies indicated that this mAChR reduction may be largely because of a decrease in M₂ subtype. A specific autoradiography study differentiating M₁ and M₂ receptors showed that while there was no change in M₁ receptors, there was a significant decrease of M₂ receptors in several regions of the hippocampus.⁴³ Also, by immunohistochemistry, there was decreased M₂ staining at 2 and 4 weeks after CCI in the hippocampus.⁴⁴ This delayed decrease of M₂ receptors in the hippocampus was also confirmed at 4 weeks after CCI by Western blot analysis. Because M₂ receptors are autoreceptors inhibiting ACh release presynaptically, one possible explanation of this specific decrease is a compensatory downregulation of inhibitory autoreceptors to maintain high ACh release chronically.

Pharmacological agents targeting mAChRs

It is not clear whether these changes in mAChR are compensatory changes induced by injury or if they are direct consequences of injury. Regardless of the cause, several studies have suggested that mAChR blockade acutely after injury may be neuroprotective.^{45,46} TBI leads to acute elevations of ACh,⁴⁷ and excessive muscarinic cholinergic receptor activation can lead to epileptic damage.^{48,49} In these studies, injection of cholinergic agents into limbic regions or the hippocampus induced seizure activity as well as local necrosis of the tissue.^{48,50} Thus, administration of scopolamine, a muscarinic antagonist, at early time points after TBI may be neuroprotective by preventing the effects of acute elevations of ACh. Early administration of scopolamine, which has equal affinity for all five mAChR subtypes,⁵¹ attenuates motor deficits,^{46,52} mortality, and weight loss^{52,53} after TBI in rats.

Another possible benefit of scopolamine after TBI is by enhancing ACh release at chronic time points after TBI by blockade of the M₂ autoreceptor. Several microdialysis studies showed that scopolamine administration can evoke ACh release in the hippocampus^{15,54} and neocortex¹⁴ at 14 days post-injury, likely by blocking presynaptic autoreceptors. Although the effect of scopolamine on ACh release is compromised in the setting of TBI compared with sham animals,⁵⁵ enhancing ACh release at chronic time points when there is decreased cholinergic activity may theoretically improve cognitive function. Thus, depending on the time frame of application, scopolamine can enhance cholinergic signaling by preventing injury (acute) or enhancing neurotransmission (chronic).

M₁ vs. M₂ specific agents

Similar to the effect of scopolamine administration, specific blockade of M₁ receptors using dicyclomine 15 min before injury

FIG. 1. Muscarinic acetylcholine receptor (AChR) regulation of ACh regulating enzymes. Activation of M₁, M₃, and M₅ are excitatory, leading to activation of phospholipase C (PLC), then subsequent calcium mediated C-Fos activation. This upregulates acetylcholinesterase (AChE) expression and downregulates choline acetyltransferase (ChAT) and vesicular ACh transporter (vAChT) expression. Activation of M₂ and M₄ are inhibitory, however, and can inhibit cyclic AMP (cAMP) mediated signaling. Color image is available online at www.liebertpub.com/neu

reduced motor deficits.⁵⁶ In another study, dicyclomine treatment 5 min before injury attenuated the increase in spectrin breakdown products in the CSF after TBI, but it did not reduce neuronal degeneration assessed by fluoro-jade staining.⁵⁷ As discussed by Cox and colleagues,⁵⁷ functional deficits after TBI can occur in the absence of neuronal death. Thus, the behavioral improvement by dicyclomine treatment may not be because of preventing neuronal death but rather by preventing deficits in cholinergic neurotransmission by reversing sublethal cellular dysfunction such as deficits in long-term potentiation. Although dicyclomine did not affect the level of neuronal death, these findings from behavioral and biomarker studies support the idea that attenuating the effects of acute and excessive activation of ACh receptors (specifically M₁ receptors) is neuroprotective.

Similarly, administration of muscarinic M₂ autoreceptor antagonist BIBN99 improves cognitive function as tested by Morris water maze (MWM) after FPI in rats.²⁵ Unlike the previous studies using dicyclomine, which was administered at the time of the injury, the administration of BIBN99 in this study was continuous for days after injury. Since specific block of M₂ autoreceptors can cause ACh release in the neocortex and hippocampus,^{58,59} the mechanism of BIBN99's effect on behavioral improvement may involve enhancement of ACh release. The therapeutic effect of BIBN99, however, is likely a combination of preventing ACh receptor activation both initially as well as chronically after injury. There was a therapeutic effect when animals were injected from 24 h after TBI and during the time of the MWM task from days 11–15, but not if injections were performed just during the days of MWM task. Previously mentioned scopolamine studies also used early administration after injury to achieve neuroprotection,^{45,52,53}

indicating that muscarinic antagonism early after injury is important in inhibiting secondary injury.

Targeting both M₁ and M₂ receptors further enhances the therapeutic effect. A partial M₁ agonist and M₂ antagonist, Lu 25-109-T, reduces TBI-induced choline acetyltransferase (ChAT) loss in the forebrain⁶⁰ and improves performance in the MWM⁶¹ after TBI. This agent would theoretically activate understimulated M₁ receptors while inhibiting M₂ autoreceptors, thereby exogenously activating ACh receptors and amplifying endogenous ACh release at the same time. In this study, Lu 25-109-T was subcutaneously injected until 15 days post-injury, indicating that maintaining the cholinergic tone may be beneficial during the recovery phase of trauma.

In summary, the inhibition of cholinergic receptors at acute time points after TBI by using mAChR specific antagonists such as scopolamine would inhibit the damage induced by excessive ACh release. At later time points, when there is general cholinergic hypofunction, agents with agonist properties for each subtype of receptor should be used to enhance ACh signaling.

Nicotinic Receptors

Activation of nicotinic ACh receptor (nAChR) by binding of an agonist leads to influx of Na⁺ and Ca²⁺ (Fig. 2). The degree of permeability of Ca²⁺ depends on the subunit composition of the nAChR, because the $\alpha 7$ subunit containing nAChRs has a much higher Ca²⁺ permeability than the $\alpha 3$ or $\alpha 4$ subunit containing receptors.^{62,63} This property of regulating the intracellular Ca²⁺ level makes it an important mediator of learning and memory, which depends on signal transduction pathways involving extracellular

signal-regulated kinase 1/2 (ERK 1/2) and cAMP response element binding protein (CREB).⁶⁴ In addition, Ca^{2+} permeability makes $\alpha 7$ receptors also a possible contributor to excitotoxicity, because excessive influx of Ca^{2+} activated by glutamate release can lead to neuronal death.

Alterations of nAChRs after TBI

Many previous studies have explored nAChR changes in various brain regions after TBI using autoradiography similar to studies exploring mAChR changes. Although one study reported no change in binding of [³H]epibatidine, which nonselectively binds to $\alpha 3$ and $\alpha 4$ subunit containing receptors in newborn piglets,³⁷ other studies report significant alteration in receptor levels. A rat model of injury using [³H]epibatidine showed reduction of $\alpha 3$ and $\alpha 4$ subunits in various regions such as the thalamus, hypothalamus, olfactory tubercle, gigantocellular reticular nucleus, and motor cortex⁴⁰ as well as subregions within the hippocampus.⁴¹

Similar reduction of $\alpha 7$ nAChR levels were reported by a decrease in [¹²⁵I] α -bungarotoxin binding in newborn piglets after FPI in the CA1 region of the hippocampus, thalamus, and superior colliculus.⁶⁵ This finding was also correlated with a generalized decrease in $\alpha 7$ nAChR levels in all the brain regions of adult rats after FPI at 2 and 24 h post-injury in the same study. Decreased binding of $\alpha 7$ receptors was also shown after CCI in adult rats in the hippocampus, somatosensory cortex, stratum oriens, and superior colliculus as early as 1 h to 21 days after injury.⁴¹ Compared with this, $\alpha 3$, $\alpha 4$ reduction is delayed, occurring at 72 h to 21 days.

These changes in $\alpha 7$ receptors have numerous implications on the mechanisms of secondary injury. Several studies showed that activation of $\alpha 7$ receptors attenuates release of inflammatory mediators from macrophages and microglia.^{66–68} Also, $\alpha 7$ receptors have an anti-apoptotic effect mediated by pathways involving

phosphatidylinositol 3-kinase-Akt⁶⁹ as well as by ERK1/2.⁷⁰ Based on these studies reporting TBI induced changes in nAChR levels, many groups have used pharmacological agents targeting these receptors, aiming to improve molecular and behavioral outcomes.

Agents targeting nAChRs

Nicotine administration, which would nonspecifically activate both $\alpha 7$ as well as $\alpha 3$ and $\alpha 4$ containing nAChRs, improves spatial learning and memory retention⁷¹ and reverses $\alpha 7$ receptor deficits.⁷² Other studies showed therapeutic potential of agents that specifically activate $\alpha 7$ receptors—namely, choline. Dietary treatment using choline, a selective $\alpha 7$ nAChR agonist, for 2 weeks before TBI leads to improved spatial memory function, reduced cortical tissue loss, and microglial activation after TBI.⁷³

There are multiple effects of choline, however, besides $\alpha 7$ nAChR activation that may aid in neurorecovery. Choline is also an intermediary in ACh formation, and its administration may enhance ACh synthesis. Accordingly, injection of a naturally occurring compound, cytidine 5'-diphosphocholine (CDP-choline), which is metabolized to form choline, attenuates TBI induced ACh surge in the hippocampus and neocortex as well as enhances spatial memory in rats.²⁷ In addition, CDP-choline treatment can reduce hippocampal neuronal loss and cortical lesion volume.⁷⁴ In a double-blind, placebo-controlled, multicenter trial using CDP-choline for 90 days,⁷⁵ no significant cognitive benefit was found, however. The reasons for this lack of agreement between animal studies and human trial are unclear, but may be because of the complexity of human TBI and heterogeneity of the injury mechanism.

Choline is also a main building block for phospholipid, which is important for membrane formation, and it is released from damaged cellular membranes after TBI,⁷⁶ likely because of phospholipid

FIG. 2. Activation of nicotinic $\alpha 7$ receptor and its effects. There are multiple effects of $\alpha 7$ receptor activation, including downstream pathways activating long-term potentiation as well as anti-apoptotic effects. In microglia, there is inhibition of pro-inflammatory cytokine expression. Color image is available online at www.liebertpub.com/neu

degradation and decreased circulatory clearance. Thus, its abundant supply after TBI by CDP-choline treatment is believed to aid in stabilization and repair of damaged membranes.⁷⁷ Other mechanisms to explain the neuroprotective effects of CDP-choline have been suggested, however, such as increased levels of antioxidant glutathione,⁷⁸ which reduces oxidative damage.

$\alpha 7$ receptor specific agents

Newer pharmacological agents with specific affinity to $\alpha 7$ receptors were used in various brain injury studies.^{79–84} In rats with intracerebral hemorrhage in the striatum, intraperitoneal injection of $\alpha 7$ agonist PNU-282987 reduced the number of activated microglia/macrophages and neuronal loss.⁸² In contrast, $\alpha 4\beta 2$ specific agonist RJR-2403 did not ameliorate these neuronal losses induced by intracerebral hemorrhage, supporting the crucial role the $\alpha 7$ receptor has in attenuating brain injury. Because the majority of nAChRs in the striatum are composed of $\alpha 4\beta 2$ and $\alpha 6\beta 2$ subunits, $\alpha 7$ is a minority in neuronal cells. The beneficial effect of $\alpha 7$ activation is thus likely because of its effect on microglia, reducing inflammatory response. Similarly, PNU-282987 was neuroprotective in a subarachnoid hemorrhage model of rats.⁸³ Its administration decreased cleaved caspase-3 and neuronal cell death. In addition, it improved neurological deficits assessed by spontaneous activity, limb movements, climbing, and various other functions.

Aside from enhancing ACh neurotransmission, attenuating inflammation, and enhancing membrane synthesis, $\alpha 7$ receptor activation may be important targets for improving learning and memory. The $\alpha 7$ receptors have high Ca^{2+} permeability and are known to activate ERK $\frac{1}{2}$ signaling⁷⁰ and CREB signaling⁸⁵ pathways, which are central components of learning and memory.⁶⁴ A wide variety of $\alpha 7$ agonists such as choline, GTS-21, SSR-180711A, and PNU-282987 are able to activate this pathway,⁸⁶ and positive allosteric modulator PNU-12596 enhances this $\alpha 7$ receptor activation effect, which will be reviewed later. Accordingly, behavioral experiments with $\alpha 7$ agonist AR-R 17779 given to adult rats resulted in improved learning and memory tested in radial maze.⁸⁷ These agents have not been tested in a TBI setting but could serve as potential candidates for cognitive function enhancing agents during recovery.

Alterations in Intracellular Cholinergic Enzymes after TBI

Changes in choline acetyltransferase and vesicular acetylcholine transporter

Changes among the cholinergic neurons have been described in many TBI studies. A commonly used marker of cholinergic neuron is ChAT, a presynaptic enzyme for ACh synthesis and a marker of integrity of presynaptic cholinergic function and structure. Most of the regions of the brain have decrease in either ChAT levels or its activity after TBI. In rats, there is a moderate reduction of ChAT activity in the dorsal hippocampus, frontal and temporal cortices 1 h after injury (25, 32, and 23%, respectively), but there is greater than a 50% increase in ChAT activity in the parietal cortex.⁸⁸

Similarly, other studies in rats after FPI showed decreased ChAT positive neurons in the basal forebrain¹⁶ and medial septal nucleus, nucleus of the diagonal band of Broca, and nucleus basalis of Meynert in 10–15 days.¹⁷

Human postmortem studies also show decrease in ChAT activity—there is a 50% reduction of ChAT activity in the inferior temporal gyrus samples after TBI.³⁶ Reduction of ChAT activity

was also found in bilateral cingulate, inferior temporal, and posterior parietal regions.⁸⁹ Histological analysis of neurons in the nucleus basalis of Meynert showed significant damage in patients who died after TBI, and decreased intensity of ChAT immunoreactivity was also found in these damaged neurons (survival times 1–300 h, median = 27 h).¹⁸

Such decrease in ChAT levels and activity may not only indicate cholinergic neuronal death; ChAT immunoreactivity decrease was not accompanied by changes in cresyl violet-staining in rats after TBI.⁶⁰ In support of this, the decrease in ChAT immunoreactive cells after TBI in rats was only transient, and there was no significant difference by 28 days.¹⁶ The decrease in level and activity of ChAT is likely a combination of cholinergic neuronal loss as well as downregulation of the ChAT protein.

Another important regulator of ACh neurotransmission is vesicular ACh transporter (vAChT), an enzyme that is responsible for loading ACh into secretory vesicles. This enzyme also has time dependent alteration in levels after TBI: downregulation acutely, but upregulation at subacute to chronic time points. At acute time points after injury (2 h–72 h) vAChT expression is reduced in multiple brain regions including the thalamus, hypothalamus, motor cortex, and basal forebrain in adult rats.⁴⁰

Starting at weeks after injury, however, there is an upregulation. Increased hippocampal vAChT protein has been reported using immunohistochemistry and Western blot at 2–4 weeks (but not at 1 day or 1 week after injury).⁴⁴ At 4 weeks after injury, there is an increase in mRNA as well as protein levels of vAChT in the hippocampus.⁹⁰ This increase is also present at least up to 1 year after injury in the hippocampus as well as the cortex of rats.⁹¹

Both ChAT and vAChT are crucial for the function of presynaptic ACh release. The reported alterations in these studies may reflect a combination of pathological process and compensatory changes. Given the ACh surge at immediate time points after injury, the decrease in vAChT levels may reflect compensatory downregulation to prevent excessive ACh receptor activation. Also, a direct effect of the injury leading to compromised function of presynaptic cholinergic neuronal function may underlie these changes. The increase in vAChT levels at weeks to 1 year after injury, however, correlates with the general behavioral recovery of animals at chronic time points,⁹¹ indicating the compensatory nature of this change.

AChE Changes after TBI

AChE is a crucial regulator of cholinergic neurotransmission that metabolizes acetylcholine after its release in the synaptic terminals. By decreasing or increasing its function, the level of ACh activating its receptors can be changed, and thus the strength of ACh signaling is affected. Because the activity level of AChE has been shown to be associated to attention and working memory,⁹² there have been several efforts to characterize activity of AChE in different brain regions after TBI to understand the basis of TBI-induced cognitive deficits. Immediately after TBI, massive release of ACh and glutamate causes excitotoxic damage as well as compensatory changes in the regulators of the cholinergic system such as AChE.

Alterations in AChE after TBI

Several studies reported short-term alterations in AChE activity after TBI. Acutely after injury, basal forebrain showed an increase in AChE activity at 2–24 h, which normalized by 72 h. This increase in basal forebrain AChE activity also occurred in newborn

pigs at 6 h after TBI.⁹³ After exposure to acute stress, such as a forced swim protocol, acute ACh release in the basal forebrain leads to upregulation of AChE mRNA to possibly restore physiological levels of ACh.⁹⁴ Thus, AChE expression and activity may be upregulated in the basal forebrain as a compensatory response to acutely increased cholinergic neurotransmission.

Stress from restraining also transiently increases ACh levels in the hippocampus of rats.⁹⁵ In addition, cold and immobilization stress increased AChE activity in the cerebrum of rats.⁹⁶ This increase in AChE activity may have an important contribution to functional losses, because transgenic mice that overexpress AChE have spatial learning and memory deficits.⁹⁷ Because application of AChE inhibitors in patients with Alzheimer's disease and enhancing ACh signaling can improve cognitive function,⁹⁸ this increase in AChE and subsequent decrease in ACh signaling may underlie post-TBI cognitive deficits.

Although the explanation of this AChE activity change has not been thoroughly explored by TBI researchers, a possible mechanism can be gleaned from a previous study using corticohippocampal brain slice. Application of AChE inhibitors to a corticohippocampal brain slice to increase ACh levels and subsequent ACh signaling caused alterations in cholinergic enzymes.⁹⁴ AChE mRNA levels were increased, but ChAT and vAChT mRNA were decreased. These alterations occurred 20 min after increase in c-Fos levels, correlating with the expression of enzymes that regulate cholinergic signaling. This bidirectional modulation of cholinergic gene regulation was presumed to be because of the increase in c-Fos levels. The increase in c-Fos has previously been shown to occur in other studies where muscarinic agonists were administered: c-Fos was increased in frontal, cingulate, and retrosplenial cortex.^{99,100} Thus, after TBI, acute increase in ACh have been shown to correlate with signaling cascades leading to AChE overexpression and ChAT and vAChT underexpression via c-Fos.

Another study, however, has shown that hippocampus, hypothalamus, and motor cortex has decreased AChE activity acutely (between 2–24 h).¹⁰¹ AChE activity deficit was also present in the hippocampus in a blast injury model of TBI.¹⁰² AChE activity may not be regulated just by transcriptional change affecting its concentration, but it likely involves additional mechanisms such as release of soluble form of AChE, which has been reported in the setting of hypoxic damage.¹⁰³ The regional differences in AChE activity may reflect different mechanisms of control, as well as different levels of cholinergic neurotransmission in these regions.

At chronic periods after TBI, there is a general hypofunction of the cholinergic system as evidenced by reduction of ACh synthesis¹⁵ and release.⁵⁴ In addition, there is a general decrease in the AChE activity in several cortical areas, likely as a compensatory change for decreased ACh release. PET imaging determined the decrease in AChE activity in human subjects with TBI more than 1 year after injury, with the most prominent decrease found in parieto-occipital regions of the neocortex.¹⁰⁴ These cholinergic deficits may be in part because of dysfunction of the basal forebrain cholinergic system, which has been demonstrated in the past.^{16–18}

To reverse these deficits in ACh neurotransmission, cholinesterase inhibitors have been used to increase the availability of cortical ACh by inhibiting enzymatic catabolism of ACh. These agents have been well characterized and studied widely in Alzheimer's disease animal models and clinical patients. These include ENA713, rivastigmine, physostigmine, tacrine, and do-

nepezil. These agents have various differences in central nervous system (CNS) specificity, hepatotoxicity, and other peripheral side effects.

AChE inhibitor studies in animals and humans

The efficacy of physostigmine in treating cognitive deficits after TBI has been observed in many animal studies as well as patients with TBI. In rats with TBI that were given physostigmine by continuous infusion, there was an improvement in locomotor function assessed by rotarod task,¹⁰⁵ attenuation of brain tissue loss, as well as spatial learning and memory deficit assessed by MWM.¹⁰⁶ This study also indicated that not only AChE inhibition alone, but AChE inhibition in combination with continuous training is important for reversing cognitive deficits. Early case studies of TBI patients with physostigmine enhanced several aspects of cognitive function such as improving verbal recall and attention, as well as reducing confusion.^{107–109} Physostigmine also improved memory after TBI assessed by standardized neuropsychological tests.¹¹⁰

One possible mechanism of physostigmine induced behavioral improvement after TBI is regulation of cerebral blood flow (CBF). As previously shown by Scremin and associates,¹¹¹ measurement of CBF with autoradiography techniques showed that the site of impact after TBI has decreased CBF at 2–24 h after injury. Cerebral cortex contralateral to focus of contusion was shown to have hyperemia in this study. Physostigmine reversed this decrease in CBF after TBI in the ipsilateral side and also increased CBF in the contralateral side to the trauma.

Because cholinergic activation enhances CBF in the neocortex,¹¹² hyperemia contralateral to contusion was postulated to be because of increased ACh synthesis and turnover.^{76,113} Thus, the behavioral benefit of physostigmine after TBI may be because of enhancing perfusion after TBI and enhancing neurological function secondarily. Physostigmine, however, has an extremely short half-life and narrow therapeutic window. It has significant systemic side effects, such as reducing heart rate and blood pressure, which has discouraged its use in patients and led to developments of newer AChE inhibitors that have more CNS specific effects.

Another AChE inhibitor, ENA713, which diffuses mainly into the CNS, has been used in animal TBI experiments. Its administration improved reflex and motor function in a closed head injury model in rats.²⁸ Aside from increasing neuronal cholinergic function, ENA713 reduced the disruption of the blood–brain barrier after injury and thus reduced vasogenic edema. The mechanism of this reduction in blood–brain barrier disruption has not been clarified, but this effect is also found with rivastigmine treatment, another AChE inhibitor with central specificity.

Rivastigmine ameliorates spatial memory impairments, motor deficits, and edema in a closed head injury in mice.²⁹ This neuroprotection was dependent on both nicotinic and muscarinic receptors, because using either mecamylamine (nicotinic antagonist) or scopolamine (muscarinic antagonist) prevented neuroprotection. These studies by Chen and coworkers^{28,29} used one-time injection of AChE inhibitor acutely after injury (5 min–2 h). Because these agents would have inhibited AChE for a short term, the improvement in spatial learning ability was attributed to attenuation of damage in the cholinergic system, rather than increased intrasynaptic ACh levels during the days of behavioral testing.²⁹

Rivastigmine, however, has only a very modest effect that was shown in clinical studies. A randomized, prospective, double-blind study showed no difference between the placebo and rivastigmine

treated group.¹¹⁴ Only a subgroup analysis among moderate to severe injured patients treated with rivastigmine showed improvements in verbal learning/memory and information processing. Similarly, in an open-labeled, multicenter study that was performed as a follow-up, rivastigmine has shown mostly no benefit except in a rapid visual information processing test, a measure of sustained attention, using a subgroup analysis.¹¹⁵

Tetrahydroaminoacridine (tacrine) is also a centrally acting AChE inhibitor with disappointing results in the previous studies. Administration of tacrine did not improve MWM performance in rats after TBI.²⁶ Even in sham rats, tacrine worsened MWM performance in a dose dependent manner. This result, however, may be because of the NMDA receptor antagonist property of tacrine.¹¹⁶ Because inhibition of NMDA receptor after TBI results in profound memory deficits,^{117,118} tacrine's failure to enhance memory is possibly because of its interaction with the NMDA receptors. In addition to this lack of effects, it also has poor tolerance and significant hepatic toxicity, discouraging clinical trials with this drug in patients with TBI.¹¹⁹

Donepezil: centrally specific agent with less systemic effects

Donepezil, a centrally acting AChE inhibitor with a much milder side effect profile than other AChE inhibitors,¹²⁰ has recently gained attention as a treatment option for patients with TBI in several initial case studies more than a decade ago.^{121,122} The clinical use of donepezil for cognitive deficits in Alzheimer's disease and anecdotal evidence from several case reports^{122,123} have indicated a therapeutic effect of donepezil in improving cognitive function after TBI.

As extensively reviewed by Ballesteros and colleagues,¹²⁴ the effect of donepezil was assessed by various neuropsychological tests in several small case series and case reports, as well as randomized controlled trials.¹²⁴ Donepezil was shown to improve cognitive function by mini-mental status examinations¹²² as well as subjective improvement in vigilance and attention.¹²⁵ Other tests have also shown improvements in intelligence quotient,¹²⁶ visual memory,¹²⁷ verbal learning and memory,¹²⁸ affective-behavioral function,¹²⁹ and attention and auditory/visual memory.¹³⁰

Several studies, however, reported no significant change in cognitive functions after donepezil treatment.^{131–133} The evidence for effectiveness of donepezil was deemed uncertain because of scarcity of data and poor methodological quality of many of these studies.¹²⁴ Despite its specificity to central AChE and low toxicity, more studies are needed to validate donepezil's efficacy in patients with TBI.

Among these studies using AChE inhibitors, simply increasing the intrasynaptic concentration of ACh or activation of ACh receptors may not be sufficient to result in behavioral improvements after TBI. Repeated AChE inhibitor administration may reduce ACh synthesis, because constant activation of the presynaptic M₂ autoreceptors may inhibit presynaptic release of ACh.²⁶ Also, excessive stimulation of mAChRs may lead to M₁ receptor downregulation.^{134,135} Because normal signaling leading to cognitive benefit will involve complex and synchronous neurotransmitter release, simply increasing the absolute concentration of synaptic ACh may not be therapeutic. As such, continuous physostigmine infusion with subcutaneous osmotic pump resulted in progressive impairment of locomotor performance at higher doses, whereas a lower dose was able to improve the performance.¹⁰⁵ Also, a higher rate of physostigmine infusion did not lead to improvement in

MWM performance after TBI, but there was improvement compared with controls only at lower rate of infusion.¹⁰⁶

With chronic treatment using high dosages, cholinergic neurotransmission may not be enhanced but instead compromised possibly because of the effects on M₂ autoreceptors as well as M₁ receptor downregulation. Aside from several studies having poor design or too few subjects, the heterogeneity of the effects of AChE inhibitors in human trials are also likely because of some studies using optimal doses and other using excessive doses, which compromises cholinergic signaling.

Time dependent changes in the cholinergic system: excitotoxicity and chronic traumatic encephalopathy

Time dependent changes in cholinergic signaling were mentioned in previous sections of this review. At early time points around TBI, blockade of mAChR was shown to be neuroprotective.^{45,46,52,53,56}

Antagonists to muscarinic receptors such as scopolamine and dicyclomine were shown to reduce mortality or behavioral deficits. An important explanation of this neuroprotection is that mAChR blockade prevented cholinergic excitotoxicity. With the excessive activation of these receptors, there is depolarization and elevation of intracellular Ca²⁺ that leads to damaging effects to the neuron.

Cholinergic excitotoxicity may also have a role in other major pathologies of the CNS such as ischemic stroke and epilepsy. Application of AChE inhibitors or muscarinic receptor agonists can induce seizures, which can lead to neuronal injury^{48,136,137} suggesting a possible role of excessive cholinergic signaling in epilepsy. In addition, a major pathologic mechanism in ischemic stroke is glutamate excitotoxicity, which can in turn lead to secondary release of excessive ACh and neuronal injury.¹³⁸ Although ACh excitotoxicity has been implicated in epilepsy and stroke, the role of this pathologic mechanism will need further future research.

At chronic time points, the opposite effect occurs in the cholinergic signaling, because there is a general hypofunction of the system. Reduction of scopolamine evoked ACh release⁵⁴ and decrease in nAChR that contain $\alpha 7$, $\alpha 3$, and $\alpha 2$ subunits have been reported.^{40,41} These changes show that there is a degeneration of specific components of the cholinergic neuronal circuitry. After TBI, chronic traumatic encephalopathy (CTE) ensues. Thus, degeneration of specific components of cholinergic signaling (e.g., $\alpha 7$ nAChR) may occur in CTE. The details of which components of the cholinergic signaling pathway degenerate in CTE and the timeline of these events will need further clarification in future studies.

Future Directions: Using Novel Agents to Enhance Cholinergic Function

Future research with agents targeting the cholinergic system will show significant progress as newer agents with receptor specific agonist/antagonist properties are developed. Muscarinic agents that have receptor subtype specific properties, such as BIBN99 and dicyclomine, can be optimally used during times of recovery and rehabilitation. Agents that have a combination of agonist and antagonist properties, such as Lu 25-109-T, could also be used to inhibit M₂ autoreceptors while activating M₁ postsynaptic receptors. In addition, many studies are now showing cognitive enhancement and attenuation of neuroinflammation by selective targeting of $\alpha 7$ nAChRs. Agents such as GTS-21, SSR-180711A, AR-R1779, and PNU-282987 may be useful in future TBI studies to show cognitive enhancement. In addition, novel agents such as

allosteric modulators that have even more complex function in activation of cholinergic signaling may show significant benefit.

Simple injection of nicotinic agonists into an animal may lead to nonspecific activation of cholinergic neurotransmission throughout all the regions of the brain that contain nicotinic receptors. Positive allosteric modulators (PAMs), however, cause more specific activation of cholinergic receptors by enhancing a pre-existing cholinergic signaling. These agents function by enhancing the potency of activation by endogenous nicotinic receptor activation without directly activating or desensitizing the target receptor.

There are specific advantages of enhancing cholinergic signaling by PAMs than nonspecific activation by a direct application of nicotinic agonist, as previously reviewed.¹³⁹ For example, activation by $\alpha 7$ nAChR specific PAMs preserves the spatiotemporal patterns of endogenous $\alpha 7$ nAChR activation. In addition, unlike direct activation of nicotinic receptors by nicotinic agonists, which is prone to desensitization, the neurocognitive effects induced by $\alpha 7$ PAMs are not reduced by desensitization. Various benefits of PAMs have been demonstrated in previous studies.^{140–147}

There are two subtypes of PAMs: Type I, which increases the peak amplitude of agonist induced response, and Type II, which increases the peak in addition to prolonging the current decay. Several of these agents have been used in either hippocampal slice study¹⁴¹ or behavioral studies to improve cognitive function.^{142,146,147} In addition, $\alpha 7$ nAChR PAM can reduce infarct volume¹⁴³ and attenuate motor deficit¹⁴⁴ in a mouse model of stroke. The exact mechanism of PAM in reducing stroke-induced damage has not been clarified, and future studies are needed to clarify this.

These novel agents have not been used in the setting of TBI, but may be potential candidates for future studies given their unique advantages over cholinergic agonists. In the past, nonspecific receptor agonists have been used in an attempt to modulate the cholinergic system after TBI. Newer pharmacological developments, however, continue to provide a variety of agents that are receptor subtype specific and have varying agonist/antagonist properties. Using these agents individually or in combination may be helpful in preventing secondary injury as well as reversing chronic cognitive deficits after TBI.

Several lines of clinical and laboratory evidence demonstrate that TBI can produce acute and chronic alterations in cholinergic systems. While therapies that target the cholinergic system have met with some laboratory success, improved therapies are needed for clinical translation in the future.

Author Disclosure Statement

The authors thank The Pittsburgh Foundation Walter Copeland Fund for their support of our TBI research. No competing financial interests exist.

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