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Region-specific disruption of synapsin phosphorylation following ethanol administration in brain-injured mice

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Abstract:

Introduction: Civilians and military personnel develop a range of physical and psychosocial impairments following traumatic brain injury (TBI), including alcohol abuse. As a consequence, increased rates of alcohol misuse magnify TBI-induced pathologies and impede rehabilitation efforts. Therefore, a developed understanding of the mechanisms that foster susceptibility of the injured brain to alcohol sensitivity and the response of the injured brain to alcohol is imperative for the treatment of TBI patients. Alcohol sensitivity has been demonstrated to be increased following experimental TBI and, in additional studies, regulated by presynaptic vesicle release mechanisms, including synapsin phosphorylation. **Materials and Methods:** Mice were exposed to controlled midline impact of the intact skull and assessed for cortical, hippocampal, and striatal expression of phosphorylated synapsin I and II in response to high-dose ethanol exposure administered 14 days following injury, a time point at which injured mice demonstrate increased sedation after ethanol exposure. **Results and Discussion:** Immunoblot quantitation revealed that TBI alone, compared to sham controls, significantly increased phosphorylated synapsin I and II protein expression in the striatum. In sham controls, ethanol administration significantly increased phosphorylated synapsin I and II protein expression compared to saline-treated sham controls; however, no significant increase in ethanol-induced phosphorylated synapsin I and II protein expression was observed in the striatum of injured mice compared to saline-treated TBI controls. A similar expression pattern was observed in the cortex although restricted to increases in phosphorylated synapsin II. **Conclusion:** These data show that increased phosphorylated synapsin expression in the injured striatum may reflect a compensatory neuroplastic response to TBI which is proposed to occur as a result of a compromised presynaptic response of the injured brain to high-dose ethanol. These results offer a mechanistic basis for the altered ethanol sensitivity observed following experimental TBI and contribute to our understanding of alcohol action in the injured brain.

Key words:

Alcohol, diffuse brain injury, phosphorylated synapsin, presynaptic, striatum

Introduction

Traumatic brain injury (TBI) is classified as a head injury that results in decreased levels of consciousness, loss of memory, intracranial lesion, and/or other abnormalities.[1] Reports estimate that 1.7 million new cases of TBI arise annually.[2] Of these new cases, 275,000 result in hospitalizations and 52,000 result in death.[2] An excessive use of alcohol or other drugs following TBI is common and can result in an increased likelihood of mortality, seizures, additional TBIs, and diminished neuropsychological performance.[3-6] It is estimated that up to 50% of persons with TBI have significant issues with alcohol or other drugs post-injury.[4,7,8] TBI commonly causes damage to the frontal lobe, which is instrumental in emotional regulation and planning.[9] Impaired frontal lobe functionality resulting from TBI may increase a patient's likelihood of engaging in alcohol misuse behaviors.[9] In addition, the striatum, which is involved in motivation, reward, and drug reinforcement, is vulnerable to various types of injury caused by TBI, including neurodegeneration, axonal injury, and ischemia.[10-13] Finally, significant cell loss has been found in the hippocampus, which plays an integral role in learning and memory, following TBI.[14,15]

Previous studies of altered synaptic plasticity may help elucidate a connection between TBI and

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post-injury alcohol abuse. TBI patients with post-injury alcohol abuse patterns have shown a greater frequency of disruptions in neural circuits that govern addictive behavior.[16] Importantly, studies examining the effects of induced brain lesions have demonstrated reduced spine density in both prefrontal cortex and nucleus accumbens regions leading to increased sensitivity to abused substances in the adult animal.[17] In addition, dendritic spine analysis reveals structural modifications resulting from TBI. Experimental TBI has been shown to reduce dendritic branching and dendritic density in rodents,[18] along with preventing increases in cortical thickness and cognitive performance during development.[19] TBI has also been shown to promote a 60% loss in CA1 hippocampal synapses[20] and a shortening of dendritic arbors at 4 months post-injury.[21] Similar disruptions in the dendritic structure have been demonstrated in mice with altered ethanol sensitivity. Larger spine areas and perimeters, larger axon terminals, and longer synaptic appositions were found in mice that demonstrated increased sedation following ethanol exposure (long-sleep mice).[22] Further studies revealed that long-sleep mice manifested a greater complexity in dendritic spine shape, decreased spine density, and decreased synaptic appositions following ethanol exposure.[23] These studies indicate that varied sensitivity to ethanol exposure may be influenced by alterations in dendritic arborization and/or spine morphology.

Similarly, ethanol has been shown to modulate synaptic proteins. For example, ethanol regulates both synapsin phosphorylation and clustering.[24-26] Notably, studies have demonstrated that impairments in phosphorylation of synaptic vesicle proteins, such as synapsin, are associated with an increase in behavioral sensitivity to ethanol.[26,27] As synapsin phosphorylation dissociates it from synaptic vesicle membranes,[28] these findings suggest that vesicle mobilization may be integral to the neuronal response to ethanol.

Our group's previous studies have demonstrated that TBI of moderate severity delivered via controlled cortical impact to the closed skull results in reactive astrogliosis in a subregion of the striatum, the nucleus accumbens, and increases behavioral sensitivity to sedating doses of ethanol.[29] Considering the involvement of synaptic vesicle protein phosphorylation with behavioral sensitivity to ethanol,[26,27] we posit that the injured brain's inability to further phosphorylate synapsin following ethanol exposure may represent a cellular mechanism that subserves enhanced behavioral sensitivity to ethanol in TBI mice.

Methods

Animals

All mice were progeny of C57BL/6 mice bred in the laboratory colony and were backcrossed at least nine generations to C57BL/6J mice from The Jackson Laboratory (Bar Harbor, ME, USA). All experiments were performed using male mice, aged 6–7 weeks, and mice were provided *ad libitum* access to food and water on a 12-h light/dark schedule. Animal use protocols were approved by the Institutional Animal Care and Use Committee at Wayne State University and followed the National Institutes of Health criteria.

Traumatic brain injury

According to previous studies,[29] mice were anesthetized with 5% isoflurane (Baxter Healthcare Corporation, Deerfield, IL, USA) using an anesthesia induction chamber. For maintenance during surgery and injury, 2.5% isoflurane was administered using a nose cone secured to the stereotaxic frame. Fur was removed from the surgical area using an electric razor. Sterile lubricant ophthalmic ointment was used to prevent drying of the eyes. On loss of pain reflex following anesthesia (assessed using paw pinch), mice were secured in a stereotaxic frame. Using sterile techniques, the skull was exposed with a 1-cm midline incision. The periosteum was reflected after prepping the scalp with betadine and isopropyl alcohol solutions. The animal's head and body were properly aligned with the nose cone and the animal was supported with a soft pad. To induce injury, an electromagnetically driven impactor tip (Leica Microsystems, Northbrook, IL, USA) was positioned over the exposed skull. The stainless steel hemispherical indenter (5-mm diameter) was displaced 2 mm at 5 m/s. The impact was directed at the midpoint between lambda and bregma along the midline suture. Automatic retraction of the impactor occurred 100 ms following impact. Ear bars were released, anesthesia was removed, and animals were monitored for respiration time, seizure behaviors, and length of apnea. Skulls of injured animals were examined for hematoma, hairline fractures, and herniation. Sham-injured animals (control) were subjected to all procedures except the impact. Tissue adhesive (3M, St. Paul, MN, USA) was used to close scalp incisions and triple antibiotic ointment was applied to prevent infection at the incision site. The animals were placed on a 37°C heating pad during recovery from anesthesia/injury. After recovery, mice were returned to their home cages. The total time from anesthesia initiation to the removal of the nose cone following injury was approximately 15 min. The injury was the first procedure to occur in all experimental animals.

Ethanol administration and tissue preparation

At 14 days post-injury, mice received an intraperitoneal injection of saline or ethanol (20% v/v in saline) to achieve a dose of 4.0 g/kg. Thirty minutes following saline or ethanol administration, mice were sacrificed by cervical dislocation. Subregions of the brain were rapidly dissected and frozen in liquid nitrogen. Using an electric pestle, brain regions were homogenized in a buffer containing 4% (w/v) 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate, 2 M thiourea, 7 M urea, and 30 mM Tris, pH 8.5. One tablet of complete protease inhibitor mixture (Roche Products, Indianapolis, IN, USA) was added to 50 mL of lysis buffer. Following homogenization, samples were centrifuged at 10,000 ×*g* for 10 min at 5°C, and the supernatants were collected. A Pierce 660nm Protein Assay kit was used to determine protein concentrations (Thermo Scientific, Rockford, IL, USA).

Immunoblotting

Equal amounts of protein were submitted to 4–12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. Membranes were probed with primary antibodies against the following proteins: phosphorylated synapsin and synapsin, 1:1000 (2311 and 2312, respectively; Cell Signaling Technology, Beverly, MA, USA), actin, 1:5000 (A5060; Sigma, St. Louis, MO, USA). Antibodies

were detected using horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies (7074; Cell Signaling Technology; 1:5000 for phosphorylated synapsin, 1:2000 for synapsin and actin). Signals were visualized using chemiluminescence (SuperSignal West Dura kit; Pierce, Rockford, IL, USA). Densitometric analysis was performed using ImageJ software (NIH, Bethesda, MD, USA). For each sample, phosphoprotein signals were normalized to total protein signals and averaged within groups. In addition, phosphoprotein and total protein signals were each normalized to actin signals and averaged within groups. A Two-way ANOVA using SigmaPlot software (Systat Software Inc, San Jose, CA, USA) (treatment and injury as factors) was performed on immunoblot data.

Results

The two-way ANOVA of phosphorylated synapsin immunoblots demonstrated significant main effects of injury in the striatum as well as significant interactions of injury and treatment for both phosphorylated synapsin I and II (injury [F (1, 13) =25.242, $P < 0.001$] and [F (1,13) =15.280, $P = 0.003$]; interaction [F (1, 13) =7.132, $P = 0.023$]; [F (1, 13) =9.955, $P = 0.01$], respectively). *Post hoc* analysis revealed that among saline-treated animals, TBI alone significantly increased striatal phosphorylated synapsin I and II expression compared to sham controls [Figure 1]. In addition, within sham controls, ethanol administration significantly increased expression of phosphorylated synapsin I and II [Figure 1] in the striatum. However, ethanol administration in injured mice did not result in significant alterations in phosphorylated synapsin I or II protein compared to saline-treated TBI mice [Figure 1]. No significant change in synapsin II protein expression was observed following injury; however, a significant main effect of treatment was observed for synapsin I (data not shown; [F (1, 13) =6.295, $P = 0.031$]). Despite the increased synapsin I expression following ethanol exposure, the ratio of phosphorylated synapsin to total synapsin was also significantly increased in the ethanol-treated sham group, indicating a more substantial increase in the phosphorylated form of synapsin I.

Immunoblot analysis of cortical section parallel findings observed in the striatum although effects were limited to phosphorylated synapsin II. A significant main effect of treatment as well as a significant interaction of injury and treatment were found for phosphorylated synapsin II (treatment [F (1, 13) =17.120, $P = 0.002$]; interaction [F (1, 13) =6.270, $P = 0.031$]). *Post hoc* analyses revealed that among sham controls, ethanol administration resulted in a significant increase in phosphorylated synapsin II expression [Figure 2]. In addition, among saline-injected animals, a statistically significant increase in phosphorylated synapsin II expression was observed following TBI [Figure 2]. However, no significant changes in phosphorylated synapsin I expression were observed in the cortex, regardless of ethanol exposure or injury status [Figure 2]. No significant changes in synapsin I and II expression were observed as a result of injury (data not shown). However, significant main effects of treatment were found for synapsin I and II expression ([F (1, 13) =9.817, $P = 0.011$)] and [F (1, 13) =6.662, $P = 0.027$], respectively), with ethanol administration resulting in significantly greater synapsin I and II expression compared to saline-treated controls (data not shown). Subsequent analysis

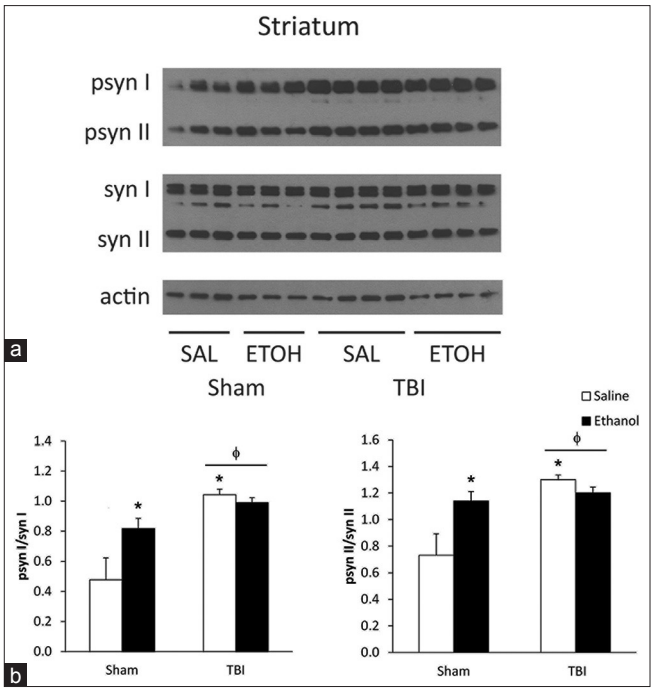

Figure 1: *Moderate TBI or acute ethanol exposure increases phosphorylation of synapsin I and II in the striatum. (a) Immunoblot data reveals that exposure of sham-injured mice to 4.0 g/kg ethanol increased the phosphorylation of synapsin I and II 30 min after injection compared to saline-treated sham animals. Moderate TBI also increased the phosphorylation of synapsin I and II compared to saline-treated sham controls. Two immunoreactive bands at ~ 77 kDa, representing the phosphorylated or total synapsin Ia and Ib proteins and a single immunoreactive band at ~60 kDa, representing the phosphorylated or total synapsin II protein were detected in all samples. Equal loading conditions were verified by immunodetection of actin. (b) Densitometric analysis of phosphorylated synapsin I/total synapsin I and phosphorylated synapsin II/total synapsin II expressed in arbitrary units. Mean ± standard error of mean, n = 3–4 per group, *P < 0.05 versus saline-treated sham mice; ϕP < 0.01 main effect of the injury. TBI: Traumatic brain injury*

of hippocampal sections revealed no significant changes in phosphorylated synapsin I and II expression resulting from ethanol administration or injury [Figure 3]. In addition, no significant changes were observed in synapsin I and II protein expression (data not shown).

Discussion and Conclusions

Here, we demonstrate that TBI either in the presence or absence of ethanol regulates phosphorylation of synapsin in a region- and isoform-specific manner. While minimal effects of TBI or ethanol exposure were observed in the hippocampus, either ethanol or TBI significantly increased phosphorylated synapsin II levels in the cortex. A similar pattern was observed in the striatum, which was extended to include effects on the phosphorylated synapsin I isoform. Furthermore, the addition of ethanol following TBI did not additionally increase phosphorylated synapsin levels in any region examined.

Synapsin functions to secure vesicles to cytoskeletal components, creating distinct vesicular pools, active and

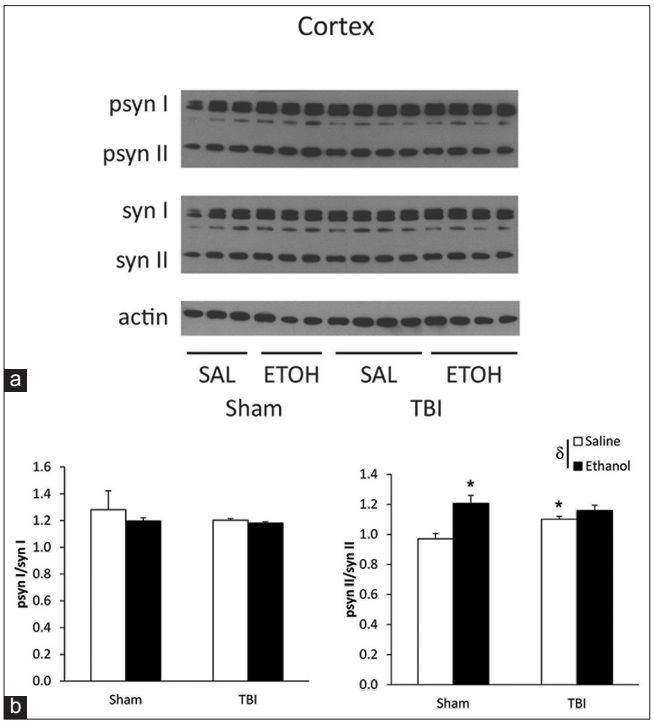

Figure 2: *Acute ethanol exposure increases the phosphorylation of synapsin II in the cortex of sham but not injured mice. (a) Immunoblot analysis of phosphorylated and total synapsin I and II and actin demonstrates that exposure of sham-injured mice to 4.0 g/kg ethanol significantly increased phosphorylation of synapsin II in cortical protein extracts obtained 30 min after ethanol injection compared to saline-treated sham mice. TBI alone also increased phosphorylated synapsin II compared to saline-treated sham animals with no further effect of ethanol treatment. No significant effects of ethanol or injury were found for phosphorylation of synapsin I. Two immunoreactive bands at ~77 kDa, representing the phosphorylated or total synapsin Ia and Ib proteins and a single immunoreactive band at ~60 kDa, representing the phosphorylated or total synapsin II protein were detected in all samples. Equal loading conditions were verified by immunodetection of actin. (b) Densitometric analysis of phosphorylated synapsin I/total synapsin I and phosphorylated synapsin II/total synapsin II expressed in arbitrary units. Mean ± standard error of mean, n = 3–4 per group, *P < 0.05 versus saline-treated sham mice; $^{\delta}$P < 0.01 main effect of treatment. TBI: Traumatic brain injury*

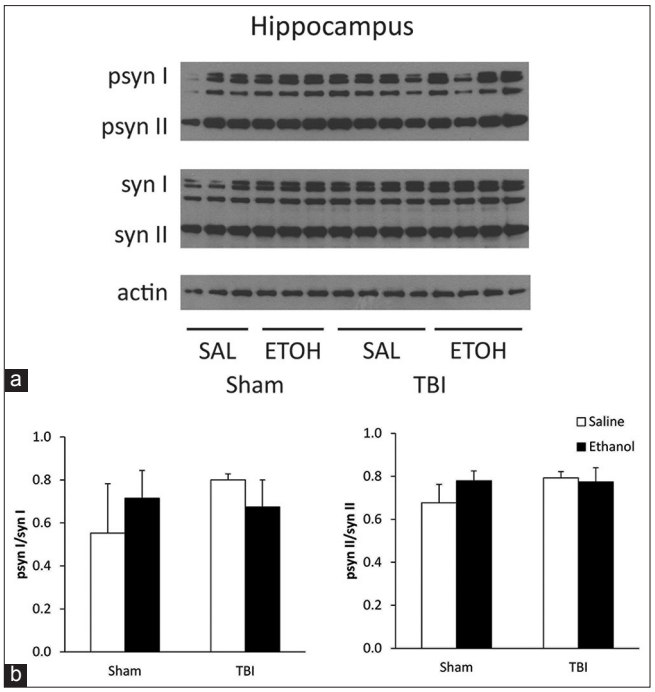

Figure 3: *Neither moderate TBI nor acute ethanol exposure affected the phosphorylation of synapsin I or II in the hippocampus. (a) The phosphorylation of synapsin I and II was not altered by acute ethanol injection (4.0 g/kg) or moderate TBI as measured by immunoblotting. Two immunoreactive bands at ~77 kDa, representing the phosphorylated or total synapsin Ia and Ib proteins and a single immunoreactive band at ~60 kDa, representing the phosphorylated or total synapsin II protein were detected in all samples. Equal loading conditions were verified by immunodetection of actin. (b) Densitometric analysis of phosphorylated synapsin I/total synapsin I and phosphorylated synapsin II/total synapsin II expressed in arbitrary units. Mean ± standard error of mean, n = 3–4 per group. TBI: Traumatic brain injury*

releasable.[30,31] Phospholipid binding is prevented upon phosphorylation, resulting in dissociation of synapsin from synaptic vesicle membranes.[28]

Previous studies have demonstrated that TBI results in disruption of synaptic proteins, including synaptophysin and synapsin. Focal cortical contusion injury (CCI) in adult rats demonstrate a significant reduction of total and phosphorylated synapsin I in the injured hippocampus as early as 48 h post-injury and extending to 7 days post-injury.[32-34] Lateral fluid percussion injury also resulted in decreased synapsin I expression in the hippocampus of rats 7 days after injury.[35] Focal CCI in adult mice also resulted in reductions in synaptophysin and total synapsin I in the hippocampus 7, 14, and 21 days post-injury.[36,37] Similarly, reductions in synapsin I protein were observed in the injured cortex as early as 24 h and were sustained to 96 h post-injury.[32,33] Together, these data demonstrate the ability of the injured brain to modify synaptic protein expression in a regionally distinct manner in response to injury. In the current study, no changes in total or phosphorylated synapsin were observed in the hippocampus while increases only in phosphorylated synapsin II were detected in the cortex post-TBI. These differences could represent a unique synaptic response to diffuse, closed-head injury, as previous studies used unilateral contusion or lateral fluid percussion models of TBI. No reports have documented alterations in synapsin phosphorylation in the striatum after TBI, underscoring the novelty of the current work. Increased phosphorylated synapsin expression in the injured striatum may reflect a compensatory neuroplastic response to axonal injury induced by TBI. A comprehensive study of neuronal morphology, including dendritic spine analysis of medium spiny neurons, may clarify this possibility. Interestingly, alterations in TBI-induced striatal synapsin phosphorylation correspond to the profound astrogliotic response in the nucleus accumbens and the increased sedative effects to ethanol previously reported using this TBI model.[29] This finding suggests that the injured striatum is unable to mount the appropriate phosphorylation response to activity blockade, preventing the restoration of the presynaptic homeostatic response to ethanol.[26,27]

Previous studies have demonstrated the ability of ethanol to modulate synapsin phosphorylation and clustering,[24-26] which aligns with the observations that synaptic inactivity, such as that induced by ethanol, increases the size of the presynaptic active zone and the quantity of both total and docked vesicles.[38] Therefore, elevations in synapsin phosphorylation indicate that vesicle mobilization may be germane to the neuronal response to ethanol. It has been suggested that phosphorylation of synaptic vesicle proteins, such as synapsin, serves to restore the presynaptic homeostatic response to ethanol.[26,27] In this way, ethanol-induced activity blockade (manifested as sedation following high-dose ethanol exposure) is reversed as synaptic homeostasis is restored and the animal awakens. In the case of impaired synapsin phosphorylation as observed in the injured animal, homeostatic restoration is disrupted, resulting in increased sedation duration following acute high-dose ethanol administration.

In contrast to the previous reports in control animals, no elevations in phosphorylated synapsin were detected in the hippocampus of sham controls following ethanol exposure in the current study.[26] This could be attributable to age differences, as the present study examined young adult mice, variability among samples or exposure to isoflurane, which can significantly impact hippocampal synapsin levels.[39] Ethanol-induced increases were observed in the cortex of sham mice; however, this was restricted to the phosphorylated synapsin II isoform. The most profound effects of ethanol on synapsin phosphorylation were observed in the striatum. Both phosphorylated synapsin I and II were significantly increased in sham controls after ethanol exposure. These findings reveal a novel role for synapsin phosphorylation in the striatum. In all regions examined, ethanol was unable to induce further phosphorylation of either synapsin isoform following TBI. This may represent a ceiling effect in the cortex and striatum, such that the already elevated levels of phosphorylated synapsin after TBI preclude further increases. In addition, it must be considered that the enhanced sensitivity to ethanol in TBI mice may be regulated by additional factors not revealed by these studies as striatal synapsin I and II phosphorylation in ethanol-treated TBI mice did not differ from ethanol-treated sham controls. Since the cortex and striatum play major roles in alcohol intoxication, seeking, and dependence,[40] one implication of this finding is that the inability of these injured regions to demonstrate further phosphorylation of synapsin following ethanol exposure may represent a cellular mechanism that subserves enhanced behavioral sensitivity to ethanol in TBI mice as has been shown in other rodent studies.[26] Together, these data offer promising insight into our understanding of the progression of alcohol misuse in the TBI population.

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Conflicts of interest

There are no conflicts of interest.

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