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Preclinical assessment of onabotulinumtoxinA for the treatment of mild traumatic brain injury-related acute and persistent post-traumatic headache

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Abstract

Objective: Investigation of onabotulinumtoxinA in a murine model of acute and persistent post-traumatic headache.
Methods: Mild traumatic brain injury was induced with a weight drop method. Periorbital and hindpaw cutaneous allodynia were measured for 14 days. Mice were then exposed to bright light stress and allodynia was reassessed. OnabotulinumtoxinA (0.5 U) was injected subcutaneously over the cranial sutures at different post-injury time points.
Results: After milt traumatic brain injury, mice exhibited periorbital and hindpaw allodynia that lasted for approximately 14 days. Allodynia could be reinstated on days 14–67 by exposure to stress only in previously injured mice. OnabotulinumtoxinA administration at 2 h after mild traumatic brain injury fully blocked both transient acute and stress-induced allodynia up to day 67. When administered 72 h post-mild traumatic brain injury, onabotulinumtoxinA reversed acute allodynia, but only partially prevented stress-induced allodynia. OnabotulinumtoxinA administration at day 12, when initial allodynia was largely resolved, produced incomplete and transient prevention of stress-induced allodynia. The degree of acute allodynia correlated positively with subsequent stress-induced allodynia.
Conclusion: Mild traumatic brain injury induced transient headache-like pain followed by long lasting sensitization and persistent vulnerability to a normally innocuous stress stimulus, respectively modeling acute and persistent post-traumatic headache. Administration of onabotulinumtoxinA following the resolution of acute post-traumatic headache diminished persistent post-traumatic headache but the effects were transient, suggesting that underlying persistent mild traumatic brain injury-induced maladaptations were not reversed. In contrast, early onabotulinumtoxinA administration fully blocked both acute post-traumatic headache as well as the transition to persistent post-traumatic headache suggesting prevention of neural adaptations that promote vulnerability to headache-like pain. Additionally, the degree of acute post-traumatic headache was predictive of risk of persistent post-traumatic headache.

Keywords

Post-traumatic headache, acute post-traumatic headache, persistent post-traumatic headache, mild traumatic brain injury, concussion, botulinum toxin, cutaneous allodynia

Introduction

The International Headache Society describes Post-traumatic headache (PTH) as a secondary headache that begins within seven days of a traumatic brain injury (TBI) and is considered to be persistent (PPTH) when it lasts for longer than three months (1). Paradoxically, the incidence of PTH is greater

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after a mild, rather than a moderate or severe, TBI (2,3). The most common characterization of PTH and PPTH among those evaluated in clinical settings is a migraine-like headache (4,5). Despite apparent phenotypic overlap between PTH and migraine, current headache treatments typically provide little relief for PPTH (6). Thus, effective mechanism-based therapies for PTH and for prevention of transition to PPTH are urgently needed.

TBI is thought to initiate a sequence of events that promote activation of the trigeminovascular system (7). Calcitonin gene-related peptide (CGRP) is causally involved in migraine pathophysiology (8,9) and has also been implicated in PTH (10,11). Two small open label studies demonstrated that some patients with PPTH achieve reduction in their monthly headache days after receiving erenumab, a CGRP receptor monoclonal antibody (mAb) (12,13). The possible contributions of CGRP to PTH has also been supported by preclinical studies. We recently characterized a model of mild TBI (mTBI) that is elicited by a weight dropped onto the head of a lightly anesthetized mouse (14). This concussive injury effectively reproduces many of the biomechanics associated with human mTBI including unrestrained head impact with linear and rotational acceleration (15–19). In this model, mice develop both acute transient cutaneous allodynia (CA) that models acute PTH (APTH), as well as later bright light stress (BLS) induced reinstatement of CA (i.e., a model of PPTH) (14). Fremanezumab, an anti-CGRP mAb, administered early after mTBI blocked acute APTH and BLS-induced PPTH (14). However, administration of fremanezumab after the resolution of the initial APTH no longer prevented PPTH (14). These findings are consistent with preliminary clinical observations from open label studies and a recently completed placebo-controlled trial with fremanezumab (20) and suggest that after the establishment of long-lasting mTBI-induced maladaptations, pain responses to normally subthreshold provocative stimuli may involve CGRP-independent mechanisms. Targeting both CGRP-dependent and independent mechanisms may therefore provide more effective treatment of PPTH.

OnabotulinumtoxinA (BOTOX, onabotA) (21) is approved for prevention of chronic migraine (22). Its effects include inhibition of mechanical nociceptive activation of dural nociceptors by blocking SNARE-dependent release of vesicle-bound neuropeptides such as CGRP and inhibition of cell surface expression of transient receptor potential (TRP) receptors (23,24). We hypothesized that onabotA targets both CGRP-dependent and CGRP-independent mechanisms that may underlie PTH and the transition to, and maintenance of, PPTH. We therefore assessed the anti-allodynic efficacy and duration of a single onabotA intervention at different times following mTBI in a preclinical model of APTH and PPTH.

Materials and methods

Animals

Adult male ICR mice weighing 17–22 g were housed five per cage on a 14/10-hour light/dark cycle (5 am–7 pm lights on) with food and water *ad libitum*. Experiments were conducted during the light cycle in accordance with the ARRIVE reporting guidelines and with approval of the Mayo Clinic Institutional Animal Care and Use Committee. A total of 125 mice were used. Group size requirements to obtain significance at the $\alpha = 0.05$ and statistical power 0.9 were determined from previous experiments using G-power analysis. The investigator (JO) was blinded to the group assignment.

Induction of mild traumatic brain injury

The mouse model of experimental mTBI was adapted from Kane et al. (15) for use in mice as described (14). Briefly, mice were lightly anaesthetized with 3% isoflurane and then laid in a prone position with the head unrestrained on an elevated tissue paper stage capable of supporting body weight. The paper stage was placed over a plexiglass box with a soft, landing sponge at the bottom. A metal guide tube was directed to the top of the mouse skull between the ears to ensure standardized placement of the weighted drop. The weight (100 g) released from a height of 94 cm, results in a concussive head impact, pushing the mouse down through the tissue paper and flipping it over to land on the sponge so that the mTBI elicits both linear and rotational head forces. After impact the weight falls away from the mouse avoiding a second hit. Following the procedure, mice were returned to their home cages to recover. Sham animals were anaesthetized but did not undergo the weighted drop. All mice awoke within five minutes of the procedure and were observed to confirm that no visual signs of neurological complications arose. Mice remained grouped housed five to a cage in their same cohorts throughout the duration of the experiment.

Bright light stress (BLS) challenge

Unrestrained mice previously undergoing mTBI or sham injury, were exposed for 15 minutes to BLS induced by LED strips (1000 lux output) placed on both sides of their home Plexiglass cages. The same parameters of BLS protocol as in our previous publication (14) were used to produce mild psychological

stress that does not elicit significant cutaneous allodynia in naïve or sham mice.

Drug administration

OnabotulinumtoxinA (onabotA) was provided by Allergan, an AbbVie company, (North Chicago, Illinois, USA). The dose and site of onabotA administration was determined in pilot studies with graded doses of 0.125, 0.25, 0.5, and 1 U administered subcutaneously over the cranium. We observed toxic effects with the highest dose, but not following the lower doses. For that reason, the 0.5 U dose was used in the study. A single onabotA (0.5 U in 50 µl, s.c.) or saline (50 µl, s.c.) dose was injected under light isoflurane anesthesia into the cranial region over the sagittal/lambdoid sutures. As off-site controls, injections were placed in the thoracic region on the back, behind the shoulder blades. The time of treatment in relation to the mTBI/sham injury varied in different experiments as described.

Behavioral assessment of cutaneous allodynia

Cephalic (periorbital) and extracephalic (hindpaw) CA was the primary outcome measure to assess APTH and PPTH. Mice were placed individually in elevated Plexiglass chambers with mesh flooring and allowed to acclimate for 2 hours each day on 3 consecutive days. CA was measured using von Frey filaments starting on day 0 (pre-mTBI baseline) and periodically over the entire experimental period after mTBI/sham injury. For assessment of periorbital allodynia, a 0.4 g (size 3.61) von Frey filament was applied to the midline periorbital region 10 times with just enough pressure to cause the filament to arch with a time of 20–30 s between each application. A positive response was considered swiping of the face, shaking of the head, and/or turning away from the stimuli. Running away or rearing up were not counted as positive responses as these behaviors sometimes occurred in uninjured control animals. For assessment of hindpaw allodynia, a 0.6 g (size 3.84) von Frey filament was applied to the left hindpaw 10 times. Sharp withdrawal of the paw, shaking and/or licking the paw were considered a positive response, while lifting of the paw with the filament or running away were not. Frequency response was calculated as [(number of positive responses/10) * 100%] and plotted as a function of time. Areas under the curve (AUC) of frequency response were calculated for individual mice and averaged for the treatment group to estimate cumulative amount of allodynia. AUC for mTBI/saline and mTBI/onabotA groups were normalized by subtracting the average AUC for the corresponding sham groups. Comparison between AUC of saline and onabotA treated mTBI groups allowed an assessment of onabotA efficacy. Normalized AUC during the initial 14-day APTH are referred to as "APTH AUC", normalized AUC during the 5-h testing after BLS are referred to as "PPTH AUC".

Data analysis

Data are plotted as the mean and standard error of the mean (SEM). All statistical analyses were performed in GraphPad Prism 9 (GraphPad Software, CA). Two-way repeated measures (RM) analysis of variance (ANOVA) was performed with "time" as within subject factor and "treatment" as between subject factor. Sidak's *post-hoc* test for multiple comparisons was used to assess differences between the groups within each time point. One-way ANOVA was used for comparisons of APTH and PPTH across experiments. Linear regression was performed to assess correlation between APTH and PPTH. Statistical significance was established a priori at 95% ($p < 0.05$). Results of the statistical analyses are summarized in Supplementary Table 1.

Experimental timelines

Five different experiments involving 25 mice each were conducted. In each experiment, mice were randomly separated into 4 groups: Sham/Saline (n = 5); Sham/onabotA (n = 5); mTBI/Saline (n = 5); mTBI/onabotA (n = 10). After baseline periorbital and hindpaw tactile measurements, mice underwent either the sham or mTBI protocol. Mice received a subcutaneous injection of either saline or onabotA according to their group assignment at two hours post-procedure either to the cranial region over the sagittal/lambdoid sutures (Experiments 1 and 2), or to an offsite location into the thoracic region (Experiment 3). Additional mice were treated with saline or onabotA into the cranial region three days (72 h) (Experiment 4), or 12 days post-procedure (Experiment 5). Periorbital and hindpaw CA was assessed periodically over a time course of 14 days. In all experiments, mice were exposed to BLS on day 14 after mTBI/sham injury and then again on day 28 (Experiments 1 and 4), 67 (Experiment 2) and 30 (Experiment 5); no additional BLS exposure was done for mice in Experiment 3. CA was assessed hourly for 5 h following BLS.

Results

OnabotA treatment 2 h after injury blocks immediate mTBI-induced CA (APTH) and prevents subsequent BLS-induced allodynia (PPTH)

In Experiments 1 and 2, mTBI and sham mice received either saline or onabotA treatment 2 h after the

procedure and periorbital and hindpaw CA was measured one day later (approximately 22 h) and again periodically over 14 days (see the injection location and timeline in Figs. 1A, 1B). Combined data from both experiments were plotted. Compared to Sham/Saline mice, mTBI/Saline mice demonstrated significantly greater responsiveness to periorbital and hindpaw stimuli at days 1–7 post-mTBI returning to

baseline by day 14 (*p < 0.05, Figure 1C, 1D) reflecting a period of transient APTH-like response. Post-mTBI, mice that were treated with onabotA at 2 h showed significantly decreased tactile responses compared to mTBI/Saline mice (+p < 0.05, Figure 1C, 1D). There was no difference in periorbital and hindpaw tactile sensitivity between mTBI/onabotA and Sham/onabotA mice except on days 1 and 3 when
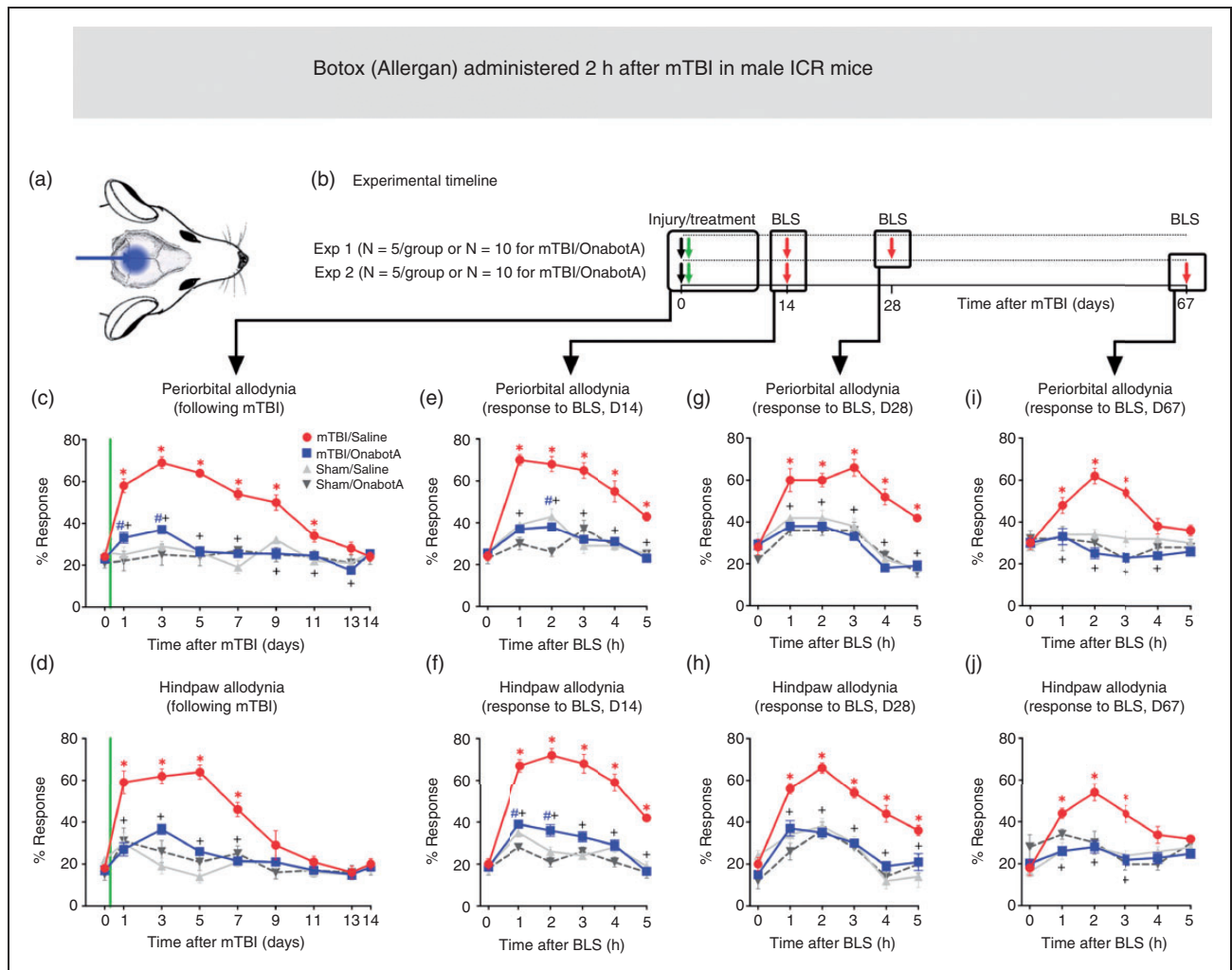

Figure 1. OnabotA treatment 2 h post mTBI blocks acute cutaneous allodynia (APTH) and prevents the development of a sensitized state reflected by allodynia induced by bright light stress (PPTH). (a) Illustration of onabotA injection site over the saggital and lambdoid suture intersection on the mouse skull. (b) Timeline of testing and group numbers in Experiments 1 and 2. After measuring baseline periorbital and hindpaw responses, mice received mTBI or sham injury and were administered saline or onabotA 2 h after the injury (green arrow/line). Periorbital (c) and hindpaw (d) Cutaneous allodynia (CA) was measured over a time course of 14 days after mTBI. On day 14, all mice were exposed to BLS and periorbital (e) and hindpaw (f) CA was measured over a 5 h time course. In Experiment 1, mice were exposed a second time to BLS on day 28 and periorbital (g) and hindpaw (h) CA was evaluted. In Experiment 2, the second BLS exposure was done on day 67 followed by assessment of periorbital (i) and hindpaw (j) CA. Two-way repeated measures ANOVA with Sidak's multiple comparison test shows significantly elevated frequency of tactile responses in the mTBI/Saline group compared to Sham/Saline mice at times indicated by red asterisk (*). Significantly different frequency of tactile responses in the mTBI/onabotA group compared to Sham/onabotA mice is indicated by blue hashtag (#). Compared to saline treated mTBI mice, frequency of response was reduced in mTBI/onabotA mice at times indicated by black + sign. Data are plotted as means and SEM; both sham groups and mTBI/saline group: N = 10 mice/group in panels C–F, N = 5 mice/group in G–J; mTBI/onabotA group: N = 20 (pooled d14 data from both experiments) mice in C–F and N = 10 mice in G–J.

mTBI/onabotA mice displayed a slight but significant increase of periorbital tactile sensitivity (#p < 0.05, Figure 1C, 1D). The data suggest that onabotA effectively inhibits APTH.

On day 14, all groups were exposed to bright light stress for 15 min, after which CA was measured every hour for five hours (Figure 1E, 1F). Compared to Sham/Saline mice, mTBI/Saline mice demonstrated significantly greater responsiveness to periorbital and hindpaw stimuli during the 5-hour time course (*p < 0.05, Figure 1E, 1F) reflecting PPTH. Tactile responses of onabotA-treated mTBI mice were consistently and significantly reduced compared to the mTBI/Saline group at all timepoints throughout the 5-hour testing (+p < 0.05, Figure 1E, 1F). Small but significant increases over Sham/onabotA mice were observed at some timepoints (#p < 0.05, Figure 1E, 1F). PPTH AUC in saline and onabotA treated mTBI groups were calculated to estimate the cumulative PPTH allodynia and analgesic efficacy of onabotA treatment (see Methods). OnabotA reduced periorbital and hindpaw PPTH AUC by $97.7 \pm 18.1\%$ and $82.8 \pm 14.0\%$, respectively. Treatment intervention with onabotA at 2 h post-mTBI is therefore effective in both APTH and PPTH.

To determine the duration of effect of a single onabotA administration, in Experiment 1, all groups of mice were exposed to BLS again on day 28. Following the second BLS, mTBI/Saline mice demonstrated significant CA compared to Sham/Saline mice that persisted longer than 5 h (*p < 0.05, Figure 1G–1H). mTBI/onabotA mice showed significantly reduced tactile responses compared to mTBI/Saline mice (+p < 0.05, Figure 1G–1H) that were not different from responses of Sham/onabotA animals. OnabotA reduced PPTH AUC for periorbital and hindpaw measurements on day 28 by $107.7 \pm 11.7\%$ and $89.7 \pm 23.3\%$, respectively. In Experiment 2, we did not expose the animals to a second stress until day 67 after mTBI. Animals were regularly handled in between days 14 and 67, and tactile responses were tested weekly to ensure the absence of unprovoked allodynia during this time. After BLS exposure on day 67, mTBI/Saline mice again developed significant periorbital and hindpaw CA lasting more than 3 h, while Sham/Saline mice did not show CA (*p < 0.05, Figure 1I, 1J). Importantly, mTBI/onabotA mice did not develop CA after BLS exposure on day 67 and their tactile responses were significantly lower than those of mTBI/Saline mice (+p < 0.05, Figure 1I, 1J) and indistinguishable from sham animals. Therefore, onabotA administered soon after mTBI prevents PPTH.

Offsite administration of onabotA has no effect on development of APTH and PPTH

To exclude the possibility that onabotA could act systemically to block tactile responses, in another cohort of mice (Experiment 3), 2 h post mTBI/sham injury we administered the same dose of onabotA or saline in the thoracic region at (i.e., offsite administration; Figure 2A). Regardless of the treatment, both mTBI groups developed periorbital and hindpaw CA that was significantly larger than in the corresponding sham groups (*p < 0.05, mTBI/Saline vs. Sham/Saline; #p < 0.05, mTBI/onabotA vs. Sham/onabotA; Figure 2B, 2C). Exposure of mTBI mice to BLS on day 14 reinstated CA in both saline and onabotA treated groups as demonstrated by significantly increased tactile responses compared to the corresponding sham groups (*p < 0.05, mTBI/Saline vs. Sham/Saline; #p < 0.05, mTBI/onabotA vs. Sham/onabotA; Supplementary Figure 2D, 2E). There was no difference between onabotA treated and saline treated mTBI groups at any time tested, confirming no systemic effects of onabotA on PTH.

OnabotA treatment at 72 hours post mTBI reverses established APTH but has only a partial effect on PPTH

In Experiment 4, after mTBI or sham injury, mice were tested for CA on days 1 and 3 before any onabotA or saline treatments. Mice with mTBI demonstrated significantly increased periorbital and hindpaw responses compared to sham controls (*p < 0.05, Figure 3A, 3B). Mice were then treated with saline or onabotA on day 3. Saline treated mTBI mice continued displaying both periorbital and hindpaw CA until day 7, demonstrated by significantly increased responses in comparison to Sham/Saline controls (*p < 0.05, Figure 3A, 3B). In contrast, mTBI/onabotA mice showed complete reversal of periorbital allodynia on day 4 and of hindpaw allodynia on day 5 (+p < 0.05, Figure 3A, 3B) demonstrating efficacy of onabotA against established APTH.

After exposure to BLS on day 14, mTBI/Saline mice revealed significantly greater CA compared to Sham/Saline mice during the 5-hour time course (*p < 0.05, Figure 3C, 3D). OnabotA treated mTBI mice also showed significant CA compared to Sham/onabotA mice (#p < 0.05, Figure 3C, 3D), albeit both periorbital and hindpaw CA was significantly reduced compared to the mTBI/Saline group (+p < 0.05, Figure 3C, 3D). PPTH AUC was reduced in onabotA treated mice by $71.8 \pm 16.3\%$ and $67.8 \pm 8.0\%$, for periorbital and hindpaw measurements respectively, indicating a partial effect on PPTH with delayed treatment.
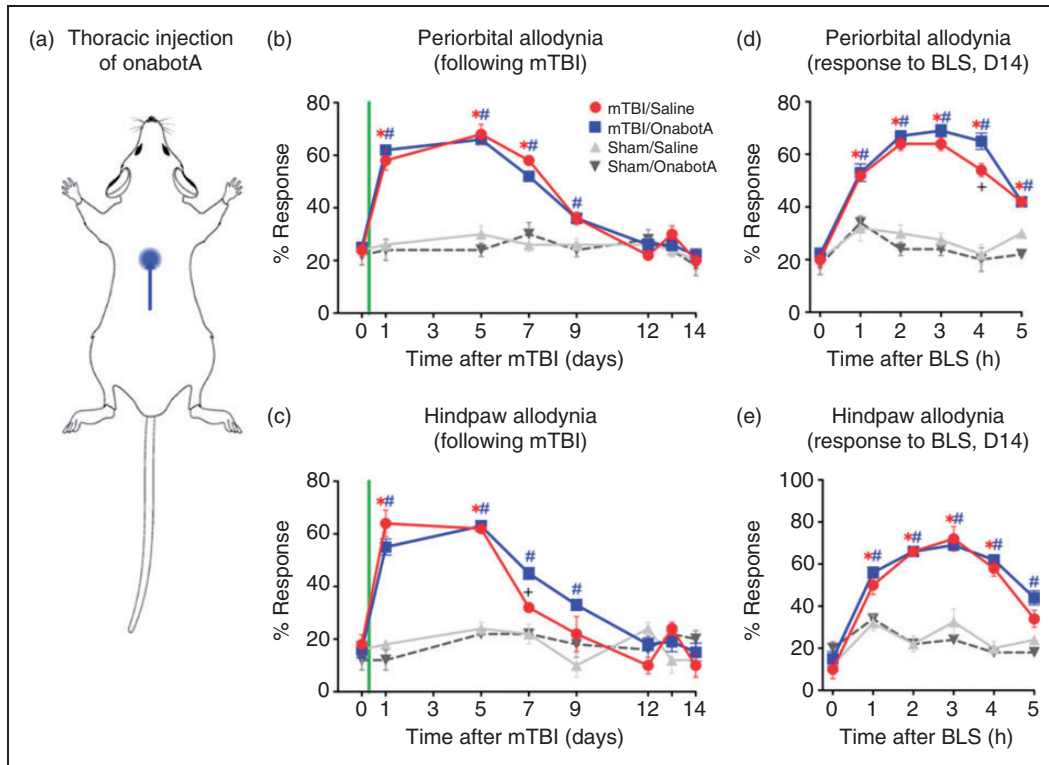
Figure 2. No effect of onabotA on CA of mTBI groups was observed when onabotA was administered in the thorax (offsite) region. Mice were given mTBI or sham injury and 2 h after the injury were administered saline or onabotA subcutaneously into the thoracic region (green line). Periorbital (a) and hindpaw (b) Cutaneous allodynia (CA) was measured over a time course of 14 days after mTBI. On day 14, all mice were exposed to BLS and periorbital (c) and hindpaw (d) CA was measured over a 5 h time course. No effect was observed between saline-treated and onabotA treated mTBI groups. Two-way repeated measures ANOVA with Sidak's multiple comparison test shows significantly elevated frequency of tactile responses in the mTBI/Saline group compared to Sham/Saline mice [times indicated by red asterisk (*)] and in the mTBI/onabotA group compared to Sham/onabotA mice [indicated by blue hashtag (#)]. Significant, yet not meaningful, difference was observed between saline treated and onabotA treated mTBI mice at times indicated by black + sign. Data are plotted as means and SEM; both sham groups and mTBI/Saline group: N = 5 mice/group; mTBI/onabotA group N = 10 mice.

In the same mice, we investigated if a subsequent BLS exposure on day 28 post mTBI would still show an analgesic effect of onabotA treatment against BLS-induced CA. OnabotA treated mTBI mice developed periorbital CA post-BLS that was significantly greater than that of Sham/onabotA mice (#p < 0.05, Figure 3E, 3F), but significantly less than CA of mTBI/Saline mice (+p < 0.05 Figure 3E, 3F). In contrast, hindpaw allodynia was comparable and not significantly different between mTBI/Saline and mTBI/onabotA groups (p > 0.05, Figure 3E, 3F). OnabotA reduced PPTH AUC for periorbital and hindpaw measurements on day 28 by $70.0 \pm 34.0\%$ and $30.0 \pm 9.9\%$, respectively. Thus, administration of onabotA during established APTH does not fully protect the mice against development of vulnerability to a subthreshold stress stimulus and expression of PPTH.

OnabotA treatment after resolution of APTH on day 12 only partially blocks BLS-induced allodynia on day 14 and has no effect on the maintenance of PPTH

In Experiment 5, onabotA or saline was not administered until day 12 after mTBI/sham injury, a timepoint when tactile thresholds were at baseline levels in this experiment. Before administration, both mTBI groups developed significant CA compared to sham groups that was indistinguishable between the two mTBI groups and lasted approximately 9 days (*p < 0.05, Figure 4A, 4B). On day 14, all mice were exposed to BLS. As in previous experiments, mTBI/Saline mice demonstrated significant BLS-induced periorbital and hindpaw CA compared to the Sham/Saline group, lasting for more than 5 h (*p < 0.05, Figure 4C, 4D). In comparison to Sham/onabotA mice, mTBI/onabotA mice also showed significantly larger tactile responses
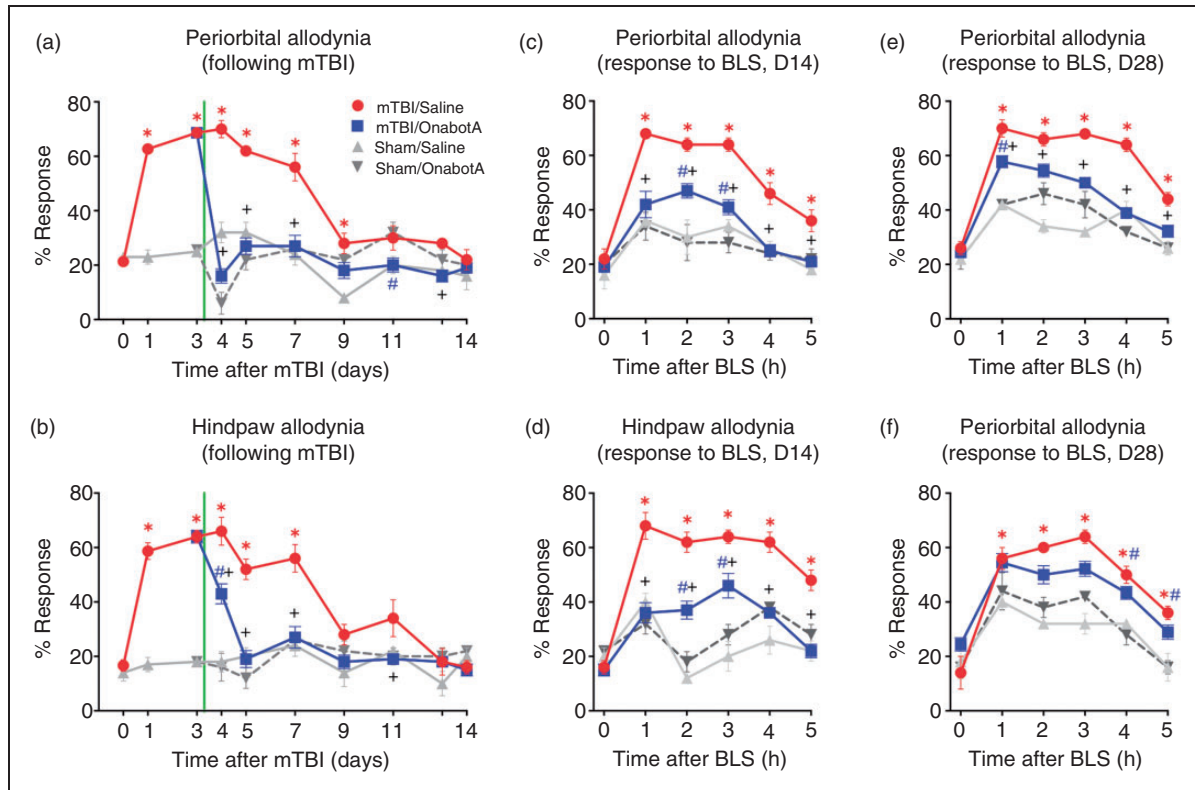
Figure 3. OnabotA treatment 72 h post mTBI reverses APTH but has only a partial effect on the development of a sensitized state. After baseline measurements, mice were given either mTBI or sham injury and periorbital (a) and hindpaw (b) Cutaneous allodynia (CA) was assessed on days 1 and 3. OnabotA or saline was administered on day 3 after the CA assessment (green line). CA measurements continued until day 14. On day 14, all mice were exposed to BLS and periorbital (c) and hindpaw (d) CA was measured over a 5 h time course. The same mice were exposed a second time to BLS on day 28 and periorbital (e) and hindpaw (f) CA was evaluted. Two-way repeated measures ANOVA with Sidak's multiple comparison test shows significantly elevated frequency of tactile responses in the mTBI/saline group compared to Sham/Saline mice at times indicated by red asterisk (*). Significantly different frequency of tactile responses in the mTBI/onabotA group compared to Sham/onabotA mice is indicated by blue hashtag (#). Compared to saline treated mTBI mice, frequency of response was reduced in mTBI/onabotA mice at times indicated by black + sign. Data are plotted as means and SEM; both sham groups and mTBI/Saline group: N = 5 mice/group; mTBI/onabotA group N = 10 mice.

(#$p < 0.05$, Figure 4C, 4D), however, these responses were significantly lower than in mTBI/Saline mice (+$p < 0.05$, Figure 4C, 4D). Periorbital and hindpaw PPTH AUC was reduced in onabotA treated mice by $79.7 \pm 12.9\%$ and $36.2 \pm 15.7\%$, respectively. Thus, onabotA treatment at day 12 reduced established PPTH, but the magnitude of the effect was smaller than with earlier treatments. Next, we investigated how long the effect of onabotA on established PPTH would last. We exposed the same mice to BLS again on day 30 after mTBI (18 days after onabotA administration). mTBI mice showed significant CA regardless of onabotA or saline treatment (*$p < 0.05$, mTBI/Saline vs. Sham/Saline; #$p < 0.05$, mTBI/onabotA vs. Sham/onabotA; Figure 4E, 4F) with no significant reduction in PPTH AUC (reduction by $6.1 \pm 19.8\%$ and $0.0 \pm 8.7\%$ for periorbital and hindpaw measures, respectively). This finding suggests that the effect of onabotA on established PPTH was lost after 18 days.

Cumulative APTH predicts the level of pain provoked by BLS in the PPTH state

By comparing behavioral outcomes with different times of onabotA intervention, our results suggested that the earlier the intervention (i.e., less overall acute "pain"), the more effective prevention of PPTH. We therefore investigated if cumulative allodynia during the 14-day APTH period may predict the level of pain provoked by BLS in the PPTH state. Normalized APTH AUC were calculated for all individual mTBI mice in Experiments 1 and 2 (2 h), 4 (72 h) and 5 (12D). AUC data from saline treated mTBI mice from all these experiments were combined into one mTBI/Saline group. Compared to the mTBI/Saline group, APTH AUC was decreased in mice treated with onabotA at 2 h post mTBI nearly completely (i.e., by $89.7 \pm 12.8\%$ for periorbital and $88.2 \pm 19.7\%$ for hindpaw measurements) but the decrease was smaller for mice treated at
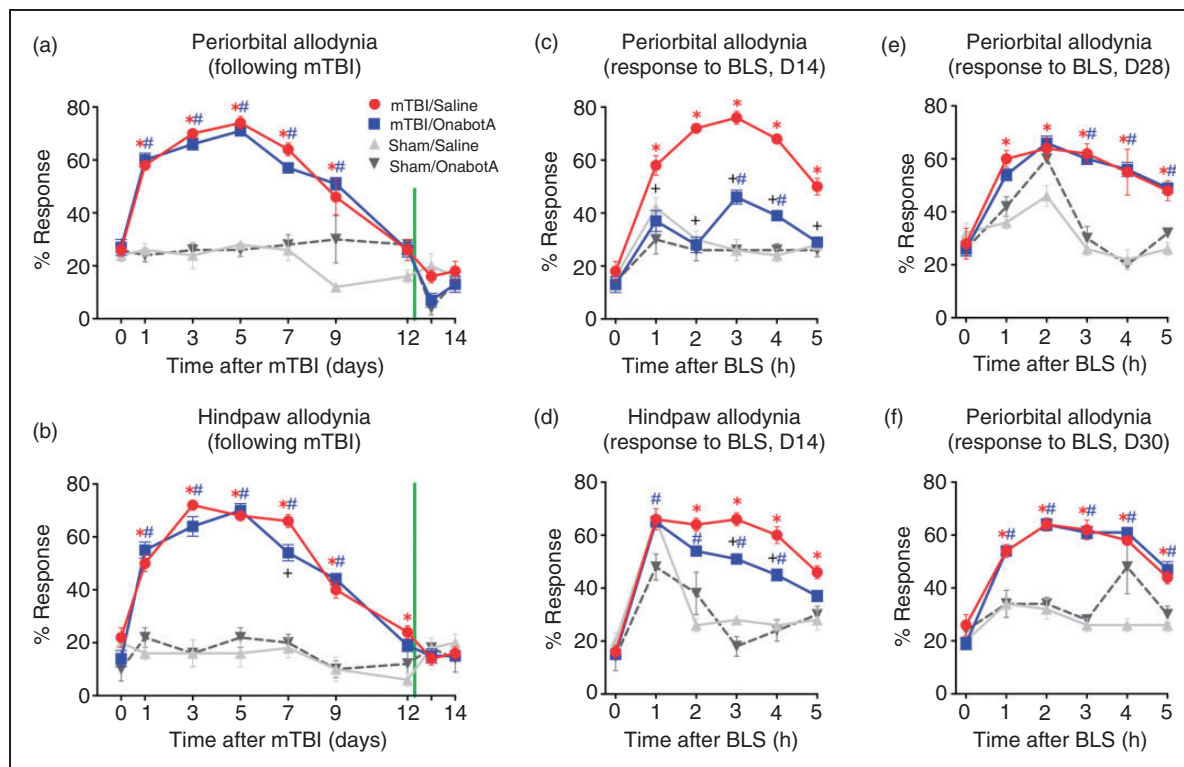
Figure 4. OnabotA treatment on day 12 post mTBI partially blocks BLS-induced allodynia (PPTH) on day 14 but has no effect on the maintenance of the sensitized state. Mice were given either mTBI or sham injury and periorbital (a) and hindpaw (b) Cutaneous allodynia (CA) was assessed over 12 days. OnabotA or saline was administered on day 12 after the assessment of tactile responses (green line) and CA measures taken on day 13 and 14. On day 14, all mice were exposed to BLS and periorbital (c) and hindpaw (d) CA was measured over a 5 h time course. Mice were again exposed to BLS on day 30 and periorbital (e) and hindpaw (f) CA was assessed. Two-way repeated measures ANOVA with Sidak's multiple comparison test shows significantly elevated frequency of tactile responses in the mTBI/Saline group compared to Sham/Saline mice at times indicated by red asterisk (*). Significantly elevated frequency of tactile responses in the mTBI/onabotA group compared to Sham/onabotA mice is indicated by blue hashtag (#). Compared to saline treated mTBI mice, frequency of response was reduced in mTBI/onabotA mice at times indicated by black + sign. Data are plotted as means and SEM; both sham groups and mTBI/Saline group: N = 5 mice/group; mTBI/onabotA group N = 10 mice.

72 h (by $69.2 \pm 14.3\%$ for periorbital and $55.3 \pm 9.4\%$ for hindpaw measurements). APTH AUC significantly depended on the time when onabotA was administered ($p < 0.05$ for all group comparisons except for the mTBI/saline and mTBI/12D onabotA groups) for both periorbital and hindpaw measurements (Figure 5A, 5B). We also calculated PPTH AUC for periorbital (Figure 5C) and hindpaw (Figure 5D) allodynia on day 28 (day 30 for Experiment 5), as an estimate of pain during a PPTH episode. We selected day 28 rather than day 14 for evaluation of the effects of onabotA on central sensitization during PPTH in order to eliminate confounds arising from sustained peripheral effects. PPTH AUC was decreased in mTBI/onabotA groups compared to the mTBI/Saline group and again, this decrease depended on the time of onabotA administration ($p < 0.05$ for all group comparisons except for the mTBI/saline and mTBI/12D onabotA groups). Correlation analysis between the individual mouse APTH AUC and subsequent PPTH

AUC found strong linear relationship for both periorbital (Figure 5E; $R^2 = 0.7317$) and hindpaw (Figure 5F; $R^2 = 0.8105$) measurements. These findings suggest the speculative possibility that both the intensity and duration of the initial "pain" (i.e., APTH AUC) may contribute to the development of maladaptive changes and subsequent vulnerability to headache triggers in this rodent model.

Discussion

Animal model of APTH and PPTH

Assessment of "headache" in rodents is commonly based on surrogate measures such as cutaneous allodynia (14). Pain from a normally non-painful tactile stimulus is also often observed during human migraine (25–27) and has been reported in approximately 50% of PTH patients (5,28,29). Both cephalic and extracephalic allodynia has been measured in migraine
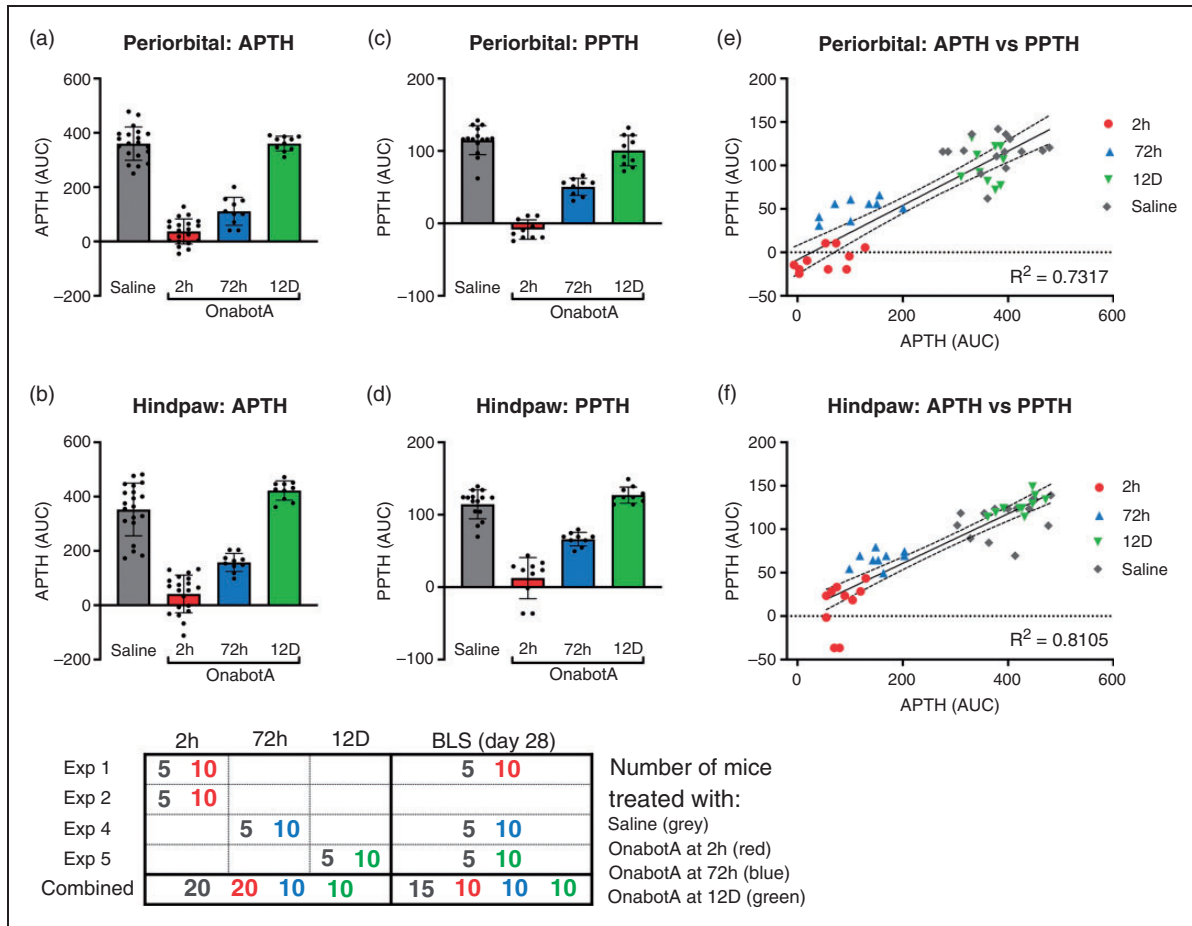
Figure 5. Amount of pain over the 14-day period of APTH determines sensitivity to BLS during PPTH. Total amount of pain in the APTH phase was estimated by calculating the Areas Under the Curves (AUC) of periorbital and hindpaw allodynia for individual mice from Experiments 1–3. AUC during the 14 day period of APTH for mTBI mice treated with saline or onabotA was normalized to the average AUC of the sham groups and plotted for periorbital (a) and hindpaw (b) measurements. Bar graphs represent individual AUC, group average and standard deviation. AUC for mTBI mice during the 5-h BLS-induced PPTH at day 28 (day 30 for Experiment 3) normalized to the average AUC of sham groups was calculated as a measure of PPTH pain and periorbital (c) and hinpaw (d) data are plotted. Graphs E and F show linear correlation between the amount of pain in the APTH phase (X-axes) and amount of PPTH pain (Y-axes) for periorbital (e) and hindpaw (f) regions. Bar graphs represent the means and SEM; data for individual mice are shown by symbols; linear regression with 95% confidence intervals are plotted in E and F. The number of animals in the saline, 2 h, 72 h and 12D groups combined from experiments 1–5 are shown on the bottom.

patients (30). We previously described a mouse model of mTBI-induced PTH characterized by transient acute CA followed by a persistent state of vulnerability to BLS (14). This mTBI model reproduces many mechanistic aspects of human concussion injuries including blunt force, closed-skull direct impact with acceleration and shearing forces from unrestrained head movement. Consistent with human classification of mTBI, there is no structural brain damage detectable by imaging, or by gross and histological examination (15–18). The presence of CA in patients with PTH suggests that transient CA following mTBI in our mouse model is relevant to human APTH. Similarly, the development of a sensitized state where CA can be triggered by an external stress stimulus from exposure to BLS appears relevant to PPTH as both stress and bright lights are known to exacerbate PTH in humans (5). Here, we demonstrated mTBI-induced CA lasting for approximately 10 days (i.e., APTH) and re-instatement of CA following BLS (i.e., PPTH) for at least 67 days in a different mouse strain.

Summary of findings on onabotA efficacy

The efficacy of cranial onabotA was studied at 2 h, 72 h and 12 days after mTBI. Administration at 2 h after mTBI significantly reduced the periorbital and hindpaw CA to levels comparable to those of sham mice

both during APTH and in PPTH even when measured as early as one day post-dosing consistent with previous preclinical and clinical observations (31–34). In chronic migraine patients, the effect of onabotA in reducing headache days per week was significant compared to placebo as early as one week after treatment, the earliest timepoint assessed in the PREEMPT trials (35). Additionally, a single onabotA administration at 2 h after injury completely prevented BLS-induced CA even 67 days later, well after the expected duration of onabotA effects on neurotransmission presumed to be 15-25 days (36,37) in rodents. In contrast, administration of saline in the scalp, or of onabotA in the thoracic (offsite) region had no effect on CA in mTBI or sham mice.

When onabotA was given 72 h after mTBI, after APTH had been verified, CA was reversed for the rest of the APTH time course. However, in these mice, BLS-induced CA was only partially inhibited on day 14 (11 days after onabotA) and even less so on day 28 (25 days after onabotA). These findings suggest ongoing blockade of trigeminal signaling by onabotA at 11 days after administration but diminishing effects at 25 days. When onabotA was administered on mTBI day 12, after APTH was resolved, BLS-induced allodynia on day 14 (2 days after onabotA) was partially inhibited, however, no analgesic effect was observed when these mice were re-exposed to BLS on day 30 (18 days after onabotA).

Interpretation of findings on onabotA efficacy

Collectively, these results suggest that: 1) OnabotA acts locally, rather than systemically, to inhibit the trigeminal nociceptive pathway; 2) Early onabotA administration is sufficient to completely inhibit APTH throughout its duration; 3) Complete inhibition of APTH is sufficient to prevent the establishment of a sensitized state and transition to PPTH; 4) The estimated duration of inhibitory effects of onabotA on the trigeminal system in mice in our studies ranged from approximately 11–18 days, consistent with a reported faster degradation of onabotA and faster recovery of SNAP25 protein in rodents than in humans (36,37). Therefore, the long-term effect of the toxin observed up to 67 days is likely due to the consequences of blockade of APTH; 5) After establishment of a sensitized state, onabotA still effectively inhibits BLS-induced CA, albeit partially, apparently without reversing the maladaptive changes that underlie the sensitized state. It should be noted, however, that neural mechanisms of latent sensitisation are not well understood.

Correlational analysis of APTH AUC with AUC of BLS-induced PPTH for individual animals across four experiments revealed that the magnitude and duration of mTBI-induced allodynia (APTH) is a predictor of BLS-induced allodynia in the PPTH state. These findings suggest the speculative conclusion that pain in the acute phase contributes cumulatively to the development of maladaptive changes that establish vulnerability to subsequent headache triggers. Therefore, effective treatment of the acute condition may provide the best opportunity for PPTH prevention.

Although our study did not directly investigate the neural mechanisms of action of onabotA, inhibitory effects on the peripheral trigeminal signaling are consistent with previous studies (24). OnabotA has been shown to preferentially inhibit nonmyelinated peripheral nerve fibers (i.e., C-fibers) by blockade of neurotransmitter release or by interfering with the insertion of pronociceptive ion channels into the neuronal plasma membrane (38). Consistent with the inhibition of TRPV1 positive C-fibers, pretreatment of human volunteers with onabotA reduced flare, hyperalgesia and pain induced by intradermal injection of capsaicin, a TRPV1 agonist (39). In preclinical studies, administration of onabotA into extracranial sutures selectively inhibited meningeal C-fibers following dural activation with TRPV1 and TRPA1 agonists or following induction of cortical spreading depression (24,40,41). Consistent with the inhibition of C-fibers, our results demonstrate full blockade of cephalic allodynia during the APTH phase. Ongoing trigeminal nociceptor activation results in sensitization of the trigeminocervical complex (42) and possibly neurons in higher brain areas including the thalamus, parabrachial area, periaqueductal grey, hypothalamus, and cortical regions. Central sensitization is reflected in the spread of CA to extracephalic regions (e.g., hindpaw) that we have observed in saline treated mTBI animals and that was also fully blocked during APTH by onabotA.

By contrast, administration of onabotA in the PPTH phase was only partially effective in inhibiting BLS-induced allodynia, indicating that untreated mTBI promotes long-term changes in the CNS (i.e., priming). Under these conditions, animals may be sensitized to sensory stimuli that may also trigger migraine attacks, including BLS, and outcomes are not fully responsive to onabotA. We observed that allodynia as a consequence of mTBI priming could be elicited by BLS for up to 67 days following mTBI (i.e., the maximum time-point tested). The pathophysiology underlying priming and the mechanisms of how BLS can trigger generalized allodynia are not well understood, but likely involve engagement of the central stress systems resulting in altered perception of trigeminal somatosensory stimulation. Once priming is established, onabotA can still partially inhibit BLS-induced CA, however, ongoing blockade of peripheral afferent

activity is necessary, since BLS-induced CA is fully reinstated once the antinociceptive effects of onabotA are diminished.

Consistency of preclinical findings with clinical observations

Our preclinical studies suggest that immediate or early treatment of APTH with onabotA may be most effective in prevention of PPTH in rodents. This is similar to our previously reported effects of early administration of anti-CGRP mAb in a mouse mTBI model which blocked acute transient allodynia (i.e., APTH) and prevented PPTH development (14). However, we also demonstrated partial inhibition of BLS-induced allodynia (i.e., PPTH) with onabotA administration after the resolution of APTH in rodents. This contrasts to the lack of effect of anti-CGRP mAb on BLS-induced allodynia in mice when given at this timepoint (14).

In patients, onabotA treatment resulted in favorable outcomes when administered at later timepoints after mTBI and in established PPTH. A study involving 64 active-duty military patients with headaches related to concussions reported significant improvement after onabotA which was administered an average of 10.8 months after injury (43). OnabotA has also been shown to diminish allodynia over time in patients with chronic migraine (44) and there is evidence of cumulative benefit over multiple injection cycles with onabotA in these patients (45), many of whom have ictal and interictal allodynia (25). Thus, it is possible that repeated injections in patients with PPTH may have a cumulative benefit even when administered after the onset of PPTH. The recent positive and negative placebo-controlled trials in patients with PPTH with onabotA (43,46) and fremanezumab (20), respectively, is also consistent with outcomes with these agents in our preclinical studies. These findings, together with the wide range of presumed antinociceptive and anti-allodynic mechanisms of onabotA, suggest that onabotA may provide benefit not only in APTH but may also represent a treatment option in already established PPTH.

Article highlights

- Mild traumatic brain injury (mTBI) in a mouse model induces transient cephalic allodynia followed by long lasting sensitization to a normally innocuous stress stimulus, respectively modeling acute and persistent post-traumatic headache (PTH).
- Administration of onabotulinumtoxinA (onabotA) early (2 h) after mTBI fully blocked both transient acute and stress-induced PTH in mice.
- Administration of onabotA following the resolution of acute PTH (day 12) diminished persistent PTH but the effects were transient, suggesting the presence of persistent maladaptations in mice subjected to mTBI.
- The degree of acute allodynia was predictive of the level of persistent PTH in this mouse model.

Abbreviations

CA – Cutaneous allodynia
OnabotA – Onabotulinumtoxin type A
mTBI – Mild traumatic brain injury
PTH – Post-traumatic headache
APTH – Acute post-traumatic headache
PPTH – Persistent post-traumatic headache
CGRP – Calcitonin gene related peptide
BLS – Bright light stress

Author Contributions

FP, TA, RSB, MFB, EJVC, SRS, TJS, and DWD conceived and designed the study, JO acquired the data, EN analyzed the data and drafted the figures; EN, FP and DWD wrote the manuscript.

Data and materials availability

All raw data, methods and analyses will be available upon request to the corresponding author.

Declaration of conflicting interests

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Supplemental material

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References

1. Headache Classification Committee of the International Headache Society (IHS) The International Classification of Headache Disorders, 3rd edition. *Cephalalgia* 2018; 38: 1–211.
2. Defrin R. Chronic post-traumatic headache: clinical findings and possible mechanisms. *J Man Manip Ther* 2014; 22: 36–44.
3. Ashina H, Porreca F, Anderson T, et al. Post-traumatic headache: epidemiology and pathophysiological insights. *Nat Rev Neurol* 2019; 15: 607–617.
4. Lucas S, Hoffman JM, Bell KR, et al. A prospective study of prevalence and characterization of headache following mild traumatic brain injury. *Cephalalgia* 2014; 34: 93–102.
5. Ashina H, Iljazi A, Al-Khazali HM, et al. Persistent post-traumatic headache attributed to mild traumatic brain injury: Deep phenotyping and treatment patterns. *Cephalalgia* 2020; 40: 554–564.
6. Ashina H, Eigenbrodt AK, Seifert T, et al. Post-traumatic headache attributed to traumatic brain injury: classification, clinical characteristics, and treatment. *Lancet Neurol* 2021; 20: 460–469.
7. Mayer CL, Huber BR and Peskind E. Traumatic brain injury, neuroinflammation, and post-traumatic headaches. *Headache* 2013; 53: 1523–1530.
8. Tepper SJ. History and review of anti-Calcitonin Gene-Related Peptide (CGRP) therapies: from translational research to treatment. *Headache* 2018; 58: 238–275.
9. Edvinsson L. The trigeminovascular pathway: Role of CGRP and CGRP receptors in migraine. *Headache* 2017; 57: 47–55.
10. Daiutolo BV, Tyburski A, Clark SW, et al. Trigeminal pain molecules, allodynia, and photosensitivity are pharmacologically and genetically modulated in a model of traumatic brain injury. *J Neurotrauma* 2016; 33: 748–760.
11. Bree D and Levy D. Development of CGRP-dependent pain and headache related behaviours in a rat model of concussion: Implications for mechanisms of post-traumatic headache. *Cephalalgia* 2018; 38: 246–258.
12. Ashina H, Iljazi A, Al-Khazali HM, et al. Efficacy, tolerability, and safety of erenumab for the preventive treatment of persistent post-traumatic headache attributed to mild traumatic brain injury: an open-label study. *J Headache Pain* 2020; 21: 62.
13. VanderEnde J, Bateman EA, MacKenzie HM, et al. Use of CGRP receptor blocker erenumab in the management of post-traumatic headache: a case series of 5 women. *Brain Inj* 2020; 34: 1431–1434.
14. Navratilova E, Rau J, Oyarzo J, et al. CGRP-dependent and independent mechanisms of acute and persistent post-traumatic headache following mild traumatic brain injury in mice. *Cephalalgia* 2019; 39: 1762–1775.
15. Kane MJ, Angoa-Perez M, Briggs DI, et al. A mouse model of human repetitive mild traumatic brain injury. *J Neurosci Methods* 2012; 203: 41–49.
16. Mychasiuk R, Farran A, Angoa-Perez M, et al. A novel model of mild traumatic brain injury for juvenile rats. *J Vis Exp* 2014; 94: 51820.
17. Bharadwaj VN, Rowe RK, Harrison J, et al. Blood-brain barrier disruption dictates nanoparticle accumulation following experimental brain injury. *Nanomedicine* 2018; 14: 2155–2166.
18. Goddeyne C, Nichols J, Wu C, et al. Repetitive mild traumatic brain injury induces ventriculomegaly and cortical thinning in juvenile rats. *J Neurophysiol* 2015; 113: 3268–3280.
19. Bodnar CN, Roberts KN, Higgins EK, et al. A systematic review of closed head injury models of mild traumatic brain injury in mice and rats. *J Neurotrauma* 2019; 36: 1683–1706.
20. Spierings EL, Silberstein S, Najib U, et al. A Phase 2 study of fremanezumab as a treatment for posttraumatic headache in adult patients. *Neurology* 2021; 96(15 Supplement).

21. Cai BB, Francis J, Brin MF, et al. Botulinum neurotoxin type A-cleaved SNAP25 is confined to primary motor neurons and localized on the plasma membrane following intramuscular toxin injection. *Neuroscience* 2017; 352: 155–169.

22. Diener HC, Dodick DW, Aurora SK, et al. OnabotulinumtoxinA for treatment of chronic migraine: results from the double-blind, randomized, placebo-controlled phase of the PREEMPT 2 trial. *Cephalalgia* 2010; 30: 804–814.

23. Matak I, Bolcskei K, Bach-Rojecky L, et al. Mechanisms of Botulinum Toxin Type A Action on Pain. *Toxins (Basel)* 2019; 11: 459.

24. Zhang X, Strassman AM, Novack V, et al. Extracranial injections of botulinum neurotoxin type A inhibit intra-cranial meningeal nociceptors' responses to stimulation of TRPV1 and TRPA1 channels: Are we getting closer to solving this puzzle? *Cephalalgia* 2016; 36: 875–886.

25. Bigal ME, Ashina S, Burstein R, et al. Prevalence and characteristics of allodynia in headache sufferers: a population study. *Neurology* 2008; 70: 1525–1533.

26. Dodick DW, Reed ML, Fanning KM, et al. Predictors of allodynia in persons with migraine: Results from the Migraine in America Symptoms and Treatment (MAST) study. *Cephalalgia* 2019; 39: 873–882.

27. Burstein R, Yarnitsky D, Goor-Aryeh I, et al. An association between migraine and cutaneous allodynia. *Ann Neurol* 2000; 47: 614–624.

28. Markus TE, Zeharia A, Cohen YH, et al. Persistent headache and cephalic allodynia attributed to head trauma in children and adolescents. *J Child Neurol* 2016; 31: 1213–1219.

29. Defrin R, Gruener H, Schreiber S, et al. Quantitative somatosensory testing of subjects with chronic post-traumatic headache: implications on its mechanisms. *Eur J Pain* 2010; 14: 924–931.

30. Ashkenazi A, Sholtzow M, Shaw JW, et al. Identifying cutaneous allodynia in chronic migraine using a practical clinical method. *Cephalalgia* 2007; 27: 111–117.

31. Marinelli S, Luvisetto S, Cobianchi S, et al. Botulinum neurotoxin type A counteracts neuropathic pain and facilitates functional recovery after peripheral nerve injury in animal models. *Neuroscience* 2010; 171: 316–328.

32. Luvisetto S, Marinelli S, Cobianchi S, et al. Anti-allodynic efficacy of botulinum neurotoxin A in a model of neuropathic pain. *Neuroscience* 2007; 145: 1–4.

33. Beer KR, Boyd C, Patel RK, et al. Rapid onset of response and patient-reported outcomes after onabotulinumtoxinA treatment of moderate-to-severe glabellar lines. *J Drugs Dermatol* 2011; 10: 39–44.

34. Jankovic J, Schwartz K and Donovan DT. Botulinum toxin treatment of cranial-cervical dystonia, spasmodic dysphonia, other focal dystonias and hemifacial spasm. *J Neurol Neurosurg Psychiatry* 1990; 53: 633–639.

35. Dodick DW, Silberstein SD, Lipton RB, et al. Early onset of effect of onabotulinumtoxinA for chronic migraine treatment: Analysis of PREEMPT data. *Cephalalgia* 2019; 39: 945–956.

36. Bach-Rojecky L, Relja M and Lackovic Z. Botulinum toxin type A in experimental neuropathic pain. *J Neural Transm (Vienna)* 2005; 112: 215–219.

37. Xiao L, Cheng J, Dai J, et al. Botulinum toxin decreases hyperalgesia and inhibits P2X3 receptor over-expression in sensory neurons induced by ventral root transection in rats. *Pain Med* 2011; 12: 1385–1394.

38. Shimizu T, Shibata M, Toriumi H, et al. Reduction of TRPV1 expression in the trigeminal system by botulinum neurotoxin type-A. *Neurobiol Dis* 2012; 48: 367–378.

39. Gazerani P, Staahl C, Drewes AM, et al. The effects of Botulinum Toxin type A on capsaicin-evoked pain, flare, and secondary hyperalgesia in an experimental human model of trigeminal sensitization. *Pain* 2006; 122: 315–325.

40. Melo-Carrillo A, Strassman AM, Schain AJ, et al. Exploring the effects of extracranial injections of botulinum toxin type A on prolonged intracranial meningeal nociceptors responses to cortical spreading depression in female rats. *Cephalalgia* 2019; 39: 1358–1365.

41. Melo-Carrillo A, Strassman AM, Schain AJ, et al. OnabotulinumtoxinA affects cortical recovery period but not occurrence or propagation of cortical spreading depression in rats with compromised blood-brain barrier. *Pain* 2021; 162: 2418–2427.

42. Wang XY, Zhou HR, Wang S, et al. NR2B-Tyr phosphorylation regulates synaptic plasticity in central sensitization in a chronic migraine rat model. *J Headache Pain* 2018; 19: 102.

43. Yerry JA, Kuehn D and Finkel AG. Onabotulinum toxin a for the treatment of headache in service members with a history of mild traumatic brain injury: a cohort study. *Headache* 2015; 55: 395–406.

44. de Tommaso M, Brighina F and Delussi M. Effects of Botulinum Toxin A on Allodynia in Chronic Migraine: An Observational Open-Label Two-Year Study. *Eur Neurol* 2019; 81: 37–46.

45. Dodick DW, Turkel CC, DeGryse RE, et al. OnabotulinumtoxinA for treatment of chronic migraine: pooled results from the double-blind, randomized, placebo-controlled phases of the PREEMPT clinical program. *Headache* 2010; 50: 921–936.

46. Lippert-Gruner M. Botulinum toxin in the treatment of post-traumatic headache – case study. *Neurol Neurochir Pol* 2012; 46: 591–594.