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Mild fluid percussion injury induces diffuse axonal damage and reactive synaptic plasticity in the mouse olfactory bulb

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Abstract

Despite the regenerative capacity of the olfactory bulb (OB), head trauma causes olfactory disturbances in up to 30% of patients. While models of olfactory nerve transection, olfactory receptor neuron (ORN) ablation, or direct OB impact have been used to examine OB recovery, these models are severe and not ideal for study of OB synaptic repair. We posited that a mild fluid percussion brain injury (mFPI), delivered over mid-dorsal cortex, would produce diffuse OB deafferentation without confounding pathology. Wild type FVB/NJ mice were subjected to mFPI and OB probed for ORN axon degeneration and onset of reactive synaptogenesis. OB extracts revealed 3d postinjury elevation of calpain-cleaved 150kD α II-spectrin, an indicator of axon damage, in tandem with reduced olfactory marker protein (OMP), a protein specific to intact ORN axons. Moreover, mFPI also produced a 3d peak in GFAP+ astrocyte and IBA1+microglial reactivity, consistent with postinjury inflammation. OB glomeruli showed disorganized ORN axons, presynaptic degeneration, and glial phagocytosis at 3 and 7d postinjury, all indicative of deafferentation. At 21d after mFPI, normal synaptic structure re-emerged along with OMP recovery, supporting ORN afferent reinnervation. Robust 21d postinjury upregulation of GAP-43 was consistent with the time course of ORN axon sprouting and synapse regeneration reported after more severe olfactory insult. Together, these findings define a cycle of synaptic degeneration and recovery at a site remote to non-contusive brain injury. We show that mFPI models diffuse ORN axon damage, useful for the study of time-dependent reactive synaptogenesis in the deafferented OB.

Keywords

traumatic brain injury; olfactory bulb; axon injury; synaptic plasticity

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Introduction

Axotomy is a hallmark of diffuse traumatic brain injury (TBI), and the axons of olfactory receptor neurons (ORNs) are particularly vulnerable to this form of axonal damage. Anosmia can result and affect up to 30% of TBI victims, compromising quality of life (Costanzo and Zasler, 1991; Sumner, 1964; Hagan, 1967), with the majority of those affected failing to recover (Costanzo et al., 2012). Further, olfactory input to cognitive circuitry (Babizhayev et al., 2011) may also contribute to broader deficits in behavioral outcome after TBI. The physical forces generated by TBI can sever ORN axons as they course through the bony cribriform plate and proceed to target synaptic sites in the glomeruli of the olfactory bulb (OB). Such axon damage can deafferent the OB, inducing ORN axon regeneration, where these neurons regenerate within the olfactory epithelium and lead to reactive synaptogenesis within OB glomeruli (Graziadei et al., 1978; Koster and Costanzo, 1996; Costanzo, 1985; Morrison and Costanzo, 1995; Yee and Costanzo, 1995; Oley et al., 1975; Jennings et al., 1995). Despite the high regenerative capacity of the olfactory system, it is not yet clear how such regeneration proceeds after TBI and why recovery may be poor.

Prior studies have examined the extent of OB deafferentation and time course of synaptic recovery using a broad range of focal ORN insults, distinct from the more complex, diffuse pathology produced by head injury. These include methyl bromide gas and zinc sulfate gavage of the olfactory epithelium, killing large numbers of ORNs and their targeted axon projections to the OB (Schwob, et al., 1999; Herzog and Otto, 1999; 2004; Chang, et al., 2003). Other models employ precise knife cuts to generate complete ORN axotomy proximal to the OB, maximizing the induction of ORN axon regrowth and synaptogenesis within an intact, non-traumatized OB (Graziadei and Graziadei, 1980; Costanzo, 1985; Yee and Costanzo, 1995; Oley et al., 1975; Jennings et al., 1995). In some reports, ablation of the OB has been used to probe repair in remnant olfactory tissue (Cizkova et al., 1997). Although these studies can model deafferentation induced synaptic repair, they employ significant focal, often severe damage to ORN axons, not consistent with the diffuse axonal damage often present with TBI. Relatively few reports directly address the effects of such injury on OB synaptic reorganization. Of the TBI models where direct OB damage was investigated, early focus was on the effects of brain contusion in the context of sub-ventricular zone (SVZ) stem cell proliferation (Radomski et al., 2013) or the effects of closed impact and penetrating OB insult on olfactory behavioral recovery (Siopi et al., 2012; Steuer et al., 2014). In the latter studies, direct impact lesion of the OB has been used to map molecular response in both the OB and olfactory epithelium, as well as time course of functional recovery under conditions of anti-inflammatory therapy. More recently, direct N-Methyl-D-Aspartate (NMDA) excitotoxicity induction was used to demonstrate that severe OB cell loss is correlated with postinjury decrease in olfactory function, and that dopaminergic neurogenesis may play a role in functional recovery (Marin et al., 2017). We posit that understanding the process of OB reactive synaptogenesis after trauma will require models of partial OB deafferentation without confounding contusion, penetrating lesion or cell loss. Notably, even mild diffuse TBI in humans can induce persistent anosmia (Costanzo et al., 2012; Proskynitopoulos et al., 2017), indicating that molecular mechanisms which influence OB synaptic recovery are affected even by diffuse axotomy. To date, no reports

have directly addressed the time course of synaptic degeneration/regeneration caused by such diffuse damage to ORN axon terminals.

In the present study we examined the utility of mild midline fluid percussion injury (mFPI) as a model of diffuse ORN axon insult, OB deafferentation and reactive synaptogenesis. Based on the published time course of OB reinnervation (Graziadei et al., 1978; Graziadei et al., 1979), we administered mFPI to FVB/NJ mice and sampled OB response during postinjury periods of acute/subacute degeneration (1, 3, 7d) as well as during the early phase of ORN axon regeneration (21d). Here we report several lines of evidence that support mFPI induction of ORN axonal damage and the ensuing OB synaptogenesis. Such injury was confirmed by both a rise in α II-spectrin lysis, a common method for verifying TBI axotomy (Hall et al., 2005; Newcomb et al., 1997; Park et al., 2007; Reeves et al., 2010; Saatman et al., 2003; Serbest et al., 2007), and the concomitant loss of olfactory marker protein (OMP), a protein specific to intact, mature ORN axons (Monti-Graziadei et al., 1977). Robust astrocyte and microglial reactivity was seen within deafferented glomeruli, accompanied by evidence of degenerating ORN axons and active glial phagocytosis. Onset of OB reinnervation was supported by normalized α II-spectrin and OMP levels, reduced glial reactivity and elevated GAP-43 expression when ORN synapses re-emerged. Together, these data demonstrate that mFPI in the mouse can be used as a model for directly exploring mediators of sensory neuroplasticity after TBI, replicating ORN axonal damage without confounding factors of local contusion or focal tissue damage.

Experimental Procedures

Experimental Animals

All procedures met national guidelines for care and use of laboratory animals, and all experimental protocols were approved by the VCU Institutional Animal Care and Use Committee. FVB/NJ WT adult male mice (The Jackson Laboratory, Bar Harbor, ME) were housed (4 littermates/cage) under a temperature (22°C) and humidity controlled environment, with food and water *ad libitum*, and subjected to a 12h dark-light cycle. WT mice (20-30g; 8-11 weeks old) were randomly selected and subjected to midline mild FPI. WT sham-injured cases served as control. Subsets of mFPI and sham-injured groups [WT Sham (n=48), WT FVB TBI (n=53)] were allowed to survive for either 1, 3, 7 or 21d post-injury prior to molecular or histological analysis.

Surgery Preparation and Injury

Mice were anesthetized with isoflurane (4% in 100% O₂ carrier gas) and maintained on 2.5% isoflurane in carrier gas delivered by nose cone. Once stabilized in a stereotaxic frame, heads were shaved, body temperature maintained at 40°C by Gaymar T/Pump water pump (Gaymar Industries Inc., Orchard Park, NY) and heart rate (bpm), arterial oxygen saturation (percent O₂), breath rate (brpm) and pulse/breath distension (in μ m) monitored by pulse oximetry (MouseOx; Starr Life Sciences, Oakmont, PA). Mice then received a midline incision and a 2.7 mm craniectomy prepared over the midline, centered between bregma and lambda. Without damaging the underlying dura, a Leur-Loc syringe hub was cemented to the skull surrounding the craniectomy and dental acrylic poured around the hub to stabilize

the site. Topical anesthetic/antibiotic was applied to the incision and the mice housed in recovery cages. After one hour, mice were anesthetized for 4 mins (4% isoflurane, 100% O₂), and subjected to mFPI as previously described (Dixon et al., 1987; Reeves et al., 2012). The device consisted of a 60 × 4.5 cm Plexiglas water filled cylinder, fitted at one end with a piston mounted on O-rings, with the opposite end housing a pressure transducer (EPN-0300A; Entran Devices, Inc., Fairfield, NJ). At the time of injury, the Leur-Loc fitting, filled with saline, was attached to the transducer housing. Injury was produced by a metal pendulum striking the piston, transiently injecting a small volume of saline into the cranial cavity and briefly deforming the brain tissue (20 millisecond pulse duration). The resulting pressure pulse was recorded extra cranially and registered 1.3±0.1 atm pressure. After injury, all mice were promptly ventilated with room air until spontaneous breathing resumed. The duration of suppression of the righting reflex (5.0±2.0 mins) was used as an index of traumatic unconsciousness. Once righting reflex was determined, mice were re-anesthetized for hub removal, scalp suture and topical anesthetic/antibiotic application. Sham-injured controls received the same surgical preparation, anesthesia and connection to the injury device, except that the intracranial pressure pulse was not applied. All animals were returned to their home cages and assessed for weight loss, locomotion, and eye/nose exudate once per day until weights stabilized.

Protein Extraction

WT mice were anaesthetized with 4% isoflurane in carrier gas of 100% O₂ for 4 min, then sacrificed by decapitation at 1, 3, 7 or 21d after mFPI or sham injury (n=4-7/group), with bilateral OBs dissected for assessment of protein expression. Tissue samples were homogenized on ice in 100 µl of RIPA Lysis Buffer (EMD Millipore, Billerica, MA), and centrifuged at 14,000 × g for 20 min at 4°C. Supernatant was aliquoted and stored at -80°C. Prior to WB analysis, protein concentration was determined using Pierce BCA Protein Assay Reagent (Thermo-Fisher, Waltham, MA) and the FLUOstar Optima plate reader (BMG Labtech, Inc., Cary, NC).

Western Blotting

WB analysis was carried out utilizing Bio-Rad products (Hercules, CA). Twenty µg of protein was prepared in WB XT Sample Buffer and reducing agent (Bio-Rad Laboratories), then denatured at 95°C for 5 mins. Samples were electrophoresed on 4-12% or 12% Bis-Tris Criterion XT gels (200v × 45 min in MOPS running buffer), then protein transferred onto polyvinylidene fluoride (PVDF) membranes (1h at 100V). Post-blotted gels were stained with 0.1% Coomassie Brilliant Blue (Sigma-Aldrich, St. Louis, MO) in 40% MeOH+10% glacial acetic acid, then de-stained at RT to confirm protein load and even transfer. Membranes were rinsed with deionized water and Tris-buffered saline (TBS) before blocking with 5% milk TBS-Tween (mTBS-T). Blots were then incubated in 5% mTBS-T overnight (4°C) with individual primary antibodies to αII-spectrin (1:2,000, Enzo, Farmington, NY, olfactory marker protein (OMP; 1:20,000, Wako, Richmond, VA), and growth associated protein-43 (GAP-43; 1:1,000, Santa Cruz, Dallas, TX). After primary incubation, membranes were washed with mTBS-T, then incubated with appropriate HRP-linked secondary antibodies [IgG bovine anti-goat (1:15,000, Santa Cruz, Dallas, TX), IgG goat anti-mouse (1:15,000, Rockland Immunochemicals Inc., Limerick, PA)] in mTBS-T for

1h at RT. Finally, blots were washed with mTBS-T and antibody binding visualized using Super Signal Dura West chemiluminescence substrate (Thermo-Fisher, Waltham, MA). WB images were captured with Syngene G: Box and positive band signal subjected to densitometric analysis (relative optical density, ROD) with Gene Tools software (Syngene, Frederick, MD). Protein data were expressed as percent change relative to paired WT Sham control cases run on the same transferred gel. Either cyclophilin A (EMD Millipore, Billerica, MA) or beta actin (Sigma-Aldrich, St. Louis, MO) was used as a load control.

Immunohistochemistry

At 3, 7 and 21d survival WT injured and WT sham mice (n=4/group) were prepared for fluorescent IHC analysis according to published protocol (Warren et al., 2012). Animals were anaesthetized with sodium pentobarbital (400mg/kg, i.p.), transcardially perfused with 0.9% saline followed by 4% paraformaldehyde in phosphate buffer (0.1M NaHPO₄, pH=7.4), after which brains with attached OBs were extracted and placed in fixative for 24 h before transfer to 0.03% NaN₃ in 1.0 M phosphate buffered saline (PBS). For IHC, fixed brains were cryoprotected in 30% sucrose for 3d, with sucrose solutions exchanged after each day. Frontal lobes were blocked and attached bulbs mounted in Tissue Tek media (Thermo-Fisher, Waltham, MA) and stored at -80°C. Coronal cryostat OB sections (13µm) were collected using a CryostarTMNX70; CryotomeTMFSE; HM525 NX cryostat (Thermo-Fisher, Waltham, MA) and prepared for immunofluorescence visualization.

Free floating sections were first permeabilized in 5% peroxidase for 30 mins. After a wash with PBS, tissues were pre-incubated in Blotto blocking buffer (fish gelatin in PBS + 0.05% Triton X-100) to prevent non-specific binding, and then sections were individually incubated overnight in primary antibody (OMP, 1:20,000, Wako, Richmond, VA; glial fibrillary acidic protein [GFAP], 1:20,000, Dako; ionized calcium binding adaptor protein [IBA1] for microglia, 1:300, Wako, Richmond, VA) at 4°C. Sections were PBS washed, placed in blocking buffer for 30 mins, and then incubated with secondary fluorescent antibody (Alexa-Fluor 488 donkey anti-goat, 1:1000 and Alexa-Fluor 594 donkey anti-rabbit, 1:1000, Thermo-Fisher, Waltham, MA) in Blotto for 1h at RT. Slices were next PBS washed, equilibrated in phosphate buffer, and mounted on Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA) with Vectashield + DAPI (Vector Laboratories, Burlingame, CA). IHC signal was visualized on the Zeiss LSM 700 (Carl Zeiss, Thornwood, NY) confocal microscope (VCU Microscopy Core).

Transmission Electron Microscopy

Following mFPI, WT injured and WT Sham-injured mice were randomly assigned to either 3, 7 or 21d survival perfused groups (n=3/group). At time of sacrifice, mice were anesthetized with sodium pentobarbital (400mg/kg, i.p.), transcardially with mixed aldehyde fixative (2% paraformaldehyde and 2.5% glutaraldehyde) in 0.1M phosphate buffer, pH-7.2. Brains were removed and post-fixed overnight at 4°C. OBs were next blocked in the sagittal plane and placed in 1% osmium tetroxide (0.1 M cacodylate buffer), and processed for embedding with Epon resin (Embed; Electron Microscopy Sciences, Hatfield, PA). After curing, OB areas containing glomeruli were mounted and both semi-thin (0.5µm) and ultrathin (silver, 600 Å) sections were cut with a Leica EM UC6i ultramicrotome (Leica

Microsystems, Wetzlar, Germany). Semi-thin sections were used to guide subsequent ultrastructural sampling. Ultrathin sections were collected on Formvar-coated slotted grids and observed on a JEOL JEM-1230 electron microscope (JEOL USA, Inc., Peabody, MA), equipped with a GatanUltraScan 4000SP CCD camera (Gatan, Inc., Pleasanton, CA).

Statistical Analyses

Changes in protein levels induced by injury were evaluated using the GLM and MANOVA routines in SPSS v.22 (International Business Machines, Corp., Armonk, NY). For data generated from WB measurements, normality (Kolmogorov-Smirnov Test) and homogeneity of variance (Levene Statistic) were confirmed prior to ANOVA analyses. The Duncan Multiple Range Test was used for post hoc pairwise group comparisons. WB results are reported as mean $\pm$ SEM. An alpha level of 0.05 was used in all analyses.

Results

Alpha II-spectrin proteolysis in the OB is increased after mFPI

In order to determine the extent of ORN axonal damage within WT FVB/NJ mice following mFPI, we first examined injury-induced production of α II-spectrin breakdown within the OB. The increased expression of α II-spectrin fragments has been used to confirm the extent of axonal injury in several different models TBI (Pike et al., 1998; Buki et al., 1999; Ringger et al., 2004; Thompson et al., 2006; Reeves et al., 2010). Here we examined change in OB caspase and calpain-mediated α II-spectrin proteolysis at 1, 3, 7, and 21d postinjury, mapping predicted periods of axon degeneration (1-7d) and early synapse regeneration (21d) induced by OB deafferentation. Overall, our results indicate that diffuse mFPI does produce OB axonal damage, however, α II-spectrin proteolysis was limited with respect to fragment size and postinjury interval of generation (Fig. 1). For the calpain-derived 150, 145kD peptides, there was a nonsignificant trend toward reduction at 1d relative to sham controls [$52.68 \pm 11.90\%$, $F_{(1,39)} = 0.24$; $p = 0.62$; $69.83 \pm 12.87\%$, $F_{(1,39)} = 1.85$; $p = 0.18$], but no detectable shift in caspase-derived 120kD α II-spectrin signal [$90.00 \pm 11.71\%$, $F_{(1,39)} = 0.37$; $p = 0.54$]. Notably, at 3d we found significant, nearly 4-fold elevation in postinjury 150kD α II-spectrin fragment relative to sham injured controls ($380.77 \pm 129.58\%$, $F_{(1,39)} = 11.28$; $p < 0.01$). By contrast, levels of the 3d 145kD ($107.02 \pm 10.20\%$, $F_{(1,39)} = 0.23$; $p < 0.64$) and 120kD [$104.14 \pm 8.18\%$, $F_{(1,39)} = 0.08$; $p < 0.77$] α II-spectrin peptides were not different from sham controls. Acute 3d postinjury α II-spectrin proteolysis is consistent with delayed onset of OB axon degeneration after olfactory nerve transection (Costanzo et al., 2006; Costanzo and Perrino, 2008; Graziadei and Monti Graziadei, 1980) and olfactory epithelium sensory neuron ablation (Bakos et al., 2010; Nathan et al., 2001). Interestingly, the 3d increase in 150kD α II-spectrin fragment production matched significant 3d reduction of ORN axon marker protein OMP (see Fig.3 below). For the later degenerative (7d) and early regenerative (21d) phases, we found no change in α II-spectrin fragments at 150kD [$130.50 \pm 26.46\%$, $F_{(1,39)} = 0.05$; $p = 0.82$; $107.21 \pm 38.98\%$, $F_{(1,39)} = 0.01$; $p = 0.91$] and 145kD [$109.77 \pm 25.42\%$, $F_{(1,39)} = 0.22$; $p = 0.64$; $102.98 \pm 16.50\%$, $F_{(1,39)} = 0.01$; $p = 0.92$], or at 120kD [$119.27 \pm 18.85\%$, $F_{(1,39)} = 0.87$; $p = 0.36$; $101.26 \pm 16.44\%$, $F_{(1,39)} = 0.01$; $p = 0.94$]. However, 150kD α II-spectrin exhibited significant time-dependent changes in expression [$F_{(3,20)} = 3.19$; $p < 0.05$], and post hoc comparisons of the different survival times revealed this was due

to a significant upward shift between 1 and 3d postinjury ($p < 0.05$). From these data we conclude that axonal damage does occur within the OB following mFPI, however, there is both a delayed and more limited postinjury generation of α II-spectrin fragments within the OB, where calpain-derived 150kD breakdown product predominates. Failure to find postinjury effect on the caspase-produced 120kD α II-spectrin also suggests that caspase related cell death is likely not a major contributor to OB pathology after mFPI.

OB glia change morphology in response to mFPI

Postinjury accumulation of a calpain-produced α II-spectrin fragment supports damage to ORN axons after mFPI consistent with OB glomeruli (GL) deafferentation, and subsequent initiation of synaptic repair. Early stages of this process require removal of degenerating presynaptic terminals through reactive gliosis. Endogenous microglia and astrocytes shift from resting to activated forms, clearing damaged axon terminals and reorganizing the postsynaptic environment for synaptic repair. We next examined OB glial reactivity after mFPI, focusing on 3, 7 and 21d postinjury. IHC with antibodies marking astrocytes (GFAP) and microglia (IBA-1) showed that both cell types were highly reactive within the GL when compared with paired sham controls (Fig. 2). Interestingly, reactive astrocytes were primarily localized to the GL, while microglial response was observed in both GL and external plexiform layer of the OB. By 3d after mFPI, astrocytes exhibited prominent hypertrophy, their thickened processes interdigitating along the surface of deafferented GL (Fig. 2, panel A). Microglia also shifted from a resting, ramified morphology to an activated form by 3d, with enlarged cell bodies and lobular cytoplasmic processes (Fig. 2, panel B). At 7d postinjury, reactivity of each glial type was reduced relative to the 3d response, but remained above that of sham injured animals. By 21d, when early synaptic regeneration is reported in other models of OB deafferentation (Graziadei and Monti-Graziadei, 1980; Cummings et al., 2000), glial phenotypes were not different from sham cases. These results show a time course of endogenous OB glial activation similar to that reported following olfactory nerve transection (Bailey and Shipley, 2003; Lazarini et al., 2012) and is consistent with the observed 3d increase in 150kD α II-spectrin fragment generation. Together, these results support the hypothesis that mFPI produces OB axonal pathology and deafferentation, thereby inducing distinct phases of reactive gliosis over time postinjury.

Expression of ORN marker OMP changes after mFPI

In order to test for ORN-specific axon damage after mFPI, we next probed the same OB protein extracts for change in expression of olfactory marker protein (OMP). OMP is a cytoplasmic protein selective for mature ORN axons, and has been routinely used to identify ORN axon damage in more severe models of OB deafferentation (Monti-Graziadei et al., 1977; Cummings et al., 2000; Griff et al., 2000; Holtmaat et al., 1995; Kasowski et al., 1999; Stone et al., 1994; Costanzo et al., 2006). Taking OMP reduction as a metric for ORN axon damage, we observed significant changes in protein level across the 1d-21d postinjury period [$F_{(3,17)} = 3.82$; $p < 0.05$], and post hoc analyses showed the mean OMP level at 3d to be significantly below those found at 1d ($p < 0.05$) and 21d ($p < 0.05$) [Fig. 3A]. This was consistent with predicted induction of diffuse axonal damage in our model and the rather delayed time course of ORN degeneration seen in other models of OB deafferentation (Morrison and Costanzo, 1995; Bakos et al., 2010). At 1d postinjury, OMP protein was

equivalent to sham control levels [$100.84 \pm 11.17\%$; $F_{(1,37)} = 0.01$; $p = 0.91$], but by 3d we found a significant reduction in the 19kD signal to approximately 62% of control [$61.84 \pm 10.09\%$, $F_{(1,37)} = 5.94$; $p < 0.05$]. Interestingly, at 7d after injury, OMP protein was no longer different from sham values [$90.76 \pm 11.34\%$; $F_{(1,37)} = 0.35$; $p = 0.56$] and reached a mean slightly higher than controls by 21d onset of the regenerative phase [$108.69 \pm 7.54\%$; $F_{(1,37)} = 0.23$; $p = 0.64$]. The significant drop in OMP levels at 3d, identifies this time point as critical in the evolution of OB postinjury axon degeneration. Interestingly, the fact that 7d OMP level approached sham controls, but was not different from the 3d expression also suggests that the postinjury interval between 3 and 7d is a critically active phase of ORN axon recovery after mFPI. Notably, we observed that OMP recovery was achieved over a postinjury time frame shorter than that required for distal ORN axon regeneration. This result opens the possibility that the time course of OB reinnervation may be a function of injury extent and that local sprouting contributes significantly to reinnervation in our model. Parallel IHC supported such local GL presynaptic disorganization, showing detectable loss of OMP signal at 3d postinjury (Fig. 3B), while OMP staining remained strong in the olfactory nerve layer (ONL). Together, these OMP findings support diffuse ORN axon damage by 3d after mFPI, and suggest that resulting synaptic regeneration emerges as early as 7d postinjury. This time course of OMP reduction and restoration is similar to that observed during postnatal injury and ORN reinnervation of OB glomeruli (Gonzalez and Silver, 1994), as well as that described for OB synaptogenesis after ORN lesion and olfactory epithelium chemical damage (Costanzo et al., 2006; Cummings et al., 2000; Bakos et al., 2010).

GL synaptic disruption and reorganization in GL after mFPI

The observation that mFPI increased α II-spectrin proteolysis and glomerular glial reactivity within the OB warranted a more precise exploration of GL synaptic morphology. In order to establish how injury affects this morphology, we employed transmission electron microscopy (TEM) to examine OB glomerular structure between 3 and 21 d after injury (Fig. 4). Low magnification imaging of 1 μ m thick plastic embedded sections (panel A) revealed the typical organization of OB glomeruli in sham controls, where large groups of electron dense ORN axons interdigitate among postsynaptic dendrites. At 7 and 21d after mFPI, pre-synaptic axons were reduced in size relative to control. Ultrastructure (panel B) of sham controls revealed the predicted pattern of ORN axon bundles encircling groups of mitral and tufted cell dendrites. At 7d after injury, these ORN axon bundles appeared collapsed and reduced in size. Higher magnification (panel C) evidence of disrupted synaptic cytology by 3d postinjury, where ORN axons exhibited early signs of degeneration. At 7d postinjury, the same axons remained shrunken and collapsed, with evidence of membranous tissue degeneration. Notably, GL glial processes were reactive at 7d after mFPI and contained cell debris. By 21d postinjury some GL tissue damage remained, however, normal axon and dendrite structure began to reemerge. These TEM results are consistent with the time course of GL glial reactivity after mFPI and support a protracted degenerative response previously reported for damaged OB, here with delayed onset regeneration following diffuse ORN deafferentation. This delayed pattern of axon loss (>3d after insult) matches that seen after knife cut ORN axotomy (Costanzo et al., 2006) and chemical ORN lesion (Cummings et al., 2000; Bakos et al., 2010).

OB GAP-43 increases during postinjury regeneration induced by mFPI

As a probe for tracking potential OB pre-synaptic sprouting and synapse reinnervation, GAP-43 expression was assayed over time postinjury. This growth factor is critical to axon growth and extension, as well as synaptic replacement in the OB (Cizková et al., 1995; Griff et al., 2000). It may also be regulated by OMP expression (Griff et al., 2000), where, as axons are lost, OMP levels fall, removing OMP inhibition of GAP-43 production. After mFPI, we found significant time-dependent changes in GAP-43 expression [$F_{(3,17)} = 8.52$; $p < 0.01$], and the observed temporal pattern was highly correlated with the predicted onset of synaptic replacement at 21d postinjury. Importantly, we found no significant change in the GAP-43 signal over the 1-7d postinjury period, covering the first week of the degenerative phase (Fig. 5). GAP-43 was equivalent to sham controls at 1d [$95.69 \pm 24.18\%$; $F_{(1,34)} = 0.13$; $p = 0.72$] and 7d postinjury [$105.20 \pm 10.99\%$; $F_{(1,34)} = 0.05$; $p = 0.82$]. At 3d after mFPI we observed a small, but non-significant, 36% rise in GAP-43 protein relative to sham control [$136.28 \pm 9.06\%$; $F_{(1,34)} = 0.53$; $p = 0.47$]. The failure to show significant rise in GAP-43 associated with significant loss of OMP (see again Fig. 3) could be attributed to the diffuse ORN axonal damage observed after mFPI. The prominent change in GAP-43 expression was detected as a nearly 3-fold rise over sham control at 21d postinjury [$297.19 \pm 58.77\%$, $F_{(1,34)} = 20.69$; $p < 0.001$], a time interval documented to correspond with the onset of ORN axon regeneration after olfactory nerve axotomy (Cizková et al., 1995; Graziadei et al., 1978; Graziadei et al., 1979). Post hoc comparisons showed this 21d elevation of GAP-43 was also significantly different from 1d ($p < 0.001$), 3d ($p < 0.01$), and 7d ($p < 0.001$) expression, further supporting the importance of its role during the early regenerative phase of OB synaptic repair induced by TBI. Overall, postinjury OMP and GAP-43 expression, markers of OB neuroplasticity, support the induction of both OB axon injury and synaptic repair after mFPI.

Discussion

Clinical olfactory dysfunction after TBI is well documented, yet the capacity for postinjury OB synaptic reorganization after this type of brain insult is not understood. Here we addressed how diffuse OB deafferentation affects synaptic recovery. We hypothesized that mFPI causes ORN axon damage, deafferenting OB synapses to elicit reactive synaptogenesis. We also posited that diffuse damage to ORN axons would be a valuable tool for dissecting the molecular basis of olfactory recovery after brain injury. Overall, our results showed that mFPI produces OB deafferentation, with a 1-21d time course of synaptic reorganization similar to ORN transection or olfactory epithelium disruption (Bakos et al., 2010; Costanzo et al., 2006; Cummings et al., 2000; Graziadei et al., 1978; Graziadei et al., 1979; Graziadei and Okano, 1979; Kobayashi and Costanzo, 2009; Graziadei and Monti Graziadei, 1980; Morrison and Costanzo, 1995). In the present study we report time-dependent postinjury change in markers of axonal injury and regeneration consistent with the process of reactive synaptogenesis. Acute spectrin proteolysis at 3d was correlated with loss of ORN axon marker OMP, and the reactivity of both astrocytes and microglia was elevated at the same postinjury interval. OB ultrastructure supported onset of ORN axon degeneration at 3d and glial phagocytosis of GL debris at 7d. Notably, reemergence of synaptic structure was observed by 21d after mFPI. Elevated GAP-43, a molecule previously

associated with OB synapse regeneration, was also elevated at 21d onset of ORN reinnervation. Together, these data indicate that mFPI is a workable model for the induction of OB reactive synaptogenesis, with uncomplicated, diffuse insult permitting detailed study of sensory synaptic reorganization following TBI.

Mild diffuse FPI causes lysis of OB α II-spectrin and loss of OMP

We first assessed α II-spectrin breakdown, a traditional marker of axotomy and axolemma destabilization. Postinjury increase in $[Ca^{2+}]_i$ activates calpain and caspase proteases, increasing α II-spectrin lysis and breakdown peptides, which gauge cytoskeletal disruption due to TBI-induced axonal injury (Pike et al., 1998; Buki et al., 1999). These biomarkers of axonal injury can be detected in the cerebrospinal fluid of TBI patients (Ringger et al., 2004), as well as in brain tissues from rodent moderate and severe traumatic injuries (Thompson et al., 2006; Reeves et al., 2012). Following mFPI, we found α II-spectrin lysis elevated, but limited to 3d postinjury change in the 150kD calpain fragment. Since calpain-generated 145kD α II-spectrin was unaffected, we conclude that ORN axon degeneration in our model is less robust and likely peaks during the early (1-3d) postinjury interval. An attenuated response would be predicted based upon OB distance from injury site. Further, since the caspase-produced 120kD α II-spectrin did not change after injury, we also conclude that diffuse mFPI does not generate α II-spectrin fragments commensurate with OB cell death. A predominant change in 150kD α II-spectrin suggests that OB calpain proteolysis may be more sensitive to diffuse mFPI. This result is in contrast to other brain regions subjected to TBI (e.g., cerebral cortex, hippocampus, corpus callosum), where injury induces up to 10-fold elevation of 150 and 145kD α II-spectrin, as well as minor increases in caspase-derived 120kD peptide (Harris and Morrow, 1988; Pike et al., 1998; Buki et al., 1999; Wang et al., 2000; Ringger et al., 2004; Thompson et al., 2006; Aikman et al., 2006; Reeves et al., 2010). Several factors could explain this difference in OB α II-spectrin proteolysis. Foremost, the rostro-caudal dissipation of injury forces from a mid-dorsal, centralized injury site likely results in damage to a smaller subset of ORN axons. It is also possible that more robust OB α II-spectrin breakdown occurs prior to 1d postinjury sampled here. In fact, Reeves et al. (2010) has reported significant increase of cortical 150, 145 and 120kD α II-spectrin at 3h postinjury. A third, technical consideration would be the dilution of ORN axon-derived α II-spectrin fragments when assayed in whole OB preparations, where the extent of ORN axon damage might be overshadowed by intact α II-spectrin from non-GL OB cells. Finally, while less likely, it remains possible that increase in 150 kD α II-spectrin is the result of modest cytoskeletal disruption within axons and dendrites of other OB neurons injured with mFPI. Mitral and tufted cells, the principal post-synaptic targets of ORNs, would be candidates for such injury sensitivity, as reported for a model of transient ischemia (Hwang et al., 2008). OB TEM analysis following mFPI would argue against this latter possibility, since the predominant GL pathology occurs within the afferent ORN axon terminals. Further analysis of additional postinjury intervals and specific OB cell damage will be required to determine the exact source(s) of our α II-spectrin effects.

In order to more specifically confirm the extent of GL pre-synaptic damage we assayed OMP, a 19kD peptide selectively expressed in ORNs and their axons (Monti-Graziadei et al., 1977). Cytoplasmic OMP is specific for mature ORNs, and is conserved across species

(Keller and Margolis, 1976). It is a good marker for olfactory synaptic function (Kass et al., 2013) and OMP KOs show attenuated response to odor stimuli (Buiakova et al., 1996, Lee et al., 2011, Youngentob and Margolis, 1999, Youngentob et al., 2001). OMP is linked to maturation of ORN axons/synapses, and it is not present within immature ORNs (Verhaagen et al., 1989). Tracking OMP loss and points to the protein as a metric of the axon damage/recovery cycle. Here we found significant 3d postinjury reduction of OMP protein, indicating ORN axon damage, and correlating with increase in a 50kD α II-spectrin fragment. IHC confirmed a 3d GL OMP signal reduction, notably within ORN axons penetrating the OB. This pattern is consistent with OMP protein reduction after olfactory nerve transection (Costanzo et al., 2006), indicating that OMP serves as a metric for lost ORN axon input. The time frame of this reduction can range between 3 and 15d after deafferentation, depending on lesion type (Bakos et al., 2010; Inamitsu et al., 1990; Kobayashi et al., 2013; Nathan et al., 2001; Tsukatani et al., 2003). By 21d postinjury, we found that onset of synaptic regeneration was matched by full recovery of OMP expression, a result similar to other OB deafferentation models. However, diffuse mFPI clearly produced a more limited OMP effect, with the protein beginning to approach control levels by 7d postinjury. This early return of OMP signal could reflect a more rapid ORN terminal reorganization and earlier synaptic recovery when the injury damage affected only a subset of axon terminals. As for α II-spectrin lysis, it is important to consider that we sampled whole OB extracts, which could similarly affect the detection of deficits in OMP signal at some postinjury intervals. Nevertheless, our α II-spectrin and OMP analyses support acute ORN axon damage after mFPI, likely within a subpopulation of pre-synaptic olfactory sensory axons. To the best of our knowledge, the present study provides the first documentation of OB synaptogenesis induced by diffuse mFPI, directly linking markers of axotomy with evidence of olfactory deafferentation.

Both OB glial reactivity and synaptic reorganization follow mild diffuse FPI

TBI generates a rapid and localized glial reactivity, concurrent with acute inflammatory response and reactive synaptogenesis (Phillips et al., 2014; Chan et al., 2014). While OB glial reactivity after diffuse axonal damage had not been examined, reactive astrocytes and microglia that support synaptic reorganization are reported following more severe ORN insult (Bailey and Shipley, 1993; Chang et al., 2003; Lazarini et al., 2012). Our IHC results confirm that mFPI activates both OB astrocytes and microglia, showing the predicted astrocyte hypertrophy and microglial shift from ramified to reactive phenotype. This reactivity peaked at the same postinjury interval as α II-spectrin lysis and OMP reduction, providing further support for mFPI induction of local OB deafferentation. The time course of OB glial reactivity in our model indicates the presence of a mild deafferentation, with return to normal glial morphology by 21d after injury. This suggests a principal role of OB reactive neuroglia during acute removal of degenerated ORN axonal terminals, preparing the deafferented zone for subsequent synaptic reorganization.

OB astrocytes are concentrated within the GL, considered the principal route of glial response in more severe models of OB deafferentation (Bakos et al., 2010; Kobayashi et al., 2013). During development these GL astrocytes mediate axon growth and synaptogenesis (Bailey and Shipley, 1993). Our IHC results show acute astrocyte hypertrophy after mFPI

indicating that these glia are first responders to local ORN deafferentation. Their unique position relative to OB synapses allows them to mediate ORN terminal degeneration to synapse reinnervation. These cells exhibit cytoplasmic processes wrapping individual glomeruli and finer extensions spreading into the glomerular structure, allowing direct contact with degenerating presynaptic terminals. This pattern is consistent with GL fine structure where astrocyte processes generate boundary zones between the axo-dendritic synapses of the central glomerular region and juxtglomerular interneurons surrounding the core (Chao et al., 1997). However, it is important to note that, unlike other reports of OB astrocyte response to olfactory epithelium ablation or ORN transection, the duration of astrocyte response to mFPI appears attenuated. For example, methyl bromide destruction of olfactory epithelium increased expression of the astrocyte marker GFAP for a period of 15d (Bakos et al., 2010), while full ORN transection steadily increased GFAP for over 60d postinjury (Kobayashi and Costanzo, 2009; Kobayashi et al., 2013; Cummings et al., 2000).

It is also well documented that pro-inflammatory cytokines peak early after TBI, secreted by reactive microglia and invading immune cells. Despite the diffuse nature of our injury, we also found maximal reactive phenotype in OB microglia at 3d postinjury. A similar time course was reported by Lazarini et al. (2012), where intranasal 2, 6-Dichlorobenzonitrile caused peak OB microglial response at 3d after lesion. Similar microglial phenotype was also detected after olfactory epithelium injury with zinc sulfate (Chang et al., 2003) and methyl bromide (Bakos and Costanzo, 2010), inducing macrophage-like reactivity. Unlike astrocytes, activated OB microglia were not restricted to the deafferented GL after mFPI, but also distributed among mitral and tufted cell dendrites in the external plexiform layer. This distribution likely facilitates microglial generation of pro-inflammatory molecules to mediate autocrine/paracrine cell signaling, a response seen after CNS insults (Shin et al., 2005; Doty, 2012). Although prolonged microglial reactivity can occur in other brain regions after TBI (Johnson et al., 2013; Smith et al., 2013; Gentleman et al., 2004; Faden et al., 2011; Engel et al., 2000; Loane et al., 2014; Ramlackhansingh et al., 2011), we found OB microglial response to be normalized by 21d postinjury. This would be consistent with the mild, diffuse nature of our mFPI, and the distance between the OB and injury site. Together, these IHC results further demonstrate that mFPI induces OB deafferentation, with the time course of reactive glial response consistent with their role in reactive synaptogenesis.

Given that we found evidence of OB diffuse axon injury after mFPI, we looked for visible change in glomerular synapto-dendritic structure. Overall, qualitative assessment supported axon degeneration and reactive synaptogenesis within a subset of ORN sensory synapses. These morphological changes reflected the predicted time course of events for deafferentation induced synaptogenesis: acute presynaptic degeneration, followed by synaptic reorganization. Consistent with other TEM studies (Chao et al., 1997; Valverde and Lopez Mascaraque, 1991; Kasowski et al., 1999) OB sham controls showed electron dense ORN axon terminals grouped around dendrites of mitral and tufted cells. Pre-synaptic vesicles were found opposed to synaptic membranes with thick post-synaptic densities. As early as 3d after injury this profile became less organized, matching loss of OMP and increased α II-spectrin lysis. Disrupted axonal cytoskeleton and pre-synaptic vesicle arrangement also point to early phases of deafferentation. Since injury did not produce this pathology in all ORN terminals, we conclude that mFPI generates a diffuse, partial

deafferentation of the OB. Interestingly, this model may also generate an additional subset of synapses that undergo disorganization and remodeling without junctional separation. Such a model permits a more detailed examination of ORN axonal populations with differential vulnerability to TBI, mapping the process of synaptic repair under different conditions. Indeed, such differential vulnerability has been extensively reported in other cortical regions and white matter following diffuse FPI (Singleton and Povlishock, 2004; Stone et al., 2004; Reeves et al., 2005). Ultrastructural images further confirmed the active role of astrocytes in phagocytic clearance of degenerating ORN axons, commensurate with a reduced glial filament load over time postinjury. While the majority of glomerular glial response was astrocytic, reactive microglia did exist in the peripheral glomerular zones, potentially serving as additional phagocytes within damaged axon bundles as seen after bullectomy (Smithson and Kawaja, 2010). Notably, no detectable change in morphology was found within the periglomerular interneuron population after mFPI (*data not shown*).

Overall, TEM results suggest that mild, diffuse mFPI induces ORN terminal degeneration and reactive synaptogenesis. This is supported by the acute postinjury change in α II-spectrin and OMP, indicating presynaptic axon damage, as well as the subsequent recovery of OMP signal and GAP-43 elevation between 7-21d after mFPI, marking reinnervation. However, the present study did not assess ORN axon morphology within the OB nerve layer, leaving open the question as to whether the observed synaptic recovery is a function of ORN axons re-entering the OB or a local axon retraction/reinnervation of GL sensory synapses. Based upon our molecular data and the observation that terminal degeneration and axon shrinkage were visible in the OB, we posit that mFPI likely induces both processes. First, prior lesion studies report clearance of degenerative processes and arrival of regenerating ORN axons 15-35d after injury (Graziadei et al., 1978; Graziadei et al., 1979; Morrison et al., 1995), the latter correlated with increased growth factor expression (Margolis et al., 1991; Rodriguez-Gil et al., 2015; Steuer et al., 2014). This pattern is consistent with our observation of 7d glial phagocytosis, along with a 21d postinjury re-emergence of normal ORN synaptic morphology and GAP-43 induction. Nevertheless, we also observed the reemergence of OMP signal at 7d, an earlier postinjury interval than reported following more severe ORN transection (Costanzo et al., 2006). Therefore, it is reasonable to assume that a subset of ORN axons may be damaged to a lesser degree after mFPI, possibly causing local OB retraction or shrinkage of presynaptic ORN processes. These axons could either regain their structural organization or be replaced by collateral sprouts of adjacent ORN axons within a week after injury. In order to clarify the details of OB reinnervation after mFPI, future studies should map the extent of ORN axon damage in the OB nerve layer, probe for time dependent change in other axon/synaptic markers, and examine postinjury survival intervals beyond 21d.

Growth factor induction accompanies OB synaptic recovery after mild diffuse FPI

Finally, we assessed GAP-43 expression to probe for further evidence of OB synaptic reorganization after mFPI. We found evidence for significant time-dependent elevation of the growth factor. GAP-43, a protein which is upregulated in growth cones after both peripheral (Knyihár-Csillik et al., 1992) and central (Stroemer et al., 1993; Benowitz and Routtenberg, 1997; Christman et al., 1997; Hulsebosch et al., 1998; Gorup et al., 2015) axon

injury, can be used to confirm ORN axon sprouting and synapse reformation. Notably, it is elevated when ORN axons grow from olfactory epithelium to the OB, then downregulated at later postinjury intervals as the axons establish fixed OB targets (Margolis et al., 1991; Rodriguez-Gil et al., 2015; Steuer et al., 2014). In the mFPI model, a nearly three-fold induction of GAP-43 induction was seen at 21d postinjury. Following olfactory nerve transection, the first regenerating axons reenter the GL between 15-35d postinjury (Morrison and Costanzo, 1995; Graziadei et al., 1978; Graziadei et al., 1979). Thus, the significant increase of this growth-regulating protein at 21d suggests its role in the phase of synapse reformation, even following mFPI. The fact that high GAP-43 levels can occur in new glomeruli exhibiting axonal re-growth (Cizková et al., 1995) further supports our interpretation that the rise in GAP-43 we observe is associated with deafferentation induced synaptogenesis.

Alternatively, it could be reasoned that the observed 21d GAP-43 elevation is generated by injury-induced neurogenesis. The integration of SVZ progenitors to replace OB granule cells and GL peri-glomerular GABA neurons (Lazarini et al., 2014) could potentially contribute to high GAP-43 at this time. Indeed, Marin and colleagues (2017) provide evidence that cell markers of neurogenesis (Ki67, PSANCAM) correlate with increased OB dopaminergic immunostaining in a model of severe NMDA neural degeneration. Interestingly, following more restricted OB NMDA lesions, entry of SVZ adult born NeuN+ cells into the OB was delayed, observed 5 weeks after the insult (Liu and Guthrie, 2011). These differences may be a function of trauma model and could reflect the dependence of OB response on mode of injury. For example, the CCI model of contusional TBI increases SVZ proliferation, but markers of OB neurogenesis are reduced, with neuroblast progenitors diverted from the OB rostral migratory stream to other, more injured areas (Radomski et al., 2013). Further, Sundholm-Peters and colleagues (2005) reported that, following cortical lesions, SVZ neuroblasts emigrate toward the lesion site, while OB SVZ migration is not altered. This pathway shift is also common for certain neurodegenerative and stroke models (Christie and Turnley, 2013). Moreover, acute (1-3d) microglial activation, as we observed after mFPI, can also impair OB neurogenesis (Lazarini et al., 2012). Given the mild level of our FPI and the delayed postinjury induction of growth factor observed, we conclude that the 21d GAP-43 elevation is less likely due to an increased presence of SVZ replacement cells, being more in line with periods of glomerular synaptic recovery. Clearly, additional studies focusing on OB region-specific GAP-43 response and protein expression during later postinjury intervals are needed to clarify its OB role after mFPI.

In conclusion, we show that a mild midline FPI produces demonstrable damage to ORN axons and induces deafferentation of OB glomeruli. Reactive gliosis accompanies the injury, contributing to removal of degenerative debris, and likely producing molecules which direct synaptic repair. These events occur despite the diffuse nature of the injury, as well as the distance of the OB from injury epicenter. Such synaptic disruption is consistent with olfactory deficits reported in clinical TBI. We posit that this model of diffuse OB insult can be used to address the interaction between neurons, glia and local proteins that regulate the recovery process.

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Abbreviations

TBI	traumatic brain injury
mFPI	mild fluid percussion injury
OB	olfactory bulb
ORN	olfactory receptor neuron
GL	glomerular layer
OMP	olfactory marker protein
GFAP	glial fibrillary acidic protein
IBA-1	ionized calcium-binding adaptor molecule 1
GAP-43	growth associated protein 43
NMDA	N-Methyl-D-Aspartate
SVZ	subventricular zone
TEM	transmission electron microscopy

Mild midline concussive brain injury induces distal olfactory bulb (OB) deafferentation.

OB deafferentation exhibits concurrent α II-spectrin proteolysis and loss of olfactory axonal marker protein OMP.

Astrocyte and microglial reactivity within OB glomeruli matches the predicted postinjury time course of glial activation.

Fine structure of OB glomerular synapses shows a degeneration/regeneration cycle commensurate with reactive synaptogenesis.

GAP43 is elevated at a postinjury interval where OB glomerular synapses present structural recovery.

Figure 1. OB αII-spectrin expression after mFPI

At 3d postinjury, 150kD αII-spectrin was elevated nearly 4-fold, supporting FPI-induced axon pathology. The same fragment was reduced by 50% at 1d, but the effect not statistically significant. No other change in calpain-derived αII-spectrin fragments (150 or 145kD) was detected. Time-dependent elevation of 150kD αII-spectrin was observed between 1 and 3d postinjury. Caspase-cleaved 120kD αII-spectrin was unchanged. Results expressed as percent of sham control R.O.D. cases (*100% dashed line*) run on the same transferred gel. Blot images, with cyclophilin A (3, 7, 21d) and β-actin (1d) load controls (LC), are illustrated for each independent time point experiment. #p<0.05, **p<0.01 Sham vs. TBI. n=4-8/group.

Figure 2. IHC of OB glial phenotypes after mFPI

A Sham cases show GFAP+ astrocytes within the GL, interdigitated among synaptic-rich zones. At 3d postinjury, reactive GL astrocytes had thick and hypertrophic cell processes (*arrows*). Over time postinjury, astrocyte hypertrophy and GFAP signal was reduced (7, 21d). **B.** IBA1+ microglia were ramified in sham GL and EPL, with thin cytoplasmic processes. At 3d after injury, GL and EPL microglia were activated with rounded cell bodies and thick, bushy processes (*arrows*). At 7d, microglial response was attenuated, with less amoeboid cells. By 21d postinjury, microglia regained ramified structure similar to sham animals. Scale bar = 50µm. n=3-4/group.

Figure 3. mFPI reduces OMP in OB

A At 3d postinjury, OMP was 60% of controls and significantly lower than both 1d and 21d expression, each of which achieved control values. This OMP reduction temporally matches 3d elevation of 150kD α II-spectrin and GL glial reactivity. Notably, 7d OMP was lower, but not different from controls. Results expressed as percent of sham control R.O.D. cases (100% dashed line) run on the same transferred gel. Blot images, with cyclophilin A (3, 7, 21d) and β -actin (1d) load controls (LC), are illustrated for each independent time point experiment. Blot images with cyclophilin A (1, 3, 21d) and β -actin (7d) load controls (LC) below. # $p < 0.05$, ## $p < 0.01$, * $p < 0.05$ Sham vs. TBI. $n = 5-6$ /group. **B.** IHC of OMP in sham animals shows labeled ORN axons in olfactory nerve layer (ONL) and pre-synaptic terminals within OB glomerular layer (GL; arrow). At 3d postinjury, OMP is reduced in the GL, punctate and diffuse suggesting altered OMP+ ORN projections (arrows). This change is consistent with 3d OMP reduction. Scale bar = 50 μ m. $n = 3$ /group.

Figure 4. Ultrastructure of ORN axon damage in OB after mFPI

A Semi-thin (0.5µm) OB sections illustrate sham ORN axons (*dark processes*) within a glomerulus (*black arrow*) and bundled together entering GL (*white arrow*). At 7d postinjury, ORN axon bundles are compressed and elongated. By 21d, control GL structure begins to reemerge. **B.** Ultrathin sections (600Å) show dark sham ORN axons (*ax*) interdigitated among post-synaptic dendrites (*d*), making multiple synaptic contacts (*arrows*). At 7d after mFPI, ORN axons appear irregular and collapsed (*white arrows*), with evidence of cytoplasmic degeneration (*black arrows*). **C.** Postinjury time course of GL axodendritic structural change. Sham control ORN axons (*black arrow*) display aggregates of pre-synaptic vesicles at synaptic junctions (*Inset sham, black arrows*) and thick postsynaptic densities (*white arrows*). At 3d after mFPI, synapse organization is disrupted (*white arrows*), with early signs of axon degeneration (*black arrow*). Some recovery of ORN axon structure is detected at 7d (*black arrow*), but poorly organized synaptic junctions remain (*white arrows*) and glial phagocytosis of degenerative debris (*Inset 7d, black arrow*) is visible. By 21d, GL synaptic structure mimics sham controls (*black arrows*), although ORN axons remain less electron dense and retain evidence of tissue degeneration (*white arrow*). Bar in A=10 µm; B sham= 4 µm; B 7d=2 µm; C=1µm. n=3/group.

Figure 5. GAP-43 is elevated after mFPI

GAP-43 protein expression in whole OB extracts failed to show change during the acute postinjury degenerative phase (1, 3d) or at 7d after mFPI. By 21d, the predicted time point of active ORN reinnervation and synapse reformation, GAP-43 was elevated by 3-fold over sham controls. This change was significantly different from all prior time points. Results expressed as percent of sham control R.O.D. cases (*100% dashed line*) run on the same transferred gel. Blot images, with cyclophilin A (3, 7, 21d) and β -actin (1d) load controls (LC), are illustrated for each independent time point experiment. # $p < 0.01$, ### $p < 0.001$, *** $p < 0.001$ Sham vs. TBI. $n = 5-6/\text{group}$.