Neonatal infraorbital nerve crush-induced CNS synaptic plasticity and functional recovery

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Running Head: Peripheral nerve-injury and developmental brain plasticity

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Infraorbital nerve (ION) transection in neonatal rats leads to disruption of whisker-specific neural patterns (barrelettes), conversion of functional synapses into silent synapses and reactive gliosis in the brainstem trigeminal principal nucleus (PrV). Here we tested the hypothesis that neonatal peripheral nerve crush injuries permit better functional recovery of associated central nervous system synaptic circuitry compared to nerve transection. We developed an in vitro whisker pad-trigeminal ganglion (TG)-brainstem preparation in neonatal rats and tested functional recovery in the PrV following ION crush. Intracellular recordings revealed that 68% of TG cells innervate the whisker pad. We used the proportion of whisker pad-innervating TG cells as an index of ION function. The ION function was blocked by ~64%, immediately after mechanical crush, then it recovered beginning after 3 days post-injury and was complete by 7 days. We used this reversible nerve-injury model to study peripheral nerve injury-induced CNS synaptic plasticity. In the PrV, the incidence of silent synapses increased to ~3.5 times of control value by 2-3 days post-injury and decreased to control levels by 5-7 days post-injury. Peripheral nerve injury-induced reaction of astrocytes and microglia in the PrV was also reversible. Neonatal ION crush disrupted barrelette formation and functional recovery was not accompanied by de novo barrelette formation, most likely due to occurrence of recovery post critical period (P3) for pattern formation. Our results suggest that nerve crush is more permissive for successful regeneration and reconnection (collectively referred to as “recovery” here) of the sensory inputs between the periphery and the brainstem.
Peripheral nerve injuries are common in neonates and children, but relatively few studies have focused on long-lasting effects on structural and functional development of the central nervous system (CNS) that normally receive information from the injured nerve. Studies focusing on pain sensitization mechanisms in neonates revealed that neonatal tissue injury can have profound effects later in life on pain sensitivity through altered spinal cord pain circuitry and descending pain modulatory pathways (Beggs et al. 2012; Hathway et al. 2009; Hermann et al. 2006; Ren et al. 2004; Walker et al. 2009; Wollgarten et al. 2009; Zhang et al. 2010). We know very little about how sensory nerve insults in developing mammals affect other modalities of sensory information processing in the CNS.

Whisker-related trigeminal central pathway of rodents is an established model for studying activity-dependent synaptic remodeling during development. The spatial distribution pattern of whiskers is replicated all along the central trigeminal pathway by discrete neural patterns. The development and consolidation of whisker-related patterns depend on the sensory periphery during a critical window after birth. For example, transection of the infraorbital branch (ION) of the trigeminal nerve up to first three days after birth leads to disruption or prevention of neural patterning in the brainstem (“barrelettes”), thalamus (“barreloids”) and cerebral cortex (“barrels”) (For review see Erzurumlu 2010; Erzurumlu and Gaspar 2012).

Previously, we showed that transection of ION in neonatal rats results in substantial synaptic reorganization in the trigeminal principal nucleus (PrV), the first
relay station of the trigeminal pathway. Along with disruption of barrelette modules, most of the functional synapses convert to silent synapses by losing functional AMPA receptors (Lo and Erzurumlu 2007). In addition, single barrelette neurons receive substantially more convergent synaptic inputs from TG cells through astrocyte-mediated reactive synaptogenesis (Lo et al. 2011). An important question of clinical significance is whether these structural and functional changes are reversible following regeneration of ION fibers. Here we tested the extent of recovery following crush injury of the neonatal ION.

In the nerve transection paradigm, regeneration is a slow process and it is never complete (Jacquin, 1989; Jacquin et al. 1989; Jeno and László 2002; Kis et al. 1999; Renehan and Munger 1986; Waite 1984; Waite and Cragg 1982). However, nerve crush leads to a partial blockade of nerve function and relatively rapid functional recovery, evidenced by the appearance of postsynaptic responses to peripheral stimuli (Kis et al. 1999; Renehan and Munger 1986; Waite and Cragg 1982). To further investigate reversibility of nerve injury-induced synaptic changes and potential underlying mechanisms we developed an in vitro preparation of a cranial cup with the peripheral and central connections of the TG between the whisker pad and the PrV intact. This preparation allowed us to determine responsiveness and action potential (AP) characteristics of TG neurons at varying intervals after ION crush and also enabled us to correlate these changes with synaptic characteristics of the PrV barrelette neurons.

Primary sensory neurons are classified in numerous ways such as expression of specific ion channels, dependence on NGF family of neurotrophins, expression of calcium binding proteins, somatic size, myelination, conduction velocity etc. Previous
studies showed that neonatal mouse TG neurons can be classified into two groups based on the shape of the AP: S-type neurons which show an inflection in the repolarizing phase of their AP and F-type neurons which do not show this inflection (Cabanes et al. 2002; Yoshida and Matsuda 1979). We identified these two types of TG cells by using intracellular recordings from the whisker-related TG area (Klein et al. 1986). In normal animals, 69% of the impaled TG cells were responsive to ION stimulation, indicating that they innervate the whisker pad. We took this proportion as an index of normal ION function in the TG. We found that mechanical crush of the ION blocked ~64% conduction function in the TG. The function started recovering after 3 days post-injury and completely recovered by 7 days post-injury. Both S- and F- type TG cells were affected by the crush equally. We used this reversible nerve-injury model to investigate recovery of normal synaptic function in the PrV. Our results indicate that nerve crush, initially leads to conversion of functional synapses to silent synapses and glial reaction, much like more permanent nerve injury (transection). However, rapid recovery of the crushed ION reverses the glial and synaptic responses in the PrV, albeit failure in barrelette formation.

MATERIALS AND METHODS

Infraorbital nerve (ION) crush. Sprague-Dawley rat pups of postnatal day 0 (P 0) were anesthetized by hypothermia. The infraorbital nerve (ION) on the right side was exposed through a microincision of the skin and connective tissue between the eye and the whisker pad, where the nerve exits the infraorbital foramen. The ION was crushed with sterile reverse action forceps (Fine Science Tools Inc. 11262-30) for 5 sec. Due to its mechanical properties, reverse action forceps produce a relatively constant force on
the nerve and all the crush procedures were done at P0 (the size of the nerve is similar between pups). Thus, we think that there was no significant variability of the extent of crush from case to case. Sham operation without ION crush was performed on the left side as control. The wound was sutured, and the pup was returned to the home cage after recovery from anesthesia. All surgical procedures followed NIH guidelines and were approved by the UMB IACUC. A total of 45 pups underwent this procedure.

In vitro intact trigeminal pathway cranial cup (Abbr. In vitro TG pathway)

We chose to assess functional recovery of the crushed nerve by recording TG neuron responses following stimulation of the ION distal to the crush site. For this approach, we developed an in vitro TG pathway preparation in postnatal rats (Fig. 1). In this preparation, TG cells innervating the whisker pad are not mechanically damaged and can be kept alive in vitro for more than 8 hours. However, it is important to keep in mind that as in other brain slice preparations, many central axonal tracts are axotomized; in our preparation, most notably the trigeminothalamic projection cells. While there may be axotomy-related short term changes in the PrV neurons, we are recording from the ganglion cells with intact axonal projections to both the whisker pad and the PrV.

Furthermore, the same preparation was used for both the controls and nerve crush experiments, from the same animal which obviates possible confounds related to axotomy of the trigeminothalamic projection cells.

Neonatal rat pups (P0-P9) were anesthetized with isoflurane and decapitated. The head was immersed in ice-cold artificial cerebrospinal fluid (ACSF, in mM: NaCl, 124; KCl, 2.5; NaH2PO4, 1.25; MgSO4, 2; NaHCO3, 26; glucose, 10; CaCl2, 2, pH=7.4). The skull was opened and trigeminal ganglia on both sides were exposed by removing the
forebrain rostral to the superior colliculus. On each side, the ION branch of the maxillary trigeminal nerve was dissected to the whisker pad, thus allowing direct visualization of the ION between the whisker pad and the TG and leaving the central TG root to the brainstem PrV intact. The cranial cup preparation was placed in a recording chamber perfused (3 ml/min) with oxygenized ACSF at room temperature. A pair of stimulating electrodes was placed on the ION.

Intracellular recording from TG cells. We performed intracellular recordings from TG cells in TG pathway preparations from P0 to P9 pups (n= 14) that have undergone P0 unilateral ION crush (and sham operation on the other side) with sharp electrodes filled with 3 M KAc (~70 MΩ). Because TG cells that innervate whisker pad are widespread in the central part of the TG (Klein et al. 1986), we randomly impaled TG cells in the central TG. Passing biphasic current pulses (±0.7 nA) induced action potentials. Whisker pad –innervating TG cells were identified by the action potential with fixed latency evoked from ION stimulation (1 ms, <1 mA).

Brain slice preparation. Rat pups that have undergone unilateral ION crush on P0 were anesthetized with Isoflurane and euthanized by decapitation at various time points between P2 to P7 (n = 26). The brainstem was removed and immersed in cold (4°C) sucrose-based ACSF (in mM: Sucrose, 234; KCl, 2.5; NaH2PO4, 1.25; MgSO4, 10; NaHCO3, 24; glucose, 11; CaCl2, 0.5) bubbled with 95% O2 and 5% CO2 (pH=7.4). The brainstem was then embedded in 2% agar and cut into 400 µm thick transverse sections with a vibratome (Campden 7000smz) in the sucrose-based ACSF at 4°C. Slices
containing the PrV were selected under a dissecting microscope; the sham and ION
crushed side of each slice was marked. After 1 hr incubation in normal ACSF at 34°C, the
slices were stored at room temperature. For recording, each slice was transferred into a
submerged-type recording chamber (27L, Warner Ins.) and continuously perfused (>2
ml/min) with normal ACSF at room temperature. During electrophysiological recording,
50 µM picrotoxin was added into the ACSF to block GABAergic responses.

Whole-cell recording from the PrV. Whole-cell-patch micropipettes were pulled
horizontally in four stages from borosilicate glass with a P-87 puller (Sutter Instrument
Co.). The patch electrodes were backfilled with a cesium-based solution (in mM:
CsMeSO$_3$, 115; NaCl, 10; KCl, 1; MgCl$_2$, 4; CaCl$_2$, 1, EGTA, 10; HEPES, 20; Na$_2$-ATP,
3; Na$_3$-GTP, 0.5; Spermine, 0.1; pH=7.25, >290 mOsm ), with a tip resistance of 6-9
MΩ. Inclusion of spermine in the recording pipette prevented loss of inward rectification
of AMPAR-mediated EPSC during whole-cell recording (Donevan and Rogawski 1995).
Neurons in the ventral part of the PrV (barrelette region) were blindly patched (Lo et al.
1999). After "break-in", the serial resistance was completely compensated with bridge
balance of AxoClamp 2B amplifier. We only collected data from cells with resting
membrane potential negative to -55 mV and input resistance >200 MΩ with an
InstruTECH ITC-16 interface unit and stored on a Dell DM061 computer with PULSE
(HEKA) software program. A pair of fine-tip stimulating electrodes (0.5 MΩ, WPI,
IRM33A05KT, tip diameter: 2-3 µm, separated by 300 µm) was inserted into the
trigeminal tract (TrV) lateral to the ventral PrV (barrelette region). Electrical pulses (0.2-
0.3 ms duration) were passed through the electrodes to evoke excitatory postsynaptic currents (EPSCs).

Test for silent synapses. We first voltage-clamped neurons at +60 mV. We stimulated the TrV by 60 pulses at 0.2 Hz. Stimulus intensity was adjusted to around threshold, so that some trials failed to evoke EPSCs. Then we clamped the neurons at -70 mV to investigate changes in failure rate between the two membrane potentials. We measured the amplitude of EPSCs at +60 mV 30 ms after stimulus where NMDA receptor-mediated EPSCs reach their peak. The amplitude of EPSCs at -70 mV was measured at 8 ms after stimulus (the peak of AMPA receptor-mediated EPSCs, Lo and Zhao 2011). The peak amplitude less than two times of noise standard deviation (S.D.) was determined as the failure of synaptic transmission (Lo and Erzurumlu 2007).

Morphological studies. P0 ION crushed rat pups were anesthetized with Isoflurane at P3, P10 (n=19) and perfused with saline followed by phosphate-buffered 4% paraformaldehyde. The brains were fixed overnight and cytoprotected in 30% sucrose. Coronal sections of 20–30 μm thickness were cut in a cryostat and collected in serial order. Alternate sections containing the PrV were processed for cytochrome oxidase (CO) histochemistry and glial fibrillary acidic protein (GFAP, astrocyte marker) and Iba-1 (microglia marker) and VGluT1 (trigeminal afferent terminal marker) immunohistochemistry.

For CO histochemistry, the sections were incubated with PBS containing 0.5 mg/ml cytochrome C, 0.5 mg/ml diaminobenzidine (DAB; both from Sigma), and 50 mg/ml
sucrose for 6–7 h at 37°C in a shaker incubator. For GFAP and Iba-1 immunochemistry, the sections were incubated in 0.6% H₂O₂ in PBS to block endogenous peroxidase activity. After rinses in PBS, the sections were incubated at 4°C in rabbit anti-GFAP (DAKO 1:20K), and Anti Iba-1, Rabbit (Wako 1:2000) for 48 h, followed by incubation in biotinylated donkey anti-rabbit antibody (Jackson ImmunoResearch). The detection of GFAP and Iba-1 immunoreactivity was performed using the ABC kit (Vector Labs) and visualized with DAB. Iba-1 immunostained sections were counterstained with cresyl violet.

For VGluT1 immunofluorescence, brainstem sections from P3 and P10 rats were incubated in polyclonal anti-vesicular Glutamate Transporter 1 (Millipore 1:3K ) in 0.01 M PBS containing 1% normal Donkey serum, 2% BSA, and 0.3% Triton X-100 overnight at 4°C. Next, the sections were rinsed with PBS and then incubated with FITC-conjugated donkey anti-guinea pig (H+L) (Jakson Immunoresearch) at room temperature for 2 h. The sections were rinsed with PBS, mounted onto glass slides and coverslipped with fluorescence mounting medium.

Data analyses. 1. Physiology. The duration of spike and AHP were measured at their half amplitude. All data are expressed as mean±SEM, A Student’s t test and one-way ANOVA test were used. Linear regression test was used to show developmental changes in the latency.

2. Morphology. Glial reaction to nerve injury in the PrV is evident as a conspicuous increase in both astrocyte number and process complexity that can be visualized with GFAP immunocytochemistry. Similar changes in the numbers and morphologies of
microglia were observed with Iba1 immunohistochemistry. We used a stereological approach and counted the number of astrocytes and microglia within a pre-defined square area (124238 \( \mu \text{m}^2 \) or 0.1mm\(^2\)) placed over the ventral PrV. This region of interest (ROI) occupied a large sector of the ventral PrV, where barrelettes are located. Both the astrocyte and microglial cell bodies are small and cell counts from alternate sections from 4 different animals at each age were made. In addition ~20 astrocytes and microglia from representative sections of 4 pups at P3 and P10 were drawn under 60X magnification in two-dimensions using a drawing tube attached to a Nikon light microscope. Astrocyte spans in all these cases were around or less than 30 \( \mu \text{m} \) and cell bodies measured around 5-7 \( \mu \text{m} \), thus the cellular processes can be traced in two dimensions and traced cells were selected from mid-depth of 40 \( \mu \text{m} \) thick sections.

Microscopic observations of the GFAP-immunostained sections gave us the impression that in addition to increased numbers astrocytes displayed more complex process branching patterns in P3 cases following neonatal ION lesion. We performed Sholl analysis (linear method, Sholl, 1953) by placing 10 \( \mu \text{m} \) spaced concentric rings centered on the cell body and counted the number of intersections. We also counted the number of branch points in each drawing. Such measures are routinely used for quantitative analyses of dendritic morphology, here we used them as measures of astrocyte process complexity.

We also analyzed the intensity of GFAP expression with a Nikon Microphot microscope (Nikon Americas) under bright-field \( \times10 \) magnification. Microscope images were captured with a Nikon Digital Sight camera mounted to the microscope, and labeling intensities were obtained using NIS-Elements software (Nikon Americas). Two
circular regions of interest (ROI) of 106068 μm² were placed on the dorsal and ventral
(barrelette region) PrV separately. Mean pixel intensities were measured inside each ROI.
The intensity of the dorsal ROI was taken as a control. The difference between the dorsal
and ventral ROI represented GFAP response to nerve injury in the ventral PrV where the
ION central axons terminate.

We did not perform Sholl analysis or count process branch points of microglia
because their processes were veil-like with few “spike” protrusions. Instead we used NIS
Elements software and measured the area of individual Iba-1-labeled microglia in
representative sections from different pups at P3 and P10.

RESULTS

Classification of TG cells. We found that biphasic (hyperpolarizing-depolarizing)
electrical pulse (±0.7 nA) is more effective than monophasic depolarizing pulse for
inducing action potential (AP) in TG cells. Unlike monophasic depolarizing pulses, with
biphasic pulses the membrane is hyperpolarized first and then depolarized, so that the
amplitude of the membrane potential change is two-folds larger than monophasic
depolarization. In addition, some ion channels (for example T-type Ca²⁺ channels) are
inactivated at resting membrane potential. Preceding hyperpolarization releases the
inactivation (also called de-inactivation), and depolarization activates them.

Similar to the mouse TG (Cabanes et al. 2002), the neonatal rat TG cells can be
classified as F-type and S-type according to the shape and duration of the AP and after
hyperpolarization (AHP). F-type cells showed a narrow AP without a "hump" in the AP
repolarization phase and a narrow AHP (Fig. 2 A) while S-type cells showed a wide AP
and AHP with a marked inflection (hump) in the AP repolarization phase (Fig. 2 B). The
half-amplitude duration of AP of F-type cells was 1.23±0.13 ms (n=10) while that of S-
type cells was 3.04±0.35 ms (n=33) that was significantly different (p<0.0001) from F-
type cells (Fig. 2 C, Action Potential, black vs. white bar). The half amplitude of AHP of
F-type cells was 3.72±1.50 ms (n=10), while that of S-type cells was 9.23±0.60 ms
(n=33) that was significantly (p<0.0001) longer than F-type cells (Fig. 2 C, After
hyperpolarization, black vs. white bar). Next, we calculated the ratio of the two cell
types, we found that neonatal rat TG contained 23.3% F-type cells and 76.7% S-type
cells (n=43).

Identification of whisker pad-innervating TG cells. Because TG cells that innervate
whisker pad are widespread in the central part of the TG (Klein et al. 1986), we randomly
impaled TG cells in the central TG. Then, we identified the whisker pad-innervating TG
cells by testing the response to stimulation of the ION. As shown in Fig. 3 A and B,
stimulation of the ION induced an AP. Note that each record is made of 5 superimposed
traces. In both F-type and S-type cells, the latency of the AP was fixed. This result
indicates that the AP originated from peripheral branch of TG axon (ION) rather than
from current spread to the cell body in the TG.

We plotted the latencies of both cell types against postnatal days up to P9 (Fig. 3 C).
There was no developmental change in the latencies (linear regression R=0.40, p>0.43)
of ION fibers between P2 to P9. We pooled latencies from all time points to get averaged
latency for the two types of cells. The averaged latency for F-type cells was 1.59±0.25
ms, while that of S-type cells was 1.65±0.22 (Fig. 3 D), which is about the same as F-type cells (p=0.086). Therefore, the conduction velocity of peripheral branch of both F-type and S-type TG cells is about the same. This conclusion was further supported by the fact that the latencies from both types of cells were overlapped at each time point (Fig. 3 C). In the central TG, 29 out of 43 (69 %) recorded cells were identified as whisker pad-innervating cells.

Mechanical crush affects both F- and S-type TG cells equally. A couple of days after ION crush, the ION and the maxillary branch of the trigeminal nerve appear thinner (Fig. 1). We wanted to determine whether ION crush selectively affected one of the two classes of TG cells. In the normal TG, 76.7% of 43 recorded cells were S-type cells while the rest 23.3% were F-type cells. Immediately after ION crush, 80.0% of the recorded cells were S-type cells (n=20), while post-injury 3 days, 76.2% (n=21), 5 days, 73.1% (n=26) and 7 days, 75.0% (n=20) recorded cells were S-type cells. There was no significant difference (ANOVA test p>0.98) among them (Fig. 4 A). Thus ION crush affects both cell types.

ION crush partially and temporarily blocks conduction function. As mentioned above, in the normal TG, 69 % of 43 recorded cells were identified as whisker pad-innervating cells. Immediately after ION crush, stimulation of ION distal to the crush site induced APs in only 25% recorded cells (n=20) that was significantly lower than the normal TG (p<0.005), suggesting that ~64% ION fibers were blocked. The proportion of responded TG cells increased to 38% 3 days after the crush (n=21) that was not
significantly difference from immediately after ION crush (p>0.30). At post-injury day 5, 54% recorded TG cells (n=25) innervated whisker pad, which was significantly higher than that of post-injury day 0 (p<0.03), suggesting that the conduction function of ION recovered to ~78%. At post-injury day 7, 80% of recorded cells (n=20) responded to the stimulation of the distal ION, which did not differ from the normal TG (p>0.40). We interpret this as complete (100%) functional recovery of ION (Fig. 4 B). In the next series of experiments we investigated the effects of nerve crush and recovery on associated synaptic plasticity in the principal sensory nucleus of the trigeminal nerve (PrV) in the brainstem.

Nerve injury-induced silent synapses are reversible after ION functional recovery. In neonatal rats, most of the synapses in the PrV are functional and show NMDA- and AMPA-mediated EPSCs at holding potentials of +60 mV and -70 mV (Fig. 5 A). We previously reported that neonatal ION transection (permanent nerve damage) leads to conversion of functional synapses into silent synapses without AMPA receptor-mediated EPSCs, indicated by high failure rate at -70 mV (Fig. 5 B, Lo and Erzurumlu 2007). Here we tested for the incidence of silent synapses following nerve crush. In normal PrV, only 20% cells (n=10) reveal silent synapses with higher failure rate at -70 mV than that at +60 mV (Fig. 5 C, donated by stars). The incidence of silent synapses increased to 69% (n=13, Fig. 5 D) 2-3 days after ION crush, that was ~3.5 times of the control (p<0.02, Fig. 5 F). At post-injury day 5-7, the incidence of silent synapses dropped to 27% (n=15, Fig. 5 E and F) that was significantly different from post-injury day 2-3 (p<0.03) but about the same as control (p>0.71). Thus, the changes in the incidence of
silent synapses depend on the conduction function of ION. The activity-dependent AMPA receptor trafficking is reversibly regulated within the first postnatal week.

Disruption of barrelettes after ION crush is not reversible after ION functional recovery. The central axons of TG cells that contribute to the ION in the periphery develop discrete clusters of terminals and together with small diameter thalamocortical projection cells of the PrV form a neural pattern ("barrelettes") that replicates the punctate distribution of the whiskers on the snout (Bates et al. 1982; Erzurumlu et al. 1980; Ma and Woolsey 1984). The PrV is a peanut shaped nucleus laterally capped by the trigeminal tract. The barrelette region, or the ION central projection zone of the nucleus is confined to the ventral half of the nucleus whereas the dorsal portion receives inputs from the mandibular division of the trigeminal nerve. To delineate the effects of ION crush and functional recovery on barrelette patterns we used immunocytochemistry for vesicular glutamate transporter 1 (VGlut1) to label sensory afferent terminal patterns and cytochrome oxidase (CO) histochemistry, a generic barrelette and barrel marker. On the sham side, whisker-related sensory axon terminal patches and CO-positive barrelettes could be seen at P3 and more distinctively at P10. (Fig. 6 A, C, E, G; barrelettes corresponding to whisker rows a-e are labeled in G). After ION crush, TG terminals failed to form whisker-specific patches and CO-positive barrelettes were absent both at P3 and P10 (Fig. 6 B, D, F, H). These observations confirm previous reports that damage to the ION during a sensitive period up to P3, irreversibly abolishes barrelette patterns (Belford and Killackey 1980; Durham and Woolsey 1984) and even functional recovery of the crushed ION does not rescue whisker-related neural patterning in the PrV.
Previously, we showed that neonatal ION transection leads to significant gliosis in the PrV, and ensuing synaptic plasticity is mediated by astrocytes (Lo et al. 2011). Neonatal ION crush also resulted in upregulation of GFAP (an astrocyte marker) expression in the barrelette region of the PrV at P3 (Fig. 7 A-H). A dramatic increase in astrocyte numbers, length and thickness of their processes were evident in the ION-recipient zone of the PrV under light microscopic observation. After ION functional recovery at P10, the expression of GFAP returned to normal levels (Fig. 7 E-H). We used stereological quantification to compare the numbers of GFAP-positive astrocytes, Sholl analysis for astrocytic process complexity and quantitative analysis of the intensity of GFAP labeling (Fig. 7 M-P). In sham-operated side astrocyte processes showed increased complexity from P3 to P10 (Fig. 7 I, K) and this was confirmed with quantitative analyses (Fig. 7 M, N). At P3, nerve crushed side showed highly arborized astrocyte processes (Fig. 7 J) and quantitative analyses showed significant increase in astrocyte process complexity (Fig. 7 M, N). However, this difference was no longer evident at P10 when functional recovery has taken place (Fig. 7 M, N). At P3, the PrV on the nerve crushed side had a dramatically increased GFAP-positive astrocyte numbers and immunostaining intensity compared to controls and also in comparison to P10 (Fig. 7 O, P).

Figure 8 illustrates our findings with IBA-1 immunostaining of microglia. As with astrocytes, we saw a pronounced increase in the numbers of microglia in the PrV on the nerve crushed side compared to sham-operated side (Fig. 8 A-D). This was notable at P3 but no longer evident by P10 (Fig. 8 E-H). There is also a marked change in the
morphism of microglia between P3 and P10. Early on, microglia have rotund cell bodies and few lamellar processes (Fig. 8 I). At P10 characteristic sentient appearance of microglia is evident with ramified processes (Fig. 8 L). On the nerve-crushed side, microglia in the ventral PrV had larger cell bodies in addition to increased numbers (Fig. 8 J, M, N) compared to sham-operated side at P3. No difference was observed in these parameters between the two sides on P10 (Fig. 8 K, L, M, N).

**DISCUSSION**

How does the CNS respond to peripheral nerve injuries during the normal developmental period of synaptic wiring of somatosensory pathways? In the mature CNS, deafferentation results in a rapid synaptic loss followed by a prolonged period of new synapse formation. This form of neural plasticity has been referred to as "reactive synaptogenesis," implying that it is a reaction to deafferentation (Cotman et al. 1981; Hamori 1990; Matthews et al. 1976). In the mature state, the time course of reactive synaptogenesis varies among different central pathways. For example, in the entorhinal cortex-dentate gyrus pathway, reactive synaptogenesis starts on post-lesion day 6 and persists for more than a month (Cotman et al. 1981; Marrone et al. 2004). In the dorsal column lemniscal pathway, reactive synaptogenesis begins in the thalamus about a month after lesion and continues for several weeks (Wells and Tripp 1987a, b).

Not much is known about reactive synaptogenesis during development of the somatosensory pathways, particularly following peripheral nerve injuries. A number of studies have noted that experimentally-induced inflammation of the skin and abnormal
activation of “pain” fibers in the dorsal root ganglia in newborn animals cause long-lasting, and often irreversible, expansion of dorsal root afferent terminal zones in the spinal cord with significant behavioral and physiological consequences in the adult (Peng et al. 2003; Ruda et al. 2000; Walker et al. 2003). Obstetric injuries to the brachial plexus during birth and orofacial injuries and fractures in young children are common occurrences, which can lead to permanent brachial plexus palsy or trigeminal nerve pathologies (see reviews by Alcalá-Galiano et al. 2008; Eggensperger Wymann et al. 2008; Sandmire et al. 2008; Vyas et al. 2008). While these neurological cases are extensively studied at the peripheral nerve level, CNS consequences are unknown.

Regeneration of the transected ION fibers is a slow process and it is never complete in neonates. Central effects of neonatal ION transection have been reported and include cell death, expansion of representation for non infraorbital vibrissae, sprouting of axon terminals in laminae I and II in the medullary trigeminal dorsal horn (Golden et al. 1997; Jacquin et al. 1993). Previously, we found that ION transection in newborn rat pups induces rapid reactive synaptogenesis within the PrV as early as 2 days after injury, then reaches a peak by P5, and remains at that level for two weeks (Lo et al. 2011). Furthermore, ION lesions during the critical period for barrelette formation initially produce “silent” synapses. High frequency stimulation, which mimics sensory afferent activity, rapidly switches silent synapses into functional synapses (Lo and Erzurumlu 2007) via activity-dependent AMPAR exocytosis. Our experiments were designed to test the hypothesis that neonatal peripheral nerve crush injuries permit better functional recovery of associated central synaptic circuitry compared to nerve transection. We found that nerve crush is more permissive for successful regeneration and reconnection.
(recovery) of the synaptic communication between the periphery and the brainstem. We sought to determine whether regenerative peripheral nerve injury (nerve crush) in neonates would arrest reactive synaptogenesis, permit de novo synaptic refinement and resume synaptic function. Unfortunately, in the present study we could not use the MII assay because the time course of recovery after mechanical crush injury is short and overlaps with the normal course of synaptogenesis. Our results show that impulse conduction along the ION is blocked by about 64% immediately after mechanical crush but it began recovering 3 days post-injury and was complete by 7 days. Similar to ION transection effects in the PrV, initial response to ION crush was conversion of functional synapses to silent synapses (~3.5 times of control value by 2-3 days post-injury) but these silent synapses did not persist, they became functional synapses paralleling ION conduction recovery. Thus, activity-dependent AMPAR trafficking in response to peripheral nerve injury in neonates is reversible depending on the recovery of function along the injured nerve.

Earlier studies on neonatal ION crush in rats recorded whisker-evoked responses in the cortex 2-3 days post injury (Waite and Cragg 1982); in adult rats evoked responses could be evoked in the PrV only 7-9 days post injury (Kis et al. 1999). Our in vitro preparation allows for quantitative measurement of ION functional recovery at the level of the TG. In this preparation we can perform the nerve crush and immediately assess the effects in the TG or several hours thereafter or use this preparation from rat pups that have undergone ION crush on P0 at different postnatal days. In assessing the CNS effects of peripheral nerve crush, we took the incidence of silent synapses as an index of synaptic plasticity in the PrV as a function of changes in ION conduction. This index provides a
quantitative way to show synaptic plasticity after ION crush, especially before ION functional recovery. We found recovery of conduction function along the crushed ION and regain of functional synapses (from silent synapses) in the PrV following nerve recovery. However, CNS effects of neonatal peripheral nerve crush injuries are not fully compensated after nerve recovery. For example, barrelette patterning in the PrV is not restored even though ION function returns and functional synapses between the trigeminal afferents and barrelette neurons are established. Earlier studies on the effects of neonatal ION crush in rat pups also found that somatotopic representation is modified both in the brainstem and cortical levels and ION input recipient cells show larger, more complex receptive fields, altered latencies and following frequencies (Waite 1984). Most likely, upon recovery from crush injury central axons of ION contributing TG cells converge onto multiple barrelette neurons leading to poor spatial resolution of whisker inputs along the central pathways leading to the neocortex.

Our morphological results provide hints on the mechanisms underlying CNS synaptic plasticity after peripheral nerve damage. Both astrocytes and microglia reacted to ION crush during blockade of ION conduction and returned to quiescent status (at the morphological level) after recovery of ION function suggesting that astrocytes and microglia are involved. Astrocytes have long been considered as the main player in the inhibition of CNS repair via formation of glial scar, but now it is accepted that astrocytes play an important role in CNS repair via promoting maturation of oligodendrocyte progenitors and removing damaged myelin debris (Barnett and Linington 2012; Baumgartner and Stangel 2013; Moore et al. 2011; Skripuletz et al. 2013). Previously, we demonstrated that neonatal ION transection induces reactive astrocytes that promote
synaptogenesis in the PrV. Pharmacological blockade of astrocyte function and related synaptogenesis events prevents the formation of new synapses (Lo et al. 2011). Similarly, Microglial activation has been considered harmful for neurons, but also has neuroprotective effects, such as phagocytosis of dead neurons and clearance of debris. Recent data support the protective function of microglia through the release of neuroprotective molecules (Aldskogius 2011; Doring and Yong 2011; Polazzi and Monti 2010; Walter and Neumann 2009). While our present morphological results are correlative, they suggest a role for astrocytes and microglia in CNS response to peripheral nerve injury in neonates.

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DISCLOSURES

No conflicts, financial or otherwise, are declared by the authors.

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AUTHOR CONTRIBUTIONS

F.-S. L, designed and performed electrophysiological experiments, drafted the manuscript. S.Z carried out immuno- and histochemistry and quantitative microscopy. R.S. E performed cellular analyses, reviewed, evaluated the results and wrote the final manuscript.
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Do burn injuries during infancy affect pain and sensory sensitivity in later childhood?


FIGURE LEGENDS

Fig. 1. Photograph of an in vitro trigeminal pathway preparation on the third day after right infraorbital nerve crush. WP: Whisker pad, ION: infraorbital Nerve, TG: trigeminal ganglion and SC: Superior colliculus. Note that the right TG and ION are thinner than the left control.

Fig. 2. Classification of TG cells in rat pups. A and B: Biphasic electrical pulse (upper traces) induces an action potential (AP) with different shape. Note that F-type (A) cell shows a narrow AP and after hyperpolarization (AHP) and S-type cell, wide AP and AHP. C: Comparison of half-duration of AP and AHP between F- and S-type TG cells showing significant difference between the two classes.

Fig. 3. Identification of whisker pad-innervating TG cells. A and B: Stimulation of ION induces an AP with fixed latency from both F- and S-type TG cells. Note that each record is formed from 5 superimposed traces. C: Plot latencies from both types of TG cells against postnatal days showing that there are no developmental changes in latency. D: Averaged latency of F- and S-type TG cells showing no significant difference between them.

Fig. 4. Effects of ION crush. A: The percentage of recorded S-type TG cells from normal TG (control) and those at different ages after ION crush. There is no significant difference between them indicating that mechanical crush affect both types of TG cells. B: The proportion of recorded whisker pad-innervating cells shows the time course of ION
functional recovery. Immediately after the crush, about 2/3 ION fibers are blocked and functional recovery starts after post-injury 3 days, finishes at post-injury day 7.

**Fig. 5.** Deafferentation-induced silent synapses are reversible after ION functional recovery. *A:* Example record of functional synapses from a PrV neuron. *B:* Example record of silent synapses from a PrV neuron. *C:* Plot of failure rate at holding potential of +60 mV and -70 mV for normal PrV showing silent synapses from two cells (denoted by stars) with high failure rate at -70 mV (lacking AMPA receptor-mediated EPSCs). *D:* At post-injury day 2-3, more PrV cells reveal silent synapses. *E:* At post-injury day 5-7, just a few PrV cells show silent synapses. *F:* The incidence of recorded silent synapses demonstrates that deafferentation-induced silent synapses are reversible after ION functional recovery.

**Fig. 6.** ION crush-induced disruption of barrelettes in the PrV is not reversible. The PrV is a peanut shaped nucleus and the barrelettes are confined to the ventral half of the nucleus. In the brainstem, most sensory afferent terminals are positive for VGluT1. VGluT1 labeled TG fiber terminals form whisker-related patches (barrelettes) in the sham side PrV at both postnatal day 3 (*A*) and 10 (*C*). Three (*B*) and ten (*D*) days after ION crush, the patchy terminal distribution is disrupted. CO staining reveals barrelette patterns in P3 (*E*) and P10 (*G*) sham-operated side better. The barrelettes are absent in nerve crushed side at P3 (*F*) and P10 (*H*). DCN: dorsal cochlear nucleus, TrV: trigeminal tract, MV: motor trigeminal nucleus. Asterisks outline the ventral, barrelette region of the PrV. In G whisker barrelette rows a-e are marked. Scale bar = 250 μm.
Fig. 7. Astrocytosis in response to ION crush. A-D show low and high power micrographs of GFAP labeling in the sham side PrV (A, C) and ION crushed side PrV (B, D) on P3. E-H: Similar series of micrographs showing GFAP labeling at P10. Note that increased glial response is diminished on the ION crush side (F, H) compared to that seen at P3 (B, D). I-L show representative camera lucida drawings of individual astrocytes from control (I, K) and nerve crushed (J, L) PrV at P3 (I, J) and P10 (K, L). TrV: trigeminal tract. Asterisks outline the barrelette region of the PrV. Scale bar in F = 250 μm for all low power micrographs and in G = 20 μm for all high power micrographs; in L = 10 μm for all camera lucida drawings. Sholl analysis of number of intersections is shown in M and number of astrocyte process branch points in N. Bar graphs in O and P show comparisons of astrocyte numbers and GFAP staining intensity, respectively.

Fig. 8. Microglial response to ION crush. A-D show low and high power micrographs of Iba-1 immunolabeling in the sham-operated side PrV (A, C) and ION crushed side PrV (B, D) on P3. E-H: Similar series of micrographs showing Iba-1 immunolabeling at P10. Note that increased glial response is diminished on the ION crush side (F, H) compared to that seen at P3 (B, D). I-L show representative camera lucida drawings of individual astrocytes from control (I, K) and nerve-crushed (J, L) PrV at P3 (I, J) and P10 (K, L). TrV: trigeminal tract. Asterisks outline the barrelette region of the PrV. Scale bar in B = 250 μm for all low power micrographs and in G = 20 μm for all high power micrographs; in L = 10 μm for all camera lucida drawings. Bar graphs in M and N show comparisons of microglia numbers and sampled microglia size (including the processes), respectively.