Functional rehabilitation of cadmium-induced neurotoxicity despite persistent peripheral pathophysiology in the olfactory system


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Abstract

Intranasal exposure to the heavy metal cadmium has been linked to olfactory dysfunction and neurotoxicity. Here we combine optical imaging of in vivo neurophysiology, genetically-defined anatomical tract tracing, mass spectrometry, and behavioral psychophysical methods to evaluate the persistent harmful effects of acute intranasal exposure to cadmium in a mouse model and to investigate the functional consequences of sensory rehabilitation training. We find that an acute intranasal instillation of cadmium chloride leads to an accumulation of cadmium in the brain's olfactory bulb that persists for at least four weeks. This is accompanied by persistent severe pathophysiology of the olfactory nerve, a gradual reduction in axonal projections from the olfactory epithelium, and complete impairment on an olfactory detection task. Remarkably, two weeks of odorant-guided operant conditioning training proved sufficient to restore olfactory detection performance to control levels in cadmium-exposed mice. Optical imaging from rehabilitated mice showed that this training did not cause any detectable restoration of olfactory nerve function, suggesting that the recovery of function was mediated by central neuroplasticity in which the brain learned to interpret the degraded sensory input. These data demonstrate that sensory learning can mask even severe damage from neurotoxicants and suggest that explicit sensory training may be useful in rehabilitation of olfactory dysfunction.

Keywords:

rehabilitation, sensory learning, heavy metal, olfactory, neuroplasticity, neurotoxicity
Introduction
The mammalian nervous system is capable of remarkable recovery after injury. The peripheral nervous system exhibits axonal regeneration, proliferation of glia, and remyelination (Liu et al. 2011; Chen et al., 2007). While the brain and spinal cord have limited capacity for physical repair, they can reorganize to partially compensate for damage (Chen et al., 2002). For example, after a peripheral motor nerve lesion the corresponding parts of the motor cortex are repurposed to represent other, uninjured body parts (Bruehlmeier et al., 1998; Huntley, 1997; Sanes et al., 1990). These changes are typically compensatory, for instance, after traumatic injury the spinal cord's central pattern generation circuitry can adapt to maintain proper sensorimotor integration by using load and speed information derived from local proprioceptive inputs in the absence of descending sensory input from the brain (Edgerton et al. 2004; Harkema et al. 1997). This capacity for central plasticity is a potential confound for behavioral assays of neurotoxicity (Hastings, 1990; Moser, 2011; Rohlman, 2008).

The olfactory system is especially vulnerable to neural damage because its primary sensory neurons are in direct contact with the external environment. Perhaps in consequence, olfactory receptor neurons (ORNs) in the nasal epithelium are replaced constantly from a local population of adult stem cells that differentiate into neurons and project their axons through the olfactory nerve to the brain's olfactory bulb (Graziadei and DeHan, 1973; Graziadei and Monti Graziadei, 1979; Schwob et al., 1995). This poses a unique challenge for the brain because it must interpret sensory information from an unstable population of neural inputs. Olfactory structures downstream of the ORNs may thus be capable of adaptive plasticity that permits the interpretation of degraded sensory inputs that survive even severe neurotoxicant exposures.
Cadmium is a heavy metal that is frequently encountered in aerosolized form in cigarette smoke and in some industrial workplaces, including battery manufacture and smelting (ATSDR 2008). ORNs may be particularly vulnerable to cadmium accumulation because they express high levels of metallothioneins and other metal-binding proteins (Himeno et al., 2009; Sunderman, 2001). Exposure to aerosolized cadmium compounds has been associated with olfactory dysfunction in humans, notably the elevation of olfactory thresholds and increased incidence of parosmia (Adams and Crabtree, 1961; Mascagni et al., 2003; Rose et al., 1992; Sułkowski et al., 2000). We have recently reported (Czarnecki et al., 2011) that acute intranasal instillations of cadmium chloride disrupt odorant-evoked neurotransmitter release from the olfactory nerve in a mouse model, even at modest doses (0.2 - 2 µg). Higher doses (20 µg - 400 µg) also induce histopathology in the olfactory epithelium in the nose and in the ORN projections to the olfactory bulb (Bondier et al., 2008; Czarnecki et al., 2011).

Behavioral studies of olfactory sensory function after intranasal cadmium exposure in rodent models have produced three different results: immediate anosmia (the inability to detect odorants; Czarnecki et al., 2011), temporary anosmia (Bondier et al., 2008), and no impairment (Sun et al., 1996). In the study that reported no impairment the subjects were continuously trained on the olfactory discrimination task throughout the period of cadmium exposure (Sun et al., 1996). Though the authors interpreted their results as showing no harmful effect of cadmium, the design leaves open the possibility that cadmium exposure disrupted the physiology of the ORNs but that the ongoing training allowed the animals to learn how to compensate. Similarly, the study by Bondier et al. (2008) showed a complete recovery of a spontaneous
odorant-avoidance behavior despite an only partial recovery of the olfactory epithelium, which might also be attributable to central plasticity.

In a previous report (Czarnecki et al., 2011), we used acute intranasal cadmium exposure to illustrate the utility of *in vivo* optical imaging for assessing neurotoxicity in the mouse olfactory system. Here we extend those findings to investigate the time course of cadmium-induced pathophysiology over four weeks and the consequences of explicit post-exposure rehabilitation training. First, we use inductively coupled plasma mass spectrometry (ICPMS) to investigate transport of cadmium chloride to the olfactory bulb and its residence time therein in C57BL/6 mice. To assess the effects of cadmium exposure on ORN synaptic physiology, odorant-evoked neurotransmitter release from the olfactory nerve into olfactory bulb glomeruli was visualized *in vivo* at various post-exposure and/or rehabilitation time points in a line of C57BL/6-background transgenic mice that expresses the fluorescent exocytosis indicator synaptopHluorin in the synaptic terminals of essentially all ORNs (Bozza et al., 2004; Czarnecki et al. 2011; McGann et al., 2005). After imaging, the olfactory bulbs of these mice were sectioned and ORN projection density was quantified to show effects of cadmium exposure on olfactory nerve structure. Olfactory sensory function was assessed in parallel behavioral assays in C57BL/6 and OMP-spH mice that were trained to nose poke for a sucrose reward if and only if they detect an odorant at a separate sampling port. Operant conditioning continued following cadmium exposure to reveal the sensory and (in OMP-spH mice) physiological consequences of rehabilitation training.

**Materials and Methods**

*Subjects*
Subjects in all imaging and histological experiments were a total of 27 homozygous male and female OMP-spH mice, which express the spH construct (Miesenböck et al., 1998) from the locus for Olfactory Marker Protein (Bozza et al., 2004). The mice used in this study are on an albino C57BL/6 background as previously reported (Czarnecki et al., 2011). Subjects in behavioral and mass spectrometry experiments were 23 and 15 adult male and female C57BL/6 mice. All animals were group housed with a 12:12 h light:dark cycle with food and water available *ad libitum*. All procedures were performed in accordance with protocols approved by the Rutgers University Animal Care and Use Committee.

**Intranasal infusions**

Mice in Cd-exposed groups received intranasal instillations (see Czarnecki et al., 2011) of 6 µL pH 7.4 buffer solution containing 200 mM HEPES, 0.9% NaCl, and 18.18 mM CdCl₂, yielding a 20 µg CdCl₂ physical dose per naris. Mice in all behavioral experiments randomly received a bilateral infusion of 20 µg CdCl₂ or vehicle solution (without CdCl₂) while animals in the time course imaging experiments received unilateral instillations with side counterbalanced between subjects. Reflux of solution was not observed. The experimenter was blind to the contents of all infusates.

**Inductively-coupled plasma mass spectrometry**

Cadmium has been previously shown to transport down the olfactory nerve to the ipsilateral olfactory bulb following intranasal instillations (Bondier et al., 2008; Tjälve et al., 1996), but it was unknown how long Cd levels remain elevated following an acute exposure. Fifteen male C57BL/6 mice, five animals per group, were given unilateral intranasal instillations of 20 µg CdCl₂. Two, 10, or 28 days after exposure, animals were lightly anesthetized with
isoflurane and decapitated. The olfactory bulbs were dissected out, weighed and stored in an Isotemp -20 °C freezer until tissue samples were processed. The tissue was analyzed for Cd using inductively coupled plasma mass spectrometry (ICPMS; Thermo Elemental X-5) after microwave (CEM Mars X) digestion. Approximately 0.01 to 0.03 g of olfactory bulb tissue was weighed into 15 ml centrifuge tubes and digested with concentrated nitric acid (0.25 ml). After approximately 15 min, the samples were sonicated for at least 1 hr before introduction into the microwave. Samples were digested five times using 300W power, 75% duty cycle, 5 min. Samples were cooled before their final dilution to 7.625 ml using 1.6% v/v HNO3. This gave a final acid concentration of 5%. All the standard solutions were prepared in 5% HNO3. The m/z =111 Cd isotope was used to quantify cadmium. All of the necessary QA/QC protocols were maintained throughout the extraction and analysis process. Roughly 20% of the samples were QC samples including blanks (laboratory and solvent) spikes and SRMs. Recoveries of >90% of the laboratory spike were deemed acceptable.

In vivo imaging of neurotransmitter release

Three time points were chosen in reference to the approximate four week life cycle of cells in the olfactory epithelium (Graziadei and Monti Graziadei, 1979). Two, seven or twenty-eight days after intranasal cadmium exposure, four mice per group were anesthetized with 0.01 ml/g of 10 mg/ml pentobarbital (i.p.), with 0.05 ml boosters as needed to maintain anesthetic plane. Body temperature was maintained using a rectal temperature probe and feedback-regulated heating pad. Mice were administered 0.01 – 0.02 ml/g 0.1% atropine (s.c.) to reduce nasal secretions and ~0.25ml of 0.25% bupivacaine (s.c.) as a local anesthetic along the scalp. The scalp was shaved, then surgically opened with a midline incision. The periosteaial membrane
was removed and the skull dried with a 70% ethanol solution. A headbar was fixed to the skull using dental acrylic to rigidly mount the mouse’s skull to a custom headholder. Using a micromotor dental handpiece, the skull overlying both olfactory bulbs was thinned until transparent when wet. Ringer’s solution containing 140 mM NaCl, 5 mM KCl, 1 mM CaCl$_2$, 1 mM MgCl$_2$, 10 mM HEPES and 10 mM dextrose was applied over this cranial window, then topped with a glass coverslip.

Optical imaging was performed using a custom apparatus as previously reported (Czarnecki et al., 2011). Images were acquired using a RedShirtImaging monochrome, back-illuminated CCD camera (NeuroCCD SM256) at 256 x 256 pixel resolution and frame acquisition rate of 7 Hz. The mouse was positioned under the microscope using a custom optomechanical stage. The entire apparatus floated on a TMC vibration isolation table.

Odorants were presented by a custom eight-channel, air dilution olfactometer controlled by a computer running software written for MatLab (Mathworks). Nitrogen served as a carrier vapor that was saturated with odorant and then diluted into ~500 ml/minute ultrazero-humidity compressed air by computer-controlled mass flow controllers. Source gases were filtered by a hydrocarbon/moisture gas purification system (Chromatography Research Services). Stimulus onset and offset were controlled by a computer controlled valve that shunted a vacuum from and to an odorant-removal tube concentric with the odorant delivery tube. The odorant delivery tube was placed within 2 cm of the mouse’s nose. Odorants included methyl valerate, 2-methyl-2-butenal, hexanone and butyl acetate, which are known to evoke transmitter release on the dorsal aspect of the olfactory bulb (Bozza et al., 2004; Wachowiak and Cohen, 2001). Six 6 s trials of
each odorant were presented at a 2-6% dilution of saturated vapor. The minimum intertrial interval was 60 s. The experimenter was blind to the experimental condition of the mouse.

Imaging data were analyzed as described previously (Czarnecki et al., 2011). Briefly, blank trials on which no stimuli were presented were subtracted from each stimulus-present trial to correct for bleaching, a time-dependent reduction in fluorescence independent of stimulus presentation. Stimulus-evoked glomerular responses were measured as the average of 15 frames centered on the peak of the fluorescence increase minus the average of 15 baseline frames immediately prior to stimulus onset. Trials were treated individually for amplitude measurements and averaged within odorants to create spatial maps of odorant-evoked responses. Candidate regions of interest corresponding to olfactory glomeruli were initially selected by hand and then confirmed statistically. A glomerulus was operationally defined as responding to an odorant if its average response across trials of that odorant was at least three standard errors greater than zero. The left and right nares are divided by a septum, and the ORNs on each side project exclusively ipsilaterally to the corresponding olfactory bulbs, thus permitting within-subjects comparisons following unilateral intranasal toxicant exposure (Czarnecki et al., 2011). Accordingly, within animal ratios were computed (Cd-exposed/vehicle-exposed) and averaged across all odorants. Analysis was performed using Neuroplex (RedShirtImaging) and custom software written in MatLab and exported to Excel, SPSS, and SigmaPlot for statistical analysis.

**Olfactory bulb histology**

In histological sections, vesicular pH is neutralized, permitting spH fluorescence to serve as a green fluorescent protein (GFP)-based marker for ORN axon terminals (Czarnecki et al.,
2011). Five additional animals that did not complete the full odorant battery during optical imaging and were not included in the analysis of olfactory nerve physiology were included in histological analyses. Immediately after imaging, seventeen animals were intracardially perfused with 0.1 M phosphate-buffered saline (PBS) followed by 4% paraformaldehyde. Brains were removed and post-fixed in 4% paraformaldehyde. Tissue was transferred to PBS at least 24 h before sectioning. Brains were blocked to include both olfactory bulbs and the frontal cortex and sectioned horizontally at 50 µm on a vibratome. Sections were mounted in ProLong Gold antifade agent (Invitrogen) containing 4',6-diamidino-2-phenylindole (DAPI) on glass slides and sealed under a glass coverslip. DAPI is a membrane-permeant fluorescent nuclear stain that enables the visualization of periglomerular cell bodies that delineate the borders between glomeruli.

Photographs of olfactory bulb sections were taken at a resolution of 1360 x 1024 pixels and 14 bit analog-to-digital conversion with a Jenoptik MFcool Peltier-cooled CCD camera mounted on an Olympus BX41 microscope at 4x (0.16 NA). Images were collected with both DAPI- and GFP-appropriate filter sets. Images were opened in ImageJ (NIH) and the glomerular layer of each olfactory bulb was selected as a region of interest based on the rings of periglomerular interneurons visualized by the DAPI stain. The optical density of these regions of interest was then measured in the corresponding image taken using the GFP-appropriate optical filter. Optical densities were recorded in Excel and exported to SPSS for statistical analysis. Experimenters were blind to the experimental condition of the animal until after the quantification was completed.
Olfactory detection assessment

Twenty three C57BL/6 and 8 OMP-spH mice were trained in operant conditioning chambers (Coulbourn Apparatus Habitest system) enclosed within sound attenuating cubicles (Med Associates or Coulbourn Apparatus). Reinforcements were delivered through a reward port, where 0.01 ml of 2% sucrose solution was delivered by a liquid dipper when the mouse broke a nose poke photobeam on rewarded trials. Olfactory stimuli were presented through a custom controlled-access odor port consisting of a nose poke operandum with odorant and vacuum ports and a guillotine door on the front to prevent odorant access during the intertrial interval. A house light and ventilation fan were also included. Odorants were presented using custom computer controlled liquid dilution olfactometers, which passed room air through odorant vials containing a 1:100 dilution of the odorant in mineral oil and then on to the odor port. The rewarded olfactory stimulus was butyl acetate. Actual concentrations in the odor ports were measured and standardized across chambers and days of training using a ppb photoionization detector (HNU DL-101, HNU Systems). Each chamber and floor was washed with 70% ethanol after every session.

Prior to training, mice were water restricted to 90% of initial weight and maintained at this level throughout training to maintain consistent motivation. Training began with conventional magazine training, in which mice received a liquid reward upon poking into the dipper port, as cued by the magazine light. The odor port remained closed throughout magazine training. Mice completed magazine training after 60 successful trials. In the second phase, mice were trained to nose poke into the odor port (in the absence of odorant) when the door opened, and then move to the reward port for reinforcement. Over at least 4 training sessions, mice were
shaped to hold the initial nose poke for at least 1 s (based on the break of a photobeam across the odor port) in order to receive a reinforcement. Each session lasted 60 successful trials or 60 min, whichever came first. After the mice achieved 60 successful trials in a single session, they were advanced to the odorant detection training. In this final phase of the training, mice were trained to nose poke in the odor port when the door opened (this poke initiated a trial) and then to poke for reward if and only if they received the odorant butyl acetate. Mice had 5 sec to respond (or not) at the reward port on each trial. The intertrial interval after correct responses was 5 s, and a false alarm triggered a “time-out” punishment of 25 s added to the intertrial interval and the conclusion of the trial (only one false alarm was possible per trial). All training was performed daily. Mice received their daily ration of water at the conclusion of training to maintain body weight.

Performance was operationally defined by a Discrimination Metric (DM), the proportion of trials the mouse correctly entered the reward port in presence of the target odorant (“hits”) minus the proportion of trials the mouse incorrectly entered the reward port in the absence of the odorant (“false alarms”). Potential DM scores range from -1 (no hits, all false alarms) to 1 (all hits, no false alarms), with 0 representing chance responding. DMs were measured in the same odorant detection go/no-go operant procedure as described above. After reaching a DM of at least 0.5 (equivalent to 75% correct responses across trial types) for three consecutive days, animals were randomly assigned to receive a bilateral intranasal instillation of cadmium chloride or vehicle control. Animals then returned to the odorant detection task either two (C57BL/6 n = 11; 4 vehicle, 7 cadmium; OMP-spH n = 8, all received cadmium) or ten days (C57BL/6 n = 12; 6 vehicle, 6 cadmium) after infusion.
Statistical analysis

When applicable, descriptive statistics are reported mean (± standard error). Analysis of variance is performed to test statistically for differences between the means of experimental groups following random assignment of subjects, as described below. Along with F and \( p \) values, we also include the \( \eta^2_p \) estimate of effect size (proportion of variance attributable to variable of interest). To compare distributions of glomerular response amplitudes (which may differ along dimensions other than central tendency) between groups we use the nonparametric Kolmogorov-Smirnov test to test the model that both distributions are samples from the same underlying distribution. All statistical testing was performed using SPSS.

Results

Cadmium accumulates in the olfactory bulb after intranasal instillation

We used inductively-coupled plasma mass spectrometry to measure absolute cadmium levels in the olfactory bulb ipsilateral to a unilateral intranasal instillation of pH-neutral 20 µg CdCl\(_2\) solution two, seven, and twenty-eight days following the instillation in 15 randomly-assigned male C57BL/6 mice. Cd levels were significantly elevated in the olfactory bulb at all three time points (Fig. 1A), peaking at nearly 800 ng/g at 7 days post-instillation. Analysis of the cerebellum as a control revealed negligible levels of Cd (Fig. 1A). Remarkably, the measured olfactory bulb Cd concentration was nearly the same at the 2 and 28 day time points, and no significant effect of time was observed (one-way ANOVA; \( F[2, 12] = 1.5, p = 0.26 \)). This indicates that cadmium persists in the olfactory bulb for at least 28 days after instillation.

Acute cadmium exposure induces persistent disruption of odorant-evoked neurotransmitter release from the olfactory nerve over 28 days
To evaluate in vivo pathophysiology following an acute intranasal exposure to cadmium chloride, twelve adult OMP-spH mice were randomly assigned to receive unilateral intranasal instillations of pH-neutral 20 µg CdCl$_2$ solution, with vehicle solution instilled into the contralateral naris. Two, seven, or twenty eight days later, odorant-evoked neurotransmitter release from the olfactory nerve into olfactory bulb glomeruli was visualized as the increase in spH fluorescence during the presentation of a panel of four odorants (six trials each). SpH signals on the cadmium-exposed and vehicle-exposed sides were compared within each mouse to reveal the effects of intranasal cadmium exposure at each time point.

As shown in Fig. 1, cadmium exposure significantly reduced the number of glomeruli receiving odorant-evoked synaptic input relative to contralateral vehicle controls at all time points (Fig. 1B & C), with no significant main effect of time (one-way ANOVA; $F_{[2,9]} = 1.74, p = 0.23, \eta^2_p = 0.28$). Among the glomeruli that did still receive measurable input following cadmium exposure, the distribution of input amplitudes (Fig. 1D) were significantly smaller on the cadmium-exposed side than on the vehicle-exposed side at all three time points (Kolmogorov-Smirnov tests, $p < 0.001$ in each case). There was no evidence of recovery of response amplitudes over the 28 days following instillation (Fig. 1D).

Intranasal cadmium exposure causes persistent damage to ORN projections from the epithelium to the olfactory bulbs

After each imaging experiment mice were fixed and histological sections through both olfactory bulbs were prepared and counterstained with DAPI. Olfactory bulb glomeruli were identified by their characteristic rings of periglomerular interneurons in the DAPI stain (Fig. 2A,
upper), while ORN projections from the olfactory nerve were visible innervating each glomerulus from their GFP fluorescence (Fig. 2A, middle). The density of ORN projections to olfactory bulb glomeruli was measured optically and expressed as a ratio of GFP fluorescence on the cadmium-exposed side to that on the vehicle-exposed side. Two days after cadmium exposure, GFP fluorescence (Fig. 2B) was modestly reduced to 80 ± 4% of control on the cadmium-exposed side as previously reported (Czarnecki et al., 2011). However, over the following four weeks there was a significant time-dependent reduction in ORN axonal projections to the bulb as shown in Fig. 2B & C (one-way ANOVA; $F[2,14] = 23.84, p < 0.001, \eta^2_p = 0.77$), declining to 41 ± 3% of control by 28 days post-infusion. This indicates that significant histopathology occurred in the olfactory nerve following cadmium exposure, but also that a considerable portion of the axons survived.

Cadmium-induced olfactory deficits are reversed by odorant detection training

In order to investigate the perceptual impairments corresponding to the severe cadmium-induced pathophysiology, animals were trained to perform an operant go/no-go odorant detection task. After achieving criterion performance of three consecutive days with a Discrimination Metric above 0.5 (equivalent to 75% correct), mice were randomly assigned to receive bilateral instillations of either 20 µg CdCl$_2$ or vehicle solution and to be tested either two days or ten days post-instillation (Fig. 3A).

Cd-exposed C57BL/6 animals were significantly impaired on the odorant-detection task at both time points (Fig. 3B). A mixed-model ANOVA (with assessment day as a within subjects variable and instillation type and time post-instillation as between subjects variables)
revealed a significant assessment day by instillation type interaction \( (F(1, 19) = 27.12, p < 0.001, \eta_p^2 = 0.59) \), a significant effect of assessment day \( (F(1, 19) = 39.42, p < 0.001, \eta_p^2 = 0.68) \) and a significant effect of instillation type \( (F(1, 19) = 27.45, p < 0.001, \eta_p^2 = 0.59) \). At both the two day and ten day post-instillation time points, Cd-exposed mice exhibited no ability to detect the odorant, with a DM that was significantly reduced compared to both their own baseline (paired t-test, \( t(6) = 5.08, p = 0.002 \) and \( t(5) = 6.64, p = 0.001 \), respectively) and vehicle-exposed mice (independent samples t-test, \( t(9) = 4.06, p = 0.003 \) and \( t(10) = 3.77, p = 0.004 \), respectively) and not significantly different from zero, indicating chance performance (one-sample t-test, \( t(6) = 1.88, p = 0.11 \) and \( t(5) = 1.89, p = 0.12 \), respectively). The pattern of errors in Cd-exposed mice indicated a large increase in the false alarm rate (i.e. guessing), which is the optimum strategy to maximize reward for an anosmic subject in this paradigm. Vehicle-exposed mice exhibited no change in discrimination performance relative to their own baseline at either time point (paired t-test, \( t(3) = -0.02, p = 0.99 \) for 2 day group and \( t(5) = 1.77, p = 0.14 \) for 10 day group).

To assess the effect of rehabilitation training on olfactory abilities, cadmium-exposed mice from the Day 2 test group received daily odorant detection training until they again achieved criterion performance of DM > 0.5 for three consecutive sessions or up to fifteen days. Despite their initial inability to discriminate between odorant-present and odorant-absent trials, cadmium-exposed mice continued to initiate trials and to receive rewards when they guessed correctly. As shown in Fig. 3D, after three days of post-instillation training, 75% of vehicle-exposed animals had reached criterion, whereas 0% of cadmium-exposed animals had reached criterion. By day 4, 100% of vehicle-exposed animals and only 43% of cadmium-exposed animals reached criterion. By fifteen days of post-instillation training, 100% of cadmium-
exposed animals had been rehabilitated to criterion. The average number of days for cadmium-exposed animals to reach criterion after infusion was not significantly different than the number of days to reach criterion when first beginning the task (Fig. 3E, paired samples t-test, $t(6) = -1.21, p = 0.27$), consistent with the idea that rehabilitated mice may be re-learning the task using whatever sensory inputs survived cadmium exposure.

To decouple the effects of the passage of time from the effects of rehabilitation training, the performance of re-trained mice was also assessed on Day 10 post-instillation (Fig. 3A). To control for the size of the initial performance deficit, only the mice that exhibited no hint of discrimination between the odorant-present and odorant-absent conditions on Day 2 were included (DM < 0.1, n = 5). As shown in Fig. 3C, on Day 10 following cadmium exposure, mice that underwent rehabilitation training exhibited significantly increased detection performance compared to their own performance two days after instillation (paired samples t-test, $t(4) = -3.37$, $p = 0.03$). Detection performance after rehabilitation did not significantly differ from baseline performance (paired samples t-test, $t(4) = 1.61, p = 0.18$), while cadmium-exposed mice who received no additional training after intranasal infusion until Day 10 remained just as impaired as animals that were tested on Day 2 (Fig. 3B). To confirm that these rehabilitated mice were indeed using their olfactory systems to perform the task, they were run for one additional session that was identical to the others except that no odorants were placed in the olfactometer. Performance on this control task declined to zero (Fig. 3D).

*Odorant detection ability recovers without increased neurotransmitter release from the olfactory nerve*
The cadmium-induced pathophysiology of the olfactory nerve does not spontaneously recover during the two weeks after exposure (Fig. 1B) and ORN innervation of the olfactory bulb declines (Fig. 2B), yet all mice could be fully rehabilitated on the olfactory detection task during this time (Fig. 3C). This raised the question of whether the improved sensory performance of mice that received rehabilitation training reflected sensory learning or an actual reversal of the neural damage. To investigate this possibility, 8 OMP-spH mice were trained to perform the olfactory detection task as described above. After reaching criterion, they received bilateral instillations of 200 µg cadmium chloride, and their performance on the detection task was assessed on Day 2 after the instillation. Similar to wild-type mice, OMP-spH mice exhibited a detection performance that was greatly reduced (Fig. 4B) relative to their baseline performance (paired samples t-test, \( t(7) = 6.59, p = 0.001 \)) and not significantly different from zero (one-group t-test, \( t(7) = 2.07, p = 0.08 \)). To standardize the size of the initial sensory deficit, the worst performing mice (DM < 0.1, \( n = 5 \)) were selected to undergo 8 additional training sessions, during which time their average detection performance significantly improved from a pre-rehabilitation baseline of 0.02 ± 0.02 to 0.36 ± 0.13 (paired samples t-test, \( t(4) = -2.89, p = 0.045 \)). As shown in Fig. 4C, this improvement was comparable to the post-rehabilitation DM exhibited by a subset of the worst-performing wild-type mice at the same time point (independent samples t-test, \( t(8) = 0.83, p = 0.43 \)). As with the wild-type mice (Fig. 3C), after 8 days of training some of the cadmium-exposed OMP-spH mice were performing well on the detection task, while others were not. All mice underwent optical imaging of olfactory nerve function as described above. Surprisingly, the patterns of odorant-evoked neurotransmitter release observed in these mice were similar to those observed at this time point in untrained mice (Fig. 1 and Fig. 4A), including a limited number of glomeruli receiving odorant-evoked synaptic
input from the olfactory nerve and greatly reduced amplitudes. There was no evidence that rehabilitation training improved the function of the olfactory nerve.

**Discussion**

These experiments demonstrated that cadmium accumulates in the olfactory bulb after intranasal exposure and persists for at least four weeks. Throughout this time, odorant-evoked neurotransmitter release from ORN synaptic terminals into the olfactory bulb remains severely disrupted (< 20% of control levels) and the density of olfactory nerve projections into the olfactory bulb gradually declines until leveling off at about 40% of control levels. Two days after cadmium infusion, performance on a behavioral olfactory detection task was not significantly different from chance. However, after 8 days of explicit odorant detection training, cadmium-exposed mice performed no differently from vehicle-exposed controls (while untrained cadmium-exposed animals continued to perform at chance, showing no evidence of spontaneous recovery of function). These cadmium-exposed, rehabilitated mice were confirmed to be using their olfactory systems to perform the task because their performance returned to chance in a control test session without odorant. Physiological imaging of odorant-evoked neurotransmitter release from the ORNs of cadmium-exposed mice at various levels of rehabilitation showed no evidence that training improved ORN function. We conclude that this recovery of olfactory ability must be the result of learning in a brain structure downstream from the ORNs. These findings demonstrate that learning can mask even severe pathological effects of neurotoxicants, and, conversely, that basic olfactory function can be rehabilitated despite persistent damage.

Our mass spectrometry data indicate that cadmium accumulates in the olfactory bulb following acute intranasal instillation, consistent with previous reports in rodent models.
(Hastings and Evans, 1991; Evans and Hastings, 1992; Bondier et al., 2008; Tjälve et al., 1996; Sun et al., 1996). Surprisingly, we found that cadmium levels remain elevated for at least 28 days following a single acute instillation, perhaps because of the high concentration of metallothioneins and other metal-binding proteins in ORNs (Himeno et al., 2009; Sunderman, 2001). As a divalent cation, cadmium can disrupt neuronal calcium signaling, including blockade of transmembrane calcium channels (Chow, 1991; Wang et al., 2008). We have previously shown that neurotransmitter release from the ORN presynaptic terminals in the olfactory nerve requires the conduction of calcium ions through N-type calcium channels and is a power-law function of extracellular calcium concentration (Wachowiak et al., 2005). This suggests if cadmium causes even a modest reduction in presynaptic calcium influx it would greatly impact the total transmitter release from the nerve. The harmful action of cadmium in the olfactory system may thus be a combination of persistent pharmacological effects and outright damage to the olfactory nerve (see Figs. 1 and 2).

In these experiments we observed rehabilitation of olfactory detection abilities despite a reduction of ORN projections to the olfactory bulb and sustained, severe pathophysiology of the olfactory nerve in those very animals (Fig. 4). In principle even just a single intact ORN could be sufficient to detect an odorant, which is likely why rehabilitation is possible at all in this circumstance. However, in practice the population of surviving, functional ORNs may not initially be sufficient to activate downstream brain regions without adaptive plasticity to "recalibrate" the olfactory system to its new, weakened input. It is not clear where in the brain this plasticity occurs, but it may well be downstream of the olfactory bulbs because rodents can
learn to perform odorant detection and discrimination tasks even after large bulbar lesions (Lu and Slotnick, 1998; Slotnick, et al., 2004).

The present findings help to explicate inconsistent previous reports of olfactory deficits after cadmium exposure. In the study by Sun et al. (1996) that found no olfactory impairment even after 20 weeks of cadmium oxide exposure, the subjects were constantly being trained on odorant-guided tasks throughout the exposure period. The data presented here demonstrate that one training session a day for eight days can be sufficient to rehabilitate odorant detection performance all the way from chance to baseline levels, suggesting that the ongoing training in that study could have prevented the authors from detecting cadmium's pathophysiological effects. Similarly, Bondier et al. (2008) reported that after an acute intranasal instillation of cadmium mice no longer spontaneously avoided the unpleasant odorant butanol, but returned to normal avoidance behavior by 18 days post-exposure. In light of the present findings, it is likely that the olfactory systems of those mice had not returned to "normal," but rather had undergone some form of compensatory plasticity to adapt to ongoing peripheral damage.

While the masking of peripheral pathology by sensory learning is a confounding problem for behavioral assessment and toxicant screening, it would be a desirable consequence of rehabilitation therapy after insult to the olfactory system. The clinical loss of olfactory function is widespread and can have many causes, both peripheral and central, including upper respiratory tract infections, physical trauma, toxicant exposure, aging, and neurodegenerative diseases such as Parkinson's diseases and Alzheimer's disease (Haehner et al., 2009; Kern et al., 2000; Mascagni et al., 2003; Mesholam et al., 1998; Rose et al., 1992; Schubert et al., 2009).
Interestingly, olfactory impairments sometimes seem to improve spontaneously, even in cases of Parkinson's disease where the underlying neuropathology is likely progressing (Herting et al., 2008). Moreover, exposure-based rehabilitation programs, where dysosmic patients are exposed to labeled odorants twice daily, have been shown to increase olfactory function in individuals with olfactory loss (Hummel et al., 2009). Without epithelial or bulbar histology from patients, it is unknown whether this rehabilitation therapy is facilitating peripheral recovery or triggering central plasticity, but the data presented here demonstrate that functional recovery can occur despite continued peripheral pathology.

In a previous paper we illustrated the experimental power of our in vivo physiological imaging technique, showing that it could detect pathophysiological effects of cadmium instillation at 1/100th of the minimum dose required to detect histological changes in the same animals (Czarnecki et al., 2011). The present data from rehabilitated mice similarly reinforces the value of using a physiological assay such as imaging, which showed a massive loss of neural function, in conjunction with behavioral assays that seemed to show no effect of cadmium in those same animals. Outside the laboratory, some toxicant exposures may be extended in time while being accompanied by constant sensory use (complete with feedback on errors). Our data suggest that under these circumstances simple behavioral assays can miss real toxicant-induced damage to sensory systems. Consequently, sensory testing should be supplemented by physiological measures when possible. In the human olfactory system, this means that if sensory learning could be masking underlying neural deficits then behavioral measures of sensory function like the University of Pennsylvania Smell Identification Test (UPSIT; Sensonics, Inc., Haddon Heights, NJ) and Sniffin' Sticks (Burghart Instruments, Wedel, Germany) tasks could be
profitably followed up by physiological measures like intranasal electro-olfactograms, olfactory event-related potentials (ERP), and functional magnetic resonance imaging (Lötsch and Hummel, 2006; Scott and Scott-Johnson, 2002; Zald and Pardo, 2000).

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**References**


Figure Captions

Figure 1. Acute intranasal instillation of cadmium chloride results in elevated olfactory bulb cadmium levels and reduced neurotransmitter release from the olfactory nerve for at least 28 days after exposure. (A) Inductively-coupled plasma mass spectrometry revealed elevated levels of cadmium in the olfactory bulbs at every time point measured, but not in cerebellum controls. This accumulation was not seen in cerebellum. (B) Ratio of the number of glomeruli receiving synaptic input from ORNs on the cadmium-exposed side compared to the vehicle-exposed side. The dashed line at one represents an equal number of responding glomeruli on each side. (C) Leftmost panel shows a sample baseline fluorescence image outlining the dorsal region of the olfactory bulbs viewable during in vivo imaging through a cranial window. Three following panels are pseudocolored response maps showing the increase in fluorescence in response to an odorant compared to pre-odor onset baseline. Callouts show the odorant-evoked response amplitude of the selected glomerulus during baseline and odorant presentation (denoted by horizontal black bar) portions of the trial. (D) Cumulative distributions of individual glomerular response amplitudes.

Figure 2. Reduction in ORN axonal projections from the olfactory epithelium to the olfactory bulbs following an acute exposure to cadmium chloride. (A) High magnification (10x) image of control horizontal sections of olfactory bulb tissue. (Upper) DAPI nuclear stain (blue) marks rings of periglomerular interneurons surrounding olfactory bulb glomeruli. (Middle) ORN afferents (green) course over the surface of the olfactory bulb and project to receptor-specific glomeruli. (Lower) Merge of DAPI and GFP images. (B) Ratio of GFP optical density on the cadmium-exposed compared to vehicle-exposed bulbs. Dashed line at 1 represents no difference.
between bulbs. (C) (Upper) DAPI nuclear stain in representative sections of vehicle- and cadmium-exposed olfactory bulbs at 2, 7, and 28 days after intranasal cadmium exposure. (Lower) GFP glomerular marker in vehicle- and cadmium-exposed olfactory bulbs at 2, 7, and 28 days after intranasal cadmium exposure.

Figure 3. Cadmium-induced deficits in odorant detection performance persist following acute exposure but can be reversed by sensory rehabilitation training. (A) Experimental design schematic. In the 2-day and 10-day delay groups, animals were given one or nine rest days after intranasal instillation, respectively. In the rehabilitation training group, the worst performing mice from the 2-day delay group were given eight additional days of training before their detection metric was again assessed on day ten after intranasal instillation. (B) Odorant detection ability of cadmium- and vehicle-exposed animals before and either two or ten days after intranasal instillation. (C) Odorant detection metric of worst performing mice from the 2-day delay group from panel B measured before and two days following intranasal exposure to cadmium, then after eight additional days of training and without odors presented to ensure animals were not responding to an additional cue. (D) Percentage of animals to reach criterion after intranasal instillation. Criterion is defined as 3 consecutive days with a discrimination metric over 0.5, therefore day 3 is the first eligible day to reach criterion. (E) Average number of days to reach criterion before and after cadmium exposure.

Figure 4. Olfactory detection performance was restored without recovery of olfactory nerve function. (A) Pseudocolored maps of glomeruli receiving odorant-evoked neurotransmitter release from the olfactory nerve in response to a butyl acetate presentation. The leftmost four
panels are from animals measured on day ten after bilateral intranasal instillation of cadmium and run continually after post-instillation rest day. Detection metrics from each mouse's final training session appear inset in the pseudocolored maps. The map of a cadmium-exposed but behaviorally untrained animal appears second from the right as a control. A map of the response to butyl acetate in a mouse that received bilateral instillation of vehicle solution appears in the last column for comparison. (B) Detection metric of OMP-spH mice before and two days after cadmium-exposure. (C) Detection metric of worst performing OMP-spH mice two days after intranasal instillation and C57BL/6 and spH mice on day ten after rehabilitation training.
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