

INTRANASAL CADMIUM EXPOSURE INDUCES OLFACTORY
PATHOPHYSIOLOGY AND SENSORY DEFICITS

By

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ABSTRACT OF THE THESIS

Intranasal cadmium exposure induces olfactory pathophysiology, disruption of sensory afferents, and sensory impairment in the mouse olfactory system

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Intranasal exposure to cadmium has been related to olfactory dysfunction in humans and to nasal epithelial damage and altered odorant-guided behavior in rodent models. The pathophysiology underlying these deficits has not been fully elucidated. Here we use optical imaging techniques to visualize odorant-evoked neurotransmitter release from the olfactory nerve into the brain's olfactory bulbs *in vivo* in mice. Intranasal cadmium chloride instillations reduced this sensory activity by up to 91% in a dose-dependent manner. In the olfactory bulbs, afferents from the olfactory epithelium could be quantified by their expression of a genetically-encoded fluorescent marker for olfactory marker protein. At the highest dose tested, cadmium exposure reduced the density of these projections by 20%. In a behavioral psychophysical task, mice were trained to sample from an odor port and make a response when they detected an odorant against a background of room air. After intranasal cadmium exposure, mice were unable to detect the target odor. These experiments serve as proof of concept for a new approach to the study of the neural effects of inhaled toxicants. The use of *in vivo* functional imaging of the neuronal populations exposed to the toxicant permits the direct observation of

primary pathophysiology. In this study optical imaging revealed significant reductions in odorant-evoked release from the olfactory nerve at a cadmium chloride dose two orders of magnitude less than that required to induce morphological changes in the nerve in the same animals, demonstrating that it is a more sensitive technique for assessing the consequences of intranasal neurotoxicant exposure. This approach is potentially useful in exploring the effects of any putative neurotoxicant that can be delivered intranasally.

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Introduction

The primary sensory neurons of the olfactory system, olfactory receptor neurons (ORNs), reside in the olfactory epithelium lining the nasal cavity and are exposed to the external environment. Inhaled odorants bind to a subset of ORNs, which project their axons to the olfactory bulb. ORN exposure to the environment may also provide a route of entry for aerosolized toxicants to enter the central nervous system. Following toxicant exposure (Adams & Crabtree, 1961; Hastings & Evans, 1991; Mascagni et al., 2003; Rose et al., 1992) and in some neurodegenerative diseases such as Alzheimer's and Parkinson's diseases (Meshulam et al., 1998; Haehner et al., 2007; Ross et al., 2008), olfactory dysfunction may present as one of the earliest or most prominent symptoms. It has been suggested that the olfactory system may act as a vector in these disease states, as the olfactory bulb projects to numerous brain regions. The olfactory system may serve as a vector by either transporting toxicants into the brain via the olfactory nerve or, being particularly sensitive to neurodegeneration, inducing pathological processes in brain regions receiving bulbar projections, thus producing clinical hallmarks and classic symptoms (see Doty, 2008). Here, we focus on the pathophysiological, histopathological and perceptual deficits induced by acute intranasal exposure to the heavy metal cadmium. The current experiment used a genetically encoded exocytosis indicator, synaptopHluorin (spH) to visualize odorant-evoked neurotransmitter release from ORNs, quantified ORN innervation of the olfactory bulb, and tested olfactory function in mice exposed to intranasal cadmium. We found that *in vivo* optical imaging revealed a significant functional impairment at a dose 1/2000th of that seen in previous work

analyzing histological sections (Bondier et al., 2008) and at a dose 1/100th of that which produced histopathology in the same animals.

1. Olfactory bulb

Interaction with environmental stimuli and basic structure

Olfactory transduction begins when volatile compounds bind to ORNs located in the olfactory epithelium lining the inside of the nasal cavity. A multigene family codes for approximately 1000 different receptor types in the rodent (Buck & Axel, 1991). Each of the millions of ORNs in the olfactory epithelium expresses only one receptor type. Receptor types differ in binding affinities for odor molecules of varying chemical features (Cagan & Zeiger, 1978; Caprio & Byrd, 1984; Malnic et al. 1999). From the epithelium, axons from ORNs expressing the same receptor type segregate as they project to the olfactory bulb (via the olfactory nerve) such that ORNs expressing the same receptor converge onto one or a few corresponding ipsilateral olfactory bulb glomeruli (Ressler et al., 1994; Potter et al., 2001). Olfactory bulb glomeruli are neuropils composed of ORN axons, mitral/tufted cells, top-down inputs from higher brain areas and are surrounded by a ring of juxtglomerular interneurons (see Fig. 1) (Pinching & Powell, 1971; Shepherd, 2004). When an odorant enters the nasal cavity, it is sorbed into the mucosa and binds to a subset of ORNs in the epithelium depending on the odorant-affinity of the receptor each expresses. Odorant binding activates an intracellular signaling cascade that induces the firing of an action potential, which propagates down the axon to the olfactory bulb glomerulus targeted by ORNs expressing that odorant receptor type. Because odorants drive sensory input into corresponding, odorant-specific

subsets of glomeruli, there is a “spatial map” of activity in the olfactory bulbs that codes for odor quality (Malnic et al., 1999; Stewart et al., 1979; Sharp et al., 1975).

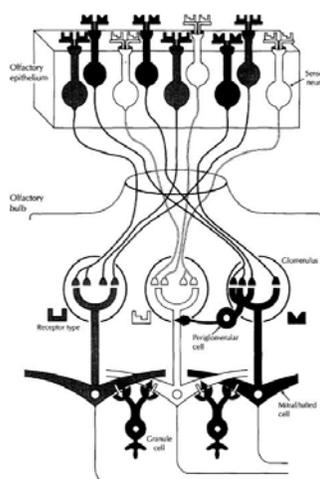


Figure 1. Olfactory receptor neurons projecting to the olfactory bulb. Modified from Mori (1995). Similar olfactory receptor neurons interspersed in the olfactory epithelium converge into the same glomerulus in the olfactory bulb.

The consistency of glomerular innervation by ORN axons is remarkable considering that cells in the olfactory epithelium are constantly being turned over. The cycle of generation and turnover normally occurs on a 28-day timescale (Carr & Farbman, 1993; Graziadei & Monti Graziadei, 1979) and continues throughout an animal’s life. Neuronal precursor cells in the olfactory epithelium either reside in the epithelium or migrate as proliferated ORNs (Calof et al., 1995). This adult neurogenesis in the olfactory epithelium may be particularly beneficial in a system that is continually exposed to environmental toxicants.

2. Cadmium

Cadmium (Cd) is a heavy metal found as an airborne particulate in cigarette smoke and industrial settings such as smelters and battery manufacturing plants. The OSHA permissible limit in workplace air is $5 \mu\text{g}/\text{m}^3$ averaged over 8 hours (ATSDR,

2008). Cadmium toxicity is characterized clinically by olfactory dysfunction, osteoporosis, and renal failure (ATSDR, 2008). Intranasal exposure to cadmium may impair ORN signaling due to damage to the olfactory epithelium or olfactory bulb tissue, either by direct pharmacological actions of cadmium or through cadmium's neurotoxic effects.

Cadmium has been shown to transport to the olfactory bulbs after aerosolized exposure (Evans & Hastings, 1992; Tallkvist et al., 2002; Tjälve et al., 1996). Once inside the cell, cadmium can trigger inflammatory, necrotic and apoptotic processes. *In vitro*, CdCl₂ exposure has been shown to upregulate p38 MAPK in endothelial cells (Jung et al., 2008) and increase reactive oxygen species in cortical neurons (López et al., 2006). Cadmium increases intracellular IP₃, thus increasing calcium concentration by releasing it from intracellular stores (Wang et al., 2008). Resulting cytosolic calcium concentration alterations can lead to cellular apoptosis (Wang et al., 2008; Jung, et al., 2008). In cortical neurons, cadmium incubation resulted in apoptosis through increased caspase-3 activity as well as necrotic cell death at higher concentrations (Kim, et al., 2000; López et al., 2003). Both L- and T-type voltage-sensitive calcium channels are expressed in olfactory ORNs and may provide a potential mechanism for cadmium-induced olfactory pathology (Gautam et al., 2007; Miyamoto et al., 1992). Through disruption of calcium channels or inducing cell death, cadmium may impair neurotransmitter release *in vivo*, a potential mechanism of cadmium induced dysosmias.

Cadmium and Olfaction

Olfactory deficits linked to cadmium exposure have been documented since the 1950's (Friberg, 1950). Industrial workers exposed to airborne cadmium for as little as 4

years have an increased olfactory detection threshold (Adams & Crabtree, 1961; Mascagni et al., 2003; Rose et al., 1992; Sułkowski et al., 2000) and impaired ability to identify odors (Sułkowski et al., 2000). With longer exposure durations, more than 30 years in one population, over 90% of workers presented with olfactory dysfunction (Potts, 1965). Because cadmium may become aerosolized and inhaled and there are numerous clinical reports of olfactory deficits after cadmium exposure, the olfactory system may be a powerful model system to study the effects of cadmium on neural function.

Rodent models of cadmium exposure report conflicting results. Findings vary from no olfactory impairment (Sun et al., 1996) to immediate but reversible anosmia (Bondier et al., 2008). The inconsistent reports apply both to varying degrees of pathology and behavioral impairment in odorant-guided tasks (Bondier et al., 2008; Hastings, 1990; Sun et al., 1996). In one such study, histological analysis revealed that one intranasal instillation of 400 μ g CdCl₂ reduced epithelial cell thickness to 3 or 4 cell layers, compared to 20 cell layers in control tissue. Behaviorally, the animals exposed to cadmium showed no difference in preference between butanol (aversive) and water containing arms in a y-maze. This suggests an inability of animals exposed to Cd to discriminate between the aversive and neutral olfactory stimuli (Bondier et al., 2008). When allowed to recover, cadmium-exposed animals began showing an increase towards water arm preference at 10 days post instillation and normalized around 18 days post instillation, but at this time point epithelial thickness had recovered only to about half of control thickness. This disconnect between epithelial histopathology and sensory function suggests that although a subset of cells in the epithelium remained after Cd-

exposure, they may not have been functional or may not be sufficient to drive activity in areas receiving bulbar projections, leading to disrupted odorant perception. Physiological measurements from remaining ORNs could reveal important aspects of the pathophysiology underlying cadmium-induced olfactory dysfunction.

3. Techniques

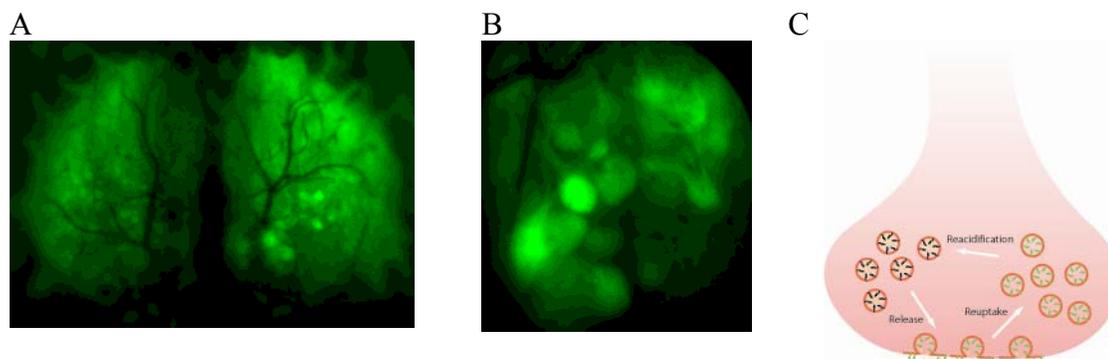


Figure 2. SynaptopHluorin (spH) is a linear indicator of neurotransmitter release. (A) *In vivo* resting light image of the dorsal olfactory bulbs visualized through a cranial window in the OMP-spH mouse. (B) 20x magnification visualizing individual axons converging into glomeruli. (C) Schematic of spH activity. pH-sensitive GFP is bound within each synaptic vesicle, where its fluorescence is normally quenched by the acidity of the intravesicular lumen. When the vesicle releases its transmitter, the neutralization of the vesicular pH causes a rapid increase in GFP fluorescence. The fluorescence then gradually returns to baseline fluorescence levels with vesicle reuptake.

SynaptopHluorin (spH) is a construct composed of pH-sensitive green fluorescent protein (GFP) bound to vesicle-associated membrane protein (*vamp*) and used to visualize exocytosis (Fig. 2) (Miesenböck et al., 1998). The pH-sensitive GFP is sequestered inside the lumen of synaptic vesicles, and consequently quenched by the intravesicular acidic pH. During exocytosis, docked vesicles and the spH therein are exposed to the neutral extracellular pH, causing a rapid increase in fluorescence (see Fig. 2C). This change in fluorescent spH signal is a linear indicator of neurotransmitter release from the olfactory nerve into the olfactory bulb glomeruli (Wachowiak et al., 2005). In the mice used here, spH is expressed under the control of olfactory marker protein (OMP) promoter (Bozza et al., 2004). OMP is normally expressed in mature

ORN axons. While *in vivo*, spH acts as an exocytosis indicator and a quantification of odorant-evoked neurotransmitter release from ORNs, as shown in Figure 3, in histological sections neutralized spH can act as a marker of ORN axonal connectivity from the olfactory epithelium to the olfactory bulbs.

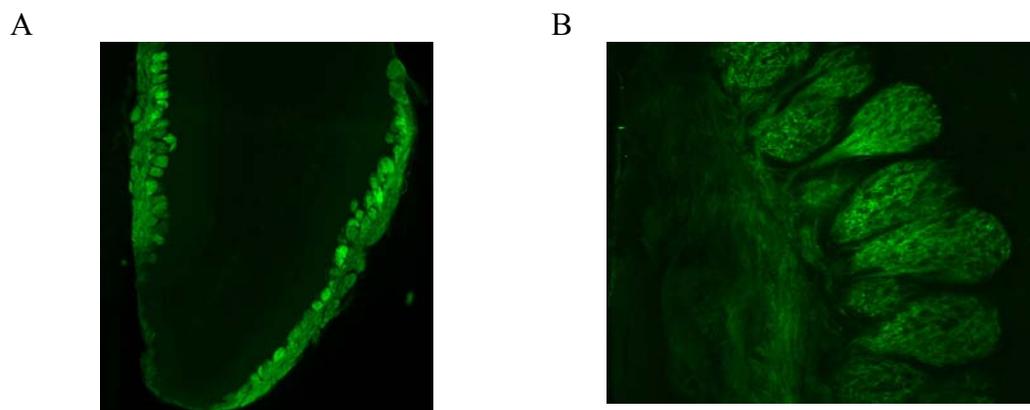


Figure 3. pH-neutral histological sections from OMP-spH mice serve as a genetically encoded anatomical tract tracer. (A) Horizontal section of one olfactory bulb under 4x magnification. GFP optical density can be analyzed as a measure of axonal connectivity. (B) Sample 2-photon high magnification image of GFP expression in olfactory bulb horizontal histological section. Individual ORN axons converge into glomeruli expressing the same ORN receptor type.

To gain insight into the actions of cadmium in the central nervous system, the current experiments quantified odorant-evoked neurotransmitter release from the olfactory nerve to measure ORN pathophysiology. The same animals were then used to measure the density of ORN axonal projections into olfactory bulb glomeruli. Using these two techniques should delineate impairment due to neuronal dysfunction above and beyond loss of ORNs. Additional animals were tested in an odorant detection go/no-go operant training paradigm to measure olfactory perceptual changes after acute intranasal cadmium instillation. The goal of the current experiment is to assess the magnitude of pathophysiology after acute intranasal exposure to a range of cadmium concentrations and to evaluate the utility of *in vivo* optical imaging as a technique to evaluate damage caused by neurotoxicants.

4. Materials and methods

4.1 Subjects

OMP-spH mice express the spH construct from the locus for OMP. Homozygous OMP-spH are on an albino C57BL/6 background. Animals heterozygous for OMP-spH were homozygous OMP-spH mice crossed with a 129 strain. Previous reports show no differences between mice homozygous and heterozygous for spH (Bozza et al., 2004; McGann et al., 2005). Imaging experiments were conducted on 12 male homozygous and heterozygous OMP-spH mice aged 6 to 10 weeks. Subjects in behavioral experiments were eleven female C57BL/6 mice initially aged 6 weeks, purchased from Charles River Laboratories. All animals were group housed with a 12:12 h light:dark cycle. Mice used for imaging procedures had food and water available *ad libitum*. Animals used in behavioral testing were water restricted and maintained at 90% of original body weight. All procedures were performed in accordance with protocols approved by the Rutgers University Animal Care and Use Committee.

4.2 Intranasal infusions

Mice in cadmium-exposed groups received intranasal instillations of 6 μ L of pH 7.4 buffer solution containing 200 mM HEPES, 0.9% NaCl, and either 18.18 mM, 1.818 mM, or 0.182 mM CdCl₂ as noted. These concentrations yield individual infusions of 20 μ g, 2 μ g, and 0.2 μ g of cadmium chloride, respectively. Mice used in imaging experiments received an infusion of cadmium solution into one external naris and vehicle solution (without CdCl₂) into the contralateral naris, with side randomly counterbalanced across subjects. Because the nasal passages on the left and right sides are separated by a nasal septum and the ORN projections are strictly ipsilateral, this design permitted

within-subjects, left vs. right comparisons. Mice used in behavioral experiments received bilateral infusions of either cadmium or vehicle solution. Reflux of the instillate was not observed.

For the intranasal instillation procedure, animals were lightly anesthetized with 1000 μ g/kg metomidine and 70mg/kg ketamine administered intraperitoneally (i.p.). An Eppendorf microloader attached to a 10 μ L Hamilton syringe containing the infusate was inserted 7mm into one naris, the mouse was placed on its back, and the infusion was delivered. The animal remained on its back for five min and was then rotated to its side for another 20 min to ensure coverage across the epithelium. The procedure was repeated for the other naris. Anesthesia was then reversed by subcutaneous (s.c.) injection of 1mg/kg atipamezole hydrochloride. The mouse was maintained on a heating pad and given food and water *ad libitum* during overnight recovery from anesthesia. The experimenter was blind to the contents of all infusates.

4.3 In vivo imaging of neurotransmitter release

4.3.1 Surgical implantation of cranial window

Two days after intranasal cadmium exposure, mice 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 0.005mL/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 periosteal 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 CaCl₂, 1 mM MgCl₂, 10 mM HEPES and 10 mM dextrose was applied over this cranial window, then topped with a glass coverslip.

4.3.2 Imaging apparatus

Optical imaging was performed using a custom apparatus composed of an Olympus epifluorescence illuminator coupled to an Olympus XLFluor4X macro objective (0.28 NA). Illumination was provided by an Opti-Quip 150W Xenon arc lamp with a 25% neutral density filter and controlled by a Uniblitz shutter. Macro-format fluorescence filter cubes included an HQ480/40 excitation filter, Q505LP dichroic mirror, and HQ535/50 emission filter (Chroma Technology). 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 3-axis optomechanical stage. The entire apparatus floated on a TMC vibration isolation table.

Odorants were presented by a custom built eight-channel, air dilution olfactometer controlled by a computer running software written for MatLab (Mathworks). Nitrogen was passed through vials of pure odorant to produce a saturated carrier vapor that was then diluted into ~500 mL/minute ultrazero-humidity compressed air by computer-controlled mass flow controllers at a user-specified ratio. Wetted parts downstream of the odorants were made of PTFE or PEEK, and 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 after the manner of Kauer and Moulton (1974). The odorant delivery tube was placed within 2cm of the mouse's nose. Odorants included methyl valerate, 2-methyl 2-butanal, hexanone and butyl acetate, which are known to evoke transmitter release on the dorsal aspect of the olfactory bulb (Bozza et al., 2004; Wachowiak & Cohen, 2001). They were presented in 6 sec trials at a 2-6% dilution of saturated vapor. The minimum intertrial interval was 60 sec. The experimenter was blind to the experimental condition of the mouse.

4.3.3 Imaging data analysis

Imaging data were analyzed as described previously (McGann et al., 2005). Briefly, blank trials on which no odorants were presented were subtracted from each odorant trial to correct for bleaching. Odorant-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 odorant 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 greater than zero by three standard errors or more.

Analysis was performed using custom software written in MatLab and exported to Excel, SPSS, and SigmaPlot for statistical analysis.

4.4 Olfactory bulb histology

4.4.1 Histological procedures

Immediately after imaging, animals were intracardially perfused with 0.1M phosphate-buffered solution (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 μ m on a vibratome. Slices were mounted in ProLong Gold antifade agent (Invitrogen) containing DAPI on glass slides and sealed under a glass coverslip.

4.4.2 Quantification of glomerular afferents

Photos of approximately 20 sections from each olfactory bulb 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 (D350/50x excitation filter, T400LP dichroic mirror, and ET460/50m emission) and GFP (ET470/40x excitation filter, T495LP dichroic mirror, and ET525/50m emission) 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.

4.5 Behavioral experimentation

4.5.1 Apparatus

Eleven wild type 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 nosepoke 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, NHU Systems, Inc.). Each chamber and floor was washed with 70% ethanol after every session.

4.5.2 Training and testing protocols

Mice underwent two to three days of water restriction prior to initial training to achieve 90% of initial weight. 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 sec (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 and then to poke for reward if and only if they received the odorant butyl acetate. The intertrial interval after correct responses was 5 sec, and incorrect responses triggered a “time-out” punishment of 25 sec added to the intertrial interval. All training was performed daily. Mice received their daily ration of water at the conclusion of training to maintain body weight.

4.5.3 Testing and data analysis

Detection performance was measured by the discrimination metric (*DM*), defined as the proportion of trials in which the mouse poked at the reward port when the odor was presented (hits) minus the proportion of trials in which the mouse poked at the reward port when the odor was absent (false alarms). Mice were considered to have reached

criterion performance when they exhibited a *DM* of 0.5 or greater (equivalent to 75% correct performance) on three consecutive days. The average *DM* of these three days to criterion served as the baseline measurement. Immediately after reaching criterion, mice received bilateral intranasal instillations of either cadmium or vehicle solution. Mice received *ad libitum* water access overnight following intranasal infusion to support their recovery from anesthesia, and were given a single recovery day on water restriction after the infusion. They were then tested for performance on the second day post-infusion. A mixed model ANOVA was run with discrimination index as the dependent variable using time point (baseline measurement, test measurement) as a within-subjects independent variable and instillation group (cadmium, vehicle) as a between-subjects independent variable.

5. Results

5.1 Cadmium exposure disrupts odorant-evoked neurotransmitter release from the olfactory nerve

To assess the effects of intranasal cadmium exposure on olfactory physiology, we used optical imaging techniques to visualize odorant-evoked neurotransmitter release from the olfactory nerve *in vivo* in OMP-spH mice (Bozza et al., 2004). Consistent with previous results (Bozza et al., 2004; Soucy et al., 2009), the patterns of odorant-evoked activity were typically bilateral and approximately symmetric. In five control mice, averaging across a panel of four odorants, the number of glomeruli receiving synaptic input was not different between the left and right olfactory bulbs (average ratio 0.95 ± 0.10 , one-sample t-test, $p = 0.66$) nor was the distribution of glomerular response amplitudes (two-sample Kolmogorov-Smirnov test, $p = 0.07$).

To assess the effects of intranasal cadmium exposure, we measured odorant-evoked neurotransmitter release from the olfactory nerve in twelve mice that received intranasal instillations of cadmium chloride in one naris and vehicle in the other, thus allowing for a within-subjects left-right comparison. We varied the dose of cadmium chloride across mice to include 20, 2, or 0.2 μg . For each odorant tested in each mouse, we measured the number of olfactory bulb glomeruli receiving synaptic input from ORNs and computed the ratio of glomeruli receiving input on the cadmium-exposed side to that on the vehicle-exposed side. We then averaged these ratios across odorants to generate an overall metric of asymmetry for each mouse. We also compared the amplitude distributions of the observed glomerular inputs between cadmium-exposed and control-bulbs across odorants.

As illustrated in Figures 4 and 5, intranasal exposure to 20 μg cadmium chloride greatly reduced both the number of odorant-responsive glomeruli and the amplitude of the observed responses. As shown in Fig. 5B (upper), the overall distribution of glomerular response amplitudes was significantly different between the olfactory bulbs on the cadmium-exposed and vehicle-exposed sides (two-sample Kolmogorov-Smirnov test, $p < 0.001$). On average, the olfactory bulb on the cadmium-exposed side included only $9.3 \pm 3.8\%$ as many glomeruli receiving measurable input from ORNs as the contralateral, vehicle-exposed bulb (Fig. 4 left, and Fig. 5A), a significant decrease (one-sample t-test, $p < 0.001$). Among glomeruli that did receive measurable ORN input, the response amplitudes were significantly smaller on the cadmium-exposed side (Mann-Whitney U-test, $p < 0.001$).

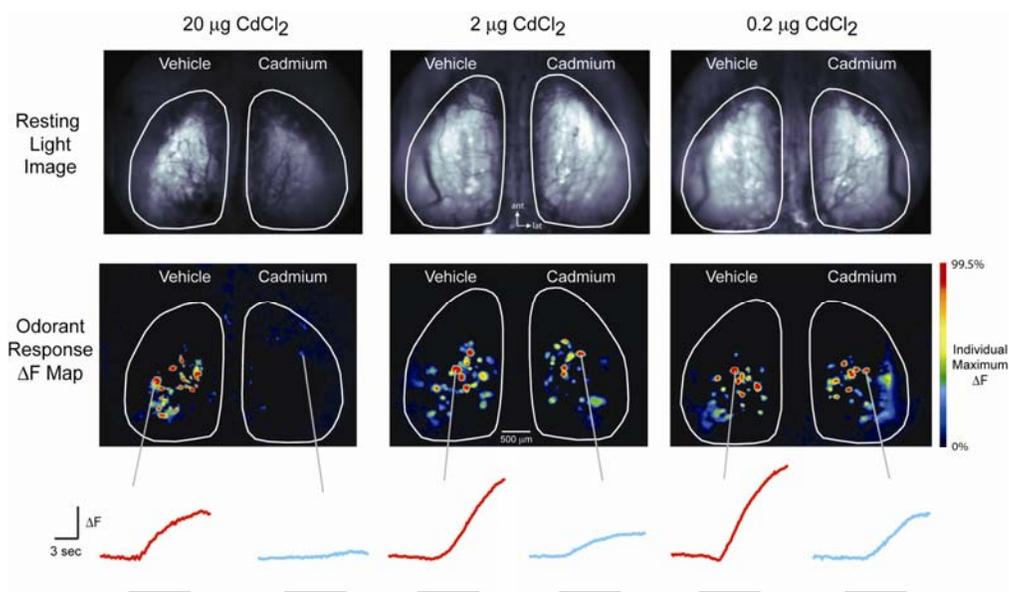


Figure 4. Acute intranasal cadmium instillation reduces neurotransmitter release from the olfactory nerve into olfactory bulb glomeruli in a dose dependent manner. (Upper) Baseline fluorescence images of the dorsal olfactory bulbs visualized *in vivo* through a cranial window. (Lower) Pseudocolored response maps visualizing the relative increase in fluorescence during odorant presentation (horizontal black line) compared to pre-odorant baseline. Each spot corresponds to an olfactory bulb glomerulus. Traces show change in fluorescence for the specified glomerulus during entire trial.

The pathophysiological effects of cadmium exposure were strongly dose-dependent (one-way ANOVA, $p = 0.001$). As shown in Figures 4 and 5A, intranasal exposure to 2 μg cadmium chloride significantly changed the distribution of glomerular responses between cadmium-exposed and vehicle-exposed bulbs (Fig. 5B, middle; Kolmogorov-Smirnov test, $p = 0.013$). The number of responsive glomeruli in the cadmium-exposed bulb was significantly reduced to $53\% \pm 9\%$ of the number on the vehicle-exposed side (one-sample t-test, $p = 0.012$). The amplitude of the observed responses was also significantly reduced (Mann-Whitney U-test, $p < 0.001$). Exposure to 0.2 μg cadmium chloride did not significantly change the overall distribution of responses, which takes into account both the number and size of the observed responses (Fig. 5B, lower; Kolmogorov-Smirnov test, $p = 0.054$). The number of glomeruli receiving ORN input was not reduced (one-sample t-test, $p = 0.51$). However, the

amplitudes of these responses were slightly but significantly reduced (Mann-Whitney U-test, $p = 0.032$).

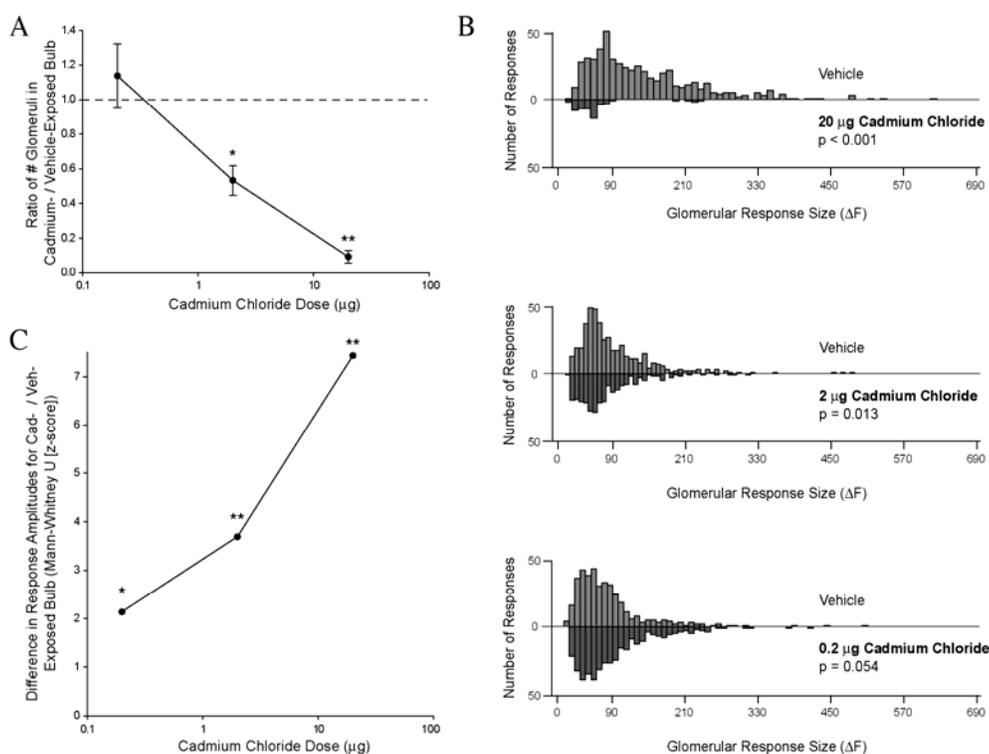


Figure 5. Intranasal cadmium instillation reduces the number of glomeruli receiving odorant-evoked sensory input and the magnitude of the synaptic input. (A) Ratio of the number of olfactory bulb glomeruli receiving sensory input from the olfactory nerve on the cadmium-exposed side compared to the vehicle-exposed side, as a function of cadmium chloride dose. The dashed line at 1 represents no difference between olfactory bulbs. (B) Frequency-distribution of glomerular response amplitudes in vehicle-exposed (above x-axis) and cadmium-exposed (below x-axis) olfactory bulbs broken out by cadmium chloride dose. (C) Plot of Mann-Whitney U |z-scores| for the distributions shown in part B with dose across the x-axis. * denotes $p < 0.05$, ** denotes $p < 0.001$.

5.2 Cadmium exposure mildly damages the connectivity between the olfactory epithelium and olfactory bulb

In histological sections the vesicular pH gradient is neutralized, permitting the use of spH fluorescence as a simple indicator of OMP expression and thus a useful marker for ORN axonal projections (Slotnick et al., 2001). The DAPI stain visualizing rings of periglomerular interneurons around glomeruli provided an unbiased marker for the

selection of glomerular layer regions of interest. As shown in Fig. 6, in mice exposed to 20 μg cadmium chloride, the optical density of GFP fluorescence in the glomeruli of cadmium-exposed olfactory bulbs was significantly reduced to $80 \pm 4\%$ of that in the contralateral, vehicle-exposed bulbs (one-sample t-test, $p = 0.003$). No significant reductions were observed in mice exposed to the 2.0 μg ($p = 0.185$) or 0.2 μg ($p = 0.253$) doses (Fig. 6B). These results suggest that at our highest concentration, intranasal cadmium exposure can induce a reduction in axonal projections from the olfactory epithelium to the olfactory bulb.

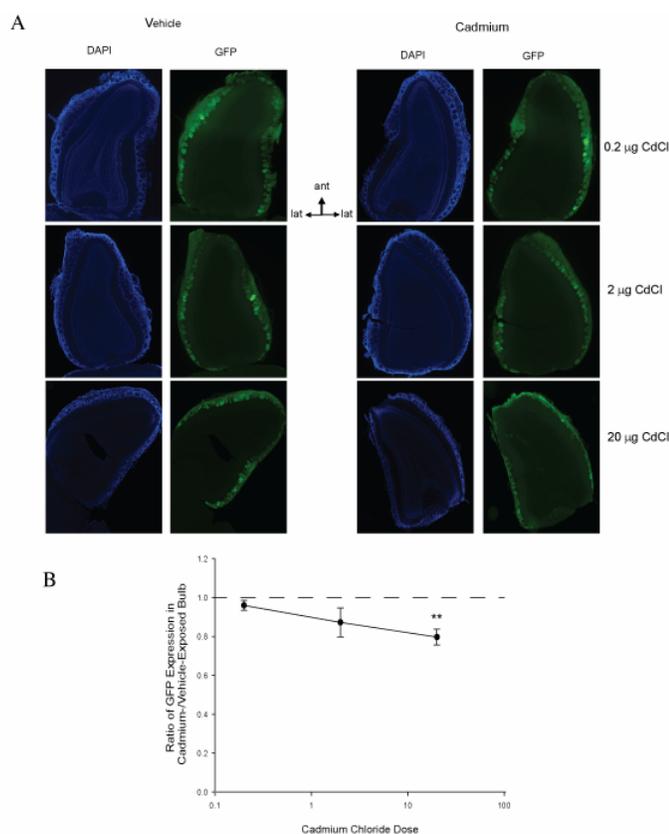


Figure 6. Axonal projection density from the olfactory epithelium to the olfactory bulbs is reduced at the highest cadmium dose tested. (A) Representative sections of cadmium- and vehicle-exposed olfactory bulbs across cadmium chloride doses. DAPI nuclear stain marks rings of periglomerular interneurons, while GFP is exclusively expressed in ORN axons from the olfactory epithelium into olfactory bulb glomeruli. (B) The ratio of GFP optical density from cadmium- compared to vehicle-exposed olfactory bulbs as a function of cadmium chloride dose. The dashed line at 1 represents to change in GFP expression between bulbs. ** denotes $p < 0.01$.

5.3 Intranasal cadmium exposure profoundly impairs odor detection

To investigate the functional and perceptual significance of the cadmium-induced pathophysiology, we evaluated the effects of intranasal cadmium exposure on mice trained to perform an odor detection go/no-go task. After reaching a criterion DM of 0.5 (equivalent to 75% correct) for three consecutive days, each mouse was randomly assigned to receive a bilateral intranasal infusion of either 20 μg CdCl_2 or vehicle. Mice were returned to the operant task on the second day after the infusion, and their detection performance was recorded.

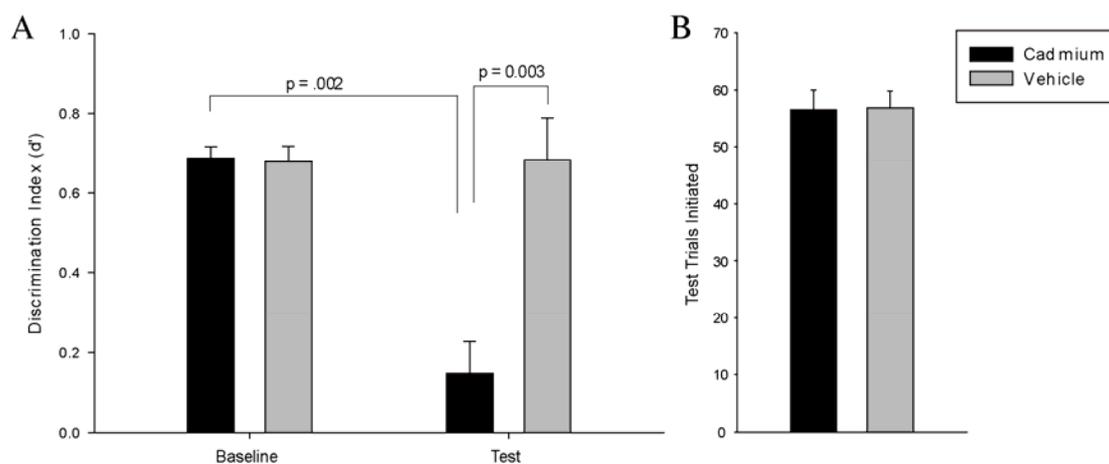


Figure 7. Performance on an olfactory detection task approaches zero in mice receiving bilateral intranasal instillations of cadmium. (A) Performance of cadmium- and vehicle-exposed mice on an odorant detection task before and after intranasal instillations. (B) Average number of trials initiated during the testing session two days after instillations.

As shown in Fig. 7A, intranasal exposure to cadmium chloride reduced performance on the detection task from an average pre-instillation DM of 0.69 ± 0.03 to an average of 0.15 ± 0.08 , while vehicle infusion had no effect. A mixed model ANOVA on the results (test day within subjects, instillation group between subjects) revealed the hypothesized test day by instillation group interaction ($F = 10.43$, $p = 0.01$, $\eta_p^2 = 0.54$) and simple main effects of instillation group ($F = 25.38$, $p = 0.001$, $\eta_p^2 = 0.74$) and test day ($F = 10.24$, $p = 0.011$, $\eta_p^2 = 0.53$). To follow up on the interaction, an independent

samples t-test revealed that the average baseline discrimination index of the cadmium animals was not significantly different ($p = 0.87$) from the baseline average performance of control animals. On test day after instillation, the average *DM* of the cadmium-treated animals was significantly lower ($p = 0.003$) than the average performance of control animals indicating impaired odor detection. This represents a significant reduction in cadmium-treated animal's odor detection performance on test day compared to their own average baseline performance (paired t-test, $p = 0.002$). Vehicle instilled animals did not show this reduction on test day compared to baseline (paired t-test, $p = 0.985$). The performance of the cadmium-infused animals was not significantly different from zero (one-sample t-test, $p = 0.11$), demonstrating a complete lack of discrimination between odorant-present and odorant-absent cues.

In principle, our experimental design controls for global changes in motivation because each odor discrimination trial only occurs when the mouse initiates it by nose-poking into the odorant-delivery port. We also observed no significant differences in the number of trials initiated by cadmium-infused mice compared to vehicle controls (independent samples t-test, $p = 0.96$), see Fig. 7B. Because the cadmium-infused mice were just as engaged in the discrimination task but entirely failed to discriminate despite being punished for wrong answers, we conclude that they were no longer able to detect the odorant.

6. Discussion

Here, we used *in vivo* optical imaging to show that one intranasal instillation of 20 μg cadmium chloride reduces odorant-evoked neurotransmitter release from the olfactory nerve by 91% compared to control. At the same dose, there is only a 20% reduction in

ORN axonal innervation from the olfactory epithelium to olfactory bulb glomeruli. While there was a significant reduction in the number of glomeruli receiving odorant-evoked neurotransmitter release after exposure to 2 μg cadmium chloride and a small, but significant reduction in the amplitude of odorant-evoked responses after 0.2 μg exposure, neither of these doses caused a reduction in the density of ORN axonal projections when analyzed in histological sections. Behaviorally, mice bilaterally exposed to 20 μg cadmium chloride were significantly impaired in a previously trained go/no-go odorant-detection task two days following instillation. This was not an effect of sickness behavior, as the cadmium and vehicle-exposed animals initiated the same number of trials during the post-instillation testing session. Taken together, these data show a significant effect of acute exposure to cadmium chloride in both pathophysiology and odorant-guided behavior.

Cadmium can pharmacologically block voltage-sensitive calcium channels (Swandulla & Armstrong, 1989; Chow, 1991) or enter neurons through L- or T-type calcium channels (Hinkle et al., 1987; Hinkle et al., 1992; Leslie et al., 2002), both of which are expressed in the olfactory epithelium (Gautam et al., 2007; Miyamoto et al., 1992). Though speculative, this may inform future investigations of the mechanism causing the cadmium-induced dysfunction of neurotransmitter release presented here. Our results showing a reduction in neurotransmitter release by glutamatergic ORNs is consistent with previous work using *in vivo* microdialysis in the central amygdaloid nuclei to show a significant reduction of glutamate release in response to perfusion with 30 μM CdCl_2 in artificial CSF (Minami et al., 2001). The current experiments extend this idea and visualize first order sensory neurons in order to elucidate the mechanism behind

hyposmia and anosmia documented in human cases of cadmium toxicity through inhalation exposure.

The OSHA permissible limit of cadmium in workplace air is $5 \mu\text{g}/\text{m}^3$ averaged over 8 hours. While cadmium exposure occurs in many industrial settings, perhaps the most widespread route of exposure is through cigarette smoke. Cadmium content in cigarettes varies, from about $0.28 - 3.38 \mu\text{g}$ per cigarette (ADSDR, 2008). It has been estimated that smokers are exposed, on average, to $1.7 \mu\text{g}$ of cadmium per cigarette and about 10% of that content is inhaled (ATSDR, 2008). Smoking increases the incidence of olfactory deficits, with severity of impairment related to duration and number of cigarettes smoked (Frye et al., 1990; Katotomichelakis et al., 2007; Vennemann et al., 2007). It is difficult to draw a direct dose comparison between human exposure from chronic inhalation and direct intranasal instillation in liquid dilution performed here. Species also differ in tidal volume, epithelial size, and other variables that may impact transport of inhaled cadmium. However, with the large range of doses investigated here and the estimates of cadmium contained in cigarette smoke or industrial exposure, we believe environmentally relevant concentrations were encompassed in this experiment.

Perhaps the most important finding presented here is the disparity between functional and anatomical changes after acute exposure to cadmium chloride. Previous experiments have shown reversible impairments in odorant-guided behavior tasks and histological damage following intranasal instillation of $400 \mu\text{g}$ cadmium chloride (Bondier et al., 2008). Here, we show pathophysiological effects after an acute exposure to $0.2 \mu\text{g}$ cadmium chloride. The use of optical imaging revealed a deficit in olfactory nerve functioning at a lower dose compared to previous reports and even when compared

to histological measurements within this experiment. Optical imaging was one hundred fold more sensitive to the effects of cadmium than histological measurement of axonal projections in the same animals. While morphological connections may be intact, they may not be functional. Therefore, a histological evaluation showing no difference in anatomical connections in control versus exposed tissue may be missing potentially large deficits in neuronal functioning. It is these functional deficits that may be most explanatory of toxicant-induced sensory deficits, as the one shown here in an odorant detection go/no-go task. The sensitivity of *in vivo* optical imaging makes it a potentially invaluable technique to evaluate damage after exposure to any putative toxicant that can be delivered intranasally.

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