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Vanadium Exposure Induces Olfactory Dysfunction in an Animal Model of Metal Neurotoxicity

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Abstract

Epidemiological evidence indicates chronic environmental exposure to transition metals may play a role in chronic neurodegenerative conditions such as Parkinson's disease (PD). Chronic inhalation exposure to welding fumes containing metal mixtures may be associated with development of PD. A significant amount of vanadium is present in welding fumes, as vanadium pentoxide (V_2O_5), and incorporation of vanadium in the production of high strength steel has become more common. Despite the increased vanadium use in recent years, the neurotoxicological effects of this metal are not well characterized. Recently, we demonstrated that V_2O_5 induces dopaminergic neurotoxicity via protein kinase C delta (PKCδ)-dependent oxidative signaling mechanisms in dopaminergic neuronal cells. Since anosmia (inability to perceive odors) and non-motor deficits are considered to be early symptoms of neurological diseases, in the present study, we examined the effect of V_2O_5 on the olfactory bulb in animal models. To mimic the inhalation exposure, we intranasally administered C57 black mice a low-dose of 182 μg of V_2O_5 three times a week for one month, and behavioral, neurochemical and biochemical studies were performed. Our results revealed a significant decrease in olfactory bulb weights, tyrosine hydroxylase (TH) levels, levels of dopamine (DA) and its metabolite, 3, 4-dihydroxyphenylacetic acid (DOPAC) and increases in astroglia of the glomerular layer of the olfactory bulb in the treatment groups relative to vehicle controls. Neurochemical changes were accompanied by impaired olfaction and locomotion. These findings suggest that nasal exposure to V_2O_5 adversely affects olfactory bulbs, resulting in neurobehavioral and neurochemical impairments. These results expand our understanding of vanadium neurotoxicity in environmentally-linked neurological conditions.

Keywords

vanadium; metals; olfactory system; neurotoxicity; non-motor symptoms; risk assessment; Parkinson's disease

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Conflict of interest
The authors declare that there are no conflicts of interest.

Introduction

Metal exposure has been considered a major chemical risk factor in the pathogenesis of chronic neurodegenerative conditions such as Parkinson's disease (PD) (Dobson et al., 2004, Aschner et al., 2009, Furbee, 2011, Caudle et al., 2012, Kanthasamy et al., 2012). PD imposes an estimated economic burden of $23 billion per year in the United States alone (Weintraub et al., 2008). Multifactorial etiology is associated with progressive and substantive degeneration of nigral dopaminergic neurons and extra-nigral neurons underlying PD (Anglade et al., 1997, Braak and Braak, 2000, Braak et al., 2000, Allam et al., 2005, Przedborski, 2005, Przedborski and Ischiropoulos, 2005). The cause and mechanism of the disease's progression are poorly understood and have not yet been exhaustively explored. Recently, many non-motor symptoms have been determined to precede the onset of motor symptoms and are considered hallmark in the early stages of PD. A non-motor, early stage symptom of PD is impaired olfactory function (Ansari and Johnson, 1975, Langston, 2006, Goldstein et al., 2010). However, the effect of environmental neurotoxic metals on the olfactory system has not been well characterized.

Case-control and epidemiological studies have linked metal exposure to the increased incidence of PD (Fleming et al., 1994, Schulte et al., 1996, Gorell et al., 1997, Liou et al., 1997, Marder et al., 1998, Smargiassi et al., 1998, Taylor et al., 1999, Priyadarshi et al., 2000, Ritz and Yu, 2000). Studies have shown that welders have an increased risk of developing PD (Racette et al., 2001, Park et al., 2005). Manganese (Mn), which is typically present in welding fumes mixed with other metals including vanadium, is the major metal that has been studied with respect to PD (Aschner et al., 2007, Aschner et al., 2009, Guilarte, 2010). Mn mainly targets the basal ganglia comprising the caudate nucleus, putamen, globus pallidus, substantia nigra, and subthalamic nucleus (Eriksson et al., 1992, Calne et al., 1994, Brenneman et al., 1999, Nagatomo et al., 1999, Aschner et al., 2009). Neurotoxicity resulting from excessive Mn exposure is distinct from sporadic PD in that the globus pallidus appears to be the most severely affected of all of the basal ganglia regions (Verity, 1999). However, studies have shown that dopamine (DA), which is the principal neurotransmitter in the striatum that is severely depleted in PD patients, is also decreased by Mn, with both *in vivo* (Parenti et al., 1986) and *in vitro* (Vescovi et al., 1991) exposure paradigms in animals. Dorman et al. reported the accumulation of $MnSO_4$ in the olfactory bulb and striatum of inhalation-exposed rats relative to controls (Dorman et al., 2001).

The rapid growth and modernization of U.S. cities are dependent on ever-changing infrastructures. Central to the evolution of these structures is welding, one of the primary anthropogenic sources of environmental metals. Vanadium, typically present in welding fumes as vanadium pentoxide (V_2O_5), is emitted by welding rods commonly used in construction. Vanadium is also widely used in various steelmaking industrial applications, such as plane and ship building, in the production of temperature-resistant alloys and glass, and in pigment and paint manufacturing (McNeilly et al., 2004). Also, large quantities of vanadium compounds are released into the environment mainly through the burning of fossil fuels, with vanadium reported as the most abundant trace metal in petroleum samples (Amorim et al., 2007). Vanadium accumulates in soil, groundwater, and plants, and is consumed by animals and humans (Pyrzynska and Weirzbicki, 2004). The processing of

vanadium slag (about 120 g/kg of vanadium pentoxide) generates dust, with vanadium concentrations ranging from 30 to 120 mg/m^3 (IARC, 2006). Crude oil from Venezuela is believed to have the highest vanadium concentration, ranging up to 1400 mg/kg. Fifty percent vanadium pentoxide has been discovered in flue-gas deposits from oil-fired furnaces (IARC, 2006). Elevated levels of vanadium (4.7 mg/m^3) have been found in the breathing air of steel industry workers (Kiviluoto et al., 1979). Vanadium exposure to humans has been shown to cause motor deficits (Done, 1979, WHO, 2000). Thus, the growing use of vanadium in a wide variety of applications warrants the full characterization of its neurotoxicological properties.

Chronic exposure to environmental toxicants, including herbicides, pesticides, solvents, and heavy metals, can alter the ability to smell (Doty and Hastings, 2001), with the best documented metal in this regard being cadmium, chromium, nickel, and manganese. Further, Avila-Costa et al. observed that inhaled V_2O_5 damages the nigrostriatal dopaminergic systems in rodent models (Avila-Costa et al., 2004). In a recent study, we showed that vanadium is neurotoxic to dopaminergic neurons in cell culture models (Afeseh Ngwa et al., 2009). In the present study, we further examine the neurotoxic properties of vanadium, specifically focusing on its effects on the olfactory bulb to determine whether subchronic nasal exposure impairs neurobehavioral and neurochemical processes associated with olfactory function.

Materials and Methods

Chemicals

Vanadium pentoxide (V_2O_5) salt, protease cocktail inhibitor, phosphatase inhibitors and anti-β-actin antibody were purchased from Sigma (St. Louis, MO). A Bradford protein assay kit was purchased from Bio-Rad Laboratories (Hercules, CA). Mouse monoclonal antibodies against tyrosine hydroxylase (TH) and GFAP were obtained from Millipore (Upstate, Billerica, MA, USA) and Cell Signaling Technology, Inc. (Danvers, MA), respectively. The anti-mouse and anti-rabbit secondary antibodies (Alexa Fluor 680 conjugated anti-mouse IgG and IRdye 800 conjugated anti-rabbit IgG) were purchased from Invitrogen and Rockland Inc., respectively.

Treatment paradigm

Six to eight week old male C57BL/6 mice were housed at room temperature under a 12 h light/dark cycle. The control and treatment animals were age-matched. Food and water were provided *ad libitum* and animal weights were monitored. Animals were cared for in accordance with institutional animal care guidelines. A previous study exposed mice to 5–20 mM V_2O_5 through inhalation route and examined neurotoxic effects of the metal (Avila-Costa et al., 2005, Fleming et al., 2008). In the present study, we used a low dose of 182 μg of V_2O_5 in 50 μL of de-ionized water and administered intranasally three times a week for period of one month. The vanadium pentoxide was administered to mice intranasally using micropipettes after briefly anaesthetizing the mice with isoflurane to prevent a gag reflex. The control animals received equal volumes of pH-adjusted deionized water (pH ~ 2.0 for V_2O_5 solution). Following the treatment, mice were subjected to behavioral and

neurochemical tests one week following last dose of V_2O_5. Intranasal delivery was chosen because vanadium exposure mostly occurs through inhalation route. Intranasal delivery of chemicals takes advantage of an incomplete blood brain barrier in the olfactory epithelium (Graff and Pollack, 2005). The olfactory nerves bypass the blood brain barrier, therefore chemicals can be taken up by these neurons and transported directly into the brain (Graff and Pollack, 2005).

Olfaction test

The ability of mice to detect pheromones from female bedding by sniffing was used as a measure of olfaction, as described in previous studies (Fleming et al., 2008, Kim et al., 2011). This test combines the principle behind the wooden block test, which relies on the ability of mice to discriminate between self and non-self odors (Fleming et al., 2008, Kim et al., 2011), and on the knowledge that female body odor and urine attract males (Lucas et al., 1982, Singer et al., 1988). For this test, the bedding from a mouse cage housing pregnant females was introduced in the cage of the male mice used in this study. The amount and location of bedding were kept constant each time. The total time spent sniffing the female bedding material was measured using a stop watch during a five minute testing session. Both vanadium-treated and control mice were subjected to the test at the same day. This test is an easy and effective measure of an animal's olfactory capacity to detect a novel odor.

HPLC detection of dopamine and its metabolites in olfactory bulb

Following the completion of treatment, mice were sacrificed and the olfactory bulbs were dissected out at the junction between the olfactory bulbs and the rest of the brain for each mouse. The dissected olfactory bulbs were weighed. The differences in weight of olfactory bulb between control and vanadium exposed animals were determined. Levels of dopamine (DA) and its metabolite DOPAC in olfactory lobe tissues were determined by high-performance liquid chromatography (HPLC) with electrochemical detection. The samples were prepared as described previously (Zhang et al., 2007, Ghosh et al., 2013). Briefly, neurotransmitters were extracted from olfactory bulbs using an antioxidant extraction solution (0.1 M perchloric acid containing 0.05% Na_2EDTA and 0.1% $Na_2S_2O_5$). The extracts then were filtered in 0.22-μm spin tubes, and 100 μl of each sample were loaded for analysis at a 1:2 dilution in mobile phase buffer. DA and DOPAC were separated isocratically by a reversed-phase column with a flow rate of 0.7 mL/min. An HPLC system (ESA Inc., Bedford, MA) with an automatic sampler equipped with a refrigerated temperature control (model 542; ESA Inc.) was used for these experiments. The electrochemical detection system was composed of a Coulochem model 5100A with a microanalysis cell (model 5014A) and a guard cell (model 5020) (ESA Inc.). Standard stock solutions of catecholamines were prepared at 1 mg/ml in antioxidant solution, and then further diluted to a final working concentration of 50 pg/μL before injection. Data acquisition was performed using EZChrome HPLC Software (ESA Inc.) and analyzed using Microsoft Excel and Prism 4.0 software (GraphPad Software Inc., San Diego, CA). The DA and DOPAC levels were quantified as ng/mg of protein.

Western blot

Olfactory bulb lysates from control and treatment groups, containing equal amounts of protein, were loaded in each lane and separated on a 10 to 12% SDS-polyacrylamide electrophoresis gel, as described previously (Kanthasamy et al., 2006, Jin et al., 2011a, Jin et al., 2011b, Latchoumycandane et al., 2011). After this separation, the proteins were transferred to a nitrocellulose membrane, and nonspecific binding sites were blocked by treating with Odyssey blocking buffer (Licor Biosciences). The membranes with transferred proteins were then incubated with primary antibody directed against TH (mouse monoclonal; 1:1000). The primary antibody incubations were followed by incubation with either Alexa Fluor 680 conjugated anti-mouse or IRDye 800 conjugated anti-rabbit secondary antibody for 1 h at room temperature. To confirm equal protein loading in each lane, membranes were probed with β-actin antibody (1:5000 dilution). Western blot images were captured and analyzed with an Odyssey IR Imaging system (LI-COR). Densitometric analysis was performed on the TH bands.

Locomotor activity

Locomotor behavioral data were collected using VersaMax animal activity monitors (model RXYZCM-16; Accuscan Instruments Inc., Columbus, OH), as previously described by (Zhang et al., 2007, Ghosh et al., 2013). The clear Plexiglas chamber has dimensions of 40 × 40 × 30.5 cm, and is covered with a ventilated Plexiglas lid. Infrared monitoring sensors are located every 2.54 cm along the full perimeter of the square chamber (16 infrared beams along each side). On two opposite walls, sensors are 2.5 cm above the floor and on the other two opposite walls, sensors are located 8.0 cm above the floor. Data were collected and analyzed by a VersaMax analyzer (model CDA-8; Accuscan Instruments Inc.). Several parameters of locomotor activity are presented here. All raw data are expressed as percentage of the vehicle control group (mean ± S.E.M.; $n = 5$) and were obtained one week post-treatment.

Immunohistological analysis of olfactory bulb sections

Tyrosine hydroxylase immunolabeling was performed in olfactory bulb sections. Briefly, one week after the last dose of vanadium, mice were sacrificed and intracardiac perfusion was performed with 4% paraformaldehyde (PFA) and subsequently post-fixed with PFA and 30% sucrose. The fixed olfactory lobes of the perfused brains were then cut using a cryostat into 30-μm coronal sections and kept at −20 °C in a cryosolution of 30% sucrose-ethylene glycol. Sections were rinsed with PBS on the day of staining, and the free-floating sections blocked with 2% bovine serum albumin, 0.5 % Triton X-100 and 0.05% Tween-20 in PBS for 1 h at room temperature. Following blocking, the sections were incubated in either anti-GFAP or anti-TH primary antibody (Calbiochem mouse anti-rabbit, 1:1600) overnight at room temperature. The sections were washed in PBS and incubated for 90 min at room temperature with Alexa Fluor 488/568 anti-mouse secondary antibody. After washing the sections in PBS, they were incubated with 10 μg/ml Hoechst 33342 for 5 min at room temperature stain the nucleus. Sections were carefully mounted on Poly-L-lysine coated slides with the organic solvent DPX, then dehydrated by being kept for one minute each in water, 70% ethanol, 95% ethanol, 100% ethanol and Xylene in that order. The sections were

viewed under an inverted fluorescence microscope (Nikon TE-2000U; NIKON, Tokyo, Japan). Images (2×, 30× and 60×) were captured with a SPOT digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI) using MetaMorph software, version 5.0 (Molecular Devices, Sunnyvale, CA).

Data analysis

Data analysis was performed using Prism 4.0 software (GraphPad Software Inc., San Diego, CA). Raw data were analyzed using a two-tailed unpaired student's t-test. Statistically significant differences are indicated by asterisks as follows: * $p<0.05$, ** $p<0.01$, and *** $p<0.001$.

Results

Intranasal vanadium exposure induces locomotor deficits

C57 black mice were administered V_2O_5 (182 μg) intranasally three times a week for one month. One month after initiating intranasal administration of vanadium, we evaluated the effect of the treatment on locomotor activity by measuring motor deficits using the VersaMax automated activity monitor. As shown in Fig. 1, significant decreases were observed in the various locomotor activities in the vanadium treatment group relative to the control. Fig. 1A shows the representative locomotor activity map of control and vanadium treated mice. Quantitative analysis indicated a decrease in motor activities in the vanadium treated mice relative to controls: 68% decrease in total vertical movement (Fig. 1B), 27% decrease in total horizontal movement (Fig. 1C), 57% decrease in total distance travelled (Fig. 1D), 54% decrease in total movement time (Fig. 1E), and a 7% increase in rest time (Fig. 1F).

Intranasal vanadium exposure induces olfaction deficits

After measuring motor deficits, we determined the effect of vanadium on olfaction. We tested the ability of the male mice to detect female pheromones by measuring their ability to find and sniff bedding taken from pregnant female cages. As shown in Fig. 2, relative to controls, the vanadium treatment group spent 63% less time sniffing female bedding during a 5-minute testing session, indicating impaired olfaction following vanadium exposure.

Intranasal vanadium exposure causes a decrease in the weight of olfactory bulbs

It has been observed that olfactory bulb volumes tend to decline with decreased smell function (Yousem et al., 1999, Rombaux et al., 2006). To determine the effect of intranasally administered vanadium on the volumes of olfactory bulbs, we dissected the olfactory bulbs and weighed each side. As shown in Fig. 3, we observed a 26% reduction in olfactory bulb weights in the vanadium treatment group relative to the control group.

Intranasal vanadium exposure causes a decrease in tyrosine hydroxylase (TH) levels in the olfactory bulb

The glomerular layer of the olfactory bulb has been reported to have an abundance of dopaminergic neurons (Halasz et al., 1981, Davila et al., 2003). Dopamine is believed to

play an important role in olfaction (Hsia et al., 1999). Tyrosine hydroxylase (TH) is the rate-limiting enzyme responsible for synthesizing dopamine, and therefore, TH is used as a marker of dopaminergic neuronal integrity. First, we used Western blots to measure changes in TH levels in the dissected olfactory bulb, following intranasally administered vanadium. As depicted in Fig. 4A, we observed a dramatic reduction in TH levels in the vanadium-treated olfactory bulbs. The changes in TH levels corresponded to an 85% decrease in the vanadium-treated olfactory bulbs compared with the control group, as shown by densitometric analysis of the Western blot (Fig. 4B). Beta-actin was used as a loading control for Western blot studies.

Next we examined the vanadium exposure on the integrity of dopaminergic neurons in the glomerular layer of olfactory bulb. As shown in Fig. 5A, a dense distribution of TH positive neurons was noted throughout the glomerular layer in control mice. However, a dramatic loss of TH positive neurons was observed in the glomerular layer of vanadium treated mice. Higher magnifications (Fig. 5B: 30×, Fig. 5C: 60×) clearly revealed the extent of dopaminergic neuronal loss in vanadium treated olfactory bulb compared to control olfactory bulb. Collectively, both Western blot and immunohistochemical studies indicated that vanadium exposure dramatically affects dopaminergic neurons in olfactory bulb.

Intranasal vanadium exposure induces dopamine depletion in the olfactory bulb

Since we found significant reductions in TH in the olfactory bulb following intranasally administered vanadium, we proceeded to measure the neurochemical changes in the dissected olfactory bulbs using HPLC. We observed that the level of DA was reduced by 82% (Fig. 6A), and that of its metabolite DOPAC by 88% (Fig. 6B), relative to control group. Together, these results show that vanadium induces a significant dopaminergic neurochemical deficit in the olfactory bulb.

Subchronic intranasal exposure to vanadium induces migration and accumulation of astrocytes in the glomerular layer of the olfactory bulb

Having shown the degeneration of olfactory bulb dopaminergic neurons in the glomerular layer, we probed for increased presence of astroglia in the same layer, as a marker and indicator of neurodegeneration and neuroinflammation. As shown in Fig. 7, GFAP immunostaining revealed a dramatic increase in astroglial proliferation in the glomerular layer of vanadium exposed olfactory bulb. No GFAP staining was detected in control animals. These results further confirmed that vanadium exposure induced degeneration processes in olfactory bulb.

Discussion

The effects of neurotoxic metals on the olfactory system are not well characterized. In light of recently observed correlations between olfactory dysfunction and environmentally linked neurodegenerative disorders, identification of the effects of neurotoxic metals on the olfactory bulb was warranted. The main findings of our study are that intranasal administration of a low dose of vanadium caused a significant reduction in a) tyrosine hydroxylase protein levels, b) dopamine levels and c) weight of olfactory bulbs. These

effects were accompanied by decreased ability of animals to detect pheromones in pregnant female bedding. In addition, intranasal vanadium exposure produced significant locomotor deficits. To our knowledge, this is the first report demonstrating adverse effects of vanadium exposure on the olfactory system.

Vanadium is one of the light metals with very high strength, and this unique property makes the metal desirable for high strength steel alloy industrial applications, including ship and aircraft manufacturing (Korchynsky, 2001). Elevated levels of vanadium in the air (4.7 mg/m^3) were reported in the breathing zones of steel industry workers (Kiviluoto et al., 1979) as well as in welding fumes (IARC, 2006). Vanadium is also used in the production of temperature-resistant alloys and glass, and in pigment and paint manufacturing. Through the Vanadium Technology Partnership, the United States Army is partnering with the vanadium industry to explore the potential applications of high-strength, light-weight and low-cost vanadium micro-alloyed steel in creating a stronger and lighter military. The program demonstrates that military support structures like vehicles, trailers, barriers and buildings can be improved in terms of protection and greater mobility through vanadium-alloying and hot-rolled steel technology. Another major source of environmental vanadium exposure is through use of fossil fuels. It has been reported that 12000 – 24000 tons of vanadium per year are generated from the burning of fossil fuels (Bertine and Goldberg, 1971), and given that fossil fuel demand has risen considerably since then (Huntington, 2010), the emission rate attributable to fossil fuel burning has likely risen dramatically. Also, vanadium concentrations have been reported in cigarette smoke to range from 0.49 to 5.33 μg/g (Adachi et al., 1998), and smoking has been linked to olfactory dysfunction (Frye et al., 1990, Martin et al., 2009).

Vanadium is known to be present in the earth's crust in average concentrations of about 150 μg/g, with soil concentrations ranging from 3 to 310 μg/g (Waters, 1977). In fact, concentrations as high as 400 μg/g have been documented in fly ash polluted areas (Bengtsson and Tyler, 1976). Urban areas have annual airborne vanadium concentration averaging from 0.05 to 0.18 $\mu g/m^3$, with maxima as high as 2 $\mu g/m^3$ occurring on the coldest winter nights in the most densely populated areas. These airborne vanadium concentrations have been on the rise in Europe, due mostly to the increased combustion of crude oil residues in community-heating systems and power plants (WHO, 2000).

Vanadium concentrations in drinking-water range from 0.2 to 100 μg/L (Vouk, 1979), with the estimated mean dietary intake of being 20 μg/day (Myron et al., 1977). Using the exposure commitment method of analysis, Davies and Bennett reported that a person living in a rural environment with an assumed airborne concentration of 8 ng/m^3 vanadium will have a body-burden of about 100 μg of vanadium (Davies and Bennett, 1983), over 80% of which is derived from the diet. Vanadium concentrations recorded and reported in urban areas range from 50 to 200 ng/m^3, which translate to a body burden of about 142–570 μg (Davies and Bennett, 1983). In this case, the inhalation route contributes more than half of the total body burden. The use of high-vanadium fuel-oil for heating during winter escalated concentrations to about 2000 ng/m^3 in bigger cities, hence an estimated body burden of over 5700 μg of vanadium following exposure. Vanadium concentrations observed in workplace air (0.01–60 mg/m^3) far exceed those reported for the general environment. Considering

more than half of the total environmental vanadium exposure is contributed through inhalation route (Davies and Bennett, 1983), we used intranasal exposure in our experiment. Thus, the dose of 182 μg vanadium used in our study is within the environmentally relevant range.

Our results show that vanadium affects the dopaminergic neurotransmitter system in the olfactory bulb. Olfactory system is important route of various neurotoxicant exposures. Our findings are consistent with results from other studies showing that the classic catecholaminergic neurotoxicants methamphetamine and amphetamine cause dopamine depletion in the olfactory bulb (Deng et al., 2007, Atianjoh et al., 2008). Another study showed both intranasal irrigation in mice with either ZnSO4 or Triton X-100 and surgical deafferentation or axotomy in rats are associated with decreased DA, DOPAC, TH enzyme activity, and olfactory bulb weights (Baker et al., 1983). These authors also found a clear correlation between reduction in TH, DA and DOPAC levels and reduced bulb weights (Baker et al., 1983). Agents like viruses, nanoparticles and prions are thought to enter the brain through the olfactory mucosa by damaging the olfactory epithelium. Olfactory receptor neurons have been shown in animal studies to take up and transport cadmium, gold, and manganese ions toward the olfactory bulbs at rates ranging from 2.5 to 3 mm/hour (Gottofrey and Tjalve, 1991, Tjalve et al., 1995, Tjalve et al., 1996, Doty, 2009). Antunes et al. carried out a study measuring olfactory function in welders employed in the construction of the San Francisco-Oakland Bay Bridge for durations ranging from 6 to 28 months. They concluded that professional welders may be at risk of losing their ability to smell properly (Antunes et al., 2007).

The olfactory bulb contains five layers, namely, the subependymal, combined mitral and granule cells, external plexiform and the glomerular layers (Lledo et al., 2006); the glomerular layer abundantly expresses dopaminergic neurons (Halasz et al., 1981, Davila et al., 2003). In the present study we show that vanadium exposure can decrease both TH and dopamine levels in the olfactory bulb. Dopamine plays an important role in olfaction (Doty and Risser, 1989, Wilson and Sullivan, 1995, Duchamp-Viret et al., 1997, Hsia et al., 1999, Koster et al., 1999), and changes in dopamine levels affect olfaction. Hsia et al. reported that dopamine regulates transmission between the olfactory bulb epithelium and the olfactory bulb glomeruli to mediate entry of olfactory information into the brain (Hsia et al., 1999). Consistent with this notion, our results indicate that the depletion of dopamine is accompanied by significantly decreased locomotion and decreased time spent sniffing pregnant female bedding. Thus, dopamine depletion in the olfactory bulb following intranasally administered vanadium may impair olfaction (odor detection) by disinhibition of neural transmission in olfactory glomeruli, leading to impaired olfactory processing. This observation may be relevant to clinical observations that patients who suffer from Parkinson’s disease experience hyposmia, which typically precedes motor deficits (Hawkes, 2003). The clinical assessment of olfactory deficits in PD patients is conducted by testing for odor identification, odor discrimination, threshold detection and odor recognition memory experiments (Mesholam et al., 1998). Parkinson’s disease patients have demonstrated impairments in odor detection, differentiation and identification (Ward et al., 1983, Doty et al., 1992, Tissingh et al., 1998). Most occupationally related environmental neurotoxicant exposures occur through the nostrils, and Avila-Costa and coworkers have shown that

vanadium enters the brain parenchyma following inhalation exposure (Avila-Costa et al., 2005). Furthermore, prolonged inhalation exposure to vanadium can damage the nigral dopaminergic system (Avila-Costa et al., 2004). Our study shows that depletion of dopamine in the olfactory bulb may contribute to the locomotor deficits that are characteristic of PD, suggesting that vanadium may have important effects in the nigrostriatal dopaminergic system. Although we did not find any significant change in either striatal dopamine or nigral TH+ neurons, the nigra did exhibit moderately increased oxidative damage, as measured by 4-hydroxy noneneal (4HNE), as well as slightly increased alpha-synuclein protein levels (data not shown). Future studies will address whether the nigrostriatal dopaminergic pathway is also affected following prolonged intranasal exposure to vanadium.

Behavioral and neurochemical results of our study correlated well with histological analysis of olfactory bulb. TH immunohistochemical analysis revealed a severe loss of dopaminergic neurons in granular layer of olfactory bulb. In addition to dopaminergic neuronal loss, we observed the migration and accumulation of astroglia in the glomerular layer of the olfactory bulb as measured by GFAP immunostaining. The GFAP staining and TH-positive dopaminergic neuronal loss was limited to the glomerular layer, indicating that dopaminergic neurons in glomerular layer are preferentially affected by intranasal exposure to vanadium. An increased number of astroglia has been observed in many neurodegenerative conditions including PD (Forno et al., 1992, Maragakis and Rothstein, 2006). Since glial cells play a role in neuroinflammatory processes (Tansey et al., 2008), it is possible that the observed glomerular dopaminergic neurodegeneration is accompanied by neuroinflammation. Further detailed studies examining microglia activation and other proinflammatory processes are required to implicate inflammatory mechanisms in vanadium neurotoxicity.

In summary, we observed that subchronic exposure to a low dose of vanadium via the intranasal route induces olfactory dysfunction which is characterized by decreased olfactory bulb volume and the loss of dopaminergic neurotransmission to the olfactory bulb. Our study reveals the neurotoxicity of vanadium to the olfactory system, and may be useful in future risk assessments and regulation of vanadium.

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Abbreviations

V_2O_5	vanadium pentoxide
Mn	manganese
PD	Parkinson’s disease
TH	tyrosine hydroxylase
OB	olfactory bulb

DA	dopamine
DOPAC	3, 4-dihydroxyphenylacetic acid
HPLC	high-performance liquid chromatography
PFA	paraformaldehyde
PKCδ	protein kinase C delta

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Highlights

- Low dose intranasal vanadium exposure induces locomotor impairment
- Intranasal vanadium exposure also causes profound olfactory deficits
- Intranasal vanadium exposure decreases the levels of TH protein and dopamine in the olfactory bulb
- Intranasal vanadium exposure also increases astroglial proliferation in the olfactory bulb

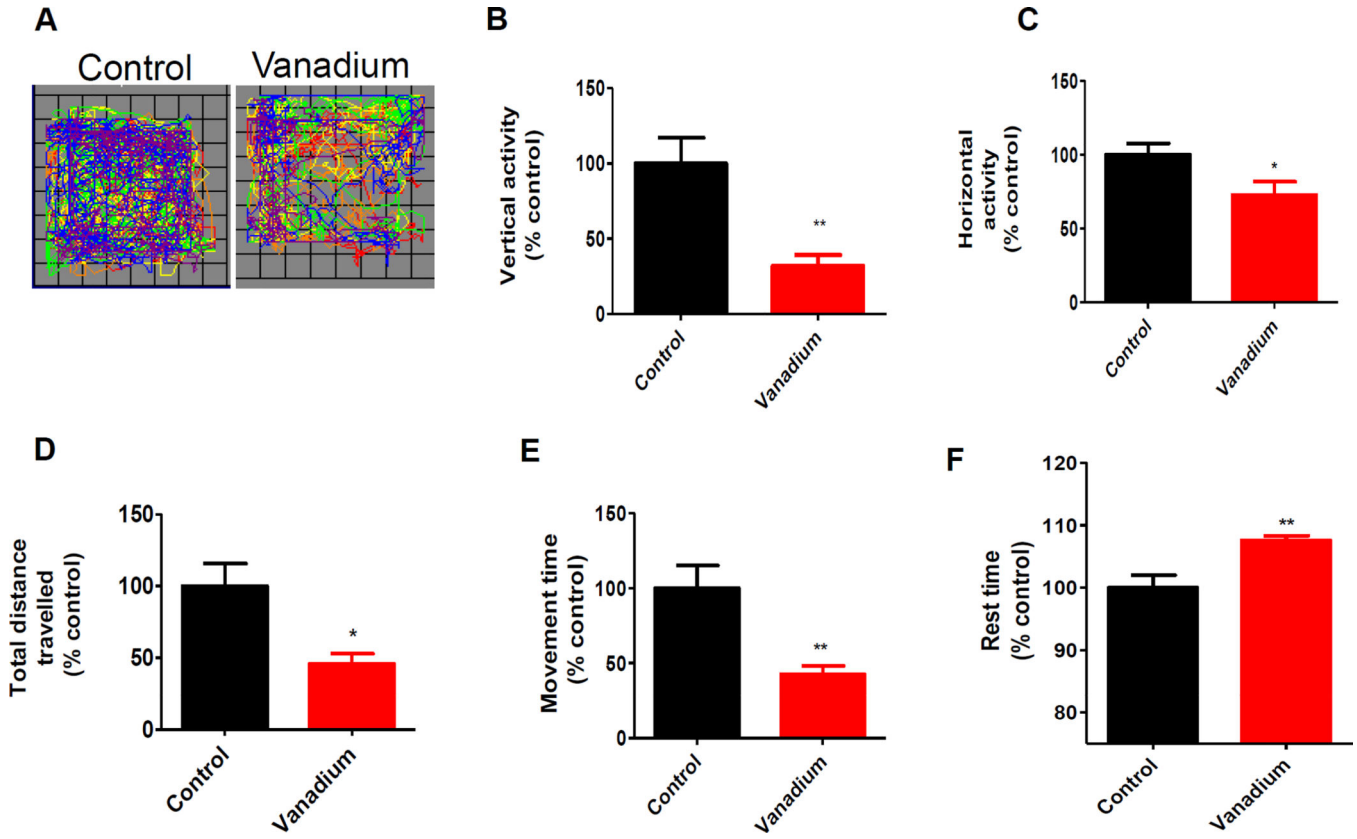

Fig. 1. Effects of intranasally administered vanadium on locomotor activity

Male C57 black mice were intranasally administered 182 μg of V_2O_5 in 50 μL of de-ionized water three times a week for one month. The vehicle control animals were administered de-ionized water. Locomotor activity was measured using an automated VersaMax locomotor activity monitor before the termination of the study. A, Representative moving tracks of mice. B, Total vertical movement. C, Total horizontal movement. D, Total distance travelled. E, Total movement time. F, Total rest time. The vehicle-treated group served as the control. Data are in percent control and represent mean ± S.E.M. from at least five animals per group. Asterisks (*, $p < 0.05$; **, $p<0.001$) indicate significant differences between treatment and the control group.

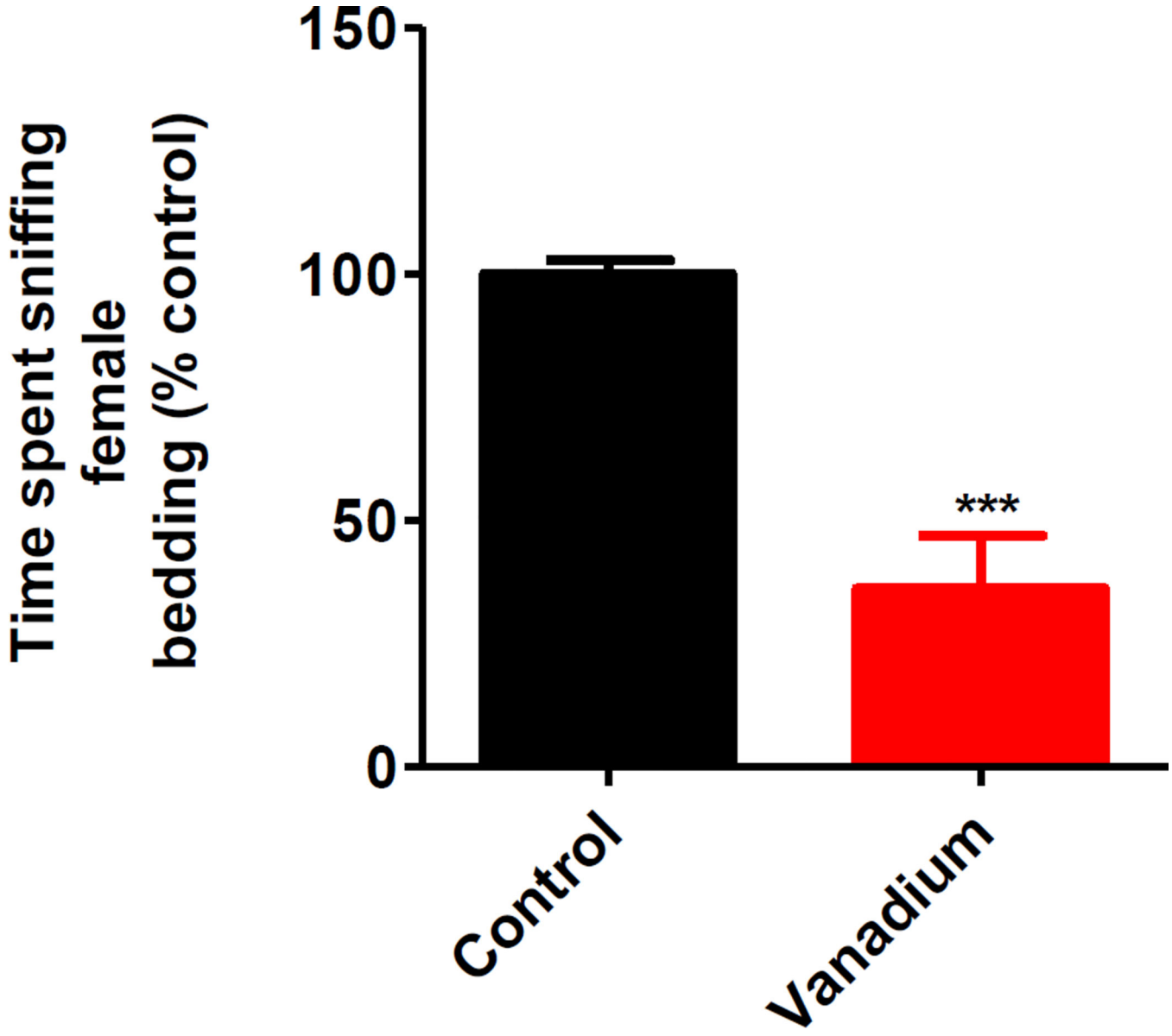

Fig. 2. Effects of intranasally administered vanadium on pheromonal olfaction (sniffing ability)

Male C57 black mice (n ≥ 5 per group) were intranasally administered 182 μg of V_2O_5 in 50 μL of deionized water three times a week for one month. The vehicle control animals were administered deionized water. At the end of the study, animals were exposed to bedding from pregnant female cages, and the amount of time males spent sniffing the bedding during a five-minute time period was recorded. Asterisks (***, $p < 0.001$) indicate a significant difference between treatment and control group means ± S.E.M.

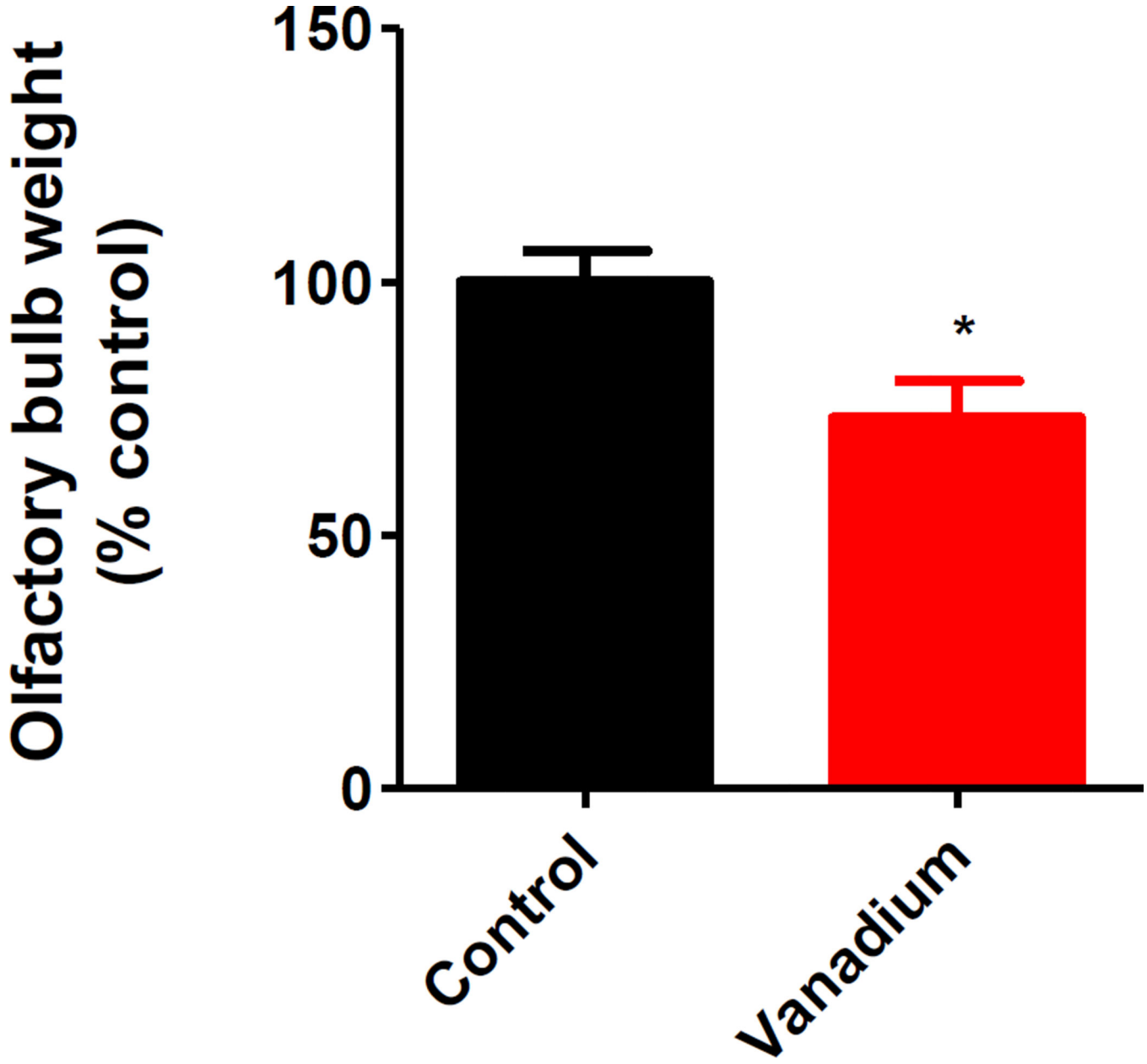

Fig. 3. Effects of intranasally administered vanadium on the size of olfactory lobes

Male C57 black mice (n ≥ 5 per group) were intranasally administered 182 μg of V_2O_5 in 50 μL of deionized water three times a week for one month. The vehicle control animals were administered deionized water. At the end of treatment, olfactory lobes were dissected out and weighed. Asterisks (*, $p < 0.05$) indicate a significant difference between treatment and control group means ± S.E.M.

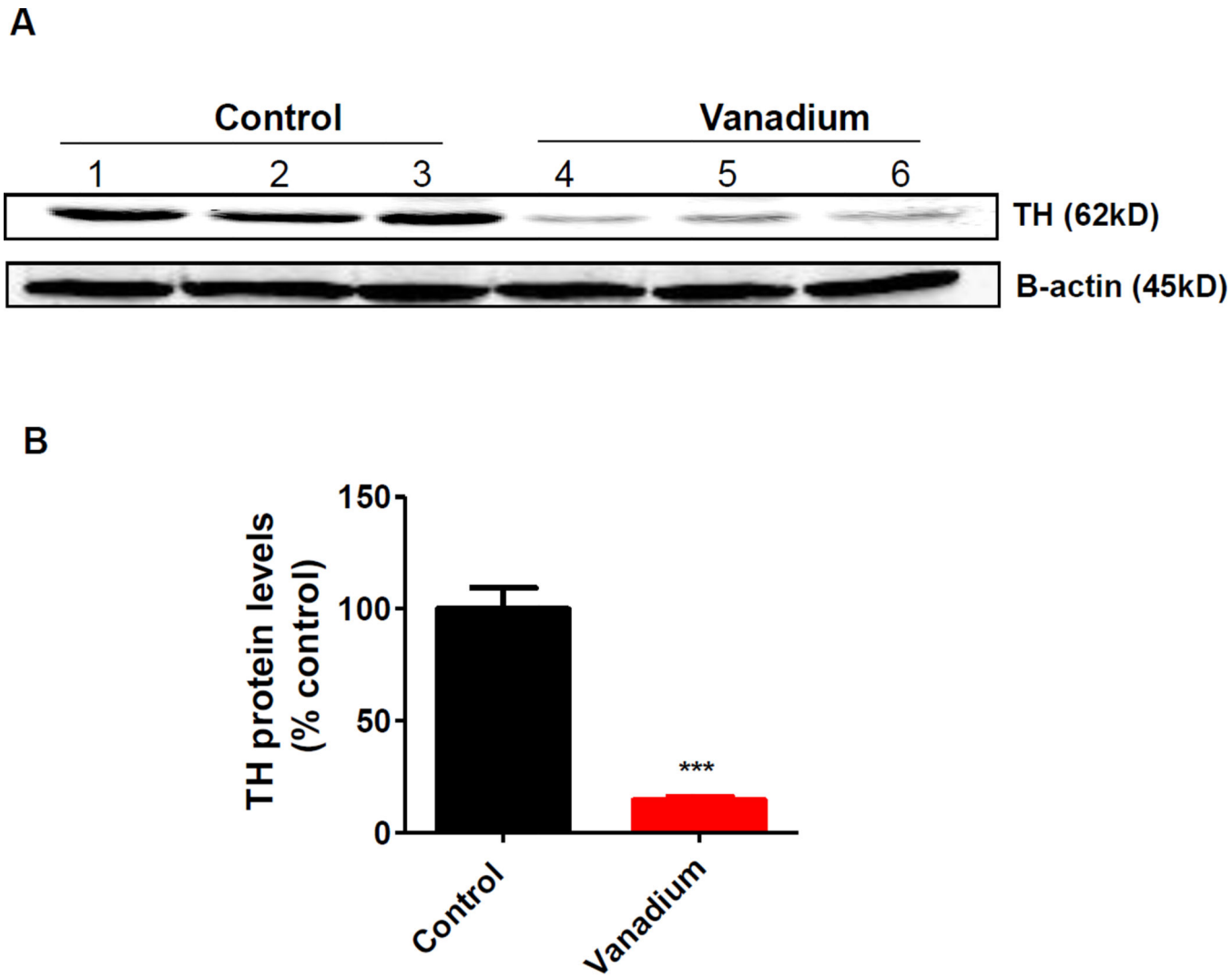

Fig. 4. Effect of intranasally administered vanadium on TH expression levels in olfactory lobes

Male C57 black mice (n ≥ 3 per group) were intranasally administered 182 μg of V_2O_5 in 50 μL of deionized water three times a week for one month. The vehicle control animals were administered deionized water. At the end of the treatment animals were sacrificed and TH expression was detected in the olfactory lobe by Western blot using mouse monoclonal antibody against TH. (A) A representative Western blot analysis of TH expression in control and vanadium treated mice. β-actin immunoblot was used to confirm equal protein loading in each lane. (B) The bands were quantified for densitometric analysis and data are expressed as a percentage of vehicle-treated bands. Asterisks (***, $p < 0.001$) indicate a significant difference between treatment and control group means ± S.E.M.

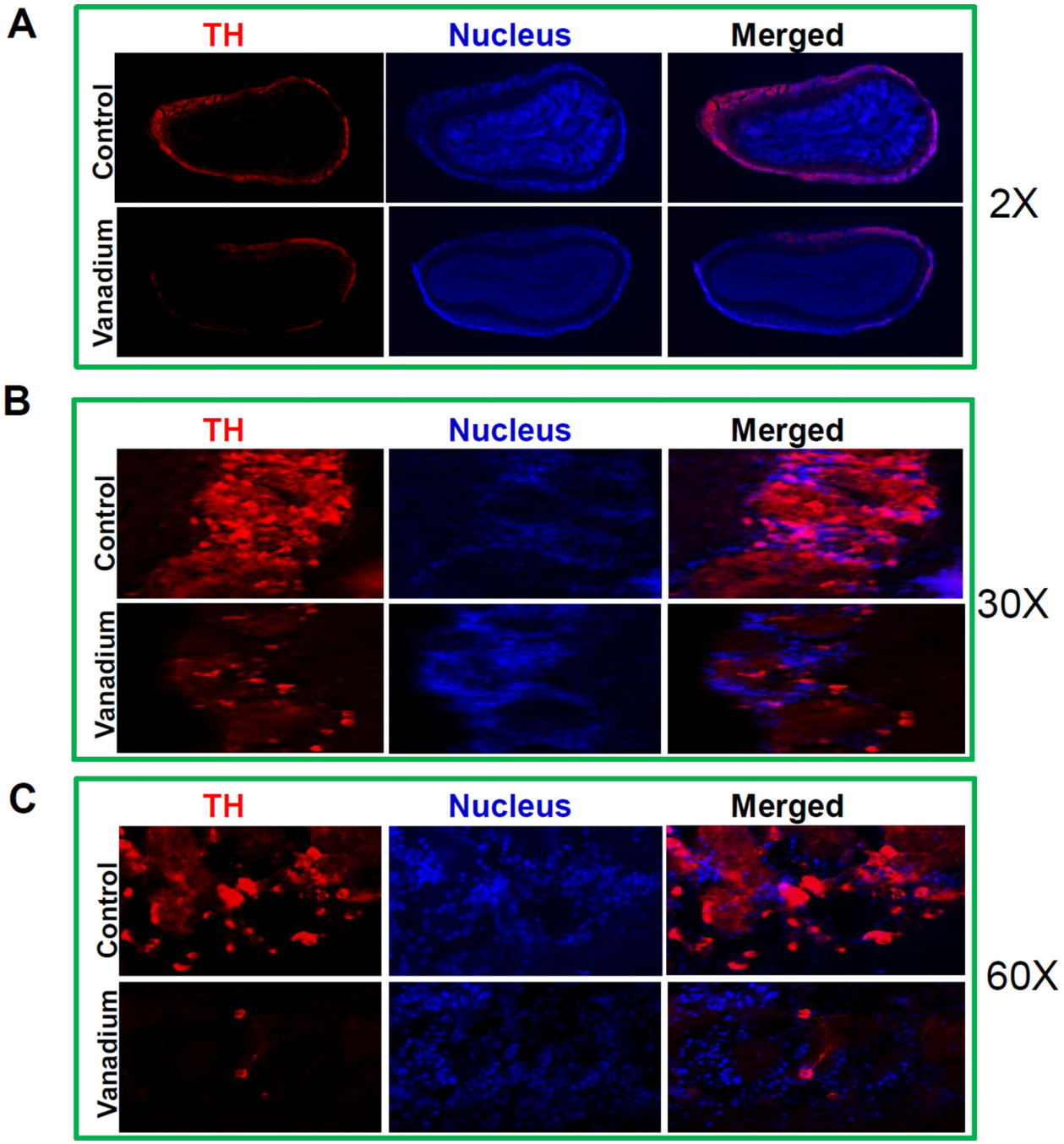

Fig. 5. Loss of glomerular TH neurons in olfactory lobes following intranasally administered vanadium

Male C57 black mice were intranasally administered 182 μg of V_2O_5 in 50 μL of de-ionized water three times a week as described in methods. The vehicle control animals were administered de-ionized water. At the end of treatment, animals were intracardially perfused and TH neurons were detected in the glomerular layer of the olfactory lobe by immunohistochemistry. (A) Representative 2× pictures of TH neurons in the glomerular layer of the olfactory lobe of control and vanadium-treated mice. (B) Representative 30× pictures of TH neurons in the glomerular layer of the olfactory lobe of control and

vanadium-treated mice. (C) Representative 60× pictures of TH neurons in the glomerular layer of the olfactory lobe of control and vanadium-treated mice.

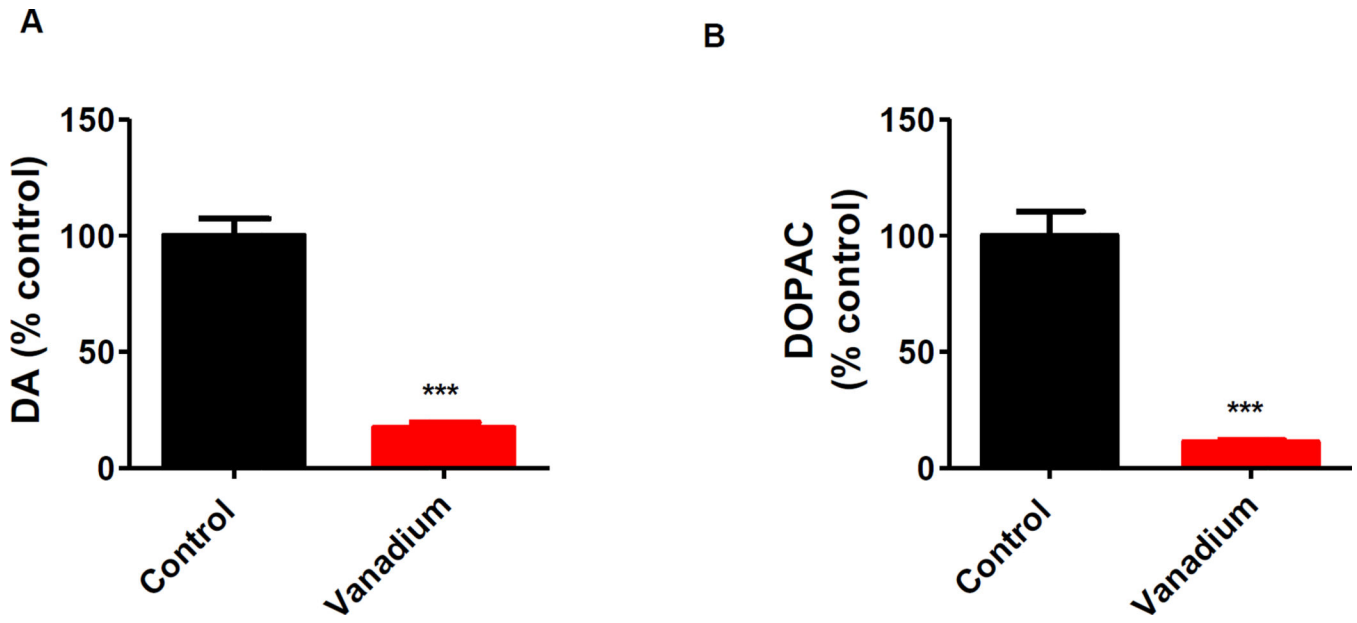

Fig. 6. Effects of intranasally administered vanadium on olfactory bulb dopamine and DOPAC levels

Male C57 black mice (n ≥ 5 per group) were intranasally administered 182 μg of V_2O_5 in 50 μL of deionized water three times a week for one month. The vehicle control animals were administered deionized water. Animals were sacrificed following the last treatment, and the neurochemical analysis (A, dopamine; B, DOPAC) was performed in olfactory lobe tissues using HPLC. Asterisks (***, $p < 0.001$) indicate a significant difference between treatment and control group means ± S.E.M.

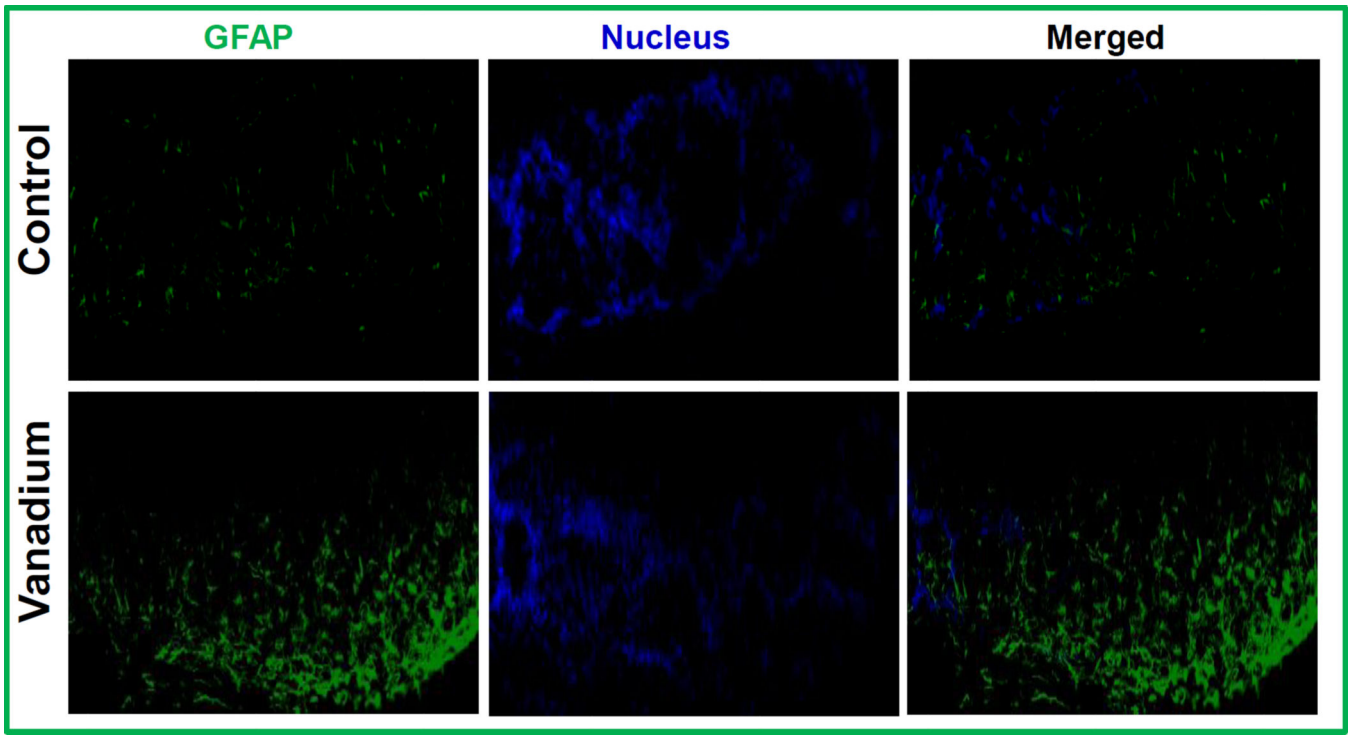

Fig. 7. Astrocyte Proliferation in the olfactory lobe glomerular layer following intranasally administered vanadium

Male C57 black mice were intranasally administered 50 μl (182 ug) of vanadium as described in the methods. The vehicle control group was administered de-ionized water. These treated animals were intracardially perfused and the astrocytes in the olfactory lobe were stained for GFAP by immunohistochemistry. A representative 20× pictures of GFAP astroglia in the olfactory lobe of the control and vanadium treated mice is shown. The data is representative of 3 mice per treatment group.