

Air pollutants reinforce autism–like behavior in the VPA-Induced – rat model of autism

Baharan Emam

Shaheed Beheshti University of Medical Sciences

ABBAS SHAHSAVANI (✉ ashahsavani@gmail.com)

Shaheed Beheshti University of Medical Sciences

Fariba Khodagholi

Shaheed Beheshti University of Medical Sciences

Philip K. Hopke

Clarkson University

Mohammad Amin Abdollahifar

Shaheed Beheshti University of Medical Sciences

Mostafa Hadei

Tehran University of Medical Sciences

Seyed Hamidreza Mirbehbahani

Shaheed Beheshti University of Medical Sciences

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Abstract

Background: Studies reporting associations between ambient air pollution exposure and autism spectrum disorder (ASD) have increased. Additionally, the effects of valproic acid (VPA) on ASD have been investigated. Pregnant women may have been simultaneously exposed to air pollution and antiepileptic drugs especially VPA. Thus, we hypothesized that simultaneous exposure to air pollution and valproic acid could reinforce ASD's behaviors in male rats. The current in-vivo study investigated the dual effects of air pollution on the VPA-induced rat model of autism using molecular and behavioral experiments.

Results: Seven exposure groups of rats included: 1) particulate matter and gaseous pollutants exposed - high dose of VPA (PGE-high), 2) particulate matter and gaseous pollutants exposed - low dose of VPA (PGE-low), 3) gaseous pollutants only exposed – high dose of VPA (GE-high), 4) gaseous pollutants only exposed – low dose of VPA (GE-low), 5) clean air exposed – high dose of VPA (CAE-high), 6) clean air exposed – low dose of VPA (CAE-low), 7) clean air exposed with no VPA injection (CAE). Rats were exposed to ambient PM_{2.5}/gaseous pollutants from embryonic day (E0) to postnatal day (PND42). Exposures to air pollutants in PGE-high, PGE-low, GE-high and GE-low groups of rat increased the ASD behaviors including: poor locomotor activity, weaker exploration activity, impaired social interaction, communication, and repetitive/restricted behavior as well as decreased oxidative stress biomarkers like catalase (CAT) activity and GSH, and decreased level of oxytocin receptor (OXTR) compared to the negative control group (CAE) and the other two control group (CAE-low and CAE-high).

Conclusions: This study suggested that simultaneous exposure to both air pollution and valproic acid contributed to ASD, and air pollution reinforces the mechanism of inducing ASD's in VPA-induced –rat model of autism, and it has provided a future field of studies on the synergistic effects of air pollution

Background

Autism Spectrum Disorders (ASDs) are developmental disorders characterized by deficits in social interactions and language as well as repetitive and restricted behaviors [1]. Autism is heterogeneous and often appears with one or more comorbidities including intellectual disability, hyperactivity, sensory processing abnormalities, and motor deficits. The cause of ASD is complex and has been related to genetic factors as well as poorly understood non-genetic causes [2]. The global incidence of ASD for 2010 has been reported 7.6 per 1000 [3, 4], and about 1 in 59 children has been identified with autism spectrum disorder (ASD) according to estimates from the U.S. Centers for Disease Control's Autism, and Developmental Disabilities Monitoring (ADDM) Network [5]. ASD is about 4 times more common among boys than girls [5]. In the last two decades, the most recent studies reported that, the prevalence of ASD increased dramatically [6, 7, 8, 9].

Studies largely in Europe and North America, reported association between ASD and exposure to traffic related air pollution (TRAP) during pregnancy. TRAP includes particulate matter, other criteria air pollutants, and air toxics (e.g., methylene chloride, chromium, lead, and volatile organic compounds (VOCs)) [10, 11, 12, 13]. Zinc, manganese, benzo(k)fluoranthene, benzo(a)pyrene, and pyrene in PM_{2.5} may have neurotoxic potential and adverse developmental outcomes [14, 15, 16, 17]. Some of these species have been associated with the production of ROS resulting in DNA damage [18].

Windham et al. [19] demonstrated that exposure to the elevated ambient air concentrations of metals (cadmium, mercury, and nickel), in diesel particulate matter was associated with a greater risk of the autism spectrum disorder. Talbott et al. [12] reported that both prenatal and postnatal exposures to fine particulate matter (PM_{2.5}) produced an increased risk of autism spectrum disorder. Volk et al. [20] found that exposure to traffic-related PM_{2.5} during pregnancy,

and during the first year of life was associated with ASD. There is some evidence that long-term exposure to ambient air pollution increases the risk of childhood ASD [19, 21, 22]. Furthermore, Allen et al. [23, 24]. reported that mice exposed to air pollution during their early postnatal period developed characteristics of autism spectrum disorder, and early postnatal exposure to airborne fine particulate matter induced autism-like phenotypes in male rats [25]. Chang et al. [26] reported that prenatal, and early-life exposure to diesel exhaust induced autism-like behavioral changes in mice.

Valproic acid (VPA) has been prescribed for epilepsy, but now it is increasingly used for psychiatric conditions, such as bipolar disease because of its modulation of GABA neurotransmission [27] Furthermore, it has been found to be associated with an increased prevalence of autism [28]. In fact, prospective, and retrospective studies reported that exposure to VPA during pregnancy was associated with an approximately three-fold increase in the rate of major behavioral anomalies [29, 30]. In utero exposure of rodents to VPA has been used to induce a phenotype with behavioral features reminiscent of those observed in ASD and provides a robust animal model for social cognitive impairment studies [31]. Other possible explanations include either the effect of VPA through an increase of fetal oxidative stress, affecting mainly the brain compared to other fetal organs [32]. It is possible to duplicate a number of human outcomes by exposing rat embryos to teratogenic agents at the time of neural tube closure [33]. Thus, in utero exposure to VPA has been used as a reliable model to increase the understanding of behavioral effects that are observed in human patients [30, 34, 35].

Approximately one in 250 pregnancies is known to be exposed to antiepileptic drugs [36], and a large proportion of these are exposed to VPA, either as part of a polytherapy drug regimen or as monotherapy [37]. The first epidemiological study with drugs as environmental risk factors of autism were described in 2000, with 57 offspring of women taking anticonvulsants, and as a result of this study, 53% of their children demonstrated poor social interaction, 49% had poor communication skills, 46% had short attention spans, 44% had insistence on routines, and 25% had hand flapping [38].

It has been reported that airborne particulate matter may act like a Trojan horse [39] representing an effective delivery system for diverse environmental toxicants to reach the brain. Water soluble compounds associated with PM may provide toxic stimuli that could be transported to the brain by the circulation system [40]. Therefore, the toxicity of particulate matter has been linked to both the particulate constituents, including oxidants, and oxidant forming species including metallic elements [41, 42] as well as the physical characteristic of particles [43]. Many compounds present in the particulate matter are neurotoxic [42]. Increased oxidative stress may result from environmental exposure to redox reactive metals such as iron (Fe), copper (Cu), manganese (Mn), aluminum (Al), and zinc (Zn) [44, 45]. The brain is vulnerable to oxidative stress because of its high level of metabolic activity and low levels of antioxidants such as catalase (CAT) [46]. Previous studies have proposed that autism could result from the interaction between genetic, and environmental factors, and oxidative stress can play a role in linking them [47]. Thus, an imbalance in the cellular redox state due to the oxidative stress or disturbing redox signaling, resulting in systemic inflammation are possible mechanisms of air pollution-induced autism [48]. Oxidative stress also plays a role in controlling and modulating the activities of receptor proteins and normal brain function [49].

Oxytocin (OXT) released within the central nervous system regulates complex behavioral functions such as social interactions [50, 51]. OXT has a key role in the mediation of pro-social behavior, including the onset of maternal care [52], pair bonding [53], and social recognition [54]. OXT produces its biological effects by attaching to and activating its receptor [55]. The OXT receptor (OXTR) is widely expressed in many nonproductive tissues such as the kidney, heart, vascular endothelium, and brain as well as in reproductive –related tissues, including the mammary gland, uterus, ovary, testis, and prostate [56, 57]. OXTRs are abundant in brain areas such as the amygdala, hippocampus, olfactory lobe, and hypothalamus [56], where they are thought to be involved in the regulation of the emotional, social, and neuroendocrine behaviors [58]. Therefore, the neuropeptide oxytocin (OXT), and its receptor (OXTR) likely regulate social functioning in animals, and humans. Initial clinical research suggests that dysregulated OXTR SNPs (single-nucleotide

polymorphism) may be biomarkers of social impairments in autism spectrum disorder [59]. Further studies showed the role of OXTR protein in ASD [60].

The number of studies reporting associations between ambient air pollution exposure and ASD have increased [12, 20, 61, 62, 63, 64]. The effects of VPA on ASD have also been studied. However, the combination has not been examined. Pregnant women may have been simultaneously exposed to air pollution and antiepileptic drugs especially VPA. The synchronous effects of these two environmental factors on their offspring have not been investigated. Therefore, we hypothesize that air pollution reinforces autism-like behavior in the VPA-induced – rat model of autism. In this first study of simultaneous exposure, rats were exposed to both VPA and air pollutants. The effects of ambient air pollution on autism spectrum disorder are poorly understood. Since initial clinical research suggests that OXTR may be biomarkers of social impairments in autism spectrum disorder [59], we used this biomarker to detect the autism spectrum disorder characteristics in rats. There is limited evidence that long time exposures to ambient air pollution increases the risk of ASD in laboratory animals. Thus, we investigated the effect of long-exposure (pre –natal, and post-natal, from E0-PND42) in this present study. In this study, we will also assess the role of aging in the rat in inducing ASD symptoms.

Results

The concentration of PM_{2.5} and gaseous pollutants

The average PM_{2.5} concentrations during the exposure period (E0 until PND 42) was $48.5 \pm 21.12 \mu\text{g}/\text{m}^3$. The average concentrations of CO, NO₂, SO₂, and O₃ were 14.5 ± 2.5 ppm, 0.382 ± 0.100 ppm, 0.322 ± 0.07 ppm, and 0.152 ± 0.010 ppm, respectively. Control rats were exposed to clean air with PM_{2.5} < $5 \mu\text{g}/\text{m}^3$, SO₂ < 0.02 ppm, NO₂ < 0.04 ppm, CO < 2.4 ppm, and O₃ < 0.02 ppm.

The concentration of metals and PAHs

Concentrations of PM_{2.5}-bound heavy metals were determined, and the mean values were ordered as: Ca > Al > Na > Cu > Fe > Cd > Cr > Ni > Pb > Zn > Mn > As > V (Supplemental Material Table S1). The mean total concentration of 16 PAHs was $45.88 \pm 21.02 \text{ ng}/\text{m}^3$ (Supplemental Material Table S2). The order of average concentrations of the observed PAHs was: phenanthrene > naphthalene > benzo(k)fluoranthene > florene > pyrene > anthracene > acenaphylen > benzo(b)fluoranthene > chrysene > fluorantene > benzo(a)anthracene > acenaphten > dibenzo (a, h) anthracene > benzo (g, h, i) perylene > benzo(a)pyrene > indeno(1, 2, 3-cd) pyrene.

Behavioral studies

CAE rats were intraperitoneally injected with normal saline on E12.5, no VPA injected, and exposed to filtered air served as the negative control. CAE-low and CAE-high groups of rats were injected with VPA intraperitoneally once on E12.5 with doses of 350 or 500 mg/kg BW, respectively, and exposed to filtered air. These rats served as controls to investigate the effects of air pollutants alone on autism spectrum disorder characteristics (Supplementary data, Table S3).

Locomotor activity

The open field test assesses locomotor activity and exploratory drive [23]. General locomotor activity representing exploratory conduct was evaluated by quantifying distance and speed in an open field arena. As shown in Fig. 1-a, a statistically significant reduction in total distance travelled was observed in all VPA- injected groups (all groups except CAE ones) vs CAE (negative control group). This reduction in CAE-low, CAE-high, GE-low, GE-high, PGE-low, and PGE-high groups of rat vs control group (CAE) showed p-values = 0.0216, p-value0.0242, p-value0.0137, p-value0.0296, p-value0.0089, p-value and 0.0066, respectively. Also, as shown in Fig. 1, b) velocity significantly decreased in all VPA-

injected groups vs CAE group. The reductions in CAE-low, CAE-high, GE-low, GE-high, PGE-low and PGE-high vs control group (CAE) showed p-values = 0.0247, p-value0.0245, p-value0.0125, p-value0.0112, p-value0.0447, and p-value0.0252, respectively.

Social behavior

In the social preference test, sociability, and ability to detect and remember social novelty were assessed using a three-chambered social apparatus. In session 1 (habituation) of this test, animals spent equal time in each chamber, suggested that subjects almost had no prior preference for either chamber before introduction of the social target (Fig. 2a). In the session 2 (sociability phase), the expected normal behavior is a preference for spending time with the occupied chamber compared to the unoccupied lateral chamber, but all groups except the CAE ones demonstrated a significant preference for spending time in the empty chamber. However, the time difference is more obvious in PGE-high and PGE-low groups of rats and the increased time spent in the empty chamber in PGE-low, and PGE-high vs. CAE group had p-values = 0.0121 and p-value0.0263, respectively (Fig. 2b). This result demonstrated a social deficit, a major characteristic of autism. In session 3 (social novelty), all groups except CAE demonstrated a clear preference for the familiar rat vs novel rat, but this time difference is most obvious in PGE-high groups. The promotion in the time spent in the chamber containing stranger1 in GE-low, GE-high, PGE-low, PGE-high vs CAE group had p-values = 0.0082, p-value0.0117, p-value0.0098, p-value and 0.0036, respectively Thus, this test indicated no preference for social novelty, whereas rats from CAE group, spent more time interacting with the novel animal (Fig. 2-c).

Repetitive behavior

One major characteristic of autism is repetitive behavior, and it was assessed by the marble burying test. Also, we assessed spontaneous alternation in the Y-maze to analyze repetitive behavior. Spontaneous alternation in a Y-maze represents a common exploratory strategy and repetitive behavior [65, 66]. Another way to demonstrate the repetitive behavior reported in VPA model, was the Y-maze test, a test designed to evaluate short-term, spatial memory [67]. However, the reduction of the Y-maze test could also indicate a restricted behavioral pattern, another core of ASD [65, 68]. As shown in Fig. 3a), all VPA injected rats demonstrated a significantly decreased level of mean spontaneous alternation vs the negative control group (CAE). This reduction in GE-low, GE-high, PGE-low, PGE-high vs CAE group had p-values = 0.0168, p-value0.0087, p-value0.006, and < 0.0001, respectively. GE-high (p-value = 0.0316), PGE-low (p-value = 0.0035), and PGE-high (p-value = 0.0006), showed significantly decreased mean spontaneous alternation behavior vs the controls (CAE-low).

In the marble-burying test (Fig. 3b), GE-high (p-value = 0.381), PGE-low (p-value = 0.0187), and PGE-high (p-value = 0.0006) rats buried significantly more marbles than the negative control rats (CAE). PGE-high rates buried more marbles than either control group (CAE-low and CAE-high with p-value = 0.0049 and p-value0.399, respectively). As shown in Fig. 3c, the time dedicated to exploring by PGE-high (p-value = 0.0005), PGE-low (p-value = 0.0315), and GE-high (p-value = 0.0315) decreased significantly vs CAE negative group. As shown in Fig. 3d, the time dedicated to interacting with the marbles in PGE-high (p-value = 0.0030) vs CAE negative controls increased significantly, and the increased interaction time in PGE-high vs VPA control groups (CAE-low and CAE-high) showed p-values = 0.0040 and p-value0.0063 respectively.

Additionally, the frequency of self-grooming events and repetitive digging behavior were quantified. As shown in Fig. 3e, the frequency of self-grooming in PGE-high (p-value = 0.0276) and PGE-low (p-value = 0.0045) increased significantly vs the negative controls (CAE). As shown in Fig. 3f, repetitive digging behavior in PGE-high (p-value = 0.0084) and PGE-low (p-value = 0.0268) increased significantly vs negative controls (CAE). Repetitive digging behavior in PGE-high increased vs controls (CAE-low and CAE-high) with p-values = 0.0249 and p-value0.0307, respectively.

Oxidative Stress

Oxidative stress was detected by measuring antioxidant enzymes i.e., catalase (CAT) and GSH in, cerebellum, hippocampus, and prefrontal cortex of brain tissue. The activity of CAT decreased significantly in all VPA-injected groups (p -value ≤ 0.0001) vs CAE ones in the cerebellum. Moreover, the activity of CAT significantly decreased in GE-low, GE-high, PGE-low, PGE-high (p -value=0.0001, p -value ≤ 0.0001 , p -value ≤ 0.0001 , p -value ≤ 0.0001 , respectively) vs. CAE-low. Furthermore; the CAT activity in the cerebellum (Fig .4a) decreased significantly in GE-low, GE-high, PGE – low, PGE-high (p -values = 0.0047, p -value ≤ 0.0001 , ≤ 0.0001 , and ≤ 0.0001 , respectively) vs. CAE-high.

The CAT activity in the hippocampus decreased significantly in all VPA-injected groups (p -value ≤ 0.0001) vs CAE. Moreover; the CAT activity significantly decreased in GE-high, PGE-low, PGE-high (p -value=0.0003, ≤ 0.0001 , and ≤ 0.0001 . respectively) vs control group CAE-low. Furthermore; the CAT activity in the hippocampus (Fig .4b) decreased significantly in GE-high, PGE –low, PGE-high (p -value = 0.0047, 0.001, ≤ 0.0001 , and ≤ 0.0001 , respectively) vs. control group CAE-high.

The CAT activity in the prefrontal cortex decreased significantly in CAE-high, GE-low, GE-high, PGE- low, and PGE-high (p -value = 0.0040, 0.0008, ≤ 0.0001 , ≤ 0.0001 , and ≤ 0.0001 , respectively) vs CAE. The activity of CAT significantly decreased in GE-low, GE-high, PGE-low, PGE-high (p -value=0.0259, p -value = 0.0009, p -value = 0.0001, p -value ≤ 0.0001 , respectively) vs control group of rats (CAE-low) in the prefrontal cortex. Furthermore; the activity of CAT decreased significantly in the prefrontal cortex (Fig. 4c) of PGE –low and PGE-high (p -value = 0.0157 and $p \leq 0.0001$, respectively) vs CAE-high.

The GSH level decreased significantly in all VPA-injected groups (p -value ≤ 0.0001) vs CAE ones in cerebellum. The GSH concentration significantly decreased in GE-low, GE-high, PGE-low, PGE-high (p -value ≤ 0.0001) vs control group of rats (CAE-low). The GSH level in the cerebellum (Fig. 5a) GSH level decreased significantly in, GE-low, PGE–low, PGE-high (p -value = 0.0266, p -value=0.0202, p -value=0.0018, respectively) vs control group of rats (CAE-high).

The GSH level decreased significantly in all VPA-injected groups (p -value ≤ 0.0001) vs CAE ones in the hippocampus. Moreover; the level of GSH significantly decreased in PGE-high (p -value = 0.040) vs control group of rats (CAE-low) in the hippocampus (Fig. 5b). The GSH level decreased significantly in CAE-low, CAE-high, GE-low, GE-high, PGE-low, PGE-high (p -value=0.0067, p -value = 0.0022, p -value = 0.0027, p -value = 0.0014, P -value = 0.0005, p -value ≤ 0.0001) vs CAE ones in the prefrontal cortex (Fig. 5c).

Expression of oxytocin receptor

Western blot tests were conducted to determine the OXTR protein expression level. All VPA-Injected groups (all group of rats except CAE), exhibited significantly decreased OXTR levels in the cerebellum, hippocampus, and prefrontal cortex compared to the negative control group of rats (CAE) (Fig. 6. a, b, c). Moreover, in the cerebellum, the OXTR level significantly decreased in GE-low, GE-high, PGE-low, PGE-high groups of rat, compared to the control group (CAE-low), (p -value = 0.0029, p -value = 0.0020, p -value = 0.0008, p -value = 0.0005 respectively), Furthermore the OXTR level dramatically decreased in control group (CAE- high) compared to another control rat (CAE-low) (p -value:0.0138) (Fig .6a). Also, in hippocampus, the OXTR level significantly decreased in PGE-high, PGE-low, and GE-high compared to control group (CAE-low), (p -value = 0.0240, p -value = 0.0098, p -value = 0.0062, respectively) (Fig. 6b).

Furthermore, in prefrontal cortex, the OXTR level significantly decreased in GE-low, GE-high, PGE-low, PGE-high compared to control group (CAE- low), (p -value < 0.0001). Besides, the OXTR level significantly decreased in GE-low, GE-high, PGE-low, PGE-high compared to control group (CAE- high), (p -value < 0.0001). Furthermore, the OXTR level dramatically decreased in control group (CAE-low) compared to another control rat (CAE-high) (p -value < 0.0001) (Fig. 6c).

Discussion

The immature brains of fetuses or toddlers are more susceptible to environmental toxicants because the nerve cells are not fully developed until around the age of two years old. The rest of the developing nervous system is also sensitive to environmental toxicants because of temporal and regional developmental processes, i.e. proliferation, migration, differentiation, synaptogenesis, myelination, and apoptosis, during this period. Toxicants that interfere with one or more of these processes may be inhalation [69]. In the rat cortex, post-synaptic glutamate receptors increase rapidly from PND5 to PND20 and reach half the adulthood's amount by PND40 followed by a continual increase to a plateau around PND50 [70]. Since glutamate receptors have been suggesting to play a role in the modulation of cell death after perinatal brain injury [71], age-specific vulnerability is somewhat dependent on the developmental expression of these neurotransmitter networks [72]. The exposure period of E0 to PND 42 covers the main neurodevelopmental events (including: the timing of neurogenesis, synaptogenesis, gliogenesis, and oligodendrocyte maturation) that occur in this developmental period.

On this subject, the immature rat's vulnerability to toxins may be related not only to the neurodevelopmental stages, but also to the failure of other protective barriers, e.g. the placental, and the blood-brain barrier. The placental should be a protective barrier against the passage of the harmful substances like environmental toxins from the mother's body. However, the placenta is not an effective protective barrier against environmental toxins during this time of fetal vulnerability [73]. In recent years, studies were conducted showing that only nano-sized particles can pass the placental barrier [74, 75]. Also, a recent study [76] found that black carbon particles were able to translocate from the mother's lungs to the placenta. However, in this subject, various potential mechanism has been proposed, including both direct particle translocation, and/or through an indirect mechanism such as intrauterine inflammation [77, 78, 79]. An indirect mechanism may also be involved because of the exposure to particulate air pollution, and its constituents e.g., metals, and PAHs, can induce oxidative stress, and inflammation that leads to developmental toxicity [80, 81] by negatively affecting placental transportation.

This study design used ambient air pollution without modification to mimic real-world exposure scenarios. Since prior studies typically used high pollutant concentrations, it was not clear that exposure at commonly observed air pollution concentrations can induce ASD or not. Comparing the internal dose of $PM_{2.5}$ per kg body weight indicates that the rat's dose in our study were about 2–3 times higher than a human dose. Therefore, these results provide more realistic exposures compared to prior studies.

To the best of our knowledge, this is the first detailed, comprehensive assessment of air pollutant's effect on the valproic acid-induced model of autism. Also, the hypothesis that air pollutants could play as an environmental trigger was investigated. $PM_{2.5}$ is an air pollutant that elicits major concerns regarding public health, and contains toxic substances, such as PAHs, metals, and organic matter. In the present study, concentrations of PAHs, and metals in $PM_{2.5}$, particularly lead, zinc, manganese, benzo (a)anthracene, benzo (k) fluoranthene, benzo (a) pyrene, and pyrene were considered potential neurotoxic risks. Some of these toxicants have been associated with the triggering of inflammation, generation of ROS, and lipid peroxidation [14, 15, 82, 83]. Mostafa, et al. [84] hypothesized that lead as a one of the main neurotoxicants acting as environmental triggers for ASD through neuroinflammation, and autoimmunity. While the $PM_{2.5}$, and components in it are likely candidates for inducing autism-like phenotype, it is possible that gaseous pollutants mainly nitrogen oxides, also contribute to induced ASD [26, 85].

Since prenatal exposure to VPA has become a reliable tool to model autism, more brain alternations were investigated in rodents exposed to this teratogen [37]. Nonetheless, different doses of this toxin have different functions in causing autism i.e. dose 350 mg/kg. BW can affect the brain by reducing the number of motor neurons from the hypoglossal,

and oculomotor nuclei [30]. A dose of 500 mg/kg.BW can altered the distribution of 5-HT neurons in the dorsal raphe nucleus [86]. Therefore, we investigated the effect of air pollutants on rates subjected to these two VPA doses.

Currently, two main clinical features are enumerated to diagnose ASD: impaired social interaction, and communication, and repetitive behaviors. Therefore, since diagnosis of autism are mainly based on clinical symptoms [87], we used clinical type behavioral tests to examine the autism characteristics in these rat groups.

In the open field arena, a hypokinetic condition that is consistent with a reduction in exploratory activity was observed, as has been previously reported by others in VPA-treated rats using the same test. All VPA-injected subjects indicated poorer locomotor activity vs negative controls (CAE). In the sociability phase of the three-chamber social test, all VPA-injected rats showed a sociability defect, a major feature of autism. Our findings in this phase were also in agreement with Dai et al..[88] In the social novelty phase of that test, all VPA-injected rats demonstrated an unwillingness to establish new social relationships. Increased marble burial activity in all VPA-injected rats indicated enhanced repetitive behavior. This finding agrees with the results of Schneider et al. [35]. Also when repetitive digging behavior in this test was examined, the PGE- high rats showed a significant increase in digging behavior vs two control group of rats, CAE-low, and CAE-high. Therefore, air pollutants can have an additive effect on repetitive behavior. Also, the reduction of mean spontaneous alternation indicates restricted behavior that is another core of the autism. In this test, PGE-low, PGE-high, and GE-high (that were in contact with gases) vs control group of rat (CAE) showed a significant decrease of mean spontaneous alternation. Therefore, air pollutants can have additive effect on restricted pattern of behavior.

The activity of CAT in the cerebellum, hippocampus, and prefrontal cortex. decreased significantly in all VPA-injected groups of rats compared to the negative controls (CAE), This finding is in agreement with Khongrum, et al.[89]. Moreover, the CAT activity in several groups of animals exposed to both air pollution, and VPA decreased dramatically, compared to control animal groups that were exposed to only low, and high doses of valproic acid. Air pollution might provide toxins that induce oxidative stress.

The GSH levels in cerebellum, hippocampus, and prefrontal cortex decreased significantly in all VPA-injected groups of rats relative to the negative controls (CAE), This finding is in agreement with Simon [90], who reported that valproic acid reduced the intracellular level of glutathione (GSH) in the human body.Our results agrees with Seckin et al. [91]. GSH levels in several groups of animals exposed to both air pollution, and VPA decreased dramatically, compared to control groups exposed only to low or high doses of VPA. Again suggesting that air pollution may trigger oxidative stress.

The level of the OXTR (as a biomarker of ASD) in the cerebellum, hippocampus, and prefrontal cortex decreased significantly in all VPA-injected rats compared to the negative controls (CAE), Our results are in agreement with Bertelsen et al. [92] who reported that VPA-induced– rat models of autism demonstrated a decreased level of OXTR compared to controls. The OXTR levels in several group of animals exposed to both air pollution, and VPA decreased dramatically, compared to control animals exposed only to low or high doses of VPA. This result suggests the possible role of air pollution on valproic acid in inducing ASD by the OXTR mechanism.

Conclusions

In the present study, our results demonstrated that, long-exposure of VPA-induced –rat to a mixture of PM_{2.5}, and gaseous pollutants (PGE-low, PGE-high), and gaseous pollutants alone (GE-low, GE-high) enhances the expression of ASD characteristics, such as behavioral deficits, including poor locomotor activity, weaker exploration activity, impaired social interaction and communication, increased repetitive behaviors, and decreased oxidative stress biomarkers like CAT, and GSH. It may modulate down-regulation of OXTR compared to the negative controls (CAE), and control groups that received valproic acid in low or high doses but were only exposed to the clean filtered air (CAE-low, and CAE-high).

The finding of this present study supports the hypothesis that air pollution plays a role in inducing ASD and provides the basis for future studies about the synergistic effects of air pollution on other environmental toxicants.

Methods

Rats

Wistar rat litters of both sexes were purchased from the Pasteur Institute (Tehran, Iran). The rats were ten weeks old at the start of the study. The rats were housed at the Shahid Beheshti University of Medical Sciences under specific-pathogen-free (SPF), standard conditions including access to supplies of water, and food ad libitum with a 12 hr. light/dark cycle. The rats were maintained under constant environmental conditions including a temperature of 20–25 °C, and relative humidity of 40–60%. The ethical use of animal models was approved by Shahid Beheshti University of Medical Sciences' Ethics Committee. They were housed in 3 chambers as described previously by Emam et al. [93] (Fig. 7). The concentrations of PM_{2.5}, and gaseous pollutants (CO, NO₂, SO₂ and O₃) in each chamber were measured using DUST TRAK model 8520 and AeroQual 500 monitors, respectively.

Drug administration

The rats were mated in-house at the age of 11 to 13 weeks, and pregnancy was determined by a vaginal plug on an embryonic day 1 (E1). To produce experimental rats based on the VPA rat model of autism spectrum disorder [94], the sodium salt of valproic acid (P4543 - Sigma) was prepared in 0.9% saline solution (100 mg/ml, pH 7.3). Because of different brain functions of different doses of VPA in causing ASD, we chose two different doses of VPA, termed low, and high. On E12.5, VPA-dams received a single intraperitoneal (i.p.) injection of either 350 mg/kg BW (as a low-dose) VPA or 500 mg/kg BW (as a high-dose) [95]. Control dams received a single injection of saline solution (i.p., 0.9%) (Fig. 8).

Animal groups, study location and method of exposure

Dams were randomly divided into seven groups (twenty-four in each group): Dams and pups were exposed to gaseous pollutants with average concentrations of CO, NO₂, SO₂, and O₃ being 14.5 ± 2.5 ppm, 0.382 ± 0.100 ppm, 0.322 ± 0.07 ppm, and 0.152 ± 0.010 ppm, respectively. These dams received a single intraperitoneal (i.p.) injection of 350 mg/kg BW VPA (GE-low). Rats were exposed to gaseous pollutants with average concentrations of CO, NO₂, SO₂, and O₃ being 14.5 ± 2.5 ppm, 0.382 ± 0.100 ppm, 0.322 ± 0.07 ppm, and 0.152 ± 0.010 ppm, respectively. These dams received a single intraperitoneal (i.p.) injection of 500 mg/kg BW VPA (GE-high). Rats were exposed to PM_{2.5}, and gaseous pollutants with an average concentration of PM_{2.5} during the exposure period (E0 to PND42) of 48.5 ± 21.12 µg/m³. The average concentrations of CO, NO₂, SO₂, and O₃ were 14.5 ± 2.5 ppm, 0.382 ± 0.100 ppm, 0.322 ± 0.07 ppm, and 0.152 ± 0.010 ppm, respectively. These dams received a single intraperitoneal (i.p.) injection 350 mg/kg BW VPA (PGE-low). Dams and pups were exposed to PM_{2.5}, and gaseous pollutants with an average concentration of PM_{2.5} during the exposure period (E0 until PND 42) of 48.5 ± 21.12 µg/m³. The average concentrations of CO, NO₂, SO₂, and O₃ were 14.5 ± 2.5 ppm, 0.382 ± 0.100 ppm, 0.322 ± 0.07 ppm, and 0.152 ± 0.010 ppm, respectively. These dams received a single intraperitoneal (i.p.) injection of 500 mg/kg BW VPA (PGE-high). Dams and pups were exposed to clean air with PM_{2.5}<5 µg/m³, SO₂ < 0.02 ppm, NO < 0.04 ppm, CO < 2.4 ppm, and O₃ < 0.02 ppm. These dams received a single injection of saline solution (i.p., 0.9%)(CAE). Rats were exposed to clean air with these characteristics: PM_{2.5}<5, SO₂ < 0.02 ppm, NO < 0.04 ppm, CO < 2.4 ppm, and O₃ < 0.02 ppm, and dams received a single intraperitoneal (i.p.) injection of 350 mg/kg BW VPA (CAE-low) Rats were exposed to clean air with PM_{2.5}<5 µg/m³, SO₂ < 0.02 ppm, NO <

0.04 ppm, CO < 2.4 ppm, and O₃ < 0.02 ppm. These dams received a single intraperitoneal (i.p.) injection of 500 mg/kg BW VPA (CAE-high). (Supplementary data, Table S3)

Rats were exposed in the animal study room (Fig. 7). It was conducted as previously described by Emam et al.[93]. The animal room was located on the roof of the School of Public Health, and Safety of the Shahid Beheshti University of Medical Sciences (35.7991° N, 51.3947° E) at a height of 20 m (fourth floor) above the ground. GE-low and GE-high rats in chamber 1 were exposed to filtered ambient air at a flow rate of 20 L/min provided by an oil-free compressor and a HEPA filter (model H13) to remove particulate matter. PGE-low and PGE-high rats in chamber 2 were exposed to ambient air at a flow rate of 20 L/min using an Echo PM_{2.5} Low Volume Sampler (LVS) (TCR Tecora Italy) without a filter to provide exposure to both PM_{2.5} and gaseous pollutants. The CAE, CAE-low, and CAE-high rats were housed in chamber 3, and exposed to ambient air at 20 L/min flow rate provided by an oil-free compressor, and two purifier systems (Model: Air Touch A5, Honeywell, and model: KAIST-AIR Home) to remove both particulate and gaseous pollutants.

Exposure periods

The pregnant dams and subsequent pups were exposed to the various air quality flows outlined above for 12 hours per day, and 5 days per week (Saturday to Wednesday) from embryonic day 0 (E0) to postnatal day 42 (PND42). The rats in the control groups were exposed to clean air during the same period.

The exposure period was chosen based on the human epidemiological studies suggesting that air pollution exposures during all the three trimesters of pregnancy, and the first 9 months of an infant's life were related to the ASD risk [4, 20]. Exposures were continued based on a human population-based cohort study [21] that suggested long-term exposures to ambient air pollution increased the risk of ASD and that was conducted on the children age less than three years. Thus, the period of E0 to PND42 was designed to cover the main neurodevelopmental events (including: the timing of neurogenesis, synaptogenesis, gliogenesis, oligodendrocyte maturation) that were happening in this window of susceptibility. And also this exposure period simulates the life span of prenatal, pregnancy and postnatal to age 3 of human life in rats (Supplementary data, Fig S4).

For the exposure of pregnant dams, and pups, female rats (11–13 week old) were time mated with males (3 females with 1 male per cage) on Friday evenings. The copulatory plug was checked the next morning before the Saturday onset of the weekly exposure. Rats with confirmed vaginal plugs were removed from the breeding cage on embryonic day zero, and housed individually in cages in either chambers one, two or three. Chambers 1, and 2 housed eighteen pregnant dams while chamber 3 housed twenty- seven dams for the duration of their pregnancy, parturition, and weaning of the litters. The use of Friday timed matings ensured that E0 occurred on a Saturday and ensured that the exposure timing was the same for all rats. All dams were exposed on the same gestational days E0-4, E7-11, E14-18, and all pups were exposed on PND1-PND7, PND10-14, PND17-PND22, PND25-29, PND32-26, PND39-42. Exposure stopped in chambers one, and two while rats were giving birth on E19 and E20. On PND14, male, and female pups were separated, and female pups were removed from their mothers. The male pups remained with their mothers until weaning occurred on PND 42. On PND 43, the pups were divided into two groups. One group were euthanized by decapitation for the biochemical tests, and the second group were transferred to housing racks to start the behavioral tests (on PND 43) and for the duration of the behavioral testing period. Behavioral tests were only performed on male rat offspring, and all behavioral tests were performed between 9:00 and 15:00. The behavioral assessments were made sequentially on a single cohort of male rats. This lack of replication is a limitation of this study (Fig. 8).

Determination of PM_{2.5} characteristics

PM_{2.5} was sampled continuously, in this study, during the exposure hours using an Echo PM Low Volume Sampler near the air intake for the exposure chambers [96]. PM_{2.5} was collected at a flow rate of 20 L/min by using 47 mm quartz

filters. Filters were changed every 2 days (48 hour samples). Before the sampling, the filters were washed with double-distilled water, and placed in an oven at 100–105 °C for 2 hours [97]. After sampling and for preventing of evaporation, and photodegradation of PM components, the filters were stored in aluminum foil at -10 °C. To control for possible contamination during sampling, field blanks were collected. A blank filter was treated in the same manner as a sample filter (placed into the sampler, placed in the aluminum foil, etc.), except that the sampler was not operated.

To determine the elemental composition of the PM, one-half of filters were used. The filters were shredded, and put into a Teflon container with 2.5 ml of concentrated of HClO₄ (70%), and 2.5 ml HNO₃ (69%), heated at 170 °C for 4 hr, and taken to dryness on a hot plate at 100°. Then 2.5 ml of double-distilled water and 2.5 ml of HNO₃ was added and, the samples were shaken at 180 rpm for 30 min [97]. Finally, the solutions were filtered through a Whatman No. 42 filter and diluted with double-distilled water to 10 ml. The samples were stored in plastic vials at 4 °C until their analysis by ICP/MS (Agilent, Model: 7900) [98]. Laboratory blanks were used to control for the effects of laboratory contaminations. The metal concentrations are provided in the Supplemental material, Table S1. The concentrations of metals were analyzed in triplicate, and the averaged values were used. To control for the effects of field and laboratory contaminations, the field and laboratory blank values were subtracted from the sample measurement. The limit of detection (LOD) and the limit of quantitation (LOQ), of Pb were about 5 and 1 µg/L, respectively. The LOD and LOQ for other elements were approximately 25 and 5 µg/L, respectively.

To determine the concentrations of polycyclic aromatic hydrocarbons (PAHs), the other section of the filter was placed in a Teflon container to which 2.5 ml of dichloromethane (CH₂Cl₂) and 2.5 ml of methanol (CH₃OH) was added to extract the PAHs in an ultrasonic bath at 20 kHz for 30 min (Elmasonic S 80 H). The extracts were filtered through 0.22 µm Millipore filters (Hesperia CA, USA). PAH concentrations were determined with GC/MS (Agilent, model: 5890 A). The concentrations of PAHs were analyzed in triplicate, and the mean concentrations were reported, PAHs were not observed in any of the the blank samples. PAH results are provided in the Supplemental Material Table S2. The LOD and LOQ values for 16 PAHs were < 2 and < 10 ng/L, respectively.

Behavioral tests

Open field

This test assesses general locomotor activity levels and willingness to explore in a novel environment. It was conducted as previously described by Chang et al. [26] with some modifications. At PND43, each rat was placed in a clean acrylic cage (60*60*60 cm) with no bedding for 30 minutes, and a video was recorded using a Microsoft LifeCam HD-6000. The rat's location during the test was tracked using the Ehtovision XT 7 system. The total distance covered and the velocity of each subject in the open field area were measured automatically by the Ehtovision software.

Social preference test

This test was conducted as previously described by Li et al. [25] with modifications. The three sessions in this test were conducted in a three chambered box (60*40*22 cm) equipped with retractable doorways that permitted access to each chamber. This test was conducted at PND46 and the sessions were as follows: in session 1 (habituation), pups were first placed in the middle chamber with the doorways open and allowed to explore the other two side chambers. For session 2 (sociability), at the end of the period of habituation, to the empty apparatus, pups were made to interact with a never-before-met, but age-matched rat (stranger 1) enclosed in a wire cup placed in a side chamber, and an empty cup placed in another side. During session 3 (social novelty), a new, and unfamiliar rat (stranger 2) was placed in the wire cup that had been empty during the previous session, and a now familiar rats (stranger 1) was placed on the other side

from the previous session. Each session lasted 10 minutes, and the time spent in each chamber was manually recorded.

Y-maze

The test was conducted as previously described by Grabrucker et al. [68]. Spontaneous alternation behavior was assessed at PND57 for each rat in a symmetrical Y Maze (3 arms, 40*9 cm with 16 cm height of walls). Rat's choices (all four paws entering one arm) were recorded while the rats were allowed to explore the Y-shaped labyrinth for 5 min. Alternation was determined by recording the order of the visited arms (A, B, or C). Overlapping triplets of three-arm visits were counted as one complete spontaneous alternation. The percentage of alternation was calculated as:

$$\text{Percentage of alteration} = \frac{\text{number of spontaneous alternation}}{\text{total number of arm visits} - 2} \times 100 \quad (1)$$

where an alternation was recorded as consecutively visiting the three-arms.

Marble Burying

The test was conducted as previously described by Ku et al. [99] with some modifications. On PND61, rats were placed in a standard Plexiglas test cage (42*24*17 cm) with a 5 cm deep layer of corncob bedding and allowed to explore freely for 10 minutes. Each rat was placed in a transfer cage, and 18 marbles (1.3 diameter, red) were placed on the bedding surface in a 3*6 pattern. The rat was then placed in the test cage and allowed to explore for 10 minutes. After ten minutes, the rat was removed from the test cage, and the number of marbles buried was counted. A marble was considered as buried only if at least two-thirds of the marble was covered by the bedding materials.

The frequency of self-grooming, and repetitive behaviors were quantified by measuring the time devoted to these behaviors.

Tissue collection and preparation

On PND43, some rats in each group were anesthetized with CO₂ and rapidly decapitated. Rats' hippocampi, prefrontal cortices, and cerebella were extracted according to Paxinos and Watson atlas (The Rat Brain in Stereotaxic Coordinates, 2013 [100]). Next, they were lysed on ice using lysis buffer [50 mM Tris-HCl (pH 8), 0.25% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 150 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, complete protease inhibitor cocktail, phosphatase inhibitor cocktail] for 2 minutes. The lysates were cleared with centrifugation at 16,100 x g for 10 minutes at 4°C. To determine the protein concentration, Bradford's method was employed [101].

GSH

The test was conducted as described by Ellman et al. [102]. To determine the level of GSH, sixty µg of protein supernatant containing 19.8 mg of DTNB (D8130-1G SIGMA) in 100 ml, 0.1% sodium nitrate, and phosphate buffer (pH 7.4) were used. Ellman's colorimetric method measures the formation of GS-TNB complex from DTNB (5, 5'-dithiobis (2-nitro benzoic acid)) in which its reduction caused the development of a yellow color. The above mixture absorbs the light at 412 nm. The absorbance was measured at 412 nm by an ELISA reader (model ELx800 BioTek).

Catalase

To determine the level of catalase activity, 20 µl of the sample were loaded in each well of the 96-well microplate. Then, 100 µl hydrogen peroxide (65 mM) was added to each well. The mixture was then incubated for 4 minutes at 25 °C. Then, 100 µl of ammonium molybdate (32.4 mM) was added in each well. Finally, the concentrations of the stable

yellow complex of ammonium molybdate and hydrogen peroxide were measured at 405 nm with an ELISA reader (model ELx800 BioTek) [103].

Western blot

Western blotting was used to assess the level of OXTR in the prefrontal cortex, hippocampus, and cerebellum of the male rats. On PND43, the prefrontal cortex, hippocampus, and cerebellum were excised. Afterward, they were lysed on ice via lysis buffer [50 mM Tris-HCl (pH 8), 0.25% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 150 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, complete protease inhibitor cocktail, phosphatase inhibitor cocktail] for 2 minutes. Centrifugation at 16, 100 Xg for 10 minutes at 4°C was used to clear the lysates. Bradford's method [101] was used to determine the protein concentration.

Bovine serum albumin (BSA) was used as the standard for denatured proteins. Equal volumes of 5X sample buffer were added to lysate proteins. Then 60 µg of total protein were loaded into SDS-polyacrylamide gel electrophoresis and afterward transferred on to polyvinylidene difluoride membranes. Then blots were blocked by adding blocking solution [2% non-fat dry milk in tris-buffered saline Tween 20 (TBST) (containing 0.2% Tween 20, 50 mM Tris-HCl pH 7.4, 150 mM NaCl)] for one hour and a half. Then, the membranes were incubated overnight with the primary antibody (Anti-Oxytocin Receptor antibody [EPR12789]-20°C) (Abcam ab181077-100 µl) at 4°C. The next day, the blots were washed with TBST three times for 10 minutes each. After incubating with the anti-rabbit horseradish peroxidase secondary antibody (Anti-rabbit IgG, HRP-linked Antibody) (cell signaling) for 90 minutes at room temperature, immunoreactivity was detected by using ECL kit and captured by Kodak x-ray film. Membranes were stripped with stripping buffer [100 mM 2-mercaptoethanol, 2% (w/v) SDS, 62.5 mM Tris-HCl (PH 7)] followed by incubating with anti-β-actin antibody to compensate for loading errors. Densitometric data of the protein bands were obtained with ImageJ1.41o software.

Statistical Analysis

All data were analyzed using Graph Pad Prism software (Graph Pad Prism v6.0). All tests except social preference test were analyzed by using one-way ANOVA followed by Tukey's test. For the social preference test, time spent in the 'stranger 1' versus 'empty' chambers (for the sociability phase) or 'stranger 1' versus 'stranger 2' chambers (for the social novelty phase) were compared with a two-way analysis of variance (ANOVA). The time spent in chamber 1, chamber 2, and chamber 3 of the apparatus were compared for each group of rats in the habituation phase of this test with a one-way ANOVA followed by Tukey's test for the multiple pairwise comparisons. Multiple comparison corrections were not done. Significance levels of 0.05, 0.01, 0.001, and 0.0001 were applied in all analyses.

Declarations

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Authors' contributions

BE participated in study design, in vivo section, experiments, data analysis, and manuscript writing. AS, FK, and SMZ participated in study design, experiments, data analysis, and manuscript writing. PKH, MH, and HMB participated in study design, parts of experiments, data analysis, and manuscript writing. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

All ethical aspects of this study were approved by Shahid Beheshti University of Medical Sciences' Ethics Committee.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no conflict of interest.

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Figures

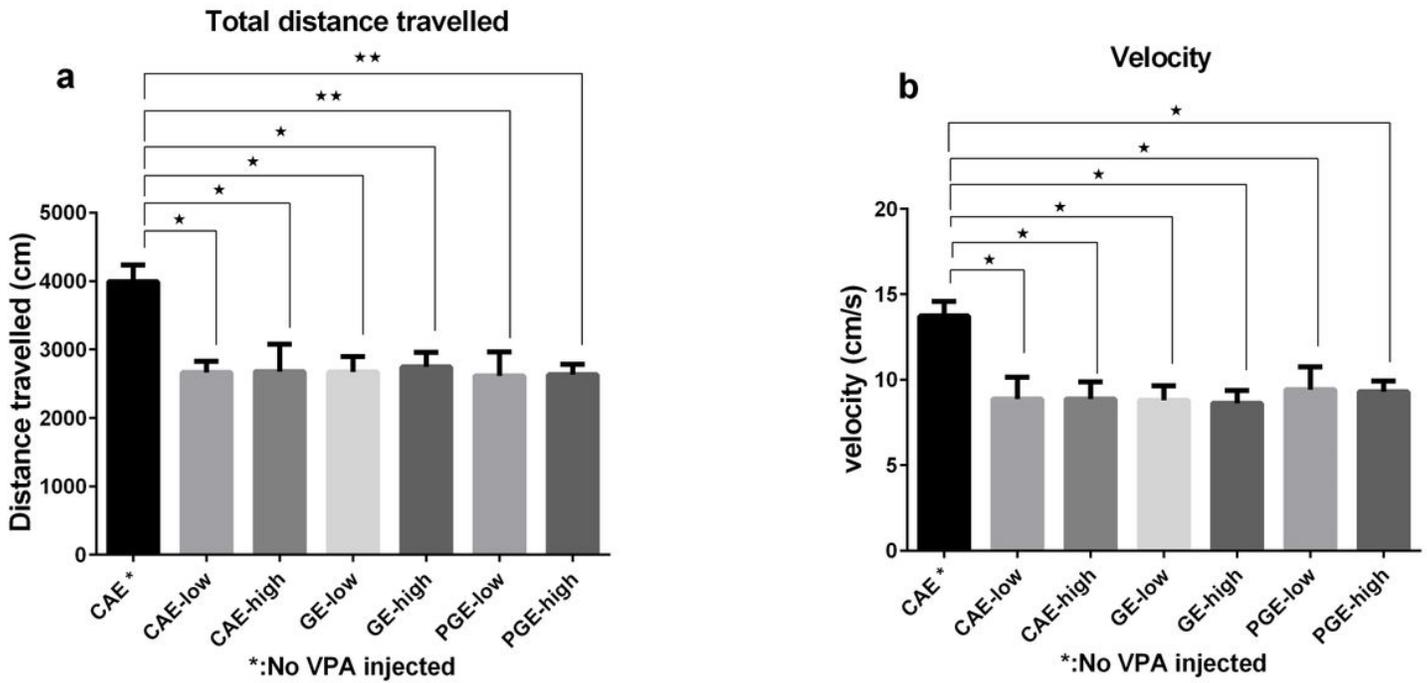


Figure 1

The total distance and velocity decreased significantly in all injected group of rat vs negative control animals(CAE*). (Fig. a, b). The error bars represent the standard error of the mean. * $p < 0.05$, ** $p < 0.01$, (Number of animal per group: CAE*=8, CAE-low=8, CAE-high=8, GE-low=10, GE-high=9, PGE-low=10, PGE-high=12).

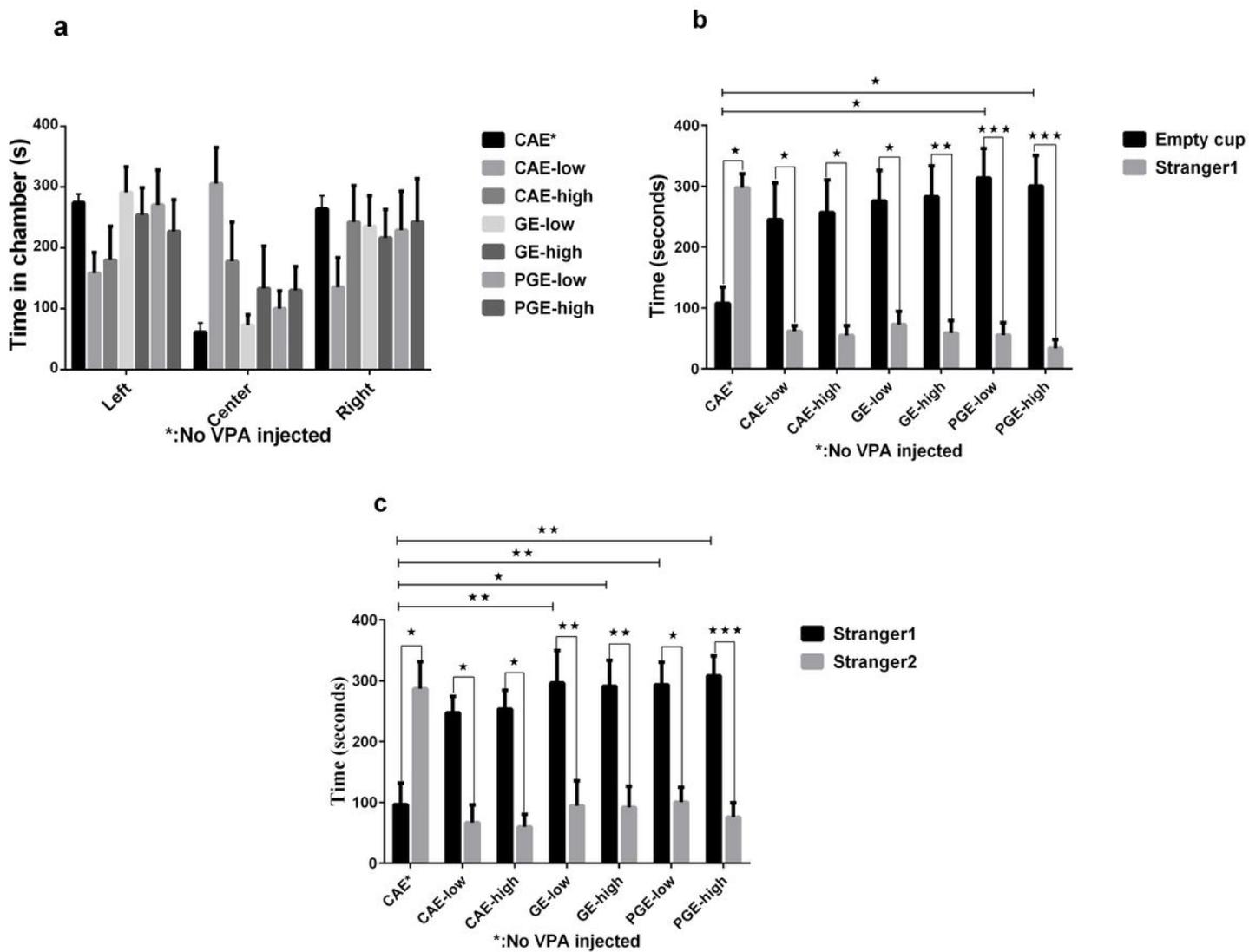


Figure 2

Three Chambered Social Preference Test. (a). Time spent in each chamber during Habituation (b). Time spent in the chamber containing the stranger rat (stranger1) and the empty cup during the sociability phase(c)Time spent in the chamber containing the novel rat (stranger 2) and familiar rat (stranger 1) during the social novelty phase. The error bars represent the standard error of the mean. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ CAE*=8, CAE-low=8, CAE-high=8, GE-low=8, GE-high=8, PGE-low=8, PGE-high=8).

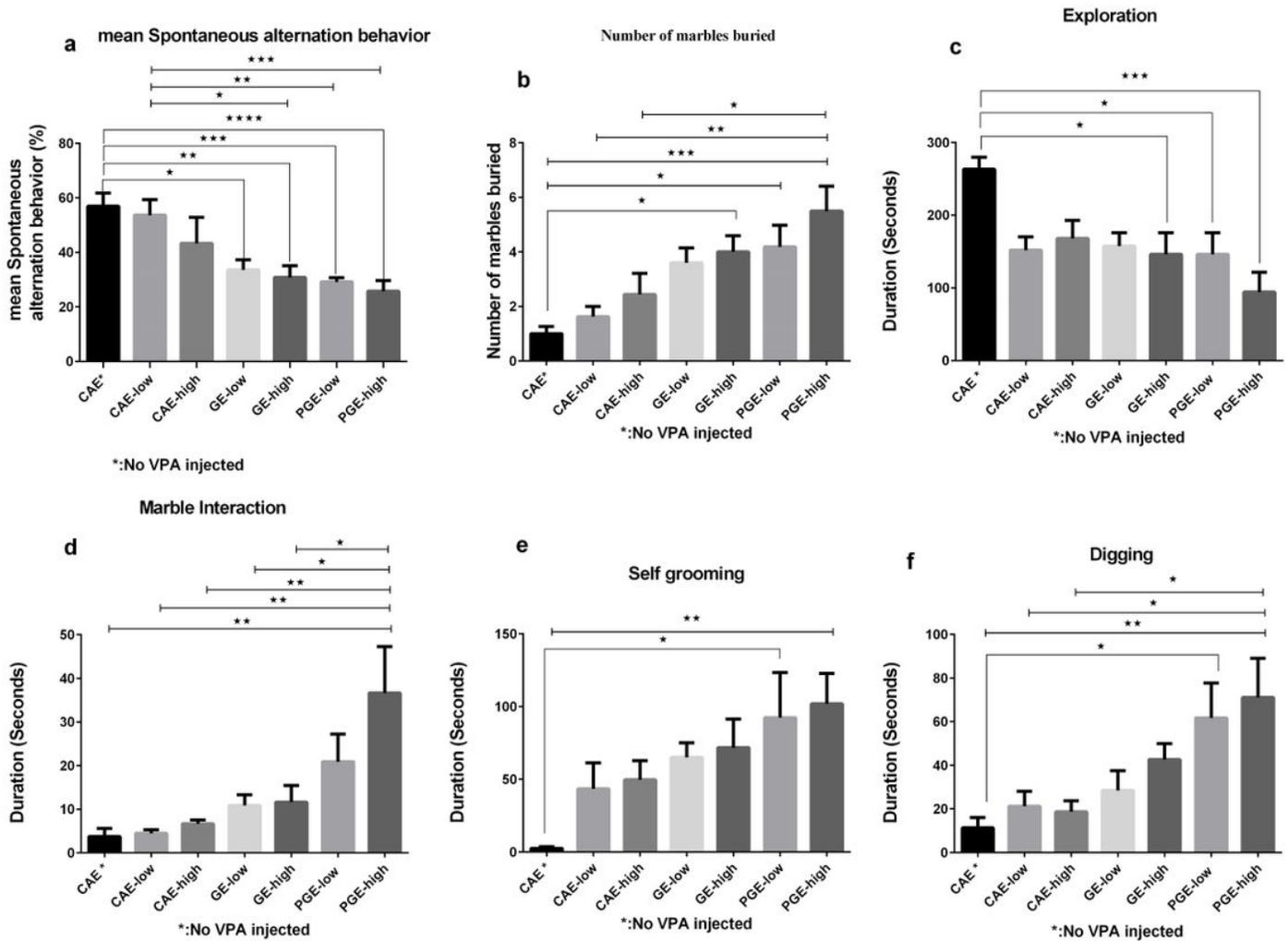


Figure 3

Repetitive behavior (a) Y maze, the percent of mean spontaneous alternation behavior decreased significantly in all VPA-injected group of rat vs negative control group (CAE*). The percent of mean spontaneous alternation behavior decreased significantly in PGE-high, PGE-low and GE-high vs CAE-low as a control group of rat. (b) PGE-high, PGE-low and GE-high buried more marbles vs CAE* and PGE-high buried more marbles vs CAE-low and CAE-high. (c) PGE-high, PGE-low and GE-high spent less time in exploring in the Plexiglas test cage vs CAE*. (d) PGE-high spent more time in interacting with more marbles vs all other group of rat (e) All VPA-injected group of rat spent more time in self – grooming as a repetitive behavior vs CAE*. (f) PGE-low and PGE-high spent more time in digging behavior as a repetitive one vs CAE*. And PGE-high spent more time in digging behavior vs CAE-low and CAE-high. The error bars represent the standard error of the mean. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ CAE*=8, CAE-low=8, CAE-high=8, GE-low=8, GE-high=8, PGE-low=8, PGE-high=8).

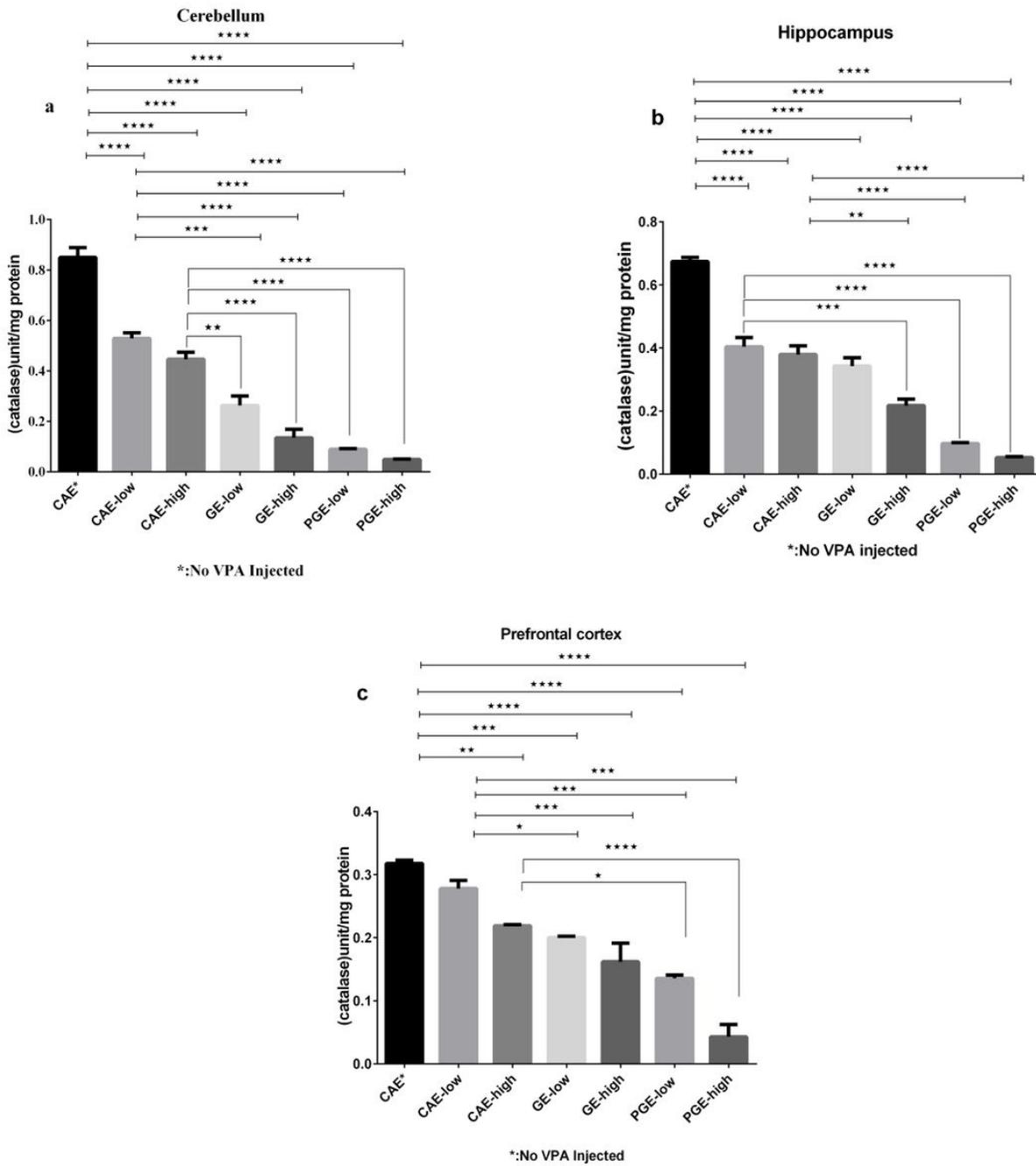


Figure 4

Catalase enzyme activity after long-time exposure of the CAE, CAE-low, CAE-high, GE-low, GE-high, PGE-low and PGE-high groups of rats in the a) cerebellum, b) hippocampus, and c) prefrontal cortex. The error bars represent the SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

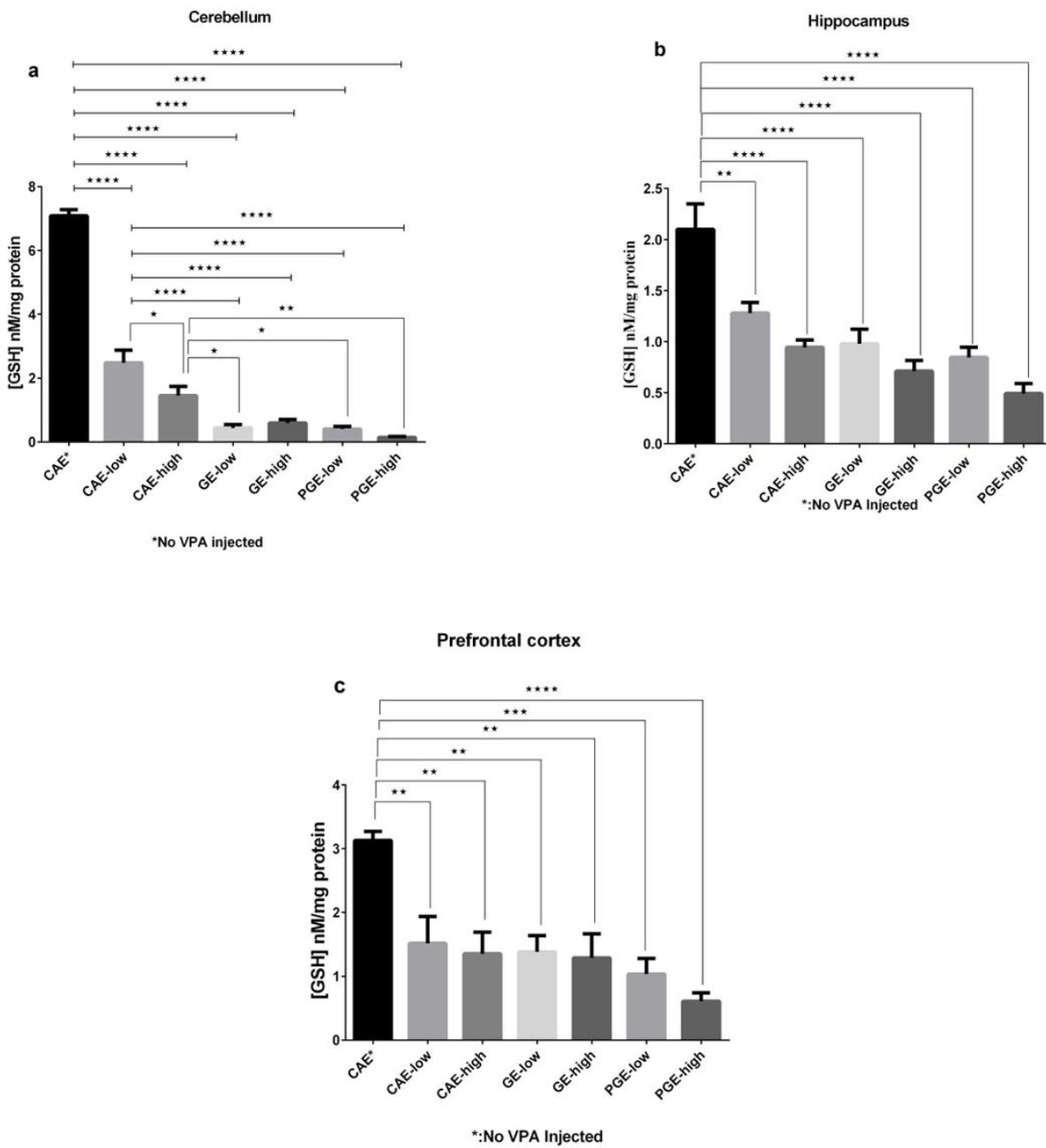


Figure 5

GSH levels after long-time exposure of the CAE, CAE-low, CAE-high, GE-low, GE-high, PGE-low and PGE-high groups of rats in the a) cerebellum, b) hippocampus, and c) prefrontal cortex. The error bars represent the SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

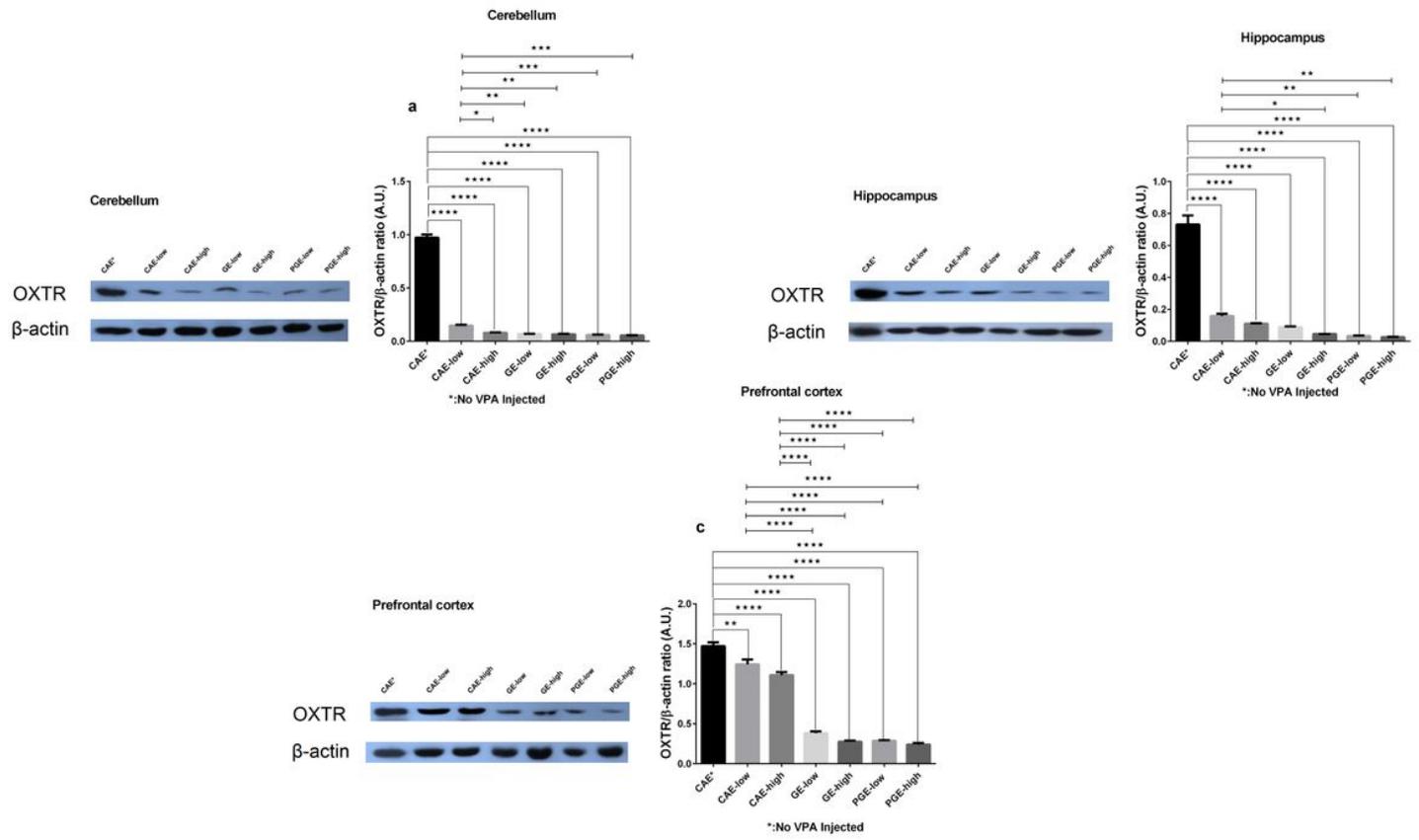


Figure 6

OXTR levels after long- time exposure in all CAE*, CAE-low, CAE-high, GE-low, GE-high, PGE-low and PGE-high the, a) cerebellum (Number of rat in each group =4, with Three times technical repetition), b) hippocampus (Number of rat in each group =4, Three times technical repetition), and c) prefrontal cortex (Number of rat in each group =4, Three times technical repetition). Error bars represent the SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; one-way ANOVA with Tukey's multiple comparisons)

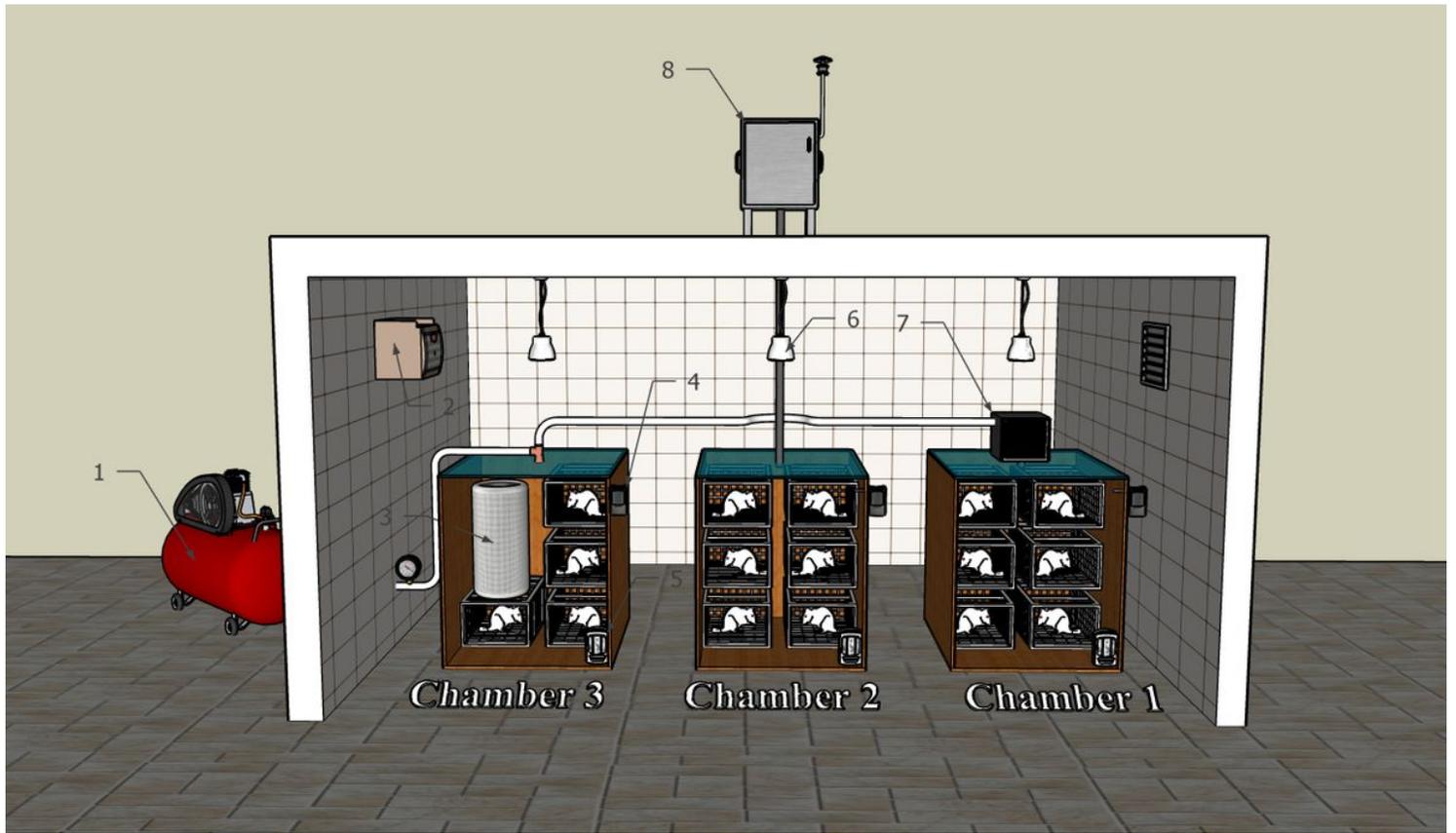


Figure 7

Schematic of exposure method: 1-Oil-free compressor, 2-Thermometer, 3-Air purifier, 4-Moisture Sensor, 5- Dust track, 6-Time lamp, 7- HEPA Filter, and 8- Echo PM.

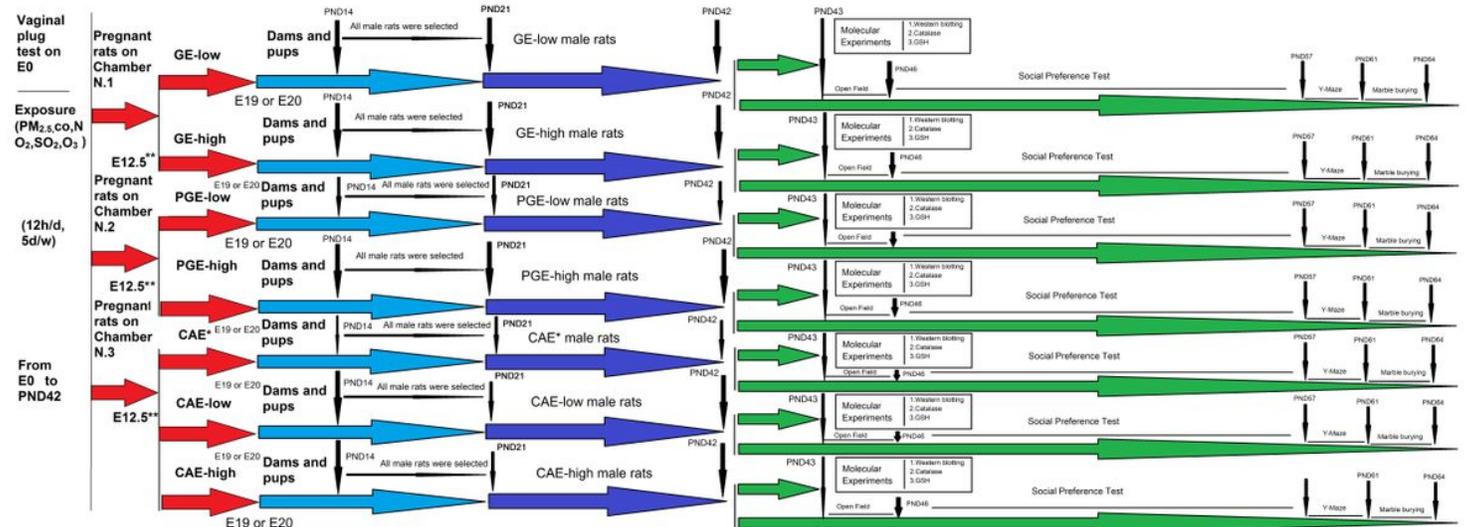


Figure 8

Experimental design of study: rats were time mated and exposed to PM_{2.5}/gases alone from E0 to PND42. Biochemical tests were stated at PND43 by extraction of brain tissue. Behavioral tests were started at PND43 and these behavioral tests continued to the PND64. **VPA-dams received a single intraperitoneal (i.p.) injection of VPA at a dose of 350 mg/kg BW. And a dose of 500 mg/kg BW. The world low in the name of rat groups is related to a VPA at a low dose of

350 mg/kg BW. And the word high in the name of rat groups is related to a high dose of 500 mg/kg BW. *No VPA Injection

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