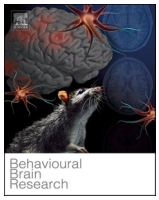
Behavioural Brain Research 468 (2024) 115040

Contents lists available at ScienceDirect

Behavioural Brain Research

journal homepage: www.elsevier.com/locate/bbr



Research article

Neuroprotective effects of Si-based hydrogen-producing agent on 6-hydroxydopamine-induced neurotoxicity in juvenile mouse model

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ARTICLE INFO

Keywords:
Si-based agent
Neuroprotection
Neurotoxin
6-hydroxydopamine
Dopaminergic neuron
Hyperactivity

ABSTRACT

Neurotoxins have been extensively investigated, particularly in the field of neuroscience. They induce toxic damage, oxidative stress, and inflammation on neurons, triggering neuronal dysfunction and neurodegenerative diseases. Here we demonstrate the neuroprotective effect of a silicon (Si)-based hydrogen-producing agent (Si-based agent) in a juvenile neurotoxic mouse model induced by 6-hydroxydopamine (6-OHDA). The Si-based agent produces hydrogen in bowels and functions as an antioxidant and anti-inflammatory agent. However, the effects of the Si-based agent on neural degeneration in areas other than the lesion and behavioral alterations caused by it are largely unknown. Moreover, the neuroprotective effects of Si-based agent in the context of lactation and use during infancy have not been explored in prior studies. In this study, we show the neuroprotective effect of the Si-based agent on 6-OHDA during lactation period and infancy using the mouse model. The Si-based agent safeguards against the degradation and neuronal cell death of dopaminergic neurons and loss of dopaminergic fibers in the striatum (STR) and ventral tegmental area (VTA) caused by 6-OHDA. Furthermore, the Si-based agent exhibits a neuroprotective effect on the length of axon initial segment (AIS) in the layer 2/3 (L2/3) neurons of the medial prefrontal cortex (mPFC). As a result, the Si-based agent mitigates hyperactive behavior in a juvenile neurotoxic mouse model induced by 6-OHDA. These results suggest that the Si-based agent serves as an effective neuroprotectant and antioxidant against neurotoxic effects in the brain, offering the possibility of the Si-based agent as a neuroprotectant for nervous system diseases.

1. Introduction

Neurotoxins, a class of substances with the intrinsic capacity to specifically target and disrupt neuronal function, represent pivotal tools in neuroscience research. Derived from diverse sources such as animals, the environment, endogenous processes, bacteria, plants, and certain medications, neurotoxins can be classified into categories including biotoxins, environmental toxins, endogenous molecules, bacterial toxins, plant-derived toxins, and drug-related toxins [1–5]. These toxins exert their effects through diverse mechanisms, including interference with neurotransmission, induction of oxidative stress, and modulation of cellular signaling pathways [1,3,5–8].

The unique ability of neurotoxins to selectively damage or impair neurons has made them invaluable in elucidating the intricate workings of the nervous system [3,6,9]. By precisely targeting specific types of neurons or neurotransmitter systems, researchers can induce controlled and localized damage, allowing for the investigation of cellular responses and the unraveling of intricate neural circuitry. Moreover,

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https://doi.org/10.1016/j.bbr.2024.115040
Received 18 January 2024; Received in revised form 2 May 2024; Accepted 3 May 2024
Available online 7 May 2024

neurotoxins serve as crucial models for understanding and replicating pathological conditions associated with neurodegenerative diseases and neurological disorders. Notably, certain neurotoxins mimic the molecular processes implicated in diseases such as Parkinson's disease (PD) [10,11], Alzheimer's disease [12,13], and Huntington's disease [14,15].

6-Hydroxydopamine (6-OHDA) is a neurotoxin commonly employed to selectively damage dopaminergic and noradrenergic neurons, making it a valuable tool in neuroscience [16–18]. 6-OHDA is taken into cells by dopamine transporters and undergoes autooxidation to produce reactive oxygen species [16–18]. By utilizing this mechanism, 6-OHDA is employed to establish animal models of PD at adult stages [10,11] and attention-deficit/hyperactivity disorder (ADHD) at juvenile stages [19,20]. ADHD is a neurodevelopmental disorder characterized by attentional deficits, hyperactivity, and impulsivity, often presenting in childhood and persisting into adulthood [21,22]. In ADHD mouse model generated by 6-OHDA, we previously reported that the shortened of axon initial segment (AIS) lengths in the medial prefrontal cortex (mPFC) and primary somatosensory barrel field (S1BF) of [23].

Medical hydrogen exhibits antioxidative, anti-inflammatory, antiallergic, and antiapoptotic effects [24–28]. It selectively reduces hydroxyl radicals (•OH) in reactive oxygen species (ROS) and reacts exclusively with hydroxyl radicals, making it a potential therapeutic agent for diseases associated with oxidative stress and inflammation, and it is devoid of side effects [29,30]. We developed a silicon (Si)-based hydrogen-producing agent (Si-based agent) capable of continually producing a substantial amount of hydrogen by reacting with water under conditions similar to those in the gut [28,31–34]. Si and its reaction product, SiO_2, are known to be nontoxic, enabling the oral administration of the Si-based agent. So far, we have reported the preventive and anti-inflammatory effects of the Si-based agent in animal models of various diseases, including maternal-fetal transmission, maternal immune activation, chronic kidney disease, ulcerative colitis, and PD [33,35–37].

In this study, we investigated the neuroprotective effect of a Si-based agent against the selective damage of dopaminergic neurons induced by 6-OHDA in the juvenile mouse brain. Utilizing a 6-OHDA-induced juvenile neurotoxic mouse model, we conducted comprehensive histological and behavioral analyses. Our results reveal the neuroprotective impact of the Si-based agent on 6-OHDA-induced neuronal degradation, structural alteration, and behavioral impairment in juvenile mice. These findings contribute novel evidence to support the neuroprotective effects of the Si-based agent.

2. Materials and methods

2.1. Mice

All procedures were conducted in accordance with the ARRIVE guidelines and relevant official guidelines, approved by the Animal Research Committee of Osaka University (#27–010) under protocols #01–040 and #27–010. C57BL/6 J (Japan SLC Inc., Shizuoka, Japan) male mice were used. Mice were housed in cage (143 mm × 293 mm × 148 mm) in the barrier facilities of Osaka University under a 12 h light–dark cycle and given free access to water and food. In this study, a total of 66 neonatal mice born to 10 mothers were used. The experiments were conducted by experimenters who were blinded to the genotypes.

2.2. 6-OHDA administration

A mouse model of ADHD was generated as previously described [19,20,23]. 6-OHDA induced ADHD mouse model shows ADHD-like behavioral abnormalities such as hyperactivity, impulsivity, and attention deficit [19,20]. Male mice were anesthetized on postnatal day (P) 5 by hypothermia. Desipramine hydrochloride (20 mg/kg) (#D3900; Sigma-Aldrich, MO, USA) was unilaterally injected subcutaneously, followed 30 min later by the administration of saline or 6-OHDA hydrobromide containing 0.1% ascorbic acid as a stabilizer (25 μg dissolved in 3 μL of saline, #H116; Sigma-Aldrich) [19,20,23]. The injection was performed intracerebroventricularly at a rate of 1.5 mL/min, with coordinates of 0.6 mm lateral to the medial sagittal suture, 2.0 mm rostral to lambda, and 1.3 mm in depth to the skin, using a 10-μL Gastight Syringe (#1701RN; Merck, Darmstadt, Germany) with a 30 G Small Hub RN Needle (#7803–07: 30GA, RN, 6PK, 16MM, 45°; Merck). After administration, pups were warmed on a heating pad at 37°C until recovery and were then randomly returned to their dams. The pups remained housed with the dam until analysis was conducted at P24.

2.3. Si-based agent and treatment

The Si-based agent and Si-based agent-containing feed were prepared as described previously [33,35,36]. The Si-based agent was produced from polycrystalline Si powder (Osaka Titanium Technologies Co., Ltd., Osaka, Japan; Si 4Nup). After milling the Si powder, surface treatment and aggregation were carried out. Therefore, the Si-based agent was composed of an aggregate of Si nanopowder. For the control laboratory chow, the AIN-93M diet (Oriental Yeast Co., Ltd., Tokyo, Japan) was used. The Si-based agent-containing laboratory chow was specially prepared by incorporating 2.5 wt% of the Si-based agent into AIN-93M. The powdery feed was provided to mothers and offspring starting at P3 until P24, with free access to food and water. Before the animal experiments, hydrogen production from the feed was evaluated using a sensor gas chromatograph, SGHA-PA (FIS Inc., Hyogo, Japan).

2.4. Immunohistochemistry

Immunohistochemistry was performed as previously described [38]. Mouse brains were fixed with 4% PFA in PBS overnight at 4°C, cryoprotected in 30% sucrose in PBS, then embedded in Tissue-Tek O.C.T. Compound (#4583, Sakura Finetek Japan Co.,Ltd., Osaka, Japan) for cryosectioning. Cryosections (20 μm thick) were placed in PBS. Sections were stained with the following primary antibody: rabbit polyclonal anti-Tyrosine hydroxylase (1:500, #AB152, Merck, Burlington, MA), mouse monoclonal anti- NeuN (1:500, # MAB377; Merck), mouse monoclonal anti-Ankyrin-G (1:200, #MABN466; Merck). For fluorescence immunostaining, species-specific antibodies conjugated to Alexa Fluor 488 (1:2000; ThermoFisher, Waltham, MA) were applied, and cover glasses were mounted with Fluoromount/Plus (#K048, Diagnostic BioSystems, Pleasanton, CA). Images were collected using Olympus microscope and digital camera system (BX53 and DP73, Olympus, Tokyo, Japan), an all-in-one fluorescence microscope (BZ-X700, KEYENCE Corporation), and Zeiss confocal laser scanning microscope (LSM 710, Carl Zeiss, Oberkochen, Germany). Fluorescence intensities and AIS lengths were quantified using ImageJ. Experimenters blinded to genotypes performed the quantifications.

2.5. Open field test

The open field test was performed as previously described [39].Male mice were placed in one of the corners of a novel chamber (W700 × D700 × H400 mm, #OF-36(M)SQ, Muromachi Kikai Co., Ltd., Tokyo, Japan) and were allowed to freely explore for 10 min. Locomotor activity was measured and tracked using ANY-maze behavior tracking software. Experimenters blinded to genotypes performed the test. Testing was performed between 10:00 and 16:00 h.

2.6. Statistical analysis

Data are presented as means of biological independent experiments with violin plot (minimum to maximum) or ± standard error of the mean (SEM). Statistical analyses (one-way ANOVA) were performed using Prism 9. A significance level of $P < 0.05$ was considered.

3. Results

3.1. Si-based agent protects 6-OHDA-induced selective neuronal degradation

To assess the neuroprotective effect of a Si-based agent against the selective damage of dopaminergic and noradrenergic neurons induced by 6-OHDA in the brain, we generated an established mouse model of ADHD, representing one of juvenile neurotoxic models. Mother and offspring mice were provided with the AIN-93M diet powder, with or without 2.5% Si-based agent by weight, from P3 until dissection at P24. To safeguard noradrenergic neurons and generate the ADHD model, desipramine was administered subcutaneously, followed by the intracerebroventricular administration of either saline or 6-OHDA in mice at P5 (see Methods). We verified the selective damage of dopaminergic neurons and loss of dopaminergic fibers in the ventral tegmental area (VTA) and their fibers in the striatum (STR) and substantia nigra pars compacta (SNc) induced by 6-OHDA (Fig. 1A, B). We observed tyrosine hydroxylase (TH)-positive immunoreactivities in the STR and VTA of control (CTR) mice (Fig. 1A-D) but not in 6-OHDA mice (Fig. 1A-D). Interestingly, TH-positive immunoreactivities were also observed in the STR and VTA of 6-OHDA mice treated with the Si-based agent (Fig. 1A-D). These results suggest that the Si-based agent protects against the 6-OHDA-induced selective damage of dopaminergic neurons and loss of dopaminergic fibers in the mouse brain.

3.2. Si-based agent protects AIS lengths in the mPFC of 6-OHDA mice

Next, we investigated whether the neuroprotective effects of the Si-based agent extended to other brain regions at P24. In a previous study, we reported the phenotype of shortened AIS lengths in layer (L) 2/3 neurons of the mPFC in the same 6-OHDA-induced ADHD model mice [23]. Consistent with a previous result, the AIS lengths in L2/3 neurons of mPFC of 6-OHDA mice were shorter than those in CTR mice at P24 (Fig. 2A, B). Cumulative frequency distribution plots of AIS lengths in mPFC L2/3 neurons exhibited leftward shifts in 6-OHDA mice

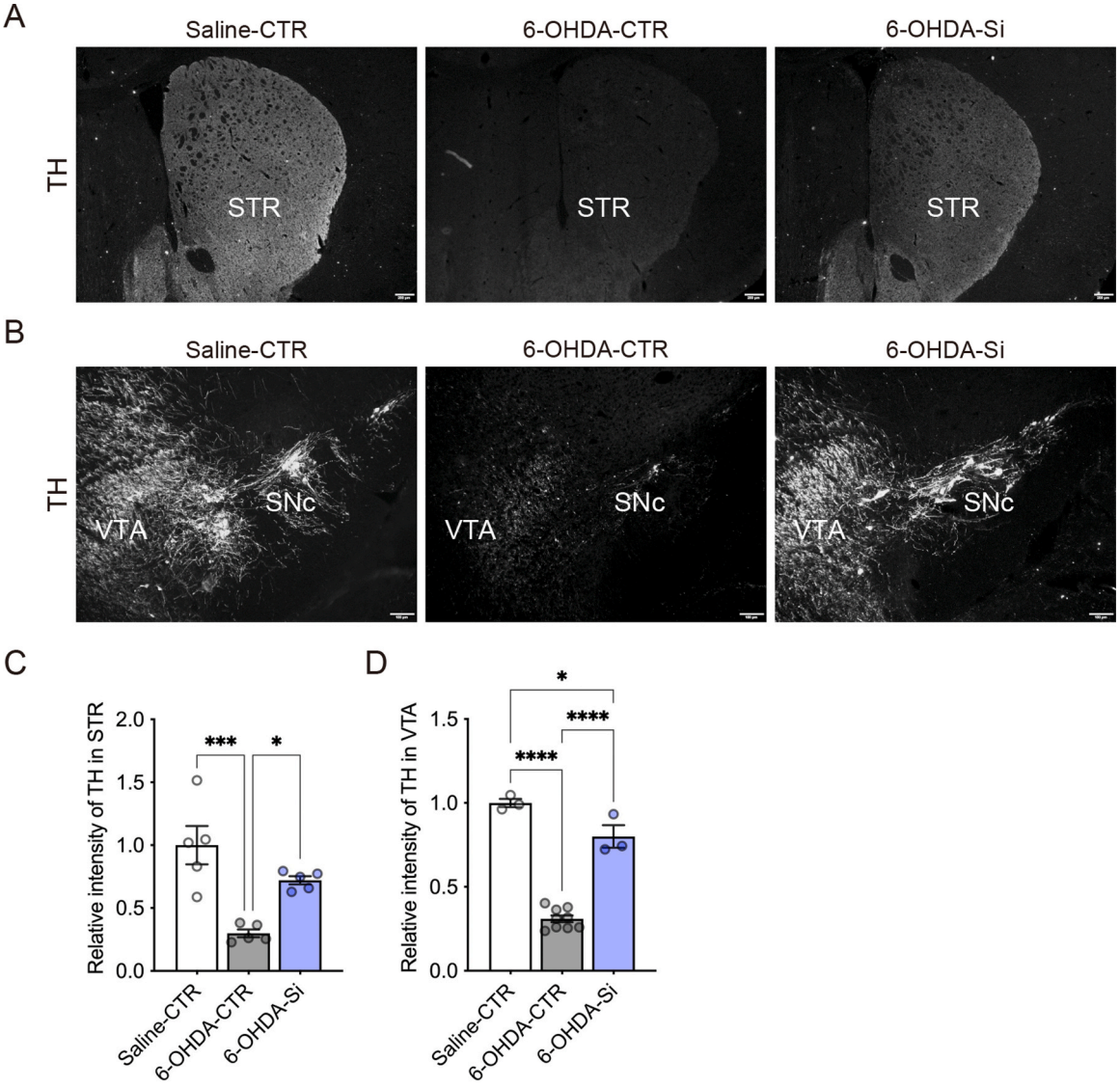

Fig. 1. Si-based agent protects 6-hydroxydopamine (6-OHDA)-induced neuronal degradation in mice. (A) Representative fluorescence images of tyrosine hydroxylase (TH)-positive immunoreactivities of the fibers from dopaminergic neurons in the striatum (STR) of mice at P24. (B) Representative fluorescence images of TH-positive immunoreactivities of dopaminergic neurons and dopaminergic fibers in the ventral tegmental area (VTA) and substantia nigra pars compacta (SNc) of mice at P24. (C, D) Quantification of TH-immunoreactivities in the STR (C) and VTA (D). 6-OHDA-induced neuronal degradation was protected by Si-based agent. Data are represented as means (±SEM). Asterisk indicates ****P<0.0001, ***P<0.001, *P<0.05, one-way ANOVA with a Tukey's multiple comparison test. n=3–9/condition. Scale bars: 100 μm.

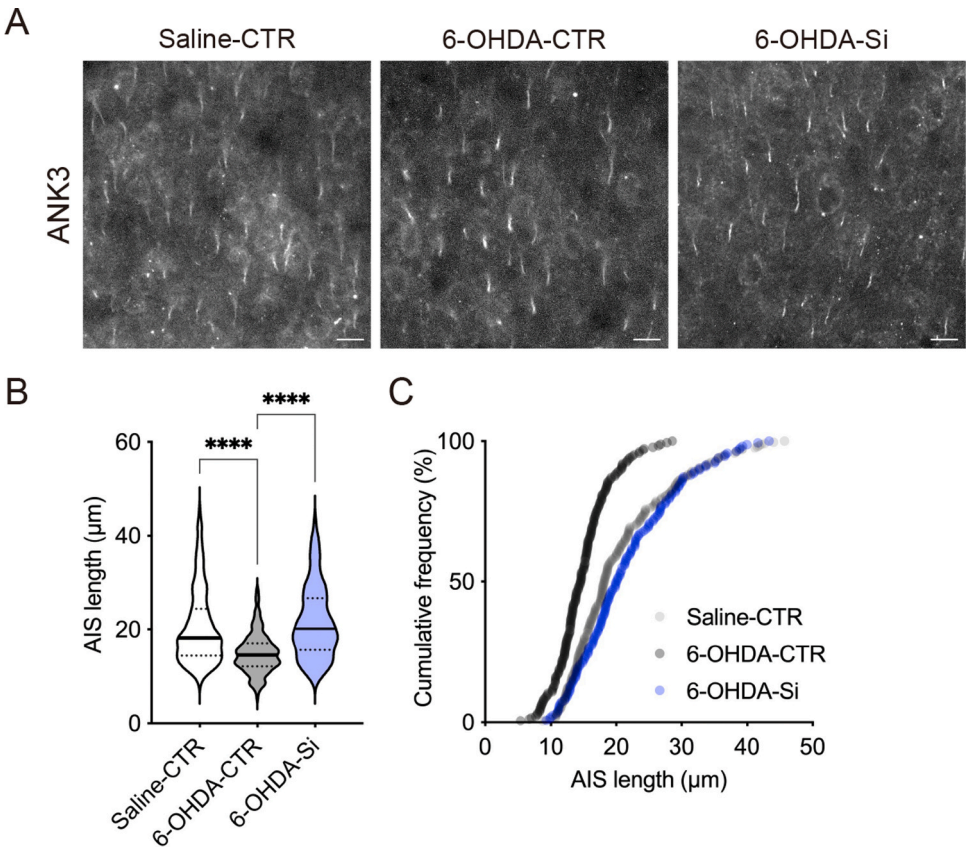

Fig. 2. Neuroprotective effect of Si-based agent on axon initial segment (AIS) in mice. (A) Representative fluorescence images of the ANK3-positive AIS of layer (L) 2/3 neurons in the medial prefrontal cortex (mPFC) of mice at P24. (B, C) Quantification and cumulative frequency distributions of AIS lengths in L2/3 neurons of the mPFC. Si-based agent showed neuroprotective effect on the AIS lengths of mPFC. Data are represented as means (±SEM). Asterisk indicates ****P<0.0001, one-way ANOVA with a Tukey's multiple comparison test. n = 165–252 cells from 3–5 animals/condition. Scale bars: 100 μm.

compared to CTR mice (Fig. 2C). In contrast, the AIS lengths in mPFC L2/3 neurons were restored in 6-OHDA mice treated with the Si-based agent (Fig. 2A, B). Cumulative frequency distribution plots of AIS lengths in L2/3 neurons of mPFC of 6-OHDA mice treated with the Si-based agent showed a similar curve to CTR mice (Fig. 2C). These results indicate that the neuroprotective effects of the Si-based agent are not limited to the damaged brain regions but also extend to neurons in other brain regions.

3.3. Si-based agent improved hyperactivity in 6-OHDA mice

Finally, we investigated whether the neuroprotective effects of the Si-based agent extended to behavioral outcomes. A mouse model of 6-OHDA-induced ADHD displays ADHD-like behaviors, including hyperactivity, impulsivity, and inattention [19,20,23]. Among these behaviors, we assessed hyperactivity using the open field test at P24. Mice treated with 6-OHDA displayed significant increases in distance traveled, mean speed, and mobile time compared to CTR mice (Fig. 3A-D). Interestingly, these phenotypes were restored in 6-OHDA mice treated with the Si-based agent (Fig. 3A-D). There was no effect on mouse weight (Fig. 3E). Together, these results suggest that the neuroprotective effect not only prevents neuronal damage but also mitigates behavioral outcomes indirectly.

4. Discussion

In this study, we demonstrated the neuroprotective effect of the Si-based agent against the selective damage of dopaminergic neurons and loss of dopaminergic fibers induced by 6-OHDA in the mouse brain. Additionally, the Si-based agent protected the lengths of AIS and mitigated hyperactive behavior in 6-OHDA mice. Our results highlight the neuroprotective role of the Si-based agent against neurotoxin-induced degradations and alterations of the central nervous system.

To explore the role of Si-based agent on the neuroprotective effect, we utilized 6-OHDA as a neurotoxin in an established mouse model of ADHD, representing one of juvenile neurotoxic models. 6-OHDA selectively degenerates and damages dopaminergic and noradrenergic neurons in the brain by inducing neuronal cell death via the productions of oxidative stress and reactive oxygen species (ROS) [40–42]. Such neurotoxic effects of 6-OHDA are commonly utilized in generating animal models for investigating the pathological mechanisms underlying diseases such as ADHD and PD. As a note, there are significant differences between these two different disease models in terms of generation method such as injection region of 6-OHDA as well as behavioral outcome phenotypes [10,11,19,20]. Indeed, PD models generated by striatal injections of 6-OHDA show decreased locomotor or normal locomotor activity in the open field test [33,43].

The Si-based agent, like hydrogen, produces neuroprotective effects due to hydrogen production in the body. In the context of redox reactions, hydrogen and Si-based agent exhibit neuroprotective effects, encompassing antioxidative, anti-inflammatory, antiallergic, and anti-apoptotic effects without side effects [24–28,34]. Moreover, we have reported that the Si-based agent modulates the expressions of antioxidant genes and suppresses the expressions of inflammation-related genes [28,35]. Among them, HMOX1 and NQO1 plays a central role in the NFE2L2/ KEAP1 redox reaction (also known as NRF2) [44–49]. In a similar study, it has been reported that apomorphine activates NFE2L2-ARE pathway and suppresses neuronal cell death due to oxidative stress induced by 6-OHDA [41,50]. Based on these findings, we speculate that the Si-based agent acts as a neuroprotectant,

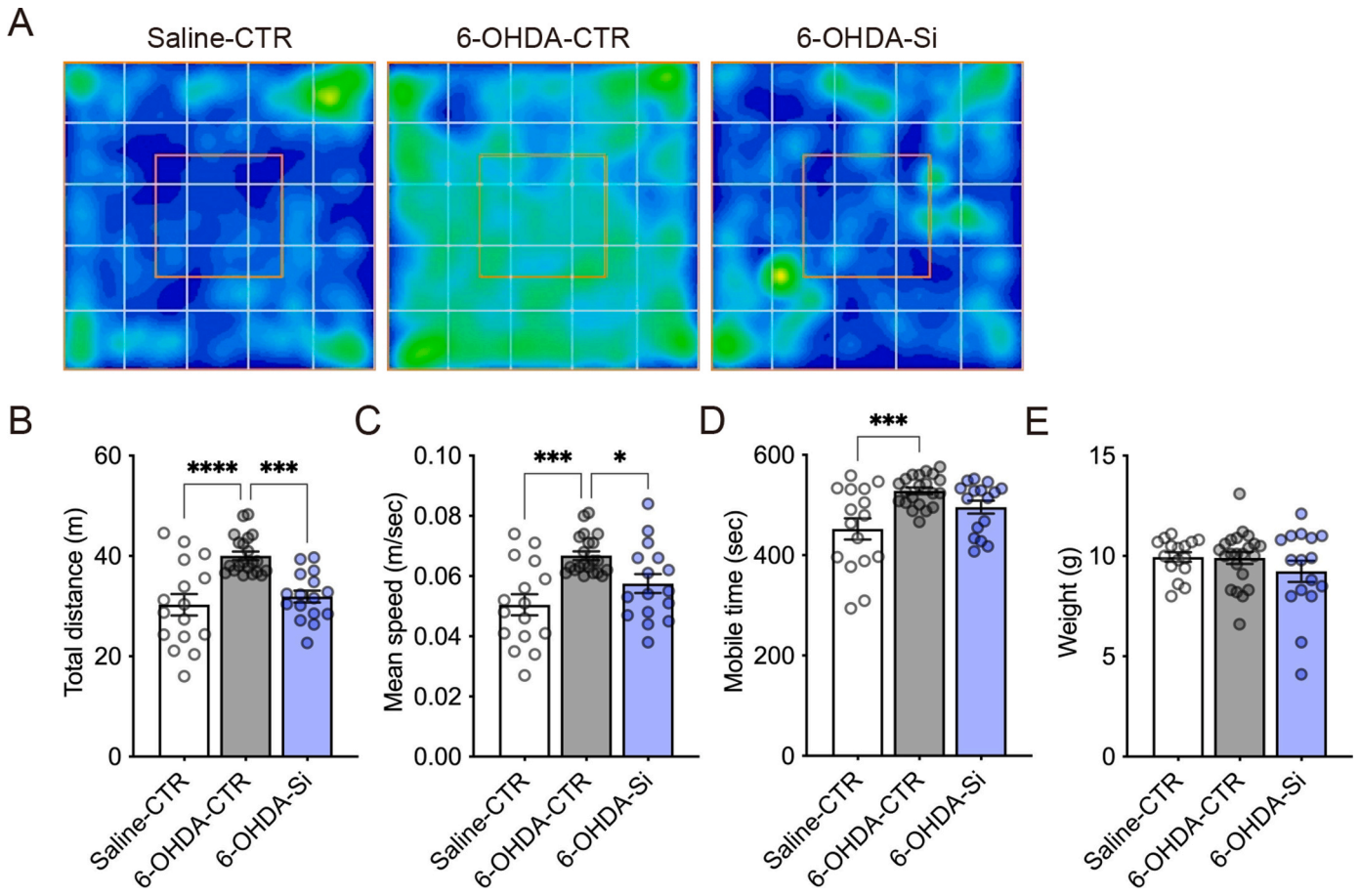

Fig. 3. Si-based agent mitigated hyperactivity induced by 6-OHDA in mice. (A) Representative heatmaps of mouse exploration activities in the open field test at P24. (B-D) Quantifications of total distance travelled (B), mean speed (C), and mobile time (D) during the open field test. Compared to the CTR mice, 6-OHDA mice exhibited hyperactivity, but such hyperactivity was not observed in 6-OHDA mice with Si-based agent. (E) No difference was observed in the weight. Data are represented as means (±SEM). Asterisks indicate ****P<0.0001, ***P<0.001, **P<0.01, one-way ANOVA with a Tukey's multiple comparison test. n=16–22/condition.

demonstrating enhanced antioxidant effects against the 6-OHDA neurotoxin.

Previously, we reported that the shortened lengths of AIS labeled with ANK3 in the mPFC and S1BF of a mouse model of ADHD induced by 6-OHDA, suggesting that shorter lengths of AIS may contribute to behavioral abnormalities in ADHD [23]. ANK3 (known as Ankyrin-G) is a main component of AIS, playing essential roles in its functions and structure, including action potential initiation and sodium channel clustering [51,52]. Mutations of *ANK3* have been identified in individuals with ADHD [53], and *Ank3* cKO mice exhibited ADHD-like phenotypes including hyperactivity and impulsivity [54]. The cortical expression of *Ank3* was strongly observed in L2/3 rather than L5 and L6, suggesting AIS lengths of L2/3 neurons contribute hyperactivity and impulsivity [54]. One reason why hyperactive phenotype of 6-OHDA mice improved with Si-based agent may be due to the improvement of AIS lengths in L2/3 neurons. A previous study has also reported that reduced cortical thickness of L2/3 to L6 and losing dendritic complexity of L2/3 pyramidal neurons in the mPFC of 6-OHDA-induced ADHD mice [20], suggesting that the loss of dendritic complexity in L2/3 neurons leads to shorted AIS lengths. In patients with ADHD, multiple studies have reported decreased volumes in brain regions and alterations in their connectivity, including the PFC, anterior cingulate gyrus, and basal ganglia influenced by dopamine from the VTA [55]. The functional magnetic resonance imaging study in children with ADHD has also reported that the functional deficits of corticostriatal circuits [56]. Thus, it is suggested that Si-based agent protects dopaminergic neurons from 6-OHDA-induced neurotoxicity and indirectly prevents morphological alterations in the mPFC of 6-OHDA mice including AIS phenotype. Collectively, these findings also suggest that the Si-based agent is an effective neuroprotective agent not only in the injured region but also in a range of its associated regions in the nervous system as a result.

Furthermore, this study has unveiled novel insights into the effects of Si-based agent, expanding our understanding of its impact. This time, Si-based agent was given during the suckling period. It is challenging for offspring mice to directly ingest the Si-based agent in the early period after birth. Thus, there is a possibility that the milk from mother mice that have ingested the Si-based agent or hydrogen elements released from the mother's body directly affects the offspring mice. It is presumed that the offspring mice experienced the neuroprotective effects of the Si-based agent from a certain period, which is attributed to both the maternally derived effects and the directly ingested effects. Although we have previously reported on the effects of Si-based agent on the fetus via the mother during pregnancy [35,36], this study is the first to report on the effects derived from both the mother's milk and the mother's body, as well as intake of Si-based agent during breastfeeding and early postnatal stages.

Several limitations of this study should be acknowledged. The detailed molecular functional mechanisms and transmission manner between mother and neonates of the Si-based agent remain largely unknown. In terms of these issues, we are exploring the further investigations as future studies. We emphasize that this study examined the effects of Si-based agent on 6-OHDA-induced neurotoxicity of dopaminergic neurons, not on ADHD. In other words, we do not claim in this study that Si-based agent is effective as a treatment for ADHD. This

is because we only evaluated the effect of Si-based agent on the AIS length of L2/3 neurons of mPFC and hyperactive behavior among ADHD-like behaviors in a 6-OHDA-induced ADHD mouse model, but not the effects of Si-based agent on attention or impulsivity. Additionally, the neural circuits that control behavior are complex and require deeper insights.

Overall, in this study, we demonstrated that Si-based agent protects the circuit formation of the developing dopaminergic nervous system and its plasticity, reducing the impact of neurotoxicity induced by 6-OHDA. This study suggests that the Si-based agent serves as an effective neuroprotectant and antioxidant against neurotoxic effects in the brain, offering the possibility of the Si-based agent as a neuroprotectant for nervous system diseases. Moreover, our findings provide a novel perspective on the neuroprotective role of the Si-based agent, particularly in the context of its use during breastfeeding and infancy.

5. Conclusion

Our study illustrates the neuroprotective effects of the Si-based agent as a neuroprotectant agent on 6-OHDA-induced neuronal degenerations in the mouse brain.

Funding

This work was supported by the Japan Science and Technology Agency (JST) Center of Innovation Program (COI Program) (JPMJCE1310) to N.U. H.K., and S.S.; the Japan Society for the Promotion of Science (JSPS) Grant-in-Aid for Scientific Research (B) (23H02837) to N.U. M.D., and S.S.; JSPS Grant-in-Aid for Scientific Research (B) (23H03002) to N.U., M.D., Y.K., Y.N., and S.S.; JSPS Grant-in-Aid for Scientific Research (C) (20K06872) to N.U.; JSPS Grant-in-Aid for Challenging Research (20K21654) to N.U., Y.K., Y.N., and S.S.; JSPS Grant-in-Aid for Early-Career Scientists (23K14443) to M.D.; Uehara Memorial Foundation to N.U.; Takeda Science Foundation to N.U.; Naito Foundation to N.U.; Mochida Memorial Foundation for Medical and Pharmaceutical Research to N.U.; Inamori Foundation to N.U.; SENSHIN Medical Research Foundation to N.U.; Osaka Medical Research Foundation for Intractable Diseases to N.U. and M.D. and Eli Lilly Japan Research Grant to N.U.

CRediT authorship contribution statement

Shogo Togawa: Investigation, Validation, Writing - Review & Editing, Visualization. **Noriyoshi Usui:** Conceptualization, Methodology, Validation, Investigation, Writing - Original Draft, Writing - Review & Editing, Visualization, Supervision, Project administration, Funding acquisition. **Miyuki Doi:** Validation, Investigation, Writing - Review & Editing, Visualization. **Yuki Kobayashi:** Resources. **Yoshihisa Koyama:** Investigation. **Yukiko Nakamura:** Investigation. **Koh Shinoda:** Writing - Review & Editing, Supervision. **Hikaru Kobayashi:** Resources, Writing - Review & Editing, Supervision, Funding acquisition. **Shoichi Shimada:** Writing - Review & Editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

Acknowledgements

This study was supported by CentMeRE, Graduate School of Medicine, Osaka University.

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