Social isolation contributes into the effect of 3-day hindlimb unloading on dopaminergic transmission in the nigrostriatal system of mice

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Abstract

The nigrostriatal system composed of the dorsal striatum and the substantia nigra (SN) is highly involved in the control of motor behavior. Various extremal and pathological conditions as well as social isolation may cause an impairment of locomotor function; however, corresponding alterations in the nigrostriatal dopaminergic pathway are far from full understanding. Here we analyzed the effect of 3-day hindlimb unloading (HU) and social isolation (SI) on the key players of dopamine transmission in the nigrostriatal system of CD1 mice. Three groups of mice were analyzed: group-housed (GH), SI, and HU. Our data showed a significant decrease in the expression of tyrosine hydroxylase (TH) in the SN and dorsal striatum of HU mice, but only in comparison with SI group that suggested attenuation of dopamine synthesis in response to HU, while TH phosphorylation was reduced in comparison with both GH and SI animals. SI also led to a decrease in TH phosphorylation in the dorsal striatum that pointed on an impact of isolation too. Expression of dopamine receptors D1 in the dorsal striatum of HU mice was increased suggesting a compensatory response, but the activity of downstream signaling pathways involving PKA and CREB was inhibited. But in the dorsal striatum of SI mice, expression of DA receptors and activity of downstream signaling was not affected. Obtained data let us to conclude that combination of short-term HU and isolation impaired dopamine transmission in the nigrostriatal system.

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Keywords *:* dorsal striatum, substantia nigra, dopamine receptors, tyrosine hydroxylase, PKA, CREB

Running title: dopaminergic transmission in muscle disuse

Abstract

The nigrostriatal system composed of the dorsal striatum and the substantia nigra (SN) is highly involved in the control of motor behavior. Various extremal and pathological conditions as well as social isolation may cause an impairment of locomotor function; however, corresponding alterations in the nigrostriatal dopaminergic pathway are far from full understanding. Here we analyzed the effect of 3-day hindlimb unloading (HU) and social isolation (SI) on the key players of dopamine transmission in the nigrostriatal system of CD1 mice. Three groups of mice were analyzed: group-housed (GH), SI, and HU. Our data showed a significant decrease in the expression of tyrosine hydroxylase (TH) in the SN and dorsal striatum of HU mice, but only in comparison with SI group that suggested attenuation of dopamine synthesis in response to HU, while TH phosphorylation was reduced in comparison with both GH and SI animals. SI also led to a decrease in TH phosphorylation in the dorsal striatum that pointed on an impact of isolation too. Expression of dopamine receptors D1 in the dorsal striatum of HU mice was increased suggesting a compensatory response, but the activity of downstream signaling pathways involving PKA and CREB was inhibited. But in the dorsal striatum of SI mice, expression of DA receptors and activity of downstream signaling was not affected. Obtained data let us to conclude that combination of short-term HU and isolation impaired dopamine transmission in the nigrostriatal system.

Keywords *:* dorsal striatum, substantia nigra, dopamine receptors, tyrosine hydroxylase, PKA, CREB

Abbreviations *:* D1R, dopamine receptor D1; D2R, dopamine receptor D2; HU, hindlimb unloading; SI, social isolation; PKA, protein kinase A; SNc, substantia nigra pars compacta; SPN, spiny projection neurons; TH, tyrosine hydroxylase.

Introduction

The striatum is the central part of the basal ganglia that integrates and processes information from the cortex, the hippocampus, the substantia nigra, and the thalamus (Lanciego *et al.* , 2012; Haber, 2016). While functions of the ventral striatum are associated with the reward system, reinforcement, and emotions, the dorsal striatum (neostriatum) composed of the caudate nucleus and the putamen mainly participates in motor control and operational learning (Lanciego*et al.* , 2012; Haber, 2016). Communication of the striatum with other brain regions is mediated by spiny projection neurons (SPNs), GABAergic striatofugal cells, which are traditionally divided into the two populations (Kreitzer, 2009). In the dorsal striatum, activation of SPNs induces direct (striatonigral) pathway of the basal ganglia that stimulates the motor cortex and promotes initiation of movements (Bateup*et al.* , 2010; Wall *et al.* , 2013). Cells of the second population initiate indirect (striatopallidal) pathway suppressing the activity of the motor cortex and locomotion (Bateup *et al.* , 2010; Wall *et al.* , 2013).

Proper activity of the striatal SPNs, in turn, is maintained by dopamine provided by neurons of the substantia nigra pars compacta (SNc) (Gantz*et al.* , 2018). According to the classical concept, bursts of nigrostriatal dopamine differentially stimulate SPNs of direct pathway and inhibit SPNs of indirect one facilitating activation of the direct circuits and suppressing indirect ones (Kreitzer, 2009; Lanciego*et al.* , 2012). Low dopamine signaling, in opposite, results in predominance of indirect pathway (Duty & Jenner, 2011). Thus, dopamine coordinates the work of stimulating and suppressing circuits and provides optimal level of striatal activation necessary for maintaining locomotor activity according to the currents needs. Plenty of data indicate that physical activity is promoted by enhanced metabolism of dopamine and its massive release in synapses between dopaminergic neurons of SNc and striatal SPNs (Korchounov *et al.* , 2010; Gepshtein *et al.* , 2014). At the same time insufficient dopamine signaling in the dorsal striatum can be associated with motor disorders, including akinesia, bradykinesia, tremor, changed muscle tone, and postural abnormalities (inability to maintain equilibrium under dynamic or static conditions), observed in patients with Parkinson's disease and in corresponding animal models (Duty & Jenner, 2011; Palakurthi & Burugupally, 2019).

It is well-known that microgravity, the main negative factor of spaceflights, strongly impairs locomotor function provoking such disorders as impaired gait, postural instability, and changes in muscle tone (Reschke *et al.* , 1998; Lacquaniti *et al.* , 2017; Tays*et al.* , 2021). Similar alterations were observed in ground-based conditions with volunteers which experienced antiorthostatic hypokinesia (head-down bed rest) (Parry &

Puthucheary, 2015) and in experimental animals exposed to hindlimb unloading (HU) (Canu *et al.* , 2007). The hindlimb unloading (HU) model is a widely used approach to simulate microgravity in ground-based animal studies. It effectively reproduces such negative effects of spaceflights as removal of the support loading from the hindlimbs, cerebrospinal fluid shift, and degeneration in bones and muscles (Morey-Holton & Globus, 2002; Globus & Morey-Holton, 2016).

Furthermore, investigation of experimental animals revealed that real microgravity significantly affects dopaminergic system of the brain (Popova *et al.* , 2015, 2020; Tsybko *et al.* , 2015). Thus, 30-day spaceflight induced a considerable reduction in mRNA of proteins responsible for dopamine synthesis (tyrosine hydroxylase, TH), degradation (catechol-O-methyltransferase, monoamine oxidases), and postsynaptic effects (dopamine receptors) in the nigrostriatal system (Popova *et al.* , 2015). In addition, studies revealed reduced nigrostriatal expression of neurotrophins GDNF (glial cell-line derived nervous factor) and CDNF (cerebral dopamine neurotrophic factor) which are necessary for optimal functioning of dopaminergic neurons (Tsybko*et al.* , 2015). However, available data concern only long-term effect of microgravity, while the main processes of adaptation are expected to occur during a shorter period of exposure. In addition, these data are associated mainly with analysis of mRNA which does not always fully correlate with protein amount and activity (Popova *et al.* , 2020). Thus, further investigations are needed to disclose detailed mechanisms of altered dopaminergic regulation in the brain under pathological and extremal conditions associated with restricted motility, loss of sensorimotor stimuli, and muscle disuse.

Ground-based animal studies could provide more information about detailed mechanisms of dysregulation in the dopaminergic system of the brain. However, the studies showed that in rodents the effect of 30-day HU on the dopaminergic system considerably differed from those of actual spaceflight (Kulikova *et al.* , 2017). Expression of some crucial markers affected by the real microgravity (TH, GDNF, CDNF) showed no changes in response to HU, while others, such as dopamine receptors D1 (D1R), demonstrated the opposite alterations. These data confirmed that HU model could not be considered as universal approach to study the physiological effects of microgravity (Kulikova *et al.* , 2017). Nevertheless, it is still can be perspective to investigate various alterations induced by ground-based pathological conditions such as inactivity, bed rest, and immobilization (Globus & Morey-Holton, 2016).

Social isolation (SI) is another factor that can produce a number of negative physiological outcomes including chronic stress, motor decline, and cognitive disorders (Arzate-Mejía *et al.* , 2020; Vitale & Smith, 2022). For example, it was shown that in elderly people, loneliness was associated with more rapid rate of motor decline manifesting as decrease in muscle strength, walking speed, and physical performance (Buchman *et al.* , 2010; Philip *et al.* , 2020). Animal models of SI also revealed dysregulation of dopamine signaling, however, main changes were observed in the ventral striatum (Benderskyet *al.* , 2021; Zhang *et al.* , 2021; McWain *et al.* , 2022), while alterations the nigrostriatal dopaminergic system still remain unclear.

On the other hand, SI is the important factor that should be mentioned both in spaceflight and ground-based experiments. Experimental data indicated that isolation contributed to negative effects of gravitational unloading including violations in the musculoskeletal apparatus and locomotor activity (Morey-Holton *et al.* , 2000; Tsvirkun *et al.* , 2012; Tahimic *et al.* , 2019). Thus, studying combined effects of SI and HU on the brain systems responsible for locomotor control is also of considerable interest.

The aim of the present work was to analyze the effect of short-term (3-day) HU and SI on the dopaminergic regulation of the dorsal striatum in mice. Obtained results showed that HU negatively affected dopamine biosynthesis and dopamine-mediated signaling in the nigrostriatal system indicating a decrease in dopaminergic neurotransmission. Contribution of SI into effects of HU was also revealed.

Materials and methods

Animals and experimental design

Adult (4-5 month old) male CD1 mice were recruited in the experiments. All animals were housed under 12/12 light–dark cycle with free access to food and water. All experimental procedures were carried out

in accordance with EC Directive 86/609/EEC for animal experiments and approved by the Institutional Animal Care and Use Committee at the Sechenov Institute of Evolutionary Physiology and Biochemistry (# 12-2/2021).

Mice were divided into three experimental groups:

1) Group-housed (control) animals (GH group, n=10) which were housed in standard vivarium cages, 5 animals per cage;

2) Mice exposed to hindlimb unloading (HU group, n=10). Each mouse was housed in the individual cage and suspended by the tail through a swivel attached to a metal rod at the top of the cage. The suspension angle was adjusted to approximately 30°, so that the hindlimbs did not contact with the floor but the animal was able to move freely around the cage with use of the forelimbs (Morey-Holton & Globus, 2002).

3) Socially isolated mice (SI group, n=10) which were housed in the individual cages similar to those for HU group, but without tail suspension and any other movement restrictions.

The duration of the experiment consisted 3 days. All animals were sacrificed on the following day.

Sample preparation

5 mice from each group were deeply anesthetized with i.p. injection of Zoletil/Xylazine mixture (60 mg/kg+10 mg/kg; Virbac, France) and then perfused transcardially with cold phosphate buffered saline (PBS) followed by 4% PFA in 0.1 M PBS (pH 7.4). After perfusion all mice were decapitated, the brains were removed, postfixed in 4% PFA (+4°C, 5 days), incubated in 20% sucrose/PBS for cryoprotection (+4°C, 3 days), then frozen, and stored at - 80°C for further immunohistochemical assay. The other 5 animals from each group were decapitated, each dorsal striatum was dissected and homogenized separately for further Western blot analysis.

Immunohistochemistry

After standard preliminary procedures cut sections were incubated overnight with primary antibodies (Table 1). Further the sections were washed in PBS and incubated for 1 hour with biotinylated anti-rabbit or anti-mouse secondary antibodies (Tabl. 1) followed by one-hour incubation with streptavidin-peroxidase complex (1:500, Supelco, #S2438). The peroxidase reaction was revealed in the buffer containing 0.05% 3,3'-diaminobenzidine (DAB, Sigma-Aldrich, #D5637) and hydrogen peroxide (0.01%).

Evaluation of sections. The frozen brains were sectioned coronally at 10 μm using Leica cryostat. Every 15thsection was selected for analysis of TH, phospho(Ser31)-TH or phospho(Ser40)-TH in the SNc, and TH, phospho(Ser31)-TH or phospho(Ser40)-TH, D1R, D2R, or phospho(Ser133)-CREB in the dorsal striatum. In total, five sections of the studied zone per each immunostaining were analyzed for each animal. The sections were processed under standardized conditions in every experiment, i.e. control and experimental groups in each experiment were collected, fixed, and processed for analysis simultaneously. To examine the specificity of staining, we performed the negative control (the same protocol without primary antibodies) that demonstrated no staining. Analysis of immunostaining in the SNc and dorsal striatum was performed with use of taken images by a 20x/0.5 objective on Zeiss Axio Imager A1 microscope (Carl Zeiss Microscopy GmbH).

Content of immunopositive substances was estimated as the optical density normalized with background with use of ImageJ software (version 6.0). Calibration was done according ImageJ recommendation (https://imagej.nih.gov/ij/docs/examples/calibration/). Each analyzed image was converted into 8-bit grayscale image and optical density of ROI was estimated. Optical density of the background was evaluated at the same image in non-immunoreactive brain tissue field.

Western blot analysis

Each striatum was homogenized in lysis buffer (20 mM Tris pH 7.5, 1% Triton-X100, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA) with protease inhibitors (Sigma-Aldrich, #P8340) and phosphatase inhibitors (Roche,

#04 906 837 001) using tissue grinder at 4°C. Insoluble materials were removed by centrifugation. Total protein content in samples was determined by Lowry assay with bovine serum albumin (BSA) as a standard. The supernatant was mixed in ratio 2:1 with 3x loading buffer (0.2 M Tris-HCl pH 6.7, 6% sodium dodecyl sulfate, 15% glycerol, 0.003% bromophenol blue, and 10% β-mercaptoethanol) and incubated for 10 min at 96°C. Equal amounts of samples (10 μg protein per line) were loaded for electrophoresis. Proteins were separated on 10% or 8% polyacrylamide gel and then transferred to nitrocellulose membrane (Santa Cruz Biotechnology, #sc-3718). The membranes were incubated in 5% non-fat milk or 3% BSA in Tris buffered saline with Tween (TBST) for 1 h and then incubated overnight with primary antibodies (Table 1). Then the membranes were washed in TBST and incubated with secondary antibodies (Table 1) for 1 hour at room temperature. Specific protein bands were visualized by chemiluminescent reaction produced by SuperSignal West Dura Extended Duration Substrate (ThermoFisher Scientific, #34075) with use of ChemiDoc MP Imaging System (#12003154, Bio-Rad Laboratories Inc., Hercules, CA, USA).

Densitometric analysis of protein content was performed using ImageJ software (version 6.0). Phosphorylation of TH (Ser31, Ser40), ERK1/2 (Thr202/Tyr204), Akt (Thr308), and GSK3β (Ser9) was estimated as ratios pTH/TH, pERK1/2/ERK1/2, pAkt/Akt, and pGSK3β/GSK3β. Phosphorylation of CAMKII (Thr286) was estimated by normalizing to actin. Overall phosphorylated substrates of PKA were analyzed on the full blot, also with normalizing to actin. Phosphorylation of specific PKA target, Ser157 of VASP (vasodilator-stimulated phosphoprotein), was estimated as ratio pVASP/VASP.

Statistical analysis

All data of Western-blot and imunohistochemical assays were processed statistically by non-parametric Kruskal-Wallis test with post hoc Dunn's test using GraphPad 7 Software. All results were presented as median ± interquartile range. Differences were regarded as significant at $p<0.05$.

Results

Expression and activity of TH in the nigrostriatal system

Firstly, we analyzed expression and phosphorylation of TH – the rate-limiting enzyme of dopamine biosynthesis. It is abundantly expressed in bodies of dopaminergic neurons localized in the substantia nigra pars compacta and in their axon terminals innervating striatal SPNs (Klein *et al.* , 2018). Immunohistochemical analysis showed that both expression of TH (Figure 1A, D) and its phosphorylation at Ser31 (Figure 1B, E) were significantly decreased in the SNc of mice in HU group compared with SI, but not GH animals. As conditions in HU and SI groups differed only by presence of unloading, we supposed that observed changes were caused rather by HU than isolation. Phosphorylation of TH at Ser40 (the main site of TH activation) was decreased in HU mice in comparison with both GH and SI groups (Figure 1C, F). Normally loaded GH and SI animals demonstrated no difference for all these markers. Thus, our data indicated that antiorthostatic unloading was the main factor which caused down-regulation of TH and dopamine biosynthesis in the SNc dopaminergic neurons.

In the dorsal striatum, TH expression estimated by immunochemical assay was as well lower in HU group in comparison with SI group (Figure 2A, D), however, TH phosphorylation at Ser31 was, in opposite, higher (Figure 2B, E). Besides, neither HU nor SI mice differed statistically from GH control. TH phosphorylation at Ser40 was reduced both in the HU and SI group in comparison with GH animals (Figure 2C, E). These data let us to suppose that both HU and SI affected dopaminergic innervation of striatal neurons.

Expression of dopamine receptors D1 and D2 in the dorsal striatum

Two main subtypes of dopamine receptors expressed in the dorsal striatum are D1R and D2R (Meador-Woodruff, 1996). Immunohistochemical analysis revealed an increase in the expression of D1R in the dorsal striatum of HU mice in comparison with both GH and SI groups, while normally loaded GH and SI animals demonstrated similar expression (Figure 3A, C). At the same time D2R expression in all investigated groups showed no significant difference (Figure 3B, D).

Activity of dopamine-linked signaling pathways in the dorsal striatum

To estimate activity of downstream signaling cascades of dopamine receptors, we analyzed activity of protein kinase A (PKA), extracellular signal-regulated kinases 1 and 2 (ERK1/2), protein kinase B (Akt), glycogen synthase kinase 3β (GSK3β), and Ca^{2+}/calmodulin-dependent protein kinase II (CAMKII) in the dorsal striatum. Western-blot analysis showed that phosphorylation of total PKA substrates was decreased in the dorsal striatum of HU mice in comparison with both GH and SI freely moving animals, while no difference between GH and SI animals was observed (Figure 4A, G). Phosphorylation of VASP at Ser157, specific PKA target (Howe *et al.* , 2005; Tejeda *et al.* , 2020), showed similar pattern of alterations (Figure 4B, G). Altogether, these data indicated decreased activity of PKA in HU mice. At the same time we did not reveal any changes in phosphorylation of ERK1/2 (Thr202/Tyr204) (Figure 4C, G), Akt (Thr308) (Figure 4D, G), GSK3beta (Ser9) (Figure 4E, G), and CAMKII (Thr268) (Figure 4F, G) indicating that 3-day HU and SI did not affect corresponding signaling pathways.

It is also known that activation of D1R and D2R is accompanied with increased phosphorylation of transcriptional factor CREB (Beaulieu & Gainetdinov, 2011). Immunohistochemical analysis showed decreased amount of active CREB phosphorylated at Ser133 in the cell nuclei of the dorsal striatum in mice exposed to HU as compared with both GH and SI groups (Figure 5). Again, GH and SI animals demonstrated no difference.

Discussion

In the present study we investigated the effect of short-term (3-day) HU and SI on the dopaminergic regulation of the dorsal striatum in mice at three levels: activity of dopamine synthesis, expression of dopamine receptors, and downstream signaling cascades.

TH expression and activity

Dopamine is the main catecholamine neurotransmitter in the brain (Marsden, 2006; Klein *et al.* , 2018). The main dopamine sources are two neighboring areas of the midbrain: the substantia nigra associated with the dorsal striatum (nigrostriatal dopaminergic pathway) and the ventral tegmental area (VTA) that sends dopaminergic afferents to the ventral striatum (mesolimbic pathway) and the prefrontal cortex (mesocortical pathway) (Gantz *et al.* , 2018). While mesolimbic and mesocortical dopamine is the key neurotransmitter of the reward system participating in goal-directed behavior and emotional reactions, nigrostriatal dopamine is mainly involved in regulation of locomotor function and operational learning (Reynolds & Flores, 2021). TH is a rate-limiting enzyme of dopamine biosynthesis which is specifically expressed in the cell bodies and processes of catecholamine-producing neurons (Klein *et al.* , 2018). Activity of TH is modulated by phosphorylation at a number of sites including Ser40 and Ser31. It was shown that Ser40 is the main activating site phosphorylation of which strongly potentiates TH activity, while phosphorylation at Ser31 activates TH in a lesser extent (Dunkley *et al.* , 2004).

Our data revealed that 3-day SI did not affect expression and phosphorylation of TH in the SNc. In the SNc of HU mice, the expression of TH phosphorylated at Ser40 was significantly reduced in comparison with both GH and SI mice. We also observed a decrease in TH phosphorylated at Ser31 and total TH in HU mice in comparison to SI. Altogether, these data indicated a decrease in the activity of dopamine biosynthesis in the SNc that was caused by HU conditions. Interestingly, changes in dopamine system in response to gravitational unloading and loss of support afferentation were observed not only in mammals (Popova*et al.* , 2015, 2020; Tsybko *et al.* , 2015; Kulikova *et al.* , 2017), but also in nematode *C. elegans* , in which both real and simulated microgravity decreased dopamine metabolism, while adding of contact stimuli reversed these changes (Sudevan *et al.* , 2022).

Moreover, our data showed decreased TH expression in the dopaminergic terminals innervating the dorsal striatum in HU mice as compared with isolated animals. Similarly to SNc, these data indicated the negative effect of unloading. However, striatal expression of pTH(Ser40) was decreased both in HU and SI mice suggesting that this indicator of TH activity was negatively affected mainly by SI. Thus, in the dorsal

striatum both HU and isolation contributed to impaired dopamine innervation.

In opposite, TH phosphorylation at Ser31 was increased in the dorsal striatum of HU mice. While phosphorylation of TH at Ser40 participates in the up-regulation of its catalytic activity (Dunkley *et al.* , 2004), the role of this post-translational modification is far from clarity. *In vivo* analysis of rats demonstrated that content of pTH(Ser31) in the terminals of dopaminergic neurons, particularly in the striatum, was larger than in SN, while there was no difference in the content of pTH(Ser40) between striatum and SN (Salvatore & Pruett, 2012). The authors supposed that pTH(Ser31) can play a role of the autonomous regulator of dopamine synthesis in the SN terminals (Salvatore & Pruett, 2012). Latest data revealed that pTH(Ser31) can be a transport form of TH which is loaded into vesicles and transferred from the cell body to the axon terminals (Jorge-Finnigan *et al.* , 2017). Moreover, in rodent model of Parkinson's disease pTH(Ser31) was decreased in the striatum, but increased in SN, against the background of progressive TH loss (Salvatore, 2014). Thus, we supposed that increased TH phosphorylation at Ser31 in the striatum of HU mice could provide an adaptive mechanism aimed to compensate the impairment of dopamine synthesis in dopaminergic neurons of SNc.

Dopamine receptors

Action of dopamine on SPNs of the dorsal striatum is mediated by two main subtypes of dopamine receptors, D1 and D2 (Meador-Woodruff, 1996). Expression of D1R and D2R is highly segregated: D1R are predominantly localized in the SPNs driving the direct pathway, while D2R are mostly presented in cells of indirect one, and only small portion of projection neurons in the dorsal striatum express both receptor subtypes (Lanciego*et al.* , 2012; Gagnon *et al.* , 2017). Binding of dopamine with D1R which are exclusively presynaptic leads to activation of SPNs of the direct pathway and results in moderate stimulation of locomotor activity (Missale *et al.* , 1998; Beaulieu & Gainetdinov, 2011). D2R are expressed both in pre- and postsynapses, so that their role in the regulation of locomotion is more complicated. Thus, it is generally assumed that activation of presynaptic D2R decreases dopamine release suppressing locomotor activity, while activation of postsynaptic D2R on the striatal cells leads to inhibition of indirect pathway SPNs and, in hence, potentiates locomotion (Beaulieu & Gainetdinov, 2011).

Our data showed that neither 3-day HU nor 3-day SI affected the expression of D2R in the dorsal striatum. At the same time, D1R expression was significantly elevated in the dorsal striatum of HU mice in comparison with both SI and GH animals. These changes might as well be compensatory and reflect the adaptation of the striatum to decreased dopamine innervation under HU. Analogously, increased dopamine receptor expression and binding was demonstrated for patients with early stages of Parkinson's disease in which extremal loss of dopamine transmission is observed (Seeman & Niznik, 1990; Kaasinen *et al.* , 2021). On the other hand, changes in expression of D1R along with unchanged content of D2R could indicate that D1R-linked signaling was more sensitive to the effect of HU.

Dopamine-dependent signaling cascades

Dopamine binding with its receptors activates a number of intracellular signaling cascades that modulate synaptic plasticity and SPN excitability. Activation of adenylyl cyclase signaling pathway in response to dopamine is best studied. Thus, dopamine binding with D1R coupled with $G_{s/olf}$ proteins stimulates production of cyclic AMP and subsequent activation of PKA (Hervé, 2011; Nishi *et al.* , 2011). In turn, PKA-mediated phosphorylation promotes multiple intracellular changes, such as activation of N-type Ca^{2+} channels and membrane expression of glutamate receptors, inhibition of protein phosphatases and up-regulation of transcriptional factors, leading to increased excitability of SPN of the direct pathway (Missale *et al.* , 1998; Nishi *et al.* , 2011). In opposite, dopamine binding with D2R coupled with $G_{i/0}$proteins suppresses cyclic AMP production and PKA activation in SPNs (Missale *et al.* , 1998).

Results of the present study indicated that 3-day HU led to a decrease in PKA activity in the dorsal striatum. According to the literature data, these changes could result either from down-regulation of D1R-linked signaling processes or from up-regulation of D2R-dependent pathways. The roles of these cascades in observed dysregulation of PKA claims further investigation. Nevertheless, our present data let us to suppose

that increased expression of D1R, as well as increased pTH(Ser31), failed to compensate decreased dopamine innervation.

Moreover, down-regulation of PKA in the dorsal striatum of HU mice was accompanied with decreased activity of CREB. This transcription factor is involved in the regulation of long-term synaptic plasticity and procedural memory and presents an important target of various dopamine-induced signaling pathways associated with both D1R and D2R (Pittenger *et al.* , 2006; Fasano *et al.* , 2009). Upon dopamine stimulation of D1R CREB is mainly activated with participation of PKA (Nishi *et al.* , 2011), while D2R stimulation can induce CREB phosphorylation through PKC and CAMKII (Yan *et al.* , 1999). ERK1/2 and Akt can mediate CREB activation downstream of both receptor types (Brami-Cherrier *et al.* , 2002). However, our data did not reveal any pronounced alterations in the activity of ERK1/2, CAMKII, Akt, and GSK3β in the striatum of all studied groups. Thus, obtained data demonstrated that in HU mice observed downregulation of TH activity and subsequent decrease in dopamine production contributed mainly to the inhibition of PKA-dependent signaling and its target - CREB.

Conclusion

Results of the present study suggested that dysregulation of the dopaminergic system in the substantia nigra was mainly associated with HU, whereas in the dorsal striatum both HU and SI contributed to the attenuation of dopamine signaling. We demonstrated that HU significantly impaired activity of TH indicating a decrease in dopamine synthesis in the nigrostriatal system of mice. In turn, reduced dopamine innervation was accompanied with significant reduction in the activity of PKA and transcriptional factor CREB in the dorsal striatum that, in hence, pointed on decrease in the activity of striatal cells. At the same time SI itself only decreased TH activity in the striatum, while no changes in dopamine-related signaling was observed.

Noteworthy, HU and SI are abnormal states which can induce stress responses (Globus & Morey-Holton, 2016; Mumtaz *et al.* , 2018; Tahimic *et al.* , 2019). However, the studies suggested that, while acute stress (hours) elevated synthesis and release of the nigrostriatal dopamine, chronic stress (days) exerted no effect (Abercrombie *et al.* , 1989; Castro & Zigmond, 2001; Baik, 2020). Thus, the effect of HU on dopamine transmission in the nigrostriatal system observed in the present work was more likely associated with a loss of sensorimotor stimuli and partial muscle disuse, while SI-induced dysregulation could be caused mainly by a decrease in locomotor activity associated with the absence of cage-mates. Nevertheless, further investigations are needed to distinguish the effects of muscle unloading and social isolation more precisely. In addition, analysis of long-term effects of these factors on the nigrostriatal system should be performed to trace the dynamics of changes that can be associated with development of locomotor disorders.

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Conflict of interest

The authors declare that they have no conflict of interest.

Author contributions

AAN: conceptualization; data curation, formal analysis, investigation, methodology supervising; writing – original draft preparation; writing – review and editing. EAO: investigation, data curation, methodology, formal analysis. AVK: investigation, formal analysis, visualization. SDN: investigation, methodology, formal analysis, visualization. EVC: methodology, project administration, writing – review and editing. MVG: conceptualization; validation; writing – review and editing. All authors contributed to manuscript revision, read and approved the submitted version.

Data availability statement

The data are available on reasonable request to the corresponding author.

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Figure legends

Figure 1: Expression and phosphorylation of tyrosine hydroxylase (TH) in the substantia nigra pars compacta (SNc) of mice after 3-day hindlimb unloading (HU) and social isolation (SI). (A) Immunohistochemical analysis revealed significant decrease in TH expression in the SNc of HU mice (n=5) in comparison with SI (n=5), but not group-housed (GH, n=5) animals. (B) Estimation of phospho(Ser31)TH in the SNc of GH (n=5), HU (n=5), and SI (n=5) mice showed similar pattern of inter-group difference. (C) Content of phospho(Ser40)TH was decreased in HU group (n=5) compared with both GH (n=5) and SI (n=5) mice. Plots show optical density of immunopositive substance (arbitrary units, a.u.). Data are shown as median ± interquartile range. * – $p < 0.05$. (D-F) Representative images of TH (D), phospho(Ser31)TH (E), and phospho(Ser40)TH (F) in the SNc of GH, HU, and SI mice. Scale bars: 100 μm.

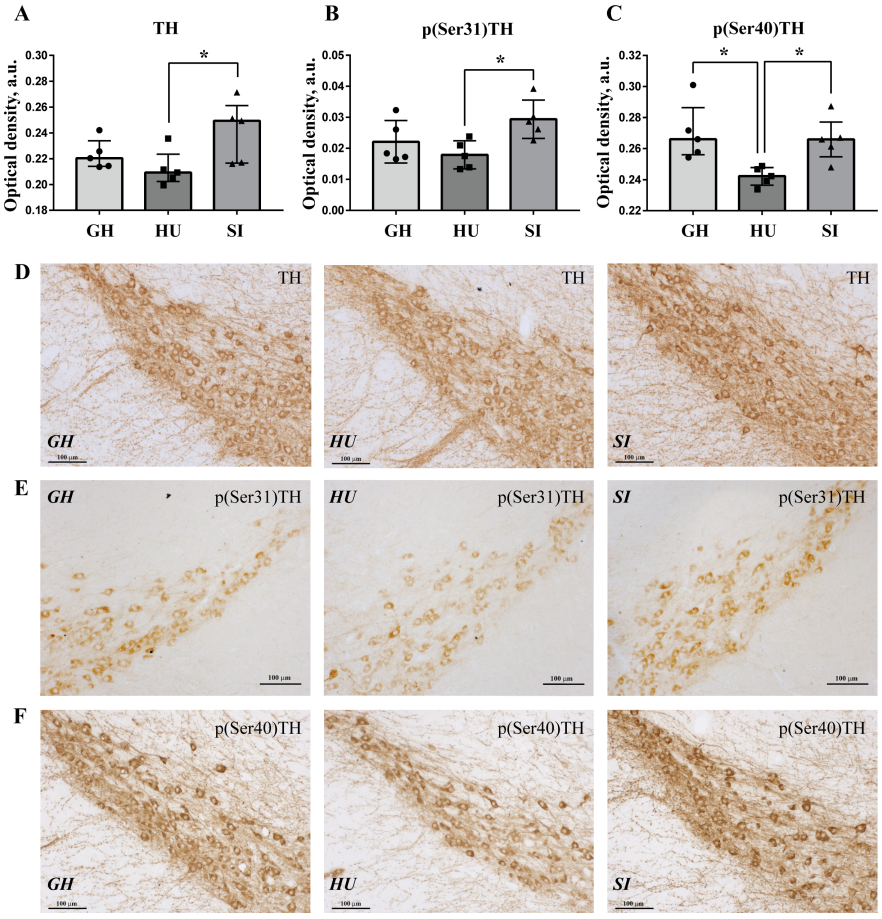
Figure 2: The effect of 3-day HU and social isolation (SI) on the expression and phosphorylation of TH in the dorsal striatum of mice. (A) Immunohistochemical assay showed reduced expression of TH in the dorsal striatum of HU mice (n=5) in comparison with SI (n=5), but not GH (n=5) mice. (B, C) Western blot analysis detected elevated striatal phosphorylation of TH at Ser31 (B) in HU mice (n=5) in comparison with SI (n=4), but not GH (n=5) group, while TH phosphorylation at Ser40 (C) was decreased in the striatum of both HU (n=5) and SI (n=5) mice compared to GH control (n=5). Plot A shows optical density of immunopositive substance (arbitrary units, a.u.); plots B, C demonstrate Western blot results in arbitrary units (a.u.). Data are shown as median ± interquartile range. * – $p < 0.05$. (D) Representative images of TH in the dorsal striatum. Scale bars: 100 μm. (E) Representative immunoblot images of total and phosphorylated TH in the dorsal striatum samples.

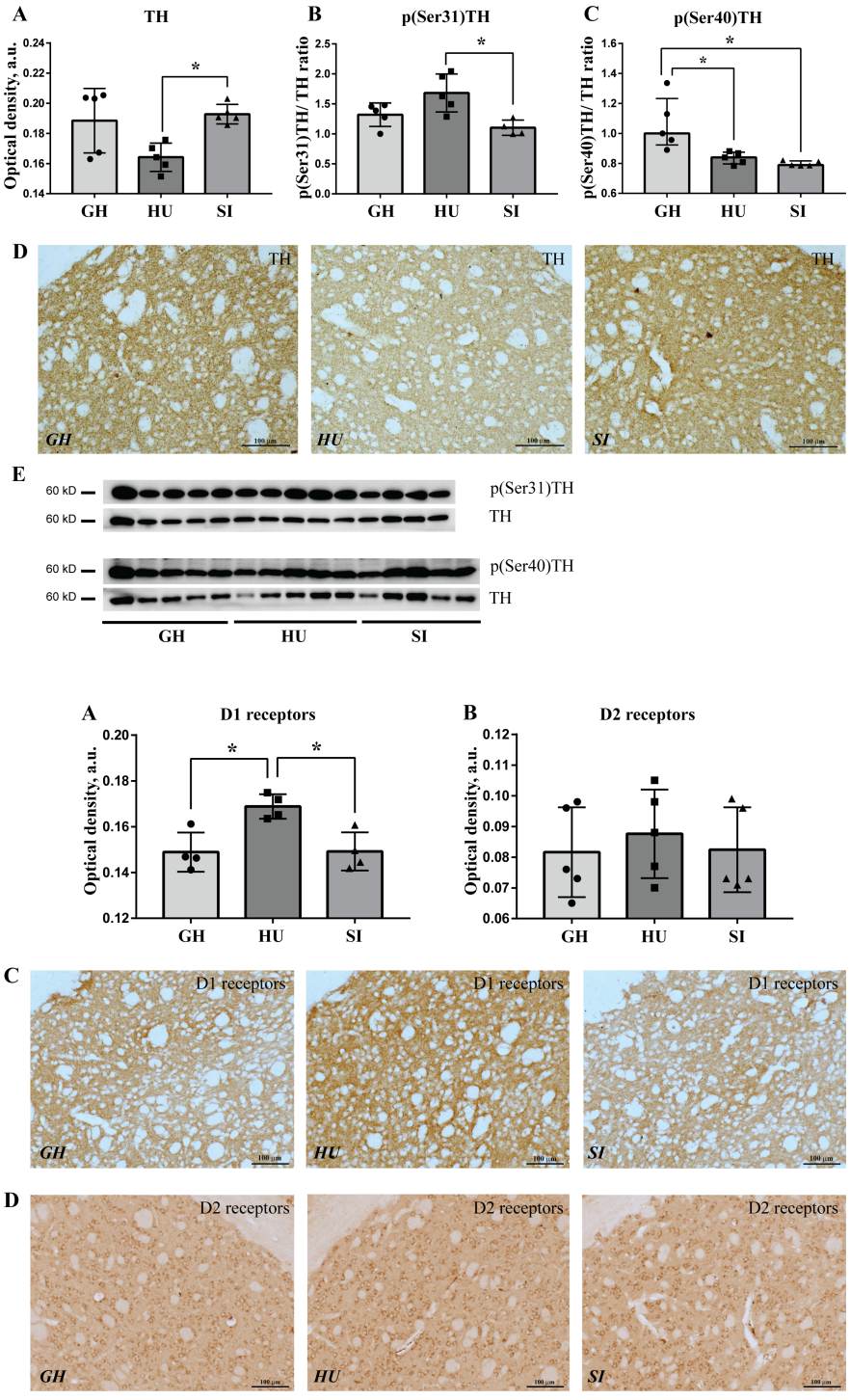
Figure 3: Immunohistochemical analysis of dopamine receptors D1 and D2 in the dorsal striatum of mice exposed to 3-day HU and SI. (A) Striatal expression of D1-receptors was significantly reduced in the HU mice (n=5) in comparison with both GH (n=5) and SI (n=5) mice. (B) D2-receptor expression was similar in all experimental groups. Plots show optical density of immunopositive substance (arbitrary units, a.u.). Data are shown as median ± interquartile range. * – $p < 0.05$. (C, D) Representative images of D1 (C) and D2 (D) receptors in the dorsal striatum. Scale bars: 100 μm.

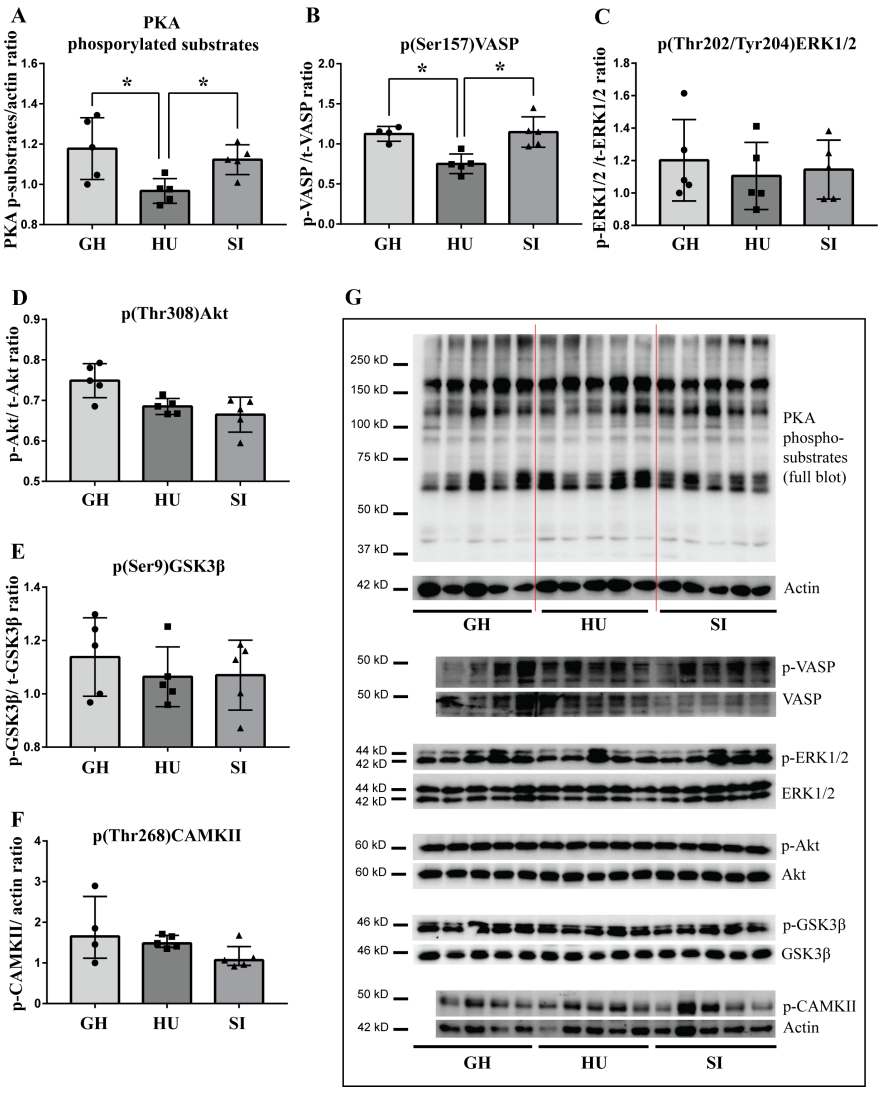
Figure 4: The effect of 3-day HU and SI on the activity of dopamine-associated signaling pathways in the dorsal striatum of mice. Western blot analysis detected a decrease in phosphorylation of protein kinase A (PKA) targets including overall substrates (A) and Ser157 of VASP (B) in the striatum of HU mice (n=5) in comparison with both GH (n=4 or n=5) and SI (n=5) groups. Phosphorylation of ERK1/2 (C), Akt (D), GSK3β (E), and CAMKII (F) did not differ between GH (n=4 or n=5), HU (n=5), and SI (n=5) animals after 3-day experiment. Plots demonstrate Western blot results in arbitrary units (a.u.). Data are shown as median ± interquartile range. * – $p < 0.05$. (G) Representative immunoblots of phosphorylated PKA substrates, phosphorylated and total forms of VASP, ERK1/2, Akt and GSK3β, phospho-CAMKII, and actin.

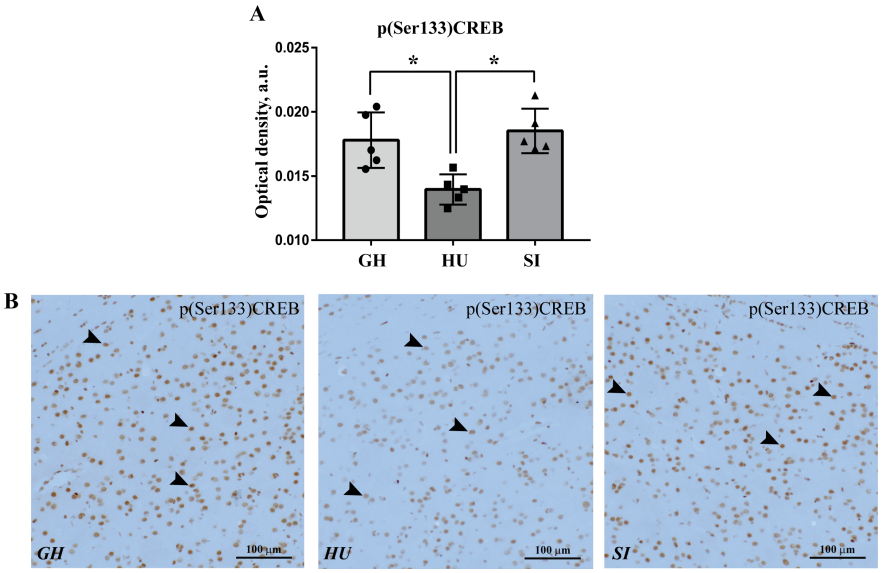
Figure 5: 3-day HU, but not SI, decreases CREB phosphorylation in the dorsal striatum of mice. (A) Immunohistochemical assay showed decreased content of CREB phosphorylated at Ser133 in striatal cell

nuclei of HU mice (n=5) in comparison with GH (n=5) and SI (n=5) animals. Data are shown as median ± interquartile range. * – $p < 0.05$. (B) Representative images of phospho-CREB in the dorsal striatum of GH, HU, and SI mice. Phospho(Ser133)CREB immunopositive cell nuclei are pointed by arrowheads. Scale bars: 100 μm.









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