Abnormal behavior, striatal dopamine turnover and opioid peptide gene expression in histamine-deficient mice

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Abstract

Hypothalamic histaminergic neurons regulate a variety of homeostatic, metabolic and cognitive functions. Recent data have suggested a modulatory role of histamine and histamine receptors in shaping striatal activity and connected the histaminergic system to neuropsychiatric disorders. We characterized exploratory behavior and striatal neurotransmission in mice lacking the histamine producing enzyme histidine decarboxylase (Hdc). The mutant mice showed a distinct behavioral pattern during exploration of novel environment, specifically, increased frequency of rearing seated against the wall, jumping and head/body shakes. This behavioral phenotype was associated with decreased levels of striatal dopamine and serotonin and increased level of dopamine metabolite DOPAC. Gene expression levels of dynorphin and enkephalin, opioids released by medium spiny neurons of striatal direct and indirect pathways respectively, were lower in Hdc mutant mice than in control animals. A low dose of amphetamine led to similar behavioral and biochemical outcomes in both genotypes. Increased striatal dopamine turnover was observed in Hdc KO mice after treatment with dopamine precursor L-Dopa. Overall, our study suggests a role for striatal dopamine and opioid peptides in formation of distinct behavioral phenotype of Hdc KO mice.

Introduction

Histamine is produced in the brain by a small group of neurons located in the hypothalamic tuberomamillary nucleus [1]. Brain histamine regulates diverse processes including maintenance of homeostasis and sustained wakefulness, modulation of motor and cognitive functions and regulation of appetite and hormones [2]. In the brain it acts via three G protein-coupled receptors - histamine receptors 1, 2 and 3 (Hrh1, Hrh2, Hrh3) [3].

Increased basal striatal dopamine release and dopamine-dependent striatal signaling are associated with the tic-like, repetitive behavior in Hdc KO mice [4,5]. These mice lack the histamine-producing enzyme histidine decarboxylase (Hdc) in peripheral tissues and central nervous system [6]. Striatal Hrh3 receptors may be a link between the hyperactive striatal dopaminergic system and tic-like behavior [7]. Hrh3 receptor activation differentially regulates intracellular signaling in medium spiny neurons of direct (expressing dopamine 1 receptors, D1-MSN) and indirect (expressing dopamine 2 receptors, D2-MSN) pathways and decreases dopamine release in ventral, but not dorsal striatum [8,9]. Mice lacking Hrh3 receptors show abnormal prepulse inhibition and dopamine receptor signaling in striatum [10]. In addition to modulating signaling cascades, Hrh3 is involved in differential modulation of mRNA levels of striatal opioids, dyorphin and enkephalin [11,12]

Open field behavior, including general locomotion and rearings of Hdc KO mice has been described previously, but results have been inconsistent [4,13-16] (see STable1). Stereotypic behavior in Hdc KO mice has been quantified previously after treatment with high amphetamine doses, which induced immobility and focused sniffing in animals [4]. Therefore, clarification of baseline behavioral phenotype of Hdc KO mice and after treatment with stimulant doses that do not produce high immobility, were needed. In addition, mice with pharmacologically inhibited synthesis of brain histamine, showed increased complexity of behavioral sequences during open-field exploration, thus suggesting a need for more detailed dissection of observed behaviors [17].

For these reasons, we aimed to perform a detailed analysis of behavioral pattern of Hdc KO mice and study possible neurochemical and molecular mechanisms associated with it. We analyzed exploratory activity in Hdc KO mice in the open field test at the baseline and after amphetamine treatment, and measured the levels of dopamine and serotonin and their metabolites in striatal homogenates after behavioral challenge.

We also treated Hdc KO and WT mice with dopamine precursor L-Dopa to further investigate striatal dopamine neurotransmission and metabolism. We measured expression levels of striatal *prodynorphin* (*Pdyn*) and *preproenkephalin* (*Penk*) genes, opioid peptides expressed by MSNs of direct and indirect pathways, respectively. Phosphorylation of striatal Creb was studied as it mediates signal transduction in both D1- and D2-MSNs and regulates expression level of *Pdyn* [18-20].

Materials and methods

Subjects

The principles of the Finnish Act on the Use of Animals for Experimental Purposes were followed and all protocols were approved by the Animal Experiment Committee of the State Provincial Office of Southern Finland.

Genetically modified mice with *Hdc* gene deletion were used in this study. The Hdc knockout (KO) mice were originally a gift from Prof. Hiroshi Ohtsu [6]. Mice were backcrossed to C57Bl/6JCrl strain for 10 generations in our laboratory as described earlier [16]. Single nucleotide polymorphism analysis of Hdc KO mouse line showed 99.5 % genetic identity with C57BL/6J strain. Heterozygous breeding was used to maintain the colony. Animals for experiments were obtained from heterozygous, heterozygote x WT or KO and homozygous mating.

Mixed breeding scheme was used because the number of male homozygous mice from heterozygous breedings was insufficient for performing behavioral experiments at the same time and under identical conditions. Genotype frequencies of mice obtained from heterozygous mating were often not following expected Mendelian ratio, with larger than expected number of heterozygous animals than wild type or knockout mice. Therefore, there is a possibility of mixed genetic and maternal care effects, although Hdc WT and KO homozygous breedings are not different in pup's survival rate.

All mice were genotyped by PCR amplification before the experiments. Fifteen- to seventeen-week-old Hdc WT and KO male mice were used in the experiments. Male mice were used in order to decrease within-group variability due to hormonal fluctuations during estrous cycle in female mice.

Housing conditions

Animals were group-housed, standard food pellets (Scanbur, Sweden) and water were available *ad libitum*. Animal rooms were maintained on a 12–12 h light–dark cycle (lights on at 6 a.m.). Temperature and humidity were controlled at 20±1°C and 50±10%, respectively.

Drug treatment

D-amphetamine sulfate (1.8 mg/kg; Dexedrine®, GlaxoSmithKline, Brentford, UK), L-Dopa methyl ester (30 mg/kg; Sigma, D1507) and benserazide (7.5 mg/kg; Sigma, B7283) were dissolved in sterile 0.9% saline. The drug doses correspond to the free base. Injections were given intraperitoneally (i.p., injection volume 10ml/kg).

Quantification of behaviors and locomotor activity in the open field

Twenty-seven naïve Hdc wild type (Hdc WT) (n=14) and KO (n=13) male mice were used for analysis of behaviour in an open field. Animals were tested between 6 p.m. and 10 p.m., during the active phase. Mice were placed in the center of an empty plastic cage (40x26x20 cm) without a habituation period in order to test the effect of novelty on the behaviour. The open field test was conducted in a dimly lit room in order to avoid the influence of light-induced anxiety on exploratory behaviour. Locomotor activity of the animals was monitored with video camera and Ethovision (Noldus) software for 30 min (n=10 animals per genotype). An additional video camera was used to record animal behaviour from a side view. Animals were sacrificed after open field test and brain tissue and blood were collected for HPLC, qPCR and corticosterone level analyses.

Video recordings were analyzed by a person blinded to the genotype and treatment. Three time intervals (0-5, 10-15 and 20-25 min) were visually analyzed and the frequencies of following behaviors quantified: rearings (free-standing or against the wall, RF and RW respectively); sitting on haunches (rearing seated free or against the wall, RSF and RSW respectively; Fig.1a); grooming (duration and count). Other counted behaviors were: paws/head/body shakes, licking and digging the floor or walls of the arena, jumping, rotations and feces eating or mouthing [17].

Grooming counts were defined as movements that are part of the grooming chain, but performed out of the full grooming sequence context. They are rapid (shorter than 3 seconds in duration), and

the same movement is often repeated several times in a row. This behaviour included forepaws touching the snout, bilateral and unilateral strokes of the face, and body licking [18].

To study the effect of amphetamine on behaviour and locomotor activity of Hdc KO mice, animals were injected intraperitoneally with saline (n=10 for both genotypes; 10 ml/kg) or amphetamine (n=13 for Hdc WT and n=10 for Hdc KO mice; 1.8 mg/kg) just before testing. Open field test was performed in the same way as with naïve mice.

Animals were sacrificed after open field test and brain tissue and blood were collected for HPLC, qPCR and corticosterone level analyses.

L-Dopa treatment

Hdc WT (n=6) and KO (n=6) male mice were used to analyze the effect of L-Dopa on striatal neurotransmission and behavior. Testing was performed between 6 p.m. and 9 p.m. Mice were habituated to the testing arena for 30 min and placed back to the home cage for 10-30 min. The animals were injected with the mixture of L-Dopa and benserazide and immediately placed to the testing arena. Horizontal and vertical locomotor activity was tracked in a chamber (27.3 cm × 27.3 cm × 20.3 cm) equipped with 48 infrared beams (MedAssociates, Georgia, Vermont, USA) for 90 minutes. Tracks were analyzed with Activity monitor software version 6.02 (MedAssociates, Georgia, Vermont, USA). Parameters such as distance traveled (in cm), vertical counts (number of times that the animal rears) and vertical time (time spent rearing in seconds) were calculated. The animals were killed after 90 min and brain tissue was collected for further HPLC and qPCR analysis.

Sample preparation

Mice were decapitated after the open field test, trunk blood was collected and striatal tissue dissected with forceps. Blood was collected in Li-heparin coated tubes on ice, centrifuged at 750g at +4C and plasma collected and kept at -20C. Brain samples were frozen on dry ice and stored at −80°C until analyzed. Striatum from one hemisphere was used for HPLC analyses and from the other one for qPCR.

Punches from dorsal striatum and nucleus accumbens regions were taken from cryostat-sectioned 300 um slices with 1 mm diameter punch tool (Fig.3c). Four to six punches per region per mouse were collected. Samples from two mice were pooled for RNA extraction and qPCR (n=4 samples for each genotype).

Striatal tissue from untreated animals (not exposed to open field test) was collected as described [21]. One striatum was used for qPCR and the other one for western blot and MAO activity analysis (n=5 per genotype).

HPLC analysis of biogenic amines

Striatal tissue from untreated, saline and amphetamine treated mice was collected after open field exposure for the analysis of biogenic amines (n=5 for all groups).

For the analysis tissue samples were sonicated on ice in 150µl of 0.3 M perchloric acid, followed by centrifugation for 30 min at 15 000 *g* at +4°C. Samples were analyzed immediately or stored at −80°C until analysis. The concentrations of dopamine, DOPAC (3,4-dihydroxyphenylacetic acid), 3-MT (3-methoxytyramine), HVA (homovanillic acid), serotonin and 5-HIAA (5-hydroxy-indoleacetic acid) were measured with a chromatographic system that consisted of a Waters 515 pump (Waters Corporation, Milford, MA), Waters 717 Plus autosampler, Waters Concorde electrochemical detector equipped with a glassy carbon 3 mm electrode and was controlled by Waters Empower software package. The SecurityGuard C18 4 × 3 mm precolumn (Phenomenex), column Phenomenex Gemini C18 4.6 × 150 5 µm and the flow cell were thermostated at +35°C. Electrode potential was set at +0.8 V. Mobile phase consisted of 50 mM citric acid, 1.5 mM 1-octanesulfonic acid sodium salt, 0.05 M EDTA, 8% methanol and 50 mM orthophosphoric acid, and pH was adjusted to 2.7. The analysis was performed at a flow rate of 1 ml/min in isocratic mode.

Measurements of striatal dopamine, serotonin and their metabolites after L-Dopa treatment were done as described previously using HPLC with electrochemical detection (n=6 for both Hdc WT and KO mice). [22].

HPLC analysis of histamine level in striatum of Hdc WT and KO mice

To verify absence of histamine in Hdc KO mice, striatal homogenates from Hdc WT and KO mice (untreated or saline treated mice after open field exposure; n=8 for both genotypes) were also

analyzed for histamine content. The concentration of histamine was determined using HPLC combined with fluorescence detection. The HPLC system consisted of four Shimadzu LC20AD pumps, an autosampler SIL-20AC, a fluorescence detector RF-10Axl, and a controller CBM-20A. LCSolution 1.21 software was used for system control and data collection/processing. The dialysis samples were analyzed without prior purification.

The histamine analysis method was based on the online postcolumn derivatization with *o*-phthalaldehyde, as described by Yamatodani et al [23]. Briefly, samples were separated on a 6*150 mm, TSK gel SP-2SW (Tosoh, Japan) column; the mobile phase consisted of 0.25 m KH_2PO_4/0.75 mm NaN_3 (flow rate 0.6 ml/min), samples were then automatically derivatized by online mixing with 0.1% OPA/2 m NaOH/0.2M H_3BO_3 reagent in a reaction coil incubated at 45°C and, finally, stabilized with 3M H_3PO_4. Fluorescence was measured at Ex360, Em 450 nm.

Plasma corticosterone measurement

Blood plasma was diluted 1:100 in the assay buffer and corticosterone was measured using Corticosterone EIA kit (ADI-900-097, Enzo Life Science, Farmingdale, NY, USA) according to manufacturer's instructions (n=8 and n=6 for Hdc WT and KO, respectively).

Quantitative real-time PCR (qPCR)

RNA was extracted from dissected striatum (RNeasy mini kit, Qiagen, Valencia, CA). To synthesize cDNA, 1-2 μg of total RNA was reverse-transcribed using SuperScript™ III reverse transcriptase (Invitrogen) according to instructions provided by the manufacturer. The primers for qPCR were designed by Primer-BLAST (NCBI). The housekeeping gene *Pgk1* was used as reference control [20]. Analyses were performed in the LightCycler 480 instrument (Roche, Mannheim, Germany) using the Lightcycler®480 SYBR GreenI Maxter (Roche).

The primers for qPCR were as follows:

Pgk1, 5'-TGATGAGGGTGGACTTCAACG-3' and 5'- CCAGGTGGCTCATAAGGACA -3';

Pdyn, 5'-TGAGTCAGAATGGCGTGGTC-3' and 5'-GGAGCAAATCAGGGGGTTGA-3';

Penk, 5'-CATGAAACGGTACGGAGGCT-3' and 5'-GCCAAGGTGTCTCCCTCATC-3';

Western blots

Striatal tissue from untreated, not exposed to open-field animals (n=5 per genotype) was homogenized with a pestle and motor mixer (VWR) in lysis buffer (50 mM Tris pH 7.4, 5 mM EDTA, 1 mM EGTA, 0.1% Triton X-100, 5 mM sodium fluoride, 1xHALT phosphatase inhibitor cocktail (Thermo Fischer Scientific, Rockford, IL, USA), Mini Complete protease inhibitor (Roche Diagnostics, Mannheim, Germany)). Homogenates were centrifuged at 13 000 x g (5 min, +4ºC) and protein concentration of the supernatant determined by Bio-Rad Protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Samples were mixed with Laemmli buffer and heated at 95°C for 3 min. Samples (15-20 µg of total protein/well) were separated on 10% SDS-PAGE gel and transferred to PVDF membrane with eBlot protein transfer system (Genscript, Piscataway, NJ, USA). Phosphorylated form of Creb was detected with rabbit-anti-pCreb S133 (1:500, PhosphoSolutions, Cat# p1010-133, RRID:AB_2492066) and total Creb was detected with rabbit-anti-Creb (1:1000, Cell Signaling Technology Cat# 9197, RRID:AB_331277). Membranes were scanned with an image scanner (FLA-9000, Fujifilm) after incubation with HRP-conjugated goat anti-rabbit secondary antibody (1:3000, Bio-Rad, AbD Serotec Cat# 170-6515, RRID:AB_11125142) and ECL Plus Western Blotting Substrate (32132; ThermoFisher Scientific, Grand Island, NY). Band intensity was quantified with ImageJ densitometry software (National Institutes of Health, Bethesda, MD).

MAO activity

Aliquots of tissue lysates prepared for western blot were diluted to 1 mg/mL using lysis buffer. Total MAO and MAOB activity were measured using the luminescence-based MAO-Glo Assay System (Promega) according to the manufacturer's instructions (n=5 per genotype). Luminescence was measured by using an EnSpire multimode plate reader (PerkinElmer Life Sciences).

Statistical analysis

Data analysis was performed in Python (Python Software Foundation, https://www.python.org/, Python 3.6 version) using pandas, NumPy and SciPy libraries. Following statistical test were used: unpaired t-test, one- or two-way ANOVA, mixed model ANOVA, MANOVA and Pearson

correlation coefficient for testing correlations. Statistical tests used in each case are indicated in the text or in STable2. Data represented as mean±SEM, if not noted otherwise.

Results

Histamine level in striatum of Hdc WT and KO mice

The lack of histamine in Hdc KO mice was confirmed by HPLC analysis on striatal samples collected after open-field test ($p=1.02e-06$, SFig.1).

Abnormal behavioral pattern in Hdc KO mice

In general, the observed behaviors in the open field consisted mostly of rearing, seated rearing and grooming. Other behaviors such as digging, licking and rotation were very rare. Several behaviors quantified from video recordings were altered in Hdc KO mice compared with WT mice (Fig.1a, SFig.2a). A significant effect of genotype on rearings was found ($F(4,22)=3.41$, $p<0.05$). While rearing free (RF), rearing wall (RW) and rearing seated free (RSF) were not significantly different in Hdc KO and control mice, frequency of rearing seated wall (RSW) was increased in mutant mice ($F(1,25)=11.52$, $p<0.01$). Frequency of RSW increased in Hdc KO mice on time intervals 10-15 and 20-25 min ($t(25)=-2.89$, $p<0.01$ for 10-15 min and $t(15.20) = -2.86$, $p=0.012$ for 20-25 min; SFig.2a).

An overall effect of genotype was found on counted behaviors ($F(6,20)=3.41$, $p<0.01$). Specifically, post-hoc analyses revealed a difference between Hdc WT and KO mice in jumping and shaking behavior ($F(1,25)=9.70$, $p<0.01$ for jumping and $F(1,25)=4.68$, $p<0.05$ for body/head/forepaws shakes).

The number of jumps increased gradually in Hdc KO mice, while it was almost absent in WT mice ($t(13.27)=-3.00$, $p=0.01$ for 10-15 min and $t(12.51) = -2.48$, $p=0.028$ for 20-25min; SFig.2b). It should be noted that jumping usually occurred after leaning on the cage wall (behaviour counted as RSW). Increased frequency of RSW in Hdc KO mice can therefore be seen as a preparation step before the actual jump. Indeed, a significant positive correlation was found between frequency of seated wall rearing and jumping (Pearson correlation coefficient $r=0.64$, $p<0.001$, Fig.1b).

Frequency of body/head/forepaws shakes was increased in Hdc KO mice on the first 5 min of observation, while frequency of the shakes increased in the WT group to the level of KO later on ($t(13.90) = -2.21$, $p<0.05$ for 0-5 min interval; SFig.2c).

Grooming behavior was not different between the genotypes.

Analysis of locomotor activity in a new environment did not show differences between the Hdc KO and WT mice. Distance traveled and maximum velocity quantified in 5 min bins for 30 min was similar in the two groups (Fig. 1c).

Effect of amphetamine on behaviour of Hdc KO mice

While amphetamine injection significantly increased distance traveled and maximum velocity in all mice, no difference was detected between the genotypes (treatment effect $p<0.001$ and $p>0.05$ for genotype and genotype x treatment effects; Fig. 1d).

Amphetamine treatment had significant effect on rearings ($F(4,36)=9.85$, $p<0.001$), but no significant effect of genotype or treatment x genotype interaction was found.

Amphetamine injection had similar effects on total frequency of counted behaviors in Hdc WT and KO mice (treatment effect $F(6,34)=13.52$, $p<0.001$, genotype and genotype x treatment effect $p>0.05$).

Grooming behavior was not different between the genotypes (treatment effect $F(2,38)=15.82$, $p<0.001$, genotype and genotype x treatment effect $p>0.05$).

Striatal dopamine and serotonin in Hdc KO mice

HPLC analysis of dopamine and serotonin and their metabolite levels revealed multiple differences in the striatum of Hdc KO and WT mice. DOPAC/dopamine and 5-HIAA/serotonin ratios were increased in the striatum of Hdc KO mice ($t(8)=-4.48$ $p<0.01$ for DOPAC/dopamine and $t(8)=-3.72$ $p<0.01$ for 5-HIAA/serotonin; Fig. 2a and SFig. 2a). Both, DOPAC/dopamine and 5-HIAA/serotonin, ratios are commonly used as characteristics of dopamine and serotonin turnover. Dopamine level in the striatal homogenates was lower in Hdc KO mice than in WT animals, while DOPAC level was higher in Hdc KO mice compared with WT mice ($t(8)=4.66$ $p<0.01$ for dopamine

and t(8)=-4.48 p<0.01 for DOPAC; Fig. 2a). In a similar manner, serotonin level was lower in Hdc KO mice than control mice (t(8)=2.53 p<0.05; SFig.3a), whereas there was no difference in 5-HIAA (SFig. 2a).

The levels of 3-MT and HVA were similar in the two genotypes (39.47±4.08 and 41.87±2,30 pmol/mg for Hdc WT and KO respectively for 3-MT; 6.22±0,24 and 6.49±0.80 pmol/mg for Hdc WT and KO respectively for HVA; p>0.05 for 3-MT and HVA).

Effect of amphetamine on striatal dopamine and serotonin

Surprisingly, saline injection per se diminished differences in the levels of dopamine and serotonin between the two groups (Tukey's multiple comparison test WT SAL vs KO SAL p>0.05 for dopamine and serotonin; Fig. 2b and SFig. 2b). Nevertheless, the level of DOPAC was still slightly higher in Hdc KO mice (Tukey's multiple comparison test WT SAL vs KO SAL p<0.05; Fig. 2b).

Amphetamine treatment decreased turnover of dopamine and serotonin in both groups (treatment effect F(1,16)=41.66 p<0.001 for dopamine and F(1,16)=45.95 p<0.001 for serotonin turnover; Fig. 2b and SFig. 2b). Hdc KO mice had a trend to increased dopamine turnover (genotype effect F(1,16)=3.85 p = 0.07), while no genotype effect was found for serotonin.

Striatal dopamine levels after amphetamine treatment did not differ significantly between the genotypes (treatment effect F(1,16)=15.00 p<0.001 and effect of genotype p>0.05; Fig. 2b). Interestingly, amphetamine increased dopamine level in Hdc WT mice as compared with saline treatment, but the effect was absent in Hdc KO mice: the level of dopamine after amphetamine injection was similar as that after saline treatment (Hdc WT SAL vs AMPH group, p<0.05; Hdc KO SAL vs AMPH group, p>0.05, Tukey's multiple comparison test; Fig. 2b). Amphetamine treatment strongly decreased the level of DOPAC in both groups equally (treatment effect F(1,16)=183.11 p<0.0001, effect of genotype F(1,16)=7.88 p<0.05; Tukey's multiple comparison test WT AMPH vs KO AMPH p>0.05, Fig. 2b).

The levels of serotonin showed similar differences as dopamine after amphetamine injection. The treatment had a significant effect on striatal serotonin levels (treatment effect F(1,16)=12.02 p<0.01 and effect of genotype p>0.05, SFig. 2b). Again, amphetamine increased serotonin level in Hdc WT mice relative to saline treatment, but the effect was absent in Hdc KO mice (Hdc WT SAL vs

AMPH group, $p<0.05$; Hdc KO SAL vs AMPH group, $p>0.05$; Tukey's multiple comparison test; SFig. 2b).

The levels of dopamine and serotonin metabolites HVA and 5-HIAA were lower in Hdc KO, but not in Hdc WT mice, after amphetamine treatment compared with saline treated animals (Hdc WT SAL vs AMPH group, $p>0.05$; Hdc KO SAL vs AMPH group, $p<0.05$, Tukey's multiple comparison test; SFig.b), although genotype effect was not significant in either case (effect of genotype and genotype x treatment $p>0.05$ for HVA; effect of genotype $p>0.05$ and genotype x treatment $F(1,16)=5.55$ $p<0.05$ for 5-HIAA).

Plasma corticosterone levels

Saline treatment abolished the neurochemical and behavioral differences between Hdc KO and WT mice (Fig.1a, 2b and SFig.3b), suggesting that mild stress affected striatal neurotransmission in Hdc KO mice. Acute stress activates the hypothalamo-pituitary-adrenal axis and leads to release of corticosterone into the bloodstream [24].

After the open field test, trunk blood was collected. We measured the level of plasma corticosterone in untreated and saline injected mice and found an increase of corticosterone level after injection, but no genotype effect (treatment effect $F(1,26)=4.22$, $p = 0.05$, genotype effect $p>0.05$, SFig.4).

Effect of L-Dopa on locomotor activity, striatal dopamine and serotonin

While analyses of distance traveled, vertical counts and time analyzed individually did not show significant differences between the L-Dopa treated Hdc WT and KO mice (unpaired t-test; $p>0.05$), a significant difference was found for overall activity in the open field test ($F(3,8)=5.18$ $p<0.05$, SFig.5).

Striatal DOPAC/dopamine ratio was higher in L-Dopa injected Hdc KO mice compared with L-Dopa treated WT group ($t(10)=-3.36$ $p<0.01$; Fig. 2c). The levels of dopamine, DOPAC, HVA, serotonin, 5-HIAA and 5-HIAA/serotonin ratio were not statistically different between the genotypes (Fig. 2c and SFig.3c). The product of L-Dopa methylation by COMT, 3-OMD was also quantified. There was no significant difference between the genotypes in striatal 3-OMD level

(420.86±32.53 and 375.95±22.65 pmol/mg for Hdc WT and KO respectively; unpaired t-test; $p>0.05$).

Striatal prodynorphin and preproenkephalin mRNA levels

Expression levels of opioid peptide genes prodynorphin (*Pdyn*) and preproenkephalin (*Penk*) are considered to reflect activity level of D1- and D2-MSNs, respectively [25,26].We studied if Hdc KO mice have abnormal striatal mRNA levels of *Pdyn* and *Penk* following open field test. Transcript levels of *Pdyn* and *Penk* were lower in Hdc KO mice than in control mice ($t(13)=3.77$ $p<0.01$ for *Pdyn* and $t(13)=2.39$ $p<0.05$ for *Penk*; Fig. 3a). Furthermore, there was a significant negative correlation between Pdyn mRNA level and dopamine turnover (Pearson correlation coefficient $r = -0.9$, $p<0.001$; Fig. 3b)

In an another batch of mice exposed to open field test as well, qPCR for *Pdyn* and *Penk* transcripts were performed separately in tissue punches from dorsal striatum (Dcpu) and nucleus accumbens area (Nacc) (Fig. 3b). Decreased *Pdyn* expression was detected again in Nacc ($t(6)=2.55$ $p<0.05$; Fig. 3c) in Hdc KO mice, while no differences were detected in Dcpu *Pdyn* and *Penk* expression in both regions (Fig. 3c).

Untreated, not exposed to open-field Hdc KO mice had decreased striatal *Pdyn* and *Penk* expression in whole striatum preparation ($t(8)=2.89$ $p<0.05$ for *Pdyn* and $t(8)=2.61$ $p<0.05$ for *Penk*; Fig. 3f).

Phosphorylation of striatal Creb in Hdc WT and KO mice

Hrh3 strongly regulates MSN signaling, thus we hypothesized that increased level of this receptor in striatum of Hdc KO mice, may also modulate Creb phosphorylation, which in turn regulates *Pdyn* gene expression [8,18]. Phosphorylation of striatal Creb in Hdc WT and KO mice was similar, as pCreb S133/Creb ratio was equal in both groups ($p>0.05$; SFig.6).

MAO activity in striatum of Hdc WT and KO mice

There was no difference in enzymatic activity of total MAO and MAOB in striatum of Hdc WT and KO mice ($p>0.05$).

Discussion

The main finding of this study is that Hdc KO mice express a distinct behavioral phenotype at the baseline and it is associated with increased striatal dopamine turnover and decreased expression of striatal dynorphin and enkephalin genes.

Previous research reported hypoactivity phenotype in the home cage and open-field test in Hdc KO mice [13,15,27,28]. In contrast to earlier reports we did not find signs of hypoactivity in Hdc KO mice challenged with exposure to novel environment [13,29]. This may be due to the differences in background mouse lines, since the studies of Dere et al. and Parmentier et al. were carried out on 129Sv mice [13,29]. On the other hand, both young (2-5 months old) and older (12-14 months old) Hdc KO mice on C57Bl/6J background were hypoactive upon the exposure to novel environment during lights-on phase, while in our previous and current study, Hdc KO mice tested during lights-off phase were not hypoactive [14,15]. The contradictory results might be due to combination of two factors: first, different background strain (129Sv vs C57Bl/6J) and second, time of the testing (light vs dark phase).

In our previous study we found decreased rearing time and frequency in Hdc KO mice, while in the current study and in the study of Castellan Baldan et al. frequency of rearing quantified manually by the observer were unaltered in Hdc KO mice [4,14]. In the first study we used an infrared beam detection system that counts rearings as breaks of the beam positioned at a the certain height above the bottom of the arena, while in the other studies rearings were identified visually by the observer. Therefore, rearing in Hdc KO mice might be of the same frequency, but lower in amplitude and duration and may be associated with increased repetitive or stereotypic behavior.

We found increased occurrence of specific types of rearing (half-rearing or seated rearing against the wall) and jumping behaviors in Hdc KO mice. Together these activities may be a part of escape behavior from an aversive environment. Indeed, standard open field test actually measures forced exploratory activity and represents the net result of two competing behavioral programs: exploration and stress-induced defensive behavior [15]. Previously we found no indication of increased anxiety in the novel open field in Hdc KO mice and in this study blood corticosterone levels after open field

test were similar in Hdc WT and KO mice, thus pointing against increased anxiety and stress in Hdc KO mice [14]. In addition, histamine-deficient mice displayed increased numbers of body/head/forepaw shakes, further indicating an increase in stereotypic behavior or behavioral complexity, rather than enhanced escape behavior.

Behavioral alterations were accompanied with abnormal dopamine and serotonin neurotransmission in the striatum of Hdc KO mice. Dopamine and serotonin levels were decreased, while DOPAC level was increased in mutant mice after open-field exposure. Increased striatal dopamine neurotransmission in Hdc KO mice was reported earlier and seems to be a stable characteristic of these mice, independently of the genetic background [4,30]. In contrast to dopamine, decreased striatal serotonin level in Hdc KO mice was not accompanied with an increased metabolite level and although the 5-HIAA/serotonin ratio was higher in Hdc KO mice, there is no previous evidence of increased serotonin release.

Histamine may indirectly modulate dopamine release through Hrh1 and Hrh3 and regulate dopamine-dependent signaling in medium spiny neurons via Hrh3 [9,31,32]. The absence of the endogenous ligand for histamine receptors in Hdc KO mice may therefore lead to increased striatal dopamine release, although the exact mechanism has not been revealed yet.

Increased DOPAC level might be also a consequence of altered activity of MAOB and COMT enzymes, rather than increased dopamine release [33,34]. To verify this hypothesis we treated animals with L-Dopa to assess the activity of MAO and COMT enzymes *in vivo*. We found no indication of impaired dopamine metabolism, but instead increased dopamine turnover in Hdc KO mice. In addition, activities of total MAO and MAOB were measured on striatal homogenates and found to be normal in Hdc KO mice. Thus, increased dopamine release, but not impaired dopamine degradation is responsible for the observed phenotype.

Surprisingly, mild acute stress induced by restrain and intraperitoneal (i.p.) saline injection was able to diminish observed behavioral and transmitter turnover changes in Hdc KO mice. Of the observed abnormalities, only an increase in DOPAC level was still present in Hdc KO mice. I.p. injection is a relatively mild stressor, but increased dopamine release in nucleus accumbens shell after single intraperitoneal saline injection has been reported [35]. Acute stress activates the hypothalamic-pituitary-adrenal axis, increasing the firing rate of dopaminergic neurons and the release of dopamine and serotonin [36-38]. We found an effect of i.p. injection of saline on plasma corticosterone

level, but not genotype effect. Therefore, we conclude that i.p. injection in combination with exposure to novel environment activated stress-related brain networks and led to similar behavioral and neurochemical outcome in both genotypes.

We also administered a low dose of amphetamine in order to investigate if Hdc KO mice are more sensitive to the stimulant. Male adult (3-9 months old) Hdc KO mice on 129Sv background have shown increased focused sniffing after administration of high doses of amphetamine (8.5 and 10 mg/kg) [4]. Low doses of amphetamine produce hyperlocomotion in C57Bl/6 mice, while higher doses induce stereotypies and a decrease in locomotion [39]. We hypothesized that Hdc KO mice will show increased stereotypic behavior already at the dose that produces locomotor hyperactivity, but not stereotypic behavior in WT mice [39]. In contrast, we found no significant differences in frequencies of different types of behaviors and no difference in locomotor activity and monoamine levels between the genotypes. We found that the levels of striatal dopamine and serotonin were significantly higher after amphetamine treatment compared with saline treated group in Hdc WT, but not in Hdc KO mice. At the same time, a decrease in the levels of HVA and 5-HIAA after amphetamine injection was more pronounced in Hdc KO mice.

In general, locomotor response and rewarding effect of addictive drugs in Hdc KO mice vary dependent on the drug and the dose used [16,28,40,41].

Endogenous opioid peptides dynorphin and enkephalin are often used as markers of D1- and D2-MSNs, respectively. Regulation of dynorphin and enkephalin gene expression by dopamine was shown in animals with lesioned nigrostriatal pathway and in pharmacological experiments with dopamine receptors antagonist/agonists [42-44]. Dynorphin binds preferentially to kappa-opioid receptors, which in turn regulates dopamine release, gates excitatory/inhibitory inputs to MSN and MSN activity itself [25,45].

There is a large body of evidence from in-vitro studies and *in vivo* studies on Hdc and Hrh3 KO mice that Hrh3 regulates striatal activity [7,10,46-48]. In addition, Hrh1 and Hrh2 are also present on the soma of MSN and other striatal cells and could contribute to adjusting MSN level of activity [49].

Given these data and studies that have shown regulation of striatal opioids by Hrh3, we hypothesized that striatal opioid systems might be altered in Hdc KO mice and analyzed gene expression of dynorphin and enkephalin. We found decreased dynorphin and enkephalin expression in the striatum of Hdc KO mice. Release of striatal opioids is activity dependent, therefore gene

expression of these opioids may reflect activity level of D1- and D2-MSNs [25,26,50]. Decreased *Pdyn* and *Penk* expression may suggest decreased activity of MSN in Hdc KO mice due to overexpression of inhibitory Gi-coupled GPCR Hrh3 [7]. Alternatively, changes in mRNA levels of opioid peptides may reflect adaptive response to increased striatal dopamine neurotransmission [26].

Intracellular dopamine-dependent signaling was found to be altered in striatum of Hdc KO mice [5,51]. Although we did not find difference in pCreb/Creb ratio between mutant and control mice, it might be still possible that alteration is evident only if D1- and D2-MSN could be studied separately [8]. On the other hand, striatal expression of dopamine receptors 1 and 2 and dopamine signaling proteins DARPP-32 and Step61 are similar in Hdc KO and WT mice [52].

We found significant negative correlation between striatal *Pdyn* level and dopamine turnover. It should be established if decreased striatal *Pdyn* expression indicates decreased dynorphin peptide release and if decreased dynorphin peptide release by D1-MSN could result in increased release of dopamine due to lack of inhibitory effect of kappa-opioid receptors, located on dopaminergic terminals [25,45].

A recent study has shown, that ablation of GABA-histaminergic neurons leads to a substantial compensation process, compared with the effects of acute chemogenic inhibition [53], which induces nonrapid eye movement sleep. In addition, factors such as genetic compensation triggered by mutant RNA decay cannot be also excluded [54]. Therefore, the phenotype of Hdc KO mice with chronically depleted histamine may reflect a mixture of gene deletion and compensation effects.

In summary, Hdc KO mice have a distinct behavioral phenotype, specifically, altered exploratory activity in the open field, which may correspond to increased stereotypies or behavioral complexity reported earlier. Biochemical alterations in striatal dopamine and transcriptional level of striatal opioids *Pdyn* and *Penk* are associated with this behavioral pattern. Alterations in striatal opioid gene expression may reflect disbalance in activity of D1- and D2-MSNs and lead to behavioral phenotype described in the current and previous studies.

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Legends for the Figures

Fig. 1

Open field behavior and locomotor activity of Hdc KO mice. (a) Ethogram of novelty-induced exploratory behavior in naïve (n=14 and n=13 for WT and KO respectively), saline (n=10 for both groups) and amphetamine (n=13 and n=10 for WT and KO respectively) treated Hdc WT and KO mice. Each horizontal section represents specific behavior quantified in the three time intervals (0-5, 10-15 and 20-15 min) during 30 min of testing. Each column represents behavior of an individual mouse (b) Correlation between seated rearing free and jumping behaviors. Each dot represents an individual mouse (n=14 and n=13 for WT and KO respectively). (c) Open field distance travelled and maximum velocity of naïve Hdc WT and KO mice (n=10 for both groups) (d) Open field distance travelled and maximum velocity in saline and amphetamine treated Hdc WT and KO mice (n=10 for both groups).

Fig. 2

Dopamine neurotransmission in striatum of Hdc WT and KO mice. (a) Level of striatal DA, its metabolite DOPAC and DOPAC/DA ratio in tissue homogenates from Hdc WT and KO mice (n=5 for both groups) (b) Level of striatal DA, its metabolite DOPAC and DOPAC/DA ratio in tissue homogenates from Hdc WT and KO mice treated with saline or amphetamine (n=5 for both groups) (c) Level of striatal DA, its metabolite DOPAC and DOPAC/DA ratio in tissue homogenates from Hdc WT and KO mice treated with dopamine precursor L-DOPA (n=6 for both groups).

Asterisk (*) indicates p-value<0.05 for differences between genotypes

Hashtag (#) indicates p-value<0.05 for differences between treatments

Fig. 3

Striatal dynorphin (*Pdyn*) and enkephalin (*Penk*) expression in Hdc WT and KO mice. (a) Relative expression of striatal *Pdyn* and *Penk* in Hdc WT and KO mice (n=7 and n=8 for WT and KO respectively) after open-field test. (b) Correlation between striatal *Pdyn* level and DOPAC/DA ratio. Each dot represents an individual mouse (n=5 for both groups). (c) Schematic representation of Dcpu and Nacc tissue punches area (n=4 for both groups) (d) Relative expression of *Pdyn* and *Penk* in DCpu region of striatum of Hdc WT and KO mice after open-field test. (e) Relative expression of *Pdyn* and *Penk* in Nacc region of striatum of Hdc WT and KO mice after open-field

test. (f) Relative expression of striatal *Pdyn* and *Penk* in untreated (no open-field exposure) Hdc WT and KO mice (n=5 for both groups).

Asterisk (*) indicates p-value<0.05

Fig.1

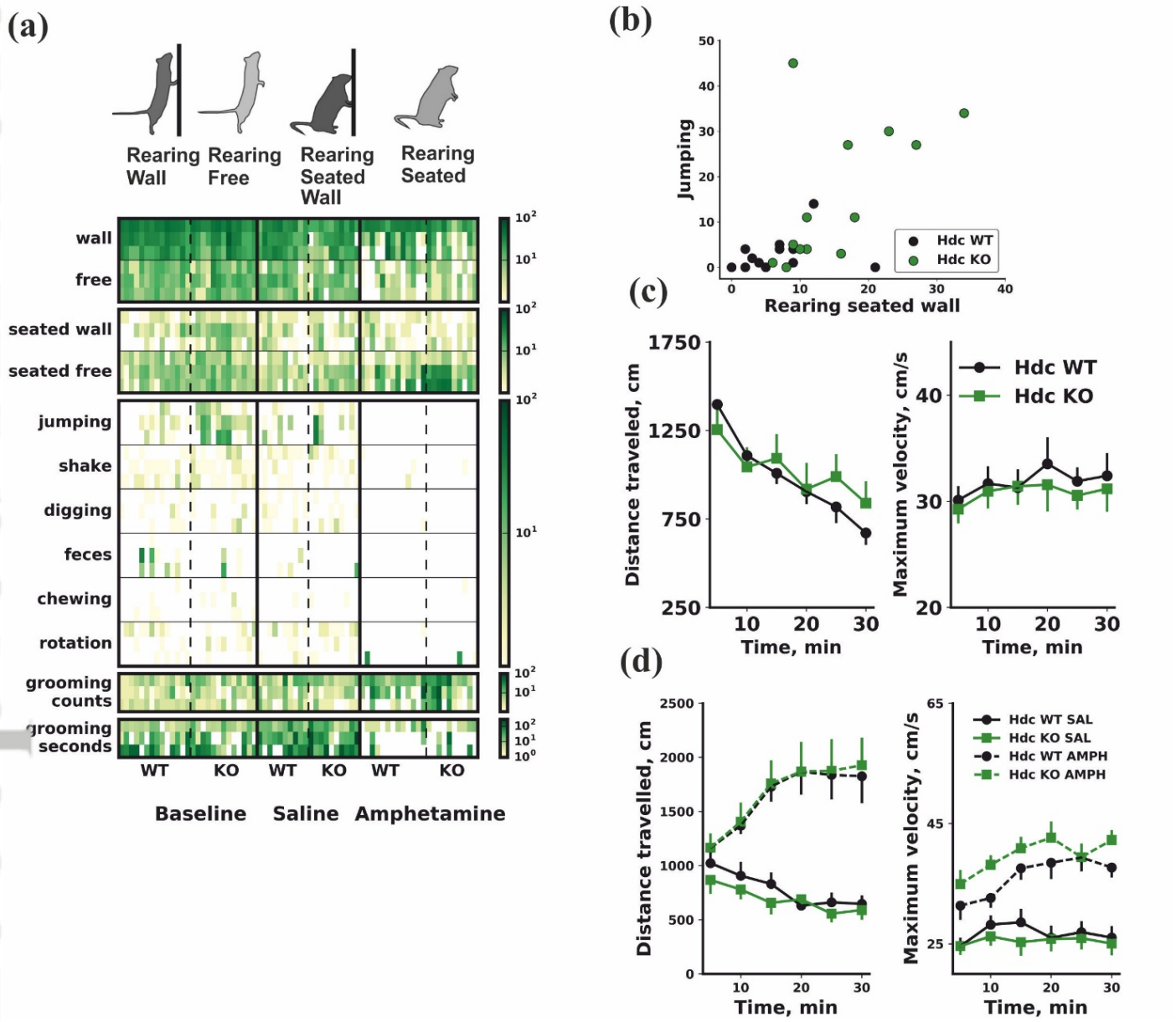

Fig.2

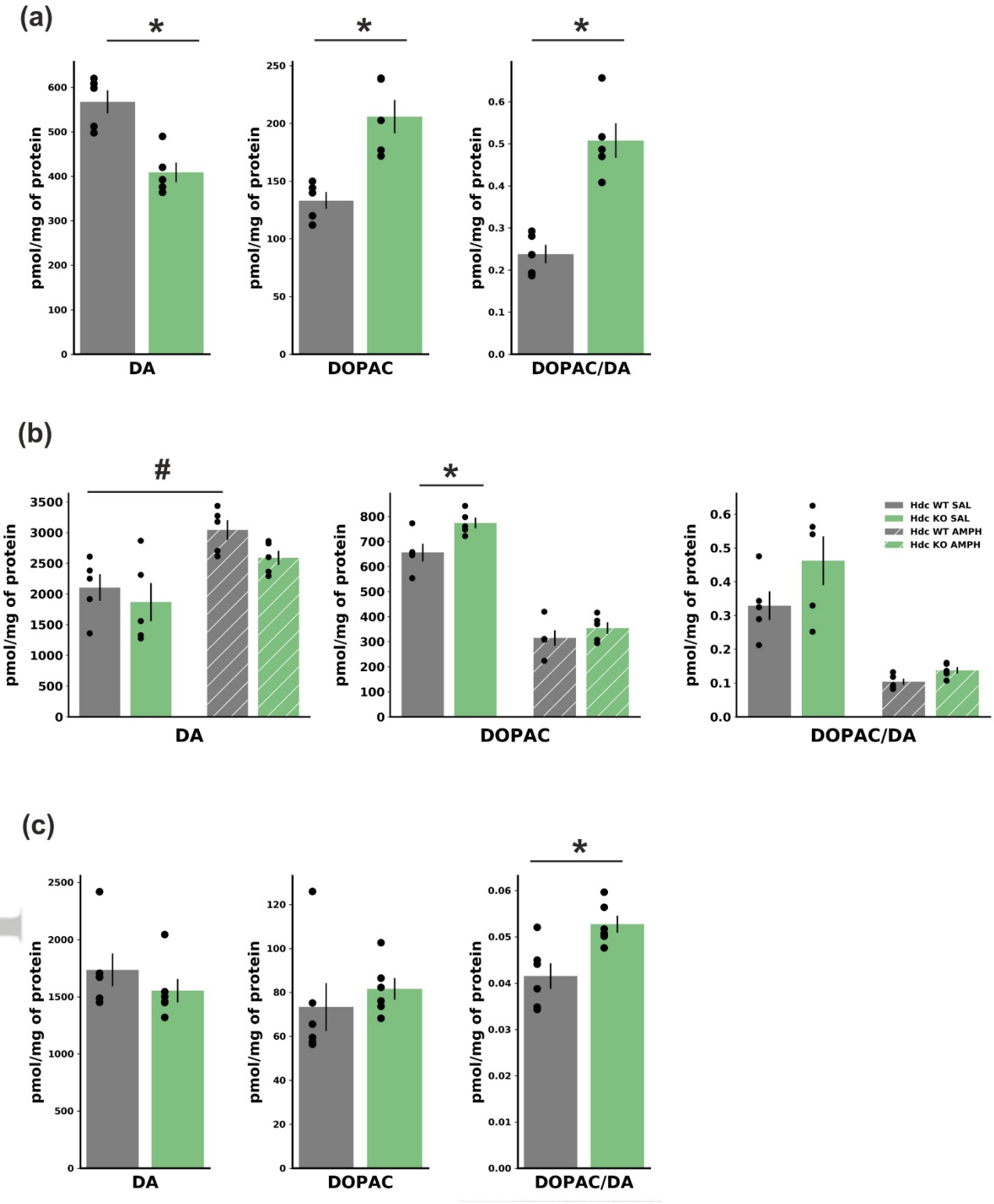

Fig.3