Research Paper

Selective genetic disruption of dopaminergic, serotonergic and noradrenergic neurotransmission: insights into motor, emotional and addictive behaviour

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Background: The monoaminergic transmitters dopamine (DA), noradrenaline (NE) and serotonin (5-HT) modulate cerebral functions via their extensive effects in the brain. Investigating their roles has led to the creation of vesicular monoaminergic transporter-2 (VMAT2) knockout (KO) mice. While this mutation results in postnatal death, VMAT2-heterozygous (HET) mice are viable and show a complex behavioural phenotype. However, the simultaneous alteration of the 3 systems prevents investigations into their individual functions. **Methods:** To assess the specific role of NE, 5-HT and DA, we genetically disrupted their neurotransmission by creating conditional VMAT2-KO mice with targeted recombination. These specific recombinations were obtained by breeding VMAT2$^{lox/lox}$ mice with DBHcre, SERTcre and DATcre mice, respectively. We conducted a complete neurochemical and behavioural characterization of VMAT2-HET animals in each system. **Results:** Conditional VMAT2-KO mice revealed an absence of VMAT2 expression, and a specific decrease in the whole brain levels of each monoamine. Although NE- and 5-HT-depleted mice are viable into adulthood, DA depletion results in postnatal death before weaning. Interestingly, alteration of the DA transmission fully accounted for the increased amphetamine response formerly observed in the VMAT2-HET mice, whereas alteration of the 5-HT system was solely responsible for the increase in cocaine response. **Limitations:** We used VMAT2-HET mice that displayed a mild phenotype. Because the VMAT2-KO in DA neurons is lethal, it precluded a straightforward comparison of the full KOs in the 3 systems. **Conclusion:** Given the intermingled functions of NE, 5-HT and DA in regulating cognitive and affective functions, this model will enhance understanding of their respective roles in the pathophysiology of psychiatric disorders.

Introduction

Monoaminergic neurotransmitters, namely dopamine (DA), noradrenaline (NE) and serotonin (5-HT), extensively modulate cognitive, affective, neuroendocrine and motor functions in the brain.[1] The role of the monoaminergic systems in psychiatric pathology has been clearly established based on the observation that effective psychotropic drugs primarily target these systems.[2–4] However, the functional subdivision of the monoamine systems indicates a disparity in cerebral distribution, receptor subtypes and mechanisms of action. Unravelling the independent roles of the monoaminergic systems in patients with neuropsychiatric disorders remains a challenge that is complicated by their large anatomic, pharmacological and functional overlaps.

At the molecular level, the monoaminergic systems share common properties. The intra- and extracellular concentrations of monoamines are primarily controlled by 2 types of transporters. The plasma membrane transporters (norepinephrine transporter [NET], serotonin transporter [SERT] and dopamine transporter [DAT]) play an essential role in the clearance and recycling of monoamines via uptake from the extracellular space, and the vesicular monoamine transporter (VMAT) accumulates monoamines in synaptic vesicles and is essential for their release.[5] Two VMAT genes have been cloned,[6,7] and although the proteins encoded by these genes are structurally related, they fulfill distinct roles. VMAT1 is primarily expressed in the peripheral nervous system; VMAT2 is primarily expressed in the neurons of the central nervous system.[8,9] The functions of VMAT2 include storage of monoamines, protection of monoamines from cytoplasmic oxidation and regulation of stimulated monoamine quantal release.[10] Thus, VMAT2 deficiency contributes to a decrease in the synaptic release of monoamines and an increase in the degradation of intracellular monoamines.

To better understand the physiologic and behavioural roles of monoaminergic signalling, several investigators have created VMAT2-knockout (KO) mice by disrupting the gene that

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encodes the neuronal isoform of VMAT.[11–13] The constitutive removal of the VMAT2 gene results in death within a few days of birth. VMAT2-KO mice display massive growth deficiency, little movement and poor feeding. However, VMAT2-heterozygous (HET) mice are viable into adulthood, with normal growth, feeding and reproductive behaviours, and they display a 50% reduction in VMAT2 expression. The levels of NE, 5-HT and DA are decreased by 94%–99% in the neonatal VMAT2-KO brain and by 34%, 23% and 42%, respectively, in VMAT2-HET mice. Behaviourally, spontaneous locomotor activity and rearing are decreased in VMAT2-HET mice compared with their wild-type (WT) littermates;[14] however, VMAT2-HET mice are more active under the effects of cocaine and amphetamine in the open-field test. This increased locomotor response to drugs is associated with a decreased sensitization to cocaine and a reduction in amphetamine–conditioned place preference (CPP).[12,13] The behavioural characterization of VMAT2-HET and WT animals did not indicate any difference in anxiety levels, although a sucrose preference test revealed that HET mice are less responsive to sucrose.[14] In conjunction with the decreased CPP in HET mice, these data suggest the presence of anhedonia in VMAT2-HET mice.

Interestingly, while attempting to produce a constitutive VMAT2-KO in a separate study, an unexpected recombination event led to the generation of a hypomorphic allele and gave rise to a VMAT2-knockdown (VMAT2-KD) that displays a 95% decrease in VMAT2 expression and function.[15] VMAT2-KD mice survive into adulthood; have a 70%–90% decrease in brain monoamines; and primarily show decreased locomotion, increased responsiveness to amphetamine-induced stereotypies[15] and increased anxiety.[16]

We used the behavioural phenotype of VMAT2-HET mice (in which the 3 monoaminergic systems are equally altered) to investigate the exact role of each monoaminergic system in these mice. We created 3 conditional VMAT2 mutant mice specific to each monoaminergic system. The floxed gene VMAT2 was specifically spliced into NE, 5-HT or DA neurons via Cre-recombinase expression under the control of the dopamine β-hydroxylase (DBH), SERT or DAT genes, respectively. We first validated and characterized our models using VMAT2 in situ hybridization and high-performance liquid chromatography (HPLC) to quantify brain monoamine levels in each specific monoaminergic area. Second, we conducted a behavioural study on each conditional VMAT2-HET mouse line to shed light on how the specific alterations in NE, 5-HT and DA neurotransmission impact motor, emotional and addictive behaviour.

Methods

Housing and breeding

Animal care and handling was performed according to the Canadian Council on Animal Care guidelines (http://ccac.ca/en_/standards/guidelines) and approved by the Animal Care Committee of the Douglas Research Centre.

The floxed VMAT2 mouse strain was produced at the Mouse Clinical Institute (Institut Clinique de la Souris, MCI/ICS) as previously described.[17] DAT-cre mice with Cre-recombinase inserted in a BAC-DAT were produced by F. Tronche.[18] SERT-cre mice [B6.FVB(Cg)-Tg(Slc6a4-cre)ET33Gsat/Mmucd, stock number 031028-UCD] and DBH-cre mice [*B6.FVB(Cg)-Tg(Dbh-cre)KH212Gsat/Mmucd*, stock number 036778-UCD] were obtained from the Mutant Mouse Regional Resource Center (MMRRC).

Heterozygous VMAT2 floxed mice ($VMAT2^{lox/+}$) were crossed with heterozygous $DBH^{cre/+}$, $SERT^{cre/+}$ or $DAT^{cre/+}$ mice to obtain double heterozygote mice, which were then crossed to generate 3 genotypes (WT, HET and KO; Appendix 1, Table S1, available at jpn.ca). After weaning and sexing, males and females were separately housed in groups of 4–5 animals per cage and maintained under standard laboratory conditions: 22° ± 1°C, 60% relative humidity and a regular 12-hour light-dark cycle (7:00–19:00 light period) with free access to food and water. The mice were used for behavioural screening at 2–4 months of age. Statistical analysis of sexes revealed no differences, therefore data for males and females were analyzed together.

VMAT2 in situ hybridization

The brains of 21-day-old mice were collected after decapitation and frozen in isopentane at –30°C. In situ hybridization was performed as previously described[19] using 10 mm-thick coronal sections. In situ hybridization experiments were run separately for each genotype. The sections were rinsed in 0.1 M phosphate-buffered saline and 10 M sodium saline citrate and treated with 0.25% ethanol. [^{35}S]-dATP oligonucleotides (5′–GAGGAACACGATGAACAGGATCAGCTTGCGCGAGT–3′; 5′–CTACGACGGTGAGCAGCATGTTGTCTAGCAGCAG–3′) were synthetized with terminal transferase (Amersham, Biosciences) to obtain a specific activity of 5×10^{-8} dpm/μg. After being covered with 70 μL of hybridization mixture and 5×10^{-5} dpm of each labelled oligonucleotide, the sections were incubated overnight at 42°C in a humid chamber. Following washes and dehydration, the slides were air-dried and exposed on a BAS-SR Fujifilm Imaging Plate for 5 days. The plates were scanned with a Fujifilm BioImaging Analyzer BAS-5000. Region identification was based on the Franklin and Paxinos Mouse Atlas.[20]

Monoamine tissue levels

After the mice were decapitated, their brains were removed, frozen in isopentane at –30°C and stored at –80°C. Whole brain ($VMAT2^{DATcre}$: 21 d old; $VMAT2^{DBHcre}$ and $VMAT2^{SERTcre}$: 2 mo old) or micropunch (2 mo old) samples were homogenized in a solution containing 225 μL or 45 μL, respectively, of 0.25 M perchlorate and 75 μL or 15 μL, respectively, of 3,4-dihydroxybenzylamine (DHBA; 100 mg/mL), which served as an internal standard. Following centrifugation at 10 000 rpm for 15 minute at 4°C, we isolated the supernatant for the detection of DA, dihydroxyphenyl acetic acid (DOPAC), homovanillic acid (HVA), NE, 5-HT and 5-hydroxyindolacetic acid (5-HIAA) using high-pressure liquid chromatography with electrochemical detection

(HPLC-EC), as previously described.[21] In parallel, pellets were reconstituted in 50 µL 0.1N NaOH for protein quantification using a BCA™ Protein Assay Kit (Fisher Scientific). The analyzed levels in each sample were expressed as micrograms per gram of protein.

Motor behaviour

Spontaneous locomotion
We measured locomotor activity with an Omnitech digiscan activity monitor. The animals' horizontal movements were measured in 5-min intervals for 30 min in open field chambers (40 cm^2) with photocells and plexiglass walls and floors.

Motor coordination
Motor coordination was tested using an accelerating rotarod (ROTO-ROD, Series 8, IITC Life Sciences). On the first day, the mice were trained in 3 trials (intertrial interval [ITI] 30 min) at rotating speeds of 6–12, 6–24 and 6–48 accelerating rpm for a maximum period of 2 min. On the test day, the mice underwent 3 trials each at rotating speeds of 6–48 accelerating rpm for a 5-min maximum period during which we recorded latency to fall.

Anxiety and depression-like behaviours

Elevated plus maze
The EPM was designed in a cross shape of 4 branching arms, with 2 opposing open arms (30 × 5 cm) and 2 opposing arms enclosed by a dark wall (30 × 5 × 11 cm). The arms radiated from a central platform (5 cm^2), and the apparatus was 50 cm above the ground. The mice were placed in the central platform, facing an open arm, and were allowed to explore the maze for 5 min. We considered the mice to be in the open or closed arm when all 4 paws were inside the arm. We recorded the percentage of time spent in the open and closed arms as a behavioural parameter.

Forced swim test
The mice were dropped into an acrylic glass cylinder (height 25 cm, diameter 9 cm) filled with water at a temperature of 21–23°C. Despair behaviour, measured using immobility time, was scored during a 6-min test. Because little immobility is observed during the first 2 min of the test, we recorded immobility only during the remaining 4 min. Immobility was scored only when the mice ceased struggling and remained floating and motionless, making only the movements necessary to keep their heads above water.

Novelty suppressed feeding test
The test was conducted in an open field (45 × 45 × 45 cm) with a sawdust-covered floor under white illumination (40 W, approximately 2400 lux) positioned immediately above the centre of the open field.[22,23] The mice were food-deprived for 24 hours before testing. At the start of the test, we placed a single food pellet on a round piece of white paper (12.5 cm diameter) at the centre of the apparatus. Each mouse was placed in a corner of the open field with its head directed toward the wall, and the latency to eat was recorded up to a maximum testing period of 10 min. Immediately afterwards, we transferred each animal to its home cage for 3 min, and we measured the amount of food consumed to assess changes in appetite as a confounding factor.

Sucrose preference test
Sucrose preference testing was carried out in the animal's home cage, in which 2 bottles were presented. The mice were habituated to the presence of 2 drinking water bottles for 2 days before being given the free choice of drinking either a 1% sucrose solution or regular water for a period of 4 days. We measured water and sucrose solution intake daily by weighing the bottles, and we switched the locations of the 2 bottles daily to reduce any confound produced by a bias in side preference. Sucrose preference was calculated as a percentage of the weight of the sucrose bottle over the total weight of both the water and sucrose bottles; this was averaged over the 4 days of testing.

Behavioural response to psychostimulants

Acute locomotor drug response
To evaluate the effects of cocaine (5, 10, 20 mg/kg) or amphetamine (1, 3, 5 mg/kg) on locomotor behaviour, the mice were first habituated to a locomotor activity monitor cage (21 cm^2) for 1 hour and then recorded for 2 hours after intraperitoneal drug administration. We measured horizontal activity in 5-min intervals for the 3-hour experiment.

Cocaine locomotor sensitization
To initiate sensitization, the mice were injected intraperitoneally with cocaine (20 mg/kg) on 6 consecutive days. After 1 day of withdrawal, on day 8, the mice were challenged with an intraperitoneal injection of cocaine (20 mg/kg) to test the expression of sensitization. We tested the mice for locomotor activity at days 1 and 8. The mice were habituated to the activity monitor cage (21 cm^2) for 1 hour and then recorded for 2 hours after cocaine administration. Horizontal activity measured at 5-min intervals was compared between days 1 and 8 for the first 30 min of cocaine challenge.

Amphetamine-CPP
We assessed CPP in a 2-compartment apparatus (27 × 22 × 22 cm) distinguished using a white wall and corncob bedding on 1 side and black walls and a wire mesh (2 mm) floor in the other compartment. The 2 compartments were separated by a 5 cm-wide door. On the first day, the mice were habituated for 15 min to the apparatus with the door open to allow exploration of both compartments. We performed a 15-min preconditioning session the next day to determine side preference. Animals that spent less than 30% of the total time in 1 compartment were eliminated from the analysis. During the 4 days of twice-daily conditioning sessions (45-min sessions separated by a 4-hr delay), the animals were restricted to 1 side of the 2-sided compartment and given intraperitoneal injections of either amphetamine (1 or 3 mg/kg) or saline (0.9%). Conditioning was elicited by pairing

amphetamine to the initially nonpreferred side of the apparatus and saline with the opposite side. A CPP assessment session during which the mice had access to both compartments for 15 min without any drug or saline administration was conducted 24 hours after the last conditioning session. The proportion of time spent during the preconditioning session in the drug-reinforced compartment was subtracted from the proportion of time spent there during the postconditioning session and used as a measure of drug-induced CPP.

Statistical analysis

The results are expressed as means ± standard errors of the mean (SEM). Since the sample sizes were small ($n < 30$) and/or the variables did not follow a normal distribution (Shapiro–Wilk test) and/or the variances were not equal among groups (Leven test), we used a nonparametric statistical analysis. We performed a multiple groups comparison (WT v. HET v. KO) using the Kruskall–Wallis test, followed by the Mann–Witney U test for 2 × 2 comparisons when appropriate. We used the Scheffe method to correct the p value for multiple comparisons ($p = 0.1/$(no. of comparison)). For the HPLC study (WT v. HET v. KO) or for the drugs study (3 concentrations), we adjusted the significance threshold to $p < 0.03$ to indicate differences between groups.

To compare 2 independent groups (WT v. HET), we used the Mann–Witney U test, whereas we used the Wilcoxon test to compare 2 dependent groups (repeated-measures). We considered results to be significant at $p < 0.05$. Statistical analyses are detailed in Appendix 1, Table S2.

Optic density of VMAT2 mRNA labelling was quantified using Image J.

Results

Specific ablation of the VMAT2 gene in NE, 5-HT or DA neurons

Conditional ablation of the VMAT2 gene was obtained by crossing VMAT2$^{lox/lox}$ mice with mice in which the DBH, SERT or DAT promoter drives the expression of bacterial Cre recombinase. The Cre recombinase spliced out the VMAT2 floxed gene specifically in NE (VMAT2^{DBHcre}), 5-HT (VMAT2^{SERTcre}) or DA (VMAT2^{DATcre}) neurons (Fig. 1A).

To validate the conditional VMAT2 transgenic mouse lines, we assessed the efficiency of Cre-mediated splicing via radioactive VMAT2 in situ hybridization (Fig. 1B). In all of the WT mice, we observed VMAT2 mRNA labelling in NE neurons of the locus coeruleus (LC), in 5-HT neurons of the raphe nucleus and in DA neurons of the ventral tegmental area (VTA) and the substantia nigra pars compacta (SNc). The efficiency of selective VMAT2-KO was evident from the selective absence of VMAT2 mRNA labelling in the NE neurons of the LC in VMAT2^{DBHcre}-KO mice, the 5-HT neurons of the raphe nuclei in VMAT2^{SERTcre}-KO mice and the DA neurons of the VTA and SNc in VMAT2^{DATcre}-KO mice. Conversely, VMAT2^{DBHcre}-HET, VMAT2^{SERTcre}-HET and VMAT2^{DATcre}-HET mice displayed a 38%, 41% and 44% reduction in VMAT2 mRNA expression in the LC, raphe, and VTA and SNc, respectively. In all of the mouse lines, VMAT2 mRNA levels were unchanged in the histaminic neurons of the tuberomammillary nucleus (data not shown).

Effects on monoamine metabolism of specific VMAT2 ablation in NE, 5-HT or DA neurons

The absence of VMAT2 mRNA observed in the KO mice was associated with a marked decrease in the tissue levels of NE, 5-HT or DA in the entire brain, as measured via HPLC (Fig. 1C). In the VMAT2^{DBHcre}-KO mice, a 70% decrease in NE tissue levels was observed compared with WT and HET at 2 months of age (WT v. KO: $U_{10} = 0$, $p = 0.006$; HET v. KO: $U_{10} = 0$, $p = 0.006$); the DA and 5-HT levels were unchanged. In the VMAT2^{SERTcre}-KO mice, only the tissue levels of 5-HT were significantly decreased (by 88%) compared with WT and HET mice (WT v. KO: $U_9 = 0$, $p = 0.009$; HET v. KO: $U_9 = 0$, $p = 0.009$). Finally, in the VMAT2^{DATcre}-KO mice, only the DA tissue levels were decreased (by 94%) compared with WT and HET mice (WT v.KO: $U_{10} = 0$, $p = 0.014$; HET v. KO: $U_7 = 0$, $p = 0.025$).

Remarkably, no changes in the whole brain levels of DOPAC, HVA or HIAA were observed in the 3 KO mouse lines. We observed a drastic increase in the DOPAC:NE ratio of the VMAT2^{DBHcre}-KO mice (WT v. KO: $U_{10} = 0$, $p = 0.006$; HET v. KO: $U_{10} = 0$, $p = 0.006$), the HIAA:5-HT ratio in the VMAT2^{SERTcre} line (WT v. KO: $U_9 = 0$, $p = 0.009$; HET v. KO: $U_9 = 0$, $p = 0.009$) and the DOPAC:DA ratio (WT v. KO: $U_{10} = 0$, $p = 0.014$; HET v. KO: $U_7 = 0$, $p = 0.025$) and HVA:DA ratio (WT v. KO: $U_{10} = 0$, $p = 0.014$; HET v. KO: $U_7 = 0$, $p = 0.025$) in the VMAT2^{DATcre} mice. This suggests that the amounts of NE, 5-HT or DA were produced normally in each mouse line but quickly degraded due to the lack of VMAT2-dependent accumulation and protection in the vesicle (Fig. 1D).

We did not detect any significant differences in the various HET mice in the whole brain levels of monoamines (Fig. 1C). Nevertheless, by targeting specific brain regions, we observed a significant decrease between HET and WT mice in each strain (Fig. 2A). In the VMAT2^{DBHcre} mice, the level of NE was significantly lower in the LC of the HET mice than in that of their WT littermates ($U_7 = 0$, $p = 0.034$). Compared with WT mice, the VMAT2^{SERTcre}-HET mice had significantly less 5-HT in the raphe nucleus ($U_9 = 1$, $p = 0.05$). Finally, the DA levels in the VMAT2^{DATcre}-HET mice were significantly lower in both the VTA ($U_{19} = 24$, $p = 0.05$) and SNc ($U_{18} = 10$, $p = 0.004$).

Effects on survival of specific VMAT2 ablation in NE, 5-HT or DA neurons

In the 3 mouse lines, KO mice were born from double heterozygote crosses at the expected Mendelian ratio of 3:16 (18.75%), indicating survival through embryonic development. However, while the VMAT2^{DBHcre}-KO mice developed normally until adulthood with a survival rate of 100%, the VMAT2^{SERTcre}-KO and VMAT2^{DATcre}-KO mice displayed a growth deficiency until the age of 3 weeks. At week 3, the weight of the VMAT2^{SERTcre}-KO mice was 8.5 ± 0.5 g compared

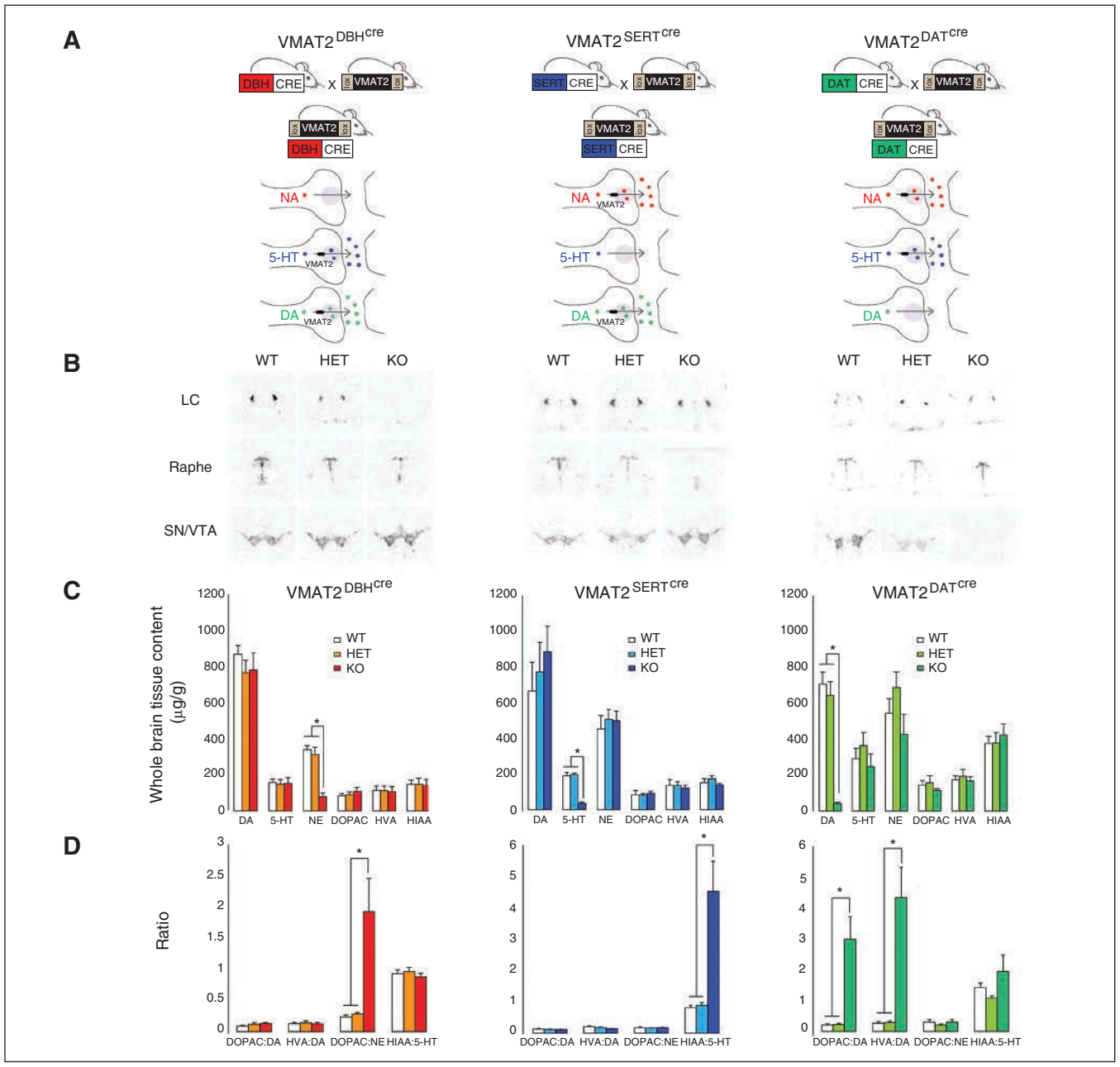

Fig. 1: Characterization and validation of vesicular monoaminergic transporter-2 (VMAT2) conditional knockout (KO) mice in dopamine β-hydroxylase (DBH)-, serotonin transporter (SERT)- or dopamine transporter (DAT)-positive neurons: $VMAT2^{DBHcre}$, $VMAT2^{SERTcre}$ or $VMAT2^{DATcre}$. (**A**) $VMAT2^{lox}$ mice were crossed with DBH^{cre}, $SERT^{cre}$ or DAT^{cre} mice to produce conditional VMAT2 transgenic mice specifically for norepinephrine (NE), serotonin (5-HT) and dopamine (DA) signalling, respectively. (**B**) The autoradiographic distribution of VMAT2 mRNA in in situ hybridization shows the effectiveness of VMAT2 Cre excision in the locus coeruleus (LC) of the $VMAT2^{DBHcre}$-KO mice (left), in the raphe of the $VMAT2^{SERTcre}$-KO mice (middle), and in the substantia nigra (SN) and ventral tegmental area (VTA) of the $VMAT2^{DATcre}$-KO mice (right). For each mouse line, the absence of VMAT2 mRNA staining in the KO mice and a 50% decrease in the heterozygous (HET) mice were specific to the targeted monoamine area. (**C**) The whole brain level of monoamines measured via high-performance liquid chromatography indicates a specific NE alteration in the $VMAT2^{DBHcre}$-KO mice (left; wild type [WT] mice, $n = 5$, HET mice, $n = 6$, KO mice, $n = 6$), a 5-HT alteration in the $VMAT2^{SERTcre}$-KO mice (middle; WT mice, $n = 5$, HET mice, $n = 5$, KO mice, $n = 5$) and a DA alteration in the $VMAT2^{DATcre}$-KO mice (right; WT mice, $n = 8$, HET mice, $n = 5$, KO mice, $n = 3$), with unchanged monoamine levels in the HET mice in each condition. (**D**) The decreased level of NE in the $VMAT2^{DBHcre}$-KO mice is associated with an increase in the dihydroxyphenyl acetic acid (DOPAC):NE ratio (left), whereas decreased 5-HT in the $VMAT2^{SERTcre}$-KO mice is associated with an increase in the 5-hydroxyindolacetic acid (5-HIAA):5-HT ratio (middle), and the decreased level of DA in the $VMAT2^{DATcre}$-KO mice relates to an increase in the DOPAC:DA and HVA:DA ratios (right). The ratios are unchanged in the HET animals of the 3 mouse lines. *$p < 0.033$ indicates a significant difference between KO and WT and HET animals.

with 9.7 ± 0.2 g for the WT mice (U_{28} = 55.5, p = 0.042). While the VMAT2^{SERTcre} mice recovered and lived through adulthood, the VMAT2^{DATcre}-KO mice did not survive past 3 weeks and had a weight that did not exceed 5 ± 0.2 g, (compared with 9 ± 0.3 g for the WT animals; Fig. 2B).

To attempt a chemical rescue of the VMAT2^{DATcre}-KO mice, we treated them postnatally with amphetamine (Fig. 2C), which enhances DA release through a reversal of the DAT directional uptake.[24,25] Paradoxically, we observed a worsening of survival, which decreased to 12 and 5.5 days after chronic daily subcutaneous administration of 2 mg/kg or 10 mg/kg d-amphetamine, respectively, compared with 22.5 days in the VMAT2^{DATcre}-KO mice treated with saline.

In contrast, the 3 HET mouse strains appeared to be indistinguishable from the WT mice throughout the observation period. They moved vigorously, fed well and grew normally. Although the expression of VMAT2 is therefore necessary for viability past the 3-week postnatal period, mice with one-half the WT levels of VMAT2 expression (HET) are able to develop. Next, we performed the behavioural analysis of each conditional VMAT2-HET mouse line to investigate the individual contribution of each monoamine to the effects observed with the constitutive VMAT2-HET mice in which all 3 systems are compromised.

Behavioural study of the specific conditional knockdown of NE, 5-HT or DA in VMAT2-HET mice

Motor functions

We evaluated spontaneous locomotion in a new environment and motor coordination in a rotarod in the 3 HET strains. The VMAT2^{DATcre}-HET mice took slightly more time to habituate than the WT mice, with decreased locomotor activity in a new environment observed throughout the 30-min test (T_{10}: U_{16} = 14, p = 0.034; T_{20}: U_{16} = 15, p = 0.043; T_{25}: U_{16} = 12, p = 0.021; T_{30}: U_{16} = 13, p = 0.027). We observed similar spontaneous locomotor activity among the WT mice, VMAT2^{DBHcre}-HET and VMAT2^{SERTcre}-HET mice (Appendix 1, Fig. S1A**).** In contrast,

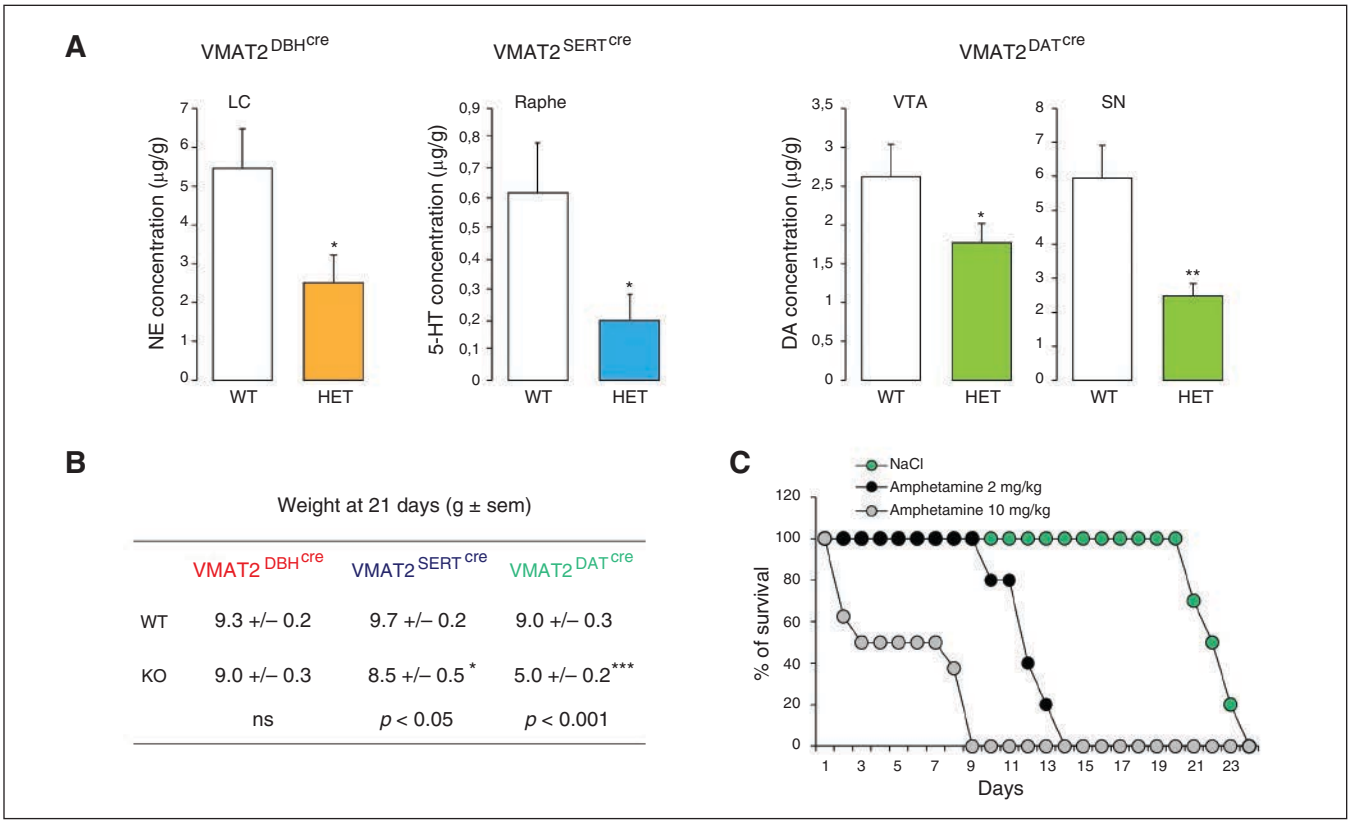

Fig. 2: Development and survival rate of the validation of vesicular monoaminergic transporter-2 (VMAT2)^{DATcre} knockout (KO) mice. (**A**) The level of norepinephrine (NE; µg/g) in the locus coeruleus (LC) measured via high-performance liquid chromatography (HPLC) in the VMAT2^{DBHcre}-heterozygous (HET) mice (n = 3) was significantly decreased compared with wild type mice (WT; n = 4; left). In the VMAT2^{SERTcre}-HET mice (n = 6), the level of serotonin (5-HT) was significantly decreased in the raphe nucleus compared with WT mice (n = 4; middle). Finally, the level of dopamine (DA) was significantly decreased in the ventral tegmental area (VTA) and substantia nigra (SN) in the VMAT^{DATcre}-HET mice (n = 10) compared with WT mice (n = 9; right). *p < 0.05 and **p < 0.01 indicate a significant difference between the WT and HET animals. (**B**) At 21 days of age, while the weight of the VMAT2^{DBHcre}-KO mice was similar to that of the WT mice, the weights of the VMAT2^{SERTcre}-KO and VMAT2^{DATcre}-KO mice were significantly lower than those of the WT mice with a huge growth deficiency in the VMAT2^{DATcre}-KO animals. *p < 0.05 and **p < 0.01 indicate a significant difference between the WT and KO mice. (**C**) Chronic amphetamine treatment started at birth in the VMAT2^{DATcre}-KO mice decreased the survival rate in a dose-dependent manner. The survival rates did not exceed 14 days with 5 mg/kg of amphetamine or 9 days with 10 mg/kg of amphetamine (compared with 23 d in the saline-treated animals).

motor coordination observed on a rotarod in 3 consecutive sessions was unaltered in the 3 strains (Appendix 1, Fig. S1B).

Anxiety and depression-related behaviours

We evaluated the behaviour of heterozygous VMAT2^{DBHcre}, VMAT2^{SERTcre} and VMAT2^{DATcre} mice in standard anxiety and depression paradigms. The VMAT2^{DBHcre}-HET, VMAT2^{SERTcre}-HET and VMAT2^{DATcre}-HET mice spent the same amount of time exploring the open arms as the WT mice in the EPM (Fig. 3A). In the novelty-suppressed feeding test (NSFT), the latency to chew the food pellet was similar among the HET and WT animals in the 3 mouse lines, indicating no individual effect for monoamine alteration on emotional reactivity (Fig. 3B). In the forced swim test (FST), the HET mice in the 3 lines spent the same amount of time immobile as their respective WT littermates, indicating no effect on resignation (Fig. 3C). Finally, in the sucrose preference test, no preference differences for 1% sucrose were observed between the HET and WT mice in the 3 mouse lines (Fig. 3D).

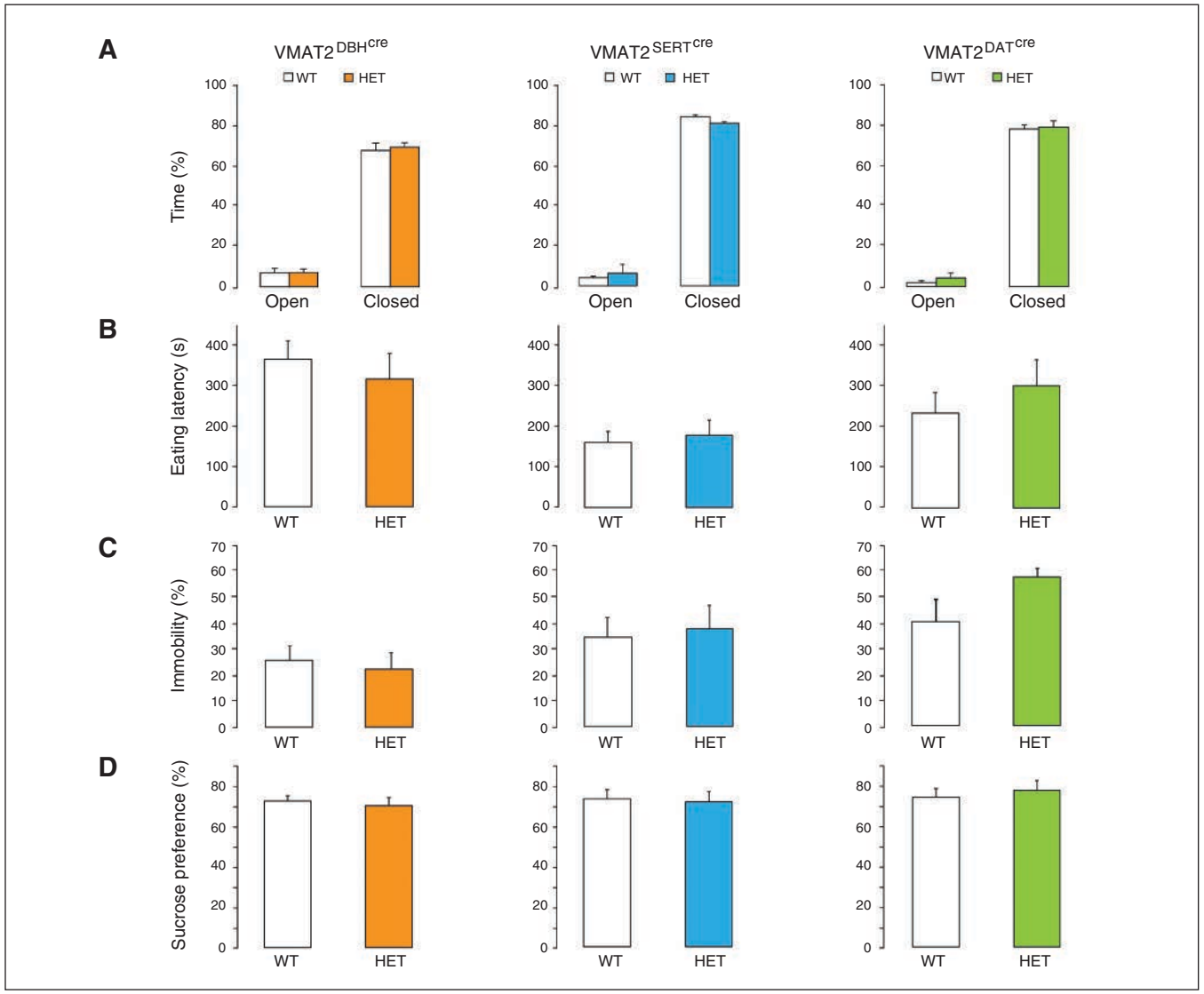

Fig. 3: Anxiety and depression-related behaviour. (**A**) In the elevated plus maze (EPM), the percentage of time spent in the open and closed arms was similar among the heterozygous (HET) mice and their respective wild type (WT) littermates in the vesicular monoaminergic transporter-2 (VMAT2)^{DBHcre} (right; WT mice, *n* = 19, HET mice, *n* = 20), VMAT2^{SERTcre} line (middle; WT mice, *n* = 14, HET mice, *n* = 12) and VMAT2^{DATcre} lines (left; WT mice, *n* = 9, HET mice, *n* = 9). (**B**) In the novelty suppressed feeding test (NSFT), the latency to chew a regular pellet was unmodified in the HET mice compared with their respective WT littermates in the 3 mouse lines (VMAT2^{DBHcre}: WT mice, *n* = 10, HET mice, *n* = 10; VMAT2^{SERTcre} line: WT mice, *n* = 8, HET mice, *n* = 6; VMAT2^{DATcre}: WT mice, *n* = 8, HET mice, *n* = 8). (**C**) In the forced swim test (FST), the percentage of time spent inactive was identical among the HET and WT mice of the 3 mouse lines (VMAT2^{DBHcre}: WT mice, *n* = 16, HET mice, *n* = 16; VMAT2^{SERTcre} line: WT mice, *n* = 8, HET mice, *n* = 7; VMAT2^{DATcre}: WT mice, *n* = 9, HET mice, *n* = 9). (**D**) The mean percentage of sucrose preference over 4 consecutive days was similar among the HET mice and their respective WT littermates in the 3 mouse lines (VMAT2^{DBHcre}: WT mice, *n* = 10, HET mice, *n* = 9; VMAT2^{SERTcre} line: WT mice, *n* = 10, HET mice, *n* = 8; VMAT2^{DATcre}: WT mice, *n* = 10, HET mice, *n* = 10).

Response to psychostimulants drugs

Given the implication of the monoamine systems in the response to drugs, we tested the effects of the individual alteration of each monoamine signalling pathway on the acute locomotor response to drugs. In the VMAT2^{DBHcre} and VMAT2^{DATcre} mice, the locomotor response to acute cocaine injection was similar among the HET and WT mice regardless of the concentration of cocaine used. However, the hyperlocomotor response induced via 10 mg/kg of cocaine in the WT mice was significantly increased in the VMAT2^{SERTcre}-HET mice (U_{16} = 12, p = 0.024; Fig. 4A and Appendix 1, Fig. S2). In response to acute amphetamine injection, we observed a similar pattern of locomotion in the HET mice compared with their respective WT littermates in the VMAT2^{DBHcre} and VMAT2^{SERTcre} lines regardless of the concentration. However, the VMAT2^{DATcre}-HET mice demonstrated increased hyperlocomotion to 3 mg/kg of amphetamine compared with the WT animals (U_{19} = 13, p = 0.005; Fig. 4C and Appendix 1, Fig. S3). Therefore, while altering the DA system is sufficient and necessary to modify the acute motor response to amphetamine, an alteration of the 5-HT system is sufficient and necessary to modify the acute motor response to cocaine (Table 1).

To measure psychomotor effects of chronic drug treatments, mice were subjected to a cocaine sensitization paradigm (Fig. 4B). Cocaine (20 mg/kg) was injected intraperitoneally on a daily basis for 6 days. Two days after the last injection, the mice were challenged with the same dose of cocaine. This paradigm consistently resulted in a significantly heightened locomotor response to the challenge dose of cocaine in the WT animals. However, the VMAT2^{SERTcre}-HET mice showed no further increase in locomotion on day 8 beyond the already enhanced response to cocaine on day 1 (day 1 v. day 8: T_{17} = 30, p = 0.008 in WT mice; T_{15} = 32, p = 0.33 in HET mice). These results clearly demonstrate that these mice were presensitized to cocaine administration. In contrast, the VMAT2^{DBHcre} and VMAT2^{DATcre}-HET mice showed the same enhanced locomotor response to the challenge dose as the WT animals.

Finally, we examined the response to 1 mg/kg or 3 mg/kg of amphetamine in the CPP paradigm, a prominent murine model for measuring aspects of drug reward and reinforcement (Fig. 4D). Both WT and HET mice of the 3 strains displayed similar preferences for the conditioning compartment in which they received conditioning amphetamine doses.

Discussion

Modifying the homeostatic tone of a given neurotransmitter is a very powerful method to ascertain its role in integrated functions. As such, the genetic removal of plasmic transporters for DA,[26] 5-HT[27] and NE[28] has provided animal models with increased transmission, which enabled many important findings in recent years.[4,5] The targeted deletion of the monoamine vesicular transporter has now enabled us to engineer mice with the opposite phenotype: a constitutive decrease (or even absence) of the releasable monoamine pool (Table 1).

The anatomic and biochemical validation of these models demonstrated an absence of VMAT2 in specific monoaminergic areas and a marked depletion of the targeted monoamine in the whole brain. Rather than accumulating in vesicles, monoamines become subject to enhanced degradation, as reflected by the lack of decline in metabolite levels. The high level of metabolites suggests an increase of both the time spent in the cytoplasm and the exposure to enzymes, such as monoamine oxidase (MAO),[29] as demonstrated by the increased 5-HT levels observed following MAO-A inhibition in VMAT2-KO mice.[11]

The deletion of VMAT2 in DA neurons is solely responsible for postnatal death

Whereas the constitutive deletion of VMAT2 is lethal 2–3 days after birth,[11–13] only its specific deletion in DA neurons reproduces this phenotype. Mice with a conditional deletion of VMAT2 in NE or 5-HT neurons are viable and reach adulthood, although retarded growth has previously been observed in VMAT2^{SERTcre}-KO mice.[17] Interestingly enough, VMAT2^{DBHcre}-KO mice did not show any sign of growth retardation, whereas in DBH-KO mice, embryos died in utero, an effect prevented by a treatment with dihydroxyphenylserine before birth (that can be converted to noradrenaline in the absence of DA β-hydroxylase). This finding confirms the major role of NE at the periphery during in utero development and parturition;[30,31] in the VMAT2^{DBHcre}-KO mice, where NE transmission is affected only in the brain, we did not have any of these effects. The lethality in VMAT2^{DATcre}-KO mice occurring during the third postnatal week is fully consistent with the course of death observed in DA-deficient mice[32] that do not express tyrosine hydroxylase in DA neurons. Interestingly, whereas the 5% of VMAT2 that were observed in the VMAT2-KD mice is sufficient for survival,[15] our results clearly show that the absence of VMAT2 in DA neurons prevents the minimal neurotransmitter release necessary for survival. The transmission of NE and 5-HT, which was operational in the VMAT2^{DATcre}-KO mice, could prevent the mice from dying immediately after birth. The genetic rescue of VMAT2 expression in the NE neurons of constitutive VMAT2-KO mice extended the lifespan by up to 3 weeks,[33] indicating a protective role of NE transmission in the early life of the mice that lack DA transmission. Additionally, compared with the constitutive VMAT2-KO mice, histamine neurotransmission remained intact in our models. The presence of histamine, a potent regulator of digestive function,[8,9,34,35] could also explain the lengthened survival from a few days to 3 weeks in the VMAT2^{DATcre}-KO mice. Whereas the monoamine releaser amphetamine was able to rescue the death of constitutive VMAT2-KO mice before weaning,[11] it had deleterious effects on the survival of the VMAT2^{DATcre}-KO mice. The effect of amphetamine on emptying the vesicular stocks of NE and 5-HT could explain this adverse outcome in a background where NE and 5-HT are still being released.

VMAT2 is an essential regulator of the quantal size of neurotransmitter release,[11,36] and its downregulation by 50% induced a strong phenotype in the constitutive

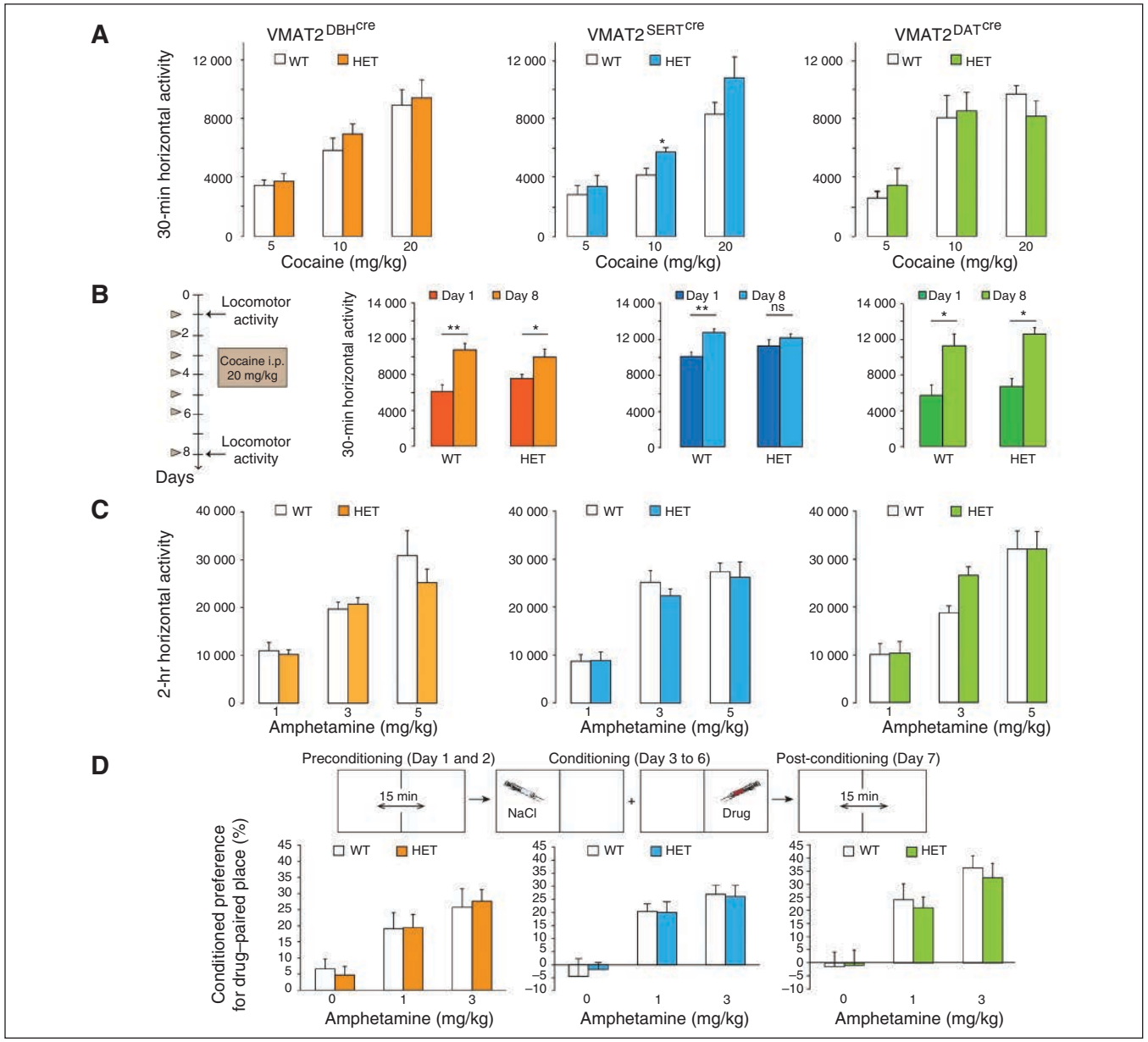

Fig. 4: Motor and reward response to drugs. (**A**) Acute locomotor response to cocaine during the 30 min following intraperitoneal injection of 5, 10 or 20 mg/kg in the wild type (WT) and heterozygous (HET) mice of the vesicular monoaminergic transporter-2 (VMAT2)^{DBHcre} (left; WT mice, $n = 6$, HET mice, $n = 6$), VMAT2^{SERTcre} (middle; WT mice, $n = 7$–10, HET mice, $n = 7$–9) and VMAT2^{DATcre} lines (right; WT mice, $n = 5$–6, HET mice, $n = 6$). An enhanced hyperlocomotor response to 10 mg/kg of cocaine was observed only in the VMAT2^{SERTcre}-HET mice compared with the WT animals. *$p < 0.033$ indicates a significant difference between the WT and HET animals. (**B**) The repeated intraperitoneal administration of 20 mg/kg cocaine over 6 days induced a greater locomotor response to cocaine beyond the already enhanced response observed at day 1 (measured on the test day [day 8] after 48 hours of withdrawal [left] in the WT and HET mice of the VMAT2^{DBHcre} [left; WT mice, $n = 10$, HET mice, $n = 10$] and VMAT2^{DATcre} lines [right; WT mice, $n = 8$, HET mice, $n = 8$]). However, although locomotor sensitization was observed in the WT mice of the VMAT2^{SERTcre} line (middle), no sensitization appeared in the HET mice (WT mice, $n = 9$, HET mice, $n = 8$). *$p < 0.05$ and **$p < 0.01$ indicate a significant difference between days 1 and 8. (**C**) The acute locomotor response to amphetamine during the 2 hours following the intraperitoneal injection of 1, 3 or 5 mg/kg in the WT and HET mice of the VMAT2^{DBHcre} (left; WT mice, $n = 5$–6, HET mice, $n = 6$–7), VMAT2^{SERTcre} (middle; WT and HET mice, $n = 6$–7) and VMAT2^{DATcre} lines (right; WT and HET mice, $n = 5$–10). An enhanced hyperlocomotor response to 3 mg/kg of amphetamine was observed only in the VMAT2^{DATcre}-HET mice compared with their WT littermates. *$p <$ 0.033 indicates a significant difference between the HET and WT animals. (**D**) In the amphetamine-conditioned place preference (CPP) test, the mice were allowed to explore 2 compartments that were separated by an open door for 15 min during a preconditioning session (day 2) and a post-conditioning session (day 7). On the conditioning days (days 3–6), the mice received intraperitoneal injections of amphetamine (0, 1 or 3 mg/kg) in 1 compartment and 0.9% saline in the other (upper schema). The %CPP for the drug-paired compartment in the WT and HET mice of the VMAT2^{DBHcre} (left; WT mice, $n =$ 5–6, HET mice, $n = 6$–7), VMAT^{SERTcre} (middle; WT mice, $n = 5$–8, HET mice, $n = 6$–8) and VMAT2^{DATcre} lines (right; WT mice, $n = 5$, HET mice, $n = 5$–7) indicate a dose-dependent increase in preference for the drug-paired compartment for both the WT and HET animals in each line.

VMAT2-HET mice. Therefore, in the following sections, we attempt to decipher the respective involvement of DA, NE or 5-HT transmission in each phenotype.

An altered DA system is necessary to induce a deficiency in motor function

Despite previous studies showing no alteration in motor activity in constitutive VMAT2-HET mice,[12,13] 1 study[14] revealed a significant impairment of locomotion in the open-field test. Therefore, the motor deficit observed in the VMAT2^{DATcre}-HET mice, an effect that was not seen when either 5-HT or NE transmission was decreased, suggests that the impaired locomotor activity observed in VMAT2-HET constitutive mice can be attributed to DA control.

Altering 1 monoaminergic system is not sufficient to induce depression-related behaviour

The absence of increased anxiety in VMAT2-HET constitutive mice[14] was reproduced in our conditional models. These results are similar to those of hypertensive patients treated with the VMAT2 blocker reserpine in whom anxiety did not appear to accompany a depressive state.[37]

More specifically, we did not observe any effect in the anxiety levels of the VMAT2^{SERTcre}-HET mice. We could have seen such an effect, in the light of the increased anxiety observed in SERT-KO mice[38] as well as to the decreased anxiety observed in both the VMAT2^{SERTcre}-KO mice[17] (decreased latency to eat in the NSFT) and in the Tph2-KO mice[39] (decreased time spent in the closed arms of the EPM). These later studies indicate a fairly straightforward association between anxiety state and extracellular 5-HT levels, both of which were lowered in VMAT2^{SERTcre}-KO and Tph2-KO mice and increased in SERT-KO mice.

In the VMAT2^{SERTcre}-KO and SERT-KO models, the mice did not appear to be affected in any depression-related paradigms.[40] Although they are often comorbid, anxiety and depression are 2 independent affective-related disorders. A depressive-like phenotype without changes in anxiety level has been observed in VMAT2-HET mice,[14] and this particular trait could not be observed in any of the specific mice we engineered. The sucrose preference and CPP alteration in VMAT2-HET mice was not replicated in our conditional models, suggesting that the alteration of only 1 monoamine system is not sufficient to induce anhedonic behaviour. Because the NET-KO mice have reduced anxiety as well as a depression-resistant phenotype[28] that is similar to that induced by antidepressant treatment,[41] the depressive phenotype of VMAT2-HET mice could be explained by the NE component. However, the absence of depressive-like behaviour in our experiments with VMAT2^{DBHcre}-HET mice does not support this hypothesis, and further studies using the homozygous deletion of VMAT2 in NE neurons will be needed to better understand the role of NE in the regulation of affective behaviour.

Differential roles of altered DA and 5-HT systems on locomotor responses to amphetamine and cocaine

Despite a lower amphetamine-induced DA release in vivo[13] and in vitro,[11] cocaine- or amphetamine-induced hyperlocomotion are increased in constitutive VMAT2-HET mice.[13] A possible explanation for this could be that VMAT2 ablation leads to the upregulation and sensitization of both D1 and D2 receptors, as suggested by the fact that this effect is observed after chronic treatment with the VMAT blocker reserpine.[42,43]

Interestingly, while the locomotor effect of amphetamine was observed in the VMAT2^{DATcre}-HET mice, only the VMAT2^{SERTcre}-HET mice displayed an increased ambulatory response to cocaine. While the motor response is enhanced at low doses of amphetamine in VMAT2-HET animals (0.5–1 mg/kg),[12,13] the same effect was observed for an intermediate dose of amphetamine (3 mg/kg) in our VMAT2^{DATcre}-HET mice. Although amphetamine elicited a dose-dependent increase in motor ambulation in the WT mice, the

Table 1: Changes in motor behaviour, anxiety- and depression-related behaviour, and addiction-related behaviour in constitutive VMAT2-HET mice*

		VMAT2	VMAT2^{DBHcre}	VMAT2^{SERTcre}	VMAT2^{DATcre}
Behaviour type	Change observed	NE, 5-HT, DA	NE	5-HT	DA
Motor behaviour	Spontaneous locomotion (horizontal activity)	↘	Ø	Ø	↘
	Rotarod (latency to fall)	Ø	Ø	Ø	Ø
Anxiety	Elevated plus maze (time in open arm)	Ø	Ø	Ø	Ø
	Novelty suppressed feeding (feeding latency)	Ø	Ø	Ø	Ø
Depression	Forced swim test (immobility %)	Ø	Ø	Ø	Ø
	Sucrose preference test (% of preference)	↘	Ø	Ø	Ø
Addiction	Acute motor response to cocaine	↗	Ø	↗	Ø
	Acute motor response to amphetamine	↗	Ø	Ø	↗
	Cocaine sensitization	↘	Ø	↘	Ø
	Amphetamine-conditioned place preference	↘	Ø	Ø	Ø

Ø = no change; ↘ = significant decrease; ↗ = significant increase; 5-HT = serotonin; DA = dopamine; HET = heterozygous; NE = norepinephrine; VMAT = vesicular monoaminergic transporter-2.
*The 3 monoaminergic systems are affected compared with the conditional VMAT2^{DBHcre}-HET, VMAT2^{SERTcre}-HET and VMAT2^{DATcre}-HET mouse lines (in which only 1 specific monoaminergic system is altered.

VMAT2^{DATcre}-HET mice already reached a plateau at 3 mg/kg. However, the increased response to cocaine in VMAT2-HET mice was observed for a high dose of cocaine (20 mg/kg), while this effect was observed with a lower dose of 10 mg/kg of cocaine in the VMAT2^{SERTcre}-HET mice in our model. These results indicate a difference in sensitivity to the motor effect of psychostimulants when only 1 monoaminergic system is affected.

There is some evidence indicating that the stimulation of dorsal raphe 5-HT$_{1A}$ receptors potentiates cocaine-induced locomotion and cocaine-induced DA release.[44] A substantial reduction in the expression of these receptors is observed in SERT-KO mice, which show decreased locomotor response to cocaine.[45] In contrast to SERT-KO mice, which have increased levels of 5-HT, the VMAT2^{SERTcre}-HET mice displayed decreased 5-HT levels. A possible explanation for the increased sensitivity to the locomotor effect of cocaine could therefore be that VMAT2 ablation in 5-HT neurons leads to an upregulation and sensitization of the 5-HT$_{1A}$ receptor. Given the anatomic interaction of the DA and 5-HT systems, it is important to mention that the response to cocaine observed in the VMAT2^{SERTcre} mice could also be due to increased DA release. Although a recent study demonstrated that the majority of dorsal raphe neurons that project to the VTA are nonserotoninergic,[46] neuroanatomical studies have revealed 5-HT immunoreactive fibres in the VTA,[47] and there is evidence that the mesolimbic dopaminergic system is under the control of the 5-HT system.[48,49]

Alteration of the 5-HT system alters cocaine sensitization

As a psychostimulant, cocaine elicits a dose-dependent increase in motor activity in rodents.[50,51] Additionally, cocaine-induced motor effects depend on the number and duration of administrations, where repeated administrations result in increased motor responses (behavioural sensitization).[52,53] In constitutive VMAT2-HET mice, cocaine-induced locomotor sensitization is abolished, indicating that these mice are already maximally sensitized to cocaine.[13] Surprisingly, this presensitization to cocaine was fully replicated only in the VMAT2^{SERTcre}-HET mice. The modulation of cocaine-induced behavioural sensitization by 5-HT receptor ligands has been previously observed in mice.[54] For example, 5-HT receptor antagonists[55–58] inhibit cocaine-induced behavioural sensitization, suggesting that the central 5-HT system plays an important role in this effect.

There was no differential effect of cocaine on the CPP paradigm in VMAT2-HET mice versus WT mice, whereas amphetamine produces a decreased preference to the paired compartment.[12] None of the specific alterations in monoamine neurotransmission in our models could replicate this effect. Considering that the diversity of reward mechanisms has been well recognized, it is important to note that the different methods used to assess the rewarding properties of drugs are not equivalent measures of a single construct.[59] Multiple conditioned effects can be observed independently from one another. For example, conditioned locomotor activity can be observed independently from CPP.[60–62] Given these observations, the differential behavioural response between locomotor sensitization and CPP in our study is not atypical. Moreover, constitutive VMAT2-HET mice show a decreased CPP to amphetamine that is associated with decreased sucrose preference. Taken together, these results clearly demonstrate that although an anhedonic state is observed in VMAT2 constitutive HET mice, the alteration of only 1 monoaminergic system, as observed in our selective conditional models, is not sufficient to alter responses in both CPP and sucrose preference.

Limitations

This study focused on the phenotypic differences that were observed in the VMAT2-HET mice. It may not preclude that some behavioural differences could exist in the conditional heterozygous mice that are not present in the VMAT2-HET mice. Therefore, further work is be needed to investigate a larger panel of behaviour.

Conclusion

To our knowledge, this study is the first to model independent alterations of the 3 main monoaminergic neurotransmitter systems. Analysis of the complete behavioural pattern (Table 1) suggests 3 main findings. First, some deficits that have been observed in constitutive VMAT2-HET mice are clearly replicated in the KO of single systems. As expected, changes in spontaneous locomotion and acute responses to amphetamine are solely linked to deficits in DA neurotransmission. However, it was not expected that differences in acute and chronic responses to cocaine would be fully reproduced in the VMAT2^{SERTcre}-HET mice. Second, some deficits, such as anhedonia and amphetamine-CPP deficits, could not be found in the VMAT2-HET mice, which clearly indicates that the occurrence of these effects likely requires the contribution of more than 1 aminergic system. Third, it is interesting to note that the VMAT2^{DBHcre}-HET mice showed no phenotypic changes in any of our experimental conditions. Additional studies using mice with conditional heterozygous deletions of VMAT2 that affect 2 systems (or mice with a full deletion of VMAT2 in noradrenergic neurons) will enable investigation into the respective roles of these monoaminergic systems in mood disorders.

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