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Differential expression of VGLUT3 in laboratory mouse strains: Impact on drug-induced hyperlocomotion and anxiety-related behaviors

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The atypical vesicular glutamate transporter VGLUT3 is present in subpopulations of GABAergic interneurons in the cortex and the hippocampus, in subgroups of serotoninergic neurons in raphe nuclei, and in cholinergic interneurons in the striatum. C56BL/6N mice that no longer express VGLUT3 (VGLUT3$^{-/-}$) display anxiety-associated phenotype, increased spontaneous and cocaine-induced locomotor activity and decreased haloperidol-induced catalepsy. Inbred mouse strains differ markedly in their sensitivity to anxiety and behavioral responses elicited by drugs. The purpose of this study was to investigate strain differences in VGLUT3 expression levels and its potential correlates with anxiety and reward-guided behaviors. Five inbred mouse lines were chosen according to their contrasted anxiety and drugs sensitivity: C57BL/6N, C3H/HeN, DBA/2J, 129/Sv, and BALB/c. VGLUT3 protein expression was measured in different brain areas involved in reward or mood regulation (such as the striatum, the hippocampus, and raphe nuclei) and genetic variations in *Slc17a8*, the gene encoding for VGLUT3, have been explored. These five inbred mouse strains express very different levels of VGLUT3, which cannot be attributed to the genetic variation of the *Slc17a8* locus. Furthermore, mice behavior in the open field, elevated plus maze, spontaneous- and cocaine-induced locomotor was highly heterogeneous and only partially correlated to VGLUT3 levels. These data highlight the fact that one single gene polymorphism could not account for VGLUT3 expression variations, and that region specific VGLUT3 expression level variations might play a key role in the modulation of discrete behaviors.

KEYWORDS
129Sv, anxiety, BALB/c, C3H, C57Bl6/N, DBA2, locomotion, vesicular glutamate transporter (VGLUT3)

1 | INTRODUCTION

Glutamate plays a major role in neurotransmission and around 50% to 70% of all brain synapses release glutamate.[1] To act as a neurotransmitter glutamate has to be accumulated inside synaptic vesicles, allowing its exocytotic release in the synaptic cleft. The transport of cytosolic glutamate into vesicles is operated by Vesicular Glutamate Transporters types 1, 2 and 3 (VGLUT1, −2, −3).[2] All three VGLUTs have similar functional properties and show almost complementary expression in the brain. VGLUT1 is mainly expressed by cortical areas, and VGLUT2 by subcortical areas.[3,4] While VGLUT1 and VGLUT2 are present mainly in glutamatergic neurons, VGLUT3 is observed in

Diana Y. Sakae and and Lauriane Ramet contributed equally to this study.

neurons utilizing other neurotransmitters.[2] For instance, VGLUT3 is expressed by subpopulations of GABAergic interneurons in the cortex and the hippocampus, serotoninergic neurons in the raphe nuclei, and in cholinergic interneurons in the striatum.[5,6] In these neurons, VGLUT3 allows glutamate to be released as well as potentiate vesicular filling[2,7] and therefore regulates the amount of release of other neurotransmitters, that is, γ-aminobutyric acid (GABA), serotonin or acetylcholine.[7–10] C57BL/6N mice lacking VGLUT3 (VGLUT3$^{-/-}$ mice) display higher anxiety-associated and striatal-related behaviors (such as increased spontaneous hyperactivity and cocaine-induced locomotor activity [LMA][8,9,11]). The use of knockout mice is a powerful tool to determine the contribution of selected genes in specific and complex behaviors.[12–17] However, these behaviors also depend on the genetic background of various mouse strains.[16,18,19] Several studies already showed phenotypic differences between inbred mouse strains for both anxiety and behavioral responses elicited by drugs.[20–25]

The first aim of this study was to assess whether the level of VGLUT3 expression was different in various mouse strains. The second aim was to investigate whether these variations of expression could be related to some VGLUT3-dependent phenotypic traits previously reported in VGLUT3$^{-/-}$ mice.[11] We measured VGLUT3 protein expression levels in the striatum, hippocampus and raphe nuclei of different mouse strains and correlated them to their anxiety-like and cocaine-induced locomotor behaviors. These brain regions were selected for their known involvement in the regulation of mood or reward behaviors. Five inbred mouse lines commonly used in laboratories were studied (C57BL/6N, C3HeN, DBA/2J and 129/Sv, BALB/c) and compared with VGLUT3$^{-/-}$ mice (C57BL/6N background). Substantial differences between strains' behaviors were observed, as well as variations in the level of VGLUT3 expression. However, no direct correlation could be established between the strain-specific genetic variations in *Slc17a8*, the gene encoding VGLUT3 and VGLUT3 levels. VGLUT3 levels did correlate to some extent with a few behaviors. This finding suggests that glutamatergic cotransmission and VGLUT3-dependent vesicular filling of other neurotransmitters might play a key role in modulating neuronal networks but only in some discrete behavioral aspects.

2 | MATERIALS AND METHODS

2.1 | Animals

BALB/cJ, DBA2/J and C3H/HeN mice were supplied by Janvier Labs (Le Genest St Isle, France), and 129S2/Sv by Charles River (L'Arbresle, France). Male mice were 6 weeks old upon arrival. C57BL/6N and VGLUT3$^{-/-}$ mice (C57BL/6N background) were obtained from our breeding facility. All mice were kept in groups of 4 per cage, housed in a temperature-controlled room (21 ± 2°C) with ad libitum access to water and food under a light/dark cycle of 12 hours (light ON from 7:30 AM to 7:30 PM). Three independent groups of mice were used: one for the behavior (n = 58), one for the anatomy (immunoautoradiography [IAR]) (n = 19) and one for the genetics (n = 20) (see Table 1 for details). Behavioral experiments were performed on 8 to 12 weeks old mice during light phase. All experiments were performed in accordance with the European Union guidelines (directive 2010/63/EU), and with the approval of the French *Ministère de l'Agriculture et de la Forêt*, *Service Vétérinaire de la Santé et de la Protection Animale* (authorization #01482.01 from ethics committee Darwin #5). All efforts were made to minimize the number of animals used in the course of the study and to ensure their well-being.

2.2 | Behavioral analysis

Animals were first tested in the open field (OF), a couple of days later in the elevated plus maze (EPM), and after a week delay in a circular corridor for cocaine-induced LMA. The group size varies from 7 to 10—exact numbers are presented in Table 1. All graphs represent the mean ± scanning electron microscope (SEM).

2.2.1 | OF test

The OF test was performed in a white perplex arena (43 × 43 × 26 cm) located in a 50-lx illuminated room. The virtual central compartment square represents one third of the total arena. Mice were introduced into the central area and allowed to freely explore the OF for 360 seconds. We recorded duration, frequency and time course of various behaviors (exploration, walk, rear, stretch and groom-data not shown) exhibited by mice in different regions of the OF (central vs periphery zone) using Viewpoint tracking system (Lyon, France).

2.2.2 | EPM test

The EPM consists of two white open arms (OAs) and two black-closed arm (CA) (66 × 66 cm, 50 cm high) with a central zone named choice area. The luminosity in the central zone is 50 lx. After 1 hour of habituation in the testing room, animals were placed into the choice area of the maze and tested for 360 seconds. The total time spent in each compartment (open vs CAs) and the number of arms entries were recorded using Viewpoint tracking system. The percentage of duration was calculated using arms occupancy: (OA) duration × 100/(OA + CA) duration, therefore excluding the time spent in the central zone.

2.2.3 | Locomotor activity

LMA was measured in cyclotron. It consists of a circular corridor with four infrared beams placed at 90° angles (Imetronic, Pessac, France). The device is connected to an electronic interface for data collection. The consecutive interruption of two adjacent infrared beams (ie, mice moving through one fourth of the circular corridor) was recorded and represents the activity of the animal. For cocaine-induced LMA experiment, after an hour habituation to the activity box, mice were

TABLE 1 Animals used in the different aspect of the study

n=	BalB/c	DBA/2	C57BL/6N	C3H/HeN	129/Sv	VGLUT3$^{-/-}$
OF	10	10	8	9	9	8
EPM	10	9	8	10	8	7
LMA	10	10	7	10	10	10
IAR	4	4	5	3	3	-
Genetics	5	5	5	5	5	-

Abbreviations: EPM, elevated-plus maze; Immunoautoradiography (IAR, *n*=19); LMA, locomotor activity induced by cocaine; OF, open field.
Note: Behavior (*n*=58), Genetics (*n*=20).

injected with saline (NaCl 0.9%, ip), and placed back into the cyclotron for another 1 hour. They were then injected with cocaine (10 mg kg^{-1}, ip), and recorded for an additional 90 minutes. LMA was recorded in 5-minute intervals for 210 minutes. To assess cocaine-induced hyperlocomotion, we normalized the data by subtracting LMA observed in the 30 minutes following saline injection to the LMA observed in the 30 minutes following cocaine injection.

2.3 | IAR labeling of VGLUT3

To assess whether VGLUT3 was differently expressed in various mouse lines, we analyzed multiple brain areas by IAR as previously described.[8] VGLUT3 density measurements were performed in the striatum, the hippocampus and raphe nuclei of wild-type mice from the different genetic backgrounds (n = 4) and compared with VGLUT3$^{-/-}$ mice (C57BL/6N background; n = 4). After cervical dislocation, brains were dissected and rapidly frozen in isopenthane at −30°C. Fourteen-micrometer-thick coronal brains sections were cut at −20°C, thaw-mounted on Superfrost Plus slides and stored at −80°C until use. For each mouse, four coronal sections of each brain area per strain were analyzed. Sections were fixed with 4% paraformaldehyde at room temperature for 15 minutes and washed with phosphate buffered saline (PBS) containing 3% bovine serum albumin, 1% goat serum and 1-mM NaI for an hour (named Buffer A). Sections were then incubated overnight at 4°C with buffer A supplemented with VGLUT3 antiserum (1/20000; Synaptic System), wash out and then incubated for 2 hours at room temperature (RT) in buffer A with anti-rabbit [^{125}I]-IgG (PerkinElmer, Villebon sur Yvette, France). Rinsed sections were then exposed to X-ray films (Biomax MR, Kodak) for 3 days. Standard radioactive microscales were exposed onto each film to ensure that labeling densities were in the linear range. The densitometry measurements were performed with MCID analysis software version 7.0 (Imaging Research Inc., St Catherines, ON, Canada). Background was determined on white matter areas on each section and subtracted from the densitometry measurements. Areas were identified and defined by comparing sections to the Paxinos mouse brain atlas (2001)[26]: dorsal striatum (DS) and ventral striatum (VS) were analyzed in coronal sections with +1.54 to +1.10 from bregma; dorsal hippocampus (DH) ranging −1.34 to −2.30 from bregma; ventral hippocampus (VH) from −2.80 to −3.16; dorsal raphe nuclei (DRN) and median raphe nuclei (MRN) −4.16 to −4.60 from bregma.

2.4 | Genetic analysis of VGLUT3 promoter

In order to determine whether variation of VGLUT3 expression levels in different mouse strains resulted from genetic differences, we sequenced 2540 bp spanning the promoter region and exon 1 as well as 4098 bp spanning the last coding exon and the 3′ untranslated region (3′-UTR) of *Slc17a8* (the gene encoding for VGLUT3). Five mice per strain were used. Genomic DNA was extracted from mouse tail using DirectPCR lysis reagent (Viagen Biotech Inc., Los Angeles, California) and 0.2 mg/mL Proteinase K solution, according to manufacturers' protocol. The promoter region and the exon 1 flanking regions of *Slc17a8* (NM_182959.3) were defined as 1770 bp upstream to the transcription start site, 451 bp of the exon 1 and 319 bp downstream to exon 1. The last exon and its flanking region were defined as 809-bp upstream to exon 12, 2638 bp of exon 12, including 2296 bp of 3′-UTR, and 651-bp downstream to the end of transcription. These regions were amplified by polymerase chain reaction (PCR) and sequenced using BigDye Terminator v3.1 cycle sequencing kit (Thermo Fisher Scientific, Carlsbad, California) and run on a 16-Capillary ABI PRISM 3130xl genetic analyzer after purification using the BigDye XTerminator purification kit (Thermo Fisher Scientific). Chromatograms were analyzed using Genalys 2.8.2b software.[27] All primers used for PCR amplification and sequence analyses are available on request. We used TargetScanMouse 7.1[28] to check whether identified single-nucleotide polymorphism (SNP) may affect a conserved mammalian microRNA (miRNA) regulatory target sites, and $MAPPER_2$[29] to study potential changes in binding sites.

2.5 | Statistics

Nonparametric Kruskal-Wallis H test was used to assess strain differences in VGLUT3 content and in behavioral measures. The Mann-Whitney *U* test was used to compare VGLUT3$^{+/+}$ and VGLUT3$^{-/-}$ mice data. Repeated measures analysis of variance (ANOVA) was used to calculate differences across time in the cocaine-induced hyperlocomotion experiment. For correlations analyses, Pearson correlation coefficient R^2 was calculated. All statistical analyses were performed using GraphPad Prism 6 (Graphpad Software Inc., La Jolla, CA, USA) for MacOS X. The threshold for statistical significance was set at 5%.

3 | RESULTS

3.1 | Behavioral characterization

3.1.1 | OF and EPM

In the OF, a strong strain-specific difference in anxiety-associated LMA (Figure 1A,C, kruskal wallis test (KW), $W = 26.35$; 24.04; 26.81, $P < 0.0001$) and anxiety-like phenotype (Figure 1D, KW, $W = 19.5$, $P = 0.0006$) was observed. Of all strains tested, the C57BL/6N strain was the less anxious (Figure 1D: B6 vs BALB: $P < 0.01$; B6 vs C3H: $P < 0.0001$). In this test, DBA/2 mice were globally more active than 129/Sv, BALB/c and C3H/HeN (Figure 1A-C, $P < 0.01$ for all comparisons). VGLUT3$^{-/-}$ mice show a strong reduction of LMA (boxed bar graphs for: (Figure 1A), peripheral LMA: Mann Whitney test (MW) *U* test, $U = 5$, $P = 0.0127$; (Figure 1B) central LMA: MW *U* test, $U = 0$, $P = 0.0007$; (Figure 1C) total LMA: MW *U* test, $U = 5$, $P = 0.0295$), as well as a fivefold higher center occupancy (boxed bar graph Figure 1D, MW *U* test, $U = 0$, $P = 0.0007$).

In the EPM test, strain-specific differences were observed in the time spent in OAs (Figure 2A, KW, $W = 13.33$, $P = 0.0098$), CAs (Figure 2B, KW, $W = 22.95$, $P < 0.0001$), percentage of time spent in OAs (Figure 2E, KW, $W = 10.9$, $P = 0.027$), and in the frequency of OAs (Figure 2C, KW, $W = 11.36$, $P = 0.0228$). However, no differences in CA entries were observed between the five strains (Figure 2D, KW, $W = 6.44$, $P = 0.1686$). Regarding the OAs analyses (Figure 2A,C,E), statistical differences can be explained by the overall higher exploration rate of the C57BL/6N strain. We observed that 129/Sv also present a significantly lower exploration of the

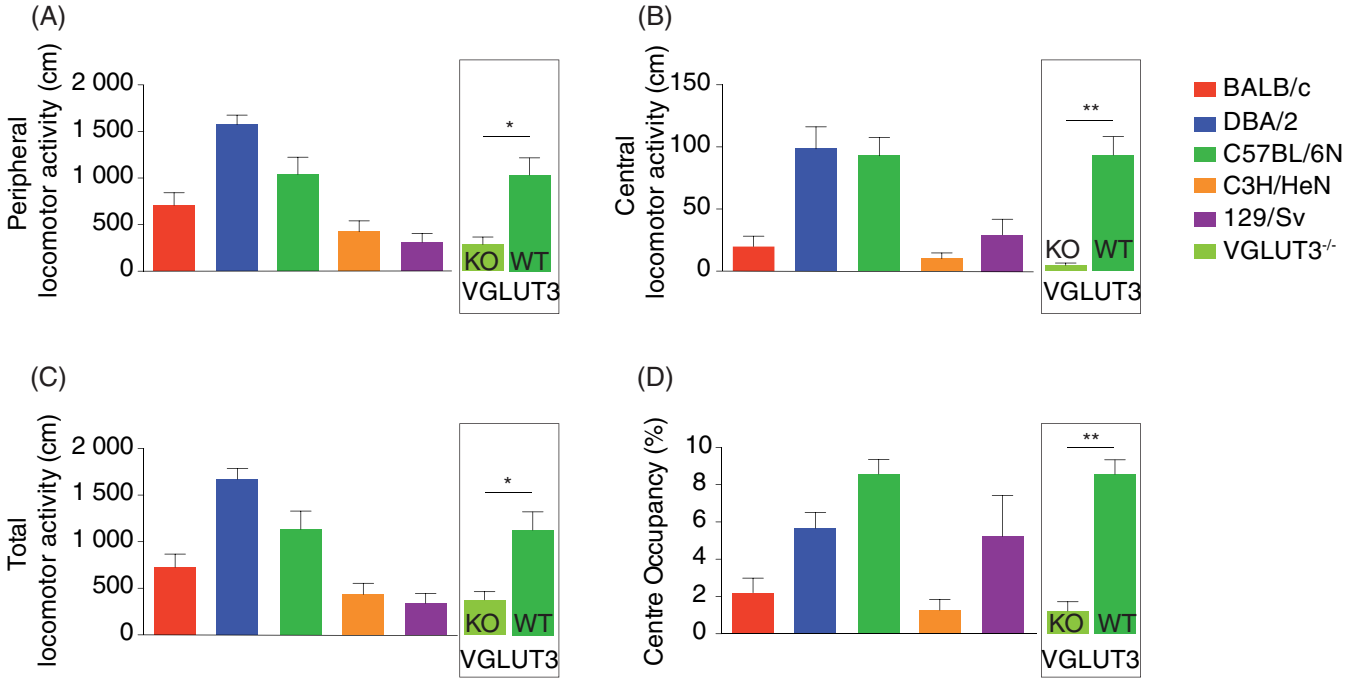

FIGURE 1 Strain effects on LMA and anxiety-related behaviors assessed in the OF test. LMA is represented by (A) the distance run in the peripheral zone, (B) central zone, and (C) the whole OF. (D) The percentage of central occupation illustrated the anxiety level of the animals. For each parameter, VGLUT3$^{-/-}$ data are compared with control littermate' in the boxed bar graphs. *P < 0.05, **P < 0.01. KO, GLUT3 knock-out; WT, VGLUT3 wild type

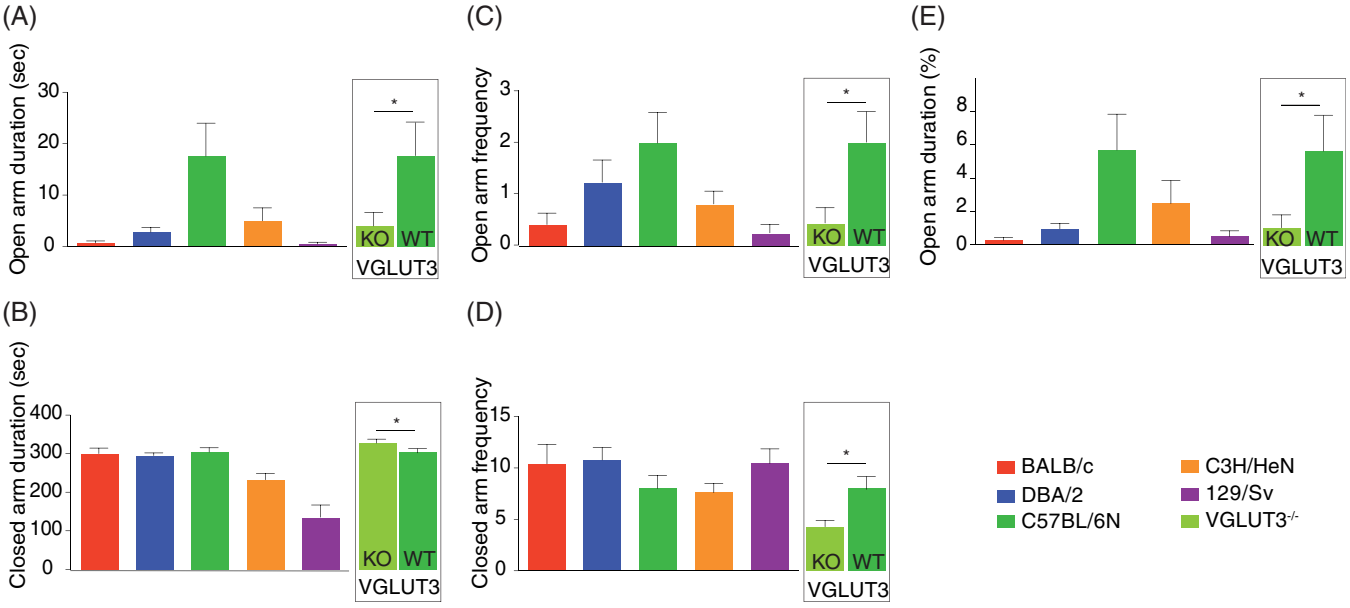

FIGURE 2 Anxiety levels assessed in the five mouse lines and VGLUT3 knock-out mice with the EPM test. The time spent (seconds) in (A) the OAs and (B) CAs is represented in seconds for the different strains and the VGLUT3$^{-/-}$. The number of entries in: (C) OAs and (D) CAs. (E) Percentage of time spent in the OAs. VGLUT3$^{-/-}$ data are compared with control littermate in the boxed bar graphs. *P < 0.05. KO, VGLUT3 knock-out; WT, VGLUT3 wild type

CAs than BALB/c, DBA/2, and C57BL/6N mice (Figure 2B; P < 0.01). As we previously published,[9] VGLUT3$^{-/-}$ mice displayed an anxiogenic-like phenotype. In the EPM, they visited less often (Figure 2C boxed bar graph, MW U test, U = 10, P = 0.0264) and spent less time in the OAs than control littermates (boxed bar graph: Figure 2A, MW U test, U = 8.5, P = 0.0183; Figure 2E, MW U test, U = 8.5, P = 0.02). VGLUT3 deletion impacts time spent and the number of visits in the CAs (respectively, boxed bar graph in: Figure 2B, MW U test, U = 11, P = 0.0485; Figure 2D, MW U test, U = 7, P = 0.0115). VGLUT3$^{-/-}$ mice spent more time in the CAs but show fewer entries, indicating a reduction in LMA in an anxiogenic environment.

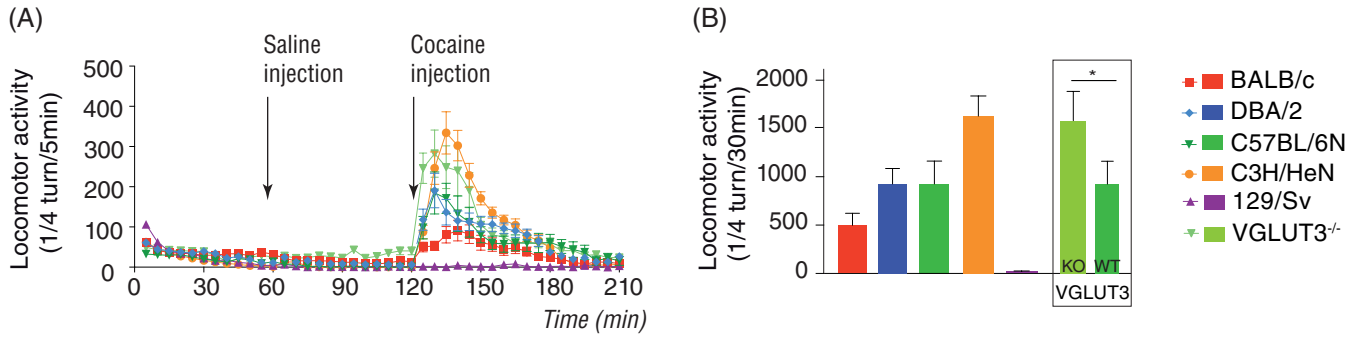

FIGURE 3 Cocaine-induced locomotion in five mouse lines and VGLUT3 knock-out mice. (A) Time course of locomotor activity (LMA) over a 3-hour 30-minute period for the different strains studied. Ip Injection of saline (NaCl 0.9%) and cocaine (10 mg/kg) are represented on the time line. (B) LMA represented by the one-fourth turn per 30 minutes the animals made after cocaine injection, minus 30 minutes after saline injection. VGLUT3$^{-/-}$ data are compared with control littermate in the boxed bar graph. *P < 0.05. KO, VGLUT3 knock-out; WT, VGLUT3 wild type

3.1.2 | Cocaine-induced LMA

None of the strains reacted to saline injection (Figure 3A at 60 minutes). In contrast, all strains except 129/Sv mice showed a hyperlocomotion after cocaine injection (Figure 3A see at 120 minutes; 2-way repeated mesures ANOVA (RM-2) ways ANOVA, all P < 0.0001: $F_{41,1722}$ = 69.97 for time, $F_{4,42}$ = 9.456 for strains, and $F_{42,1722}$ = 11.37 for time × strains interaction). The highest LMA was observed for C3H/HeN mice, whereas the lowest was observed in BALB/c, – not including 129/Sv (Figure 3B; KW, W = 31.72, P < 0.0001). Knock-out mice for VGLUT3 express a twofold increase in cocaine-induced LMA compared with control littermates (C57BL/6N), reaching the level of C3H/HeN animals (Figure 3B boxed bar graph: MW test, U = 13, P = 0.033).

3.2 | VGLUT3 expression

As expected, VGLUT3 was not detectable in the whole brain of the VGLUT3$^{-/-}$ mice[8] (data not presented). In the DS and VS, VGLUT3 expression fluctuates between strains (KW-test, Figure 4A: DS, W = 15.28, P = 0.0042, Figure 4B: VS, W = 12.83, P = 0.0121), with levels around 2 times higher in 129Sv, C3HeN and C57BL/6N than BALB/c and DBA/2 mice. In the hippocampus, VGLUT3 expression gradually decreases from high expression in 129/Sv > C3H/HeN> C57BL/6N to lower expression in DBA/2 > BALB/c (KW test, Figure 4C: DH, W = 13.18, P = 0.0104, and Figure 4D: VH, W = 8.3, P = 0.0505). In raphe nuclei, no differences of VGLUT3 expression were found between strains (KW test, Figure 4E: DRN, W = 7.46, P = 0.0874, and Figure 4F: MRN, W = 7.45, P = 0.0879).

Overall, in comparison to C57BL/6N mice (set up as the reference 100%; see supplementary data, Figure S1), 129Sv and C3HeN strains had higher VGLUT3 expression levels (respectively 126.9 ± 9.4% and 116.4 ± 3.4%, P < 0.021), while BALB/c and DBA/2 mice displayed lower VGLUT3 levels (respectively 63.9 ± 3.7% and 80.8 ± 5.7%, P < 0.035).

3.3 | *SLC17A8* regulation regions

We identified 51 SNPs in the promoter region, and 40 SNPs and 4 indels in the 3′-UTR (Supporting Information Table S1). Most of these SNPs were in linkage disequilibrium (r^2 = 1) and we thus reduced to seven haplotype-tagging SNPs (ht-SNPs) to specifically define each mouse strain (Figure 5A and Supporting Information Table S1). Only one SNP (rs29353268) in the last exon might explains the overall lower VGLUT3 expression observed both in DBA2 and BALB/c (see Figure S1).

As the lowest expression observed was in BALB/c mice, we checked which SNPs were shared by C57BL/6N, C3HeN and 129sv, but BALB/c. Hap4 matched these criteria (Supporting Information Table S1). This haplotype spanned two SNPs in the promoter region (rs46766687 and rs29349498) and two SNPs in the last exon (rs29325887 and rs29367655). In the promoter region, only rs29349498 was conserved through evolution. Interestingly, we found this SNP was predicted to change putative binding sites for the AhR and Pax-8 transcription factors (Figure 5B). However, for both the G allele observed in BALB/c should allow transcription factor binding and thus could not explain the lower expression observed for VGLUT3. Downstream, only rs29367655 was highly conserved through evolution, but no putative miRNA binding site was found to be affected by this SNP (not shown).

3.4 | VGLUT3 expression and behavior characterization: Correlation

In order to assess the possible link between VGLUT3 protein levels and phenotypic traits, we ran correlation analyses for each parameter.

We first studied the anxiety trait. The only parameter showing correlation with VGLUT3 expression was the time spent in CAs in the EPM. We found a negative correlation between these parameters both in DRN and MRN (Figure 6E,F).

We then performed correlative analysis between the cocaine-induced LMA and VGLUT3 expression in the various brain regions studied (Figure 7). The 129/Sv line that did not respond to cocaine injection was excluded from this analysis. We found no correlation in the DS or the VS (Figure 7A,B). In contrast, we observed a weak correlation between VGLUT3 densities and LMA in the VH (Figure 7D) and the DRN (Figure 7E).

4 | DISCUSSION

The application of animal models of anxiety and drug-responses for experimentation in mice is becoming increasingly important for

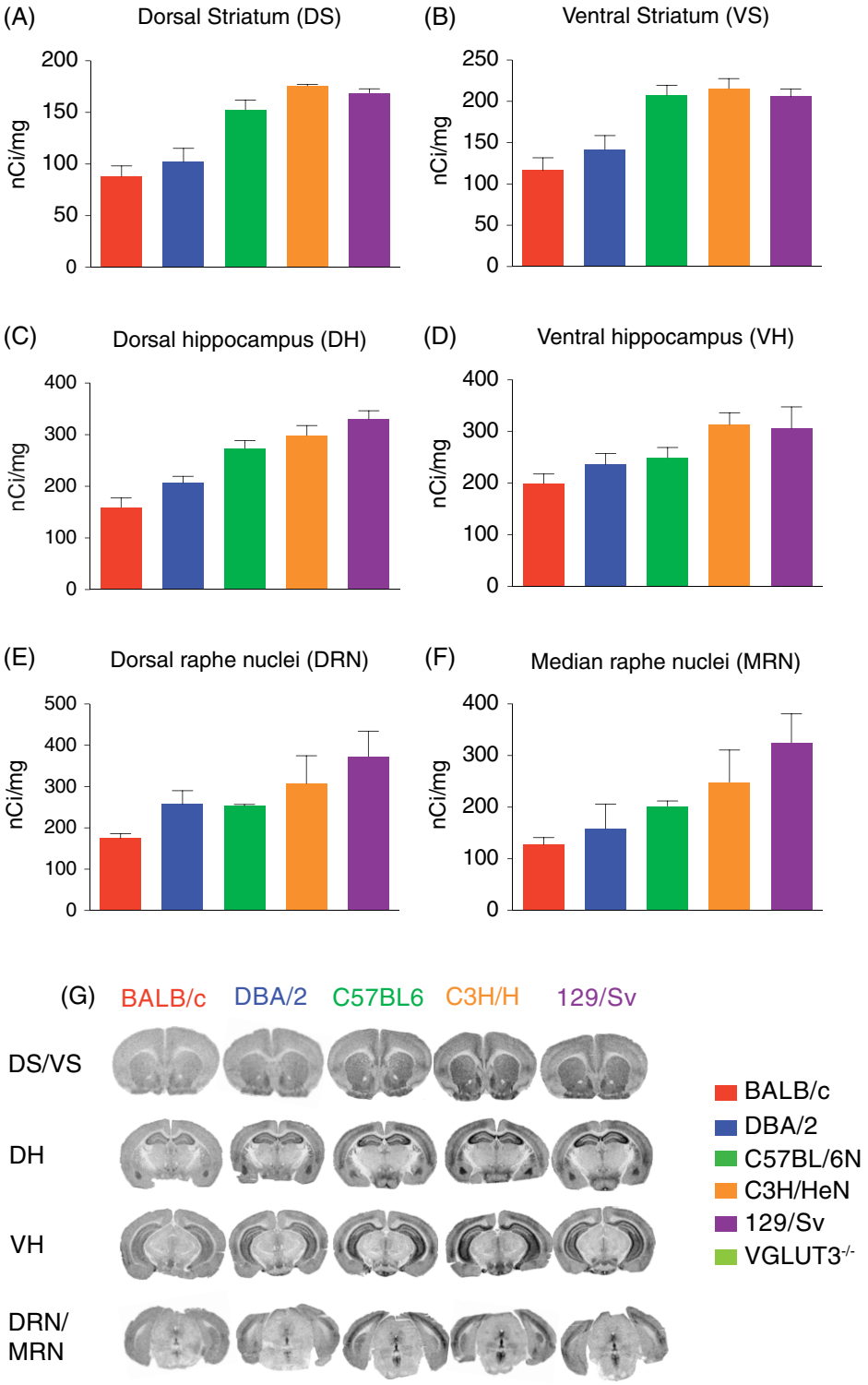

FIGURE 4 Quantification of VGLUT3 content in different brain structures of five mouse lines: (A) DS, (B) VS, (C) DH, (D) VH, (E) DRN, (F) MRN. (G) Representative immunoautoradiographies

studying the contribution of genetic differences, as well as the roles of selected genes, in specific behaviors. By running these experiments, we wanted to better understand[1] the contribution of genetic differences, as well as[2] the contribution of the VGLUT3 gene in specific behaviors of anxiety and addiction.

In previous studies, VGLUT3$^{-/-}$ mice were used to assess VGLUT3 involvement in the regulation of anxiety and addiction. The absence of VGLUT3 leads to severe changes in anxiety- and addictive-like phenotypes, including cocaine-induced locomotor hyperactivity.[9,11] To further investigate the association between VGLUT3 and anxiety- or addictive-like behaviors, we herein tested whether variation of VGLUT3 endogenous expression in different mouse strains might be correlated with differences in their anxiety- or addictive-like behaviors.

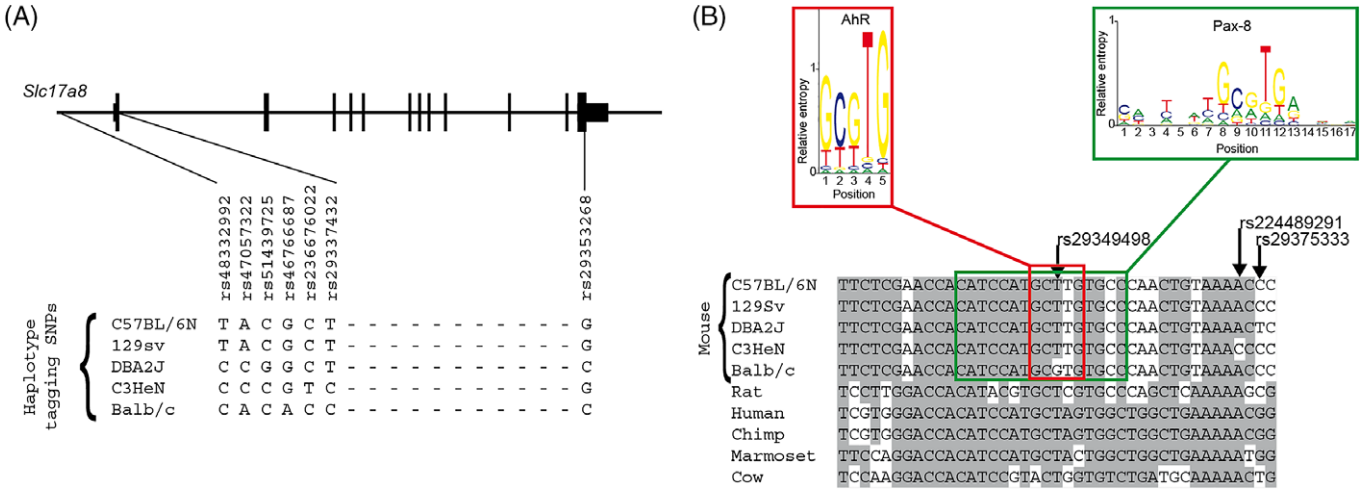

FIGURE 5 *Slc17a8* sequence: (A) gene structure of *Slc17a8*. The coding exons are shown with thick blocks, whereas the thinner represents UTRs. Six haplotype-tagging SNPs have been identified in the promoter region and one in the 3′-UTR defining specific haplotypes for each mouse strain. (B) Genomic alignment of rs29349498. This SNP is located 341-bp upstream to the transcription start site and affects a highly conserved nucleotide. This variation is observed only in BALB/c mice as compared with other mouse strains and alters putative binding site of AhR and Pax-8

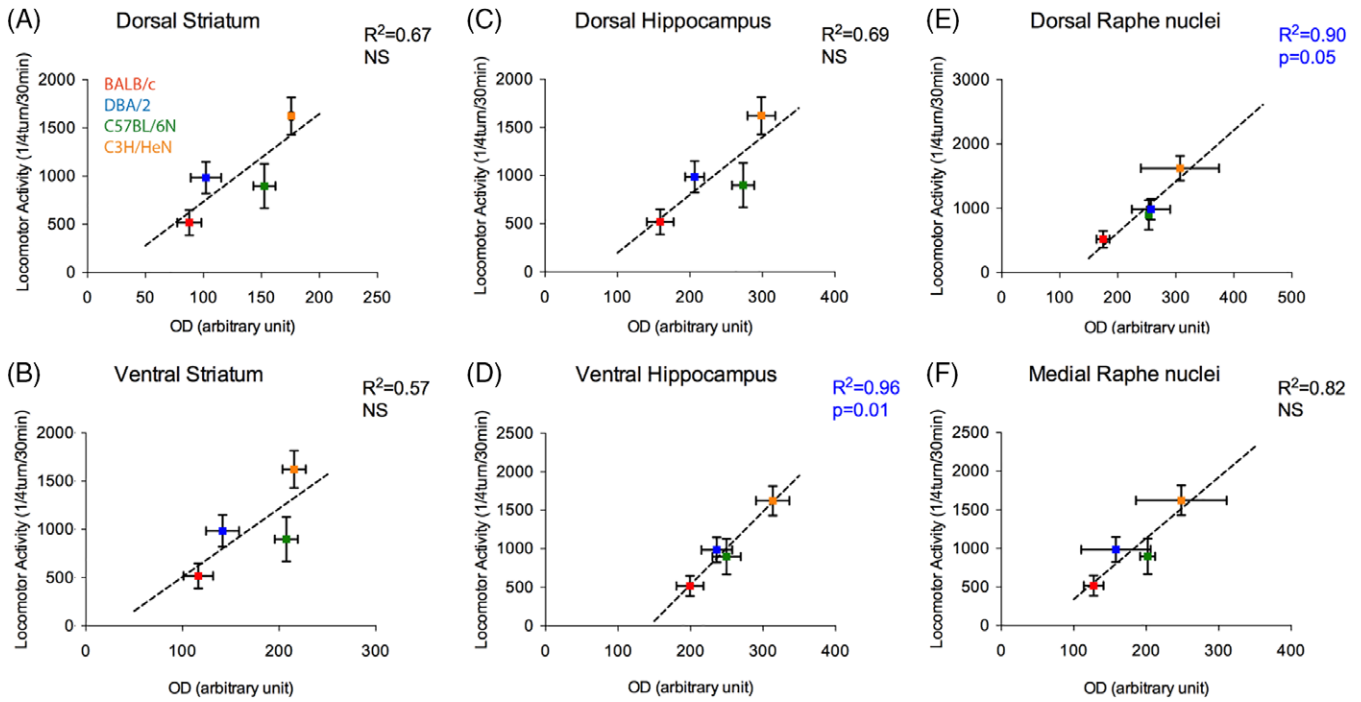

FIGURE 6 Correlation between VGLUT3 expression level in various brain regions and LMA after cocaine injection (excluding 129/Sv strain). Dots represent mean ± SEM. Pearson correlation coefficients R^2 are indicated, with *P* value when significant. (A) DS. (B) VS. (C) DH. (D) VH. (E) DRN. (F) MRN. NS, non significant; OD, optical density

Surprisingly, in our hands, the 129/Sv mouse strain did not respond to cocaine at 10 mg/kg. Published work showed controversial results concerning 129/Sv strains.[23,30–34] It seems that it is not only dependent on the study (ie, behavioral design, provider and drug concentration), but also on the substrain used (129S1/Sv, 129S6/Sv, and 129X1/Sv). For instance, Miner (1997) was the first to describe locomotor activation by cocaine in the 129S1/Sv line.[31] However, the observed increased locomotion due to cocaine injection was not that clear when compared with locomotor activation after saline injection. Crabbe et al[31] also found highly variable behaviors in 129/Sv substrains between labs following cocaine injections.[30] In our study, we used the 129S2/Sv line with a dose of 10 mg/kg that did not elicit locomotor hyperactivity. This lack of increased-LMA also found in various mutant mice targeting the dopamine receptor or transporter (D1 or dopamine transporter (DAT)) is often consistent with the absence of reinforcing effect of cocaine.[35–38] Moreover, the 129/Sv line, as the C3H, is known to be especially hypoactive. However, in our hands, C3H mice showed the highest reaction to cocaine injection, reaching the level of VGLUT3$^{-/-}$ mice. Our results, in agreement with a previous study,[34] identify C57BL6 and C3H mice

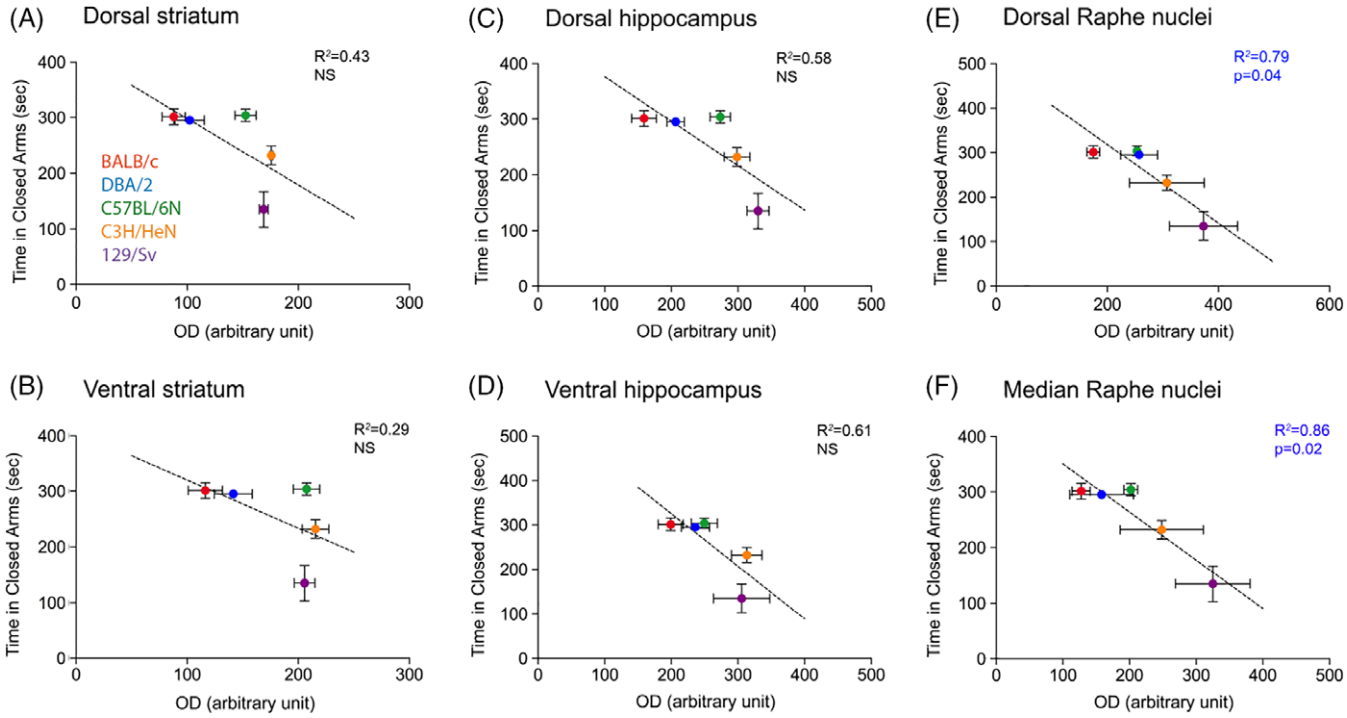

FIGURE 7 Correlation between VGLUT3 expression level in various brain regions and the time spent in CAs in the EPM test. Dots represent mean ± SEM. Pearson correlation coefficients R^2 are indicated, with *P* value when significant. (A) DS. (B) VS. (C) DH. (D) VH. (E) DRN. (F) MRN. NS, non significant; OD, optical density

as the most appropriate lines to study behavioral responses to cocaine.

Consistent with Keum et al[39], we found that C3H/He mice are low performers and very anxious, especially in the OF test (Figure 1C, D).[40] They spent less than 2% in the central zone of the OF whereas B6 mice were the less anxious spending more than 8% of their time exploring the center. In our hands, DBA/2 and 129/Sv mice display a moderate anxiety-like phenotype, with BALB/c being more anxious, as found by Lad et al.[41] These discrepancies with published studies can be explained by substrain differences because we used 129S2/Sv instead of 129S1 and S4 as in.[41,42] It could also be due to the fact that mice spent considerable time in the central compartment, a behavior that can be associated with high anxiety levels, because mice can express freezing behavior in the central zone.

In anxiety tests, the illumination intensity is known to be a prime parameter. However, we established the same ranking in anxiety response as previous work despite very different conditions of illumination.[39] They used a very high intensity (300–330 lx), compare with our lower measures (50 lx) supposedly less aversive for the mice. The ranking of the various mouse strains we obtained in EPM and OF is consistent. Indeed, in both cases, we observed the strongest and the weakest state of anxiety for the BALB/c and B6 strains, respectively, while the DBA/2 and the 129/Sv strains express a moderate anxiety. The C3H line is the only line for which we noticed discrepancies in the level of expressed anxiety in the EPM and OF test. We observed high anxiety level of C3H mice in the OF (spending less than 2% in the central zone), However, in the EPM, they explored more often the OAs, which can be interpreted as a sign of low anxiety.

As we previously highlighted, the C3H strain is hypoactive. This is also the only known strain used with visual impairments. Both these traits could undoubtedly have a noticeable impact on the EPM exploration.[41] A possible explanation for the difference in anxiety levels observed for this strain between the OF and the EPM is that LMA is recruited differently, with the exploratory activity component priming in the EPM.

In mice, there is an inverse correlation between anxiety levels and LMAs, that is clearly observed in VGLUT3$^{-/-}$ mice. In fact, VGLUT3$^{-/-}$ mice that are hyperactive when placed in normal housing conditions, turn out to be hypoactive when exposed to stressful environments.[9,43,44]

Within each strain, VGLUT3 expression varies substantially according to the brain region involved in anxiety- and drug-related behavior. Between strains, the overall VGLUT3 expression is also different, with C3H/HeH = 129/Sv, C57Bl/6N and BALB/c = DBA/2, from the highest VGLUT3 expression to the lowest, respectively. More precisely, the striatum (dorsal and ventral) is the brain region showing the highest variability in VGLUT3 expression between the mouse lines. In this area, C57BL/6N mice express the same level of VGLUT3 than C3H/HeH and 129/Sv strains.

Interestingly, the analysis of the *Slc17a8 locus* identified SNPs associated with the low expression group including BALB/c and DBA/2 strains. However, the relationships between these sequence variations and their impact on the level of VGLUT3 expression has to be elucidated.

We observed high heterogeneity of VGLUT3 expression depending on mice strain that might be partially explained by a SNP in the BALB/c and DBA/2 strains. This nucleotide change does not alter the amino acid sequence of the protein and is highly conserved through

mammalian evolution (not shown), but no known putative miRNA binding site has been described in this region.

Nevertheless, no clear correlation could be established between VGLUT3 expression and behavioral traits, highlighting the fact that even if the ablation of this gene in VGLUT3$^{-/-}$ mice lead to a clear cut phenotypic pattern (ie, cocaine-induced hyperlocomotion, anxiety-like phenotype), one single gene polymorphism could not be taken responsible for the intrinsic variability observed in various mice line. However, the correlation analysis showed that VGLUT3 could play an important role in two regions regarding LMA and anxiety traits: raphe nuclei and hippocampus. Because VGLUT3 is present in serotoninergic neurons of raphe nuclei and in GABAergic interneurons of the hippocampus[7,9] the use of mice with a floxed VGLUT3 will enable the spatio-temporal control of VGLUT3 deletion. This method will open the way to a more precise dissection of the contribution of VGLUT3 within specific neuronal populations and behaviors. Finally, this work confirms that the C57BL/6N background was the more appropriate to study the behavioral effect of VGLUT3 deletion and illustrates how critical is the choice of the genetic background when engineering new mice models.

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AUTHORS CONTRIBUTION

S.J., S.E.M. and S.D. conceived and designed the study. L.R., D.Y.S., A.H., O.P. and S.D. acquired, analyzed and interpreted data. D.Y.S., S.J. and S.D. wrote the paper. S.E.M. revised critically the article.

CONFLICTS OF INTEREST

The authors have no conflicts of interest to report.

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