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VGLUT3 gates psychomotor effects induced by amphetamine

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Abbreviations:

5-hydroxytryptamine (5HT), acetylcholine (ACh), serotonin (5HT), choline acetyltransferase (ChAT), dopamine (DA), dorsomedial striatum (DMS), medium-size spiny neurons (MSNs), Research Resource Identifier (RRID), serotonin transporter (SERT), Tonicly Active Neurons (TANs), vesicular acetylcholine transporter (VACHT), vesicular glutamate transporter type 3 (VGLUT3), ventromedial striatum (VMS).

Abstract

Several subtypes of modulatory neurons co-express vesicular glutamate transporters (VGLUTs) in addition to their cognate vesicular transporters. These neurons are believed to establish new forms of neuronal communication. The atypical vesicular glutamate transporter 3 (VGLUT3) is of particular interest since in the striatum this subtype is found in tonically active cholinergic interneurons (TANs) and in a subset of 5-HT fibers. The striatum plays a major role in psychomotor effects induced by amphetamine. Whether and how VGLUT3-operated glutamate/ACh or glutamate/5HT co-transmissions modulates psychostimulants-induced maladaptive behaviors is still unknown.

Here, we investigate the involvement of VGLUT3 and glutamate co-transmission in amphetamine-induced psychomotor effects and stereotypies. Taking advantage of constitutive and cell-type specific VGLUT3-deficient mouse lines, we tackled the hypothesis that VGLUT3 could gate psychomotor effects (locomotor activity and stereotypies) induced by acute or chronic administration of amphetamine.

Interestingly, VGLUT3-null mice demonstrated blunted amphetamine-induced stereotypies as well as reduced striatal Δ FosB expression. VGLUT3-positive varicosities within the striatum arise in part from 5HT neurons. We tested the involvement of VGLUT3 deletion in serotonergic neurons in amphetamine-induced stereotypies. Mice lacking VGLUT3 specifically in 5HT fibers showed no alteration to amphetamine sensitivity. In contrast, specific deletion of VGLUT3 in cholinergic neurons partially phenocopied the effects observed in the constitutive knock-out mice.

Our results show that constitutive deletion of VGLUT3 modulates acute and chronic locomotor effects induced by amphetamine. They point to the fact that the expression of VGLUT3 in multiple brain areas is pivotal in gating amphetamine-induced psychomotor adaptations.

Introduction

Unlike VGLUT1 and VGLUT2, the atypical glutamate transporter 3 (VGLUT3) is detected in neurons that use other neurotransmitters than glutamate. For example, VGLUT3 is present in subsets of neurons using acetylcholine (ACh), serotonin (5HT) or GABA (Herzog *et al.* 2004; Nickerson Poulin *et al.* 2006). Several recent studies highlight the pivotal involvement of VGLUT3 in striatal functions (Gangarossa *et al.* 2016; Higley *et al.* 2011; Sakae *et al.* 2015).

The striatum is centrally involved in motor functions, reward processing, procedural learning and habits formation (Kreitzer 2009). Importantly, the striatum is also critical in motor dysfunctions such as L-DOPA-induced dyskinesia and psychostimulants-induced motor stereotypies (Cenci and Konradi 2010; Saka *et al.* 2004).

The striatum is mainly composed of GABAergic medium-size spiny neurons (MSNs) that represent striatal projecting neurons. Striatal and accumbal MSNs can be further subdivided in two non-randomly distributed classes according to the expression of either the dopamine (DA) D1 or D2 receptors (D1R-MSNs and D2R-MSNs) (Gangarossa *et al.* 2016; Gangarossa *et al.* 2013a; Gerfen and Surmeier 2011; Keeler *et al.* 2014). However, the striatum also contains a fraction of interneurons which use either acetylcholine (ACh) or GABA to locally signal and control MSNs activities (Keeler *et al.* 2014). Cholinergic interneurons, also known as TANs (1-2% of striatal neurons), together with cholinergic inputs arising from the pedunculopontine and laterodorsal tegmental nuclei, represent the sole source of ACh within the striatum (Dautan *et al.* 2016a; Dautan *et al.* 2016b; Dautan *et al.* 2014). Interestingly, TANs express both the vesicular acetylcholine transporter (VACHT) and the atypical vesicular glutamate transporter (VGLUT3); consequently, TANs have the unique feature to signal with both acetylcholine (ACh) and glutamate (Gras *et al.* 2008; Gras *et al.* 2002). In addition to TANs, the striatum also contains VGLUT3-positive serotonergic terminals (Sakae *et al.* 2015). VGLUT3 contained by these fibers could potentially contribute to striatal functions. In fact, it is well established that 5-HT transmission regulates dopamine-related motor dysfunctions, amphetamine-induced as well as stress-induced stereotypies (Lees *et al.* 1979; Miguez *et al.* 2014; Schoenecker and Heller 2001).

Recent reports show that genetic inactivation of VGLUT3 strongly alters striatal physiology (Gras *et al.* 2008; Higley *et al.* 2011; Nelson *et al.* 2014a; Nelson *et al.* 2014b; Sakae *et al.* 2015). For example, VGLUT3 is critical for several striatum-dependent pathophysiological conditions such as Parkinson's disease, L-DOPA-induced dyskinesia and cocaine addiction

(Divito *et al.* 2015; Gangarossa *et al.* 2016; Sakae *et al.* 2015). In fact, locomotor and rewarding properties of cocaine are increased whereas abnormal and involuntary movements are decreased in mice lacking VGLUT3 (VGLUT3^{-/-} mice) (Gangarossa *et al.* 2016; Gras *et al.* 2008; Sakae *et al.* 2015).

Amphetamine is a well-known psychostimulant and a potent releaser of DA (Lüscher and Ungless 2006). Here, we investigate whether VGLUT3 is implicated in the psychomotor effects (locomotor responses and stereotypies) induced by acute and chronic administrations of amphetamine.

Stereotyped behavior, a non-goal directed repetitive activity, is a core feature of several neurologic and neuropsychiatric disorders such as Tourette's syndrome or obsessive-compulsive disorders and autism spectrum disorders (Burguière *et al.* 2015; Langen *et al.* 2011a; Langen *et al.* 2011b). In addition, stereotypies are a major component of behavioral syndromes induced by psychostimulants such as cocaine or amphetamine (Yates *et al.* 2007). Several lines of evidence suggest the involvement of cholinergic transmission and TANs in the development of motor stereotypies (Aliane *et al.* 2011; Crittenden *et al.* 2014; Cubo *et al.* 2008). However, these reports do not consider the fact that cholinergic and 5HT terminals express VGLUT3 and co-release glutamate in addition to ACh or 5HT, respectively. Whether VGLUT3 is specifically involved in the expression of stereotypies is still unknown. Taking advantage of different transgenic mouse lines, we tackled the hypothesis that VGLUT3 could gate psychomotor stereotypies induced by single or repeated administrations of amphetamine.

Materials and methods

Animals

Animal care and experiments were conducted in accordance with the European Communities Council Directive for the Care and the Use of Laboratory Animals (86/809/EEC), in compliance with the *Ministère de l'Agriculture et de la Forêt, Service Vétérinaire de la Santé et de la Protection Animale* (authorization number 01482.01). Male mice were housed in groups of 2- 5 per cage, in a temperature-controlled room (21 ± 2°C) with a 12:12- h dark/light cycle (lights on 7:30 a.m. to 7:30 p.m.). Water and food were provided *ad libitum*.

VGLUT3^{-/-} mice were produced as previously described (Gras *et al.* 2008). They have been backcrossed on a C57BL/6J background for more than 12 generations.

To delete VGLUT3 in specific neuronal subpopulations, mice carrying a floxed allele of the exon 2 of *slc17a8* (VGLUT3^{LoxP/LoxP}, RRID:IMSR_EM:05733), the gene coding for VGLUT3, was used (Fasano *et al.* 2017). VGLUT3^{LoxP/LoxP} mouse line was established at Phenomin - iCS (Phenomin - Institut Clinique de la Souris, Illkirch, France, RRID:SCR_011021).

VGLUT3 was ablated from cholinergic interneurons by crossing VGLUT3^{LoxP/LoxP} mice with the ChAT-IRES-Cre line (B6.129S6-Chattm2(cre)Lowl/J, stock number 006410, Jackson Laboratory, RRID:IMSR_JAX:006410). Mutant mice (ChAT-IRES-Cre::VGLUT3^{LoxP/LoxP}, or VGLUT3cKO-ACh) were compared to control littermates (ChAT^{+/+}::VGLUT3^{LoxP/LoxP}, or WT). To remove VGLUT3 from serotonergic neurons, VGLUT3^{LoxP/LoxP} mice were crossed with the SERT-Cre line (B6.129(Cg)-Slc6a4tm1(cre)Xz/J, stock number 014554, Jackson Laboratory, RRID:IMSR_JAX:014554). Mutant mice (SERT-Cre::VGLUT3^{LoxP/LoxP}, or VGLUT3cKO-5HT) were compared to control littermates (SERT^{+/+}::VGLUT3^{LoxP/LoxP}, or WT).

For the whole study (behavior, immunofluorescence and supplementary material) we used the following number of mice: n=24 (WT_{VGLUT3}), n=24 (VGLUT3^{-/-}), n=13 (WT_{5HT}), n=14 (VGLUT3cKO-5HT), n=15 (WT_{ACh}) and n=17 (VGLUT3cKO-ACh), as reported in Fig. 1.

Behavior

Male mice (aged 2-4 months) were habituated to handling prior their use for behavioral experiments. No differences in body weight were observed in all groups (Suppl. 1A). Behavioral experiments were performed during the light phase (10 am – 3 pm).

Amphetamine-induced locomotor activity and sensitization

Animals were individually placed in a Plexiglas activity box (l 24 x w 15 x h 25 cm) covered with cob bedding (Imetronic, Pessac, France). In the activity box, horizontal and vertical locomotor activity were detected by two sets of horizontal and vertical infrared photocell beams located 15 mm above the floor along the long axis. For behavioral assessments, animals were arbitrarily assigned to individual cages (no specific

randomization method). A blind investigator arbitrarily assigned the mice into two genotype-independent groups A and B. However, the same location for each mouse was attributed during the chronic protocol. All the mice received identical pharmacological treatments (saline during habituation and amphetamine during chronic treatment), thus arbitrary assignment was only used for allocation to behavioral cages.

Animals received a daily injection of saline for three consecutive days (habituation phase, Fig. 1). All mutant mouse lines (VGLUT3-KO, VGLUT3cKO-ACh and VGLUT3cKO-5HT) showed intact habituation phases to the environment (Suppl. Fig. 1B-D), suggesting normal exploratory drive. Then, mice were injected with either D- amphetamine hemisulfate (Sigma A5880, Steinheim, Germany) or vehicle (saline solution, 0.9% NaCl) for five consecutive days (Fig. 1). Amphetamine solutions were prepared at 0.5 mg/mL for a final concentration of 5 mg/kg per mouse. Drugs were administered intraperitoneally in a volume of 0.1 mL/10 g body weight, and control injections consisted in an equivalent volume of vehicle (0.9% NaCl). Each day, animals were first placed in the activity box for 120 minutes, then they were injected and placed back for an additional 90 minutes recording.

Mice were excluded only when technical issues regarding infrared photobeams occurred: 1 WT mouse (rearing behavior in Fig. 2C-D, Suppl. Table 1), 2 WT mice and 1 VGLUT3cKO-Ach mouse (rearing behavior in Fig. 7F-G, Suppl. Table 6).

Locomotor stereotypy scoring

A treatment-blind investigator manually scored amphetamine-induced stereotypies. Five minutes after drug injections, animals were sequentially observed during 45 seconds every 10 minutes. Stereotypies were evaluated using an adapted rating scale from Creese & Iversen as following: 0, asleep or stationary; 1, active; 2, constant exploratory activity with discontinuous sniffing; 3, continuous sniffing with periodic exploratory activity; 4, continuous sniffing, discontinuous biting, gnawing or licking with very brief periods of locomotor activity; 5, continuous biting, gnawing or licking with a residual locomotor activity; 6, continuous biting, gnawing or licking without locomotor activity (Creese and Iversen 1973).

Oral- based stereotypies elicited by amphetamine were evaluated using the following scale: 0, absence of orofacial stereotypy; 1, continuous sniffing with bursts of orofacial licking or biting; 2, continuous licking or biting with locomotion; 3, continuous biting in a restricted area ± bursts of licking; 4, continuous biting without locomotion (Creese and Iversen 1973).

Immunofluorescence experiments were performed to confirm the deletion of VGLUT3 in specific subsets of cells or terminals as previously described (Fasano *et al.* 2017; Janickova *et al.* 2017; Sakae *et al.* 2015). Naïve conditional knockouts mice (n=3-4 per genotype) were anesthetized with sodium pentobarbital (0.1 mL/10 g body weight) and perfused intracardially with paraformaldehyde (PFA 4%, in phosphate-buffered saline (PBS)). Briefly, free-floating coronal sections (30 µm) were rinsed in PBS and then incubated in a blocking solution of normal horse serum 4% (RRID:AB_2532168) in PBS/Triton 0.25%. Sections were then incubated with VGLUT3 (1:2000; Synaptic Systems, Cat# 135 203, RRID:AB_887886), VACHT (1:5000; (Gras *et al.* 2008)) and 5-HT (1:50; Millipore, Cat# MAB352, RRID:AB_11213564) antisera (Sakae *et al.* 2015) in an incubation buffer containing normal horse serum 1% (in PBS/Triton 0.25%). Sections were rinsed in PBS before mounting.

Images were acquired using a Leica SP5 confocal system (Leica Microsystems, Deerfield, IL; USA, RRID:SCR_008960). The same laser intensities were used to image both WT and mutant cKO mice. For quantifications of double immunolabeling, images were taken with a 63x/1.4 numerical aperture (N.A.) Plan-Apochromat, an argon laser at an excitation wavelength of 488 nm, and a diode at 561 nm. Images were then treated using Fiji (RRID:SCR_002285) and Adobe Photoshop software (RRID:SCR_014199). The quantification of fluorescence intensities was automatically performed using Fiji software. Mean optical density (OD) was measured from the whole cytoplasm (excluding the nucleus) for VGLUT3-immunolabeling in VACHT-positive TANs. Mean OD was measured from axonal varicosities for 5HT- and VGLUT3-immunolabeling. For each marker, a background value of OD was measured around immunopositive processes and subtracted to specific OD. Quantifications were performed on rostral striatal sections (bregma= +0.98/+0.74, (Franklin and Paxinos 2008)).

ΔFosB was also detected by immunofluorescence. Twenty-four hours following the last injection (amphetamine or saline), mice were deeply anesthetized and perfused intracardially with PFA 1,5% and brains were post-fixed for 24h in PFA 4% prepared in PBS as previously described (Vialou *et al.* 2010). Free-floating coronal sections (30 µm) were rinsed in PBS and then incubated in a blocking solution of normal horse serum 3% (RRID:AB_2532168) in PBS/Triton 0.3%. Immunohistochemistry against FosB was performed with a FosB rabbit antiserum (1:1.000, Santa Cruz, catalog ref. sc-48,

RRID:AB_631515) (Vialou *et al.* 2010) in an incubation buffer containing normal horse serum 3% (in PBS/Triton 0.3%). Sections were rinsed in PBS before mounting.

Images were acquired using a NanoZoomer 2.0 HT scanner (Hamamatsu Photonics) and extracted using NDP.view2 software (Hamamatsu). Quantification of immunoreactive cells was blindly performed using an image analyzer software (Image-J, RRID:SCR_003070) taking as standard reference a fixed threshold of fluorescence. FosB cell counting was performed on rostral striatal sections (bregma= +0.98/+0.74, (Franklin and Paxinos 2008)). For each mouse (N=4-6 mice/group), values from both hemispheres for each striatal territory (DMS and DVS) were averaged.

Statistics

All statistical comparisons were performed with two-sided tests in Prism 6 (GraphPad Software, La Jolla, CA, USA, RRID:SCR_002798). The distribution of data was determined with D'Agostino & Pearson omnibus normality test and Welch's correction. Student's t-test or unpaired Mann–Whitney test (nonparametric) were used when necessary. No sample size calculations were performed. To determine outliers in every experimental group we performed the Grubbs' test in Prism (RRID:SCR_002798). No outliers were identified.

Behavioral sensitization was analyzed with two-way analysis of variance (ANOVA) with repeated-measures (time, genotype). One-way ANOVA was applied when necessary. Sidak's post hoc test for multiple comparisons was performed when required unless otherwise indicated. Results are expressed as mean \pm S.E.M., and differences were considered significant for: * $p < 0.05$; ** $p < 0.01$ and *** $p < 0.001$.

Results

Genetic deletion of VGLUT3 alters amphetamine-induced locomotion and stereotypies

VGLUT3^{-/-} mice and wild-type (WT) littermates were injected with relatively high doses of amphetamine (5 mg/kg) to test the hypothesis that VGLUT3 may be involved in locomotor and stereotypic responses induced by both acute and chronic administrations of amphetamine. When acutely injected with amphetamine (5 mg/kg), VGLUT3^{-/-} mice showed

an enhanced hyperlocomotion compared to their WT littermates (Fig. 2A, A¹). These findings suggest that the absence of VGLUT3 alters the acute locomotor effects induced by amphetamine.

Chronic administration of low doses of amphetamine induces locomotor sensitization (2 mg/kg, Valjent et al., 2010). In contrast, a reduced locomotor response and a progressive stereotypic sensitization are observed when using high doses (7 mg/kg, Crittenden et al., 2014). When mice were chronically injected with high doses of amphetamine (5 mg/kg) a stronger locomotor response was observed in VGLUT3^{-/-} mice compared to their WT littermates (Fig. 2B, B¹). However, at this dose (5 mg/kg), amphetamine was not able to induce locomotor sensitization in both genotypes (Fig. 2B, B¹).

In order to fully explore the psychomotor responses induced by amphetamine, we also analyzed the vertical activity (rearing behavior) of VGLUT3^{-/-} mice and WT mice during acute and repeated administrations of amphetamine. Acute administration of amphetamine induced an enhanced vertical response in VGLUT3^{-/-} mice compared to WT littermates (Fig. 2C, C¹). Such response progressively declined following repeated administrations of amphetamine (Fig. 2D, D¹). Altogether these results indicate that genetic ablation of VGLUT3 promotes higher sensibility to acute and chronic amphetamine-induced locomotor responses.

Chronic administration of high doses of psychostimulants promotes stereotyped behaviors. This in turn may explain the lack of locomotor sensitization and the progressive decline in rearing behavior (Crittenden *et al.* 2014). We then investigated whether the deletion of VGLUT3 was able to alter the expression and the development of abnormal and repetitive locomotor responses (stereotypies). Indeed, repeated injections of amphetamine (5 mg/kg) induced motor stereotypies in WT mice. The severity of stereotypies increased during the 5 days of treatment (Fig 3A, B). In contrast, the severity of amphetamine-induced stereotypies was reduced in VGLUT3^{-/-} mice compared to their WT littermates (Fig. 3B, C). Detailed analysis revealed that orofacial stereotypies were particularly reduced in VGLUT3^{-/-} mice (Fig. 3D-F). These results indicate that amphetamine-induced stereotyped behaviors are blunted in VGLUT3^{-/-} mice.

Reduced expression of Δ FosB in the striatum of VGLUT3^{-/-} mice

Fos family proteins are critical transcription factors that regulate gene expression. Δ FosB is a highly stable FosB variant that accumulates over time with repeated stimulations and that mediates neuronal plasticity (Lobo et al., 2013). Repeated administrations of psychostimulants induce accumulation of Δ FosB, a truncated product of *fosB* gene (Nestler 2001). We therefore investigated whether the blunted amphetamine-induced stereotypies observed in VGLUT3^{-/-} mice could be mirrored by a modified expression of striatal Δ FosB. As expected, repeated administrations of amphetamine (5 mg/kg) triggered a strong increase of Δ FosB-positive cells (putatively MSNs) in both the dorsomedial (DMS) and the ventromedial (VMS) striatum of WT mice compared to mice repeatedly treated with saline (Fig. 4). Interestingly, a significant reduction of Δ FosB-positive cells was detected in both the DMS and the VMS of VGLUT3^{-/-} mice compared to WT littermates (Fig. 4B-D). Basal expression of Δ FosB in saline-treated mice did not differ between genotypes (Fig. 4C, D). Our results suggest that the absence of VGLUT3 reduces Δ FosB accumulation in the striatum of amphetamine-treated mice. Importantly, these molecular observations mirrored the behavioral phenotype mentioned above (reduced stereotypies in VGLUT3^{-/-} mice), thus providing further evidence for VGLUT3-mediated striatal adaptations.

Deletion of VGLUT3 in 5-HT neurons does not affect amphetamine-induced locomotor and stereotypic responses

The Δ FosB accumulation suggests that the striatum could be the key area where VGLUT3 modulates amphetamine-induced locomotor activation and stereotypies. In the rodent striatum, VGLUT3 is mainly observed in cholinergic and serotonergic varicosities ((Sakae *et al.* 2015) and Fig. 5A-A'' and 5A-A'''). In order to decipher the contribution of VGLUT3-positive fibers in amphetamine-induced locomotor and stereotypic responses, VGLUT3 was specifically suppressed in either 5-HT or ACh neurons.

VGLUT3 was first specifically deleted from 5-HT neurons by crossing VGLUT3^{LoxP/LoxP} mice with SERT-Cre mice (VGLUT3cKO-5HT mice). As shown in Fig. 5A-C, VGLUT3 was dramatically reduced in striatal 5HT-positive fibers. In addition, a reduction in 5HT-immunostaining was observed in VGLUT3cKO-5HT mice (Fig. 5C). This reduction is well in line with the previously described loss of vesicular synergy in VGLUT3-KO mice (Amilhon *et al.* 2010; Voisin *et al.* 2016).

We then inspected the acute and chronic locomotor effects of amphetamine (5 mg/kg) in VGLUT3cKO-5HT mice. Acute injection of a single dose of amphetamine similarly increased the locomotor activity in both VGLUT3cKO-5HT mice and WT littermates (Fig. 5D, D¹). Furthermore, chronic administration of amphetamine induced a similar horizontal locomotor

activity in both VGLUT3cKO-5HT mice and WT mice (Fig. 5E, E¹). As reported for the horizontal locomotor activity, no differences were observed in amphetamine-triggered rearing behavior (Fig. 5F-G).

Behavioral analyses of global (Fig. 6A-C) as well as orofacial stereotypies (Fig. 6D-F) induced by chronic administration of amphetamine revealed no significant differences between genotypes. Therefore, VGLUT3/5HT-positive fibers do not seem to contribute to either the increased locomotor effects of amphetamine or the reduced stereotyped behavior observed in VGLUT3 constitutive knock-out mice.

Deletion of VGLUT3 in cholinergic neurons partially modifies amphetamine-induced psychomotor and stereotyped behavior

In the rodent striatum, VGLUT3 is also highly expressed in cholinergic neurons (Gras *et al.* 2002). Therefore, VGLUT3 was then specifically deleted from ACh neurons by crossing VGLUT3^{LoxP/LoxP} mice with ChAT-IRES-Cre mice. In WT mice, VGLUT3 and VAcHT are highly co-expressed in both soma and cholinergic varicosities (Fig. 7A and A'). In contrast, VGLUT3cKO-ACh mice showed a dramatic reduction in VGLUT3 intensity in TANs (Fig. 7B, B', C).

Cholinergic transmission plays a pivotal role in the control of dopaminergic tone in the ventral striatum (Sakae *et al.* 2015). We therefore investigated the role of cholinergic VGLUT3-positive varicosities in the locomotor effects of amphetamine. As shown in Fig. 7D-D¹, no significant differences were observed in the horizontal locomotor response induced by acute administration of amphetamine (5 mg/kg) in both VGLUT3cKO-ACh mice and WT littermates. Following chronic amphetamine, VGLUT3cKO-ACh mice showed a trend of increase in their locomotor activity ($p=0.06$, Fig. 7E) compared to their WT littermates. Both genotypes showed a progressive reduction in horizontal locomotor activity during chronic amphetamine (Fig. 7E¹). We then considered the rearing behavior. As observed in VGLUT3^{-/-} mice (Fig. 2C), acute injection of amphetamine triggered an increased vertical locomotor response in VGLUT3cKO-ACh mice (Fig. 7F, F¹). These data suggest an increased locomotor sensitivity to acute amphetamine in VGLUT3cKO-ACh mice. However, following chronic treatment, both genotypes showed a blunted vertical response to amphetamine (Fig. 7G, G¹), as observed in VGLUT3^{-/-} mice (Fig. 2D).

We then decided to investigate whether ACh/glutamate co-transmission was able to modulate amphetamine-induced stereotypies. A significant reduction in amphetamine-induced stereotypies was observed at Day1 in VGLUT3cKO-ACh mice (Fig. 8A, C). However, both genotypes showed sensitization of stereotypies during the entire protocol of 5 days (Fig. 8C). These data suggest a transient decrease of stereotypies in VGLUT3cKO-

ACh mice. As shown in Fig. 8D-F, no major differences were detected for orofacial amphetamine-induced stereotypies in both genotypes. Taken together these results suggest that VGLUT3 in ACh-neurons play a transient role in the psychomotor effects of amphetamine. However, the effects observed in full VGLUT3^{-/-} mice are only partially phenocopied by the deletion of VGLUT3 in cholinergic neurons.

Discussion

Glutamatergic co-transmission is a particular neurochemical feature of a subset of modulatory neurons. Functional and behavioral investigations aimed at understanding the role of glutamate co-transmission begin to provide interesting insights, even though a clear picture is still missing. In this context, VGLUT3 has raised high interest because of its implication in several brain functions and dysfunctions (El Mestikawy *et al.* 2011). The striatum harbors two sources of glutamate release from non-glutamatergic neurons, notably *i)* the striatal cholinergic TANs and *ii)* the 5HT varicosities, both of which express VGLUT3 (El Mestikawy *et al.* 2011). Whether and how ACh/glutamate and 5-HT/glutamate co-transmissions contribute to striatum-dependent behaviors is still poorly understood. Here, we provide the first evidence showing that different VGLUT3-positive subsystems differentially gate psychomotor effects induced by amphetamine.

Our data indicate that full genetic ablation of VGLUT3 strongly increases locomotor responses whereas it significantly decreases the occurrence and severity of stereotypies. These observations are in agreement with previous reports showing increased locomotor responses following cocaine administration (Sakae *et al.* 2015) and reduced L-DOPA-induced abnormal movements (Gangarossa *et al.* 2016). In addition to our behavioral observations, we found that reduced amphetamine-triggered stereotypies in VGLUT3^{-/-} mice were paralleled by a reduction of $\Delta FosB$ -positive striatal neurons. Repeated administration of psychostimulants (e.g., cocaine and amphetamine) causes long-lasting cellular and molecular adaptations. $\Delta FosB$, a stable variant of the transcription factor FosB and a mediator of adaptive neuroplasticity, accumulates over time with repeated psychostimulants stimulations (Lobo *et al.* 2013). Interestingly, a positive correlation between $\Delta FosB$ and abnormal movements (stereotypies and/or dyskinesia) has been highlighted (Cao *et al.* 2010; Engeln *et al.* 2016; Phillips *et al.* 2016). Furthermore, striatal overexpression of $\Delta FosB$ predisposes to altered psychostimulants-induced effects (Kelz *et al.* 1999). In agreement with our results, several studies also show that dopamine and glutamate are both required

for the expression of immediate early genes (IEGs) (Ferguson *et al.* 2003; Konradi *et al.* 1996) and the activation of critical signaling pathways in striatal MSNs (Cahill *et al.* 2014). In agreement with such molecular reactivity, L-DOPA-induced dyskinesia in VGLUT3^{-/-} mice was accompanied by blunted dopamine-related signaling cascades (Gangarossa *et al.* 2016).

Since striatal VGLUT3 is present in both cholinergic and 5HT varicosities (Gras *et al.* 2002), VGLUT3 was selectively deleted in each subsystem. Immunofluorescence experiments confirmed the selective ablation of VGLUT3 from either striatal ACh-neurons or 5HT-positive varicosities. Amphetamine-induced psychomotor effects were then investigated in these mutant lines.

TANs play a critical role in shaping and regulating the activity of striatal networks, thus strongly impacting on striatal functions and dysfunctions (Bonsi *et al.* 2011). It is well established that DA is pivotal for motor functions, motivation and learning (Wise 2004). Cholinergic transmission from striatal TANs powerfully regulates DA release (Cachope *et al.* 2012; Exley *et al.* 2012; Sakae *et al.* 2015; Threlfell *et al.* 2010; Zhang *et al.* 2009). Importantly, increasing recent evidence associate TANs dysregulation with striatum-related disorders such as Parkinson's disease or addiction (Bonsi *et al.* 2011; Deffains and Bergman 2015; Gangarossa *et al.* 2016; Pisani *et al.* 2007; Sakae *et al.* 2015; Witten *et al.* 2010; Won *et al.* 2014). Our data indicate that selective deletion of VGLUT3 in ACh-neurons only partially and transiently recapitulates amphetamine-induced locomotor activity and stereotypies observed in full knock-out mice. Furthermore, no major differences in amphetamine-induced hyperlocomotor activity or behavioral sensitization were observed when VGLUT3 was specifically deleted from 5HT neurons.

These results indicate that VGLUT3 may operate through a more complex system/network to gate the psychomotor effects of amphetamine. Several not mutually exclusive explanations may account for such an apparent discrepancy and may constitute parallel and convergent processes, with complementary and/or interactive effects.

First, the striatum receives glutamatergic inputs from the cortex. Interestingly, VGLUT3 is expressed in scattered cortical GABA interneurons (Herzog *et al.* 2004; Hioki *et al.* 2004; Somogyi *et al.* 2004). These cortical cells, by signaling through both glutamate and GABA, could influence pyramidal cortical activity as previously shown for the hippocampus (Fasano *et al.* 2017). Thus, it can be hypothesized that the absence of VGLUT3 in cortical GABA interneurons could alter striatal functions. In fact, cortico-striatal projections are critical components of forebrain circuits and are highly implicated in the etiopathology of several neuropsychiatric and motor disorders (Graybiel 2008; Shepherd 2013). Several

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studies in both humans and rodents point to frontal-cortical areas as key players in repetitive and compulsive disorders (Graybiel and Rauch 2000; Mallet *et al.* 2002; van den Heuvel *et al.* 2005). This functional hypothesis is also supported by the fact that the striatum receives topographically organized cortical projections (Hunnicutt *et al.* 2016) and shows territory-specific molecular reactivity (Gangarossa *et al.* 2013b). Interestingly, striatal territories in which $\Delta FosB$ activation was blunted in VGLUT3^{-/-} mice, notably the DMS and VMS, receive massive projections from the cingulate, orbitofrontal and prelimbic cortices. These cortical regions are involved in stereotyped and repetitive behaviors as well as in psychomotor effects of drugs of abuse (Abe *et al.* 2015; Homayoun and Moghaddam 2008; Kim *et al.* 2016; Thakkar *et al.* 2008; Wearne *et al.* 2016). Modifications of cortical local microcircuits may lead to aberrant cortical-striatal connections and hence mediate dysfunctional responses. In addition to the cortical inhibitor microcircuit, it is worth to mention that a fraction of long-range cortical GABA neurons has been shown to project to the striatum (Rock *et al.* 2016), thus contributing to striatal functions (Melzer *et al.* 2017). Whether VGLUT3 regulates long-range cortical GABA neurons is still unknown. Future studies will be necessary to establish if VGLUT3 in cortical GABA interneurons can shape cortico-striatal functions and associated behaviors.

Second, the hypocholinergic phenotype of constitutive VGLUT3 KO mice may contribute to the amphetamine-induced maladaptive behaviors. A ~40% reduction of ACh release is found in the dorsomedial striatum of VGLUT3^{-/-} mice (Gras *et al.* 2008). Importantly, the ACh/DA balance is pointed as a pivotal regulator of striatal functions, striatum-related disorders and drugs-induced stereotypies (Aliane *et al.* 2011; Aliane *et al.* 2009; Benarroch 2012; Lester *et al.* 2010). Selective deletion of VACHT in TANs (VACHT^{D2-cre-flox/flox} mice) leads to a complete loss of ACh release (Guzman *et al.* 2011). However, this mutation does not cause overt motor alterations (Guzman *et al.* 2011). Furthermore, VACHT^{D2-cre-flox/flox} mice demonstrate only marginally affected cocaine-induced locomotor activity and behavioral sensitization as well as L-DOPA-induced dyskinesia (Gangarossa *et al.* 2016; Guzman *et al.* 2011). Taken together these data suggest that the behavioral responses consequent to VGLUT3 deletion are likely triggered by the absence of glutamate released by ACh-neurons rather than by a reduction of ACh tone. Despite such evidence, here we report that glutamate from cholinergic neurons is only partially involved in amphetamine-induced psychomotor effects. However, it is worth to mention that an additional source of ACh within the striatum is provided by cholinergic inputs arising from the brainstem (Dautan *et al.* 2016a; Dautan *et al.* 2016b; Dautan *et al.* 2014). Whether these extra-striatal projecting ACh neurons co-release glutamate through a VGLUT3-dependent mechanism is still unknown.

Third, cell-type specific deletion of VGLUT3 in ACh or 5HT neurons may not be fully sufficient to precisely mirror behaviors observed in VGLUT3^{-/-} mice. Indeed, unforeseen integrative functions of other VGLUT3-positive circuits may be needed to observe full effects of psychostimulants. VGLUT3 is also expressed in other brain regions than the striatum and the raphe, notably the hippocampus, the amygdala, the bed nucleus of the stria terminalis (BNST), the hypothalamus, the habenula, inner hair cells of the cochlea and in non-5HT neurons of the raphe (Herzog *et al.* 2004; Jackson *et al.* 2009; Ruel *et al.* 2008; Seal *et al.* 2008). Although largely unexplored, several studies suggest a role for some of the above-mentioned structures in repetitive and stereotypic behaviors (Al-Amin *et al.* 2000; Bauman *et al.* 2008; Dietrich *et al.* 2015; Singer 2013). To date, we cannot exclude a potential involvement of these structures in phenotypes herein observed following acute and chronic administration of amphetamine in VGLUT3^{-/-} mice. A caveat may also reside in the use of constitutive VGLUT3-KO mice that may undergo through compensatory mechanisms, although previous characterizations revealed only minor adaptive modifications (Gras *et al.* 2008).

In conclusion, our study reveals a novel modulatory function of VGLUT3 in governing psychostimulants-dependent behaviors. In particular, our results point VGLUT3 as an interesting and innovative candidate to modulate psychostimulants-induced psychomotor effects and stereotypy-like behaviors. Future studies will be required to fully elucidate the integrative mechanisms allowing VGLUT3 to gate psychomotor adaptations elicited by psychostimulants. This work adds an important piece of evidence to the role of VGLUT3 in normal or pathological brain functions.

Figure legends

Figure 1. Experimental design.

Schematic representation of the behavioral protocol used in the study. Mice were habituated to the recording chambers and saline injections for three consecutive days (H1-H3) before being daily injected with saline or amphetamine (5 mg/kg) for five consecutive days (D1-D5). Horizontal and vertical locomotor activities or stereotypies were recorded during D1-5. After behavioral assessments, immunofluorescence analyses were performed on fixed brains. Boxes (a, b, c) indicate the repartition of number (range) of mice/genotype.

Figure 2. Acute and chronic effects of amphetamine on the locomotor activity of WT mice and VGLUT3^{-/-} mice. Horizontal (A) and vertical (C) locomotor activity (counts/5 min) of WT mice and VGLUT3^{-/-} mice recorded for 90 min following acute injection of amphetamine (Amph) 5mg/kg. (A¹ and C¹) Cumulative locomotor activities. Daily cumulative (90 min) horizontal (B) and vertical (D) locomotor activity of WT mice and VGLUT3^{-/-} mice following chronic administration of Amph (5mg/kg). Cumulative horizontal (B¹) and vertical (D¹) locomotor activities following Amph injection on the first day (D1) and the fifth day (D5). N=9-12 mice/group. All data are mean ± SEM. For statistical details see Suppl. Table 1. Specific comparisons: (A) ***p*<0.01 (VGLUT3^{-/-}_{T0-90min} vs WT_{T0-90min}); (A¹) ***p*<0.01 (VGLUT3^{-/-} vs WT); (B) ****p*<0.001 (VGLUT3^{-/-}_{D0-D5} vs WT_{D0-D5}); (B¹) ****p*<0.001 (VGLUT3^{-/-}_{D5} vs WT_{D5}); (C) ***p*<0.01 (VGLUT3^{-/-}_{T0-90min} vs WT_{T0-90min}); (C¹) **p*<0.05 (VGLUT3^{-/-} vs WT); (D) ****p*<0.001 (VGLUT3^{-/-}_{D1} vs VGLUT3^{-/-}_{D0}), °*p*<0.05 (WT_{D1} vs WT_{D0}), #*p*<0.05 (VGLUT3^{-/-}_{D1} vs WT_{D1}); (D¹) #*p*<0.05 (VGLUT3^{-/-}_{D1} vs WT_{D1}), ****p*<0.001 (VGLUT3^{-/-}_{D5} vs VGLUT3^{-/-}_{D1}).

Figure 3. Amphetamine-induced stereotypies in WT mice and VGLUT3^{-/-} mice.

(A) Daily time course of general stereotypies during five consecutive days of Amph (5mg/kg) injections. (B) Maximum stereotypy score during each day of injection. (C) Distribution pattern of max general stereotypy score on the first day (D1) and the fifth day (D5). (D) Time course of orofacial stereotypies. (E) Maximum orofacial stereotypy score during each day of injection. (F) Distribution pattern of max orofacial stereotypy score on the first day (D1) and the fifth day (D5). N=14 mice/group. All data are mean ± SEM. For statistical details see Suppl. Table 2. Specific comparisons: (A) **p*<0.05, ***p*<0.01, ****p*<0.001 (VGLUT3^{-/-} vs WT); (B) ***p*<0.01 (VGLUT3^{-/-}_{D3-5} vs WT_{D3-5}); (C) **p*<0.05 (WT_{D5} vs WT_{D1}), #*p*<0.05 (VGLUT3^{-/-}_{D5} vs WT_{D5}); (D) **p*<0.05 (VGLUT3^{-/-} vs WT); (E) ***p*<0.01 (VGLUT3^{-/-}_{D3-5} vs WT_{D3-5}); (F) ***p*<0.01 (WT_{D5} vs WT_{D1}), ##*p*<0.01 (VGLUT3^{-/-}_{D5} vs WT_{D5}).

Figure 4. Effect of amphetamine (5mg/kg) on the expression of ΔFosB in the striatum of WT mice and VGLUT3^{-/-} mice. (A) Coronal mouse brain sections at the level of the striatum showing where quantifications were performed. (B) Representative ΔFosB immunofluorescence microphotographs (negative inverted) taken in the dorsomedial (DMS) or ventromedial striatum (VMS) of WT mice and VGLUT3^{-/-} mice. Scale bar in B: 200μm. Quantification of ΔFosB-immunopositive neurons in the DMS (C) and VMS (D). All data are mean ± SEM. N=4-6 mice/group. For statistical details see Suppl. Table 3. Specific comparisons: (C) ****p*<0.001 (WT_{Amph} or VGLUT3^{-/-}_{Amph} vs WT_{Sal}) and °°*p*<0.01 (VGLUT3^{-/-}

Amph vs WT_{Amph}); (D) *** $p < 0.001$ (WT_{Amph} or VGLUT3^{-/-}_{Amph} vs WT_{Sal}) and °°° $p < 0.001$ (VGLUT3^{-/-}_{Amph} vs WT_{Amph}).

Figure 5. Acute and chronic effects of amphetamine on the locomotor activity of WT mice and VGLUT3cKO-5HT mice. Immunofluorescent detection of VGLUT3 (A, B) and 5HT (A', B') in WT (A) and VGLUT3cKO-5HT (B). Colocalizations are shown in A'' and B''. Scale bar is set at 2 μm . Histograms show quantifications of 5HT (C¹) and VGLUT3 (C²) fluorescence intensities in both WT mice and VGLUT3cKO-5HT mice.

Horizontal (D) and vertical (F) locomotor activity (counts/5 min) of WT mice and VGLUT3cKO-5HT mice recorded for 90 min following acute injection of amphetamine (Amph) 5mg/kg. (D¹ and F¹) Cumulative locomotor activities. Daily cumulative (90 min) horizontal (E) and vertical (G) locomotor activity of WT mice and VGLUT3cKO-5HT mice following chronic administration of amphetamine (Amph) 5mg/kg. Cumulative horizontal (E¹) and vertical (G¹) locomotor activities following Amph injection on the first day (D1) and the fifth day (D5). N=9-12 mice/group (behavior). N_(varicosities)=31-51 from 3 mice/genotype (immunofluorescence). All data are mean \pm SEM. For statistical details see Suppl. Table 4. Specific comparisons: (C¹) * $p < 0.05$ (VGLUT3cKO-5HT vs WT) and (C²) *** $p < 0.001$.

Figure 6. Amphetamine-induced stereotypies in WT mice and in VGLUT3cKO-5HT mice. (A) Daily time course of general stereotypies during five consecutive days of Amph (5mg/kg) injections. (B) Maximum stereotypy score during each day of injection. (C) Distribution pattern of max general stereotypy score on the first day (D1) and the fifth day (D5). (D) Daily time course of orofacial stereotypies. (E) Maximum orofacial stereotypy score during each day of injection. (F) Distribution pattern of max orofacial stereotypy score on the first day (D1) and the fifth day (D5). N=10 mice/group. All data are mean \pm SEM. For statistical details see Suppl. Table 5. Specific comparisons: (C) ** $p < 0.01$ (WT_{D5} vs WT_{D1}), °°° $p < 0.001$ (VGLUT3cKO-5HT_{D5} vs VGLUT3cKO-5HT_{D1}); (F) ** $p < 0.01$ (WT_{D5} vs WT_{D1}), °° $p < 0.01$ (VGLUT3cKO-5HT_{D5} vs VGLUT3cKO-5HT_{D1}).

Figure 7. Acute and chronic effects of amphetamine on the locomotor activity of WT mice and VGLUT3cKO-ACh mice. (A-B) Immunofluorescent detection of VACHT and VGLUT3 in WT mice (A) and VGLUT3cKO-ACh mice (B). Insets (A' and B') show higher magnification of a striatal TAN. Scale bars are 10 μm . Histograms show quantification

VGLUT3 (C) fluorescence intensity in both WT mice and VGLUT3cKO-ACh mice. Horizontal (D) and vertical (F) locomotor activity (counts/5 min) of WT mice and VGLUT3cKO-ACh mice recorded for 90 min following acute injection of Amph (5mg/kg). (D¹ and F¹) Cumulative locomotor activities. Daily cumulative (90 min) horizontal (E) and vertical (G) locomotor activities of WT mice and VGLUT3cKO-ACh mice following chronic administration of Amph (5mg/kg). Cumulative horizontal (E¹) and vertical (G¹) locomotor activities following Amph injection on the first day (D1) and the fifth day (D5). N=10-12 mice/group (behavior). N_(cells)=31-32 from 3-4 mice/genotype (immunofluorescence). All data are mean ± SEM. For statistical details see Suppl. Table 6. Specific comparisons: (C) *** $p < 0.001$ (VGLUT3cKO-ACh vs WT); (E¹) *** $p < 0.001$ (WT_{D5} vs WT_{D1}), °°° $p < 0.001$ (VGLUT3cKO-ACh_{D5} vs VGLUT3cKO-ACh_{D1}); (F) * $p < 0.05$ (VGLUT3cKO-ACh_{T0-90min} vs WT_{T0-90min}); (F¹) * $p < 0.05$ (VGLUT3cKO-ACh vs WT); (G) *** $p < 0.001$ (VGLUT3cKO-ACh_{D1} vs VGLUT3cKO-ACh_{D0}), # $p < 0.05$ (VGLUT3cKO-ACh_{D1} vs WT_{D1}); (G¹) ** $p < 0.01$ (VGLUT3cKO-ACh_{D5} vs VGLUT3cKO-ACh_{D1}), # $p < 0.05$ (VGLUT3cKO-ACh_{D1} vs WT_{D1}).

Figure 8. Amphetamine-induced stereotypies in WT mice and VGLUT3cKO-ACh mice.

(A) Daily time course of general stereotypies during five consecutive days of Amph (5mg/kg) injections. (B) Maximum stereotypy score during each day of injection. (C) Distribution pattern of max general stereotypy score on the first day (D1) and the fifth day (D5). (D) Daily time course of orofacial stereotypies. (E) Maximum orofacial stereotypy score during each day of injection. (F) Distribution pattern of max orofacial stereotypy score on the first day (D1) and the fifth day (D5). N=14 mice/group. All data are mean ± SEM. For statistical details see Suppl. Table 7. Specific comparisons: (A) * $p < 0.05$ (VGLUT3cKO-ACh_{D1} vs WT_{D1}); B * $p < 0.05$ (VGLUT3cKO-ACh_{D1} vs WT_{D1}); (C) *** $p < 0.001$ (WT_{D5} vs WT_{D1}), # $p < 0.05$ (VGLUT3cKO-ACh_{D1} vs WT_{D1}), °°° $p < 0.001$ (VGLUT3cKO-ACh_{D5} vs VGLUT3cKO-ACh_{D1}); (F) *** $p < 0.001$ (WT_{D5} vs WT_{D1}), °°° $p < 0.001$ (VGLUT3cKO-ACh_{D5} vs VGLUT3cKO-ACh_{D1}).

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Authors contribution: GG, SD and SEM conceived and designed the study. GG and SEM drafted the article. VV and SD critically revised the article. NM-G, VB and EV acquired, analyzed and interpreted the data.

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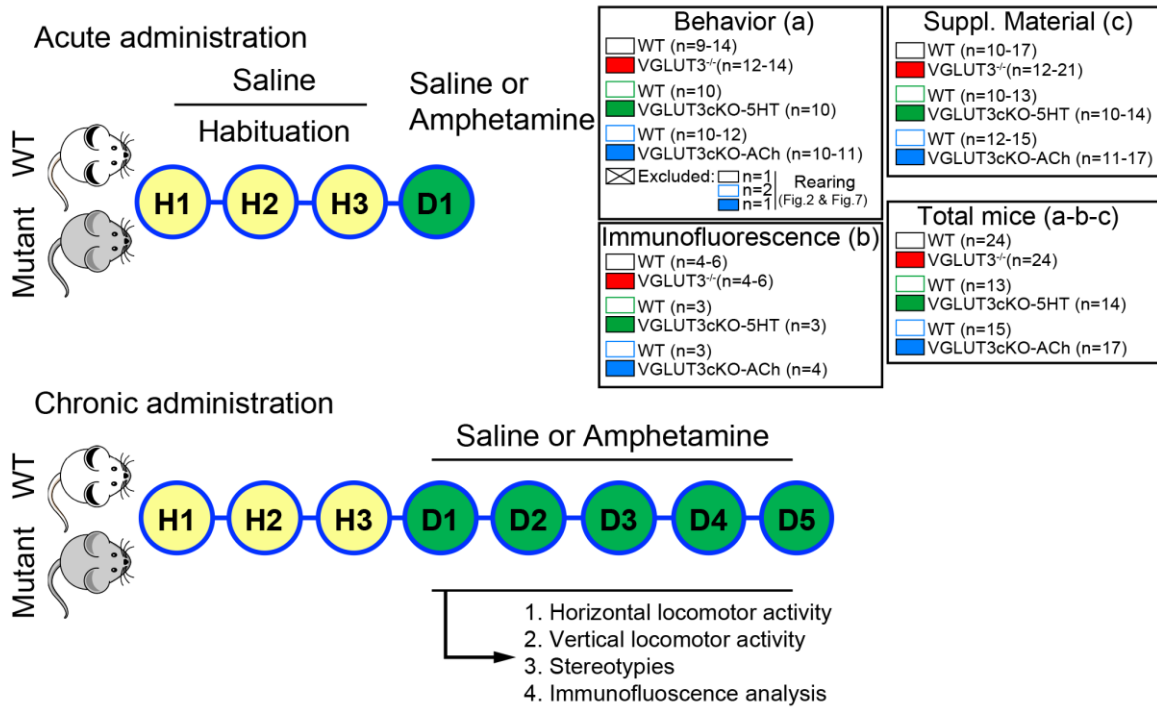


Figure 1 Mansouri-Guilani et al.

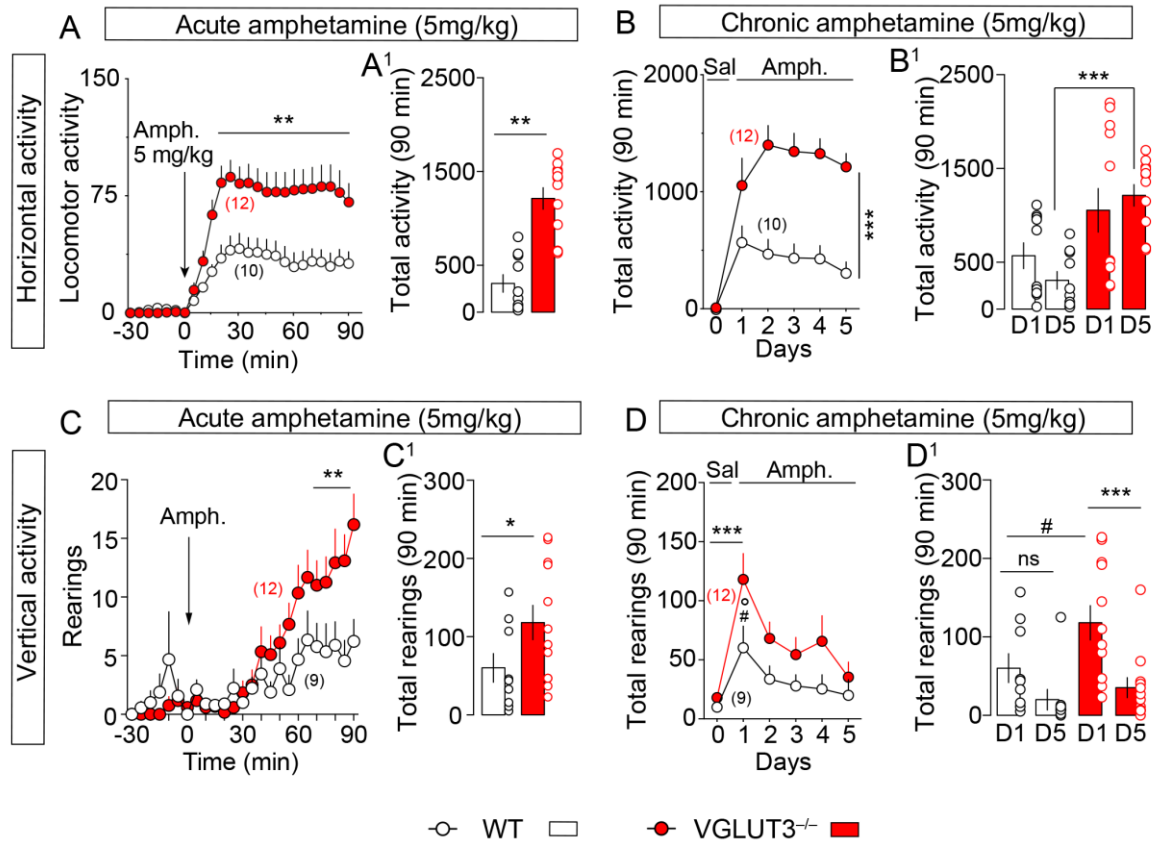


Figure 2 Mansouri-Guilani et al.

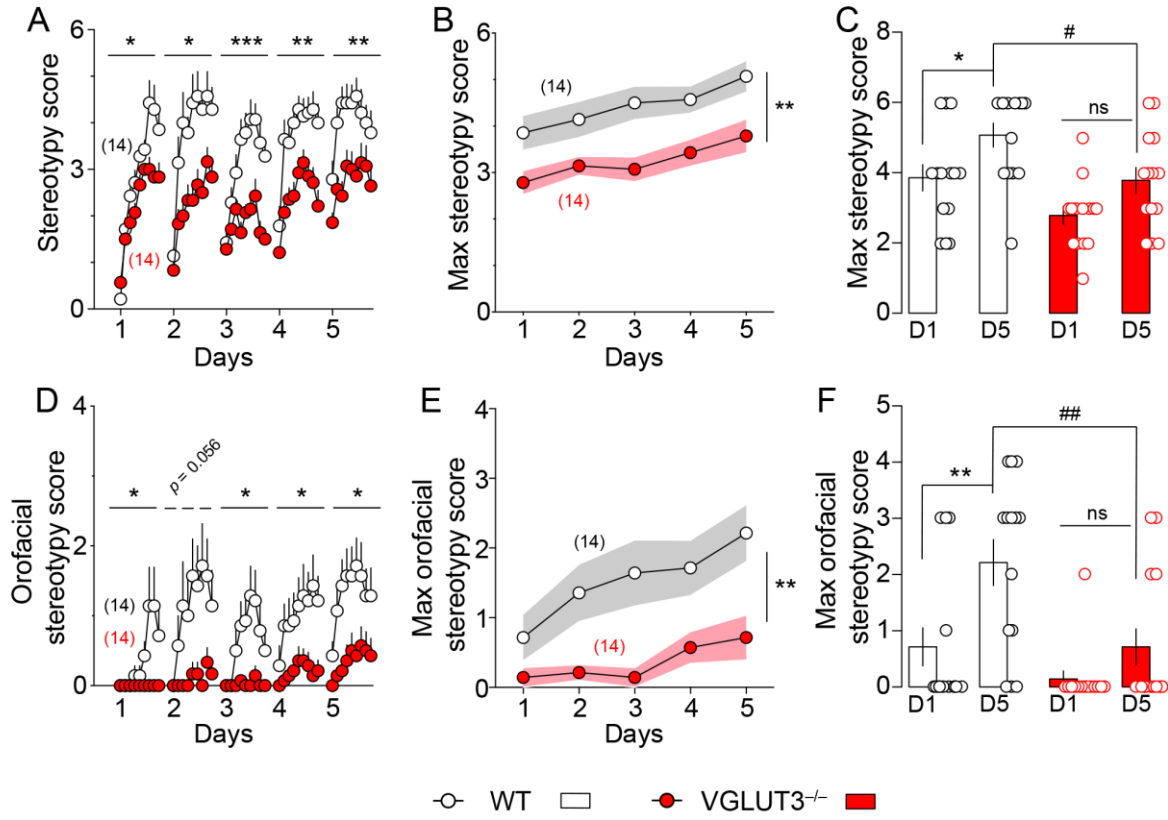


Figure 3 Mansouri-Guilani et al.

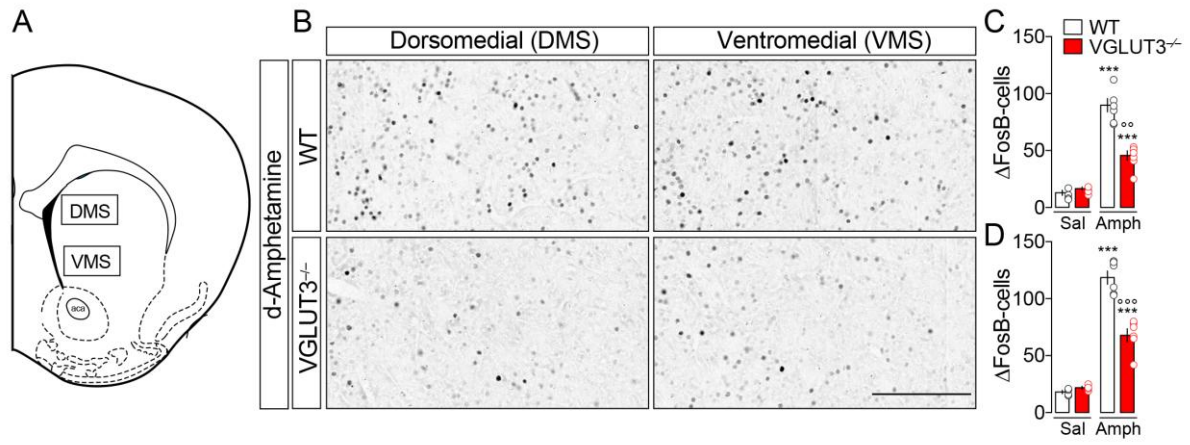


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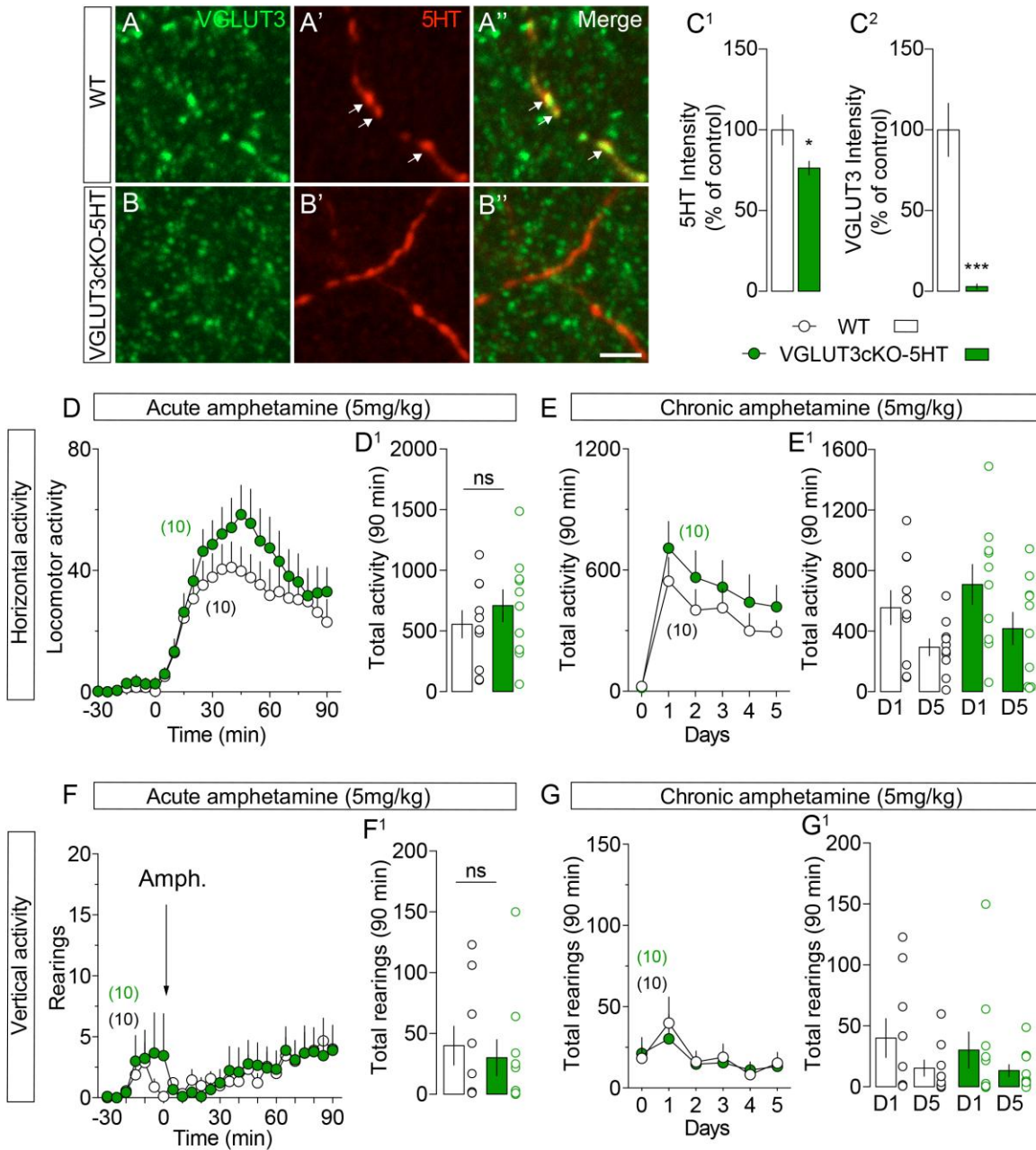


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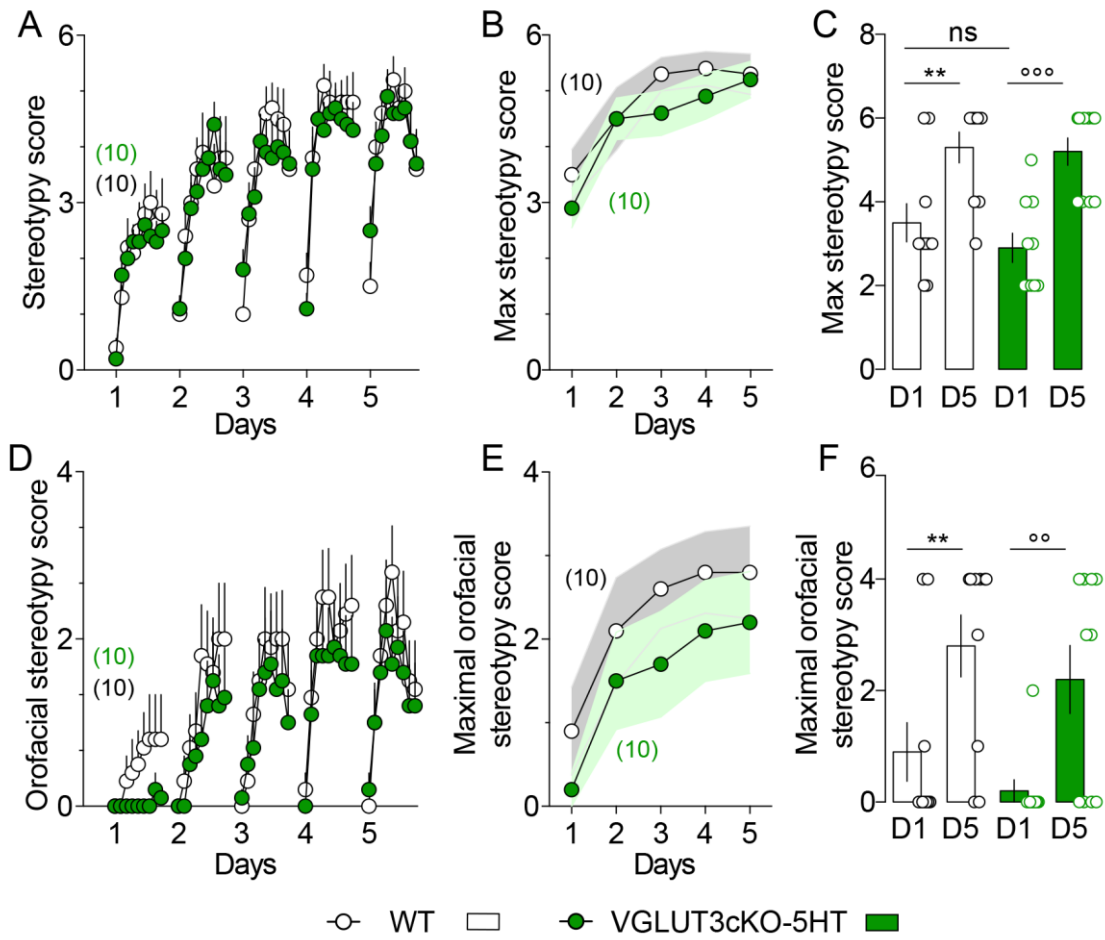


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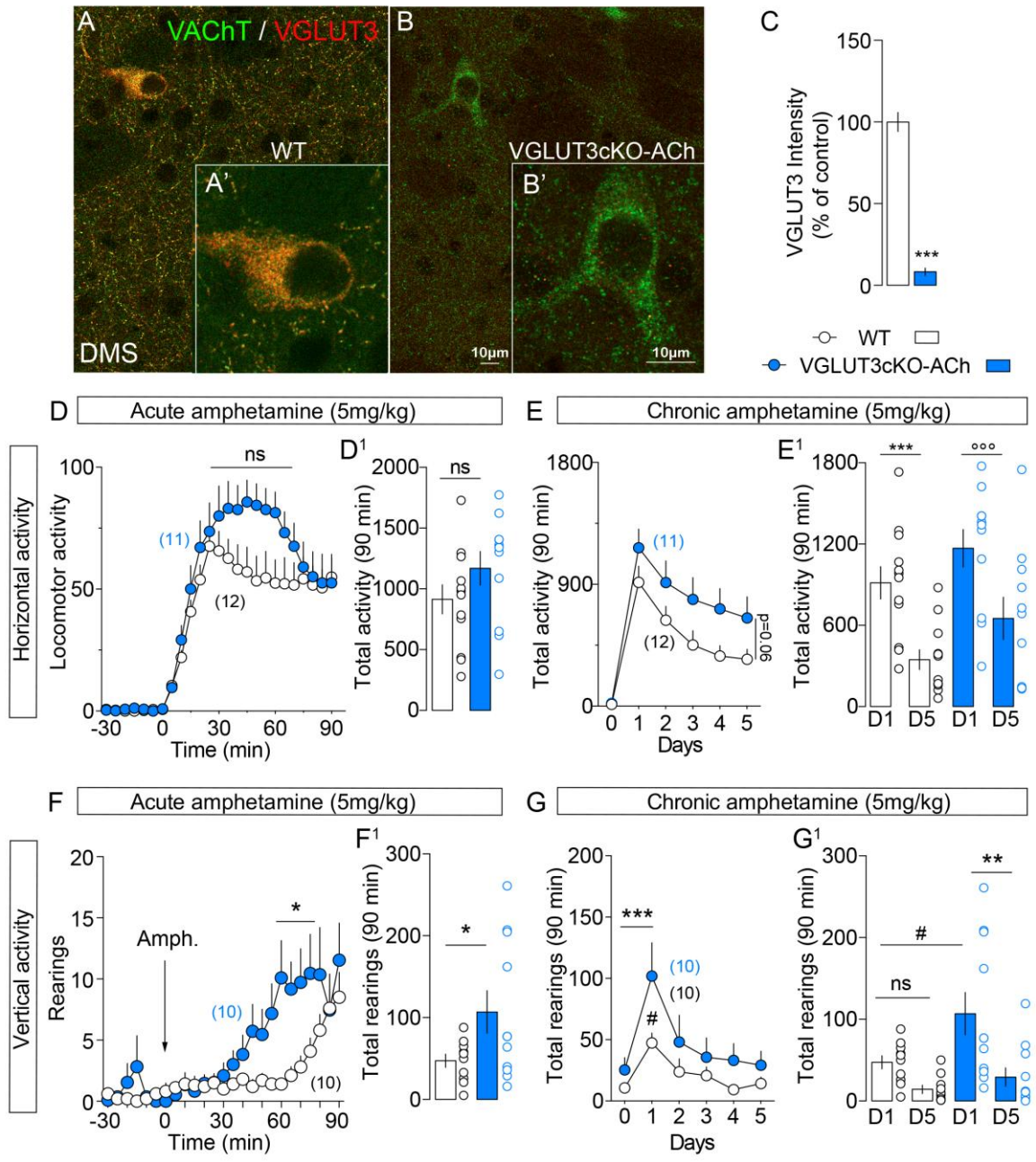


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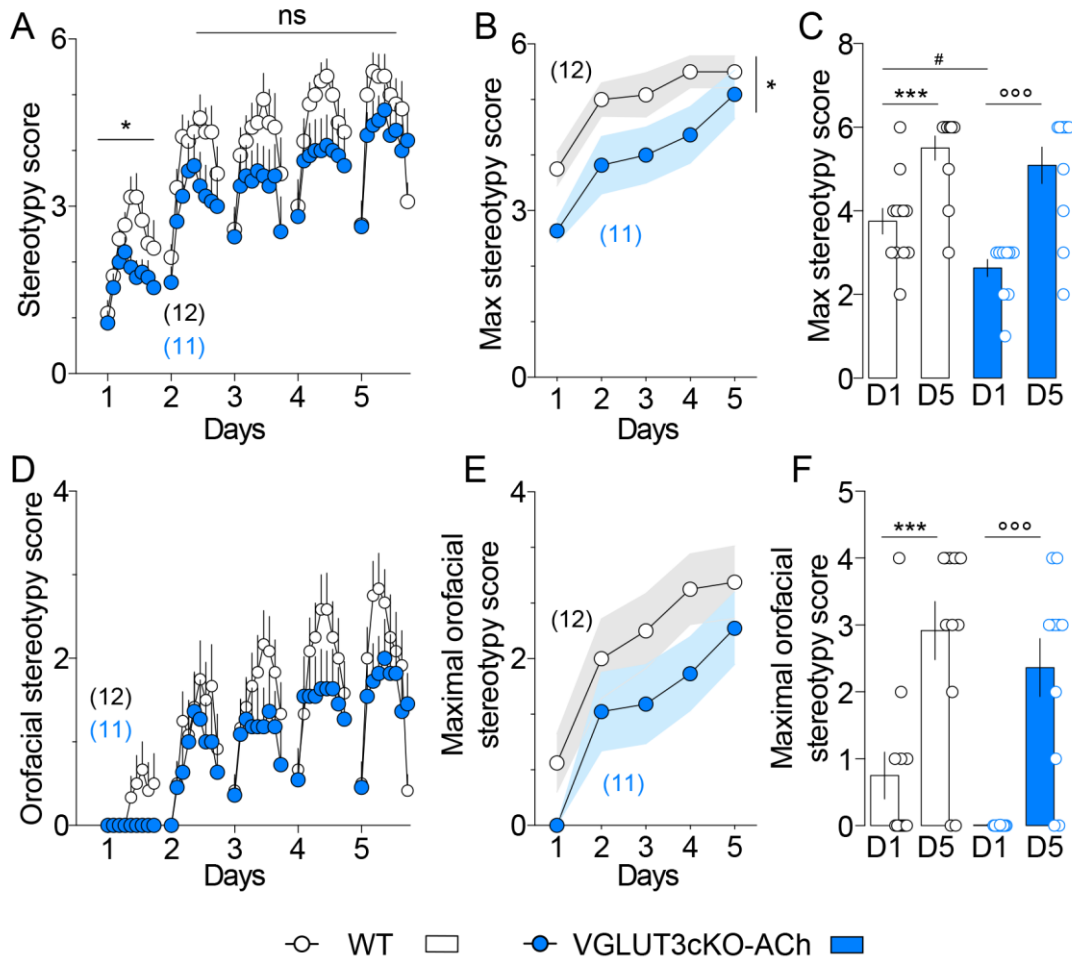


Figure 8 Mansouri-Guilani et al.