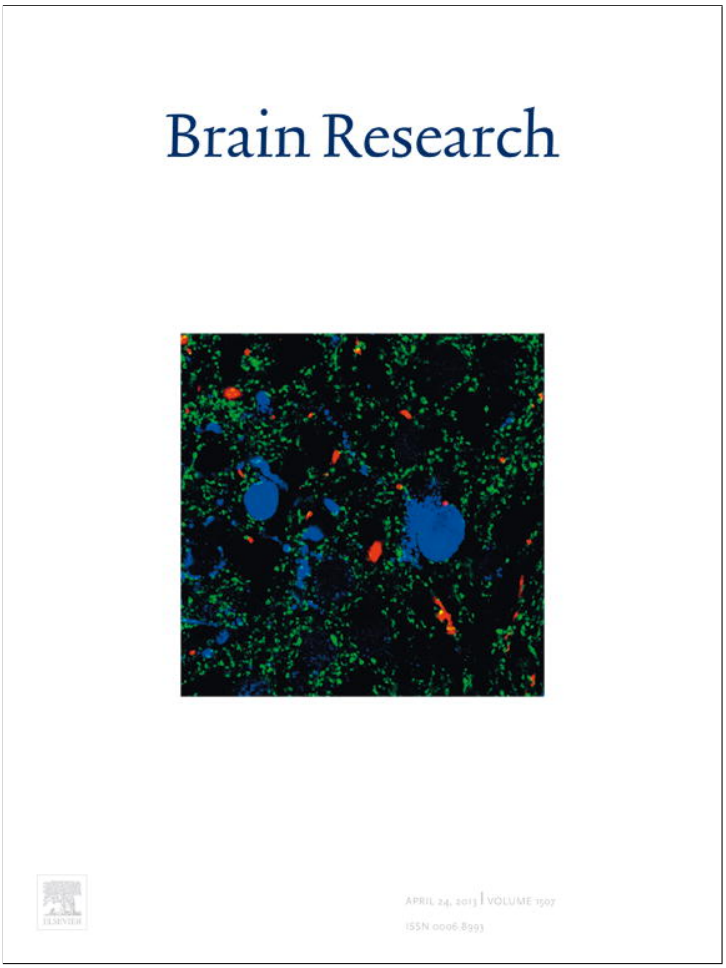

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Research Report

A role for Sv2c in basal ganglia functions

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ABSTRACT

SV2C is an isoform of the synaptic vesicle 2 protein family that exhibits a particular pattern of brain expression with enriched expression in several basal ganglia nuclei. In the present study, we have investigated SV2C implication in both normal and pathological basal ganglia functioning with a peculiar attention to dopamine neuron containing regions. In SV2C−/− mice, the expression of tyrosine hydroxylase mRNA in midbrain dopaminergic neurons was largely and significantly increased and enkephalin mRNA expression was significantly decreased in the caudate-putamen and accumbens nucleus. The expression of SV2C was studied in two models of dopaminergic denervation (6-OHDA- and MPTP-induced lesions). In dopamine-depleted animals, SV2C mRNA expression was significant increased in the striatum. In order to further understand the role of SV2C, we performed behavioral experiments on SV2C−/− mice and on knock-down mice receiving an injection of adeno-associated virus expressing SV2C miRNA specifically in the ventral midbrain. These modifications of SV2C expression had little or no impact on behavior in open field and elevated plus maze. However, even if complete loss of SV2C had no impact on conditioned place preference induced by cocaine, the specific knock-down of SV2C expression in the dopaminergic neurons completely abolished the development of a CPP while the reaction to an acute drug injection remains similar in these mice compared to control mice. These results showed that SV2C, a poorly functionally characterized protein is strongly involved in normal operation of the basal ganglia network and could be also involved in system adaptation in basal ganglia pathological conditions.

1. Introduction

In contrast to SV2A and SV2B, the SV2C isoform exhibits a strikingly restricted localization in the rodent brain (Dardou et al., 2011; Janz and Südhof, 1999). Indeed, as shown by using in situ hybridization and immunohistochemistry, the expression of this isoform of SV2 is strictly restricted in small subsets of brain regions that included the olfactory bulb, the striatum, the substantia nigra and nuclei in the pons and the medulla oblongata. In addition, a complete absence of SV2C

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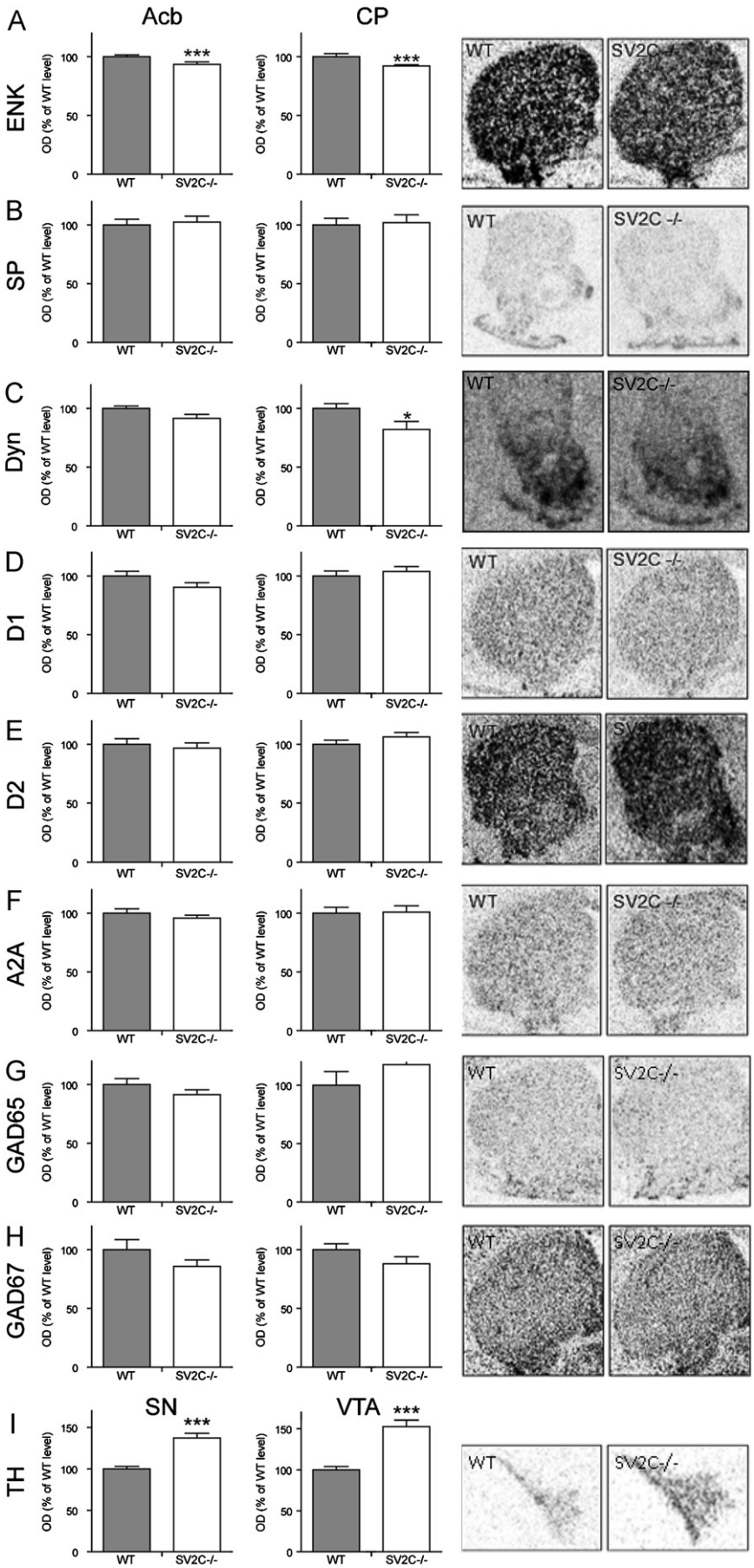

expression was noticed in the cerebral cortex and the hippocampus. The dense expression of SV2C in the striatum, pallidum, and substantia nigra could point to a specific role of SV2C in the normal and/or pathological functioning of the basal ganglia.

Expression of SV2C, both at mRNA and protein levels, is present at high level in dopaminergic neurons of the substantia nigra *pars compacta* (SNpc) and ventral tegmental area (VTA) and in cholinergic interneurons of the striatum as well as, albeit at a lower level, in the principal striatal neurons, the medium-sized spiny neurons (MSN) (Dardou et al., 2011). Despite this specific and restricted pattern of expression, the precise role of SV2C in neurons remains mostly unknown. To date, few hypotheses have been put forward about the possible functions of the SV2 protein family in mammalian cells (Brose and Rosenmund, 1999). It has been proposed that the glycosylated intravesicular loop of these proteins may trap soluble neurotransmitter to diminish the intravesicular osmotic pressure. Alternative hypotheses are that SV2 could modulate exocytosis by interacting with synaptotagmin I and converting the vesicles into a calcium and synaptotagmin-responsive state (Schivell et al., 1996; Lazell et al., 2004; Chang and Südhof, 2009), or could act as a scaffold protein that regulates vesicle shape (Janz et al., 1998). In addition, due to its sequence and conformation, an integral trans-membrane protein with three *N*-glycosilation sites in the intravesicular loop, it was also postulated that SV2 proteins could act as transporters. However, no transporter activities have been identified so far.

In the present study, we investigated what could be the role and implication of SV2C in the basal ganglia system. We explored the changes in gene expression within the basal ganglia including the substantia nigra *pars compacta* when SV2C expression was knocked-out in mice brain. On the other hand, since SV2C is expressed in brain regions known to play a key role in Parkinson's disease, we also studied the change in SV2C expression in two models of dopamine depletion, intrastriatal 6-OHDA injection and MPTP model. Finally, we investigated what could be the behavioral consequences of the loss in SV2C expression either in the whole brain in the full SV2C knock-out (SV2C−/−) mice or in a very restricted brain region using injection of AAV expressing miRNA targeting SV2C mRNA in the ventral midbrain (SNpc/VTA) of wild-type (WT) mice.

2. Results

2.1. Gene expression changes in the basal ganglia in case of complete loss of SV2C expression

In SV2C−/− mice, we noted a significant ($p<0.05$) decrease in encephalin (ENK) mRNA labeling in both the Acb and CP regions of the striatum as compared to WT (Fig. 1A). In addition, we also noted a significant ($p=0.05$) decrease of dynorphine (Dyn) mRNA expression in the SV2C−/− compared to WT in the CP (Fig. 1C); a non-significant trends to a decrease was alsa observed in the Acb. On the contrary, no changes in substance P (SP), dopamine D1 (D1), dopamine D2 (D2) or adenosine A2A (A2A) receptors or in GAD65 and GAD67 (Fig.1B and D–H) mRNA expression were observed in Acb and CP in SV2C−/− as compared to WT mice. We also noticed no change in GAD65 and GAD67 mRNA expression in the globus pallidus (GP) and ventral pallidum (VP), two targets of striatal MSN that expressed SV2C in wild-type mice (data not shown).

In the SNpc and VTA, we observed that TH mRNA expression was significantly ($p<0.05$) increased by 37% and 52%, respectively, in SV2C−/− mice as compared to WT (Fig. 1I).

These data showed that the absence of SV2C expression in the mouse brain induces a large increase in TH mRNA expression in the SNpc/VTA and a slight but significant decrease in ENK mRNA expression in the striatum.

2.2. SV2C expression changes following dopaminergic denervation

2.2.1. 6-OHDA-induced unilateral lesion

As illustrated in Fig. 2A, the expression of TH mRNA in the lesioned side was significantly (Mann Whitney *t* test, $t=4$; $p<0.05$) reduced as compared to the non-lesioned side. ISH revealed that the striatal ENK mRNA expression was significantly increased in the side of the lesion (data not shown), showing that the dopaminergic lesion is effective. Interestingly, we noticed that the SV2C mRNA expression was significantly increased in the dopamine-depleted CP as compared to the non-lesioned side (Fig. 2B and C). No difference was observed in the Acb.

2.2.2. MPTP-induced lesion

Injection of MPTP induced a large and significant decrease in DAT binding in the Acb and CP as compared to control mice, confirming the serious degeneration of dopaminergic innervation in the striatum in MPTP-injected mice (Fig. 2D–F). As detected in 6-OHDA-injected animals, we observed that, in MPTP-injected mice, SV2C mRNA expression was significantly increased in CP and also in Acb (Fig. 2G–I).

Those results pointed out that the loss of dopaminergic afferences induces an increased SV2C expression in their striatal targets.

2.3. A place for SV2C in behavior

SV2C gene inactivation strongly affects the expression of TH mRNA expression in midbrain dopamine neurons in the full KO animals suggesting that SV2C could influence dopamine

Fig. 1 – Changes in mRNA expression in basal ganglia of SV2C−/− mice. (A) significant ($p<0.001$) decrease in enkephalin mRNA expression (ENK, A) has been noted in knock-out (SV2C−/−, white bar) mice compared to wild-type (WT, grey bar) mice in the accumbens nucleus (Acb) and caudate–putamen (CP). On the contrary, tyrosine hydroxylase (TH, H) mRNA expression in the substantia nigra pars compacta (SNpc) and ventral tegmental area (VTA) is significantly ($p<0.001$) increased in SV2C−/− mice compared to WT. No changes in substance P (SP) (B), dopamine D1 receptor (D1) (C), dopamine D2 receptor (D2) (D), adenosine A2A receptor (A2A) (E), GAD65 (F) and GAD67 (G) mRNAs expression in Acb and CP have been observed. Data are reported as mean ± SEM in percent of WT mice. Significant differences are indicated by * ($p<0.001$).**

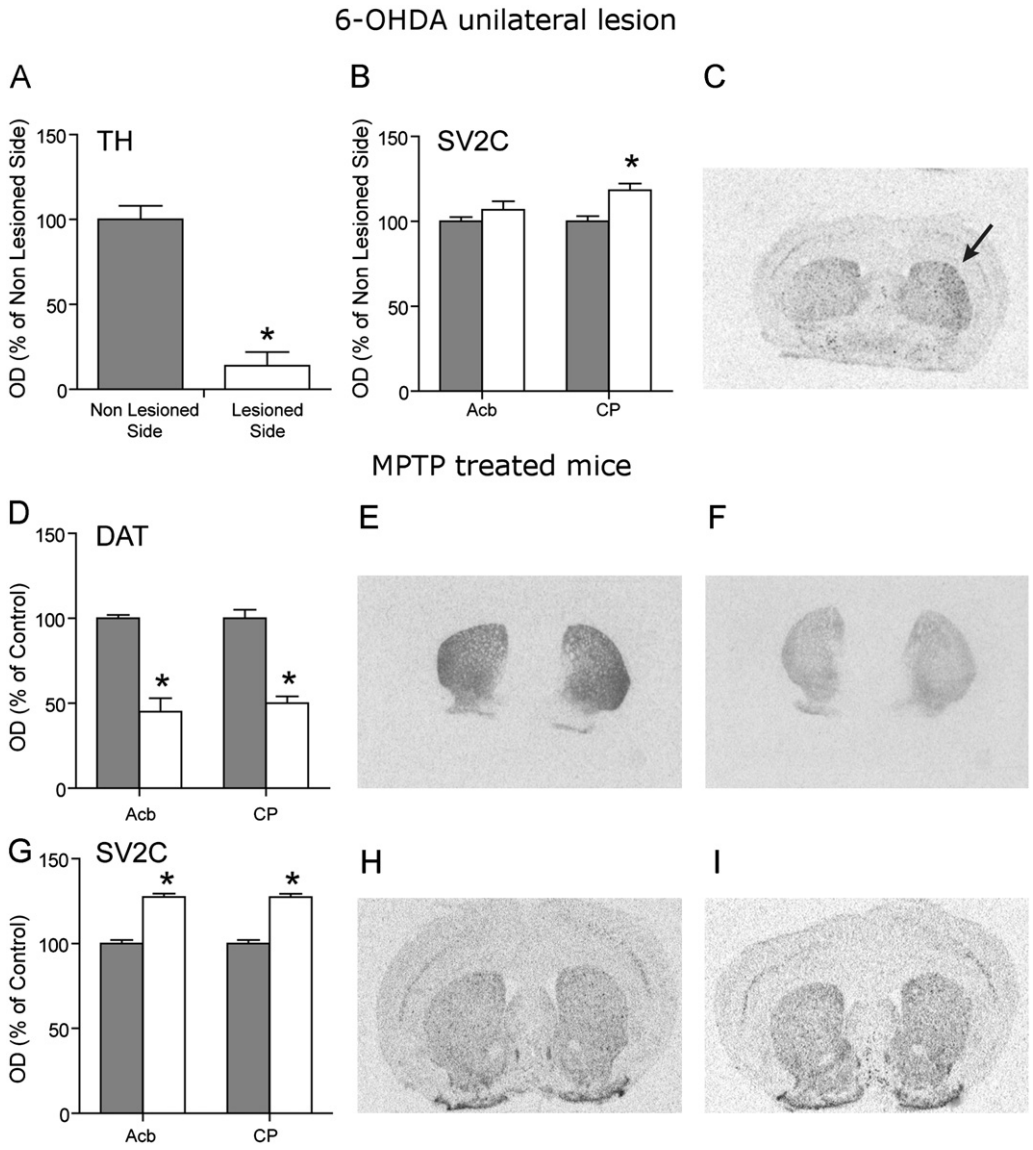

Fig. 2 – Changes in SV2C mRNA expression in animal models of dopamine depletion. In 6-OHDA unilateral lesioned mice, tyrosine hydroxylase (TH) mRNA expression in the substantia nigra (A) showed a significant ($p<0.05$) decrease in the lesioned (white bar) as compared to the non-lesioned (gray bar) sides. In these mice, we noted that SV2C mRNA expression (B), significantly ($p<0.05$) increased in the CP on the side of the lesion (arrow on (C)) compared to non lesioned side, while SV2C mRNA expression remained the same in Acb. In MPTP-treated mice (F) ((D), white), a significant ($p<0.05$) decrease in DAT binding (D) was observed in the Acb and CP as compared to the control (E) ((D), gray). SV2C mRNA expression is significantly increased ($p<0.05$) in the Acb and CP in MPTP-treated mice ((G): white bar, (H)) compared to saline-treated mice ((G): gray bar, (I)). Significant differences are indicated by * for $p<0.05$. Data are reported as mean ± SEM in percent of the non-lesioned side ((A), (B)) or of saline-treated mice ((D), (G)).

neurons-mediated behaviors. However, it also abolishes SV2C in other brain areas. To investigate more specifically the potential role of SV2C expression in the dopaminergic SNpc/VTA regions, we specifically knock-down SV2C expression in these neurons by using an artificial microRNA (miRNA) targeting SV2C gene, expressed in adeno-associated virus (AAV) that was locally injected.

2.3.1. *Animal selection*

After the completion of all behavioral experiments, brains were analyzed in order to determine the level of SV2C down-regulation. As illustrated in Fig. 3, no SV2C mRNA was observed in all brain regions of SV2C−/− mice (Fig. 3B and F).

In miSV2C mice, SV2C expression was down-regulated but not completely abolished in the SNpc/VTA regions (Fig. 3H) and was then quantified as compared to mice that received injection of the control miRNA (Fig. 3G) and that showed no effect of the injection on SV2C expression in the SNpc/VTA. Mice showing a decrease in SV2C expression in the SNpc/VTA region lower than 40% were excluded from the behavioral analysis. No change in SV2C mRNA expression was observed in the striatum (Fig. 3C and D). In both series, animals with

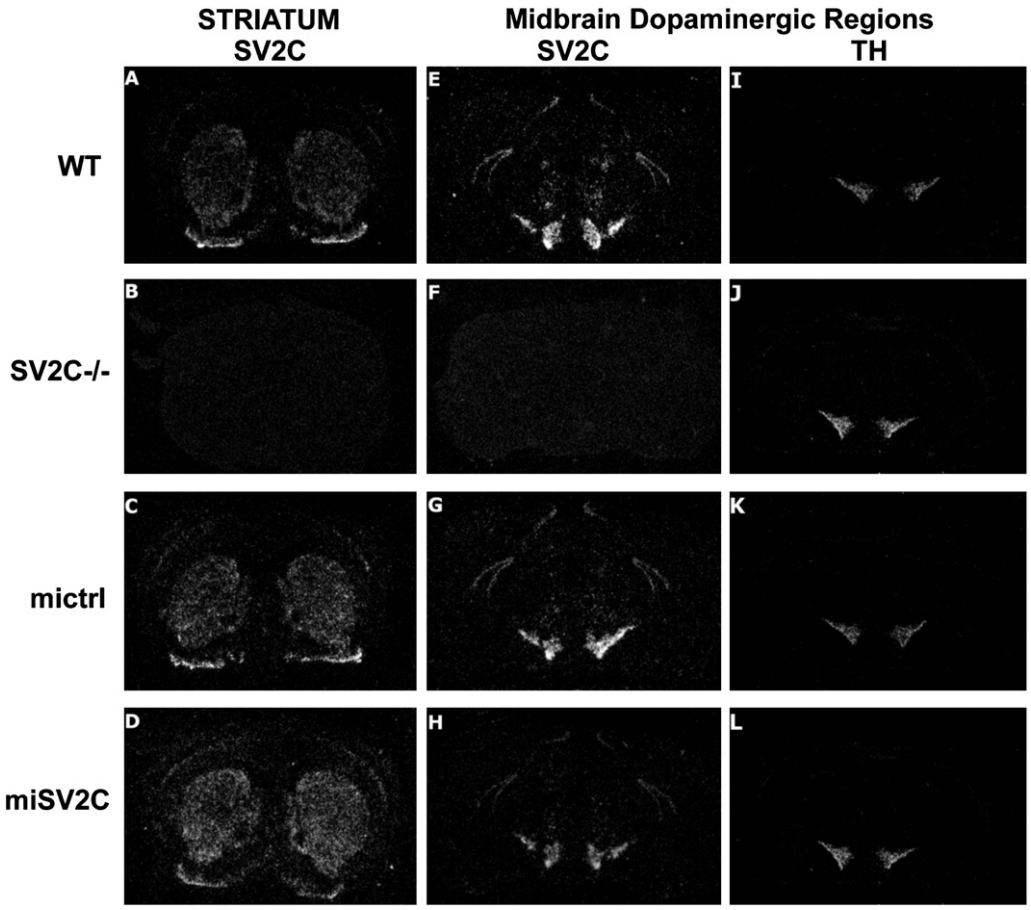

Fig. 3 – SV2C expression in striatum and dopaminergic midbrain regions. Inverted contrast microphotographs of SV2C mRNA ((A) to (H)) and TH mRNA ((I) to (L)) detected by ISH in WT ((A), (E)), SV2C−/− ((B), (F)), mictrl ((C), (G)) and miSV2C mice ((D), (H)). SV2C−/− mice show a complete abolition of SV2C mRNA expression either in striatum (B) and dopaminergic brain regions (F), while mice with miRNA injection present a downregulation of SV2C mRNA expression only in dopaminergic brain region (H) but not in the striatum (D). No changes were noted in mice receiving scramble miRNA injection ((C), (G)). TH mRNA expression is upregulated in SV2C−/− mice in dopaminergic brain regions (J) compared to other experimental groups.

Table 1 – Experimental groups. Number of mice used for each behavioral experiments and for each experimental groups. WT: wild type animals, SV2C−/−: SV2C-knock-out mice, mictrl: injection control for the miRNA animals, miSV2C: mice with injections of miRNA targetting SV2C mRNA. For further informations regarding experimental groups see Section 4.

Experimental groups	WT		SV2c−/−		mictrl		miSV2C	
Open field	34		40		18		15	
Elevated plus maze	12		40		13		15	
Injections	Saline	Cocaine	Saline	Cocaine	Saline	Cocaine	Saline	Cocaine
Conditioned place preference	13	11	18	17	5	7	5	6
Acute cocaine	7	6	4	4	8	8	7	7

midbrain traumatic lesion have been removed from the results of the study. The number of mice taken into consideration for each experiment is listed in Table 1.

Although SV2C expression was completely or partially abolished in some experimental groups, TH mRNA remains present in all groups (Fig. 3I–L). However, we observed that, as showed above, TH mRNA expression in SNpc/VTA regions is increased in SV2C−/− mice (Fig. 3J) compared to other groups.

2.3.2. *Open field*

The total distance run during the 30 min of the open field exposition is shown in Fig. 4. A one way ANOVA indicated an effect of the group on the total distance run ($F[3,103]=4.1928$; $p<0.05$). A Bonferroni post hoc test showed that the total distance run was slightly but significantly ($p<0.05$) lower in the SV2C−/− mice compared to WT. Since no modification was observed in the miSV2C group, such modest difference could be more related to the loss of SV2C protein expression in striatum.

2.3.3. *Elevated plus maze*

The four experimental groups were submitted to elevated plus maze and the times spent in protected and non-protected arms were illustrated in Fig. 5. A one way ANOVA with group as factor showed no significant effect on the time spent either in protected or non-protected arm. In each group, we compared the amount of time spent in each type of arms using a Student paired *t* test. The time spent in non-protected arms was significantly ($p<0.05$) lower compared to the time spent in the protected arms in the WT ($t=6.8$; $p<0.05$), in the SV2C−/− ($t=7.5$; $p<0.05$), in the mictrl ($t=8.84$; $p<0.05$) and in the miSV2C ($t=-11.4$; $p<0.05$) groups (Fig. 5A).

On the contrary, one way ANOVA using group as factor, showed a significant effect on the frequency of entrance in the non-protected arm (Fig. 5B) from the protected arm ($F[3,76]=3.77$; $p<0.05$). A post hoc Bonferroni test showed that this frequency was significantly ($p<0.05$) decreased in the SV2C−/− group compared to WT mice.

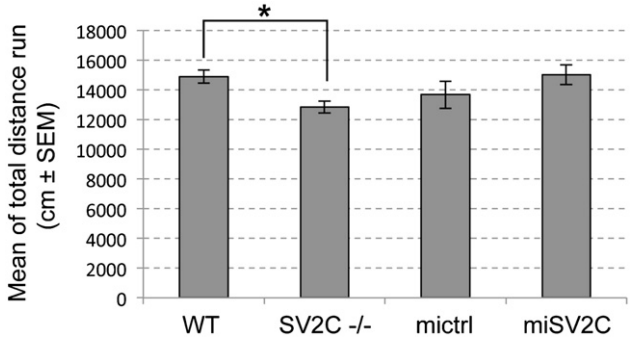

Fig. 4 – Open field behavior. Distance run in an open field arena is represented for each experimental group showed that SV2C/−/− mice explored significantly ($p<0.05$) less than WT mice. Significant differences are indicated by * for $p<0.05$. Data are reported as mean ± SEM.

2.3.4. *Conditioned place preference*

Since SV2C was mostly expressed in midbrain dopaminergic areas and basal ganglia, we have investigated how loss of SV2C can modify activity in the brain reward system and perturb the expression of addictive behavior such as conditioned place preference.

In each group, place preference scores of saline- and cocaine-treated (4 mg/kg) animals were compared using Student *t* test. We noted that in WT, SV2C−/− and mictrl groups the place preference score is significantly ($p<0.05$) higher in cocaine-treated mice compared to saline-treated mice (respectively, $t=-3.24$; $p<0.05$; $t=-3.65$; $p<0.05$ and $t=-3.91$; $p<0.05$). Animals of these three groups developed place preference when they were paired with cocaine injection (Fig. 6). On the contrary, in the miSV2C group, mice receiving cocaine did not develop place preference (no significant difference noted) (Fig. 6).

A two way ANOVA using group and treatment (saline or cocaine injection) showed a significant effect of the group ($F[3,74]=3.757$; $p<0.05$) and of the treatment ($F[1,74]=16.15$; $p<0.05$), but no significant interaction between those factors have been noted. Bonferroni post hoc test showed that score in WT, KO and mictrl were significantly ($p<0.05$) higher than the score of the miSV2C group.

The present results showed that only mice bearing a specific midbrain SV2C down-regulation through local injection of miRNA failed to develop place preference induced by cocaine. To determine if this lack of association between place and drug injection was due to a deficit of drug action, we submitted animals to an acute cocaine injection.

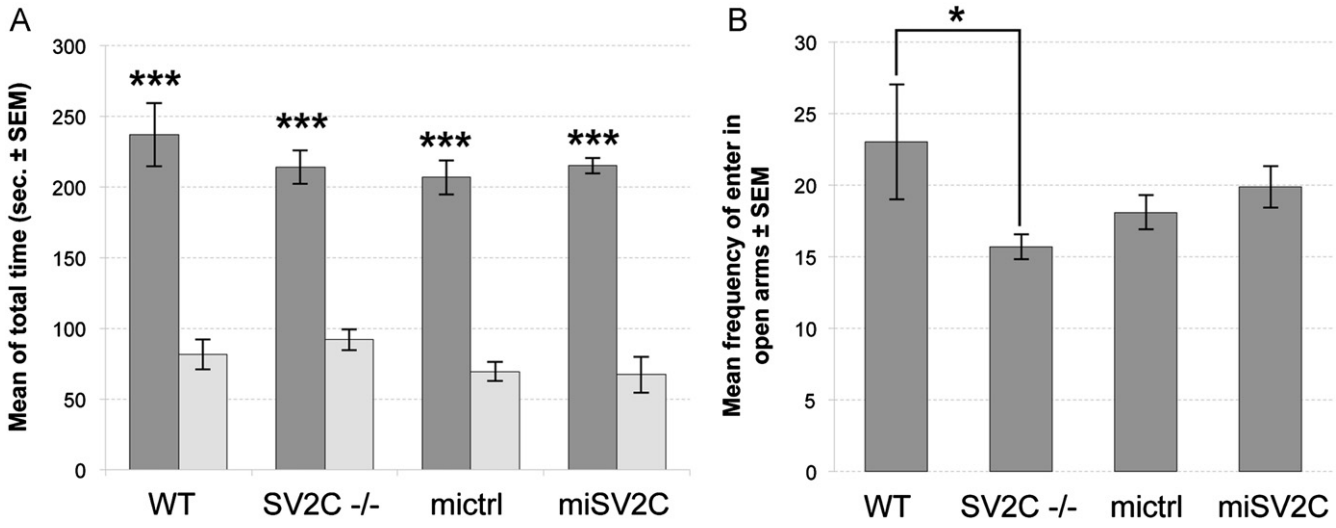

Fig. 5 – Elevated plus maze behavior. (A) Time spent in the protected arms (in deep gray) and in non-protected arms (in light gray) was represented for each experimental group. All experimental groups spent significantly ($p<0.001$) less time in the open arms. Significant differences are indicated by * for $p<0.001$. Data are reported as mean ± SEM. (B) Frequency of entrance in the non-protected arm is represented for each experimental group. We observed that SV2C−/− mice have a significantly ($p<0.05$) lower number of entries in the open arms compared to the WT. Significant differences are indicated by * for $p<0.05$. Data are reported as mean ± SEM.**

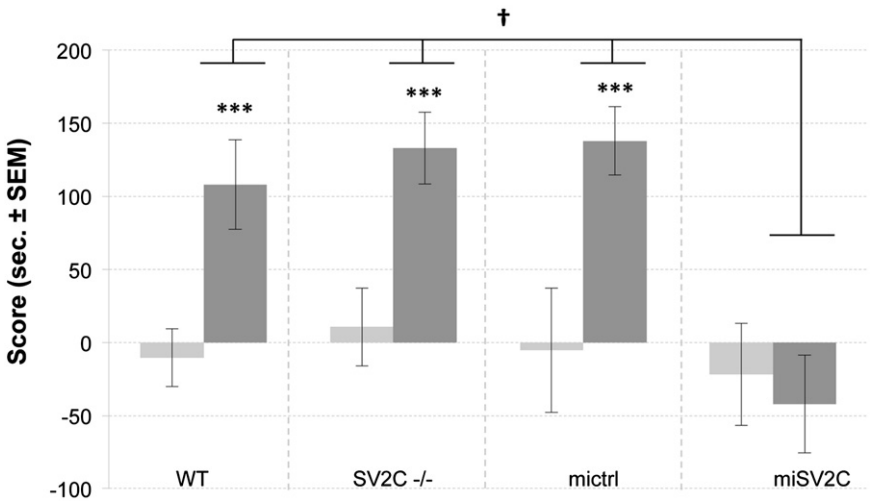

Fig. 6 – Conditioned place preference behavior. Place preference score is represented for each group, in saline- (light gray) and cocaine- (deep gray) conditioned mice. In all groups, excepted the miSV2C group, mice significantly ($p<0.001$) preferred the drug paired compartment as compared to the saline paired one. Significant differences between saline and drug conditioning in each group are indicated by * for $p<0.001$. Differences between groups are indicated by † for $p<0.001$. Data are reported as mean ± SEM.**

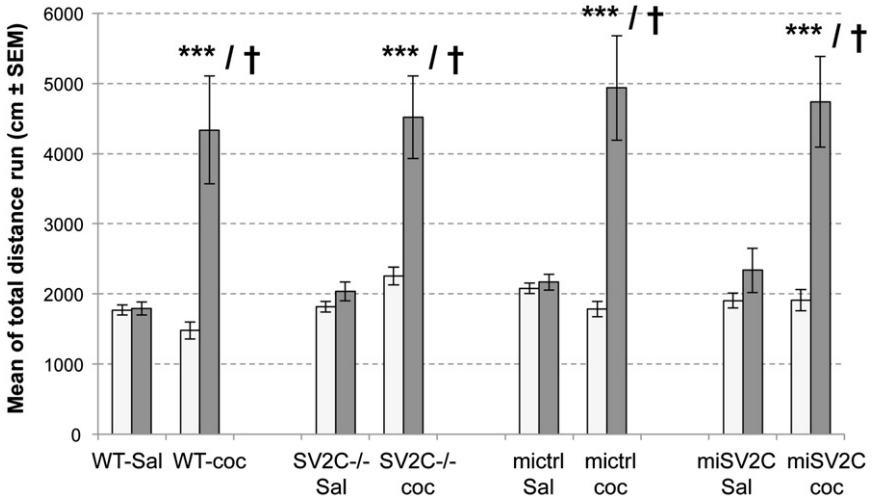

Fig. 7 – Acute cocaine injection. The total distance run by mice on the habituation day (white bars) and on the injection day (gray bars) following a single drug dose injection is reported as mean of total distance run for 30 min ± SEM. Saline-treated mice (WT-sal, SV2C−/− sal, mictrl-sal and miSV2C-sal) received saline on the second day while cocaine treated mice (WT-coc, SV2C−/− coc, mictrl-coc and miSV2C-coc) received a single dose of cocaine. Differences between saline and drug treatment in each group are indicated by * for $p<0.001$. Differences between groups are indicated by † for $p<0.001$.**

2.3.5. *Cocaine acute injection*

In this behavioral experiment, we have submitted our experimental groups to an acute cocaine injection (4 mg/kg) in order to investigate whether animals have a reaction deficit toward drug injections. After one day of habituation, mice received a single injection of saline or cocaine. Their locomotor activity was recorded during 30 min.

In all groups, there was no significant effect of saline injection on locomotor activity (Fig. 7). In the WT mice group, a significant increase in locomotor activity ($t=-4.067$; $p<0.05$) was observed between habituation and injection days in mice receiving cocaine injection. Similarly, in SV2C−/−, mictrl and miSV2C groups, mice receiving a single injection of cocaine significantly ($p<0.05$) increased their locomotor activity in the injection day compared to the habituation one (respectively: $t=-3.243$; $p<0.05$; $t=-4.471$; $p<0.05$ and $t=-3.941$; $p<0.05$) and as compared to the saline-injected mice.

A two way ANOVA with the groups and treatment as factor did not point significant effect of one of these factors.

These results showed that all mice exhibited a strong increase in locomotor activity following cocaine injections that did not significantly differ from each other.

3. Discussion

In the present study, we have investigated the involvement of SV2C expression in basal ganglia functions in mice. At the molecular level, the absence of SV2C in mice brain induced a decrease in ENK and Dyn mRNA in the striatum and an increase in TH mRNA in the SNpc/VTA. In addition, we noted that the decrease of dopaminergic innervation due to the lesion of the SNpc/VTA region induced an increase of SV2C mRNA expression in the striatum in two different models of dopaminergic denervation. We also addressed the possible involvement of SV2C in normal mice behavior. At the behavioral level, SV2C−/− mice exhibited a disturbed behavior in open field since they explored less than other experimental groups. In addition, behavior of mice in elevated plus maze have also showed a modification of their anxiety-like behavior. The most striking result came from CPP experiment. We observed that mice who have received injection of AAV expressing miRNA targeting SV2C mRNA in the ventral midbrain (SNpc/VTA) did not develop place preference induced by cocaine injection whereas all the other experimental groups (WT, SV2C−/− and mictrl) develop strong association between place and drug injection. This observation was completed by the fact that mice responded normally to one injection of the drug indicating that the failure of midbrain SV2C knock-down (miSV2C) mice to learn association between drugs and place was not due to a lack of efficiency of the cocaine injection.

To the best of our knowledge, the present study, aiming at understanding the possible role of SV2C in basal ganglia functions, is the first to use SV2C−/− and mice with a specific miRNA targeting SV2C in the ventral midbrain (miSV2C mice) in behavioral and gene expression experiments. The injection of such miRNA construct directly in midbrain regions (SNpc/VTA) would normally allow a complete decrease of SV2C mRNA expression without directly disturbing dopamine-containing neurons functioning. However, we did not observe a complete down-regulation of SV2C mRNA in the group of miSV2C mice but rather a marked decrease. Although, the SV2C knock-down was not complete, we believed that this strategy usefully extended the data obtained on the full SV2C knock-out mice. To avoid misinterpretation of our results, we have only included mice that showed at least 40% of SV2C mRNA decrease in these brain regions.

We have previously described the restricted expression of SV2C expression in the mouse brain. SV2C, gene and protein, expression is only present in olfactory bulb, dentate gyrus of hippocampus, basal ganglia and in dopaminergic midbrain regions. We noted that SV2C is highly expressed in most dopaminergic neurons within the SNpc and VTA, in MSN and in a subpopulation of cholinergic interneurons in the striatum (Dardou et al., 2011). Due to the peculiar pattern of expression of SV2C, we further investigated here if the complete absence of SV2C in mice brain could modify expression of the main markers of the striatum and SNpc/VTA. Surprisingly, only ENK and Dyn mRNA expression were decreased in the striatum of the SV2C−/− mice, and, in same time, TH mRNA was strongly increased in the SN/VTA. Enkephalin is expressed only in MSN of the indirect pathway in the striatum. Although our previous study showed that SV2C expression is important in striatal MSN, we did not show a specific expression of this protein in this motor regulation pathway as compared to the direct pathway since high level of SV2C protein is present in terminals in both the globus pallidus and the substantia nigra pars reticulata (Dardou et al., 2011).

It is well known that dopaminergic inputs to the striatum strongly and negatively regulate ENK expression contrary to Dyn expression (Gerfen et al., 1991). The increased expression of TH in SNpc dopaminergic neurons of SV2C−/− mice could therefore explain this decrease in ENK mRNA. It can be noted that MSN also received inputs from the cholinergic interneurons. These tonically active neurons are known to regulate activities of MSN via GABAergic innervation and/or through the control of dopamine release (Aosaki et al., 1994; English et al., 2011). The absence of SV2C in cholinergic neurons could elicit a change in the inhibitory regulation of these interneurons on striatal D2-MSN of the indirect pathway. Nevertheless, these hypotheses need further investigation since only ENK and Dyn mRNA expression was modified whilst no change in SP mRNA levels was noted. More specific decreases in ENK mRNA have been described in murine model of Huntington disease (Menalled et al., 2000) or in rats with ibotenic acid-induced lesions of the intralaminar thalamus (Salin and Kachidian, 1998). However, we may underline that, in SV2C−/− mice, we did not observe any morphological changes characterizing such pathological modification. In addition to this change in mRNA expression, we observed a significant large increase in TH mRNA expression in SNpc/VTA regions in SV2C−/− mice. Such increases in TH mRNA expression have been observed following chronic drug administration, ibotenic-acid induced lesion of the striatum and striatal GDNF injection (Hurd and Herkenhalm, 1992; Vrana et al., 1993; Hantraye et al., 1994; Ortiz et al., 1995; Salvatore et al., 2004). In the present results, since no clear molecular role has been defined for SV2C, it is hard to conclude whether such major change in mRNA expression are related to changes in neuronal activity, dopamine release, production of neurotrophic factors or modification in striatal input strength.

The SV2 proteins family is formed by three isoforms. Each of these have different patterns of expression, since SV2A is ubiquitously expressed in the whole brain, the SV2B isoform is widespreadly expressed in a large series of brain regions and the SV2C expression is limited to few brain regions including mostly the basal ganglia system (Janz and Südhof, 1999; Dardou et al., 2011). This protein family is amongst proteins involved in synaptic vesicle formation and/or release, and to date, it seems that SV2A also acts as receptor for botulin A toxin (Verderio et al., 2006 and Dong et al., 2008). With the possible involvement of SV2C in synaptic vesicle molecular machinery, the absence of this protein in SV2C−/− mice could disturb dopamine release or local GABA and acetylcholine release within the striatum. Change in the phasic dopamine release could disturb the tonic activity of cholinergic interneurons which make a pause when dopamine-containing neurons make phasic burst of activity (Aosaki et al., 1994). In SV2C−/− mice, two mechanisms could be disturbed. Firstly, the phasic burst activity of dopaminergic

neurons could induce some subtle modifications of cholinergic neurons activity, which in their turn could modify the activity of D2-MSN in striatum. Secondly, the loss of SV2C in dopaminergic neuron could also, by modifying dopamine release, induce the decrease in the auto-regulation process on these neurons (via dopamine auto-receptor), leading to a drastic increase of TH mRNA synthesis. To date, these hypotheses need further investigations.

Nevertheless, whatever are the mechanisms leading to the molecular changes in the basal ganglia network, these change induced by the loss of SV2C could account for the behavioral differences detected in SV2C−/− mice. In open-field, SV2C−/− showed a decrease in exploration compared to others experimental groups. In addition, these mice showed an increase in their anxiety since they entered less than WT mice in the open arms. These behavioral tasks strongly involved motor and attention capacities. It is worth to note that since no modification in these behavioral processes was observed in midbrain SV2C-knock down (miSV2C) mice; such modest difference could be more related to the loss of SV2C expression in the striatum.

The complex interconnection and regulation between cholinergic interneurons and MSN is known to be involved in various aspects of motivated and anxious behaviors in animals (Sauvage and Teckler, 2001; Yarom and Cohen, 2011). Since they seem to respond to reward or to stimuli that predicted a reward, a large number of studies have investigated this neuronal population in the striatum. To date, the tonic activity of these neurons has been implied in various aspects of behaviors ranging from detection of aversive stimuli to recognition of the context and spatial locations of stimuli (Ravel et al., 2003; Lee et al., 2006; Shimo and Hikosaka, 2001). Our results are in agreement with a part of these data about involvement of cholinergic modulation of striatal circuit in attentional aspects of behavior. It is possible that SV2C in cholinergic interneuron and/or in MSN are implied in some aspect of the normal functioning of the limbic and/or motor circuits involved in behaviors, mainly attention and anxiety, in mice. On the other hand and surprisingly, SV2C−/− mice performed well in an associative learning task, the CPP, while miSV2C groups completely failed to learn the association between drug reward and compartment. The absence of any alteration in associative learning in the full knock-out mice could be due to a paradoxical compensation of the change observed in dopamine containing neurons by the inactivation of SV2C in striatal cholinergic interneurons and/or in MSN in SV2C−/− mice.

The fact that local disruption of SV2C expression in midbrain dopaminergic fully blocked the drug associative learning strongly suggests a role for SV2C in dopamine release within the striatum. Dopamine is implied in the induction of LTP in striatum, and more particularly in the nucleus accumbens (Pennartz et al., 1993; Schotanus and Chergui, 2008) and have a modulatory effect on behavior as well as deep implication in addiction (Di Chiara, 2002). Disruption of SV2C expression in the local midbrain circuit possibly altered some aspect of dopamine release in the limbic loop, hence inducing a perturbation of the striatal memory association process. Such change could be sustained by the selective down-regulation of SV2C expression in dopaminergic neurons, since the complete absence of SV2C in SV2C−/− did not interfere with the learning of CPP due to a paradoxical compensation by SV2C inactivation in MSN or choloinergic interneurons (see above) and/or a developmentally-regulated compensation.

As demonstrated previously (Dardou et al., 2011) and strongly illustrated above, SV2C expression is clearly presynaptically linked to the dopaminergic system. We further investigated whether dopaminergic neurons could regulate the post-synaptic expression of SV2C and indeed showed that disruption of dopamine transmission increased SV2C expression in the striatum in two different models of dopamine depletion. As stated above, it is well known that lesion of the dopaminergic system induced adaptive changes in the striatum such as increase in enkephalin expression and decrease in substance P expression. Change in SV2C expression in the striatum could be related or a part of such adaptive changes.

Taken together, these results bring new information regarding the possible role of SV2C, a poorly functionally characterized protein. Based on the present results, SV2C seems to be implied in the basal ganglia working since its absence induced marked change in gene expression of specific markers and led to alterations in basal ganglia-mediated behaviors as exploratory behavior or associative learning. One of the possible hypothesis is that SV2C participate to the regulation of dopamine release and/or on electrical properties of the dopaminergic and cholinergic communication by subtly altered their phasic and/or tonic activities bursts or also directly on MSN properties. Nevertheless, even if these first data demonstrated the functional importance of SV2C in the basal ganglia network, further experiments are needed to clarify the hypotheses and to better characterize the relation between the dopamine system and SV2C expression.

4. Experimental procedures

4.1. Animals

Different series of mice have been used in the different experiments. Wild-type mice (WT) were adult male C57BL/6J weighed 25–30 g at the start of the experiments (Charles River, France). For each experimental group, the number of animals used is presented in Table 1.

4.1.1. Knock-out mice

Adult male SV2C-knock-out mice (SV2C−/−), have been generated by targeting gene disruption on a C57BL/6J background (Dardou et al., 2011). In these mice, SV2C expression has been completely extinguished in all brain areas as compared to their WT littermates (Dardou et al., 2011 and Fig. 3).

4.1.2. Knock-down mice

We also used mice with a specific knock-down of SV2C mRNA expression in the SN/VTA area and their controls by injection of AAV expressing miRNA targeting SV2C mRNA, performed

in the ventral midbrain (SNpc/VTA) of C57BL/6J background mice (miSV2C group).

For these, plasmids containing artificial microRNAs (miRNA) were based on vectors described by Boudreau et al. (2009) to minimize cellular toxicity in neuronal tissue associated with expression of short-hairpin RNAs (shRNAs). In brief, several artificial miRNAs targeting SV2C were cloned into plasmids under control of the U6 promoter (Boudreau et al., 2008). All SV2C related miRNA constructs were produced by gene synthesis (GenScript USA Inc) and confirmed by sequencing. Artificial miRNA constructs were subcloned for AAV expression into pFBGR-derived plasmids co-expressing CMV-driven green fluorescent protein (Boudreau et al., 2009). For SVC knock down in vivo the following sequence TCGATGTCGTCACGGTGGAACTGTA was inserted in the above vector. A vector contained unrelated sequence (AACUCUGACGGUUCGAUGGUA) served as negative control.

Several artificial miRNA constructs were generated and tested in vitro. HEK cells stably expressing mouse SV2C were transiently transfected with artificial miRNA constructs and knock down of SV2C mRNA was measured by qPCR. In brief, HEK cells were cultured in 6 well plates and transfected with Lipofectamine following the manufacturers guidelines. Cells were harvested 48 h post transfection and RNA extracted (Qiagen, RNAeasy kit). SV2C mRNA levels were quantified by qPCR using ABI inventoried probes using the ΔΔCq method using β-actin as endogenous control. The construct with the artificial miRNA sequence TCGATGTCGTCACGGTGGAACTGTA was providing maximum knock down in this in vitro assay and was used for consecutive in vivo studies.

University of Iowa Vector Core facility did produce rAAV serotype 2/1 in insect cell (Sf9)/baculovirus-based system as previously described (Urabe et al., 2002). AAVs were purified by a two-step purification process consisting of an iodixanol gradient followed by an ion exchange step. qPCR was used to determine rAAV titers (viral genomes/ml).

Adult (8 weeks old) male C57bl/6J mice were anesthetized by intraperitoneal injection (10 ml/kg) of Rompun (Xylazine, 8 mg/kg) and Imalgene (Ketamin, 80 mg/kg). Animals were then placed on a stereotaxic frame (Kopf 900, David Kopf Instruments, Tujunga, CA, USA) and an ophthalmic cream (Visidic) was applied on the eyes to avoid drying. Following a skull skin incision and a small skull perforation (−3.2 mm anteroposterior; + and −1.25 mm lateral from bregma (bilateral injections); −4.25 mm dorsoventral), virus containing artificial miRNA against SV2C (AAV2/1 miSV2C), or controlled virus (AAV2/1 mictrl) were injected (0.4 μl, 4 min, 3×10^{12} viral genomes/ml) using a Hamilton syringe (25 μl) coupled with 0.2 mm diameter needle. After the injection, the needle was kept in the same position for 6 min to allow diffusion of the virus in the tissue and avoid loss of virus upon removal of the pipette. After removal of the needle, bone wax was used to seal the skull, isobetadine was applied to avoid infection, tissues were sutured (Safil 4/0) and isobetadine was applied on the skin. The animals were then placed back in their home cage and health-monitored until further use. Infection of the relevant brain area with AAV was confirmed in separate pilot experiment using GFP fluorescence. The knock-down of SV2C expression was controlled by in situ hybridization (ISH) at the end of the experiments.

4.2. Dopamine depletion models

4.2.1. 6-OHDA lesion

In order to explore the change in SV2C expression in dopamine depletion models, we performed 6-OHDA- and MPTP-induced lesions in WT adult male C57BL/6J mice. 6-OHDA lesions were performed by unilateral injection of 6-OHDA (3 μg/μl dissolved in 0.02% ascorbic acid, Sigma France) into the striatum at two different points (+1 mm anterior, 2.1 mm lateral and −3.2 mm from dura and +0.3 mm anterior, 2.3 mm lateral and −3.2 mm from dura; all coordinates are from bregma). The two injections were 2 μl injection at 0.5 μl/min and needles were let in place at least 10 min. Eight mice were treated and used for this experiment. The non-lesioned sides were used as controls.

4.2.2. MPTP induced lesion

Five mice have received a total of four i.p. injections of MPTP (20 mg/kg, Sigma France) dissolved in saline solution (NaCl 0.9%) every 2 h. After a resting period of seven days, animals were killed and prepared for ISH procedure. Six mice received only saline injections and were used as control.

4.3. Behavioral tasks

4.3.1. Experimental groups

The number of animals in each group for the different experiments is indicated in Table 1.

Mice from the four experimental groups, (WT, SV2C−/−, miSV2C and mictrl) were housed in groups of 4 in clear plastic cages and maintained in a temperature - and humidity- controlled room on a 12 h light/dark schedule with food and water provided ad libitum. The number of animals used for this study was kept to a minimum, and all efforts to avoid animal suffering were made in accordance with the standards of the European Community guidelines for the use of experimental animals. In addition, the protocol described below was approved by the Institutional Ethical Committees of UCB-Pharma (Braine-L'Alleud, Belgium) and of the Faculty of Medicine of the Université Libre de Bruxelles according to Belgian law.

4.3.2. Open field

Open field experiment was conducted in 15 cm × 15 cm × 30 cm plexyglas boxes. Sixteen experimental boxes, eight per rooms, were recorded simultaneously using Ethosion XT software. Since we wanted to study the spontaneous exploratory behavior in our experimental groups, mice were not previously habituated to the open field boxes. Animals were habituated to the experimental handler during 3 days, at least 5 min per animal. The total distance run during 30 min was recorded and compared using one way ANOVA (group as main factor) followed by Bonferroni test (significance was set at $p < 0.05$).

4.3.3. Elevated plus maze.

Mice were submitted to an elevated plus maze in order to test their arousal towards environment and the expression of anxiety. Elevated plus maze is composed of four branches (30 cm × 7 cm), two branches have walls (17 cm of height) and

correspond to protected arms, the two other branches have no walls and correspond to non-protected arms. The maze was placed on white floor at 40 cm height and under bright (100 lux) illumination. All trials were recorded and analyzed using EthoVison XT software. Each mouse is placed in the center of the maze and stays 5 min in the maze. The time spent in non-protected and in protected arms, as well as, frequency of entering in open arms were recorded and analyzed using a one way ANOVA (group as factor) followed by Bonferroni test ($p<0.05$).

4.3.4. *Conditioned place preference*

Experimental system is composed of four place conditioning boxes. Each device corresponds to a rectangular box divides in two distinct compartments (each 18×20 cm) connected by a short neutral alley (6×20 cm). The first compartment has a smooth floor and black lines drawn on the wall and is described as Line compartment. The other compartment has a rough floor and has black dots drawn on the wall and is described as Dot compartment. During the first day, animals were placed in the neutral alley of the experimental box and have free access to both compartments during 18 min. Animals showing a preference for one compartment were removed from the experiment. All mice, whatever their groups were divided in two subgroups: conditioned and non-conditioned mice. For conditioning, during the six following days, mice were placed in a compartment of the boxes and received injection of cocaine (4 mg/kg) on the first, third and fifth days whilst they received injection of the vehicle (NaCl, 0.9%) in the other compartment on the second, fourth and sixth days; animals stayed in the box during 20 min. Non-conditioned mice received only saline injections whatever the compartment or the day of the experiment. On the last day, mice were placed anew in the experimental boxes with free access to the two compartments for 18 min. The time spent in each compartment was recorded. In each group of mice, 50% of the animals have been drug-paired in the Dot compartment and the other 50% have been drug-paired in the Line compartment, ensuring that all treatments were counterbalanced as closely as possible between the compartments. Results were expressed as the difference between postconditioning and preconditioning time spent in the drug-paired compartment (Score, in sec) and were compared between groups using a two way ANOVA (with group and condition as factors), followed by Bonferroni test ($p<0.05$).

4.3.5. *Acute cocaine injection*

After the conditioned place preference experiment, mice that have received cocaine injection were sacrificed, and mice that only received saline injection were submitted to a single cocaine injection to check if the drug has effect on motor activity. Mice were habituated by placing them in open field boxes during 30 min for one day and then, on the next day, they received one injection of cocaine (4 mg/kg) before being placed again in the boxes. Control mice received saline vehicle injections. Mice were left 30 min in the box after drug or saline injections and the total distance run during this period was recorded. Statistical comparisons were made using two ways ANOVA (group and injection as factors) followed by Bonferroni test.

4.4. *In situ hybridization*

Mice were sacrificed by decapitation and their brains were quickly removed and frozen in 2-methylbutane cooled by dry ice. Brains were serially cut in 18 μm coronal sections and thaw-mounted onto SuperFrost Plus slides (Menzel–Glaser). Mounted tissues were stored at −20 °C until use. Slides were fixed by immersion in a freshly-prepared buffered 4% formaldehyde for 30 min and rinsed in PBS 0.1 M. All sections were dehydrated by successive alcohol washes and dipped for 5 min in chloroform. After air drying, slides were incubated overnight at 42 °C with 0.35×10^6 c.p.m. of ^{35}S-labelled probes diluted in the hybridization buffer (Dassesse et al., 2001; Dardou et al., 2011). After hybridization, slides were rinsed for 2×5 min in SSC $2\times$ at room temperature and then for 4×15 min in SSC $1\times$ at 55 °C and finally dehydrated. Slides were covered with Hyperfilm-βmax film (Amersham) for 3 weeks. The specificity of in situ hybridization (ISH) was assessed by the absence of SV2C mRNA labelling in SV2C−/− mice, by the comparison of the localization of the different mRNAs using oligonucleotide probes of the same size and similar guanosine-cytosine content and by the fact that all distributions were in agreement with previously published reports (Schiffmann and Vanderhaeghen, 1993; Soghomonian and Martin, 1998; Dassesse et al., 2001 and references therein).

Oligonucleotide probes were synthesized by Eurogentec (Belgium) with the following condition: a GC:AT ratio between 45% and 65%. In the present experiment, the following probes were used (Dassesse et al., 2001; Dardou et al., 2011): SV2C probe (5′-CTGGGCCCGATCCACTGCCTGGTTCACCTTCTTCACCGTCTGCTT-3′) which is complementary to the nucleotides 302–347 of the SV2C sequence, SP probe (5′-GCGTCACTGCCAAGCTTGGACAGCTCCTTCATCACTGTGCTTTGC-3′), Enk probe (5′-GGCCAAGGTGTCTTCCTCATCCTTCTTCATGAAGCCGCC-3′), TH probe (5′-GGTCAGGGTGTGCAGCCCCTCTAAGGAGCGCC-3′), D1 probe (equal mix of GACAGGGTTTCCATTACCTGTGGTGGTCTGGCAGTTCTTGGCATGGAC and GCTGAGATGCGCCGGATTTGCTTCTGGGCAATCCTGTAGATACTG), D2 probe (equal mix of CGCTTGGTGTTGACCCGCTTCCGACGCTTGCGGAGAACGATGTAG and GCGGGCAGCATCCATTCTCCGCCTGTTCACTGGGAAACTCCCATT), Dyn probe (5'-ATTCTGGGATGGGCAGGGATCACAGGGACAGAGCGGTTACGCTGA-3'), A2A probe (AACTCTGCGTGAGGACCAGGACAAAGCAGGCGATGAAGAGGCAGC), GAD65 probe (GCCATCTTCAGATCCGAAGGACCAAAAGCCGGAGCCAGGAGACGC) and GAD67 (TGGGCTACGCCACACCAAGTATCATACGTTGTAGGGCGCAGGTTGGT). All these probes were labelled with [α-^{35}S]dATP (PerkinElmer) at the 3′ end by Terminal Deoxynucleotidyl Transferase (Invitrogen) and purified two times by alcohol precipitation and with a nucleic acid purification by passing on column (G35 sephadex). SV2C and TH mRNA expressions were studied in the SNpc/VTA from +1.34 mm to −0.08 mm from interaural line. SV2C, ENK, Dyn, SP, D1, D2, A2A, GAD65 and GAD67 mRNA expressions were studied in the striatum, caudate–putamen (CP) and nucleus accumbens (Acb) from +4.98 mm to +3.80 mm from interaural line. All labeling

were done on adjacent brain sections allowing a better comparison between animals.

Images were acquired from autoradiograms using camera coupled to Scion Image software. The level of background was set to be the lower as possible and the same between all images. Images were analysed using Image J software. Averaged optical densities (OD) in the areas of interest were measured and background level was subtracted to obtain corrected values. Data were expressed as optical density values of the experimental group (SV2C−/− mice, MPTP-treated mice or drug-injected side) in percent of the control group (WT mice, saline-treated mice or uninjected side).

4.5. *Dopamine transporter (DAT) binding autoradiography*

DAT binding was used to check the efficiency of the dopaminergic loss. Shortly, mice brains were serially cut in 20 μm thickness sections, thaw mounted on slides and stored at −20 °C. Slides were then incubated in a Tris 1 M buffer containing NaCl, KCl and BSA. For specific binding, we add in this buffer 50 pM of RTI [^{125}I] and 1 μM of Fluoxetine. The non-specific binding was assessed in adding 10 μM of GBR12935 in the RTI/Fluoxetine buffer, Slides were incubated in either specific or non-specific buffers for 1 h at room temperature in humid chambers. Slides were then rinsed four times in cold (4 °C) 1 M Tris buffer containing NaCl, KCl and BSA and shortly passed in cold water. Finally, slides were dried using cold air drier before being exposed to Hyperfilm-βmax film (Amersham) for 2 weeks. Image analysis was performed as described above. SV2C mRNA in situ hydridization was performed on adjacent sections as described above.

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