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Títol del treball: “Role of the SV2A protein in epilepsy and Alzheimer's mouse models”.

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Abstract:

SV2s family proteins are neuronal vesicles membrane glycoproteins that are highly conserved in evolution (Janz & Südhof, 1999). It is known that SV2A isoform is the target to Levetiracetam (LEV), an effective anti-epileptic drug (B. Lynch et al., 2004). There are certain hypotheses about his role in epilepsy. However, his physiological function and its interaction with LEV remains unknown. Specifically, it is known that a specific type of Temporal Lobe Epilepsy (TLE) presents a reduction of SV2A expression associated with an upregulation of SV2C isoform (Crèvecœur et al., 2014). The aims of this study consist in validating the proper operation of SV2A $^{lox/lox}$ sequence in order to invalidate SV2A expression and, secondly, analysing the expression of SV2B and SV2C in the absence of SV2A. For this purpose, a mouse line allowing the conditional removal of SV2A in the hippocampal region (CA3 and *dentate gyrus (DG)*) at postnatal stages 15 (P15) (Grik4:SV2A-cKO) was engineered. Unexpectedly, Grik4: SV2A-cKO transgenic mice did not present an epileptic phenotype. To determine the reason of these phenomenon, the concentration of the three isoforms was analysed and quantified. Results have shown a significant reduction of the quantity of SV2A transcript and protein in Grik4:SV2A-cKO animals hippocampus compared to the wild-type mice (WT). Together, these results confirmed the efficiency of the invalidation of SV2A in our mouse model. Parallel to these values, no significant change in SV2B or SV2C protein and transcript expression was observed implying an absence of compensation phenomenon. Nevertheless, many other hypotheses should be tested to explain this unexpected phenotype in Grik4: SV2A-cKO mice.

Finally, in order to resolve the role of SV2A in synaptic plasticity, a new nucleofection protocol for embryonic cortical neurons at the embryonic stage 16 (E16) *in vitro* was developed.

Key words: epilepsy, hippocampus, SV2A.

Resumen:

Las proteínas SV2 son una familia de glicoproteínas de membrana localizadas en las vesículas sinápticas neuronales caracterizadas por su alto grado de conservación a lo largo de la evolución (Janz & Südhof, 1999). Además, se ha descubierto que una de sus isoformas, la SV2A, es la molécula diana del principio activo antiepiléptico Levetiracetam (LEV) (B. Lynch et al., 2004). Se han propuesto varias hipótesis para entender su papel en la epilepsia. Sin embargo, la función fisiológica de esta proteína y su interacción con LEV son aún desconocidas. Recientemente, en un caso específico de "Temporal Lobe Epilepsy" (TLE), se ha caracterizado una reducción de SV2A ligada a una sobrexpresión de la isoforma SV2C (Crèvecœur et al., 2014). El objetivo de este proyecto consiste en validar el correcto funcionamiento de la secuencia $SV2A^{lox/lox}$ para eliminar la expresión de SV2A y analizar la expresión de SV2B y SV2C en ausencia de SV2A. Para ello, se ha diseñado una línea de ratón transgénico que permite la eliminación de SV2A en el hipocampo (CA3 y *dentate gyrus (DG)*) en el estado posnatal 15 (P15) (Grik4:SV2A-cKO). Sin embargo, este transgénico no presenta ningún fenotipo epiléptico como se esperaba. Para explicar fenómeno, se ha analizado y cuantificado la expresión de las tres isoformas. En comparación con el control (WT), se observa una reducción en la concentración de mRNA y de proteína SV2A en los animales Grik4:Sv2A-ckO. En conjunto, estos resultados confirman la eficacia de la invalidación de SV2A en el modelo animal propuesto. Paralelamente, no se ha observado ningún cambio en la expresión de las otras dos isoformas, SV2B y SV2C, confirmando la ausencia de un fenómeno de compensación. Aun así, muchas otras propuestas deben ser analizadas para intentar explicar este inesperado fenotipo inesperado de los ratones Grik4:SV2A-ckO.

Finalmente, para determinar el papel que tiene SV2A en la plasticidad sináptica, se ha desarrollado un nuevo protocolo de nucleofección *in vitro* para neuronas corticales de ratones en estado embrionario 16 (E16).

Palabras clave: Epilepsia, hipocampo, SV2A.

Resum:

Les proteïnes SV2, son una família de glicoproteïnes de membrana localitzades en les vesícules sinàptiques neuronals caracteritzades pel seu elevat grau de conservació al llarg de l'evolució. (Janz & Südhof, 1999). A més a més, s'ha descobert que una de les seves isoformes, la SV2A, és la molècula diana del principi actiu antiepilèptic Levetiracetam (LEV) (B. Lynch et al., 2004). Diverses hipòtesis han sigut proposades per entendre el seu paper en l'epilèpsia. Per contra, la funció biològica i com interactua amb LEV segueixen sense resposta. Recentment, en un cas específic de TLE, s'ha caracteritzat una reducció de SV2A lligada a una sobrepressió de la isoforma SV2C (Crèvecœur et al., 2014). L'objectiu d'aquest projecte consisteix en validar el correcte funcionament de la seqüència $SV2A^{lox/lox}$ per eliminar l'expressió de SV2A i analitzar l'expressió de SV2B i SV2C en absència de SV2A. Per realitzar-ho, s'ha dissenyat una línia de ratolí transgènic que permet l'eliminació de SV2A en l'hipocamp (CA3 i *dentate gyrus (DG)*) en l'estat post-natal 15 (P15) (Grik4:SV2A-cKO). No obstant, aquest transgènic no presenta cap fenotip epilèptic com s'esperava. En comparació amb el control (WT), s'observa una reducció en la concentració de mRNA i de proteïna SV2A en els animals Grik4:Sv2A-ckO. Conjuntament, aquest resultats confirmen l'eficàcia de la invalidació de SV2A en el model animal proposat. Paral·lelament, no s'ha observat cap canvi en l'expressió de les altres isoformes, SV2B i SV2C, confirmant l'absència d'un fenomen de compensació. Tanmateix, moltes altres propostes han de ser analitzades per intentar explicat aquest inesperat fenotip dels ratolins Grik4:SV2A-cKO.

Finalment, per determinar el paper de SV2A en la plasticitat sinàptica, s'ha desenvolupat un nou protocol de nucleofecció *in vitro* per neurones corticals de ratolins en estat embrionari 16 (E16).

Paraules clau: Epilèpsia, hipocamp, SV2A.

Contents:

Abbreviation used:

AEDs	Antiepileptic Drugs
CA	Cornu Ammonis
cKO	Conditional Knock out
CNS	Central nervous system
CRE	Cyclization recombination enzyme
DG	Dentatus Gyrus
DKO	Double Knock Out
EYFP	Enhanced Yellow Fluorescent Protein
E14-16	Embryonic stage 14-16
GFP	Green Fluorescent Protein
GRIK4	Glutamate Receptors Ionotropic Kainate 4
kDA	Kilo Dalton
KO	Knock Out
LEV	Levetiracetam
MTS	Mesial Temporal Sclerosis
PBS	Phosphate Buffered Saline
P7/15/30	Postnatal stage 7/15/30
qRT-PCR	quantitative Real-Time polymerase chain reaction
RT	Room Temperature
sEPSC	Excitatory Post-Synaptic Current
SIPSC	Inhibitory Post-Synaptic Current
SNARE	Soluble NSF Attachment Protein Receptor
SV2	Synaptic Vesicle protein 2 family
SV2A	Synaptic Vesicle protein 2 isoform A
SV2B	Synaptic Vesicle protein 2 isoform B
SV2C	Synaptic Vesicle protein 2 isoform C
SYT-1	Synaptotagmin 1
TRMs	Transmembrane Regions
Ub	Ubiquitin
WB	Western Blot
WT	Wild Type

1. Introduction:

The epilepsy is a chronic disease of the central nervous system (CNS) that is the fourth more common neurological disorder and affects people of all ages. It concerned approximately 50 millions people in the world and 3.4 millions in Europe. Epilepsy is characterized by abnormal discharges in short periods and unusual neuronal activity in cortex. This abnormal discharges are due to or imply the deregulation of the mechanisms involved in synaptic transmission driven by the synaptic vesicles (Casillas-espinosa, Powell, & Brien, 2012).

1.1 Nervous System and Synaptic vesicles:

The synaptic vesicles are responsible for the storage, secretion and liberation of neurotransmitters. These structures are found in the presynaptic region of the axons and release by exocytosis their content in the synaptic cleft. Discharge of neurotransmitters are due to an action potential conducted throughout all the axon by membrane depolarization due to voltage-gated sodium and potassium channels opening and inactivation (Rizzoli, 2014). A complex of proteins called SNARE - (*soluble NSF Attachment Protein) Receptor* -, responsible of fusions of synaptic vesicle's membranes to the presynaptic cell membrane, drives the synaptic vesicles exocytosis. This event will allow neurotransmitters to join specific receptors located in the post-synaptic membrane (Kiessling et al., 2015). Concretely, synaptic vesicle exocytosis is divided in four stages. During the first stage, neurotransmitters are loaded into synaptic vesicles. Loading of neurotransmitters requires a selective neurotransmitter transporter and a proton pump ATPase. Second, the vesicles dock at the active presynaptic membrane (docking) and prime at the plasma membrane (priming) in response to a higher intracellular calcium concentration raised by the action potential through the opening of voltage-gated calcium channels. Priming prepares the synaptic vesicle to fuse rapidly in response of the calcium influx involving the formation of partially assembled SNARE complex. At last, primed vesicles are triggered for exocytosis in response of Ca^{2+} elevation in the cytoplasm. Fusion is regulated by SNAREs complex by the interaction between the vesicle-SNARE and target-SNARE (Thomas C Südhof 2004; Rizzoli, 2014).

1.2 Synaptic Vesicle Protein 2 (SV2) family:

Like SNARE, other proteins are located in the membrane of the synaptic vesicle and are also implicated in the release of neurotransmitters. One of them is a family of protein called the synaptic vesicle protein 2 family (SV2), composed by three isoforms. The SV2 protein was identified for the first time using a monoclonal antibody generated against cholinergic vesicles isolated from the electric organ of the ray *Discopyge ommata*. Moreover, using antibodies raised in rats, two cDNA encoding for two proteins called SV2A and SV2B were identified. These

two amino acid sequences have 65% of identity, 80% of similarity (Bajjalieh et al., 1994) and revealed a homology to a large family of transporter proteins (Bajjalieh, Peterson, Linial, & Scheller, 1993). Specifically, five different transport activities of this protein were suspected: the acetylcholine (Ach), the biogenic amine serotonin, dopamine, epinephrine and norepinephrine. A transport of the inhibitory amino acids GABA, glycine and the excitatory amino acid glutamate was also mentioned but not fully demonstrated (Bajjalieh et al., 1994). The homology to neurotransmitters transporters is localized in the last 6 transmembrane domains and the six first domains are highly homologous to the subfamily transporters that include the human glucose transporter (Mendoza-Torreblanca et al., 2013) and bacterial and fungal proteins that cotransport sugars, citrate and drugs (Bajjalieh et al., 1993).

At the molecular level, the nucleotide sequence contains a 2226-pb open reading frame that structure a protein backbone of 82.7 KDa (Buckley & Kelly, 1985). As previously mentioned, the protein exhibits twelve hydrophobic transmembrane domains, but also three highly charged sites in the intravesicular loop (Figure 1) (Mendoza-Torreblanca et al., 2013). Other studies described a third isoform of SV2, the SV2C. This protein is more closely related to the SV2A isoform with a 62% of identity than SV2B isoform with 57% of identity. Its cDNA was isolated from a rat brain library, based on its high homology to SV2A in the N-terminus of the protein (Janz & Südhof, 1999). Specifically, the most conserved sequences of the three isoforms are transmembrane regions (TRMs) and cytoplasmic loops connecting the TRMs including the large loop between TMRs 6 and 7. On the other hand, the cytoplasmic N-terminal sequences and the large intravesicular loop are less conserved (Janz & Südhof, 1999). The differences between SV2A and SV2B isoforms are located in the N-terminal region. This portion is composed by 17 non-similar amino acids, being for the SV2A isoform from the 130 to the 120 amino acid and form the 56 to the 74 for the SV2B isoform (Figure 1) (Bajjalieh et al., 1994).

Another molecular characteristic of this protein is the three glycosylation sites in the large luminal domain between seventh and eighth transmembrane domains. It has been suggested that these three locations are respectively the amino acid 498 (N1), 548 (N2) and 573 (N3) (Figure 1). Induced mutations of any of these glycosylated sites cause an inactivation of the synaptic traffic. This result indicates that glycosylation plays thus an essential role in SV2 trafficking (Nowack et al., 2010). Moreover, the N3 domain was described as essential for the entry of Botulinum Neurotoxine E (BoNT/E) but also recognizes Botulinum Neurotoxine A (BoNT/A). This establishes that glycosylated SV2 proteins are functional receptors for BoNT/E and BoNT/A in neurons (Dong M. et al., 2008).

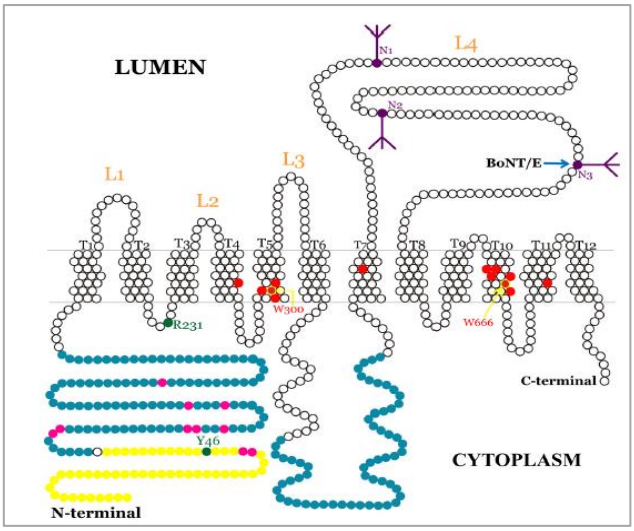

Figure 1: Schematic representation of SV2A protein. The 12 TMRs are illustrated and each circle represents one amino acid. The N-terminal domain, the first 57 amino acids in yellow, corresponds to the site of interaction with the C2B domain of SYT-1. Also, it is shown in pink the ten putative phosphorylation sites and the putative ATP-binding sites are indicated in blue. In purple are located the glycosylation sites essentials for the entry of BONT/A and BONT/E (N1, N2 and N3) (Mendoza-Torreblanca et al., 2013).

1.3 SV2 distribution:

These three isoforms are present in synaptic vesicles of all neural and endocrine secretory vesicles. Also, they are among the most abundant and conserved component of synaptic vesicles in vertebrates but no SV2-orthologuous protein could be identified in invertebrates indicating that SV2 is a relative recent acquisition during evolution. SV2C appeared to be the more ancient isoform due to its higher homology to the SV2 gene from the elasmobranch *Discopyge ommata* than SV2A or SV2B (Janz & Südhof, 1999). This idea is reinforced by its stricter distribution in evolutionarily ancient brain structures: the brainstem, basal ganglia and olfactory bulbs (Janz & Südhof, 1999). Referring to SV2A and SV2B isoforms, all brain structures express one or both forms. SV2A is expressed ubiquitously in all brain structures and in endocrine cells. However, SV2B expression exhibits varying degrees of co-expression of SV2A isoform and a more limited distribution, being undetectable in dentate gyrus of hippocampus, the globus pallidus, reticular nucleus of the thalamus and in the reticular part of substantia nigra (Bajjalieh et al., 1994).

1.4 SV2A putative role:

Nowadays, the function of SV2A is still unknown but still exist divers hypotheses. As previously said, it was suggested that SV2 proteins are synaptic-specific transporters due to their homology to transporters. However, this possibility was ruled out because of their presence in synapses with different types of neurotransmitter and the fact that the absence of SV2s does not modify the amount of glutamate package (Custer, 2006).

Other functions have then been proposed like the immobilization and subsequently liberation of neurotransmitters. SV2A glycosylated part is the main component of the internal vesicular lumen that holds and releases Ach and ATP by electrostatic interactions. It suggests thus that in early steps of vesicle fusion, this internal matrix modulate the quantity of transmitter released (Reigada et al., 2003).

Other studies raise the point that SV2 proteins have a putative ATP-binding site suggesting that the priming mediated by this protein is regulated by adenine nucleotides. Specifically, two binding site located in the cytoplasmic domain including the 1 to 7 transmembrane domains were identified (Figure 1). The first binding domain is positioned between the amino acid 59-162 that corresponds to a cytoplasmic N-terminal domain prior to the first transmembrane domain. The second domain lies between amino acids 382 and 439, corresponding to the loop between the 6 and 7 transmembrane domain (Yao & Bajjalieh, 2008).

In 2009, Chang and Südhof accumulate data demonstrating that the SV2's proteins are required for Ca^{2+}-triggering of the exocytosis but without being essential for normal priming of synaptic vesicles. This hypothesis was sustained by their study showing that the deletion of SV2 *in vitro* culture of cortical neurons causes a substantial loss of neurotransmitters release that operates downstream of synaptic vesicles priming, but upstream of Ca^{2+}-triggering of release. So, the most cautious and plausible hypothesis suggests that SV2 protein joins the maturation step of primed vesicles that convert the vesicles in Ca^{2+} and synaptotagmin-responsive state. This theory was also reinforced from the observation of SV2A KO phenotypes whose synapses do not conform to current models of release (Chang & Südhof, 2009). Even so, the exact function remains still elusive.

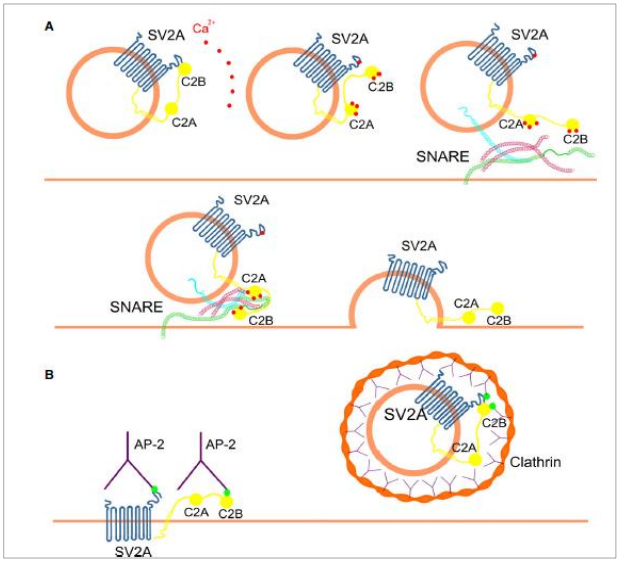

Figure 2: Schematic image of vesicle synaptic release. The potential mechanism of SV2A action as a regulator of Synaptotagmin 1(SYT-1) is showed. The interaction between the SV2A and SYT-1 could be necessary to maintain the SYT-1 inactivated before the moment of synaptic release. Ca^{2+} disrupts the union C2B – SV2A union allowing the interaction between SYT-1 and SNARE complex for synaptic vesicle fusion (A). The tyrosine (Tyr46)-base motif of SV2A and the C2B domain of SYT-1 are recognized by the clathrin adaptor protein (AP-2). During endocytosis, interaction between SV2A and C2B domain prevent SYT-1 diffusion (B). (Mendoza-Torreblanca et al., 2013).

Apart of this hypothesis, other studies indicate that SV2 performs at least two actions at the synapse that contribute to neurotransmitter release: an effect of synaptotagmin expression and an effect of synaptotagmin internalization from the plasma membrane. This internalization is due to the tyrosine-based motif in SV2 (SV2A-Y46A) that is predicted to serve as a binding site for the clathrin adaptor-protein 2 (AP-2) (Nowack et al., 2010). Thus, another potential function of the SV2 protein, as stated above, contributes in the priming of the vesicular release and in the endocytosis of the synaptotagmin precluding to the vesicle recycling (Nowack et al., 2010). SV2A may bind synaptotagmin-1 (SYT-1) in AP-2 site and restrict its diffusion after synaptic vesicles fusion and facilitate its endocytosis. In 1996, Batchelor et al. proposed that SV2A acts as a regulator of synaptotagmin-1 by the cytoplasmic N-terminal region due to the fact that neurons without SV2A have less vesicular SYT-1 and more SYT-1 in the plasma membrane. This N-terminal region is the variable region in the three isoforms of the SV2 proteins. It is composed of 57 amino acids and interacts directly with the C2B domain of the SYT-1. It is suggested that this interaction may control the initiation of synaptic vesicles fusion. The interaction between SV2A and SYT-1 through the C2B domain could be necessary to maintain the SYT-1 inactivated as it is needed during the Ca^{2+} rise in the cytoplasm of the presynaptic neuron (Figure 2). If the SYT-1 is not inhibited during this period, it could interact with the SNARE-complex in the C2B domain at a wrong moment. Moreover, this interaction between SV2A and SYT-1 suggest that SV2A is necessary for the endocytosis of SYT-1 after synaptic vesicle fusion and also, its absence reduce the size of the readily releasable pool (RRP) of SVs (Mendoza-Torreblanca et al., 2013).

1.5 Transgenic mice and study of SV2A expression:

To get a better understanding the role of this protein, some authors work with knock out (KO) animals for SV2's isoforms. No changes in the number of morphology of synapses or in synaptic vesicles were observed in this case, indicating that SV2 are not a vital structural component (Janz et al., 1999). Moreover, neurons without SV2A and SV2B showed an increase of Ca^{2+}-dependent synaptic transmission when two or more action potentials are triggered together (Mendoza-Torreblanca et al., 2013). Other studies have observed a reduced expression of SV2A in a rat hippocampus after an epileptic seizure (Venkatesan et al., 2012). In fact, deletion of SV2A alone was lethal at the early post-natal day 15 (P15) in mice while deletion of SV2B had no effect and a double KO for these two isoforms was no more deleterious than the deletion of SV2A alone. This phenotype could be explained due to the fact that all neurons apparently express SV2A but SV2B is co-expressed with SV2A in a subset of neurons. Thus, the lethality of only SV2A and SV2A/SV2B Double Knock Out (DKO) but not SV2B KO is

due to the functional changes in neurons expressing only SV2A, while SV2B has a little effect because there are few neurons expressing only SV2B. SV2C absence is not followed by lethality, probably due to is restricted to a very small subset of neurons (Janz et al., 1999). In humans, SV2A expression is decreased at 32% in the hippocampus Temporal Lobe Epilepsy (TLE) as compared in control patients (Van Vliet et al,. 2009)).

Crèvecœur et al., 2013 analyzed the expression of SV2s isoforms during rodent brain development to understand the role of this protein during brain development and their role in epileptic seizures. It was previously demonstrated that SV2A KO present epileptic seizures at P7 leading to death around the P15. Focusing the study of the expression from the embryonic day 12 to P30, it was shown an increase of the expression of SV2A and SV2B between P5 and P7 in the entire hippocampus and stabilization between P7 and P10. This increase seems to be restricted to the *Cornu Ammonis* 1 (CA1) region of the hippocampus and it is different than the SV2B expression that progressively increases. So, the increase of SV2A in CA1 in P7 correlates with the age of onset of epileptic seizures in SV2A KO animals.

The expression in SV2C in the hippocampus and olfactory bulbs during development is very low contrary to the striatum where SV2C is highly abundant. Regarding SV2A and SV2B, the expression in olfactory bulbs remains stable between P5 and P7 but significantly increased between P7 and P10 unlike the hippocampus (Crèvecœur et al., 2013).

An important effect seen in SV2A KO or SV2A/SV2B DKO mice is an abnormal action potential-dependent GABAergic neurotransmission in the CA3 of the hippocampus. Specifically, after deletion of SV2A protein, there is a significant increase of Excitatory Post-Synaptic Current (sEPSC) frequency and a significant reduction in Inhibitory Post-Synaptic Current (sIPSC) frequency and amplitude. On one hand, this increase of sEPSCs probably reflects an enhancement of glutamate neurotransmitter in the presynaptic site linking it with the idea of a calcium presynaptic regulation by SV2A. On the other hand, the reduction of sIPSCs is due to an altered GABAergic inhibitory neurotransmitter release and some studies suggest that it is occasioned by a presynaptic mechanism. Thus, this imbalance shown in SV2A KO animals is probably responsible of their phenotypic epileptic seizures (Venkatesan et al., 2012).

1.6 Levetiracetam and SV2-related pathologies:

Other studies show that decrease levels of SV2A (30-50%) are involved in the temporal lobe epilepsy (TLE). Therefore, it was shown in a specific type of TLE epilepsy, (MTS 1A; mesial temporal sclerosis 1A) the reduction of SV2A isoform is associate with an overexpression of SV2C isoform (Crèvecœur et al., 2014). Additionally, during hippocampal low-frequency stimulation (Hip-LFS) the expression of SV2A is increased and subsequently

GABA release is increased too (Wang et al., 2014), reinforcing the idea that decreased SV2A expression may contribute to the instability of neuronal network and therefore the progression of epilepsy (Mendoza-Torreblanca et al., 2013).

The epilepsy is a chronic neurological disorder having an important impact in health and quality of life. In 30% of cases, it is resistant to conventional antiepileptic drugs (AEDs), unable thus to provide effective seizure control in one third of patients (Klitgaard et al., 2016). TLE is the most common type of epilepsy and most patients with pharmacoresistant TLE are candidate to for neurosurgical resection of epileptic focus. However, this surgical resection is not an appropriate option in most cases due to the location of the starting point of seizures.

Few years ago, a new antiepileptic drug called Levetiracetam (LEV) (*α-ethyl-2-oxo-pirrolidine acetamine*) were developed and currently commercialized as Keppra® which is a potential new treatment for TLE. It is revealed that LEV possesses a unique preclinical profile leading to selective seizure protection with a high therapeutic index in epileptic animals (B.A.Lynch et al., 2004). It seems to have a completely different mechanism of action and did not act like the three current ways of AEDs such as facilitation of ɣ-aminobutyric acid (GABA)-mediated neurotransmission modulation of Na^+ channels or modulation of lower voltage-activated calcium currents (B. A. Lynch et al., 2004; B. Lynch et al., 2004).

It was demonstrated that the binding protein of the antiepileptic drug Levetiracetam is the SV2A (Nowack et al., 2011). Indeed, it was confirmed with a photoaffinity labeling of purified synaptic vesicles from mice lacking SV2A. These vesicles without SV2A do not couple LEV indicating that SV2A is necessary to LEV adhesion. Moreover, it was seen that LEV join to SV2A expressed in a fibroblast showing that SV2A is enough to LEV attachment (B. Lynch et al., 2004). However, the exact position of interaction of SV2A with LEV and the mechanism of this interaction remain unclear (Nowack et al., 2011). It is suggested that LEV binds to the SV2A's cytoplasmic side and entry into vesicles during recycling and endocytosis then escape during vesicle fusion. Therefore, it is further hypothesize that the binding region could be a region of the intravesicular side of the protein or a region that can be accessed from the vesicular side of the protein. Moreover, this binding side could be the highly glycosylated loop on the N-terminal domain with also bind to BoNT/E and BoNT/A (Meehan et al., 2011). There are few reports of the relation between SV2A and LEV in patients with epilepsy. But a study suggests that SV2A expression establish LEV efficiency, being an important parameter to decide if the patient should be treated with LEV. However, prospective controlled studies are needed to address this issue and to extend these results (De Groot et al., 2011).

2. Aims of the present study:

The main objective of this work was to better understand the role of SV2A in epilepsy. For this reason, our host laboratory developed a new type of transgenic mice: the Grik4:SV2A-cKO. This was obtained by mating mice characterized by the addition of flox sites flanking exon 3 of the SV2A gene and mice expressing the Cre-recombinase in neurons of the CA3 region and the dentate gyrus (DG) of hippocampus. This expression of the Cre-recombinase under the control of the promoter of the glutamate receptor type 4 isoform of the kainite subtype, allow the exon 3 of the SV2A gene excision between P14 and P40 in neurons of these regions, leading to an absence of the SV2A protein. Unexpectedly, it was shown a non-epileptic phenotype in this type of transgenic mice. Therefore, we want to find an explanation to this fact.

Firstly, the establishment of $SV2A^{lox/lox}$ mice line was validated with the Ub:SV2A-cKO transgenic mice as the recombination is then allowed very early during development and in all cell types leading to a phenocopy of the SV2A KO mice. The Grik4-Cre line was also validated in the Grik4-Cre/SV2Alox/EYFP transgenic mice allowing the expression of EYFP only in neurons of the CA3 and DG only after an efficient recombination step. Secondly, we hypothesized that the depletion of SV2A in hippocampus of Grik4:SV2A-cKO mice could be compensated by an upregulation of the other SV2 isoforms. To confirm or infirm this hypothesis, we analysed and quantified SV2A, SV2B and SV2C expression in Ub:SV2A-cKO and Grik4:SV2A-cKO mice using Western-blot and qRT-PCR.

The second aim of this study is to get a better idea about the neuronal adaptation to a SV2A loss. Indeed, SV2A has an important function in neuronal transmission and in neurotransmitters release. Specifically, this synaptic vesicle protein seems to be involved in pathological processes such as epileptic seizures and Alzheimer's disease, bought in connection with the hippocampus, particularly in the CA3 region. To study the changes in synaptic plasticity in SV2A KO neuronal cells, we will use the specific method of nucleofection. The aim of this method is to quantify and measure dendritic and axonal ramification in seeded neuronal cultures from SV2-floxed mice. $SV2A^{lox/lox}$ neurons from cortex will be nucleofected with a designed vector expressing the Cre-recombinase sequence to induce the deletion of SV2A and the GFP as a fluorescent marker.

3. Materials and Methods:

3.1 Animals:

In this study, we used transgenic mice with C57BL/6 strain origin. SV2A-floxed mice were obtained by Ozgene®, Ubiquitin-creERT2 and Grik4-Cre mice were purchased from Jackson Laboratory® (#007001 and #006474 respectively). Animal care was performed in accordance with the declaration of Helsinki and followed the guidelines of the Belgium ministry of agriculture in agreement with European Community laboratory animal care and use regulation (86/609/CEE, CE of J nº L358, 18 December 1986). Experimental researches on animals were performed with the approval of ethics committee of the University of Liège. The number of the file from ethic committee is 1753 and is accepted until December 31, 2020.

SV2AloxP-flanked mouse. The Cre-lox system is based on the ability of the Cre-recombinase enzyme to recombine with specific genetic sequence called lox sites. This tool allows us to inactivate the target gene flanked by lox sites only in tissues that express the Cre recombinase protein (conditional Knock-Out, cKO). Additionally, the expression of Cre can be ligand-dependent enabling us to invalidate a gene in a specific tissue and at a specific moment, when the ligand is given to the mouse. The transgenic $SV2A^{lox/lox}$ mouse displays lox sites around the third exon of the SV2A sequence. When a recombination occurs, the exon 3 deletion introduced an open reading-frame shift in the transcribed mRNA and, produced an early STOP codon that disrupted at the protein level the transporter/Major Facilitator Superfamily domain.

Grik4 Cre-recombinase mouse. This mouse line expresses the Cre-recombinase under the control of the promoter sequence *Grik4*. The *Grik4* gene code for the protein Grik4 that it is expressed specifically in the CA3 region of hippocampus and in DG. This specific localization of Grik 4 expression allows us to use its promoter to reproduce this expression pattern with the Cre-recombinase. Grik4-Cre mice are viable, fertile and they have not any physical or behavioral anomalies. The activity of Cre-recombinase is detectable at P14 in the CA3 of the hippocampus and in the DG. At 8 weeks of age the recombination is observed in the 100% of neuronal cells of CA3 approximately. To validate the proper function of Grik4-Cre mouse line, it used the Grik4-Cre/SV2Alox/EYFP transgenic mice. This type of transgenic are created by crossing a Grik4-Cre mouse/SV2Alox/lox with a reporter mouse containing in one hand, the SV2Alox/lox construction and on the other hand, a strong promoter, a stop codon floxed by two sequence lox and a sequence of the EYFP at the end.

Tamoxifen- inducible Ubiquitin-Cre-ERT2 mouse. In contrast with the Grik4-Cre system, this Ub-Cre transgenic mouse express constitutively the Cre recombinase in all cells but inactive in cytoplasm. Indeed, Cre-recombinase is linked to the mutated estrogen receptor recognized by tamoxifen and not by natural estrogen. Therefore, the nuclear translocation of the Cre-ERT2 is

only observed only after tamoxifen injection or added in the mice food. The translocation to the nucleus of the enzyme is then followed by the recombination of the floxed target gene SV2A.

When a Grik4-Cre line is crossed with a SV2A loxP-flanked mouse, the offspring presents the construction with the *Cre* gene which expression is regulated by the Grik4 promoter and the construction with the loxP sequences between the exon 3 of SV2A. So, this offspring presents de deletion of SV2A only in the CA3 and DG where the Grik4 protein is normally expressed. On the other hand, when the SV2A loxP-flanked mouse is crossed with the Ub-cre transgenic, the offspring presents, after tamoxifen administration, the deletion of the 3 exon SV2A in all cell types.

3.2 Transfection plasmid in *E. coli* and purification:

First, the plasmids GFP/Cre+(2197,1 µg/mL) or GFP/Cre- (5300,7 µg/mL) and the *E. coli* liquid culture were defrosted on ice. 1 µL of each plasmid solution were seeded separately in two Eppendorfs and 100 µL of the *E. coli* liquid culture was added in each of them. A thermic shock was done transferring tubes at 42°C for 45 seconds from ice and return it to ice for 2 minutes. Subsequently, 200µl of culture medium (LB medium; 10g Bacto-Tryptone, 5g Bacto-yeast extract and 10 g NaCl in 900mL of water, adjust pH 7.0. Sterilize by autoclave) were added and the cultures were started at 37°C during 1h under agitation. Agar plates with ampicillin were previously prepared and they were left at 37 °C overnight (O/N). 1mL of liquid culture was added in the agar plate (agar plate + LB + ampicillin 1/1000) and it was expanded with a Didralsky handle. After one night at 37 ˚C, single colonies were taken and transferred to a 3 ml liquid culture containing ampicillin (1/1000). After (overnight or one day +/-8h) at 37 °C the samples was transferred in a biggest liquid culture of 250mL and left O/N at 37°C. Finally, the culture was centrifuged at 20,000 g during 15 minutes and the supernatant was removed. Purification of the plasmid was done following the protocol of QUIAGEN Plasmid kit (Quiagen®, Venlo, Netherlands) (Quiagen plasmid, 2012).

3.3 Cortical neuronal culture and nucleofection:

Medium and plates preparations. The day before the dissection, a 24-wells cell culture plate was coated with a solution of 40 µg/ml of Poly-D-lysine (PLL) and 6µg/ml laminin diluted in sterilized water. 500 µL of the mix was added in each well and it was placed at 37°C during 2h or O/N at 4°C.

Dissection. Before the extraction of embryos in E14-E16 development state, three Petri dishes were prepared with PBS -glucose 0.1M 0.1%. The mice were sacrificed using the method of cervical dislocation. After the euthanasia, the mice belly was disinfecting with ethanol, embryos were removed, placed in a Petri dish and placentas were taken off. Then, the head of the

embryos was cut and placed in a new Petri dish. The brain was recovered keeping the head caught by eyeballs with the skull a dorsal position, the skin and the skull was removed until the brain was visible. The brain was taken off slowly putting the tweezers under the olfactory lobes and pushing back to peel it of the skull. The cortex was recovered removing the meninges, olfactory bulbs, hippocampus and striatum. Subsequently, the isolates cortices were placed in a Falcon° tube with 15 mL containing 1,5 mL of Trypsin DNase (0.25% trypsin, 0.1% DNase) and they were incubated at 37°C during +/- 20 minutes. Then, the samples were rinsed adding 2 mL of optiMEM (Dulbecco's Modified Eagle Medium, Invitrogen®, Merelbeke, Belgique) and the supernatant was aspired. 500 µL of serum FBS 10% and 2mL of optiMEM were then added. Subsequently, the serum and the optiMEM were aspired to wash. 2mL of optiMEM was re-added and washed. 3 mL of optiMEM was added slowly with a pipette of 5 mL and the sample was resuspended mechanically until all the tissue was dissociated. The number of cell per mL was assessed using a Thoma cell couting chamber and the samples were then diluted to obtain 10^6 cells per mL. 1 mL from this diluted solution was used to perform the nucleofection and the rest was put in a 24 well cell culture plate, 500µL per well. Finally, the plate was incubated at 37°C for four days.

Nucleofection. The tube with 10^6 cells per mL was centrifuge at 1,200 rpm during 5 minutes and the pellet was recovered, aspirating the supernatant. Then, 2 µg of plasmid and 100µL of Transfecting Agent were added and the content was recovered in a cuvette of the Ingenio® Electroporation Kit and Solution (Ingenio ®Electroporation , Mirus Bio LLC, Madison, Wi 53711, USA). The electroporation was done with the Amaxa Nucleofector machine. Subsequently, the solution in the cuvette was recovered in a new Eppendorf and it was added 1 mL of neurobasal medium (1ml MEM with Earle's salts, glutamine-free, 0.1ml 10%FBS 20mM and 0.0036g Glucose (Dextrose)). After a new centrifugation at 1,200 rpm during 5 minutes, the cell pellet was recovered aspirating the supernatant. Finally, 1 ml of neurobasal medium was added and the cell suspension was seeded in a 24 well culture plate and incubated at 37°C for 4 days.

3.4 Immunolabelling and histology:

To fix the cells in culture, samples were incubated at room temperature (RT) during 15 minutes with 500 µL of 4% paraformaldehyde (PFA) (NaOH 4,3 g/L, NaH2PO4 18,8g/L, PFA 4,0g/L Sigma-Aldrich®, Bornem, Belgium). After the 15 minutes, samples were washed 3 times with 0.1M phosphate-buffered saline (PBS) during 5 minutes. The blockage of the unspecific binding was done adding 5% of donkey serum (Jackson Immunoresearch Laboratories®, West Grove, PA, USA) in of PBS. Then, 100 µL of this mix was added in each sample for 30 or 60 minutes. Primary antibodies were diluted in a solution containing 5% donkey serum in PBS. Commercially available antibody directed against mouse anti-Tuj1

(1:1000 ABCAM®) were diluted with 0,1M PBS and placed on cells during 2 hours. Then, samples were washed 3 times during 5 minutes with 0.1 M PBS. Anti-rabbit secondary antibody like to Texas Red fluorophore (Jackson Immunoresearch Laboratories®) was diluted 1:500 in PBS and used during 60 minutes. Finally, three washes of 15 minutes were performed and DNA marker Hoechst (1:10000, Vector Laboratories®, Burlingame, CA, United States) was added during 10 minutes in order to visualize the nucleus. The samples were washed with milliQ water and dried. To mount the sample, two drops of SafeMount were added, covered with a coverslips and the samples were left to dry.

3.5 Image acquisition data analysis:

The inmunostained neuronal cultures were observed using a Zeiss Axiovert 10VR microscope (Carl Zeiss®) coupled with FluoView software (Olympus® Fluoview, Aartselaar, Belgium). The figures were composed using ImageJ® software (public domain Java processing program, author: Wayne Rasband, National Institute of Mental Health, Bethesda, MD, USA). Statistical analyses were performed by student t-test and the level of significant was set at 0.05.

3.6 Genomic PCR:

DNA extraction. First, cells were digested to liberate the DNA of the nucleus. The digesting solution contain Proteinase K (5µL; Promega®, V302, 100mg) in 300µL of TENS buffer containing TRIS-HCl pH 8,5 (100mM), EDTA (5mM), SDS 0,2% and NaCl (200mM). The digestion proceeds overnight at the temperature of 55°C, under slight agitation (or under strong agitation during 2 hours). After this time, 200 µL of cold isopropanol (popan-2-ol) was added in order to precipitate the DNA. The samples were centrifuged at 13,000 rpm at 4°C during 10 minutes, then the supernatant was removed. The pellet was washed with 300 µL of ethanol 70%, and the samples were centrifuged once again in the same conditions. The samples were dried at RT and the pellet was suspended with 400 µL of MilliQ water (previously sterilized in the autoclave).

Polymerase Chain Reaction (PCR). Genomic DNA was added in a mix that contains: deionized water, desoxyribonucleotides (dNTPs) (10mM Promega®), the Taq polymerase (Promega®), the Taq buffer with $MgCl_2$ 7,5mM and the specifics primers (IDT®, Belgium), forward and reverse to SV2A gene amplification (SV2Alox/lox forward: 5’ – TGG GTT GGG CTA CTG TTA GG – 3’, SV2Alox/lox reverse : 5’ – AGT TGG GAA GGA GGC AAG AT – 3’) and for Cre detection (Cre forward : 5’ – GCG GTC TGG CAG TAA AAA CTA TC – 3’, Cre reverse : 5’ – GTG AAA CAG CAT TGC TGT CAC TT – 3’, Internal control forward : 5’ – CTA GGC CAC AGA ATT GAA AGA TCT – 3’, Internal control reverse : 5’ – GTA GGT GGA AAT TCT AGC ATC ATC C – 3’).

Table 1: The table represents the quantities of the PCR products for one sample of DNA to amplify SV2aFlox and Cre gene sequence.

Product	SV2aflox	Cre
dNTPs (µL)	0,5	0,5
Primer FOR (µL)	0,5	0,05
Primer REV (µL)	0,5	0,05
Taq Buffer (µL)	5	5
Taq (µL)	0,25	0,125
H2O (µL)	16,25	17,28
DNA (µL)	2	2
Total/sample (µL)	25	25

The PCR reactions were run in a thermocycler T3000 (Biometra®). On one hand, a Cycle PCR SV2Alox was done with a denaturation of double-stranded DNA, 94°C, 10 minutes. 30 cycles; denaturation of double-stranded DNA (94°C, 30 sec), hybridation of primers on the mono-stranded DNA (46°C - 30 sec), polymerization of the new stranded of DNA (72°C, 1min 30 sec). Final polymerization, 72°C, 10 minutes. On the other hand, the Cycle PCR Cre-recombinase was done with a denaturation of double-stranded, 94°C, 3 minutes. 35 cycles; denaturation of double-stranded DNA (94°C, 30 sec), hybridation of primers on the mono-stranded DNA (55°C, 30 sec), polymerization of the new stranded of DNA (72°C, 1minutes). Final polymerization, 72°C, 5 minutes.

DNA electrophoresis. First, a gel of 1,5% agarose (Nippon Genetics®, Dueren, Germany) was prepared by dissolving solid agarose with the 100 mL of Tris-Borate-EDTA (TBE) buffer (89mM Trizma base, 89mM Boric acid and 2,5 mM EDTA, pH 7,4). The solution was heated and was complemented with 0.007% of Midori Green® 100x (LabGene Scientific®, Châtel St Denis, Swiss), which intercalates in the double stranded of DNA, produces fluorescence and allows us to see the fragments of DNA on the gel. When the gel was polymerized in the cuvette (Life Technologies®, Mount Holly, NJ, United States), it was then immerged in TBE. 10 µL of the samples were loaded, and DNA migration was performed by applying 100V during 35 minutes. 5µL of a molecular weight reference was also added (SmartLadder®, Eurogentec®, Liège, Belgium). The gel was scanned with the scan of transillumination UV (ImageQuant^{TM} 350 Healthcare®).

3.7 RNA quantification (RNA extraction, RT-PCR, qPCR):

RNA extraction. This procedure is performed using of TRIzol Reagent Invitrogen (ThermoFisher Scientific ®). First, 1mL of TRIzol was added in each sample. 200 µL of chloroform were added and correctly homogenized with others components. After 2-3 minutes

in RT, the samples were centrifuged at 12,000 g during 15 minutes at 4˚C. The supernatant containing the RNA was recovered and 500 µL of isopropanol per 1mL of Trizol were added. After 10 minutes at RT, the samples were centrifuged at 12,000g during 10 minutes at 4 ˚C, the supernatant was discarded and the pellet was washed with 1mL of ethanol 75%. After a centrifugation (12,000g during 5 minutes at 4˚C), the ethanol was discarded and the samples were dried at RT. The pellet was finally suspended in RNase-free water.

Reverse -Transcription PCR. The samples were diluted with water to obtain a concentration of 500 ng/µL of RNA. Then, 1µL of DNase and 1µL of Taq buffer with $MgCl_2$ (7,5mM) were added before incubation at 37˚C for 30 minutes. After this time, DNase I activity was stopped adding 1µL of Random primers and 1µL of Stop Solution. We allow the primers to hybridize for 10 minutes at 65˚C. A reverse transcription was then started: for each sample, 2µL of RT buffer, 0,5µL of dNTPs, 1 µL of DTT and 0,5µL of reverse transcriptase by M-MLV (Promega®) were added. For the negative controls, all the components of the mix were added but the 1µL of RT enzyme were replaced by milliQ water. 8 µL of the mix were added in each sample and incubation at 37˚C for 1hour was performed. After this reverse transcription step, a positive control was performed by running a PCR to detect the mRNA coding for actin. By sample, the mix for the PCR contains: 1µL of dNTPs, 34,5 µL of H_2O, 1µL of active diluted forward and reverse primers (10µM), 10µL of Taq Buffer and 0,5 µL of Taq polymerase enzyme. 48 µL of this mix were added for 2 µL of cDNA. The PCR of actin was performed in 40 cycles after an initial denaturation at 94˚C for 4 minutes: 94˚C 30 sec, 54˚C 30 sec, 72˚C 30 sec. A final elongation step at 72˚C for 7 minutes finishes the reaction.

Quantitative real-time PCR Reaction (q-RTPCR). SV2A expression was analysed by q-RTPCR using the expression of the housekeeping gene glyceraldehyde-3-phospate dehydrogenase (GAPDH) as normalization between samples. Also with this gene expression quantification, a calibration curve was performed using a serial dilution of a mix composed of 8µl of each sample and two negatives controls were added in the analysis: 1) adding milliQ water in the well instead of cDNA and 2) a RT-reaction (without the enzyme retrotranscriptase to synthetize the cDNA using the RNA as a matrix). The mix added in each well contains: 5 µL of SyberGreen (Eurogentec ®), 0,03 µL of the forward primer, 0,03µL of the reverse primer and 0,94 µL of milliQ water. Two different mix were prepared, one with the SV2A specific primers IDT®: (forward GTCTTTGTGGTGGGCTTTGT, reverse CGAAGACGCTGTTGACTGAG) and the other with the GAPDH primers (forward TGCAGTGGCAAAGTGGAGAT, reverse TTTGCCGTGAG TGGAGTCATA). 6µl of the mix and 4 µL of cDNA or RT reaction were added in each well. After adding all the components, a short centrifuge was done to homogenize and pellet the components at the bottom of the Eppendorf. Then the quantification was performed in the LightCycler (LightCycler® 480, Instrument II, Belgium). Cycle qRT-PCR

performed in 47 cycles after a denaturation of double-stranded DNA, 95°C, 10 minutes: 95˚C 15 sec, 60 ˚C 45 sec (amplification), 95 ˚C 15 sec, 60 ˚C 30sec (melting), 40 ˚C 30 sec (cooling). Finally, relative gene expression was calculated with the formula $E^{-\Delta\Delta Cq}$. E was calculated as the efficiency of the q-PCR and was obtained from the calibration curve.

3.8 Western blotting:

In order to quantify the expression of SV2A, SV2B or SV2C proteins, western blots were performed using proteins extracted from one half of brain for the Ubiquitin-Cre inducible mice and one hippocampus for the Grik4 mice.

Protein Extraction. The samples were taken from -80°C stock (after mouse sacrifice, brain or hippocampus were dissected and immediately put at -80°C until the protein extraction step) and 500 µL of extraction buffer (NaCl 450 mM, Tris HCl 50 mM, 1% protease inhibitor phosphatase x100) was added. Then, they were suspended with the Potter blender and after 15 minutes on ice, the samples were centrifuged at 12,000 rpm for 10 minutes at 4°C. The supernatant containing the soluble proteins was kept and stoked at -20°C or used directly to western blot.

Protein quantification and samples preparation. The protein concentration was quantified using the Bradford's method. This method is based on a colorimetric dosage created by the interaction between the Bio-Rad Protein Assay react (Bio-Rad® GmbH, Munich, Germany) with the basics and aromatics amino acids presents in proteins. When the colorant was linked to these amino acids, it becomes blue (595 nm absorbance), contrary to the free colorant that remains red and green (465 nm absorbance). The absorbance modification was thus proportional to the quantity of proteins in the sample. The compute was done with a curve of known concentrations of BSA (Bovine Serum Albumin, Sigma-Aldrich®, St-Louis, MO, United-States).

To prepare the samples for running, 15µg of protein were mixed with 3µl of Loading buffer (LB) (containing 3ml Tris 0,5 M (pH=6,8), 2,4 ml SDS 10% solution, 4,8 ml Glycerol 20%, 1,5 ml β-mercaptoetanol 5%, 1,8 mg bromophenol blue adjusted to a final volume of 100ml with the milliQ water). Subsequently, water was added up to a total volume of 15 µl. The prepared samples are then heated at 70°C for 10 minutes.

Migration in SDS-PolyAcrylamide Gel Electrophoresis. The gels were poured the day before the running. A resolving gel 10% of 10ml (Tampon Tris 1,5 M pH 8.8, acrylamide 30%, SDS 10%, TEMED, APS 10% diluted in milliQ water) is poured under a staking gel 5% of 5 ml (Tampon Tris 0,5 M pH 6.8, acrylamide 30%, SDS 10%, TEMED, APS 10% diluted in milliQ water). When the gels were polymerized, they are put into the migration bucket and bathed with the migration buffer (a 500ml 20x buffer composed by: 104,6g MOPS, 60,6g Tris Base, 10g SDS ($NaC_{12}H_{25}SO_4$) and 3g EDTA) diluted 1x. The samples were then loaded with precautions

in the wells. The migration was performed in two steps. First, we applied 40V during 15 minutes in constant voltage, so that the samples were running together through the stacking gel (5% polyacrylamide). With this first step, we ensure that all the samples start the migration properly through the resolving gel (10% polyacrylamide) at the same time. Migration through the resolving gel allows the weight-based separation of loaded proteins. This second migration step was done at 140V (constant voltage), for 90 minutes approximately.

Transfer of proteins onto a PVDF membrane. The proteins that were separated in the SDS-polyacrylamide Gel were then transferred onto a polyvinylidene fluoride (PVDF) membrane (Roche®, Basel, Switzerland) in order to allow a post-detection with antibodies. Before proper transfer, this PVDF membrane was bathed in methanol during 1 minute, then in MilliQ water for 2 minutes and at last in a transfer buffer for 15 minutes (20x, 25 ml: 2,9g Glycine, 5,8g Tris Base, 200 ml methanol), diluted in MilliQ water to obtain 1x concentrated. The sponges and the blotting paper were bathed in the transfer buffer as well. Then, we put the components of the transfer system in the next order: sponges, 2 Blotting papers, run gel, membrane PVDF, 2 botting papers, sponge. This system was placed inside a compartment that is bathed with transfer buffer. Finally, the transfer was operated using a constant voltage, 50 V, for 2 hours at 4°C.

Antibodies' incubation. When the transfer was finished, the membrane was washed with a TTBS buffer 1x (5L: 30g TrisBase, 45g NaCl (pH=4,5) and 5 ml Tween-20 in MilliQ water) and then was bathed with this TTBS solution containing 5% of powdered milk, for 1 hour in RT. This powdered milk allows us to block all the non-specific sites of the proteins that can fix non-specifically the antibody. Then, the membrane was placed in a plastic envelope with a solution of TTBS - 5% powdered milk and appropriate dilutions of polyclonal primary antibodies in appropriate dilution: 1/2,000 rabbit anti-SV2A (Abcam®), 1/2,000 rabbit anti-SV2B (Abcam®) and 1/2,000 rabbit anti-SV2C (Abcam®). Incubations were left overnight at 4°C. An antibody specific to actin was also added 1:10,000 in order to quantify also the expression of the cytoskeleton present in all cells. This control allows us a correct quantification of SV2A, SV2B and SV2C proteins. The next day, the membranes were washed three times during 5 minutes with TTBS to remove the primaries antibodies that did not attached during the night. Once again, membranes were placed in a plastic envelope with TTBS with a secondary antibody coupled with a horseradish peroxidase (HRP) anti-rabbit IgG (1:5,000, Jackson Immunoresearch Laboratories®). The membranes are incubated during 1 hour at RT. After, they were washed three times for 5 minutes with TTBS.

Revelation. In this step, the membranes were bathed (2ml) in a solution of peroxide/luminol (substrate of HRP enzyme) (Thermo Scientific®). This revelation was based on the activity of

HRP enzyme coupled to the secondary antibodies. Thanks to HRP enzyme, Luminol was then transformed in a chemoluminescent signal that it was proportional to the quantity of protein that are present in each sample. This chemoluminescent signal was revealed using ImageQuant 350 device and Elite software (Amersham Pharmacia Biotech®, GE Healthcare Lifesciences).

4. Results:

4.1 Detection and quantification of SV2 proteins:

The modifications of SV2 proteins' expression level was investigated in two types of transgenic mice: Ub:SV2A-cKO and Grik4:SV2A-cKO. Western blot and q-RTPCR were performed to quantify protein and mRNA levels respectively. In addition, fluorescence in Grik4-CRE/SV2ALox/EYFP mice was detected to check that the Cre-recombinase acts properly with the Grik4-Cre construction in order to be sure that it is not the reason of the absence of an epileptic phenotype in Grik4:SV2A-cKO mice.

4.1.1. SV2B and SV2C isoforms are not upregulated in the absence of SV2A in Ub:SV2A-cKO mice.

Experiments were performed for Ub:SV2A-cKO and WT mice based on half of a brain extract from pups at P12 that received tamoxifen during embryonic stages 18.5 (E18.5). This type of transgenic mice is used to validate the proper functioning of $SV2A^{lox/lox}$ sequence and verify that it correctly reproduces the SV2A-KO phenotype if crossed with a ubiquitous expressed cre-recombinase. As expected, Western-Blot of SV2A isoform (80 kDA) presents a strong reduction of SV2A expression in Ub:SV2A-cKO samples compared to WT (Figure 3A). To normalize and quantify the Western blot, the concentration of SV2A was divided by the concentration of actin (40kDA) that has been used as an internal loading control. Quantified values confirm the reduction observed in Westerns images (Figure 3B). To validate by another way, the depletion of SV2A in Ub:SV2A-cKO extract, we performed a qRT-PCR. Relative expression of GAPDH and of SV2A mRNA presents a strong reduction in Ub:SV2A-cKO mice compared with WT (Figure 3C). Moreover, the pups disrupted for SV2A exhibited spontaneous seizures around P12. Mice who experienced epileptic seizures were smaller and thinner compared to WT (data not shown). Altogether, these results shows that the $SV2A^{lox/lox}$ gene is efficiently recombined by Cre-recombinase and the deletion of exon 3 of *SV2A* gene mimics the SV2A KO phenotype.

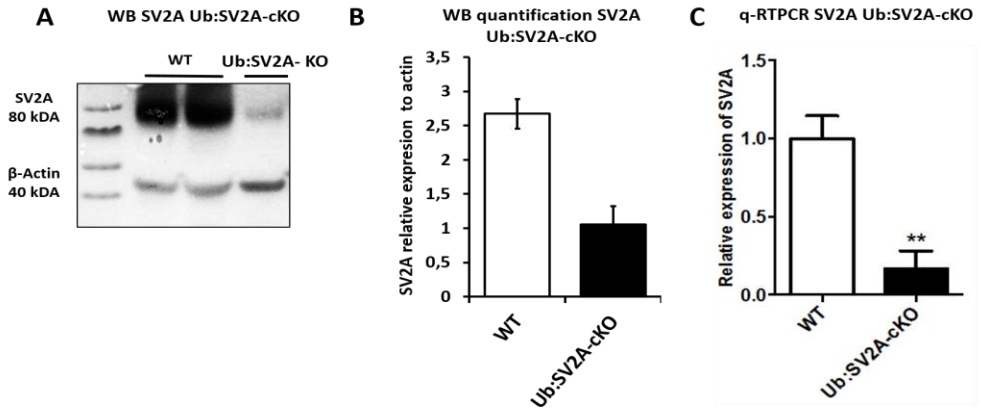

Figure 3: SV2A quantification shows a depletion of mRNA and protein levels in Ub:SV2A-cKO mice. A: Western Blot analyses showing the depletion of SV2A protein (80kDA) in Ub:SV2A-cKO mice comparing to WT mice. **B:** Quantification and normalization of detected SV2A protein by Western Blot, data are present as a mean± S.E.M **C:** Quantification of SV2A mRNA expression measured by quantitative real-time PCR of cDNA synthetized using RNA extracted WT and Ub:SV2A-cKO mice brain at P12. The level of SV2A proteins was normalized using the level of GAPDH mRNA (n=2). The data are presented as mean ± S.E.M

Next, we want to know if this decrease of SV2A protein is responsible of an up-regulation of the two other SV2 isoforms (i.e. SV2B, SV2C). Focusing on SV2B isoform, we can observe by western blot a similar protein detection between Ub:SV2A-cKO mice and WT (Figure 4A). Interestingly, quantification of the Western-Blot shows a slightly overexpression in Ub:SV2A-cKO but different between groups are not enough to be significant (Figure 4B). To confirm this lack of SV2B overexpression, a q-RTPCR of SV2B mRNA was done and it shows that SV2B mRNA relative expression is not altered having similar levels between Ub:SV2A-cKO mice and WT mice (Figure 4C).

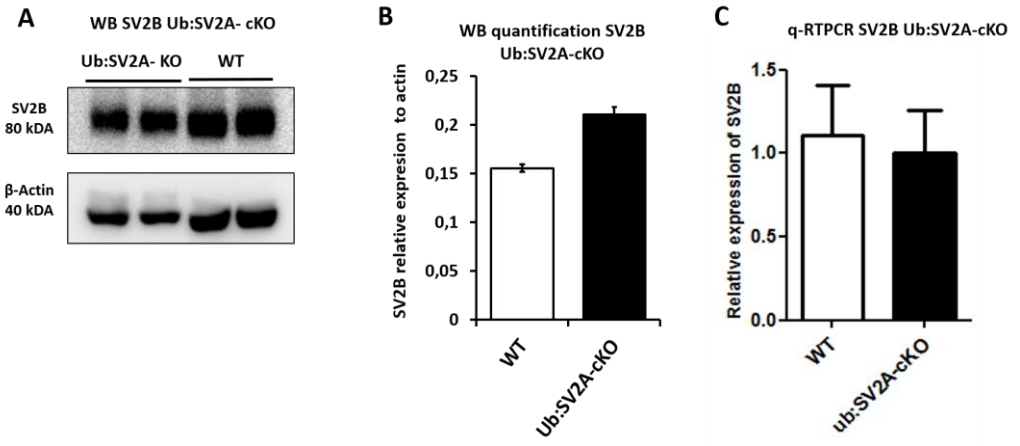

Figure 4: SV2B isoform is not upregulated in Ub:SV2A-cKO mice when SV2A is missing. A: Western blot showing the SV2B protein (80kDA) expression in Ub:SV2A-cKO samples and WT. Actin was used as a control of loaded proteins (40kDA). **B:** Relative Quantification of SV2B expression, data are present as a mean± S.E.M **C:** Relative quantification of SV2B mRNA expression to GAPDH extracted from half of a brain of WT and Ub:SV2A-cKO mice by qRT-PCR (n=2). Data are present as a mean± S.E.M

Finally, expression of SV2C isoform was also analysed in Ub:SV2A-cKO mice. SV2C expression seems to be up-regulated in Ub:SV2A-cKO compared to WT mice (Figure 5A) but this difference is not significant (Figure 5B). On the other hand, q-RTPCR attest that SV2C does not present an overexpression in Ub:SV2A-cKO samples (Figure 5C).

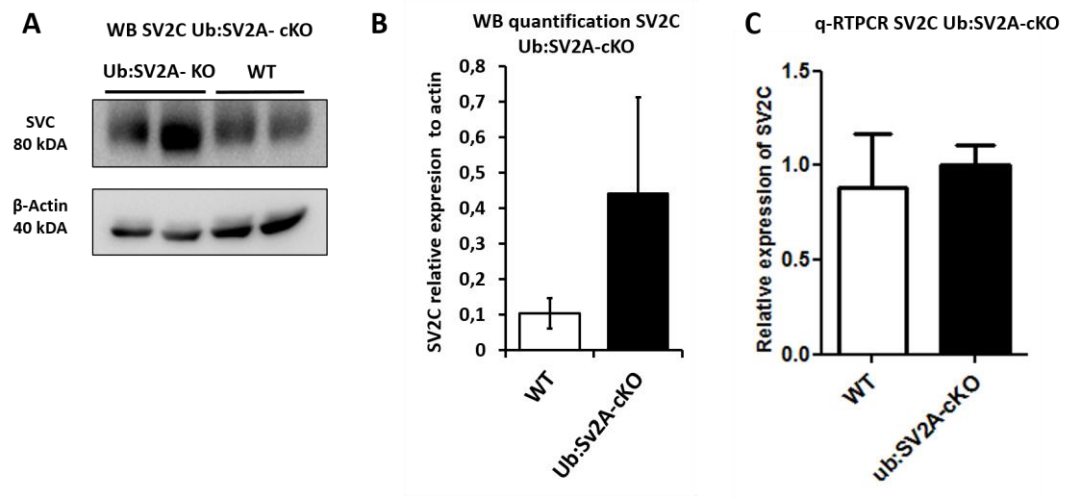

Figure 5: SV2C does not present an overexpression in Ub:SV2A-cKO mice in absence of SV2A isoform. A: Western-Blot analysis of SV2C isoform (80kDA). Beta-Actin is used as an internal loading control. **B:** Graphic representing normalized quantified protein of the Western Blot. Data are present as a mean± S.E.M **C:** Relative mRNA quantification of SV2C isoform to GAPDH by q-RTPCR. Data are present as a mean± S.E.M

4.1.2. SV2B and SV2C isoforms are not upregulated in the absence of SV2A in Grik4:SV2A-cKO mice.

Once the validation of $SV2^{lox/lox}$ sequence were done with Ub:SV2A-cKO transgenic mice, we want to investigate consequences of the depletion of SV2A in Grik4:SV2A-cKO transgenic.

First of all, a detection of EYFP fluorescent protein was done in brain slices of Grik4-CRE/SV2Alox/EYFP transgenic mice (Figure 6) to known in which specific area of hippocampus the Cre-recombinase works when Grik4 promoter rules its expression. This construction induces a recombination in hippocampus areas visually marked by the expression of EYFP. As we can see in Figure 6, the Grik4 promoter Cre-recombinase is expressed in CA3 and DG regions thank to the strong detection of EYFP. However, it seems to be less expressed in CA1 region. Normally, EYFP emit a green-yellow fluorescent light but with ImageJ programme this fluorescence is changed to red to have a harmonious comparison with the Figure 9.

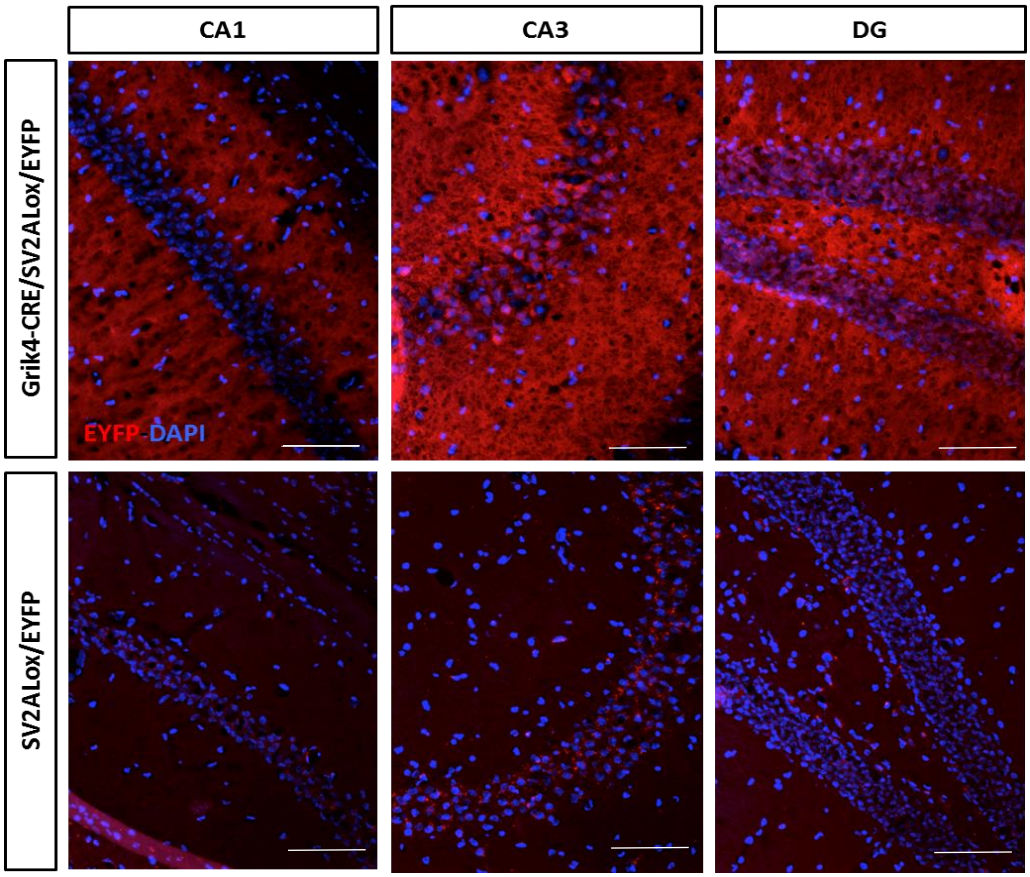

Figure 6: Grik4: Cre-recombinase protein is highly expressed in CA3 and DG of the hippocampus. EYFP labelling is in red and neurons nuclei are in blue (DAPI). In Grik4/SV2ALox/EYFP mice (Grik4: SV2A-cKO), DG and CA3 hippocampus regions present a higher EYFP expression than in CA1 region. Scale bar = 40µm.

Then, we want to confirm if SV2A isoform expressed in the hippocampus in Grik4:SV2A-cKO mice is correctly depleted. As we can see in the Figure 7A, the expression of SV2A in hippocampus is reduced in Grik4:SV2A-cKO mice showing in western-blot a decreased signal for SV2A in Grik4:SV2A-cKO mice brain protein extracts than in protein extracted from WT mice brains. Once the values have been normalized using the expression of beta-actin, a reduction of SV2A expression in Grik4:SV2A-cKO has been significantly observed compared with WT samples (Figure 7B) (*: p-value= 0,034). A q-RTPCR experiment confirm this reduction of the SV2A mRNA expression in the hippocampus of Grik4:SV2A-cKO mice (Figure 7C).

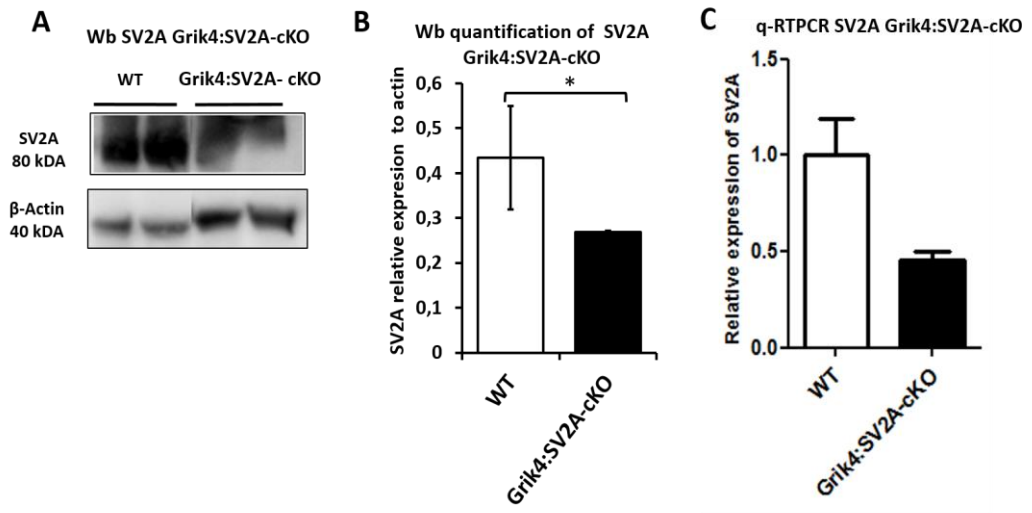

Figure 7: A reduction of SV2A expression is observed in Grik4:SV2A-cKO hippocampus. A: Western-Blot analysis showing the reduction of SV2A detection in Grik4:SV2A-cKO mice comparing to WT mice. **B:** Quantification results of Western blot demonstrate a significant reduction of SV2A expression in Grik4:SV2A-cKO mice compared with WT (student's t-test; *p<0.05). Data are present as a mean± S.E.M. **C:** SV2A mRNA expression in hippocampus measured by quantitative RT-PCR (student's t-test; p=0.056 p>0.05). The level of SV2A was normalized using the level of GAPDH mRNA expression. Data are present as a mean± S.E.M, n=3 per genotype.

Subsequently, Western-Blot on proteins extracted from hippocampus of these both types of mice followed by quantification showed that SV2B isoform is expressed at equal levels in the hippocampus between the Grik4:SV2A-cKO and the WT mice (p=0.23; p>0.05) (Figure 8A and 8B). Furthermore, quantification of mRNA extracted from hippocampus of these two types of mice confirms Western Blot results (Figure 8C) and student t-test shows a non-significant difference (p>0.05; p= 0.157) between the two groups.

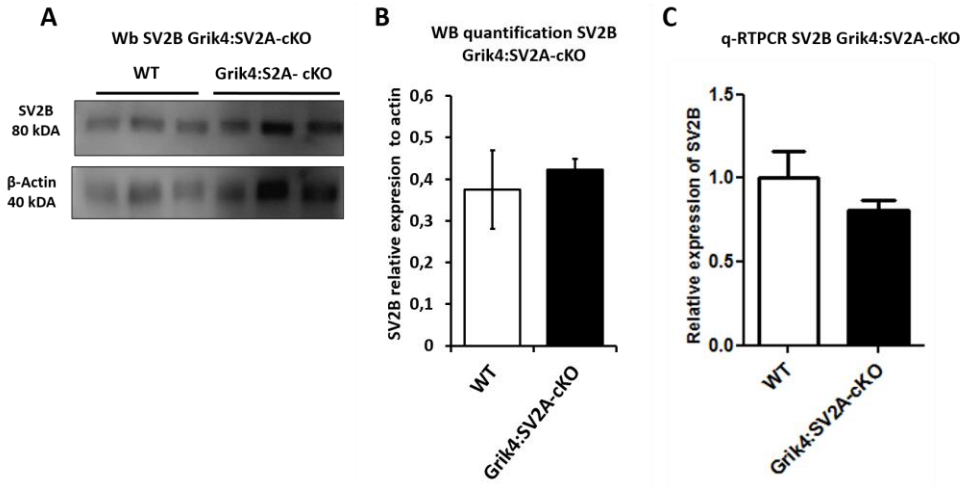

Figure 8: SV2B isoform is similarly expressed in Grik4:SV2A-cKO and WT hippocampus. A: Western Blot analysis performed to detect SV2B protein from hippocampus samples of Grik4:SV2A-cKO and WT samples. **B:** Normalized values of Western exhibit the same expression of SV2B in Grik4:SV2A-cKO mice and in WT (p>0.05; p= 0.23). Data are present as a mean ± S.E.M.; n=3 per genotype **C:** In q-RTPCR, relative expression of mRNA SV2C isoform to GAPDH also show no significant modification in Grik4:SV2A-cKO samples compared with WT (p=0.157). Data are present as a mean± S.E.M n=3 per genotype.

On the other hand, we want to know if a depletion of SV2A in hippocampus in those older mice could be responsible for an up-regulation of two other isoforms, SV2B and SV2C. First, we conduct immonulabelling experiments to observe the expression pattern of SV2C in the Grik4-CRE/SV2Alox/EYFP transgenic mice. SV2C labelling in a Grik4-CRE/SV2Alox/EYFP (Grik4: SV2A-cKO) correlated with SV2ALox/EYFP pattern (WT) in all the regions of hippocampus (CA1, CA3 and DG) (figure 9).

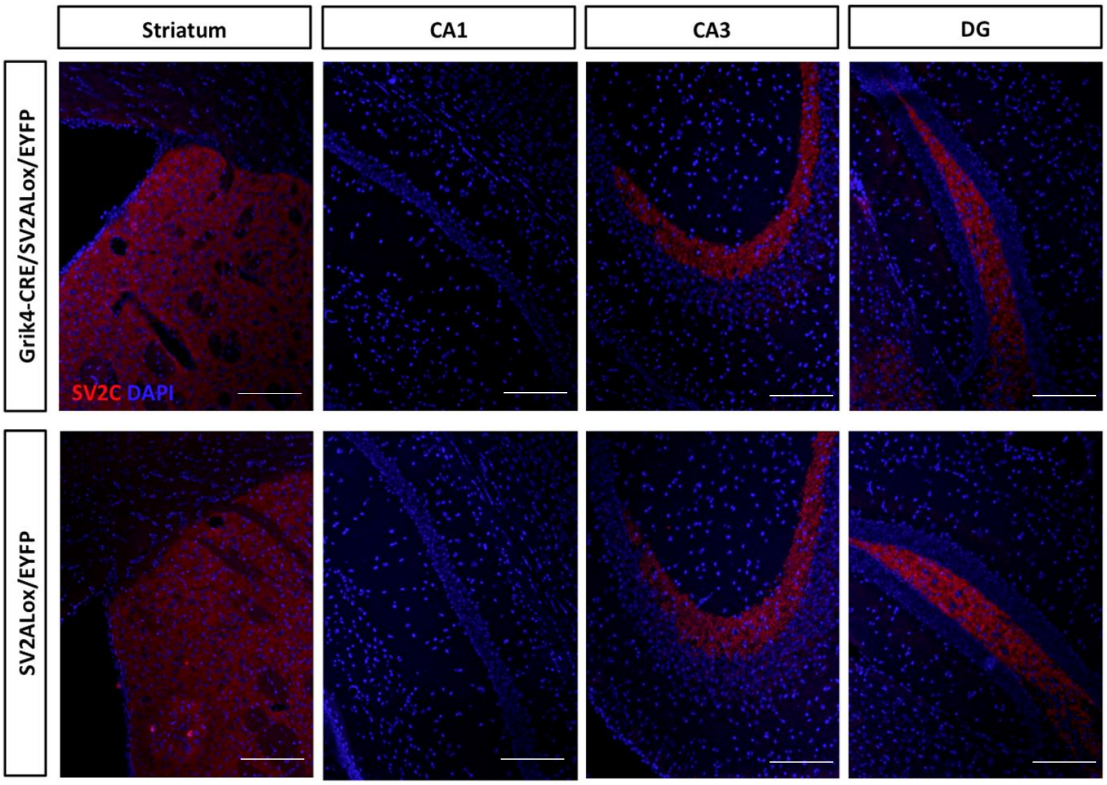

Figure 9: SV2C does not present an overexpression when SV2A protein is absent in the hippocampus. Immunolllabelings of SV2C isoform (red) in brain sections of a Grik4: SV2A-ckO and WT. CA3 and DG are the areas were SV2A protein is normally expressed in the hippocampus. SV2C is labelled in red and neurons nucleus in blue (DAPI). Striatum images are taken as a positive control of the labelling. Scale bare = 96µm.

Besides of SV2C imunollabelling showed in Figure 9, we performed a SV2C isoform quantification (Figure 10A, B). Western-blots of protein extracted from hippocampus of both types of mice showed a similar protein expression between Grik4:SV2A-cKO and WT (student t-test shows a p-value = 0.43). q-RTPCR on RNA extracted from hippocampus of these two types of mice also showed no differences in SV2C mRNA expression among Grik4:SV2A-cKO and WT ($p=0.202$; $p>0.05$). Thus, there are no changes of SV2C isoform in Grik4:SV2A-cKO mice to compensate the absence of SV2A.

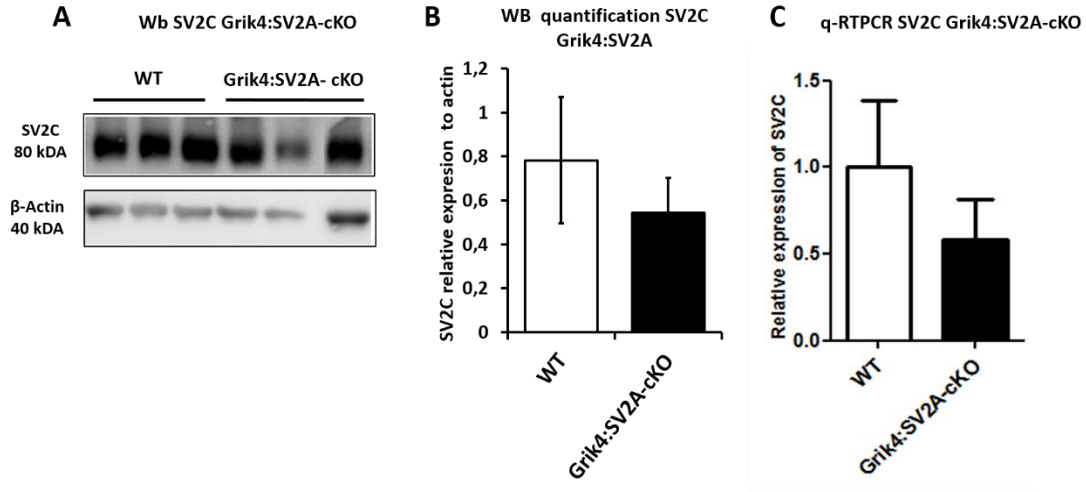

Figure 10: SV2C is not upregulated in hippocampus when expression of SV2A isoform is depleted in Grik4:SV2A-cKO transgenic mice. A: Western Blot analyses showing the detection of SV2C isoform. **B:** Quantification and normalization of SV2C expression using the beta-actin expression in western blot experiment (p= 0.43). **C:** q-RTPCR of SV2C mRNA relative expression (p=0.202). In images B and C, data are present as a mean± S.E.M, n=3 per group.

4.2 Development of cortical neurons nucleofection:

4.2.1. Cultivated E16 Neurons could be nucleofected.

Next, we wanted to know if the lack of SV2A in neurons has an effect in synaptic plasticity or complexity. To observe dendritic and axonal ramification in details, we plan to perform first neuronal cultures and a nucleofection of SV2A lox/lox cultivated E16 cortical neurons with a Cre-recombinase expressing plasmid was done. The nucleofected plasmid allows the expression of the CRE-recombinase and GFP. The negative control plasmid allows the only expression of the GFP. Plasmid insertion in SV2A lox/lox cortical neurons and GFP expression have also the advantage to view the whole neurone structure. We started to set up this experiment with E16 wild-type cortical neurons (Figure 11A). One can indeed observe that some cultivated E16 cortical neurons are indeed efficiently nucleofected. To confirm that cultured cells are neurons and not glial cells, immunolabelling using anti-βIII-tubulin antibodies (Tuj1 antibodies) (Figure 11B) or with anti-GFAP antibodies (data not showed) have been performed. As a result, we could see all cells in culture are labelled with Tuj1 and negative for GFAP staining (data not shown). The GFP expression allows us also to observe in details the dendritic and axonal ramifications.

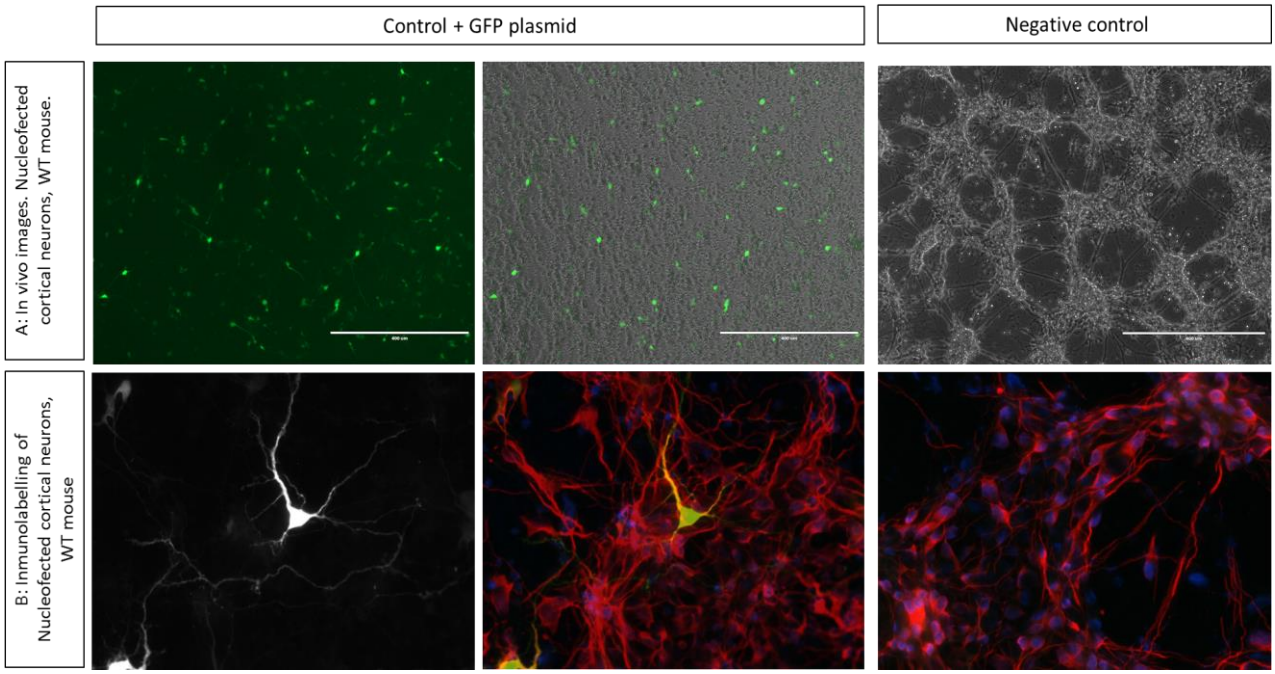

Figure 11: Nucleofection of cortical neurons (WT mice in E16) and validation that transfected cells are specifically neurons. A: Images of cortical neurons in culture after nucleofection or not (negative controls) with GFP plasmid. The nucleofected neurons with GFP plasmid are showed in green thank to GFP expression. Merge image allow seeing all neurons in culture, the nucleofected and the non nucleofected. **B:** Immunolabelling with Tuj1 in red allows us to confirm that cultured cells are neurons but not glial cells. Black and white image show only the nucleofected neurons with all his ramifications. Merge image of Control + exhibit all neurons, the nucleofected and the non nucleofected with the specific neuronal labelling, Tju1. Scale bar =25µm.

5. Discussion:

SV2 isoforms role remains elusive. It is known that their presence is critical for the function of the CNS and that SV2A is associated with the physiopathology of epilepsy (Van Vliet et al,. 2009). The three isoforms are abundantly present in synaptic vesicles and seems to contribute to neurotransmitters release and to the endocytose of SYT-1 (Nowack et al., 2010). Specifically, SV2A isoform has an important role in the control of the SVs exocytosis (Mendoza-Torreblanca et al., 2013). Interestingly, SV2A was identified as the target of an anticonvulsant epileptic drug like Levetiracetam (B. Lynch et al., 2004). Moreover, it has also been shown that levels of SV2B and SV2A protein expression (30-50%) are decreased in epileptic foci, more precisely in areas of synaptic loss in patients with TLE (Crèvecœur et al., 2014). In these regions however, a SV2C higher expression could be observed. Nevertheless, the SV2A physiologic implication and its role in epilepsy and how it interacts with LEV remain hitherto elusive.

Thus, in order to obtain a better idea of SV2A connection with epilepsy, experiments with SV2A-KO were done. Interestingly, SV2A-KO mice present epileptic seizures at P7 and died around P15 (Crowder et al., 1999). But due to their early death, one can hypothesize that SV2A could play a role in late brain development steps (the brain development is supposed to be finished around P40 in mouse) and thus, this type of transgenic mice does not allow the study of SV2A in adult state. Therefore, to deepen the study of SV2A, two type of transgenic mice were

used in this work: Ub:SV2A-cKO and Grik4:SV2A-cKO. Unlike SV2A traditional KO, Ub:SV2A-cKO was a tamoxifen inducible KO model. This type of transgenic construction allowed us to perform a validation of the $SV2A^{lox/lox}$ sequence and confirm that this construction induce a depletion of SV2A expression in brain and an epileptic phenotype when it is recombined with Cre-recombinase. The only difference between Ub:SV2A-cKO pups and full KO animals is the age of seizure, P7 for total KO and P15 for Ub:SV2A-cKO, probably due to the transgenic construction which never recombines with an efficiency of a 100%. Another possible explanation to this difference is that SV2A gene became inefficient when tamoxifen is given and so the previously synthetized SV2A protein has to disappear to observe the phenotype.

On the other hand, Grik4:SV2A-cKO transgenic mice was created to invalidate SV2A only in excitatory neurons of the CA3 and DG hippocampal regions, considering the fact that TLE patient have a significantly reduced amount of SV2A in this particular zone (van Vilet et al., 2009). It is known that a specific type of TLE (i.e. mesial temporal sclerosis 1A(MST1A)) present a reduction of SV2A expression associated with an overexpression of SV2C isoform (Crèvecœur et al., 2014). Unexpectedly, Grik4:SV2A-cKO transgenic mice do not present an epileptic phenotype. To explain that, we hypothesize that the depletion of SV2A in hippocampus of Grik4:SV2A-cKO mice could be or not associated with an overexpression of the other SV2 isoforms. In the hippocampus of Grik4:SV2A-cKO mice, SV2A isoform is well down regulated. However, SV2B and SV2C isoform are not upregulated. These results suggest thus the up-regulation of SV2C could be a necessary condition to obtain an epileptic phenotype like in MTS 1A patient. However, in the epileptic brain of Ub:SV2A-ckO transgenic mice, the expression of SV2B and SV2C isoforms was unaltered suggesting that the up-regulation of SV2C is not necessary to have epileptic seizures in mouse.

Another explanation proposes that the epileptic phenotype is associated with an altered balance between excitatory (sEPSC) and inhibitory (sIPSC) inputs. Indeed, it is known that SV2A KO and SV2A/B DKO that exhibit important seizures, present an altered balance between these both inputs (Venkatesan et al., 2012). To see if in absence of SV2A in hippocampus, the synaptic inputs were modified, in a near future we could analyze by Patch-Clam technique sEPSC and sIPSC in hippocampus of Grik4:SV2A-cKO.

In addition, it is suspected that epilepsy observed in SV2A KO mice or in Ub:SV2A-cKO is not due to the specific disappearance of SV2A in excitatory neurons of the hippocampus alone but the result of SV2A deletion in another area of the brain. To validate this hypothesis, in the lab, we will use an another type of transgenic mouse which invalidated SV2A in inhibitory interneurons (Kohwi et al., 2007). Finally, Crèvecœur et al., 2013 prove that SV2A expression

starts during embryonic stage around E14 (Crèvecœur et al., 2014). However, it is known that Cre-recombinase activity in Grik4:SV2A-cKO mice starts at P14 and has a 100% of recombination efficiency at P56 (Nakazawa et al., 2002). Consequently, the delayed activity of Cre-recombinase in Grik4:SV2A-cKO cause a normal apparition and development of SV2A and could explain the absence of epileptic phenotype. Unfortunately, it is known and we demonstrated thanks to the Rosa-eYFP expression that in Grik4:SV2A-cKO transgenic do not recombined in CA1 area of the hippocampus (Nakazawa et al., 2002).

Finally, in the last part of this work, we set up a protocol to nucleofect cortical neurons to allow us the quantification of axonal and dendritic ramification in a near future. This quantification will determine if a reduction of SV2A isoform causes an effect in neuronal synaptic plasticity, at least at the morphological level. The results obtained in this project validate that cortical neurons of E16 embryos are nucleofectable and we can observe the neuronal neuritic arborisation. In a near future the study of how SV2A deletion affect the axonal and dendritic complexity will be possible thanks to the development of this protocol.

In conclusion, this study led us think that epilepsy is a multifactorial disease. Thanks to Ub:SV2A-cKO model, we demonstrated that the SV2A lox/lox model is efficient to invalidate SV2A in specific type of cells. Thanks to Grik4:SV2A-cKO model, we demonstrated that the SV2A deletion in the CA3 area of the hippocampus is not enough to induce an epileptic phenotype at the adult stages. We also demonstrated that the absence of SV2A do not induce a modification of SV2B and SV2C expression which could be the reason explaining the lack of epilepsy in Grik4:SV2A-cKO transgenic mice.

6. Ethics and Sustainability:

Animal health and care was performed in accordance with the declaration of Helsinki and followed the guidelines of the Belgium ministry of agriculture in agreement with European Community laboratory animal care and use regulation (86/609/CEE, CE of J nº L358, 18 December 1986). All the experiments were approved by ethics committee of the University of Liège. Biosecurity and control in GMO (genetic modify organism) and pathogens was regulated by SUPHT (“Service Universitaire de Protection et d’Hygène au Travail”) and toxic and radioactive wastes by SUCPR (“Service Universitarie de Côntrole Physisque des Radiations”) of the University of Liège. Laboratory security is regulated by regions in Belgium and based in the Belgian environmental legality which determines a biosecurity responsible in all installations.

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