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Deciphering the role of Sulfotransferase 4A1 in brain development and neuronal functioning

Ph.D. Thesis of:

Lorenza Culotta

R10999

Tutor: Prof. Monica Di Luca

Co-Tutor: Dr. Chiara Verpelli

Coordinator: Prof. Alberico L. Catapano

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ABSTRACT

Cytosolic sulfotransferases (SULTs) are enzymes that transfer a sulfuryl group from the obligate donor PAPS (3'-phosphoadenosine 5'-phosphosulfate) onto a variety of exogenous and endogenous substrates (Negishi 2001). In 2000, a novel member of this family (SULT4A1) was isolated from human and rat brain (Falany 2000). To date, the exact substrate and function of SULT4A1 are not fully addressed but since it is highly conserved and expressed extensively, and almost exclusively, in the brain, it is possible that SULT4A1 may have an important role in the central nervous system. Moreover, some recent reports have associated polymorphisms in the SULT4A1 gene with susceptibility to schizophrenia (Brennan 2005; Meltzer 2008); SULT4A1 has been suggested to be associated with neurological symptoms of Phelan-McDermid Syndrome (Disciglio 2014) and altered levels of SULT4A1 protein have been observed in bipolar and Alzheimer's patients (Wang 2003; Ryan 2006).

Given this background, we decided to investigate the still unknown role of SULT4A1 within neuron development and functioning. We started evaluating the physiological expression of SULT4A1 in the brain areas mainly involved in neuropsychiatric and neurodevelopmental disorders. To this purpose, we performed western blot analyses of total lysates of hippocampus, striatum, cerebral cortex and cerebellum dissected from adult mice (P60). Our results showed that SULT4A1 is highly expressed in all the analyzed areas, especially in cortex and in cerebellum. Moreover, area-specific expression of SULT4A1 appears to be similar between adult male and female mice.

Considering the possible implication of SULT4A1 in the pathogenesis of neurodevelopmental disorders, a major point for our study was the evaluation of SULT4A1 expression during physiological neuronal maturation. To this purpose, we analyzed by western blot rat primary neuronal cultures at different stages of neuron maturation, which are Day-In-Vitro (DIV) 1, 7 and 14. From the results of these analyses, it was inferable that the expression of SULT4A1 appreciably rises during neuronal maturation, going from an almost undetectable level at DIV1 to an almost 4-fold greater level at DIV14 in cortical cultures. This result was confirmed

by immunofluorescence (IF) staining of the same cultures where the protein showed a cytoplasmic localization and its level of expression steadily increased from DIV1 to DIV14. IF results also suggested that SULT4A1 is mainly expressed in GAD67-positive inhibitory neurons, in particular in Calbindin- and Parvalbumin-positive neurons.

Therefore, to better determine SULT4A1 expression in human neurons, we obtained peripheral blood mononuclear cells (PBMCs) from control healthy individuals and reprogrammed them into induced Pluripotent Stem Cells (iPSCs). iPSC-derived neural stem cells (NSC) were differentiated into neurons for at least 50 days, time necessary to obtain MAP2-positive mature neurons. SULT4A1 expression was evaluated during neuronal maturation from NSC stage to mature neuron and the data from biochemical analysis suggested that the level of SULT4A1 protein rises during differentiation of NSCs into neurons.

Considering that abnormalities in dendritic spines and neuronal arborization are some of the most consistent anatomical correlates of neurodevelopmental disorders (Hung 2008; Glausier 2013; Jiang 2013; Moyer 2015), we characterized the effect of SULT4A1 on spine dynamics and dendrite morphology: in particular, we overexpressed or silenced SULT4A1 in cortical cultures and, interestingly, we observed that both conditions altered neuronal arborization as well as spine density and morphology.

Moreover, in light of the possibility that SULT4A1 polymorphisms may lead to a reduction of mRNA translatability (Brennan 2005) and to clarify the specific role of SULT4A1 in neuronal maturation and functioning, we further investigated the effects of SULT4A1 silencing. Biochemical and electrophysiological analyses of neurons infected or transfected with SULT4A1 shRNA demonstrated that SULT4A1 deficiency perturbs the composition and activity of excitatory and inhibitory synapses: indeed, we found an increase of GAD65 expression and a reduction of GluN1 levels. Interestingly, these data were in line with the electrophysiological recordings, where neurons lacking SULT4A1 displayed a slight augmentation of spontaneous inhibitory postsynaptic currents (sIPSC) frequency and a decrease of spontaneous excitatory postsynaptic currents (sEPSC) frequency.

ABSTRACT (Ita)

Le sulfotrasferasi citosoliche (SULTs) sono enzimi che trasferiscono un gruppo sulfurilico dal donatore obbligatorio PAPS (3'-fosfoadenosina 5'-fosfosolfato) ad un ampio numero di substrati, sia endogeni che esogeni (Negishi 2001). Nel 2000 è stato isolato dal tessuto nervoso umano e di ratto un nuovo membro di questa famiglia: SULT4A1 (Falany 2000). Ad oggi, l'esatto substrato e la funzione di SULT4A1 sono ancora sconosciuti ma, dal momento che è altamente conservata ed è espressa quasi esclusivamente nel cervello, è possibile che SULT4A1 rivesta un ruolo importante nel sistema nervoso centrale. Inoltre, dei polimorfismi del gene SULT4A1 sono stati recentemente associati alla suscettibilità alla schizofrenia (Brennan 2005; Meltzer 2008); SULT4A1 è stata anche associata ai sintomi neurologici della Sindrome di Phelan-McDermid (Disciglio 2014) e in pazienti affetti da bipolarismo e Alzheimer sono stati riscontrati dei livelli alterati della proteina SULT4A1 (Wang 2003; Ryan 2006)..

Noto questo background, abbiamo deciso di chiarire il ruolo di SULT4A1 nello sviluppo e nel corretto funzionamento neuronale. Per prima cosa, abbiamo valutato l'espressione fisiologica di SULT4A1 nelle aree cerebrali maggiormente coinvolte nei disordini neuropsichiatrici e del neurosviluppo: a tal proposito, abbiamo effettuato delle analisi biochimiche su lisato totale di ippocampo, striato, corteccia cerebrale e cervelletto dissezionati da topi adulti (P60): i nostri risultati hanno dimostrato che SULT4A1 è altamente espressa in tutte le aree prese in considerazione, specialmente nella corteccia e nel cervelletto. Inoltre, l'espressione area-specifica di SULT4A1 sembra essere simile tra topi maschi e femmine.

Considerando il possibile coinvolgimento di SULT4A1 nella patogenesi dei disordini del neurosviluppo, un punto focale del nostro studio è la valutazione dell'espressione di SULT4A1 durante la maturazione neuronale. A questo scopo, abbiamo analizzato via western blot delle colture neuronali embrionali di ratto a differenti stadi di maturazione, quali Day-In-Vitro (DIV) 1, 7 e 14. Dai risultati è stato possibile dedurre che l'espressione di SULT4A1 aumenta visibilmente durante la maturazione dei neuroni corticali, passando da un livello quasi impercettibile a DIV1 ad un livello circa quattro volte più alto a DIV14. Questo

risultato è stato confermato tramite esperimenti di immunofluorescenza (IF), in cui è stato possibile apprezzare la localizzazione citoplasmatica di SULT4A1 e l'aumento di espressione passando da DIV1 a DIV14. Dalle analisi di immunofluorescenza è stato anche possibile evincere che SULT4A1 è maggiormente espressa nei neuroni inibitori GAD67-positivi, in particolare in neuroni positivi per Calbindina e Parvalbumina.

Per conoscere più nel dettaglio l'espressione di SULT4A1 anche in neuroni di origine umana, abbiamo ottenuto delle cellule mononucleate del sangue periferico (peripheral blood mononuclear cells or PBMCs) da individui sani e le abbiamo indotte a cellule staminali pluripotenti indotte (induced Pluripotent Stem Cells or iPSCs). Le cellule staminali neurali (neural stem cells o NSCs) derivate dalle iPSCs sono state successivamente differenziate a neuroni per almeno 50 giorni, tempo necessario per ottenere neuroni maturi positivi per MAP2. L'espressione di SULT4A1 è stata valutata durante la maturazione neuronale dallo stadio di NSC a quello di neurone maturo: i dati di biochimica suggeriscono che il livello di SULT4A1 aumenta durante il differenziamento da NSC a neurone.

Dal momento che le alterazioni a livello di spine dendritiche e arborizzazione neuronale sono considerate tra i correlati anatomici più validi dei disordini del neurosviluppo (Hung 2008; Glausier 2013; Jiang 2013; Moyer 2015), abbiamo deciso di focalizzarci questo aspetto della fisiologia neuronale per chiarire il ruolo di SULT4A1 nella maturazione e nella funzionalità dei neuroni: in particolare, abbiamo overespresso o silenziato SULT4A1 in colture corticali di ratto e abbiamo riscontrato che, sorprendentemente, entrambe le condizioni risultano in un'alterazione dell'arborizzazione dendritica così come della densità e della morfologia delle spine dendritiche.

Inoltre, alla luce della possibilità che i polimorfismi del gene SULT4A1 possano portare ad una riduzione della traduzione di mRNA (Brennan 2005), siamo entrati più nel dettaglio nella valutazione degli effetti del silenziamento di SULT4A1. Analisi di biochimica e di elettrofisiologia eseguite su neuroni infettati o trasfettati con un shRNA specifico per SULT4A1 hanno dimostrato che la mancanza di SULT4A1 perturba la composizione e l'attività delle sinapsi sia eccitatorie che inibitorie: infatti, abbiamo riscontrato un aumento dell'espressione di GAD65 e una

diminuzione dei livelli di GluN1. Dato interessante è che queste variazioni di espressione proteica erano in linea con le registrazioni elettrofisiologiche, dal momento che i neuroni silenziati hanno mostrato un lieve aumento della frequenza delle correnti postsinaptiche inibitorie spontanee (sIPSC) e una diminuzione della frequenza delle correnti postsinaptiche eccitatorie spontanee (sEPSC).

INTRODUCTION

1. Sulfotransferases

Sulfotransferases (SULTs) are a superfamily of enzymes that catalyze the reaction of sulfonation, also referred to as sulfuryl transfer or sulfation, which consists of the transfer of a sulfuryl group (SO_3) from the ubiquitous doron 3'-phosphoadenosine 5'-phosphosulfate (PAPS) to an acceptor group of numerous endogenous and exogenous substrates (Negishi 2001). According to their subcellular localization, SULTs can be categorized into two main families: cytosolic sulfotransferases and membrane-associated sulfotransferases, which are bound to the Golgi apparatus.

Membrane-associated SULTs sulfonate several compounds such as glycosaminoglycans, glycoproteins, peptidyl tyrosine and heparan sulfates, and are involved in signaling processes and intercellular communication (Lidholt 1992).

By contrast, cytosolic sulfotransferases sulfonate steroids, environmental chemicals and drugs; in mammals, cytosolic SULTs have been shown to be important in the metabolism and excretion of numerous drugs and xenobiotics as well as in the homeostasis of endogenous compounds such as steroid and thyroid hormones, cholesterol and neurotransmitters (Blanchard 2004; Yasuda 2007). Intriguingly, membrane-bound and cytosolic sulfotransferases share little sequence similarity and have been reported to have no sequence elements in common.

1.1 Cytosolic Sulfotransferases

Since the late 1980s, a considerable number of cytosolic SULTs have been characterized and classified into several gene families according to the similarity of their catalytic properties and amino acid sequences (Nagata 2000, Blanchard 2004; Freimuth 2004): to date, the known SULTs families are SULT1, SULT2, SULT4, and SULT6. Even though the family members display considerable sequence homology and structural similarity, they are apparently involved in different biological processes. The SULT1 family includes nine members which form four subfamilies (1A1-4, 1C1-3, 1B1, 1E1): these enzymes catalyze the sulfonation of estradiol, simple phenols and thyroid hormones, as well as drugs

and xenobiotics. The SULT2 family comprises two genes encoding SULT2A1, SULT2B1a, and SULT2B1b proteins which sulfonate steroids like allopregnanolone, androsterone and dehydroepiandrosterone (DHEA). Lastly, both the SULT4 and SULT6 families comprise a single member encoding an orphan enzyme, respectively 4A1 and 6B1, but, out of the two proteins, only SULT4A1 protein has been characterized (Freimuth 2004).

2. Sulfotransferase 4A1

Cytosolic Sulfotransferase 4A1 (or BR-STL, brain sulphotransferase-like) was isolated from both human and rat brain in 2000 by Falany et al. (Falany 2000). Human SULT4A1 gene localizes in the 22q13.1–13.2 region and spans approximately 36.5 kb; the 852 bp coding sequence consists of seven exons and encodes a 33 kDa protein (Blanchard 2004). The intron between exon 6 and 7 is reportedly subject to incorrect splicing, resulting in a second SULT4A1 transcript variant which is translated in a truncated protein, unstable *in vivo* (Sidharthan 2014).

SULT4A1 was identified as member of the SULT family on the basis of sequences alignment and structure similarities, even though SULT4A1 is one of the human SULTs with the lowest sequence homology (<40%) (Minchin 2008). Examining SULT4A1 secondary structure (Fig.1) it is possible to appreciate that the cofactor binding site involves two different domains of SULT4A1 protein: the N-terminal phosphosulfate binding loop, also known as “P-loop”, and a small sequence located in the C-terminal region of the protein. The P-loop is responsible for the binding to the 5’-phosphate group of PAPS, and is highly conserved in other SULTs. The C-terminal domain represents the binding site for 3’-phosphate of the cofactor and includes a conserved lysine in a RKG sequence: however, SULT4A1 protein does not present the lysine residue (Allali-Hassani 2007).

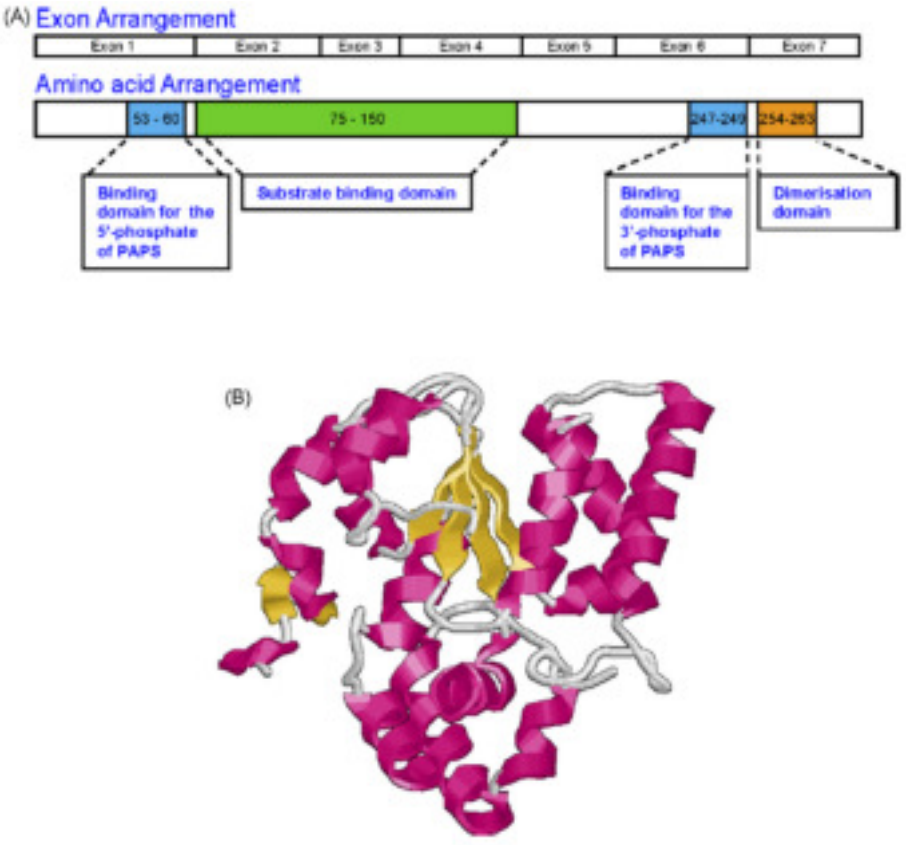


Figure 1. *(**A**) Structural motifs in the SULT4A1 protein, identified by homology with other sulfotransferases. (**B**) Tridimensional structure of human SULT4A1 protein showing the central four-stranded parallel β-sheet (yellow) flanked by α-helices (red). (modified from Minchin 2008).*

SULT4A1 showed a remarkable degree of cross-species similarity: human, mouse and rat isoforms share at least 97% amino acid sequence identity, with only nine amino acid differences at the protein level. Even human SULT4A1 and Xenopus SULT4A1 present a high degree of homology (91% amino acid identity), suggesting an important and conserved function across species (Blanchard 2004; Minchin 2008).

Nevertheless, there are some relevant differences in SULT4A1 protein compared with other mammalian cytosolic SULTs, in particular at the C-terminus end of the sequence. First, a conserved Trp in one of the three α-helices (α3) is replaced with

a Leu in SULT4A1; second, a 13-amino-acid gap is present in the PAPS binding loop, which also lacks a lysine residue (Lys259, SULT4A1 numbering), key to PAPS binding (Allali-Hassani 2007). Taken together, these differences in the otherwise highly conserved C-terminal end of SULT4A1 protein might have relevant effects on PAPS binding and substrate specificity. Indeed, Allali-Hassani et al. showed that SULT4A1 has an atypical crystal structure and its PAPS binding pocket, moderately smaller, is proposed to be unable to host the cofactor (Allali-Hassani 2007); moreover, several potential alternate sulfate donors, such as 4-methylumbelliferyl sulfate and adenosine phosphosulfate, and different prototypic SULT substrates have been tested to asses SULT4A1 catalytic activity but no sulfonation activity has been detected with any of these substrates or cofactors, suggesting the possibility that SULT4A1 may not have relevant catalytic activity in vivo or that the functional enzyme may be active as a part of a multi-enzyme complex (Falany 2000).

2.1 Tissue distribution

SULT4A1 tissue distribution has been examined in both humans and rodents: it has been widely demonstrated that it is mainly, but not solely, expressed in the brain, albeit the levels of mRNA and protein in other organs such as kidney, lung, liver and heart were much less than that in the brain (Alnouti 2006, Sidharthan 2014). This almost exclusive expression in the brain of different species suggests a relevant role for SULT4A1 protein in the central nervous system (CNS).

Throughout the brain, strongest expression has been detected in cerebral cortex, thalamus, cerebellum, and hippocampus (Liyou 2003). Within the cerebral cortex, the prefrontal cortex as well as pyramidal neurons of the motor cortex exhibited particularly marked immunolabeling; moreover, moderate SULT4A1 expression was observed in other areas including insular and cingulated cortex, subthalamic nuclei and pituitary gland.

Sidharthan et al. reported a differential expression of the wild-type transcript and the splice variant in numerous tissues and cell-lines (Sidharthan 2014). The variant transcript was detected in several tissues such as bladder, cervix and

intestine, but not in the brain where, on the contrary, the wild-type transcript was highly expressed; similarly, the wild-type transcript was observed only in cell-lines derived from neuronal tissue, such as human SH-SY5Y and mouse Neuro 2A cells, while the splice variant resulted to be almost ubiquitously expressed. Moreover, when human neuroblastoma cell-lines were differentiated into neurons, the level of variant transcript remarkably decreased while the expression of wild-type transcript increased (Sidharthan 2014). Even though SULT4A1 transcript was observed in a variety of cell-lines and tissues, the protein was only detected in those cells and tissues where the wild-type mRNA was expressed. The switch from splice variant to wild-type transcript, followed by the rise in SULT4A1 protein expression, may represent a post-transcriptional regulation of SULT4A1 expression.

Furthermore, SULT4A1 mRNA expression has been investigated during mouse brain development (Alnouti 2006): indeed, mRNA levels were very low in fetal brains and remained nearly unchanged until 30 days after birth, when, only in female animals, SULT4A1 markedly increased, reaching a fourfold higher expression compared to male mice. This diversity may indicate a possible hormone responsiveness of SULT4A1 gene.

2.2 Pharmacogenetics and possible relevance to disease

There are several genetic conditions associated with changes in the structure or number of copies of chromosome 22: in particular, the chromosomal region around the SULT4A1 gene (22q13) has been implicated in neurodevelopmental disorders including schizophrenia and the Phelan-McDermid Syndrome (Brennan 2005; Disciglio 2014).

2.2.1 Schizophrenia

Schizophrenia is an enigmatic neuropsychiatric disorder affecting about 1% of the world population (Sullivan 2003). There is a wide range of clinical symptoms associated with schizophrenia, going from positive symptoms (e.g. delusions and

hallucinations), negative symptoms (e.g. anhedonia and social withdrawal), and cognitive impairments (e.g. deficits in attention and working memory). Schizophrenia has long been known to have a strong genetic component: in fact, since no single gene has been found to have a prominent implication in schizophrenia etiology, this disease has been included in a group of pathologies known as complex genetic disorders (Gejman 2010).

Recent evidences pointed out a possible role for SULT4A1 gene in genetic predisposition to schizophrenia. Brennan and collaborators identified a new microsatellite polymorphism in the 5' untranslated region of SULT4A1 mRNA (Brennan 2005). They observed seven alleles of D22s1749E ranging in size from 198 to 216 nucleotides (nt), among which the 213 and 216 nt alleles appeared to be transmitted more often than expected to offspring affected by schizophrenia. The 213 and 216 nt alleles are believed to encode mRNA with longer 5' untranslated leader sequences, thus reducing mRNA translatability and final levels of SULT4A1 enzyme.

In 2008, three intronic single nucleotide polymorphisms (SNPs) in SULT4A1 gene were proposed to be associated with risk for schizophrenia or schizophrenia spectrum disorder (Meltzer 2008): two of these SNPs, rs138060 and rs133097, are related, respectively, to clinical symptoms and cognitive function in schizophrenia. Furthermore, SULT4A1-1 haplotype status (rs2285162 [A]-rs2285167 [G]) in schizophrenia patients have been correlated with reduced severity of clinical symptoms, superior response to antipsychotic drug olanzapine and decreased hospitalization risk in olanzapine-treated patients (Ramsey 2011 and 2014). Taken together, these evidences suggest that SULT4A1 may represent not only a candidate gene for susceptibility to schizophrenia, but also an advantageous biomarker to be evaluated prior to initiation of treatment with antipsychotic drugs.

2.2.2 Phelan-McDermid Syndrome

Phelan-McDermid Syndrome (22q13.3 deletion syndrome or PMS) is a genetic disease orphan of cure characterized by neonatal hypotonia, global developmental

delay, absent to severely delayed speech, intellectual disability, autism and minor dysmorphic features (Phelan and McDermid 2012). Although it is widely recognized that SHANK3 gene, encoding a scaffold protein of the post-synaptic density, is the major gene contributing to the neurological phenotype of PMS (Guilmatre 2014; Wang 2014), the wide clinical heterogeneity among PMS patients suggests that the haploinsufficiency of genes in the 22q13 region, beside SHANK3, might contribute to cognitive and speech development deficits associated with PMS: among others, the deletion of SULT4A1 gene has been proposed to be related to neurological symptoms of PMS patients (Disciglio 2014).

2.2.3 Other pathologies

Differential expression of SULT4A1 may indicate a possible correlation of the protein with human diseases. For instance, genome wide microarray screens revealed that SULT4A1 may be upregulated two- to threefold in brain tissues of Alzheimer's subjects (Wang 2003); by contrast, a downregulation of SULT4A1 mRNA was observed in the dorsolateral prefrontal cortex of bipolar patients (Ryan 2006), in intracranial ependymomas (Modena 2006) and in the hippocampus of aging rats (Rowe 2007).

2.3 Regulation

In absence of any substantial catalytic activity, the role of SULT4A1 in the brain is yet to be revealed. An attempt towards this issue is represented by the identification of interacting proteins involved in SULT4A1 regulation and/or function.

Several authors provided evidences that post-translational modification of SULT4A1 can occur in cells, but whether these modifications are essential for enzymatic activity is still not known. Butcher et al. identified three cAMP responsive elements (CREs) located within the first 100 bp upstream SULT4A1 transcription star site (Butcher 2010). Two complexes can possibly bind to each CRE element in the promoter: the first complex contains CREB homodimers or

CREB/ATF-1 heterodimers, while the second complex contains ATF-2/c-Jun heterodimers. Even though ubiquitously expressed, both CREB and ATF-2 are preferentially expressed in the brain, so their transcriptional regulation of SULT4A1 may be a key element to understand why the protein is found predominantly in the brain.

Furthermore, SULT4A1 stability can be regulated by events of phosphorylation and dephosphorylation. In mouse brain, SULT4A1 can be phosphorylated at Thr8 and Thr11 (Trinidad 2008), and it has been demonstrated that MAP kinase ERK1 is responsible for Thr11 phosphorylation (Mitchell 2011); in addition, the serine/threonine phosphatase PP2A, which is particularly expressed in the brain, is capable of dephosphorylating SULT4A1 through the binding of its regulatory subunit Bβ (Mitchell 2011).

2.3.1 The interaction with Pin1

SULT4A1 phosphorylations at Thr8 and Thr11 are essential for the interaction between SULT4A1 and Pin1 (Mitchell 2011). Pin1 is a ubiquitously expressed peptidyl-prolyl *cis-trans* isomerase that binds to phosphorylated serine/threonine-proline motifs and catalyzes the *cis*-to-*trans* isomerization of prolines. It has already been shown that Pin1-mediated isomerization can regulate the stability of several proteins like p53, c-Myc and c-Jun (Wulf 2001; Yeh 2004): similarly, SULT4A1 can undergo Pin1-dependent isomerization through Pin1 interaction with the two binding motifs located at SULT4A1 N-terminus. One possible outcome of SULT4A1 conformational change is the enhancement of PP2A-mediated dephosphorylation and subsequent SULT4A1 degradation via calcium-dependent calpains (Mitchell 2009). However, multiple Pin1 sites, like those in SULT4A1 N-terminus, are supposed to increase Pin1 binding affinity but reduce isomerase efficiency (Smet 2005): this implies that SULT4A1 may not be isomerized after binding to Pin1, although an isomerization-dependent destabilization of SULT4A1 has already been demonstrated (Mitchell 2009). Therefore, it is not known whether SULT4A1 destabilization is due to Pin1 isomerization or Pin1 isomerase activity is directed toward other proteins involved in SULT4A1 degradation.

2.4 SULT4A1 deficiency in animal models

The highly conserved nature of SULT4A1 has allowed the use of zebrafish as a model organism to investigate the function of the protein. Indeed, zebrafish and human SULT4A1 share considerable sequence homology (87% identity and 92% similarity) (Crittenden 2014); moreover, the early development of the nervous system in zebrafish larvae provides an excellent opportunity to gain insight into the activity of a brain-specific sulfotransferase.

Falany et al. reported that the morpholino-induced knockdown of SULT4A1 expression in zebrafish embryos significantly affected a great number of cellular pathways, leading to the deregulation of hundreds of genes (Crittenden 2014): phototransduction resulted to be the cellular process mainly dysregulated by the knockdown, while other processes, including circadian rhythm signaling and CREB signaling, were affected to a less extend.

Further investigations revealed that mutant zebrafish lacking SULT4A1 did not present any relevant morphological phenotype but exhibited excessively sedentary behaviors during wakefulness (Crittenden 2015). A possible explanation for this result is the disruption of the normal sleep cycles in the mutant strain of zebrafish: this suggests that SULT4A1 may be involved in the regulation of hypocretin and/or melatonin signaling, which are the central pathways in the regulation of sleep and wakefulness in diurnal vertebrates like zebrafish.

AIM OF THE WORK

The superfamily of cytosolic sulfotransferases (SULTs) includes enzymes capable of transferring a sulfuryl group from the obligate donor PAPS (3'-phosphoadenosine 5'-phosphosulfate) onto a variety of exogenous and endogenous substrates (Negishi 2001). In 2000, a novel member of this family (SULT4A1) was isolated from human and rat brain (Falany 2000). Initially termed "brain sulfotransferase-like" due to the almost exclusive expression in neuronal tissue, it was subsequently renamed SULT4A1 according to sequence homology to other cytosolic sulfotransferases (Blanchard 2004). Besides, SULT4A1 is the most conserved member of SULT family and it has been identified in all the species investigated to date; even species like humans and zebrafish, which do not share other homologous SULT genes, present high degree of homology in SULT4A1 genes (92% amino acid identity). However, SULT4A1 crystal structure suggests that the enzyme may not be able to catalyze the reaction of sulfonation, being the active site too small to host the cofactor PAPS (Allali-Hassani 2007). Thus, in absence of any known substrate, SULT4A1 biological function remains obscure.

Family transmission disequilibrium analyses have pointed to a possible role for SULT4A1 in schizophrenia susceptibility: indeed, specific SNPs and microsatellite polymorphisms in SULT4A1 gene appeared to be transmitted more often than expected to affected offspring (Brennan 2005) and were related to clinical symptoms and cognitive function in schizophrenia patients (Meltzer 2006); moreover, SULT4A1-1 haplotype status correlated with severity of clinical symptoms, antipsychotic drug response and hospitalization risk (Ramsey 2011 and 2014). Furthermore, SULT4A1 has been suggested to be associated with neurological symptoms of Phelan-McDermid Syndrome (Disciglio 2014) and altered levels of SULT4A1 protein have been observed in bipolar and Alzheimer's patients (Wang 2003; Ryan 2006).

Considering its highly conserved nature and the alterations in its expression observed in different physiological and pathological states, it is possible that SULT4A1 might have a key function in the central nervous system. Thus, we decided to investigate the still unknown role of SULT4A1 within neuron development and functioning.

We decided to assess its physiological expression, focusing on some of the brain areas mainly involved in schizophrenia and autism (i.e. cortex, hippocampus, striatum and cerebellum). Given the possible implication of SULT4A1 in the pathogenesis of neurodevelopmental disorders a major point for our study was the evaluation of SULT4A1 expression during physiological neuronal maturation: to this purpose, we used rat primary neuronal cultures as well as human iPSC-derived neural stem cells in order to gain a more complete picture of SULT4A1 protein expression in different species.

Moreover, considering that abnormalities in dendritic spines and neuronal arborization are some of the most consistent anatomical correlates of neurodevelopmental disorders (Hung 2008; Glausier 2013; Jiang 2013; Moyer 2015), we characterized the effect of SULT4A1 on spine dynamics and dendrite morphology: in particular, we overexpressed or silenced SULT4A1 in cortical cultures so to analyze neuronal arborization as well as spine density and morphology.

In light of the possibility that SULT4A1 polymorphisms may lead to a reduction of mRNA translatability (Brennan 2005), and to clarify the specific role of SULT4A1 in neuronal maturation and functioning we knocked down SULT4A1 in neuronal cells using a specific shRNA. Neurons infected or transfected with the SULT4A1 shRNA were analyzed by biochemistry and electrophysiology.

MATHERIALS AND METHODS

1. DNA constructs and vectors

For RNA interference, a siRNA sequence targeting SULT4A1 C-terminal (siSULT4A1) was designed following GenScript siRNA Target Finder instructions (GenScript) and exhibits the following nucleotide sequence: AAGTGTGACCTCACGTTTGAC. The sequence was used to generate a short hairpin RNA (shRNA) which was cloned into 2nd generation lentiviral transfer vector pLVTHM-GFP (Wiznerowicz and Trono, 2003) with EcoRI and ClaI restriction sites (shSULT4A1). A scrambled form of siSULT4A1 was cloned into pLVTHM-GFP so to generate the control shRNA (shCtrl).

pFlag-SULT4A1 previously described (Mitchell 2009) was modified using QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies) to generate pFlag-SULT4A1r construct: this construct is resistant to interference by siSULT4A1 and was generated by changing three nucleotides of the siSULT4A1 target site, without changing the amino acid sequence of the resultant protein.

As control, pLVTHM-GFP vector was used.

2. Animals

To prepare primary neuronal rat cultures, pregnant female rats (Rattus norvegicus) of the phylum Wistar were purchased from Charles River (Charles River Laboratories, Calco, Italy). C57BL/6 wild-type mice were purchased from Charles River (Charles River Laboratories, Calco, Italy). Mice and rats were housed under constant temperature (22 ± 1°C) and humidity (50%) conditions with a 12 h light/dark cycle, and were provided with food and water ad libitum. All experiments involving animals followed protocols in accordance with the guidelines established by the European Communities Council and the Italian Ministry of Health (Rome, Italy).

3. Primary neuronal culture preparation and transfection

Low density rat cortical neuronal cultures were prepared from embryonic day (E) 18 rat embryos (Charles River) as previously described with slight modifications (Verpelli 2010). Neurons were plated at high density (350-400 cells/mm^2) or medium density (150-200 cells/mm^2) on 6- or 12-well plates (Euroclone) with or without coverslips (VWR), coated with 0.01 mg/ml poly-L-Lysine (Sigma-Aldrich). Neurons were cultured in Neurobasal (ThermoFisher) supplemented with home made B27 which is a slight variation of a previously described formula (Chen 2008). 6- and 12-well plates without coverslips were used for protein biochemical analysis, whereas 12-well plates with coverslips were used for immunocytochemical or electrophysiological analysis. Neurons were transfected using Lipofectamine 2000 at day-in-vitro 7 (DIV7), and the experiments were performed at DIV14.

4. Lentiviral production and infection of primary rat neuronal cultures

Genetically modified lentiviruses were produced as previously described (Naldini 1996; Lois 2002) and the production was carried out with 2nd and 3rd generation lentiviral transfer vectors. Lentiviral infection took place at DIV7 and the experiments were performed at DIV14.

5. Preparation and neuronal differentiation of human iPS cells

Human blood samples were collected according to a clinical protocol approved by the local Bioethical Committees of different medical centers. Participating individuals have been informed of the objectives of the study and have signed an informed consent before inclusion in the study. Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll and growth in StemPro®-34 SFM Medium (ThermoFisher), supplemented with L-Glutamine (2mM, Euroclone), PenStrep (1%, ThermoFisher), SCF (100ng/mL, ThermoFisher), FLT-3 (100ng/mL, ThermoFisher), IL-3 (20ng/mL, ThermoFisher), IL-6 (20ng/mL, ThermoFisher). To generate induced pluripotent stem cells (iPSCs), PBMCs were transduced with 2.0

Sendai virus particles containing four Yamanaka factors using the integration-free CytoTune-iPS Sendai Reprogramming Kit (ThermoFisher). After seven days, transduced cells were plated on cultures dishes coated with hESC-qualified matrigel (Corning) and grown with Essential 8 medium (ThermoFisher). Three to four weeks after transduction, iPSC colonies were picked and transferred onto matrigel-coated culture dishes (Corning) for further expansion or analysis. Immunofluorescence and RT-PCR experiments were performed to detect pluripotency markers (Oct 3/4, Lin28, Nanog and Sox2). Then, iPSCs were differentiated to neural stem cells (NSCs) via embryoid body method (Verpelli 2013). To obtain terminally differentiated neurons, proliferating NSCs were plated on matrigel-coated 6- or 12-well plates and cultured in Neurobasal medium supplemented with B27 w/o vitA (2%, ThermoFisher), PenStrep (1%, ThermoFisher), Glutamax (2mM, ThermoFisher), NT-3 (10ng/mL, Miltenyi Biotec), BDNF (10ng/mL, Miltenyi Biotec), GDNF (10ng/ml, Miltenyi Biotec), Retinoic Acid (1uM, Sigma-Aldrich) and growth for 50 days (Borroni 2017). Medium was changed every 2–3 days thereafter.

6. Sample preparation and western blot analysis

Cells or brain lysates were collected with precooled “buffered sucrose” [0.32 M sucrose (Sigma-Aldrich)/4mM HEPES-NaOH buffer (Sigma-Aldrich), pH 7.3, protease inhibitors (Roche), phosphatase inhibitors (Roche)] and analyzed via Bradford protein assay (Bio-Rad) to assess protein concentration. For total lysate, proteins were solubilized in 2-4x loading buffer [(250 mM Tris, 8 % (w/v), 40 % (v/v) glycerol, 0.008 % (w/v) bromophenol blue (all Sigma-Aldrich)] in order to have a final protein concentration of 1 µg/µl in the sample. In other cases, fractionation took place prior to Bradford protein assay analysis to obtain a synaptosome-enriched fraction (P2) (Huttner 1983; Grabrucker 2011). Also in the case of P2 fractions, 2-4x loading buffer was added to have a final protein concentration of 1 µg/µl in the sample. Samples were then heated for 10 min at 95°C; 5-10 µg of samples were loaded in the pockets of 7.5-12% polyacrylamide gels (homemade with reagents from Bio-Rad) and proteins were electrophoretically separated by sodium dodecyl sulfate-Polyacrylamide gel electrophoresis (SDS-PAGE). Then,

proteins were electroblotted onto nitrocellulose membranes using the Trans-Blot Turbo System (Bio-Rad); membranes were then stained by Ponceau S Stain (Sigma-Aldrich) to control the efficiency of protein transfer and were subsequently washed twice in Tris-buffered saline-Tween (TBS-T) [20 mM Tris pH 7.4, 150 mM NaCl (both Sigma-Aldrich), and 0.1% Tween 20 (Bio-Rad)]. Blocking of membranes took place for at least 1.5 h at 4°C in blocking buffer (TBS-T and 5% dried nonfat milk). Primary antibodies were applied for 3-16 h in blocking buffer (here: with 3 instead of 5% dried nonfat milk); then, HRP-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories) were used in blocking buffer (3% dried nonfat milk). After each antibody incubation, 3 washes (10 min each) took place with TBS-T. Finally, chemiluminescence was induced using an ECL kit (GE Healthcare). Immunoblot band intensity was quantified manually with ImageJ (US National Institutes of Health), an open source program. Normalization took place via actin.

7. Immunocytochemistry

Cells were fixed in 4% paraformaldehyde and 4% sucrose at room temperature for 10 minutes and then washed 3 times (10 min for each wash) with PBS [136.8 mM NaCl, 2.68 mM KCl, 10.1 mM Na_2HPO_4 and 1.76 mM KH_2PO_4, pH 7.4 (all Sigma-Aldrich)]. Primary antibodies were diluted in homemade gelatin detergent buffer (GDB) [30 mM phosphate buffer, pH 7.4, 0.2% gelatin, 0.5% Triton X-100, 0.8 M NaCl (all Sigma-Aldrich)] and applied for 3 hours at room temperature or o/n at 4°C. Secondary antibodies conjugate with fluorophores (Jackson ImmunoResearch Laboratories) were also diluted in GDB buffer and applied for 1 h. After each antibody incubation, 3 washes (10 min each) took place with “high-salt buffer” [20mM Na_2HPO_4/NaH_2PO_4 and 0.5M NaCl, pH 7.4 (all Sigma-Aldrich)] and before mounting a final wash (for 10 min) was carried out with PBS. Before mounting, coverslips were briefly passed through ddH_2O to dilute salts. 4',6-diamidino-2-phenylindole (DAPI) staining (ThermoFisher) was carried out for 2 min (DAPI diluted in PBS to a final concentration of 0.5 µg/ml) and took place during the washing steps before mounting the coverslips with Mounting Medium (Vecta Shield).

8. Section Preparation and Immunohistochemistry

Animals were anesthetized with an intraperitoneal injection of Avertin (Sigma-Aldrich) and perfused with 5% sucrose and 4% paraformaldehyde. Then, brains were left overnight in 4% paraformaldehyde, followed by incubation in 30% sucrose. Finally, brains were included in cryomolds with Tissue-Tek OCT compound (Sakura) and put at –80°C until cryostat sectioning. 10µm-thick slices were collected on polysine microscope adhesion slides (ThermoFisher) and than incubated in blocking solution (3%BSA, 10% goat serum, 0.4% Triton-X-100, diluted in PBS). Primary and fluorophore-conjugated secondary antibodies were diluted in blocking solution and applied respectively o/n at 4°C and 1h at room temperature. After each antibody incubation, 3 washes (10 min each) took place with PBS. 4',6-diamidino-2-phenylindole (DAPI) staining (ThermoFisher) was carried out for 2 min (DAPI diluted in PBS to a final concentration of 0.5 µg/ml) and took place during the washing steps before mounting the coverslips with Mounting Medium (Vecta Shield).

9. Antibodies

The following primary antibodies were used: mouse anti-β-actin (Sigma-Aldrich), mouse anti- βIII-tubulin (Sigma-Aldrich), mouse anti-Calbindin D-28K (Swant), mouse anti-GABARα1 (NeuroMab), mouse anti-GAD65 (Synaptic System), mouse anti-GAD67 (Santa Cruz Biotechnology), rabbit anti-GluA1 (Millipore), mouse anti-GluA2 (NeuroMab), mouse anti-GluN1 (NeuroMab), mouse anti-GluN2A (NeuroMab), mouse anti-GluN2B (NeuroMab), mouse anti-MAP2 (Abcam), rabbit anti-mGlu5 (Millipore), mouse anti-Nestin (Millipore), mouse anti-NL1 (NeuroMab), mouse anti-Oct3/4 (Santa Cruz Biotechnology), goat anti-Parvalbumin (Swant), mouse anti-Pin1 (Santa Cruz Biotechnology), mouse anti-PSD-95 (NeuroMab), rabbit anti-Sox2 (Proteintech), rabbit anti-SULT4A1 (Proteintech), rabbit anti-VGAT (Synaptic System).

All HRP- and fluorophore-conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratories.

10. Image acquisition and processing

Confocal images were obtained using LSM 510 Meta confocal microscope (Carl Zeiss, a gift from Fondazione Monzino) with Zeiss 63x, 40x or 20x objectives at a resolution of 1024 x 1024 pixels. Images represent averaged intensity Z-series projections of 5-7 individual images taken at depth intervals of around 0.45 µm. Labeled, transfected cells were chosen randomly for quantification from 3 independent experiments for each condition and image analysis was performed under blind condition.

For dendritic spine analysis, morphometric measurements were performed using MetaMorph software (Molecular Devices). Individual dendrites were selected randomly and their spines were traced manually. Maximum length and head width of each spine were measured and archived automatically.

For neuronal arborization analyses, primary and secondary dendrites were measured manually while Sholl analysis was performed using NeuronStudio (Computational Neurobiology and Imaging Center Mountain Sinai School of Medicine, New York, NY). Sholl analysis is a method of quantitative analysis used in neuronal studies to characterize the morphological features of an imaged neuron. It creates a series of concentric circles around the soma of the neuron. Within each sphere, various metrics can be obtained such as the total length of intersecting dendrites or the number of branching points. We performed Sholl analysis to measure the number of branching points in order to evaluate the dendritic arborization complexity in adult neurons (DIV14).

11. Electrophysiological recording

Whole-cell patch-clamp recordings were performed on DIV14 neurons at room temperature using a Multiclamp 700A amplifier and pClamp 10.5 software (Molecular Devices, Sunnyvale, CA, USA) in voltage-clamp configuration; signals were filtered at 1kHz and sampled at 10 kHz. External bath solution contained (mM): 129 NaCl, 1.25 NaH_2PO_4, 35 glucose, 1.8 $MgSO_4$, 1.6 $CaCl_2$, 3 KCl and 10 HEPES, pH 7.4 with NaOH. Spontaneous excitatory postsynaptic currents (sEPSCs) were recorded at -70 mV in the presence of gabazine (12.5 µM) with an

internal pipette solution containing (mM): 120 K-gluconate, 15 KCl, 2 $MgCl_2$, 0.2 EGTA, 10 HEPES, 20 phosphocreatine-tris, 2 ATP-Na_2, 0.2 GTP-Na_2 and 0.1 leupeptin, 3 lidocaine N-ethyl bromide, pH 7.2 with KOH. Spontaneous inhibitory postsynaptic currents (sIPSCs) were recorded in the presence of kynurenic acid (3 mM) with an internal pipette solution containing (mM): 135 CsCl, 2 $MgCl_2$, 0.2 EGTA, 10 HEPES, 20 phosphocreatine-tris, 2 ATP-Na_2, 0.2 GTP-Na_2 and 0.1 leupeptin, 3 lidocaine N-ethyl bromide, pH 7.2 with KOH; in these conditions, Cl^- reversal potential was 0 mV, thus allowing to record at hyperpolarized potentials and avoid noise artifacts. The quantification of the instantaneous frequency of postsynaptic currents was performed with Clampfit 10.5 and their means and cumulative probabilities were analyzed with Origin 8.5 software.

12. Data analysis and display

Data are expressed as means ± SEM or percentage, were analyzed for statistical significance, and were displayed by Prism 5 software (GraphPad, San Diego, CA). If there were only two groups whose means were compared, a student's t-test was carried out to assess statistical significance. The accepted level of significance was $p \leq 0.05$. In all cases with three or more groups, a one factorial analysis of variance (ANOVA) was calculated for the data and if group means differed in a significant manner ($p \leq 0.05$), a post hoc Tukey test was calculated to assess statistical significance. The accepted level of significance for the post hoc test was $p \leq 0.05$. Electrophysiology measurements were compared with the Kolmogorov-Smirnov test, statistical significance level was set at $p \leq 0.05$.

13. Acknowledgement

Electrophysiological analysis was carried out by Dr. Benedetta Terragni (Carlo Besta Neurological Institute IRCCS Foundation, Milan, Italy). The pFlag-SULT4A1 construct was a gift from Prof. Rodney Minchin (University of Queensland, Brisbane, Australia).

RESULTS

1. SULT4A1 is widely and differentially expressed in mouse brain

In both human and rat, SULT4A1 is expressed throughout the central nervous system (CNS) and it appears to have a particularly strong expression in distinct areas of the cerebral cortex (e.g. prefrontal cortex), cerebellum (e.g. neuronal stroma) and brainstem (e.g. hypoglossal nucleus) (Liyou 2003). In mouse, the amount of SULT4A1 mRNA level is remarkably different between male and female, given that in female animals the mRNA level appreciably rises after 30 days after birth (Alnouti 2006). Given these premises, we decided to gain a more complete picture of SULT4A1 protein distribution, evaluating the area-specific physiological expression as well as potential changes during neuronal development.

We started assessing SULT4A1 protein levels in wild type mouse CNS, focusing on the areas mainly involved in neurodevelopmental disorders. To this purpose, we performed western blot (WB) analyses of total lysates of hippocampus, striatum, cerebral cortex and cerebellum dissected from 2-month-old male mice: our results showed that SULT4A1 is highly expressed in all the considered areas, especially in cerebral cortex and in cerebellum (*Fig.1A*). Moreover, the immunofluorescent staining of brain sections revealed a localization of SULT4A1 to neuronal cell bodies and dendrites throughout the cerebral cortex and to cerebellar Purkinje cells (*Fig. 1C*).

To confirm, at the protein level, the differences between male and female animals highlighted by Alnouti et al. (Alnouti 2006), we compared SULT4A1 protein expression in male and female mice at post-natal day 60 (P60), age at which female animals showed a significantly greater amount of SULT4A1 mRNA compared to male mice: however, we did not observe any significant difference in protein expression (*Fig. 1B*).

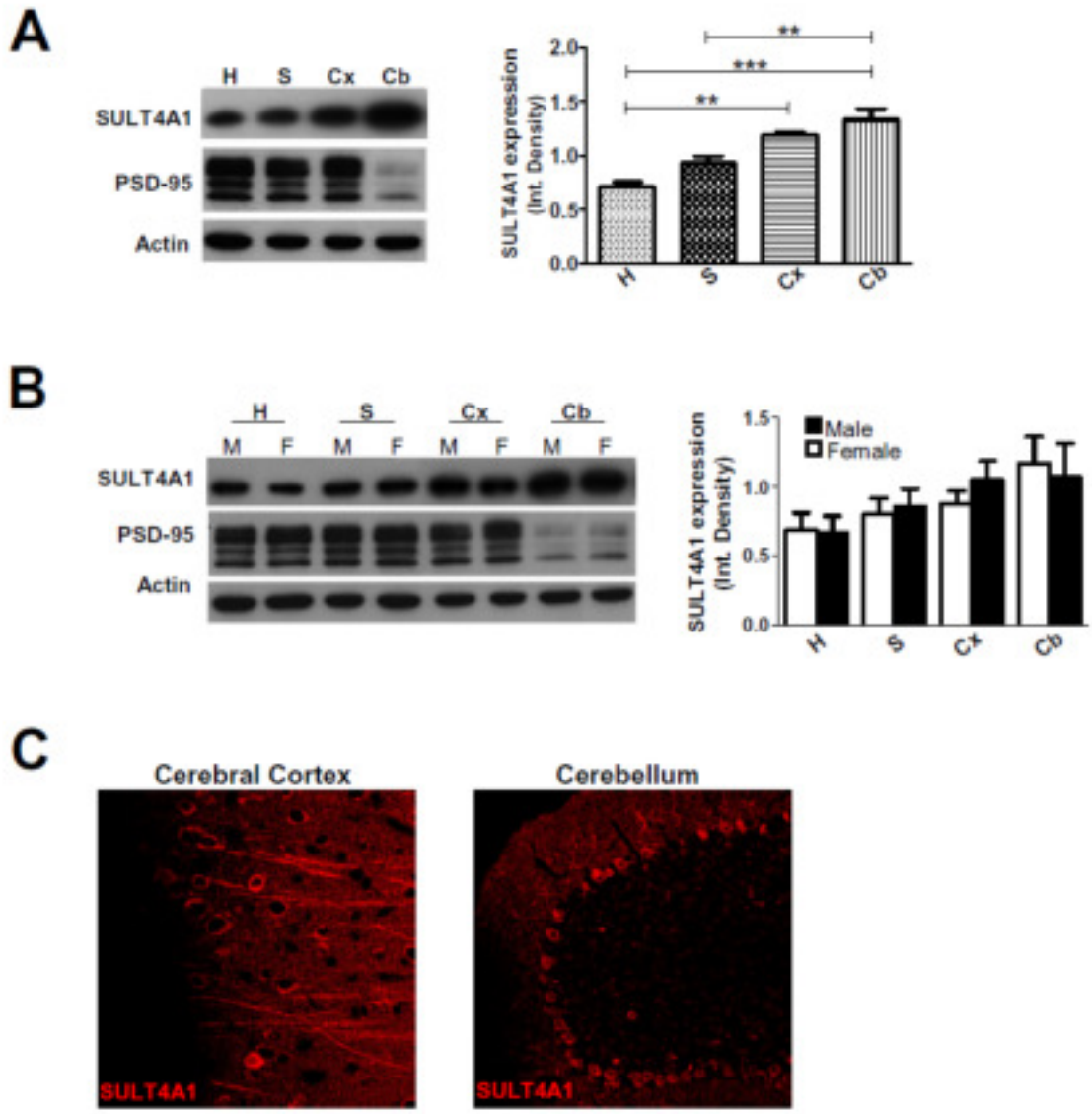


Figure 1. *The expression of SULT4A1 was studied in different brain areas of adult (P60) wild type mice. (A) Biochemical analysis of total lysates suggested that SULT4A1 is differentially expressed in hippocampus (H), striatum (S), cortex (Cx) and cerebellum (Cb). All values represent Mean ± SEM of five wild type male mice (** p<0.01; *** p<0.001; ANOVA test, Tukey post-test). (B) Area-specific expression of SULT4A1 appeared to be similar between adult male (M) and female (F) mice. All values represent Mean ± SEM of five wild type male mice and five wild type female mice. (C) Staining of mice brain sections revealed SULT4A1 localization to neuronal cell bodies and dendrites throughout the cerebral cortex (left) and to cerebellar Purkinje cells (right).*

2. SULT4A1 expression rises during neuronal maturation

Considering that SULT4A1 has been implicated in schizophrenia susceptibility (Brennan 2005; Condra 2007; Meltzer 2008) and has been suggested to be associated with neurological symptoms of Phelan-McDermid Syndrome (Disciglio 2014), a major point for our study was the evaluation of SULT4A1 neuronal expression during physiological maturation. So, we analyzed by western blot rat primary neuronal cultures at different stages of neuron maturation, Day-In-Vitro (DIV) 1, 7 and 14. From the results of these analyses, we demonstrated that the expression of SULT4A1 substantially rises during neuronal maturation, going from an almost undetectable level at DIV1 to an almost 4-fold higher level at DIV14 in cortical cultures (*Fig. 2A*). This result was confirmed by immunofluorescence staining of the same cultures where the protein showed a cytoplasmic localization and its level of expression steadily increased from DIV1 to DIV14 (*Fig. 2B*).

Immunofluorescence results also suggested that SULT4A1 is mainly expressed in GAD67-positive inhibitory neurons, in particular in Calbindin- and Parvalbumin-positive neurons (*Fig. 2C*).

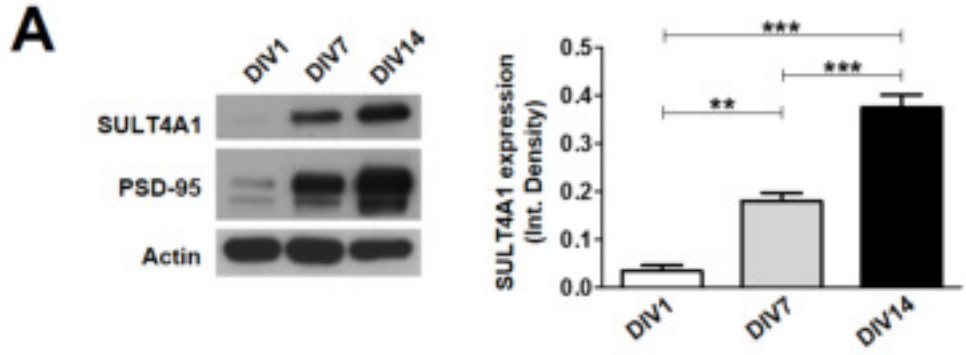


Figure 2. *Endogenous levels of SULT4A1 were evaluated in neuronal cultures at DIV1, DIV7 and DIV14.* *(Continues next page)*

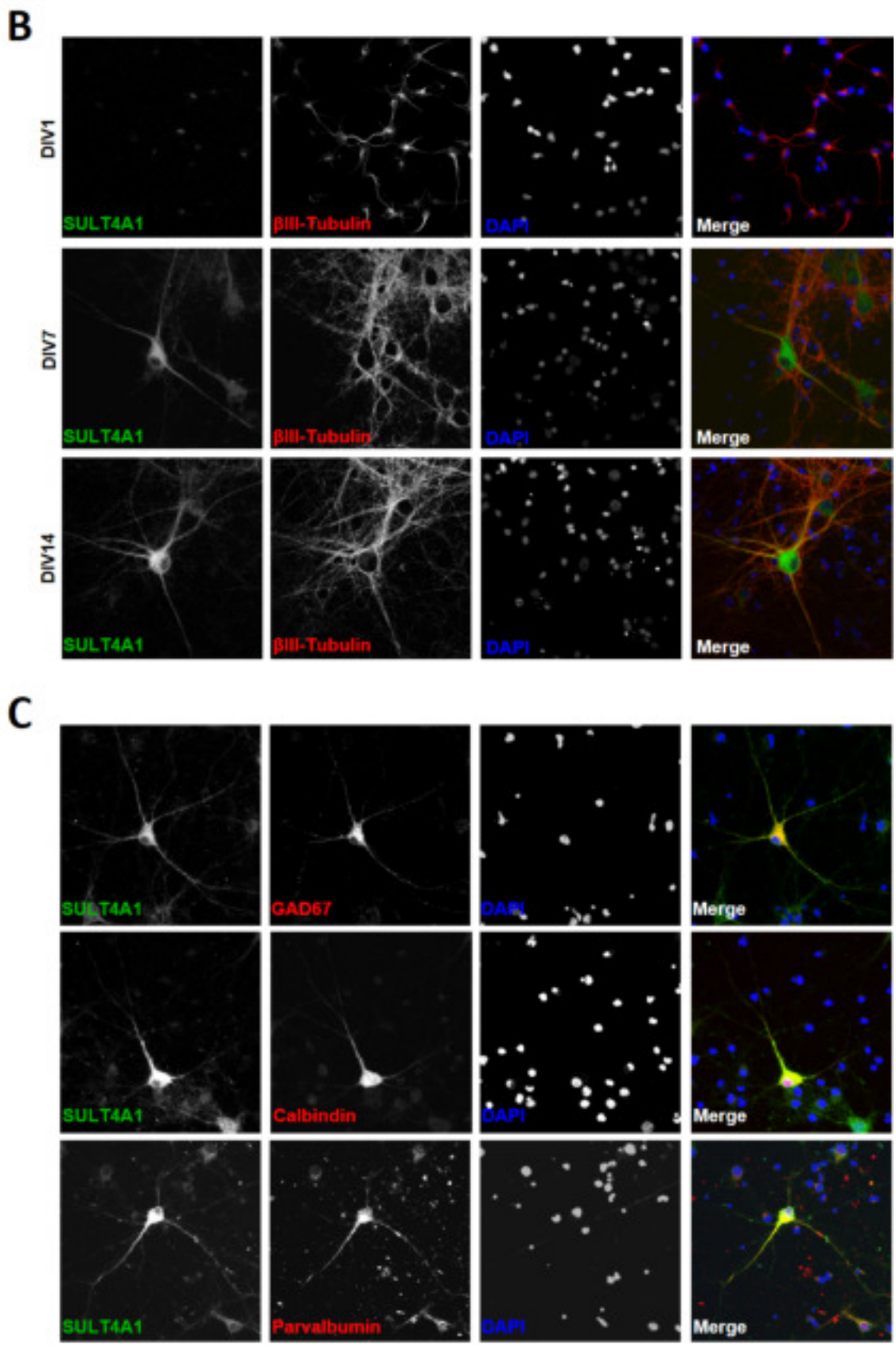

B
DIV1
DIV7
DIV14
SULT4A1
βIII-Tubulin
DAPI
Merge
C
SULT4A1
GAD67
Calbindin
Parvalbumin
DAPI
Merge

***Figure 2**. Endogenous levels of SULT4A1 were evaluated in neuronal cultures at DIV1, DIV7 and DIV14. (A) The expression of SULT4A1 in total lysates appreciably rises during neuronal maturation, going from an almost undetectable level at DIV1 to a ~4-fold greater level at DIV14. The gradual development of neurons is appreciable through the increase of PSD-95 levels (NeuroMab). All values represent Mean ± SEM of five independent experiments (** p<0.01; *** p<0.001; ANOVA test, Tukey post-test). (B) Rat cortical neurons at DIV1, DIV7 and DIV14 were stained with SULT4A1 antibody (ProteinTech) to evaluate endogenous levels of the protein. The protein showed a cytoplasmic localization, appreciable thanks to the βIII-Tubulin co-staining (Sigma-Aldrich) and its level of expression steadily increased from DIV1 to DIV14. (C) Co-staining with GAD67 (Santa Cruz Biotechnology), Parvalbumin (Swant) and Calbindin D-28K (Swant) showed that SULT4A1 is particularly expressed in inhibitory neurons.*

So far, SULT4A1 protein expression in human neurons has been scarcely investigated: in fact, beside the immunohistochemical studies on human brain slices performed by Liyou and collaborators (Liyou 2003), only few studies report the use of human cell lines (e.g. SK-N-MC) in the characterization of SULT4A1 regulation and level during neuronal maturation (Mitchell 2009; Sidharthan 2014). Therefore, to better determine SULT4A1 expression in human neurons, we obtained peripheral blood mononuclear cells (PBMCs) from control healthy individuals and reprogrammed them into induced Pluripotent Stem Cells (iPSCs) by introducing the four Yamanaka factors (Takahashi 2006). Once tested the expression of endogenous pluripotency markers (Oct3/4 and Sox2), iPSCs were differentiated into Nestin-positive Neural Stem Cells (NSCs) and subsequently into neurons for at least 50 days, time necessary to obtain MAP2-positive mature neurons. SULT4A1 expression was evaluated during neuronal maturation from NSC stage to mature neuron and the data from biochemical analysis suggested that the level of SULT4A1 protein rose during differentiation of NSCs into neurons (*Fig.3*).

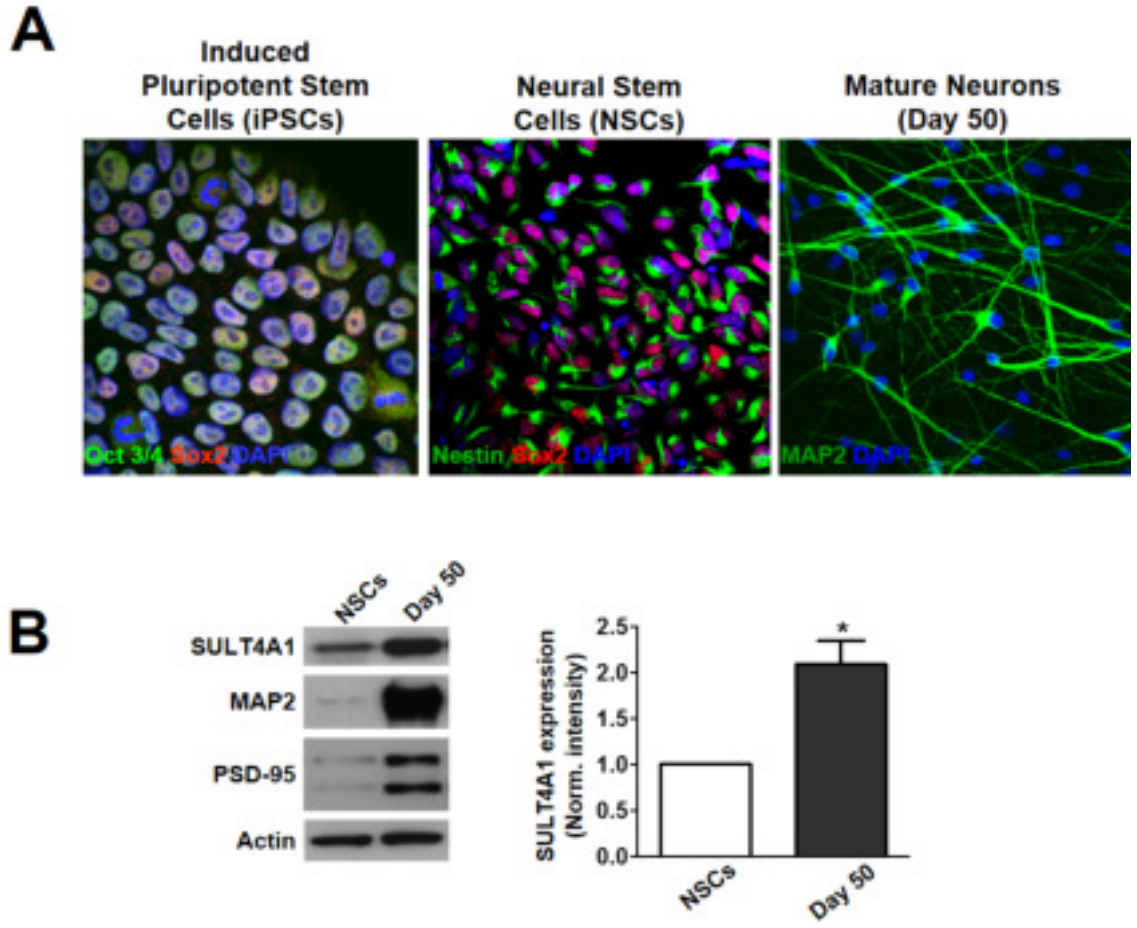


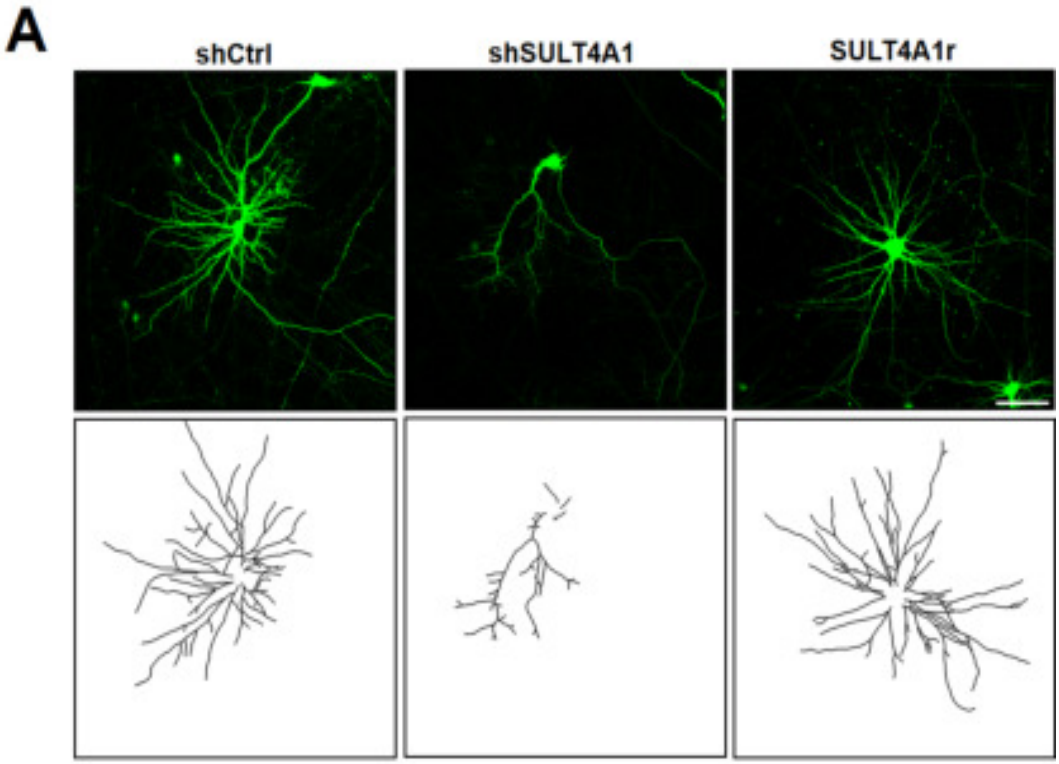
Figure 3. *Peripheral blood mononuclear cells (PBMCs) obtained from control healthy individuals were reprogrammed into induced pluripotent stem cells (iPSCs) in feeder-free conditions. iPSC-derived neural stem cells (NSCs) were differentiated into neurons for at least 50 days, time necessary to obtain MAP2-positive mature neurons (**A**). Immunoblots of total lysates of NSCs and 50-day-old neurons suggested that the level of SULT4A1 protein rises during differentiation. Neuronal differentiation can be appreciated by the increase of PSD-95 and MAP2 expression (**B**) All values represent Mean ± SEM of four independent experiments (* p<0.05; Student t test).*

3. SULT4A1 silencing reduces neuronal branching and dendritic spine density

Regardless its high level of conservation, SULT4A1 substrate and biological function remain obscure. Since it has been widely demonstrated that autism spectrum disorders and schizophrenia are often associated to alteration in neuronal arborization and dendritic spine morphology (Hung 2008; Glausier 2013; Jiang 2013; Moyer 2015), we decided to analyze dendritic branching complexity and spine density and morphology in cortical neurons overexpressing or lacking SULT4A1, so to identify the possible neuronal function of SULT4A1.

First, we generated a shRNA specific for SULT4A1 with the aim of examining the effect SULT4A1 knockdown in neuronal cultures. Briefly, rat cortical neurons were transfected at DIV7 with SULT4A1-specific shRNA (shSULT4A1) or control scrambled shRNA (shCtrl), and then fixed at DIV14 (*Fig.4A*). Dendrites quantification and Sholl analysis revealed that SULT4A1 depletion resulted in a simplification of neuronal branching, suggested by the reduction of the number of branching points, primary and secondary dendrites (*Fig.4B*). This phenotype was rescued by the overexpression of SULT4A1r, a construct resistant to interference by SULT4A1-specific shRNA *(Fig.4*).

SULT4A1 knockdown had also a remarkable effect on dendritic spine density; indeed, spines derived from neurons lacking SULT4A1 showed normal length and width but the number of those spines per 10 µm was significantly decreased. Once again, the resistant construct was able to revert the alteration of spine density (*Fig.5*).


A
shCtrl
shSULT4A1
SULT4A1r

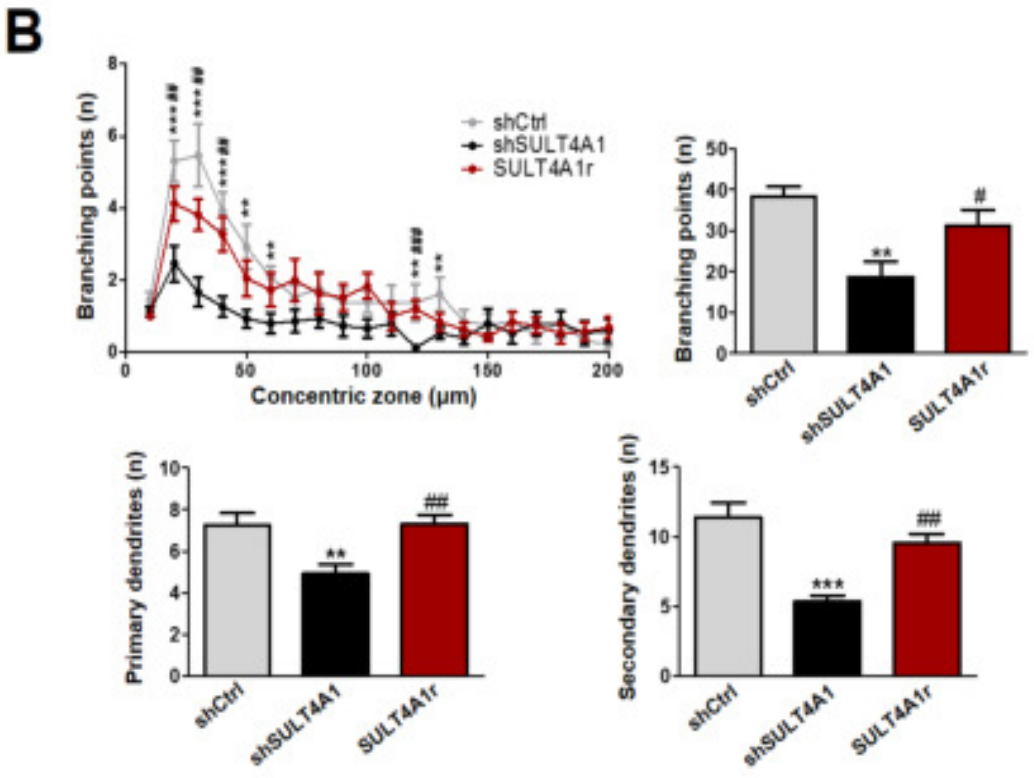

B
Branching points (n)
Concentric zone (µm)
shCtrl
shSULT4A1
SULT4A1r
Branching points (n)
Primary dendrites (n)
Secondary dendrites (n)

***Figure 4**. Confocal microscopy images showing neuronal morphology of rat cortical neurons transfected at DIV7 with a plasmid expressing shCtrl, shSULT4A1 or GFP and SULT4A1r (**A**). The quantification and distribution of number of branching points, primary and secondary dendrites revealed that SULT4A1 depletion led to a simplification of neuronal branching (**B**). All values represent Mean ± SEM (*shSULT4A1 vs shCtrl, * p<0.05, ** p<0.01, *** p<0.001; # shSULT4A1 vs SULT4A1r, ## p<0.01, ### p>0.001; Student t test). At least 10 neurons per each condition from three independent experiments were analyzed. Scale bar= 10 µm*

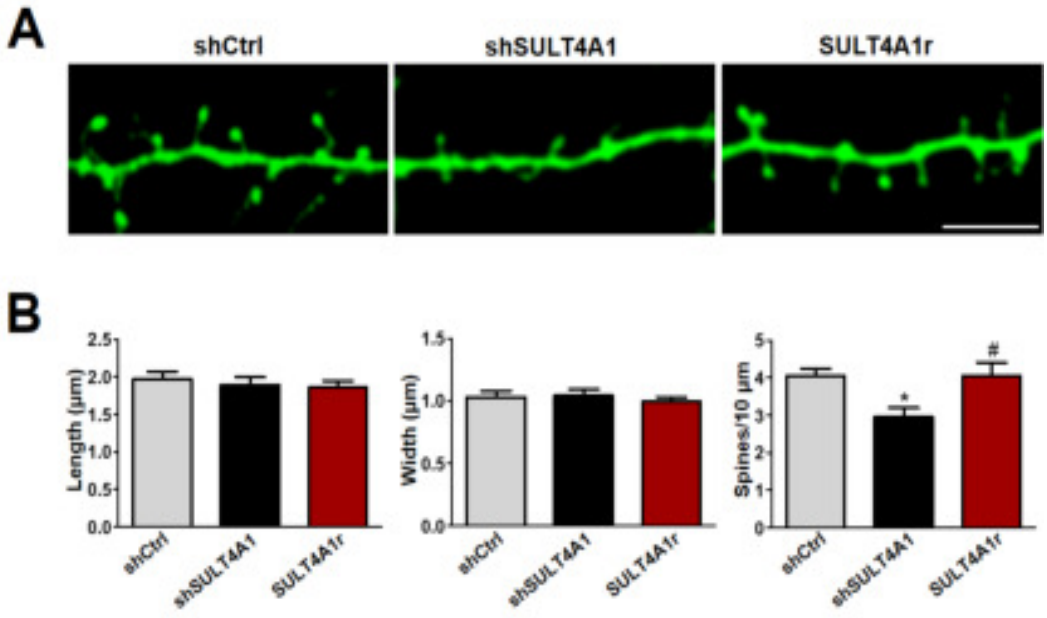


***Figure 5.** Immunofluorescence images showing dendritic spines of rat cortical neurons transfected at DIV7 with shCtrl, shSULT4A1 or GFP and SULT4A1r (**A**). Spines derived from neurons lacking SULT4A1 showed normal length and width but the number of those spines per 10 µm was significantly decreased (**B**). All values represent Mean ± SEM (*shSULT4A1 vs shCtrl, * p<0.05; # shSULT4A1 vs SULT4A1r, # p<0.05; Student t test). At least 10 neurons per each condition from three independent experiments were analyzed. Scale bar= 10 µm*

4. SULT4A1 overexpression impairs dendritic arborization and spines

To assess the effect of SULT4A1 overexpression, cortical neuronal cultures were transfected at DIV7 with GFP or GFP and SULT4A1 and were fixed and stained at DIV14 with anti SULT4A1 antibody, in order to locate SULT4A1-overexpressing neurons. The analysis of confocal microscopy images revealed that SULT4A1 overexpression led to an overall increase of the number of branching points, which appeared to have a different organization along the dendritic tree compared to wild type neurons: in fact, neuron overexpressing SULT4A1 presented a decreased number of branching points close to the soma (<50 μm from soma) and an increased number of distal branching points (>100 μm from soma) (*Fig.6*), suggesting a reorganization of neuronal arborization. However, the number of primary and secondary dendrites was apparently equal between the two conditions.

Moreover, cortical neurons overexpressing SULT4A1 displayed a slightly decreased density of dendritic spines, which were also considerably longer than wild type spines (*Fig.7*).

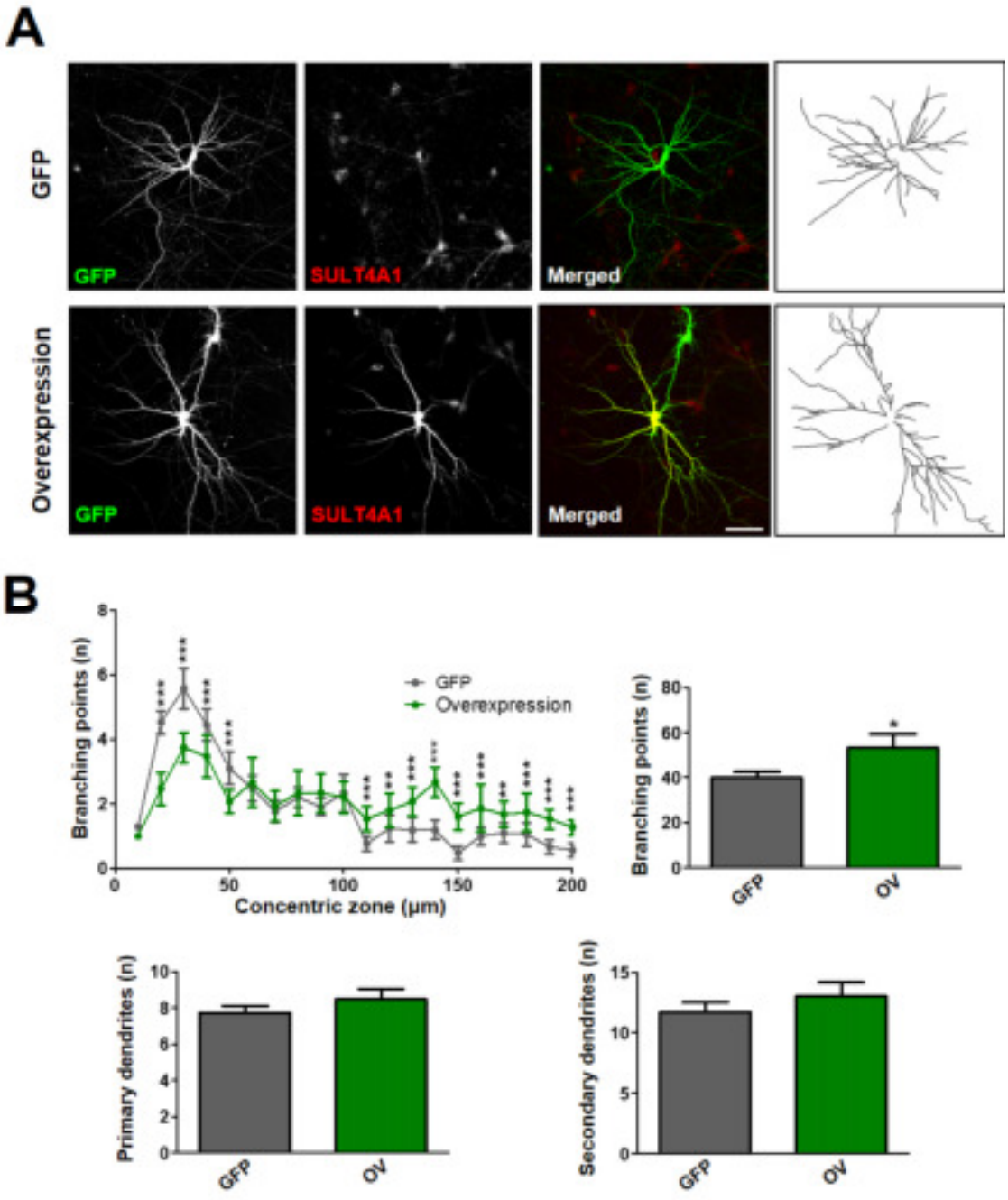


Figure 6. *Confocal microscopy images showing neuronal branching of rat cortical neurons transfected at DIV7 with a plasmid expressing GFP or GFP and SULT4A1 (**A**). Sholl analysis revealed that SULT4A1 overexpression (OV) led to a reorganization of neuronal branching, with an overall increase of the number of branching points (**B**). All values represent Mean ± SEM (* p<0.05, ** p<0.01, *** p<0.001, Student t test). At least 10 neurons per each condition from three independent experiments were analyzed. Scale bar= 10 µm*

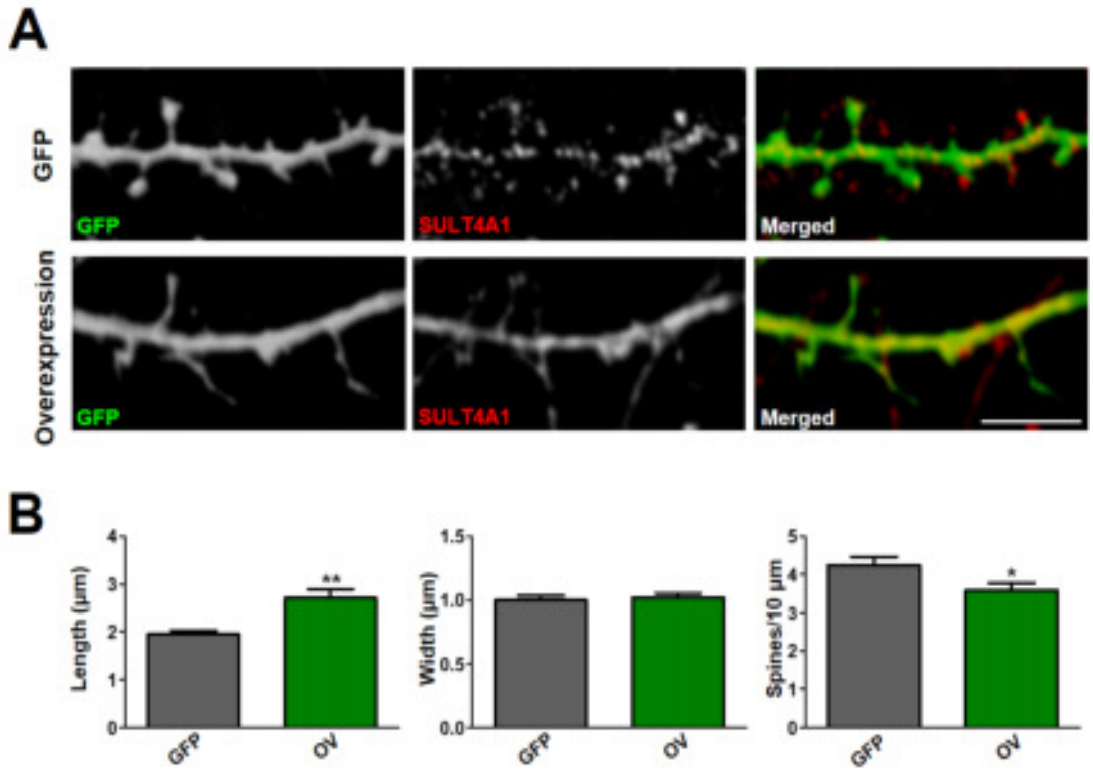


Figure 7. *Microscopy images showing dendritic spines of rat cortical neurons transfected at DIV7 with GFP or GFP and SULT4A1 (**A**). Spines derived from neurons overexpressing SULT4A1 (OV) showed increased length and decreased density (**B**). All values represent Mean ± SEM (* p<0.05; ** p<0.01, Student t test). At least 10 neurons per each condition from three independent experiments were analyzed. Scale bar= 10 µm*

5. SULT4A1 knockdown alters synaptic transmission

Several authors speculate that polymorphisms of SULT4A1 gene may be related to cognitive deficits and clinical symptoms in schizophrenia or schizophrenia spectrum disorder. For instance, the microsatellite polymorphism in the 5' end of SULT4A1 gene described by Brennan and collaborators, is believed to reduce its mRNA translatability and result in lower final levels of SULT4A1 enzyme (Brennan 2005).

For this reason, despite the intriguing results obtained through the overexpression of SULT4A1, we decided to focus on the characterization of SULT4A1 knockdown.

Based on the alterations of dendritic arborization and spine density, we examined the effect of SULT4A1 silencing on the level of relevant synaptic proteins, considering both excitatory and inhibitory synapses. The screening of total lysates of rat cortical neurons infected with shCtrl or shSULT4A1 revealed that SULT4A1 knockdown led to a significant increase of GAD65 protein level and, simultaneously, to a decrease of GluN1 amount (*Fig.8*). These data suggested that the presence of SULT4A1 might somehow influence both excitatory and inhibitory synaptic transmission.

A similar result was obtained when spontaneous post-synaptic currents were recorded from the same cultures: indeed, neurons lacking SULT4A1 displayed an appreciable reduction of spontaneous excitatory postsynaptic currents (sEPSC) frequency, reflected by the leftward shift of the curve in the cumulative probability plot, and to a slight augmentation of spontaneous inhibitory postsynaptic currents (sIPSC) frequency, reflected by the rightward shift of the curve in the range of 0.5-5 Hz (*Fig.9*).

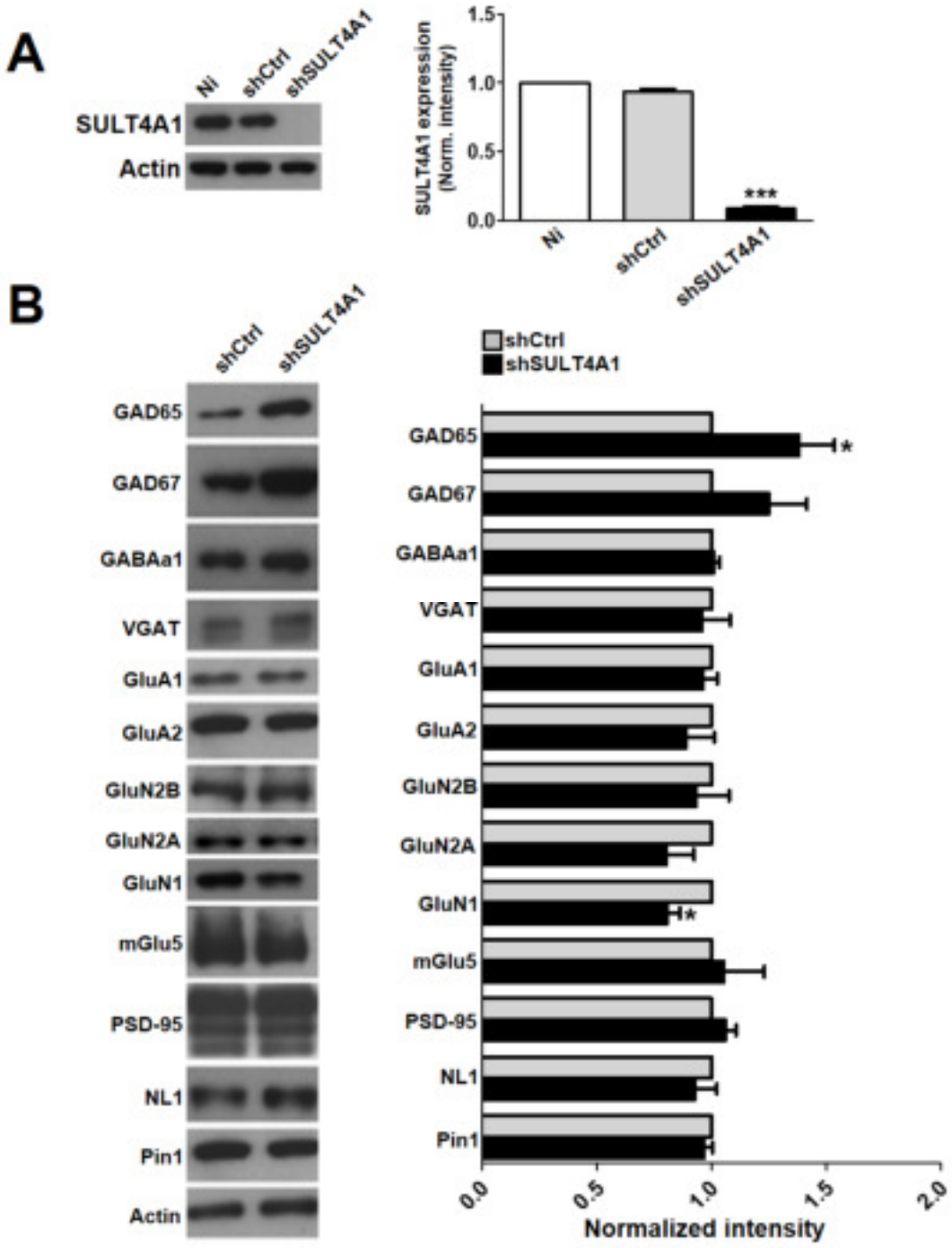


Figure 8. *The reduction of SULT4A1 expression following silencing was evaluated via biochemical analysis of lysates of cortical neurons infected with lentivirus expressing shCtrl or shSULT4A1. Both shCtrl (grey) and shSULT4A1 (black) conditions were compared with non-infected condition (Ni) so to verify whether the infection itself caused any alteration of SULT4A1 protein expression (**A**). The*

*knockdown of SULT4A1 led to a significant increase of GAD65 expression and a decrease of the levels of NMDAR subunit GluN1 (**B**). All values represent Mean ± SEM of four independent experiments (* shSULT4A1 vs Ni or shCtrl, *p<0.05, *** p<0.001, Student t test).*

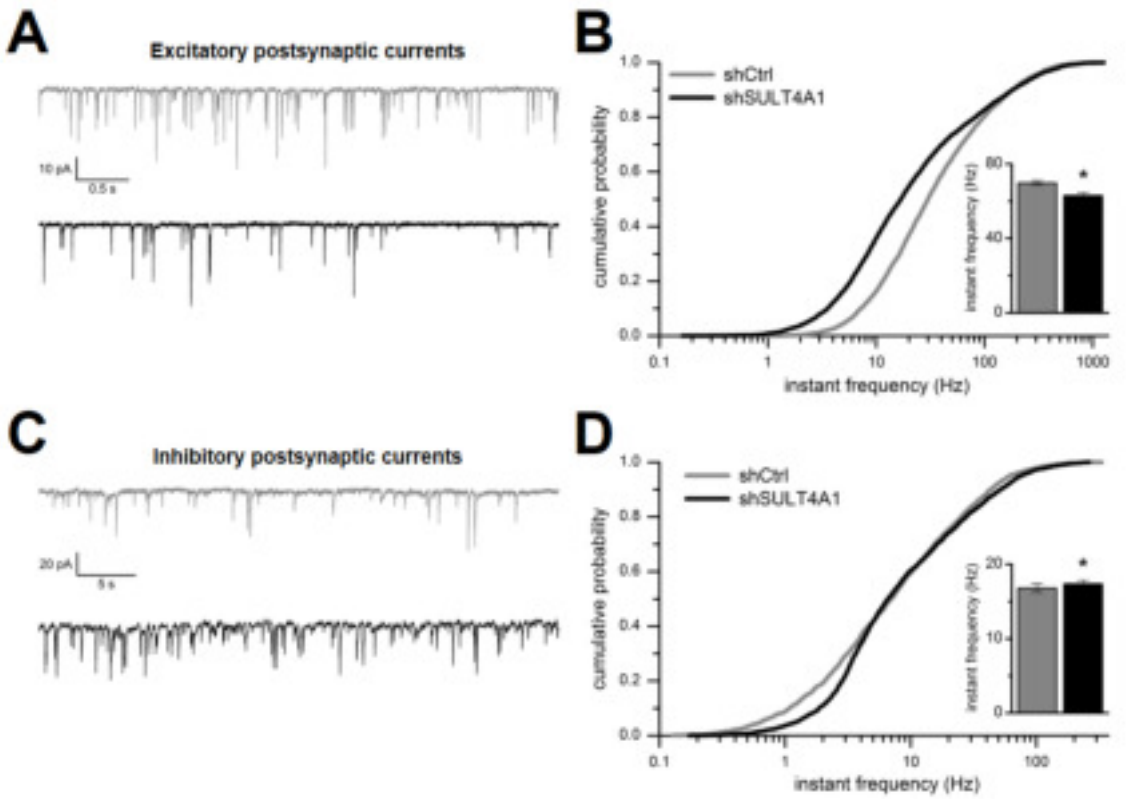


Figure 9. *Analysis of spontaneous postsynaptic currents. (**A**) Representative sEPSCs recorded from shCtrl (grey) or shSULT4A1 (black) transfected neurons. (**B**) Cumulative probability plot showing a significant decrease in sEPSCs frequency when SULT4A1 is silenced (Kolmogorov-Smirnov test, * p<0.05). Inset panel shows EPSCs mean instantaneous frequency. (**C**) Representative sIPSCs recorded from shCtrl (grey) or shSULT4A1 (black) transfected neurons. (**D**) Cumulative probability plot showing a small but significant increase in sIPSCs frequency when SULT4A1 is silenced (Kolmogorov-Smirnov test, * p<0.05). Inset panel shows sIPSCs mean instantaneous frequency. At least five neurons per each condition from three independent experiments were analyzed.*

6. The interaction with Pin1 might be the key to unveil SULT4A1 role in excitatory synapses

Peptidyl-prolyl cis-trans isomerase Pin1 is known to be recruited to excitatory synapses, where it exerts a negative action on synaptic transmission by suppressing protein synthesis and impeding the organization of crucial structural protein complexes (Westmark 2010). In particular, Antonelli and collaborators have demonstrated that Pin1 negatively affects NMDAR signaling and spine morphology by dampening PSD-95 ability to complex with the glutamate receptor subunits GluN1 and GluN2B (Antonelli 2016).

Mitchell and Minchin have proven that SULT4A1 is able to recruit Pin1 at the phosphoserine/threonine-proline motifs in its N-terminus, meaning that, potentially, SULT4A1 can be post-translationally modified (Mitchell 2009). However, multiple Pin1 sites, like those in SULT4A1 N-terminus, reportedly increase Pin1 binding affinity but reduce isomerase efficiency (Smet 2005), and this implies that SULT4A1 may not be isomerized after binding to Pin1.

Considering our data on the reduction of both GluN1 protein level and sEPSC frequency following SULT4A1 depletion, and the Pin1-driven modulation of synaptic transmission and dendritic spine dynamics proposed by Antonelli et al., we verified whether there was any detectable difference in the synaptic amount of Pin1, PSD-95 and NMDAR subunits in presence or absence of SULT4A1 expression. Interestingly, immunoblot analysis revealed that the synaptosomal preparations derived from SULT4A1-deficient neurons exhibited increased levels of Pin1, significantly lower levels of GluN1 and a reduction of GluN2B and PSD-95, albeit not statistically significant (*Fig.10*).

This observation prompted us to hypothesize that the binding of SULT4A1 to Pin1 somehow prevents Pin1 from exerting its negative effect on PSD-95/NMDAR complex formation, plausibly by binding to and seizing Pin1 or by operating as a substrate, alternative to PSD-95, for Pin1 isomerase activity.

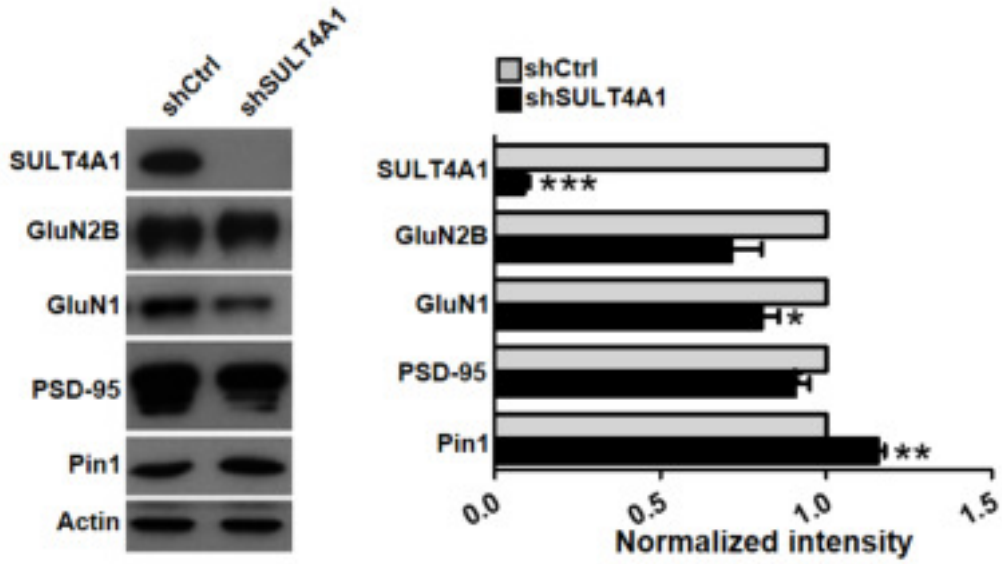


***Figure 10**. Representative immunoblots of synaptosome fractions obtained from neuronal cultures infected with shCtrl (grey) or shSULT4A1 (black). SULT4A1 silencing resulted in reduced synaptic level of GluN1 and increased level of Pin1. GluN2B synaptic level appeared to be reduced, even though the reduction was not significant (p=0.0883). All values represent Mean ± SEM of three independent experiments (*p<0.05, ** p<0.01, *** p<0.001, Student t test).*

DISCUSSION

Cytosolic sulfotransferases are a superfamily of enzymes that transfer a sulfuryl group to a wide range of endogenous and exogenous substrates; SULT4A1 is the newest member of this family. Since its identification in 2000 by Falany and collaborators (Falany 2000), SULT4A1 has presented an enigma in the field of cytosolic SULT biology: indeed, its biological function is yet to be revealed, mainly because several previous studies have failed to identify possible SULT4A1 substrates (Allali-Hassani 2007). However, given that it is highly conserved and expressed extensively, and almost exclusively, in the brain, it is possible that SULT4A1 may have a role in the central nervous system (Liyou 2003; Alnouti 2006). Moreover, some recent reports have associated polymorphisms in the SULT4A1 gene with susceptibility to schizophrenia (Brennan 2005; Meltzer 2006) and haploinsufficiency of SULT4A1 has been suggested also to be associated with neurological symptoms of Phelan-McDermid Syndrome (Disciglio 2014); additionally, altered levels of SULT4A1 protein have been observed in bipolar and Alzheimer's patients (Wang 2003; Ryan 2006).

Given this background, we decided to investigate the still unknown role of SULT4A1 within neuron development and functioning.

First, we decided to evaluate SULT4A1 tissue distribution in adult mouse brain, being the current knowledge of SULT4A1 area-specific expression restricted to human and rat tissues (Liyou 2003): immunoblots and fluorescence immunohistochemistry analyses confirmed that, even in mice, SULT4A1 is abundantly present in areas mainly associated with neuropsychiatric disorders, such as hippocampus, striatum, cerebral cortex and cerebellum. In particular, high levels of SULT4A1 were observed in cortical neurons and in cerebellar Purkinje cells (*Fig.1A-C*). We tested if SULT4A1 protein was differentially expressed between male and female brains but, in contrast to the data reported by Alnouti and collaborators (Alnouti 2006) we did not detect any significant difference. We only found a slightly, but not significantly, greater amount of SULT4A1 protein in female cortex, compared to male animals of the same age (*Fig.1-B*). This discrepancy might be due to the fact that we analyzed separately four distinct brain areas, whereas previously reported mRNA quantifications were performed on total mRNA isolated from the whole mouse brain.

Once elucidated its tissue distribution, a major point for deciphering SULT4A1 physiological function is the characterization of the enzyme expression during neuronal maturation.

To this purpose, we assessed SULT4A1 level during neuronal maturation in both rodent and human neuronal cultures: we started analyzing rat primary cortical cultures at different stages of maturation (i.e. DIV1, DIV7 and DIV14) (*Fig.2A-B*), then we differentiated human iPSC-derived neural stem cells (NSCs) into mature neurons and collected total lysates before and after neuronal differentiation (*Fig.3*). In line with previous observations regarding the increasing expression of SULT4A1 during neuronal maturation (Sidharthan 2014), we found that in both human and rodent neuronal cultures, SULT4A1 level rises during maturation and differentiation, suggesting an important role of this protein throughout CNS development.

While assessing SULT4A1 localization via immunostaining of rat cortical neurons, we noticed that some neurons were intensely labeled when stained with the antibody against SULT4A1: subsequent co-labeling assays revealed that the highly-positive cells were inhibitory, GAD67-positive neurons and more specifically Parvalbumin-positive and Calbindin-positive neurons (*Fig.2C*). This result provides an important clue for further investigation considering that, in the last few years, gabaergic circuitry disruption has been widely proposed to be an important player in the pathogenesis of schizophrenia and neurodevelopmental disorders (Pizzarelli 2011; Chattopadhyaya 2012; Nakazawa 2012).

The study of sequence homology and gene expression patterns can provide leads about gene function, but they do not reveal what is the exact physiological role of a gene. From this perspective, a powerful tool is represented by genetic engineering techniques which allow to modulate protein expression: few examples are gene overexpression, leading to abnormally high levels of gene expression (Prelich 2012), and gene silencing, achievable by RNA interference (RNAi)-based methodologies (Bantounas 2004).

Therefore, with the purpose of gaining insights into the activity of SULT4A1 gene, we verified how overexpression and silencing of SULT4A1 might affect phenotypes in neuronal cultures. Since it is widely acknowledged that

schizophrenia and autism spectrum disorders are often correlated to deficiencies in dendrites architecture and spine dynamics (Hung 2008; Glausier 2013; Jiang 2013; Moyer 2015), we started evaluating neuronal branching complexity and dendritic spine density and morphology in cortical neurons overexpressing or lacking SULT4A1.

Interestingly, both conditions altered neuronal physiology. Regarding neuronal arborization, we found a decrease in the number of branching points in absence of SULT4A1 (*Fig.4*), and, conversely, an increase of branching points number in neurons overexpressing SULT4A1, which also displayed a reorganization of branching points distribution along the dendritic tree (*Fig.6*).

Dendritic spines emerged to be influenced by SULT4A1 levels too: in fact, SULT4A1 knockdown affected spine density but without altering their morphology (*Fig.5*); on the other hand, SULT4A1 overexpression had a greater effect on spine morphology rather than on spine density, leading to a significant increase in spine length and a slight decrease in spine density (*Fig.7*). These observations suggest that the expression of SULT4A1 at the appropriate level is crucial to ensure proper neuronal development and functioning, seeing as the outcome of SULT4A1 expression below and above some critical threshold is a remarkable defect in both neuronal arborization and dendritic spines.

In the light of the possibility that SULT4A1 polymorphisms, identified in schizophrenia patients, may lead to a reduction of mRNA translatability (Brennan 2005), we further investigated the effects of SULT4A1 silencing, despite the intriguing results obtained through SULT4A1 overexpression.

Biochemical and electrophysiological analyses of cortical neurons knockdown for SULT4A1 showed that SULT4A1 deficiency perturbs the composition and activity of excitatory and inhibitory synapses. Immunoblotting for relevant excitatory and inhibitory markers unveiled an increase of GAD65 expression and a decrease of GluN1 levels in shSULT4A1-infected cortical neurons (*Fig.8*). Interestingly these data were in line with the electrophysiological recordings, where neurons lacking SULT4A1 displayed a slight increase in sIPSC frequency and a reduction of sEPSC frequency (*Fig.9*).

NMDA receptors mediate spine formation, maturation and stabilization via calcium-dependent signaling (Matus 2000): in particular, NMDAR-mediated calcium influx into dendritic spines induces forms of synaptic plasticity, such as long-term potentiation (LTP) and long-term depression (LTD) (Nagerl 2004; Tada 2006; Ultanir 2007). Given that proper expression and regulation of NMDARs is crucial for neuronal plasticity and maturation as well as learning and memory processes (Snyder 2013), it is not surprising that several authors have hypothesized a link between disrupted NMDAR function and developmental and psychiatric disorders: indeed, a large number of clinical and animal studies converge to the "NMDARs hypofunction hypothesis" as the possible root cause of diseases such as schizophrenia and autism spectrum disorders (Coyle 2012; Lee 2015).

A possible connection between SULT4A1 and NMDARs could be represented by the Peptidyl-prolyl cis-trans isomerase Pin1. The interaction between SULT4A1 and Pin1 has been demonstrated by Mitchell and collaborators (Mitchell 2009) but it is not clear whether this interaction implies SULT4A1 isomerization, being the multiple Pin1 sites in SULT4A1 N-terminus reportedly able to increase Pin1 binding affinity but reduce isomerase efficiency (Smet 2005).

Antonelli et al. demonstrated that Pin1 can be recruited to excitatory synapse by its binding to PSD-95: once isomerized, PSD-95 ability to complex with NMDAR subunits GluN1 and GluN2B is drastically reduced, thus affecting NMDA-mediated transmission and spine morphology (Antonelli 2016). Moreover, pyramidal neurons derived from Pin1-/- mice exhibits increased spine density and increased amount of GluN1 and GluN2B. These features resulted to be practically opposite to the phenotype described in SULT4A1 knockdown experiments.

Thus, this observation prompted us to hypothesize that the interaction with Pin1 might be the key to unveil SULT4A1 role in excitatory synapses. According to our hypothesis, SULT4A1 represents an alternate partner or substrate for Pin1, whether in neuronal soma or at a synaptic level. In absence of SULT4A1, a higher amount of Pin1 can be recruited to dendritic spines, where it exerts its negative effect on PSD-95/NMDAR complex formation. This hypothesis is also supported by the evidence that synaptosomal preparations derived from neurons infected

with shSULT4A1 presented increased amount of Pin1 and decreased content of GluN1, GluN2B and PSD-95 (*Fig.10*), suggesting a feasible major recruitment of Pin1 to the synapse in the absence of SULT4A1.

Validating this hypothesis requires a set of key experiments, which are currently in progress: first, a co-immunoprecipitation should be performed to demonstrate that SULT4A1 presence can interfere with Pin1/PSD-95 interaction; simultaneously, it is essential to assess NMDA-mediated currents in control and silenced conditions, so to have a functional match on NMDAR subunits decrease. Finally, the hypothesis of Pin1-based activity of SULT4A1 may offer the opportunity for a pharmacological rescue of the phenotypes caused by SULT4A1 depletion: indeed, Pin1 selective inhibitors are available and, among them, PiB (Millipore) has already been exploited to block Pin1 isomerase activity and modulate PSD-95/NMDAR complex formation (Antonelli 2016).

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