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Interaction of the brain-selective sulfotransferase SULT4A1 with other cytosolic sulfotransferases: effects on protein expression and function.

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Running title: Interaction of SULT4A1 with other SULTs

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Abbreviations: BSA, bovine serum albumin; HA, hemagglutinin; MAP2, microtubule-associated protein 2; PBS, phosphate buffered saline; RA, retinoic acid; SULT, sulfotransferase; TH, tyrosine hydroxylase

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Abstract

SULT4A1 is a brain-selective sulfotransferase-like protein that has recently been shown to be essential for normal neuronal development in mice. In the present study, SULT4A1 was found to co-localize with SULT1A1/3 in human brain neurons. Using immunoprecipitation, SULT4A1 was shown to interact with both SULT1A1 and 1A3 when expressed in human cells. Mutation of the conserved dimerization motif located in the C-terminus of the sulfotransferases prevented this interaction. Both ectopically expressed and endogenous SULT4A1 decreased SULT1A1/3 protein levels in neuronal cells, which was also prevented by mutation of the dimerization motif. During differentiation of neuronal SH-SY5Y cells, there was a loss in SULT1A1/3 protein but an increase in SULT4A1 protein. This resulted in an increase in the toxicity of dopamine, a substrate for SULT1A3. Inhibition of SULT4A1 using siRNA abrogated the loss in SULT1A1/3 and reversed dopamine toxicity. These results show a reciprocal relationship between SULT4A1 and the other sulfotransferases suggesting that it may act as a chaperone to control the expression of SULT1A1/3 in neuronal cells.

Significance statement:

The catalytically inactive sulfotransferase SULT4A1 may regulate the function of other SULTs by interacting with them via a conserved dimerization motif. In neuron-like cells, SULT4A1 is able to modulate dopamine toxicity by interacting with SULT1A3, potentially decreasing the metabolism of dopamine.

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Introduction

The aryl sulfotransferases (SULTs) are cytosolic enzymes that catalyze the transfer of a sulfonate group from the common cofactor 3' -phosphoadenosine 5'-phosphosulfate to a wide range of endogenous and exogenous substrates. In humans, there are at least 13 different SULT genes, which have been grouped into three families based on amino acid sequence homology: SULT1, SULT2, and SULT4 (Blanchard et al., 2004; Freimuth et al., 2004). The SULT1A family consists of 1A1, 1A2, 1A3 and 1A4 where the 1A3 and 1A4 genes appear to have arisen from a segmental duplication that encode the same protein (Hildebrandt et al., 2004). The SULT1A sulfotransferases are expressed widely with high abundance in the liver, lung, brain, skin, platelets, gastrointestinal tissues and kidney (Maus et al., 1982; Campbell et al., 1987; Heroux et al., 1989; Cappiello et al., 1990; Falany et al., 1990; Nakamura et al., 1990; Hume and Coughtrie, 1994; Kudlacek et al., 1995). In humans, the SULT1A subfamily members SULT1A1 and SULT1A2 primarily conjugate xenobiotics and have a role in the activation of carcinogens (Glatt, 2000), although they also sulfonated endogenous substrates (Falany, 1997; Strott, 2002). Human SULT1A3 has high catalytic activity towards catecholamines such as dopamine, noradrenaline and adrenaline (Veronese et al., 1994; Falany, 1997; Brix et al., 1999).

SULT4A1 is the only known member of the 4A family. It was originally cloned from human and rat brain (Falany et al., 2000) where it is predominantly expressed. Although SULT4A1 shares low sequence homology with other human SULTs, it is highly conserved between species (Minchin et al., 2007). SULT4A1 may be catalytically inactive because the active site is reportedly too small to accommodate 3'-phosphoadenosine-5'-phosphosulfate (Allali-Hassani et al., 2007). SULT4A1 expression is regulated by the cAMP response element binding protein and the activating transcription factor-2 (Butcher et al., 2010). Post-translationally, it is phosphorylated to generate a recognition site for the peptidyl propyl cis-

trans isomerase Pin1 (Mitchell and Minchin, 2009; Mitchell et al., 2011). The *SULT4A1* gene shows a very low rate of synonymous or non-synonymous mutations within its exonic sequence (Lewis and Minchin, 2009), and has the lowest overall mutation rate amongst all of the known humans sulfotransferases (Hildebrandt et al., 2007). Single nucleotide polymorphisms in the *SULT4A1* gene have been associated with schizophrenia susceptibility (Brennan and Condra, 2005; Condra et al., 2007; Ramsey et al., 2011). More recently, deletion of *Sult4a1* in a mouse model resulted in a severe phenotype that included absence seizures, tremors and ataxia (Garcia et al., 2018). The SULT4A1 null animals had a life-span of only 20-25 days. These experiments provide strong evidence that SULT4A1 may be an important protein in neuro-development. SULT4A1 has been identified in the mitochondrial fraction of neuronal cells and it has been suggested that it may be involved in regulating redox homeostasis and provide protection against mitochondrial dysfunction and oxidative stress (Hossain et al., 2019). This study further highlights the potential physiological importance of SULT4A1 expression.

Most mammalian cytosolic sulfotransferases are found as homodimers in solution, with the mouse estrogen sulfotransferase Sult1e1 being an exception. The physiological consequence of dimer formation is still unclear, although studies have shown differences in function and stability between the monomeric and dimeric forms (Thomae et al., 2002; Cook et al., 2010; Sidharthan et al., 2014). Crystallographic and site directed mutagenesis experiments have shown that a conserved sequence motif of 10 residues (KxxxTVxxxE) located towards the C-terminus of the protein is responsible for the interaction between the two sulfotransferase monomers (Petrotchenko et al., 2001). The two residues (T and V) at the center of the dimerization motif make strong symmetric contacts between the monomers. Dimeric human SULT1E1 contains a dimerization motif whereas monomeric mouse Sult1e1 contains the sequence PE in place of TV. Mutation of TV to PE in human SULT1E1 produced a monomer, whereas mutation of mouse PE to TV created a homodimer, establishing the validity

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of the motif as the dimerization interface (Petrotchenko et al., 2001). Interestingly, heterodimers can also form between different SULTs containing the dimerization motif (Petrotchenko et al., 2001). Enzyme kinetic studies indicated that the two monomers do not catalyze sulfonation independently when present as a dimer (Sun and Leyh, 2010; Leyh et al., 2013). Moreover, dimerization may allow for cofactor-dependent allosteric regulation of enzyme activity (Tibbs et al., 2015).

During a yeast two-hybrid screen of a human brain library with SULT4A1, interactions between SULT4A1 and a variety of cytosolic sulfotransferases were observed including SULT1A1 and SULT1A3, which are also found in abundance in the brain (Salman et al., 2009; James and Ambadapadi, 2013). These observations prompted the current study into the interaction of SULT4A1 with SULT1A1 and SULT1A3 using human cell models to identify protein-protein binding and determine whether any observed interactions affected the toxicity of the SULT1A3 substrate dopamine.

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Materials and Methods

Cell lines, transient transfections and cell differentiation. HeLa, SK-N-MC and SH-SY5Y cells were obtained from the American Type Culture Collection (Manassas, VA) and were maintained in RPMI 1640 medium (Hela), Dulbecco's modified Eagle's medium: nutrient mixture F-12 (SH-SY5Y) or minimum essential medium (SK-N-MC) supplemented with 10% fetal bovine serum and incubated at 37°C in 5% CO_2. For transient transfections, cells were seeded in 6-well plates at 0.6×10^6 cells per well. Cells were transfected for 24 h and total transfected DNA was held constant at 4 μg by addition of empty control plasmid. Plasmid DNA was transfected using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. SK-N-MC and SH-SY5Y were differentiated into neuron-like cells in normal medium supplemented with 10 μM *trans*-retinoic acid (RA, Sigma-Aldrich) for 5 days and then in medium containing low serum (1% fetal bovine serum) and RA (10 μM) for a further 5 days.

Construct cloning. The generation of the HA-tagged pHM6-SULT4A1 and FLAG-tagged pX3FLAG-CMV-7.1-SULT4A1 constructs have been described previously (Mitchell and Minchin, 2009). For SULT1A1 and 1A3, the following forward and reverse primers containing EcoRI/XbaI and HindIII/EcoRI restriction sites (underlined) were used to PCR and clone each into pFLAG-CMV-7.1 or pHM6, respectively: FP 5'-GATCGAATTCGATGGAGCTGATCCAGGAC-3' and RP 5'-TCTAGATCTAGATCACAGCTCAGAGCGGAAG-3', FP 5'-GATCAAGCTTGATGGAGCTGATCCAGGAGACC-3' and RP 5'-GATCGAATTCTCACAGCTCAGAGCGGAAGC-3'. SULT1A1 and SULT1A3 dimerization mutants (V270E) were generated using the GeneTailor site-directed mutagenesis kit (Invitrogen) and the SULT constructs described above as templates with the following

common primers; FP 5'-GACTGGAAGACCACCTTCACCGAGGCGCAGAATGAG-3' and RP 5'-CGGTGAAGGTGGTCTTCCAGTCCCCAGCCATG-3' (mutated bases underlined).

To generate p3XFLAG-CMV-10-SULT4A1 for stable transfection, SULT4A1 was excised from pFLAG-CMV-7.1 and ligated into the same sites of p3XFLAG-CMV-10 (Sigma-Aldrich), which contains a G418 selection cassette. This plasmid was then used as template to create the SULT4A1 dimerization mutant (KTV→EEE; K265E, T269E, V270E) in two steps using the GeneArt site-directed mutagenesis kit (Invitrogen). First, the T269E and V270E mutations were introduced with primers FP 5'-GGAAGGACATCTTCGAGGAGTCCATGAATGAGAAG-3' and RP 5'-CTTCTCATTCATGGACTCCTCGAAGATGTCCTTCC-3' (mutated bases underlined). Second, using the above construct as template, the K265E mutation was introduced with primers FP 5'-AGAGTTGGGCTGTGGGAGGACATCTTC-3' and RP 5'-GAAGATGTCCTCCCACAGCCCAACTCT-3' (mutated bases underlined). The lysine at position 265 is a site of polyubiquitination and was changed to stabilize the dimerization mutant SULT4A1 protein (Sidharthan et al., 2014). All clones were verified by DNA sequencing.

Stable cell-lines. To generate stable cell lines, SK-N-MC cells were transfected with p3XFLAG-CMV-10 plasmid containing either wild-type or dimerization mutant (KTV→EEE) SULT4A1 or empty vector using Lipofectamine 2000 (Invitrogen). Transfected cells were then selected with 1 mg/ml Geneticin (Thermo Fisher Scientific) and single clones were isolated and expanded. Stable cell lines were maintained in medium supplemented with 0.3 mg/ml Geneticin. Expression of the SULT4A1 proteins was verified by Western blot with anti-FLAG antibody.

Immunocytochemistry. Post-mortem human brain tissue from confirmed neurologically normal individuals was obtained from the Queensland Brain Bank in Brisbane, Australia, under the University of Queensland Ethics Committee approval (#2012001283). Frozen cryostat sections from the anterior cingulate cortex and formalin-fixed paraffin embedded sections (7 μm thick) from the substantia nigra were used. Antigen retrieval was performed using 0.05% citraconic anhydride (pH 7.4) at 90°C for 30 min followed by treatment with Historeveal solution (Abcam) according to the manufacturer's instructions. Sections were then washed 3 times in phosphate buffered saline (PBS) and incubated in blocking buffer (PBS containing 2% BSA, 5% normal donkey serum, 0.1% Triton X-100 and 0.05% Tween-20) for 2 h at room temperature. Sections were then washed once in PBS and incubated with primary antibodies for SULT4A1 (Proteintech, diluted 1:1000) and SULT1A1/1A3 (gift from Professor Mike Coughtrie, The University of British Columbia, Vancouver, Canada; diluted 1:1000) overnight at 4°C in antibody buffer (PBS with 2% BSA and 2% normal donkey serum). Tyrosine hydroxylase (TH; Cell Signaling Technology, diluted 1:2000) was used as a dopaminergic neuron marker and microtubule-associated protein 2 (MAP2; Cell Signaling Technology, diluted 1:2000) was used as a pan-neuronal marker for co-localisation studies. After 5 washes in PBS, sections were incubated with the respective Alexa dye-conjugated secondary antibodies (diluted 1:5000) for 2 h at room temperature. Sections were then washed 5 times in PBS and treated using the Autofluorescence Eliminator Reagent (Merck Millipore, Cat# 2160) for 5 min to eliminate lipofuscin-derived auto-fluorescence. Sections were then mounted using Prolong Gold antifade reagent (Molecular Probes) for confocal microscopy.

Co-immunoprecipitation assays. HeLa cells were transfected as described above with various combinations of plasmids that expressed FLAG-tagged and HA-tagged SULT proteins. Transiently transfected cells (1×10^6) were washed in PBS and whole cell extracts were

prepared by incubation in 500 µl NP-40 lysis buffer (1% Nonidet P-40, 50 mM Tris, pH 8.0, 150 mM NaCl, 5 mM NaF, 1 mM Na_3VO_4, 10 mM β-glycerophosphate, 1 mM EDTA and protease inhibitor cocktail (Sigma-Aldrich) for 15 min on ice. Lysates were cleared by centrifugation at 14,000 × *g* for 10 min at 4°C and samples were incubated with or without rabbit anti-HA antibody (Sigma-Aldrich, diluted 1:200) for 2 h at 4°C after which 30 µl protein A sepharose beads (Sigma-Aldrich) were added and the samples incubated for a further 1 h at 4°C. Beads were washed 3 × with wash buffer (0.5% Nonidet P-40, 50 mM Tris [pH 8.0], 150 mM NaCl) and then boiled for 5 min in Laemmli buffer. Samples (20 µl) were separated by SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted with mouse anti-FLAG M2 horseradish peroxidase conjugated antibody (Sigma-Aldrich, diluted 1:5000) to detect FLAG-SULT binding. To validate HA-SULT pulldown, blots were incubated with mouse anti-HA antibody (Sigma-Aldrich, diluted 1:1000) followed by anti-mouse horseradish peroxidase conjugated secondary IgG (Jackson ImmunoResearch Laboratories, diluted 1:30000). Protein bands were visualized with Immu-Star ECL reagent (Bio-Rad Laboratories) as per the manufacturer's instructions.

Western blots. At the times indicated, cells were washed twice with cold PBS and lysed directly in Laemmli buffer and boiled for 5 min. Samples (10-20 µg protein) were separated by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted for FLAG- and HA-tagged SULT proteins, endogenous SULT1A1/3 (anti-SULT1A1/3 antibody detects both isoforms and was a gift from Professor Mike Coughtrie, The University of British Columbia, Vancouver, Canada; diluted 1:1000), SULT4A1 (Proteintech, diluted 1:1000) and α-tubulin (Cell Signaling Technology, diluted 1:2000) as previously described (Sidharthan et al., 2013; Sidharthan et al., 2014).

siRNA studies. SH-SY5Y cells were transiently transfected with siRNAs directed against SULT4A1 (Origene Technologies; SR308535 A, B and C) for a total of 72 h using Lipofectamine RNAiMAX (Invitrogen) according to manufacturer's protocol. A universal scrambled siRNA (Origene Technologies; SR30004) was used as a negative control. All siRNAs were used at a final concentration of 20 nM. *SULT4A1* gene knockdown was evaluated by Western blot.

Dopamine toxicity. SH-SY5Y cells were seeded in 96-well plates at a density of 2000 cells/well and allowed to adhere overnight. The cells were then treated with up to 1 mM dopamine (Sigma-Aldrich) in culture medium for 48 h. Cell viability was then assessed using the CyQuant Cell Proliferation Assay kit (Invitrogen), according to the manufacturer's protocol.

Statistical analysis. Data are presented as mean ± SEM unless otherwise noted. Significant differences were determined by Student's *t*-test ($p<0.05$) or ANOVA as indicated. IC_{50} values were estimated by fitting the dopamine toxicity data to a dose-response curve using non-linear least-squares regression (GraphPad Prism).

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Results

Co-localization of SULT4A1 and SULT1A1/3 in human brain

The physiological relevance of any interaction between the sulfotransferases is predicated on whether the proteins are expressed in the same cell-type(s). Therefore, immunocytochemistry was performed with human brain tissue from two different regions to determine whether SULT4A1 co-localizes with SULT1A1/3 (Fig. 1). The SULT1A1/3 antibody recognizes both proteins (Riches et al., 2009) so it was not possible to determine whether the SULT1A1/3 staining represents either or both sulfotransferases. Nevertheless, in the anterior cingulate cortex, all cells that expressed SULT1A1/3 also expressed SULT4A1 (Fig. 1A). Moreover, these cells were of neuronal origin as identified by co-staining with anti-MAP2 antibody. There were some cells that expressed SULT4A1 but were negative for SULT1A1/3 (Fig. 1A, yellow arrows). These cells were also negative for MAP2 staining suggesting they may be of non-neuronal origin. A similar co-localization was seen in the substantia nigra where both of the sulfotransferases were expressed in dopaminergic neurons, which were identified by co-staining with anti-tyrosine hydroxylase antibody (Fig. 1B). These results demonstrated co-localization of SULT4A1 with SULT1A1 and/or SULT1A3 in human brain and suggest that any interaction between these proteins may produce a physiological effect.

SULT4A1, SULT1A1 and SULT1A3 proteins form homodimers and heterodimers that requires the sulfotransferase dimerization motif

Using HeLa cells that do not express SULT1A1/3 or 4A1, FLAG-tagged and HA-tagged SULTs were co-expressed and then co-immunoprecipitated with anti-HA antibody. Initially, each SULT was co-expressed with itself to determine homodimerization (Fig. 2A). Western blots for FLAG-tagged SULTs that co-immunoprecipitated with HA-tagged SULTs

showed that all 3 SULTs formed homodimers in HeLa cells. Western blots of samples prior to immunoprecipitation (Input) confirmed expression of each FLAG-tagged SULT in the cells (Figure 2A, lanes 2-3 in each blot). Furthermore, both expression (Input) and immunoprecipitation of the HA-tagged proteins were also confirmed (Fig. 2A, lower panels). Omitting the HA antibody showed there was no non-specific binding to protein A beads (Fig. 2A, lanes 4-6 in each blot).

Co-expression of the different SULTs as FLAG-tagged and HA-tagged proteins was then performed to detect heterodimerization. All three proteins interacted with each other as detected by immunoprecipitation (Fig. 2B, last lanes). To determine whether the dimerization motif was required for protein-protein interactions, both SULT1A1 and 1A3 were mutated to the sequence found in mouse Sult1e1, which does not form homodimers (T269P, V270E) (Petrotchenko et al., 2001). Mutants bound neither to SULT4A1 nor to each other (Fig. 3, last lanes). Taken together, these results suggest that SULT4A1, SULT1A1 and SULT1A3 can interact with each other in the context of the whole cell. Moreover, this interaction appears to occur via the conserved dimerization motif in the C-terminus of each protein.

A yeast-2-hybrid assay and an *in vitro* pulldown of GST-tagged recombinant proteins were also investigated to confirm the immunoassay results. In both assays, SULT4A1 was shown to bind to SULT1A1/3 (Supplemental Table 1 and Supplemental Fig. 1, respectively)

SULT4A1 protein expression decreases SULT1A1/3 protein levels

HeLa cells were transiently transfected with SULT4A1 and either SULT1A1 or 1A3 following which protein levels were determined by Western blotting. In the presence of SULT4A1, expression of SULT1A1 and 1A3 was significantly attenuated by 60% and 64%, respectively, compared to empty vector controls (Fig. 4A). To determine whether a similar effect is seen with endogenous SULTs, SK-N-MC cells were used as these cells constitutively

express both SULT1A1 and 1A3 but very low levels of SULT4A1 (Fig. 4B). In cells stably transfected with SULT4A1, endogenous SULT1A1 and 1A3 proteins were decreased by 90% and 85%, respectively (Fig. 4B). However, when cells were stably transfected with a SULT4A1 mutant, there was no change in SULT1A1 or SULT1A3 protein (Fig. 4C). These experiments show that the presence of SULT4A1 leads to a decrease in SULT1A1/3 protein, most likely following protein-protein interactions.

It has been shown previously that SULT4A1 is induced following differentiation of SK-N-MC cells with retinoic acid (Sidharthan et al., 2013). Since SULT1A1/3 are constitutively expressed in these cells, this was a good model to determine whether up-regulation of endogenous SULT4A1 affects endogenous SULT1A1/3 expression. The levels of SULT1A1/3 protein were quantified before and after treatment with 10 μM retinoic acid for 10 days (Fig. 5). There was an 11-fold increase in SULT4A1 protein following differentiation, which was accompanied by a 20% and 50% decrease in SULT1A1 and SULT1A3 proteins, respectively ($p < 0.05$, Student's *t*-test). To determine whether this change was due to the increase in SULT4A1, cells were differentiated with retinoic acid and then treated with siRNA (sequence B or C, see Experimental procedures) directed against SULT4A1 mRNA. Figure 6 (upper blot) shows efficient down-regulation of SULT4A1 by the siRNA compared to a scrambled sequence. Following treatment, the loss in SULT1A1/3 seen in Figure 5 was reversed. These results show that endogenous SULT4A1 may regulate the level of SULT1A1/3 protein expression in cells.

SULT4A1 up-regulation during neuronal differentiation enhances dopamine toxicity

Dopamine is toxic to most cells, but this can be attenuated by SULT1A3, which metabolizes dopamine to dopamine sulfate (Sidharthan et al., 2013). Thus, a change in SULT1A3 expression leads to a change in cell survival. Unlike SK-N-MC cells, SH-SY5Y

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cells express low levels of SULT4A1 and SULT1A1 but high levels of SULT1A3. Following 10 μM retinoic acid treatment for 10 days, the SH-SY5Y cells showed characteristic dendrite formation, consistent with a neuronal-like phenotype (Fig. 7A). Moreover, the neuron-specific marker NeuN was up-regulated (Fig. 7B). Similar to that seen in the SK-N-MC cells, SULT4A1 protein increased while SULT1A3 protein decreased upon differentiation. Expression of SULT1A1 was low both before and after treatment (Fig. 7B).

To determine whether the down-regulation of SULT1A3 by endogenous SULT4A1 affects dopamine toxicity, SH-SY5Y cells were treated with increasing concentrations of dopamine before and after differentiation. The toxicity of dopamine appeared to be inversely related to SULT1A3 expression levels. This is seen in Figure 8A where the IC_{50} of dopamine decreased from 137 ± 16 μM in undifferentiated cells (high SULT1A3) to 15 ± 1 μM following retinoic acid treatment (low SULT1A3). To determine whether this increase in toxicity was related to an increase in SULT4A1, the differentiated cells were treated with siRNA for SULT4A1 or a scrambled sequence as a control. Two independent siRNAs were used (B and C), both of which decreased SULT4A1 protein over 72 h (Fig. 8B). Moreover, both siRNAs reversed the increase in dopamine toxicity to that seen in the undifferentiated cells (Fig. 8C). By contrast, the scrambled sequence had no effect on toxicity.

Discussion

Despite the discovery of SULT4A1 in the brain almost 20 years ago, there has been little work to uncover its role in mammalian cells. When its expression was inhibited in a zebrafish model, distinct phenotypic changes were seen including sedentary behavior (Crittenden et al., 2015) and upregulation of genes associated with photo-transduction (Crittenden et al., 2014). In a mouse gene knockout model, a severe phenotype was observed with animals surviving only 20-25 days post birth (Garcia et al., 2018). A recent study showed that SULT4A1 expression in SH-SY5Y cells altered mitochondrial function and protected the cells from hydrogen peroxide-induced cell death (Hossain et al., 2019). Interestingly, it also reported that SULT4A1 was co-localized with the mitochondrial outer membrane translocase protein TOM20, which forms part of a complex that imports proteins from the cytosol into mitochondria. The possible interaction of SULT4A1 with the TOM complex may be important for its role in altering mitochondrial function. In the present study, SULT4A1 co-localized with SULT1A1/3 in human neurons in at least 2 separate regions of the brain. SULT4A1 was also present in non-neuronal cells in the absence of the other sulfotransferases. SULT4A1 heterodimerized with both SULT1A1 and 1A3 and this interaction required an intact dimerization motif. This is the first study to identify heterodimerization between these proteins. Using ectopic expression of the sulfotransferases, as well as changes in endogenous levels during cell differentiation, SULT4A1 was shown to decrease both SULT1A1/3 protein levels. For SULT1A3, this resulted in an increase in the toxicity of dopamine, a substrate for the enzyme. Collectively, these results show a reciprocal relationship between SULT4A1 and SULT1A1/3 in neuronal cells. The mechanism behind SULT4A1-induced SULT1A1/3 protein loss remains unclear and requires further study. One possible explanation may be that heterodimerization of the SULTs increases protein degradation, which is supported by the fact that mutation of the dimerization site prevents protein loss.

There is an emerging picture of SULT4A1 regulation as a complex cellular process involving mRNA splicing, post-translational modification and protein-protein interactions. SULT4A1 mRNA is found in most tissues in the body but primarily as an aberrant transcript that does not translate a stable protein (Sidharthan et al., 2014). Following correct splicing that removes a pseudo-exon located between exons 6 and 7, the full-length stable protein is generated. Thus, SULT4A1 protein levels are regulated at a post-transcriptional level through alternative splicing. SULT4A1 expression is also regulated by both transcriptional and post-translational processes (Mitchell and Minchin, 2009; Butcher et al., 2010). In neuronal-like cells, SULT4A1 transcription is under the control of the cAMP response element-binding protein and the activating transcription factor-2 (Butcher et al., 2010). Post-translationally, the protein is phosphorylated in an ERK1/2 dependent manner, which promotes binding of the peptidyl prolyl cis-trans isomerase Pin1 and subsequent SULT4A1 degradation. These pathways are summarized in Figure 9.

In a previous study using SK-N-MC cells, low dopamine concentrations were shown to increase SULT1A1 and 1A3 transcription through activation of the D1 receptor (Sidharthan et al., 2013). Part of this pathway involved up-regulation of MEK-ERK1/2 signaling. Thus, ERK1/2 is a point of convergence for the regulation of both SULT1A1/3 and SULT4A1 (Figure 9). However, while ERK1/2 up-regulates SULT1A1/3, it down-regulates SULT4A1. The dopamine – ERK1/2 – SULT1A3 axis may provide a feedback loop to regulate dopamine levels in the brain. Controlling SULT4A1 protein levels may be an additional pathway to ensure adequate SULT1A3 protein in the presence of excess dopamine. This may be an important observation that warrants further investigation. In addition, there are a number of drugs that can modulate ERK1/2 activity, including serotonin (Li et al., 2010), amphetamines (Choe and Wang, 2002), isoproterenol (Du et al., 2010) and cocaine (Hoffmann et al., 2011).

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Whether these treatments alter the levels of sulfotransferases in the brain remains to be determined.

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Conflict of interest. The authors declare that there are no competing interests associated with the manuscript.

Authorship contributions.

Participated in research design: Butcher and Minchin.

Conducted experiments: Idris, Mitchell, Gordon, Sidharthan, and Butcher.

Performed data analysis: Idris and Minchin

Contributed to the writing of the manuscript: Idris, Butcher, and Minchin.

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Legends for Figures

Fig. 1. SULT1A1/3 and SULT4A1 proteins co-localize in human brain. (A) Anterior cingulate cortex stained with anti-microtubule-associated protein 2 (MAP2) antibody (red) shows the presence of neurons. SULT1A1/3 staining is shown in the green panel while SULT4A1 is shown in the grey panel. White arrows indicate co-localization of SULT1A1/3 and SULT4A1 in neuronal cells. Yellow arrows show SULT4A1 expression in cells that were negative for both SULT1A1/3 and MAP2. (B) Protein expression in the substantia nigra. Dopaminergic neurons were stained with anti-tyrosine hydroxylase (TH) antibody. White scale bars = 20 μm.

Fig. 2. SULTs form homodimers and heterodimers in HeLa cells following transient expression. (A) Cells were co-transfected with either HA- and FLAG-tagged SULT4A1 (top panels), SULT1A1 (middle panels) or SULT1A3 (bottom panels). Cells were lysed and then immunoprecipitated using anti-HA antibody and protein A sepharose beads. In controls, the anti-HA antibody was omitted (-Ab). Co-immunoprecipitation of FLAG-tagged SULTs was determined by FLAG Western blot and immunoprecipitation of HA-tagged SULTs was

confirmed by HA Western blot. (B) Cells were co-transfected with HA-4A1 and FLAG-1A1 (top panels), HA-4A1 and FLAG-1A3 (middle panels) or HA-1A1 and FLAG-1A3 (bottom panels) and then treated as described in (A). Results are representative of duplicate experiments. Input = protein expression in cell cytosols before immunoprecipitation (1/20); -Ab = immunoprecipitations in the absence of anti-HA antibody; +Ab = immunoprecipitations in the presence of anti-HA antibody. Molecular weights are shown in kDa.

Fig. 3. Mutation of the SULT1A1/3 dimerization motif prevents heterodimer formation with SULT4A1 in HeLa cells following transient expression. Cells were co-transfected with HA-4A1 and dimerization mutant FLAG-1A1mut (top panels) or FLAG-1A3mut (middle panels), or with HA-1A1 and FLAG-1A3mut (bottom panels). Cells were lysed and then immunoprecipitated using anti-HA antibody and protein A sepharose beads. In controls, the anti-HA antibody was omitted (-Ab). Co-immunoprecipitation of FLAG-tagged SULTs was determined by FLAG Western blot and immunoprecipitation of HA-tagged SULTs was confirmed by HA Western blot. Results are representative of duplicate experiments. Input = protein expression in cell cytosols before immunoprecipitation (1/20); -Ab = immunoprecipitations in the absence of anti-HA antibody; +Ab = immunoprecipitations in the presence of anti-HA antibody. Molecular weights are shown in kDa.

Fig. 4. SULT4A1 expression decreases levels of SULT1A1/3 proteins. (A) HeLa cells were transiently co-transfected with FLAG-SULT4A1 (4A1) or empty vector (EV) and either FLAG-SULT1A1 (upper panels) or FLAG-SULT1A3 (lower panels). Cell lysates were prepared after 24 h of transfection and Western blotted with anti-FLAG antibody to detect expressed SULT proteins. Quantitation of Western blots was performed by densitometry using ImageJ and is shown on the graph to the right. Expression of SULT proteins was normalized

to α-tubulin. Results are mean ± SEM, n=3. Asterisks indicate significant differences (P<0.05) by one-way ANOVA. Molecular weights are shown in kDa. (B) Cell lysates were prepared from SK-N-MC cell lines that were stably transfected with plasmids for either wild-type FLAG-SULT4A1 (4A1), dimerization mutant FLAG-SULT4A1 (m4A1), or empty vector control (EV) and levels of endogenous SULT1A1/3 proteins were determined by Western blotting with a SULT1A1/3 antibody that detects both SULT isoforms. Expression of SULT4A1 protein was verified by FLAG Western blot. Quantitation of Western blots was performed by densitometry using ImageJ and is shown on the graph. Expression of SULT proteins was normalized to α-tubulin. Results are mean ± SEM, n = 3. Asterisks indicate significant differences (P<0.05) by one-way ANOVA. Molecular weights are shown in kDa.

Fig. 5. Differentiation-induced upregulation of SULT4A1 expression decreases SULT1A1/3 protein levels in SK-N-MC cells. SK-N-MC cells were treated for 10 days with 10 μM retinoic acid (+RA) and then endogenous SULT4A1 and SULT1A1/3 protein levels were quantified by Western blotting. Quantitation of Western blots was performed by densitometry using ImageJ and is shown on the graphs. Expression of SULT proteins was normalised to α-tubulin. Results are mean ± SEM, n = 3. Asterisks indicate significant differences (P<0.05) by Student's *t*-test compared to untreated control cells (-RA). Molecular weights are shown in kDa.

Fig. 6. Knockdown of SULT4A1 with siRNA in differentiated SK-N-MC cells results in increased SULT1A1/3 protein levels. Cells were treated for 7 days with 10 μM retinoic acid and then transfected with 20 nM of either scrambled (Scr) or SULT4A1 siRNA (siRNA B or C) in the presence of retinoic acid for an additional 3 days. Endogenous SULT4A1 and SULT1A1/3 protein levels were determined by Western blotting. Quantitation of Western blots was performed by densitometry using ImageJ and is shown on the graphs below. Expression

of SULT proteins was normalized to α-tubulin. Results are mean ± SEM, n = 3. Asterisks indicate significant difference ($P<0.05$) by Student's *t*-test from cells treated with scrambled siRNA (Scr). Molecular weights are shown in kDa.

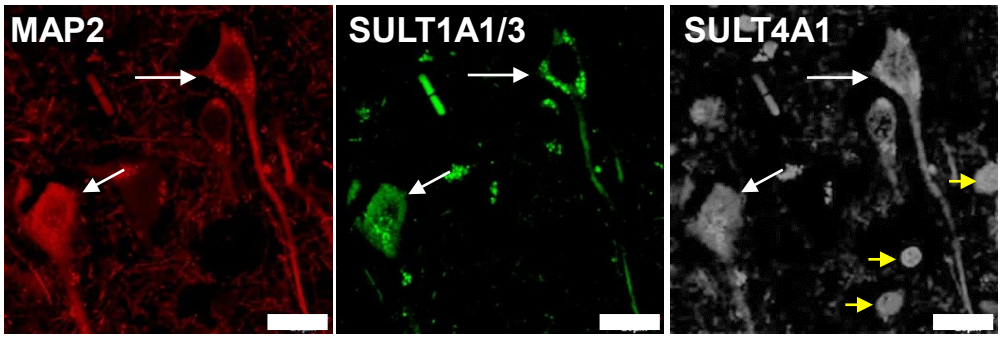
Fig. 7. SULT expression in differentiated SH-SY5Y cells. (A) Light microscopy showing SH-SY5Y cells before (SH-SY5Y) and after differentiation (dSH-SY5Y) with 10 μM retinoic acid for 10 days. (B) Cell lysates of undifferentiated (SH-SY5Y) and differentiated (dSH-SY5Y) cells were Western blotted for endogenous SULT4A1 and SULT1A1/3 proteins. NeuN was blotted as a marker of neuronal differentiation and α-tubulin was a loading control. Molecular weights are shown in kDa.

Fig. 8. Knockdown of SULT4A1 with siRNA reduces dopamine toxicity in differentiated SH-SY5Y cells. (A) Toxicity of dopamine in SH-SY5Y cells before (●) and after (○) differentiation with RA for 10 days. (B) SH-SY5Y cells were differentiated for 7 days and then transfected with 20 nM of either scrambled (Scr) or SULT4A1 siRNA (siRNA A, B or C) in the presence of retinoic acid for an additional 3 days. Cells were lysed after 72 h of siRNA transfection and subjected to Western blot to verify knockdown of SULT4A1. Quantification of knockdown is shown in parentheses below the blots and is expressed relative to Scr and normalized to α-tubulin. Only siRNA B and C showed knockdown. (C) Undifferentiated (●), differentiated (○), differentiated treated with scrambled siRNA (■) and differentiated treated with SULT4A1 siRNAs B (left graph) or C (right graph) (□) SH-SY5Y cells were seeded into 96-well plates at 2000 cells/well and allowed to adhere overnight. The cells were then treated with up to 1 mM dopamine and viability assessed using a CyQuant proliferation assay kit. Results are mean ± SEM, n = 4.

Fig. 9. Pathways for the regulation of SULT4A1 and SULT1A1/3. ERK1/2 phosphorylates SULT4A1 to generate a Pin1 binding site, which leads to protein degradation. The phosphorylated SULT4A1 is dephosphorylated by the phosphatase PP2A. ERK1/2 is also involved in the transcriptional regulation of SULT1A1/3 in a dopamine-dependent manner. Dopamine activates the D1 receptor to up-regulate ERK1/2 activity. The increased SULT1A3 then feeds back onto dopamine through its metabolism to dopamine sulfate. Finally, SULT4A1 can dimerize with SULT1A1/3 to promote its degradation and loss of activity.

Figure 1

A. Anterior Cingulate Cortex



B. Substantia Nigra

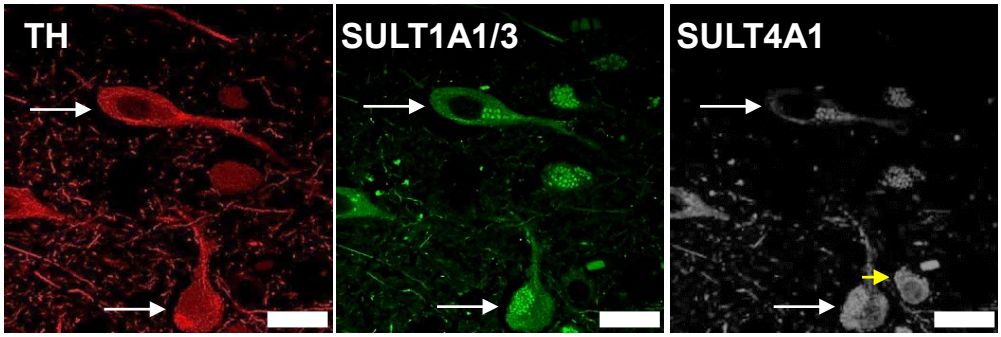
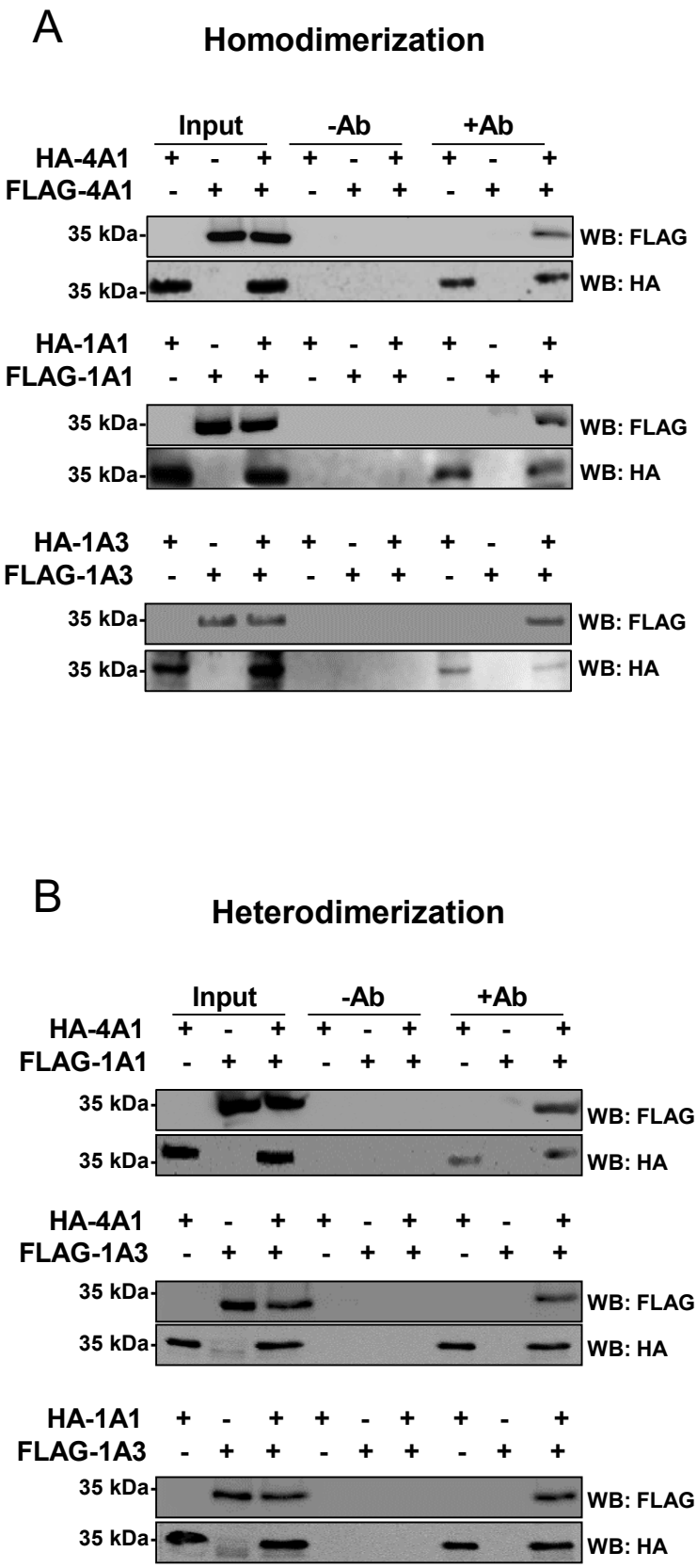


Figure 2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FLAG-4A1
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WB: HA
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HA-1A3
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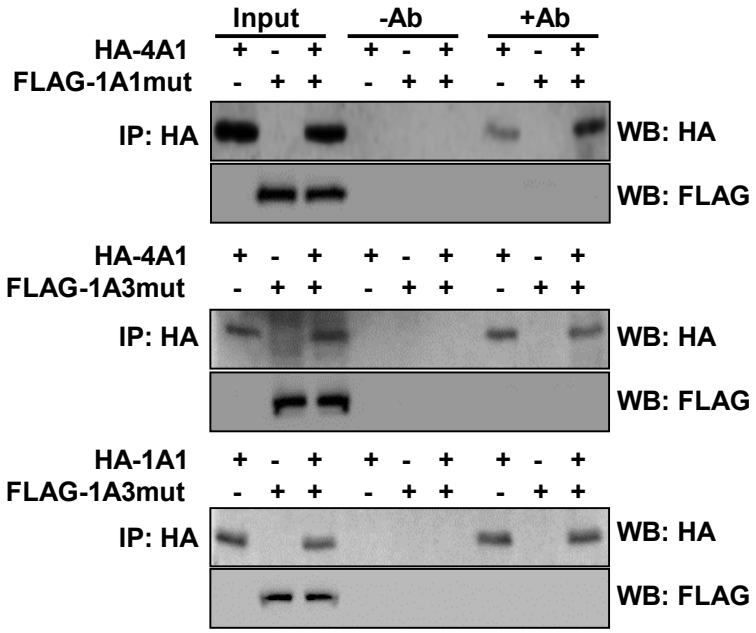
Figure 3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WB: FLAG
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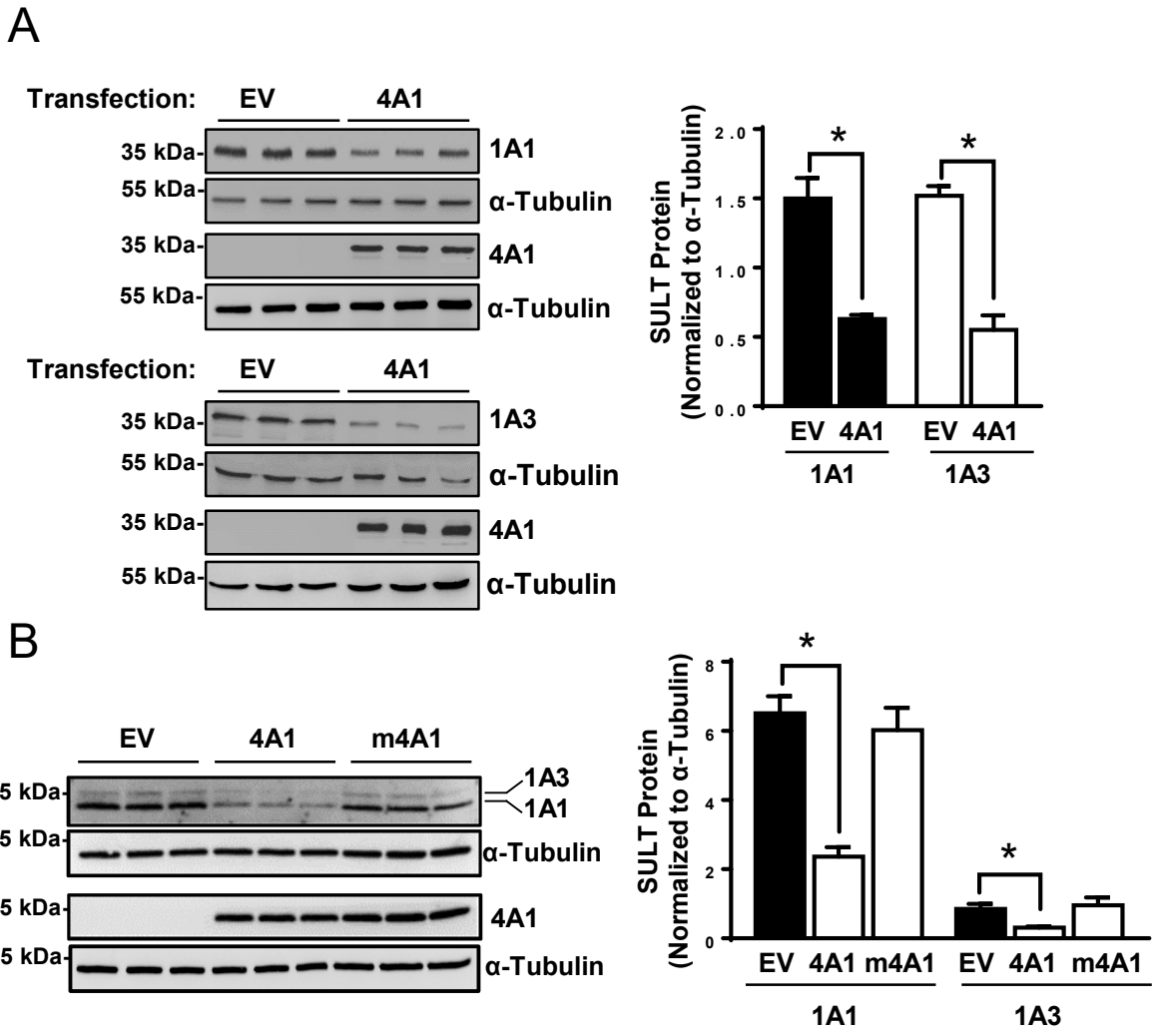
WB: HA
WB: FLAG

Figure 4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1A1
55 kDa
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55 kDa
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EV
4A1
35 kDa
1A3
55 kDa
α-Tubulin
35 kDa
4A1
55 kDa
α-Tubulin
SULT Protein
(Normalized to α-Tubulin)
2.0
1.5
1.0
0.5
0.0
*
*
EV 4A1
EV 4A1
1A1
1A3
B
EV
4A1
m4A1
35 kDa
1A3
1A1
55 kDa
α-Tubulin
35 kDa
4A1
55 kDa
α-Tubulin
SULT Protein
(Normalized to α-Tubulin)
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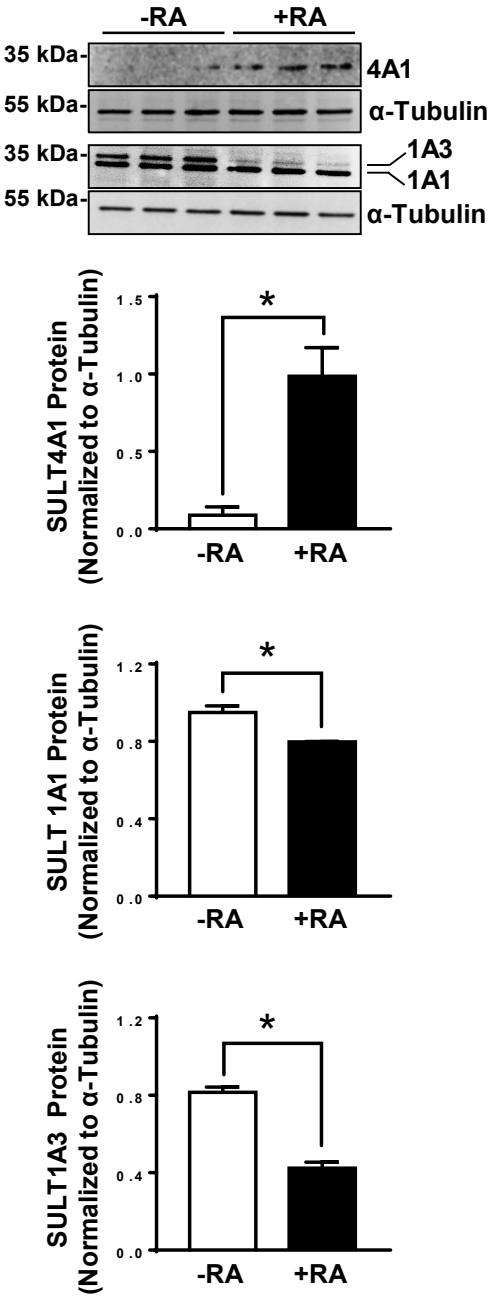
1A3

Figure 5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1A1
55 kDa-
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(Normalized to α-Tubulin)
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1.0
0.5
0.0
*
-RA
+RA
SULT 1A1 Protein
(Normalized to α-Tubulin)
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0.8
0.4
0.0
*
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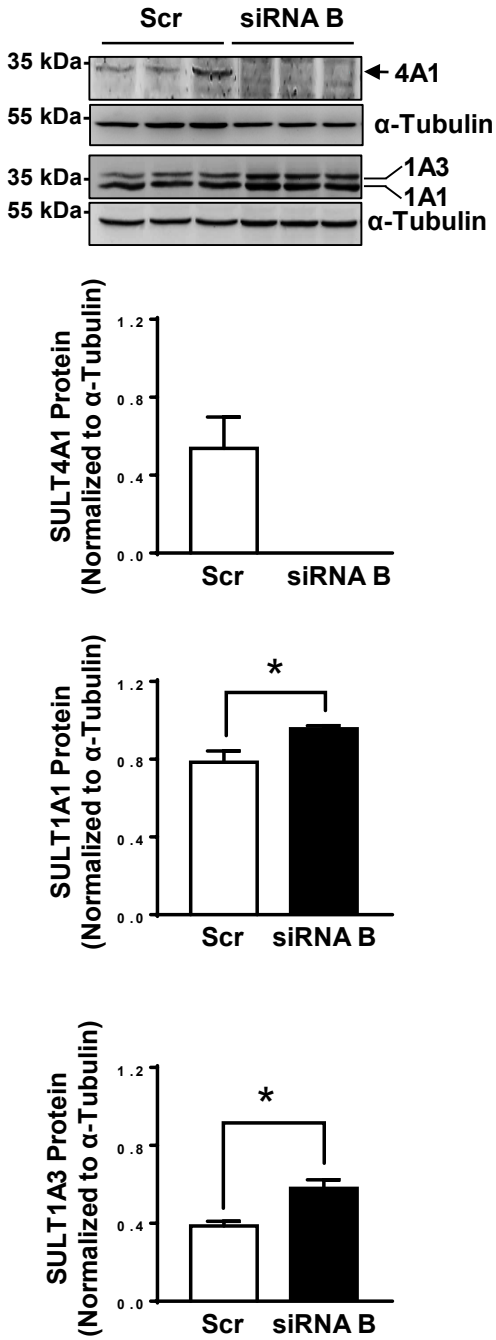
0.0
*
-RA
+RA

Figure 6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(Normalized to α-Tubulin)
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0.8
0.4
0.0
Scr
siRNA B
SULT1A1 Protein
(Normalized to α-Tubulin)
*
1.2
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0.4
0.0
Scr
siRNA B
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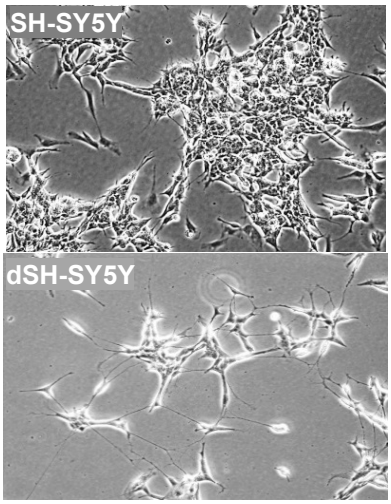
0.8
0.4
0.0
Scr
siRNA B

Figure 7

A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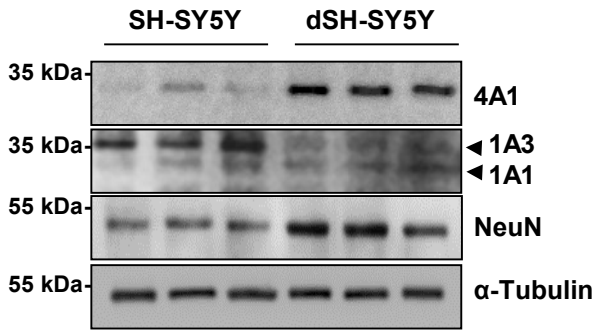
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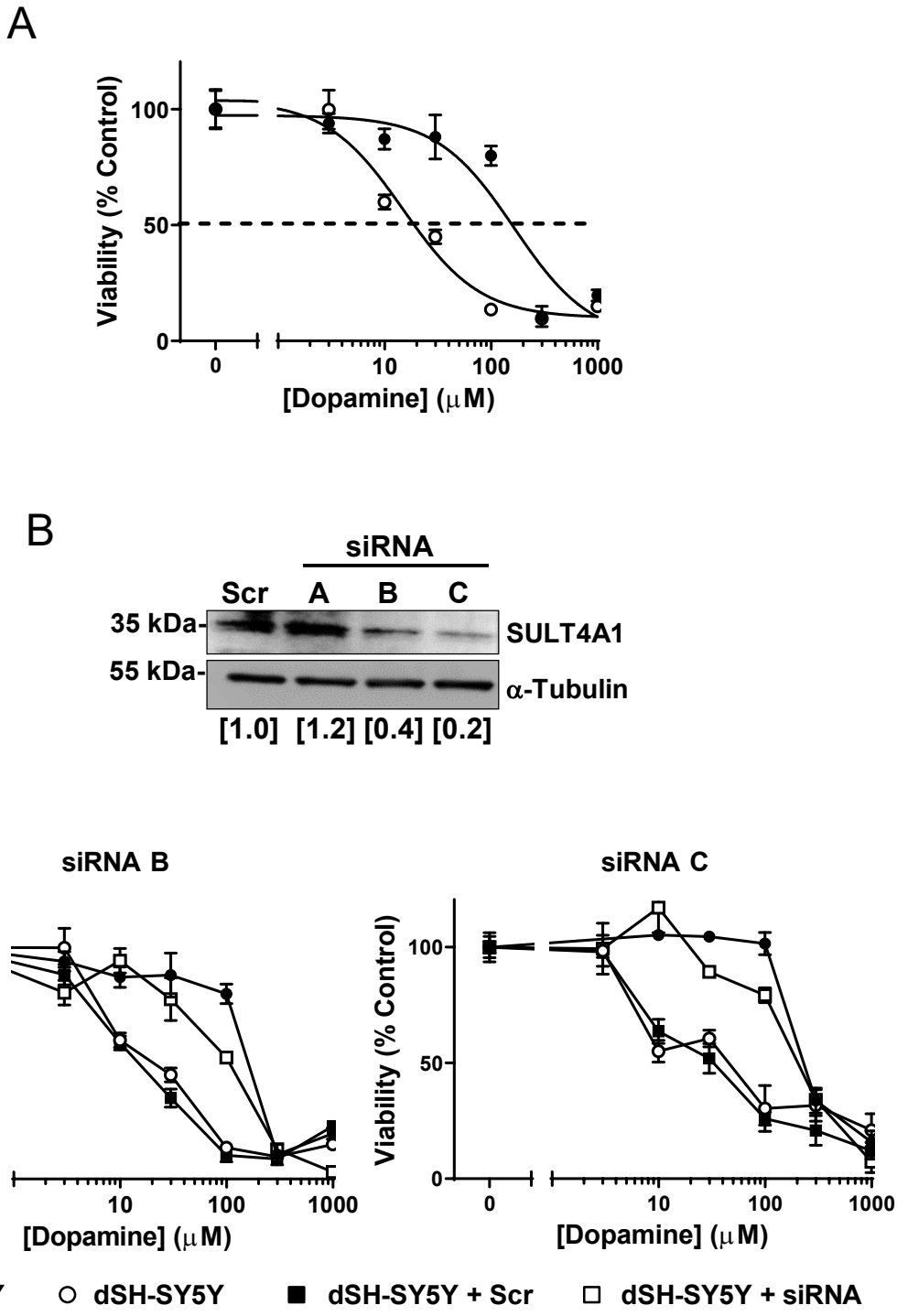
α-Tubulin

Figure 8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10
100
1000
[Dopamine] (μM)
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siRNA
Scr
A
B
C
35 kDa-
SULT4A1
55 kDa-
α-Tubulin
[1.0] [1.2] [0.4] [0.2]
C
siRNA B
siRNA C
Viability (% Control)
100
50
0
0
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1000
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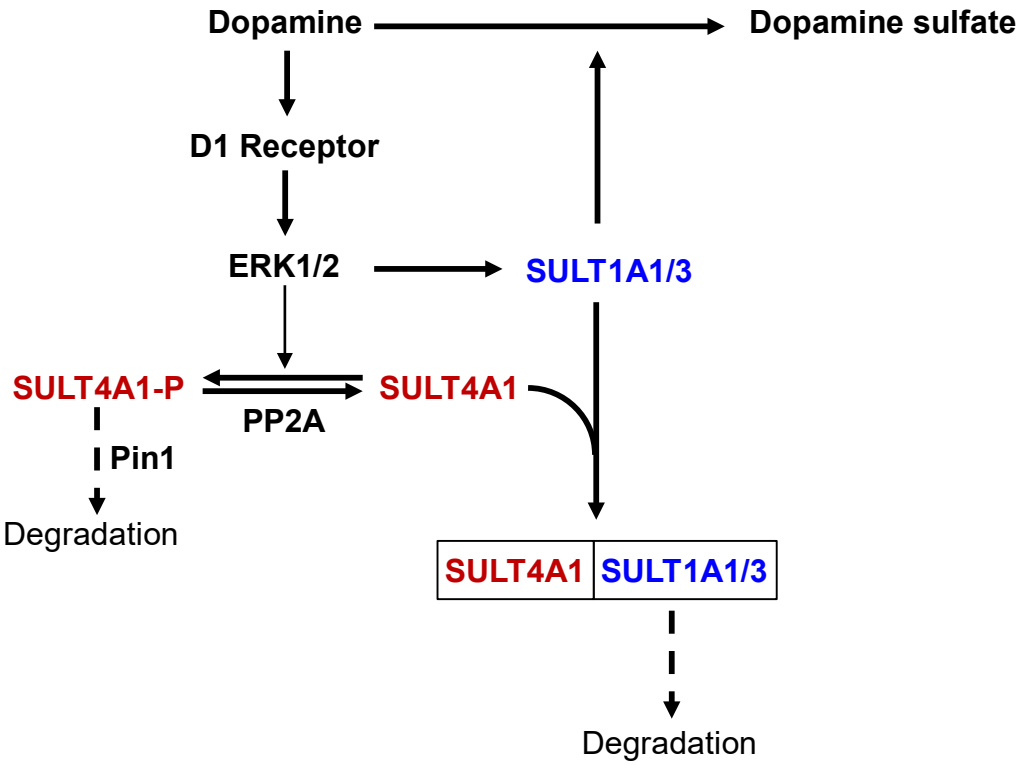
dSH-SY5Y
dSH-SY5Y + Scr
dSH-SY5Y + siRNA

Figure 9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SULT4A1
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Degradation
SULT4A1
SULT1A1/3
Degradation