Molecular characterization of the conditional deletion of Vesicular Aminergic-Associated Transporter (VAAT / SLC10A4) in serotonergic neurons of adult mice

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Certificate

This is to certify that this dissertation entitled “Molecular characterization of the conditional deletion of Vesicular Aminergic-Associated Transporter (VAAT / SLC10A4) in serotonergic neurons of adult mice” towards the partial fulfillment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents original research carried out by “Ms. Akilandeswari.B at Uppsala University under the supervision of Prof. Klas Kullander, Developmental Genetics Unit, Department of Neuroscience, Uppsala University, Sweden during the academic year 2015-2016.

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28th March, 2016

Declaration

I hereby declare that the matter embodied in the report entitled "Molecular characterization of the conditional deletion of Vesicular Aminergic-Associated Transporter (VAAT / SLC10A4) in serotonergic neurons of adult mice" are the results of the investigations carried out by me at the Department of Neuroscience, Uppsala University, under the supervision of Prof. Klas Kullander and the same has not been submitted elsewhere for any other degree.

Akilandeswari Balasubramanian
5th year BS-MS — 28th March, 2016
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Abstract

The orphan transporter, Vesicular Aminergic – Associated Transporter (VAAT/SLC10A4) was recently shown to have a modulatory role in monoaminergic and cholinergic signalling systems. These studies used constitutive knockout mice (VAAT KO) and revealed that some effects of the VAAT KO are obscured by compensatory changes. Moreover, VAAT deletion might exert different effects in different aminergic neurons. Hence, for the proper understanding of the function of VAAT, its effect on individual aminergic brain nuclei should be studied separately. Here, we have generated and characterised a conditional allele of VAAT gene for implementing the conditional knockout strategy. We used the tamoxifen-inducible CreERT2 ($SERT^{CreERT2}$) to spatio-temporally restrict the VAAT knockout in the serotonergic neurons of adult mice. Detailed analysis revealed that $SERT^{CreERT2}$ has background activity in the absence of tamoxifen resulting in 35.3% VAAT knockout in $SERT^{CreERT2/+}$; $VAAT^{lox/ko}$ animals. After tamoxifen administration, a very specific and complete deletion of VAAT was observed in the raphe nuclei, thus confirming the inducible (64.7%) and conditional deletion of VAAT (VAATicKO). Furthermore, no compensatory changes in the transcript levels of serotonergic genes were observed in VAATicKO either under normal or stressful situations. Finally, preliminary results indicate higher brain serotonin levels in VAATicKO mice under stress when compared to control (P=0.0926) while no difference was observed under normal conditions. This observation could now be further explored to validate the role of VAAT in serotonin homeostasis.

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Introduction

Membrane Transport Proteins

The lipid bilayer acts as a gatekeeper to separate intracellular and extracellular milieu in all cells. Its hydrophobic nature restricts the passage of polar small molecules across the membrane. In order to maintain the composition of the cytoplasm and to uptake essential nutrients, individual cells selectively transport small molecules through specific membrane transport proteins. There are three major families of transport proteins: Carriers, Channels and ATP driven pumps **(Fig 1)**.

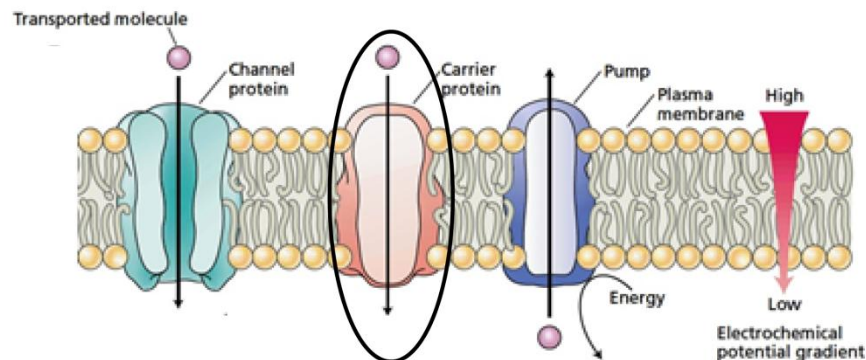


Figure 1: Schematic diagram illustrating the three major membrane transport proteins: Channels, Carriers and Pumps. The protein of interest in this study, solute carrier (carrier protein), is encircled (Adapted from Taiz, L. and Zeiger, E., 2010).

SLC gene nomenclature

Carrier proteins or Solute carriers (SLCs) are the largest group of transport proteins, and represent one third of the total proteome. The Solute Carrier gene nomenclature was established in 1990's by the HUGO Gene Nomenclature Committee (HGNC). The general nomenclature includes the family name, family number, a letter denoting the subfamily and a number indicating the individual transporter gene written in series. For example, SLC10A4 stands for Solute Carrier family 10, subfamily A, member 4. There are more than 400 members categorized into 52 families in the SLC series. The Solute Carriers are assigned to specific families based on sequence

similarity. A minimum of 20% similarity with the other members of the same family is needed for the assignment (Hediger, M. et al., 2013).

Solute Carriers in physiology and disease

Solute carriers are integral membrane transporters which are located either on the cell membrane or on organelle membranes and include both facilitative and secondary active transporters **(Fig 2)**. They transport a wide range of substrates such as inorganic ions, amino acids, glucose, metals, neurotransmitters, vitamins, organic anions, etc across the membrane (César-Razquin, A. et al., 2015). SLCs include families with a very specific type of substrate (all SLC7 family members transport amino acids) as well as families with transporters of chemically different substrates (SLC22 family transports organic anion, cations and zwitter ions) (Schlessinger, A. et al., 2013).

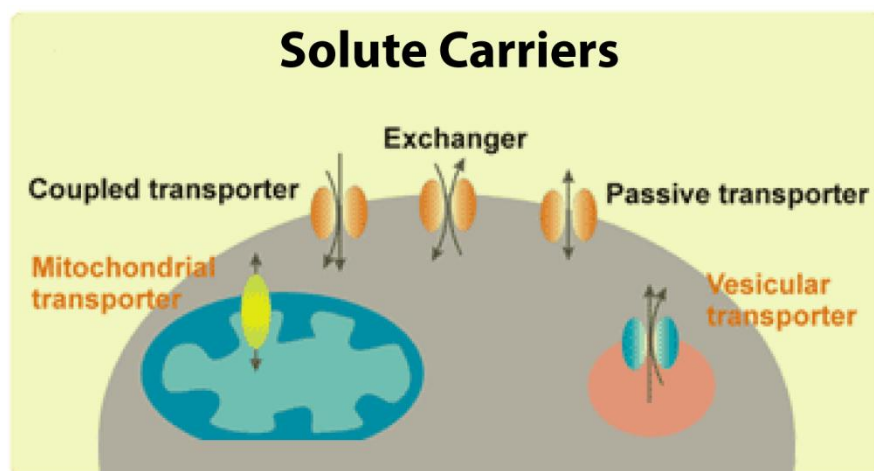


Figure 2: Schematic diagram illustrating the different categories of SLCs. (modified from Hediger, M. et al., 2013)

During the last decade, the field of SLCs has been advancing rapidly with new members and families being identified and increasing number of structural studies being published. Furthermore, over 80 SLC transporters have been associated with human disease conditions such as gout, diabetes, psychiatric disorders, inflammatory bowel disease, Rotor Syndrome, hyperbilirubinaemia, jaundice, blood pressure, etc. Slc-knockout mice studies have been crucial in studying the transport function *in vivo*, and thus improve our understanding of this physiologically important,

yet understudied family of proteins. However, owing to the overlap of substrate among different SLCs, abolishment of the transporter function does not always produce a clear phenotype. This redundancy in transport function can often be seen as a compensatory upregulation of other transporters in the knockout mice. For example, a full effect of the SLC6A4 or Serotonin Transporter (SERT) knockout is obscured by the upregulation of low affinity serotonin transporter SLC22A3 (organic cation transporter 3 –OCT3) (Lin, L. et al., 2015).

Vesicular Amine Transporters and Aminergic Neurotransmitters

This study mainly focuses on the vesicular neurotransmitter transporters in the central nervous system. Vesicular Neurotransmitter Transporters (VNTs) are responsible for the vesicular uptake of neurotransmitters and determine the quality and quantity of neurotransmitters in these secretory vesicles, thereby playing a crucial role in efficient synaptic transmission (Eiden, L.E. et al., 2000). The vesicular proteins involved in the transport of aminergic neurotransmitters such as the quaternary amine-acetylcholine and the different monoamines- dopamine, noradrenaline, and serotonin, are known as the vesicular amine transporters (Larhammar, M. et al., 2015). The two major vesicular amine transporters in the central nervous system are Vesicular Monoamine Transporter 2 (VMAT2/ SLC18A2) and Vesicular Acetylcholine Transporter (VAChT/ SLC18A3), which are involved in the storage of monoamines and acetylcholine respectively. The neuromodulatory role of neurons expressing these aminergic neurotransmitters makes them crucial players in higher cognitive functions of the brain. Hence deeper insights into the synaptic vesicle cycle and homeostasis of these neurotransmitters would contribute to new drug discoveries and better treatment of many mood related psychiatric disorders.

Novel Vesicular Transporter: SLC10A4/VAAT

Interestingly, an orphan transporter of the SLC10 family- SLC10A4 was recently discovered to have an aminergic expression pattern, covering the domains of both VMAT2 and VAChT, thereby opening new possibilities for the better understanding of amine homeostasis. SLC10A4/ Vesicular Aminergic-Associated Transporter (VAAT) was first identified in 2004 and reported to have an abundant mRNA

expression in neural tissue (Hagenbuch, B. and Dawson, P., 2004). VAAT was assigned to SLC10 family based on its 37% amino acid sequence identity to SLC10A1. However, unlike other well characterised members of SLC10 family, such as SLC10A1/2 and SLC10A6, it does not transport bile acids or steroid sulphates. Also VAAT has a vesicular expression pattern, differing further from other SLC10 family members that are localized in the plasma membrane (Geyer, J. et al., 2008; Burger, S. et al., 2011; Burger, S., 2012; Larhammar, M. et al., 2015).

VAAT constitutive knockout (VAAT KO) studies

VAAT is expressed in all aminergic neurons of central nervous system and colocalizes with both VAChT and VMAT2 vesicles **(Fig 3.a)** (Larhammar, M. et al., 2015). This expression pattern indicates that VAAT might be involved in vesicle sorting and/or vesicular transport of substances and other processes required for efficient synaptic vesicle filling and refilling of amine neurotransmitters (Borges, K., 2013).

All the studies addressing the potential role of VAAT in the synaptic vesicle cycle have been carried out using VAAT constitutive knockout (VAAT KO) mice. In this gene trap construct, a β-galactosidase – Neomycin resistance gene (Neo^R) fusion marker known as β-geo fusion marker is used to replace the coding region of VAAT gene, thus generating a reporter tagged knockout **(Fig 3.b)** (Stanford, W. L. et al., 2001).

VAAT constitutive knockout mice (KO) displayed defective cholinergic synaptic transmission at NMJ along with a compensatory upregulation of cholinergic receptor subunit transcript levels (Patra, K. et al., 2015). They were also hypersensitive to cholinergic chemoconvulsant pilocarpine (Zelano, J. et al., 2013) and dopaminergic psychostimulant amphetamine. Moreover, decreased dopamine uptake was observed in vesicle preparations from VAAT KO mice, while over expression of VAAT resulted in increased acidification of synaptic vesicles. These findings, together with the molecular reports published by Larhammar, M. et al (2015) showing reduced central monoamine levels suggest that VAAT has a global regulatory role in the homeostasis of aminergic neuromodulators.

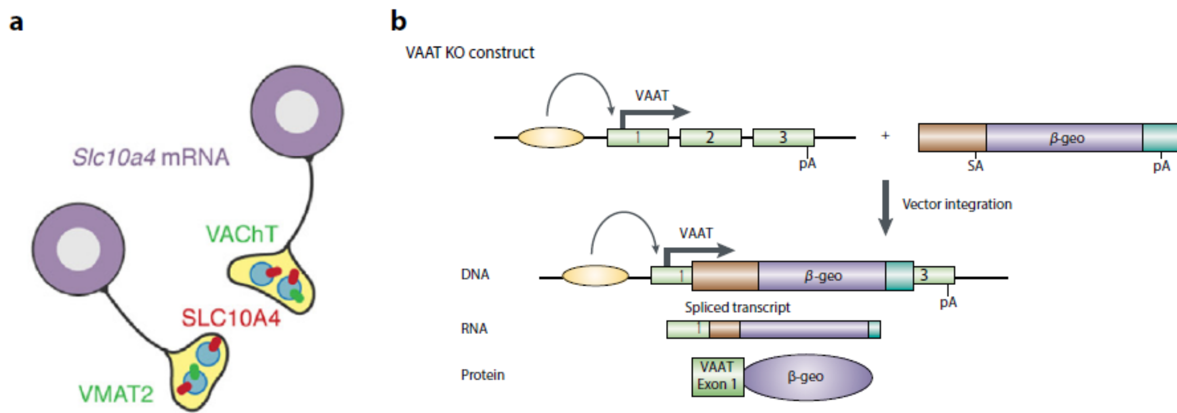


Figure 3: Schematic diagram illustrating the **(a)** expression pattern of VAAT (adapted from Larhammar, M. et al., 2015) and **(b)** VAAT constitutive knockout construct (modified from Stanford, W. L. et al., 2001).

Compensatory effects in VAAT KO

Even though VAAT KO resulted in synaptic and neural defects, a global behavioural phenotype was not observed. This can be mainly due to the changes that take place during development that compensate the loss of a protein. In a recent paper, Patra, K. et al (2015) showed that VAAT KO results in structural defects at the neuromuscular junction endplate, but no defects in motor abilities and muscle strength. However , detailed analysis showed reduction in the amount of neurotransmitters within each quantum, with a compensatory increase in the number of readily releasable pool of vesicles and increased gene expression of cholinergic nicotinic receptor complex (Chrna1). In addition to these compensatory effects, the behavioral consequences in these VAAT KO mice also result from a complex interaction all the aminergic nuclei in tandem.

In order to minimise the compensatory effects during development a conditional knockout of VAAT in each aminergic nuclei seperately, should be carried out instead of the constitutive knockout strategy.

Cre/loxP system

In order to achieve the conditional VAAT deletion strategy, here, we have employed the Cre/loxP system, which is the widely used Site-Specific Recombinase (SSR) system in mice. Cre is a tyrosine DNA recombinase from Bacteriophage P1 that recognises a 34-bp region known as loxP (locus of crossover of P1). It catalyses the recombination between two directly repeated loxP sites, resulting in the excision of the DNA fragment between the two loxP sites as a circular molecule, leaving a single loxP site behind (Deussing, J. M., 2013).

VAAT gene targeting strategy

To implement the knockout strategy, loxP flanked (floxed) conditional allele for VAAT was generated from the Conditional Ready (VAAT ConR) mouse line obtained from the Knockout Mouse Project (KOMP). $VAAT^{ConR}$ is a knockout first allele in which, a gene-trapping cassette containing Internal Ribosomal Entry Site (IRES)/LacZ and promoter driven Neo, flanked by FRT (FLP recognition target) sites, is inserted in the intron, upstream of the floxed exon 2 of VAAT gene. The gene-trapping cassette can be removed by FLP recombination to generate the VAAT conditional allele in which, the VAAT expression is restored and has its exon 2 flanked by loxP sites ($VAAT^{lox}$) (Testa, G. et al., 2004). $VAAT^{lox}$, in combination with Cre recombinase, deletes the exon 2 of the VAAT gene (Skarnes, W. C. et al., 2011) **(Fig 4).**

Serotonin and Depression

As a first step towards the VAAT conditional knockout study, we set out to investigate the role of VAAT in serotonin homeostasis. Serotonin is a phylogenetically ancient monoamine neurotransmitter that has been highly associated with depression. The 'Serotonin Hypothesis of Depression' postulates that depression is highly related to low brain serotonin levels (Coppen, A.J., 1969). This hypothesis paved the way for the discovery of Selective Serotonin Reuptake Inhibitors (SSRIs) (Claassen, V. et al., 1977), which are the widely used antidepressants in market until today. However the exact mechanism of its action is still unknown. The global aim of this study is to analyse if there are any alterations in depression tendencies upon VAAT knockout.

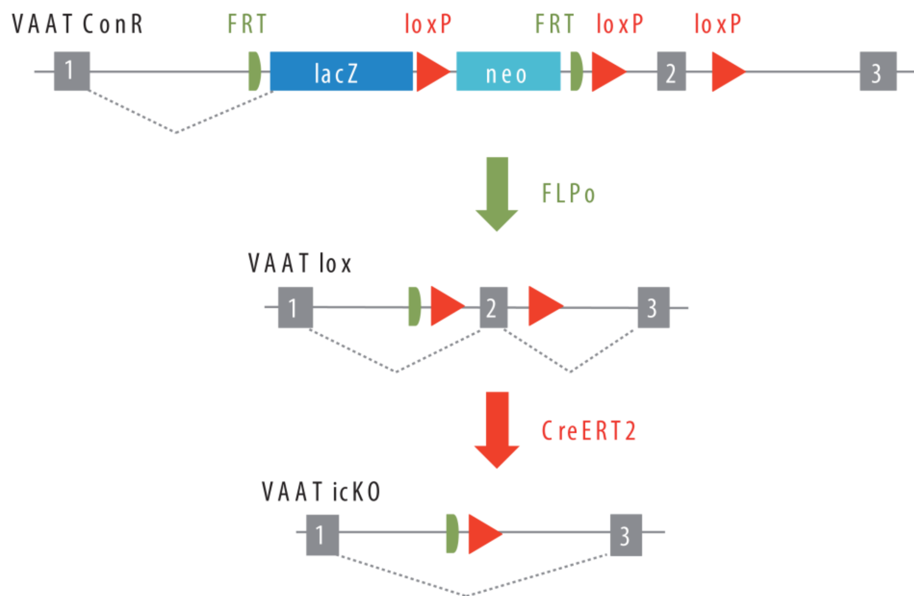


Figure 4: VAAT inducible and conditional knockout strategy. Schematic diagram illustrating the **(a)** the generation of VAAT conditional allele (VAATlox) and VAAT inducible and conditional knockout (VAATicKO) from VAAT ConR using FLPo and CreERT2 recombinase respectively. (modified from Skarnes, W.C. et al.,2011).

SERT CreERT2: Temporal control of Cre activity

Since an important aspect of this project is to eliminate the compensatory effects seen in the VAAT constitutive knockout studies, we have used the inducible Cre^{ERT2}-LoxP system to selectively delete VAAT in adult mice. Cre-ER is a fusion protein of Cre recombinase and estrogen receptor (ER) ligand domain. The interaction of ER ligand domain with the heat shock protein 90 (HSP90) restricts the fusion protein to the cytoplasm but can enter the nucleus upon interaction with the tamoxifen ligand **(Fig 5)**. Cre-ERT2 expresses a mutated version of the ER ligand binding domain and is the most effective variant. It has much less background activity and is insensitive to the endogenous β-estradiol (Deussing, J. M., 2013). This cutting edge genetic tool facilitates both spatial (conditional) and temporal (inducible) control over gene deletion. $SERT^{CreERT2}$, which expresses Cre^{ERT2} under the control of SERT promoter, was used in this study, in combination with $VAAT^{lox}$ mice, to obtain the inducible and conditional deletion of VAAT gene ($VAAT^{iCko}$) in the serotonergic neurons after tamoxifen treatment.

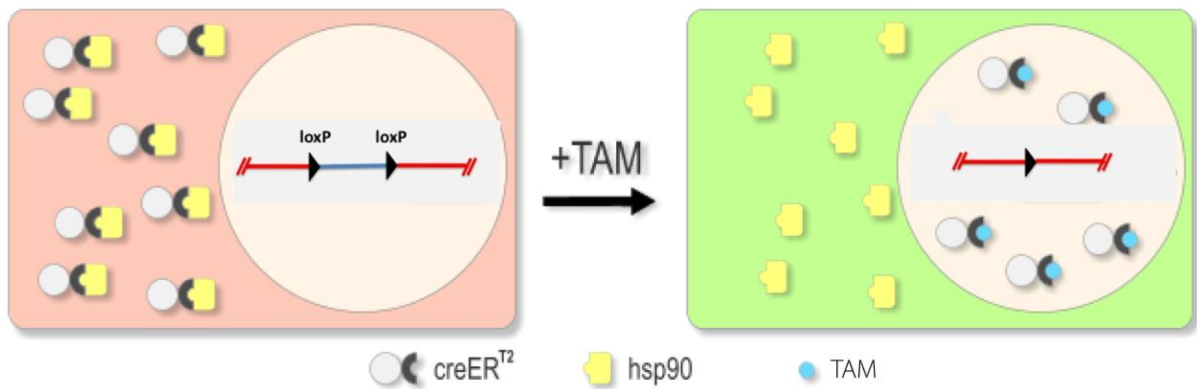


Figure 5: Mechanism of action of ligand-dependant Cre^{ERT2}- LoxP system. HSP90 restricts CreERT2 to the cytoplasm, which is replaced by tamoxifen. Upon entry into the nucleus after tamoxifen administration, CreERT2 recombinase excises the floxed target sequence (modified image from http://crezoo.crt-dresden.de/crezoo/).

In this study we aim to minimize the compensatory effects seen in VAAT constitutive knockout studies, for which we employ the CreERT2 lox system spatio-temporally restrict the knockout of VAAT in serotonergic neurons of adult mice. Hence the main goals of this study are:

- Generation and characterization of VAAT lox
- Characterization of the spatial and temporal resolution of SERT^{CreERT2}
- Characterization and validation of the VAAT inducible and conditional knockout (VAATicKO).
- Study the neuronal effects of VAATicKO under normal and stressful situations, by quantifying:
    1. Changes in serotonergic gene expression
    2. Changes in brain serotonin levels

Methods

Transgenic mice lines

All mice were housed and maintained in the animal care facility at Uppsala University and bred in C57BL/6 genetic background. The procedures carried out in this study were all approved by the Swedish ethical committee. The site-specific recombinase mouse lines, FLPo and the SERT CreERT2, were obtained from Jackson laboratory (Maine, USA). The two VAAT knockout mouse lines used in this study, VAAT null mice (KO) and VAAT Conditional Ready (ConR) were acquired from Texas A&M Institute for Genomic Medicine (TIGM) and Knockout Mouse Project (KOMP), respectively. All these mice lines have been later explained in detail.

Genotyping of mice was done using Polymerase chain reactions (PCR) for which primers targeting the transgenic loci were designed using the Primer3 Plus Software (Table 1; FLPo genotyping was done according to the genotyping protocol suggested by Jackson laboratory). The genomic DNA was extracted from ear tissue of each animal using standard HotSHOT DNA preparation protocol (Truett, E.G. et al., 2000). The PCR reactions were done either with KAPA Taq (KAPA Biosystems) or Maxima Hot Start Taq DNA Polymerase (Thermo Scientific) following the standard protocol. PCR programs/cycling conditions were optimized for the respective primers separately. A multiplex PCR was designed for genotyping VAAT WT, ConR and lox alleles, for which a step down PCR program was opted. The amplified product was visualized on 2% agarose gel stained with Ethidium Bromide.

Tamoxifen administration

For all molecular and behavioural experiments, 7-8 weeks old mice were used. For tamoxifen induction of the knockout, 2mg of tamoxifen dissolved in 90% oil & 10% ethanol was administered via intraperitoneal (IP) injections, once per day for 3 consecutive days. Mice were sacrificed one week after the last dose for immunostaining or after 3 weeks for Serotonin ELISA and qRTPCR.

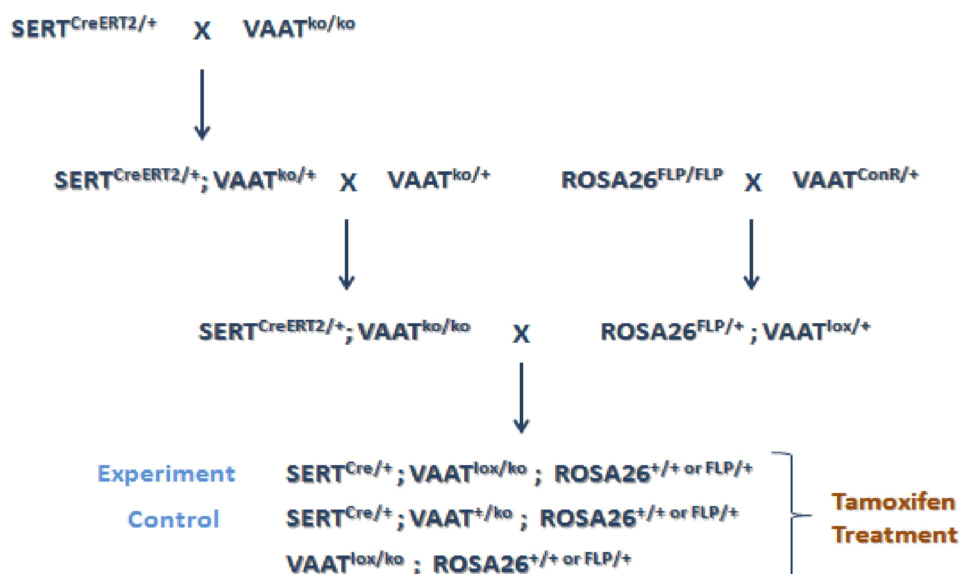
Table 1: List of primers used for genotyping

Transgene		Primer
SERT CreERT2	F	ACGAGTGATGAGGTTCGCAAGA
	R	ACCGACGATGAAGCATGTTTAG
VAAT WT	F	GGTATGGCCTCTTTCTCACC
	R	CCTTCGAGTCCTAGCTTCTGA
VAAT ConR	F	GCCATCACGAGATTTCGATT
	R	CCCGGATCCAGACATGATAA
VAAT lox	F	GGTCTGAGCTCGCCATCAGT
	R	TCCTGTCCGGGAACTCACTA
VAAT KO	F	GGACGCTTGACCAGACAGA
	R	AGACGGCAATATGGTGGTGGAAA
Tomato WT	F	AAGGGAGCTGCAGTGGAGTA
	R	CCGAAAATCTGTGGGAAGTC
Tomato Tg	F	CTGTTCCTGTACGGCATGG
	R	GGCATTAAAGCAGCGTATCC

Genetic Crossing

The VAATKO mice available in the lab were used to generate the VAATicKO mice of the genotype $SERT^{CreERT2/+}$; $VAAT^{lox/ko}$. This was preferred over $SERT^{CreERT2/+}$; $VAAT^{lox/lox}$ as less number of generations were required to obtain the VAATicKO mice. The breeding scheme implemented in this study has been shown in figure 6.

Figure 6: Breeding strategy for the generation of $VAAT^{iCko}$ mice.



Tissue Preparation

Adult mice were anaesthetized by IP injecting 0.3µl of a 1:2 mixture of Ketaminol 50 mg/ml and Domitor vet 1mg/ml. Completely anaesthetised animals were transcardially perfused with PBS, followed by 4 % formaldehyde in PBS. Spinal cord and brain were dissected out and post-fixed overnight at 4^0C in 4% formaldehyde. After three washes with 1XPBS to remove the traces of formaldehyde, the spinal cords were incubated in 30% sucrose in PBS for 24 hours for cryoprotection. Thermo Scientific™ Richard-Allan Scientific™ Neg-50™ was used to embed the tissue, which was immediately frozen in dry ice. The frozen tissue was stored at -80^0C, until cryosectioning. The cryosections (16µm) were collected on Superfrost Plus slides (Menzel GmbH, Braunschweig, Germany) and stored at -20°C. On the other hand, the brain tissue after post-fixation was embedded in 4% agarose and sectioned (60um) immediately, using the vibratome. The brain slices covering all aminergic nuclei were collected in ice cold PBS and dehydrated in methanol series (25%, 50%, 75% and 100% methanol; 15 minutes each) for long term storage at -20°C.

In situ probe preparation

Plasmid linearization: 3µg of plasmid (**Fig 7.a**) containing SLC10A4 cDNA (NCBI accession number NM_173403) was linearised using HincII Restriction Enzyme (Promega) in 1X Tango Buffer. The digestion reaction (total volume 20ul) was carried out for one hour at 37^0C and later stopped by heat inactivating the enzyme at 65^0C for 10 minutes. The linearization of the plasmid was confirmed by running 2 µl of the total mixture on an agarose gel with Ethidium Bromide and was visualised under UV light (**Fig 7.b**)

In vitro transcription: In order to produce Digoxigenin (DIG) labelled SLC10A4 anti sense probe, approximately 1.5 µg of linearised plasmid was used as a template for the DIG labelling reaction (total volume 20µl) carried out using the T3 Polymerase. Transcription was carried out for 1 hour at 37^0C, following which the reaction was stopped by heating the mixture to 65^0C for 10 minutes. The reaction mixture containing DIG labelled probe was diluted to 5 times in nuclease free water and stored as 20µl aliquots at -20^0C. 2µl of the stored aliquot was used to confirm the presence of the probe by running on a 2% agarose gel. (**Fig 7.c**)

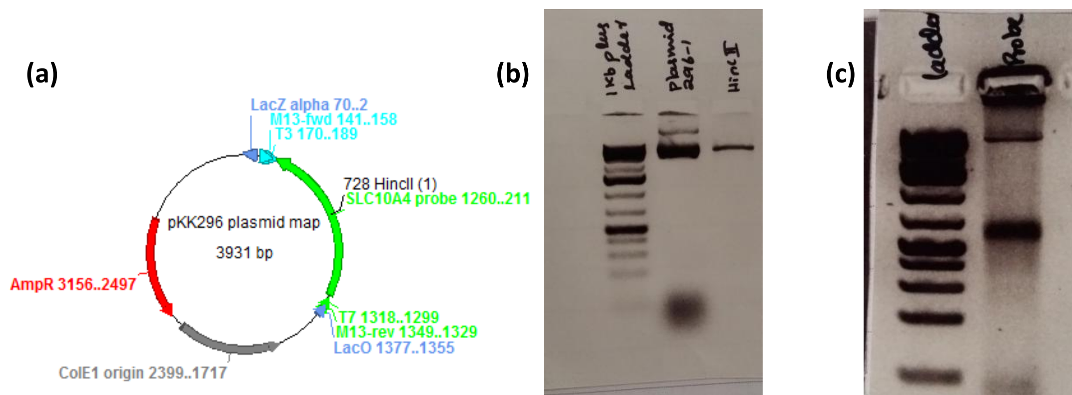


Figure 7: ***In situ* probe preparation** (**a**) Graph map of the plasmid containing SLC10A4 (Plasmid no: pkk296) gene sequence, showing the restriction site for HincII enzyme and T3 polymerase binding site. (**b**) Gel electrophoresis of the circular plasmid (plasmid 296-1) and HincII digested linearised plasmid (HincII). (**c**) Gel electrophoresis of the transcription mixture after *in vitro* transcription indicating a single band of probe.

In situ hybridisation

This protocol was modified from Schaeren-Wiemers and Gerfin-Moser., 1993.

Day 1: All slides containing cryosections of spinal cord were washed with 1XPBS for 5 minutes and fixed in 4% formaldehyde in PBS for 10 minutes, followed by another wash with 1X PBS. They were then digested using Proteinase K (Thermo Scientific) at a concentration of 6µg/µl for 8 min, refixed in 4% formaldehyde for 5min, washed twice with 1XPBS, acetylated (2.68ml of triethanolamine, 352µl of conc.HCl, 500µl of acetic anhydride and 200ml of autoclaved water, stir on a magnetic stirrer) for 10 minutes and again washed twice with 1XPBS. Sections were thereafter incubated in pre-hybridisation buffer (50% formamide, 5X Denhardts, 50µg/ml of salmon sperm DNA (ssDNA) and 50µg/ml of yeast t-RNA) for 2 hours at room temperature. Prehybridisation buffer was heated at 80^{0}C and snap cooled on ice for 5 minutes to denature the ssDNA and tRNA in the buffer, before adding onto the slides. Hybridisation mixture was prepared by adding 1:150 dilution of the probe from the aliquots that were prepared and denatured as aforementioned. 150µlof the hybridisation mixture was now carefully spread over the slides using a cover slip, avoiding bubbles and incubated for 16-20 hours at 55^{0}C.

Day 2: Coverslips were allowed to slide off by immersing in 5XSSC for 5 minutes. Stringency washes were performed in 0.2X SSC at 55^{0}C for 1hour after which, the sections were washed in 0.2XSSC for 30 minutes at room temperature. For the detection of the DIG labelled probes the sections were pre-blocked in blocking solution (5% Goat Serum, 0.3% Triton X in 1XTBS) for two hours and then incubated overnight with 1:1000 dilution of anti-Digoxigenin-AP Fab fragment (Roche Diagnostics, GmbH) in blocking solution at 4^{0}C.

Day3: The sections were washed thrice (15 minutes each) with TBST (0.3% Triton X in 1XTBS) and once with 2mM levamisole in TBST for 15 minutes. NTMT was added for 10 minutes to activate the phosphatase for the colour reaction, followed by which, the sections were incubated in BM purple ((Roche Diagnostics, GmbH) at 37^{0}C. The colour reaction was stopped after 4 hours by washing thoroughly with 1XPBS. The slides were mounted using mowiol mounting medium and images captured using ZEISS LSM 510 META confocal microscope.

Immunofluoresence staining

The vibratome sectioned brain slices were rehydrated in reverse methanol series and the cryosectioned spinal cord slices were washed with 1X PBS to remove the embedding medium. The sections were thereafter pre blocked in blocking solution (2% Bovine serum albumin, 6% goat serum and 0.3% triton X in 1XPBS) for 90 minutes, followed by overnight incubation in primary antibodies diluted with blocking solution at 4^{0}C [Table 2].

Following incubation with primary antibodies, the sections were washed thrice with blocking solution (30 minutes each) and incubated in (Alexa Fluor 488, 594, 647 conjugated) secondary antibodies (1:1000) and DAPI (1:1500) diluted with blocking solution for two hours at room temperature or overnight at 4^{0}C.

This was followed by washes with blocking (twice, 30min each), PBST (thrice, 15min each) (0.3% triton X in 1XPBS) and 1XPBS (twice, 10min each). The slices were later mounted using Mowiol® 4-88 (Sigma Aldrich) mounting medium and images were acquired using either ZEISS LSM 510 META confocal microscope or Olympus BX61WI microscope. All images were adjusted for brightness and contrast in ImageJ along with background correction. All data sets used for comparison between

different genotypes were acquired under identical settings. Number of technical replicates used for each immunostaining experiment is n=20 for spinal cord sections and n=3 for brain sections.

Table 2: Primary antibodies and their dilutions used for immunostaining

Primary antibodies	Catalogue number	Dilution
Rabbit anti-SLC10A4	Sigma HP028835	1:500
Guinea pig anti-VAChT	Millipore AB1588	1:500 (spinal cord);1:300 (brain)
Mouse anti-NeuN	Millipore MAB3777	1:400
Mouse anti-VMAT2	Santa Cruz sc-390285	1:150

Cell count

Images of 60 µm thick brain sections containing raphe nuclei were acquired using Olympus BX61WI microscope. A 100µm X 500µm rectangle, covering the midline region of dorsal raphe nuclei, was selected for manual cell counting from all images.

Behavioural scheme

A 4 week long behavioural scheme was implemented to monitor the depression tendencies upon the inducible and conditional knockout of VAAT in raphe nuclei. A battery of behavioural tests was carried out on consecutive days in week 1, 3 and 4. This battery of tests consisted of Forced Swim Test, Elevated Plus Maze, Open Field Test and Tail suspension Test. Tamoxifen was administered in the start of week 2. The week 1 behavioural tasks demonstrated the baseline activity of mice before the tamoxifen induced knockout. Animals were sacrificed immediately after the last behavioural task in week 4 for the extraction of brain regions needed for Serotonin ELISA and qRTPCR. (These behavioural experiments were conducted by my colleague Charlotte van Gelder.)

Serotonin Enzyme-Linked Immunosorbent Assay (ELISA)

1mm brain slices, between bregma values 0.5mm and 1.5mm, were acquired from animals perfused with 1XPBS. The tissues were homogenised in 1XPBS using a motor rotor homogeniser. The lysate was centrifuged at high speed for 15 minutes and the supernatant was stored at -20^{0}C. Serotonin in the supernatant of each sample was quantified (atleast thrice) using Abcam's Serotonin competitive ELISA kit (ab133053) following the recommended protocol.

Quantitative Real Time Polymerase chain reaction (qRTPCR)

Approximately 2mm thick brain slices containing raphe nuclei (between bregma values -5.74mm and -4.04mm) were acquired from animals perfused with 1XPBS and immediately transferred to RNAlater RNA Stabilization reagent (Qiagen, catalog no: 76104). From the RNA stabilised slices, midline raphe region was microdissected using a 0.5mm X 2mm rectangle made using a tungsten wire as frame and the total RNA was extracted using RNeasy Mini Kit (Qiagen, Catalog No: 74104) with an on column DNase digestion following the recommended protocol. The quality and quantity of RNA was checked using Nanodrop (ND-1000 spectrophotometer). 80ng RNA, extracted from raphe, was used for cDNA synthesis (M-MLV Reverse Transcriptase, Invitrogen). qRTPCR was carried out using 0,9ng of RNA equivalent from raphe, in 10ul of total reaction volume prepared according to the KAPA SYBR® FAST qPCR Kit protocol. Each sample was run in triplicates. NCBI Primer- BLAST software was used to design gene specific primers and their specificity was further verified by analyzing the melting curve and visualizing the amplified product using ethidium bromide stained agarose gel electrophoresis. Most of the primers were designed spanning exon-exon junctions with an amplicon size of approximately 150 base pairs. All primers used in this study are listed in Table 3. LinRegPCR software was used to calculate PCR efficiency and quantification cycle (Cq) values. Cq values for each sample were normalized over the geometric mean of the 3 reference genes- Gapdh, B2M and Pgk1 for the respective sample. The delta Cq (dCq) value that was thus generated was used to calculate the relative gene expression according to the formula 2^{-dCq} .

Statistical Analysis

All statistical tests were performed using Graphpad Prism software with α=0.05. Details of the test are given along with the results.

Table 3: Quantitative real time PCR primers

Gene		Primer
Vesicular monoamine transporter2 (VMAT2)	slc18a2_qf	GCCTATCATGGGCTACCTGG
	slc18a2_qr	CACCAGCAGAGGGACCGATA
Serotonin transporter (SERT)	slc6a4_qf	GGAACGAAGACGTGTCCGAG
	slc6a4_qr	GGCCTGCGAACGTACTATCC
Serotonin 5HT1A receptor	5ht1a_qf	CATGCTGGTCCTCTATGGGC
	5htr1a_qr	GGCTGACCATTCAGGCTCTT
Serotonin 5HT2A receptor	5htr2a_qf	GGTCATCATGGCAGTGTCCC
	5htr2a_qr	AAGGCCACCGGTACCCATAC
β2-microglobulin	b2m_qf	GCCTGTATGCTATCCAGAAAACC
	b2m_qr	CCCGTTCTTCAGCATTTGGA
Phosphoglycerate kinase 1	pgk1_qf	GGTGTTGCCAAAATGTCGCT
	pgk1_qr	CAGCAGCCTTGATCCTTTGG
Glyceraldehyde-3-phosphate dehydrogenase	gapdh_qf	CCGGGTTCCTATAAATACGGACTG
	gapdh_qr	CCAATACGGCCAAATCCGTT

Results

Validation of $VAAT^{ConR}$

Enriched expression of VAAT and its colocalisation with VAChT in the cholinergic motor neurons of spinal cord **(Fig 8.a, b)** demonstrated that the conditional ready genetic construct of VAAT is constitutively inactive. Immunofluorescence analysis of spinal cord sections revealed strong VAAT protein expression in the ventrally located motor neurons of $VAAT^{+/ko}$ and $VAAT^{+/+}$ spinal cord. On the contrary, immunolabelling of cholinergic neurons was completely absent in $VAAT^{ConR/ko}$, similar to that of $VAAT^{ko/ko}$ **(Fig 8.c)**. Similar results were obtained for the VAAT mRNA expression analysis performed using RNA *in situ* hybridization **(Fig 8.d)**, thus validating that $VAAT^{ConR}$ is a null allele of the SLC10A4 gene and is equivalent to that of $VAAT^{ko}$.

Generation of $VAAT^{lox}$ from $VAAT^{ConR}$

Since the gene trapping cassette is flanked by target sites of FLP recombinase (FRT sites), VAAT ConR mice were crossed to FLPo deleter mice to generate the VAAT conditional allele ($VAAT^{lox}$) (**Fig 6**; methods). FLPo (FLP optimized) deleter mice ubiquitously express the mouse codon optimized version of the FLP recombinase enzyme from *Saccharomyces cerevisiae,* with enhanced mRNA stability and recombination efficiency in mammalian cells (Raymond, C.S. et al., 2007), under the GT (ROSA) 26Sorpromoter. Since GT (ROSA) 26Sorpromoter drives the expression of FLPo recombinase from the embryonic stage (pre-implantation onwards), VAAT ConR is converted to VAAT lox ubiquitously, even in the germ line cells.

In order to monitor excision of the gene trapping cassette over generations, a VAAT multiplex PCR was implemented using primers that targeted the gene trapping cassette, floxed exon 2 and the endogenous region of VAAT wildtype (absent in the transgenes), which facilitated the genotyping of VAAT ConR, VAATlox and VAAT WT alleles, respectively. While designing primers for the VAAT ConR allele, it was taken care not to target the LacZ and NeoR gene sequences since they were also present in the β-geo cassette of the VAAT constitutive knockout allele (VAAT KO). The absence of amplicon corresponding to the gene trapping cassette confirmed the

generation of VAATlox allele (**Fig 9**). Through this genotyping strategy, 100% conversion rate from VAAT ConR to VAAT lox was observed in the F1 generation of the $VAAT^{ConR}$ X FLPo breeding which was consistent over generations.

Validation of $VAAT^{lox}$

Immunofluorescence analysis confirmed that the protein expression is restored in the VAAT conditional allele **(Fig 10.a)**. Moreover, the colocalization of VAAT with VAChT in motor neurons of spinal cord **(Fig 10.b)** and with VMAT2 in the serotonergic neurons of dorsal raphe nuclei **(Fig 10.c)** demonstrated that the restored VAAT expression is in accordance with the endogenous aminergic expression pattern of VAAT.

Characterisation of SERTCreERT2

For the conditional and inducible knockout of VAAT (VAATicKO) in serotonergic neurons, we used the SERT CreERT2 construct, in which a tamoxifen inducible Cre recombinase-CreERT2 is expressed under the control of SERT promoter. SERT CreERT2 was crossed to TomatoAi9 reporter mice in order to determine the distribution of Cre recombinase activity. TomatoAi9 is a Cre reporter mouse in which a floxed stop cassette controlled tdtomato fluorescent marker gene is inserted in the ROSA26 locus under the ubiquitous highly expressing CAG promoter (Madisen, L. et al., 2010). Tamoxifen administered $SERT^{CreERT2}$; Tomato mice showed a specific tdtomato fluorescent protein expression in the rostral cluster of raphe nuclei which colocalised with the immunostaining of VAAT **(Fig 11)**. This demonstrates that the CreERT2 is very specific to serotonergic neurons.

Validation of the VAAT conditional knockout

The generated VAAT conditional allele was combined with $SERT^{CreERT2}$ to study the conditional knockout. VAAT immunostaining was carried out in tamoxifen administered $SERT^{CreERT2/+}$; $VAAT^{lox/ko}$ (experiment) and $SERT^{+/+}$; $VAAT^{lox/ko}$ (control) mice, to monitor CreERT2 induced knockout. VAAT protein expression present in the raphe nuclei of the control brain was totally absent in the experimental mice **(Fig 12)**. However, VAAT immunostaining in the other aminergic nuclei such as substantia niagra (dopaminergic) and locus coeruleus (noradrenergic) was identical between

the control and the experiment, **(Fig 13)**, demonstrating the high spatial resolution and efficiency of $SERT^{CreERT2}$ induced VAAT deletion.

Inducibility of SERTCreERT2

$SERT^{CreERT2}$; Tomato mice, with and without tamoxifen administration, were analysed for tomato fluorescence in the raphe nuclei. Since CreERT2 recombinase activity is dependent on the tamoxifen drug, tomato fluorescence was expected to be completely absent in the $SERT^{CreERT2}$; tomato mice without tamoxifen and to be equivalent to that of a Cre negative tomato mice. However, comparable tomato fluorescence was observed in the raphe nuclei, irrespective of tamoxifen administration (n=3) (**Fig 14.a**), indicating that the hsp90 mediated sequestering of CreERT2 within the cytoplasm is not completely efficient and has a leaky translocation to the nucleus.

Since Cre recombinase efficiency is known to vary depending on the genetic location of the lox sites (Tronche, F. et al., 2002), expression of tomato reporter protein does not imply complete deletion of VAAT before tamoxifen administration. Immunostaining of $SERT^{CreERT2/+}$; $VAAT^{lox/ko}$ (experiment) mice without tamoxifen treatment showed VAAT protein expression similar to that of $SERT^{+/+}$; $VAAT^{lox/ko}$ (control) (n=3) (**Fig 14.b**). These results show that the weak background activity of CreERT2 is sufficient enough to excise the stop cassette of the Tomato Ai9 mice equivalent to that of tamoxifen induced CreERT2.

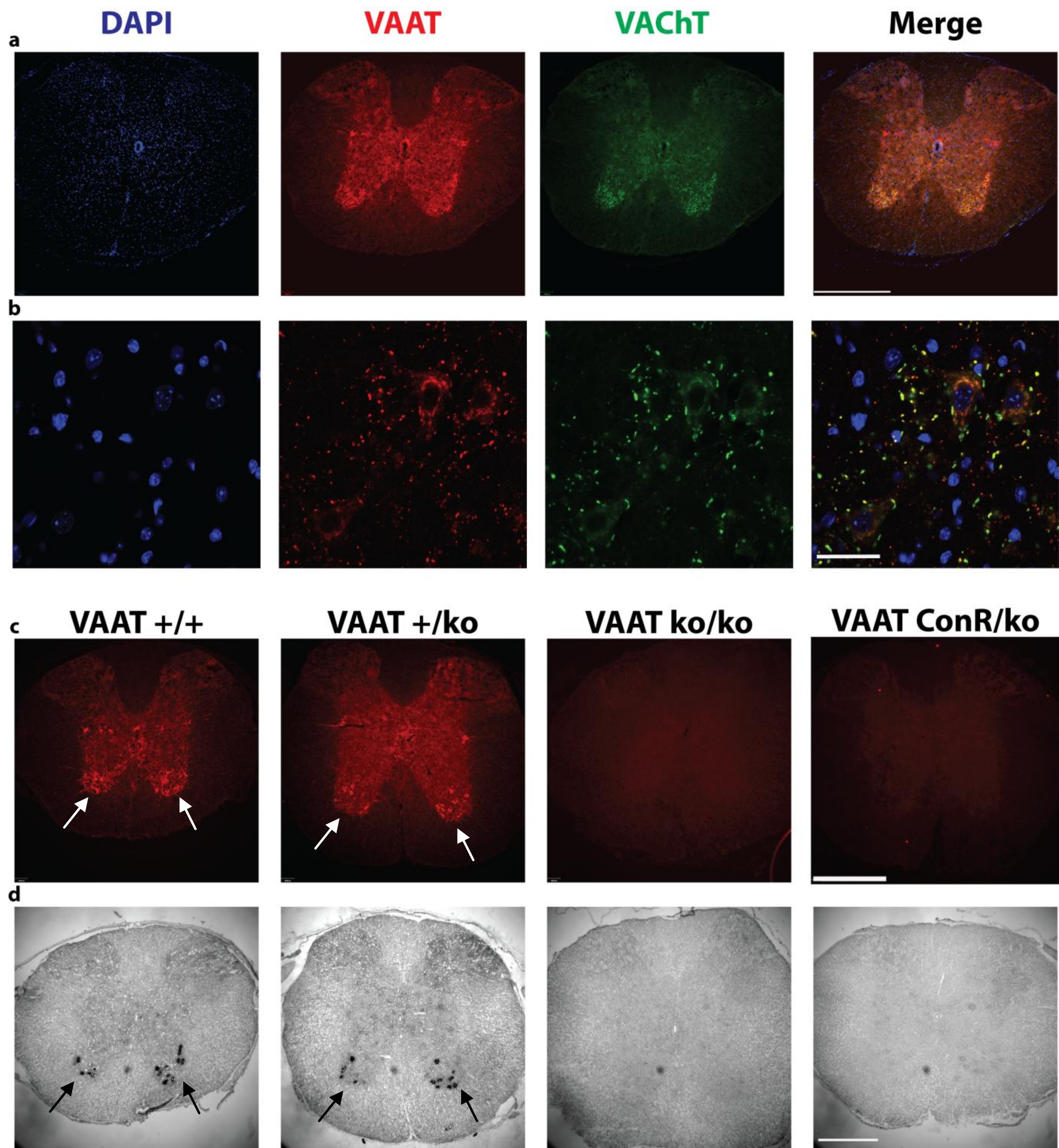


Figure 8: Validation of the knockout first property of $VAAT^{ConR}$ using VAAT cholinergic expression in the mouse spinal cord. (a) Colocalization of VAAT and VAChT immunolabelling in the ventral motor neurons of wild type mouse spinal cord. **(b)** Higher magnification confocal images of (a) show VAAT colocalization with VAChT. DAPI staining (blue) indicates cell nuclei. **(c)** Immunostaining and **(d)** RNA *in situ* hybridization of spinal cord coronal sections showing VAAT expression in ventral motor neurons (arrows) of $VAAT^{+/+}$ and $VAAT^{ko/+}$ but not in VAAT $^{ConR/ko}$ and $VAAT^{ko/ko}$. Scale bar: a) & c) 600µm, b) 30µm, d) 400µm.

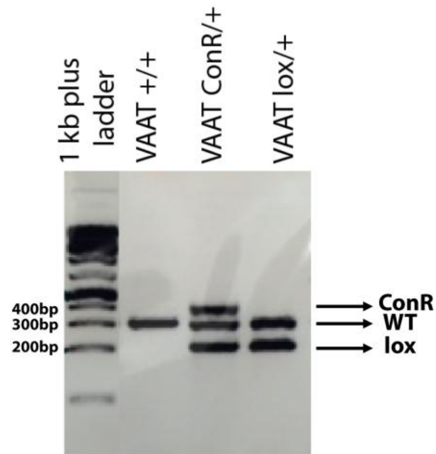


Figure 9: Genotyping strategy to monitor the generation of VAAT lox allele. The band corresponding to the gene trapping cassette indicated as ConR (400bp), was absent in all the VAAT lox mice. Also note that the 200bp band indicating the floxed exon2 is present in both VAAT ConR and VAAT lox, while it is absent in wildtype mice.

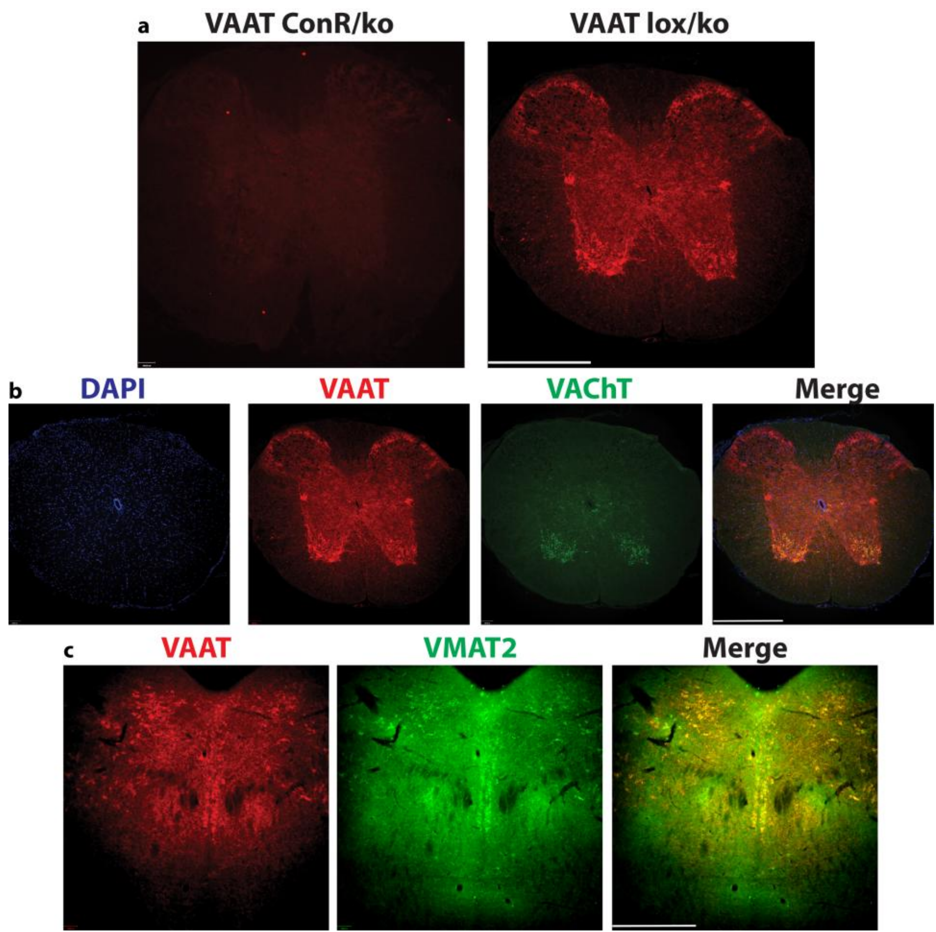


Figure 10: Validation of VAAT^{lox} allele (a) Immunostaining showing restored VAAT expression in the VAATlox construct generated from VAATConR. **(b, c)** VAAT aminergic expression of the conditional allele in the **(b)** cholinergic neurons of spinal cord and **(c)** serotonergic neurons of raphe nuclei indicated by the co-immunolabelling of VAChT and VMAT2 respectively (all scale bars- 600μm).

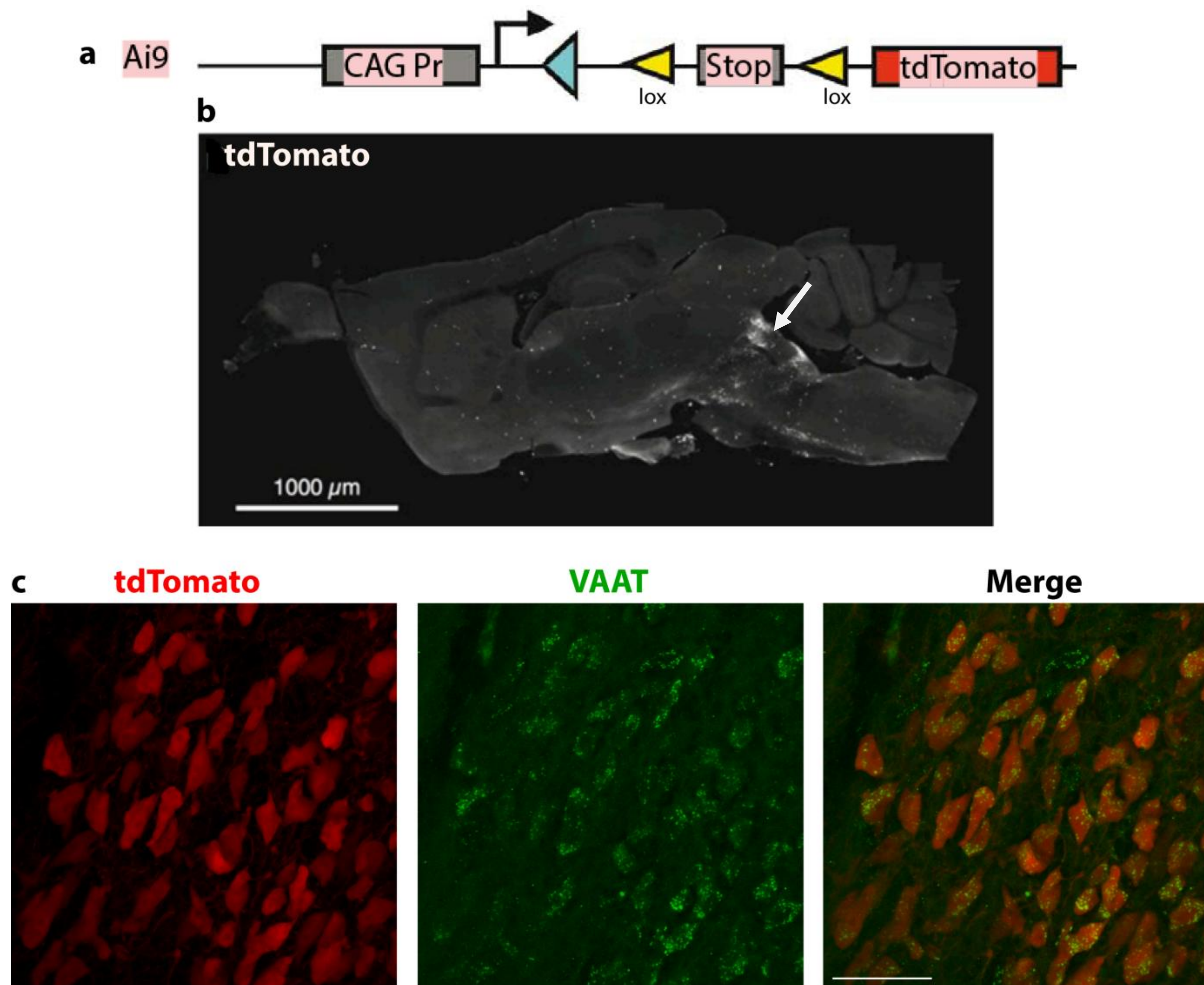


Figure 11: Characterization of SERT^{CreERT2} (a) Schematic diagram of the TomatoAi9 Cre reporter cassette in the ROSA26 locus (adapted from Madison, L. et al., 2010). **(b)** Sagittal brain section of SERT Cre^{ERT2}; tomato mouse showing tdtomato expression in the rostral cluster of raphe nuclei (arrow pointing to dorsal raphe nuclei) **(c)** Immunostaining illustrating vesicular expression pattern of VAAT in tomato expressing cells in dorsal raphe. Scale bar: b) 1000 μm; c) 30μm.

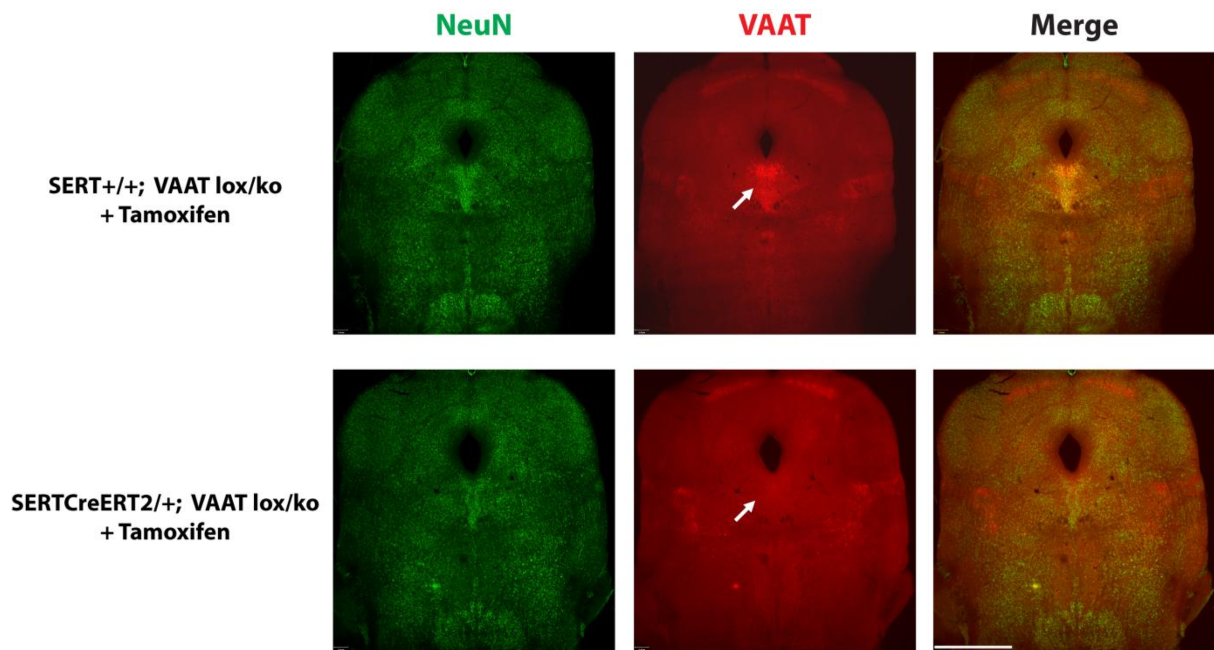


Figure 12: VAAT conditional knockout upon tamoxifen administration. Arrow indicates the dorsal raphe nuclei. Scale bar: 1000µm.

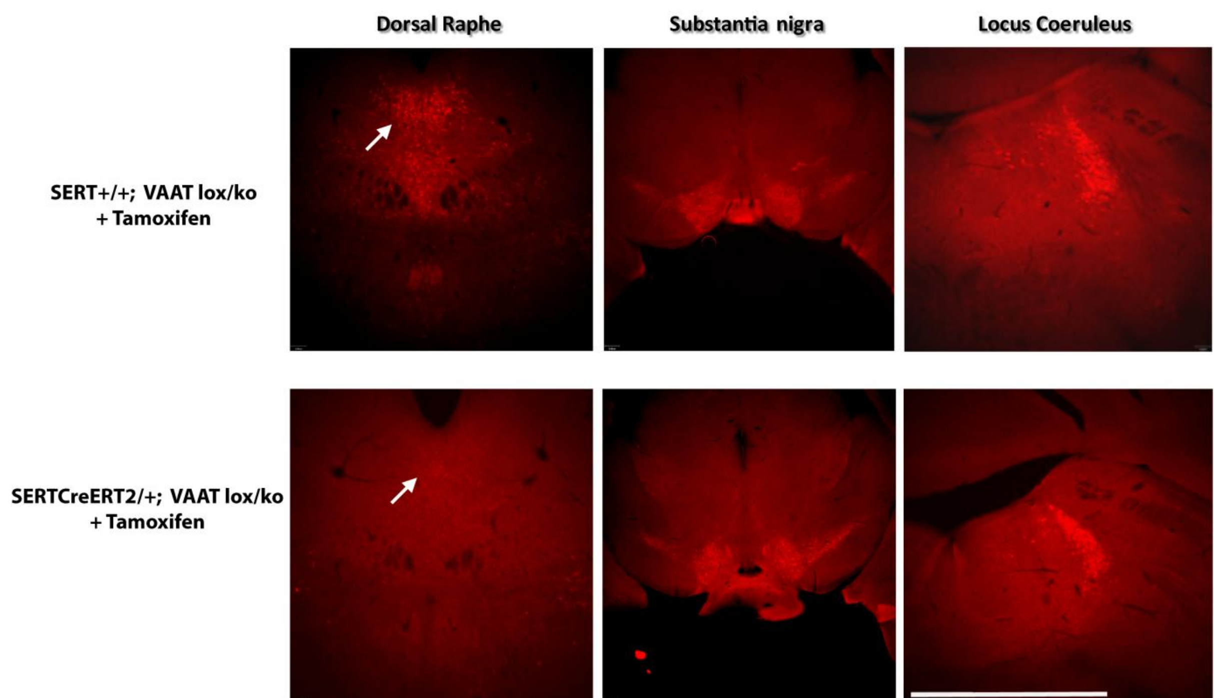


Figure 13: Spatial resolution of VAAT deletion. VAAT immunolabelling of different aminergic nuclei demonstrating that the knockout is very specific to serotonergic neurons (arrow). Scale bar: 900µm

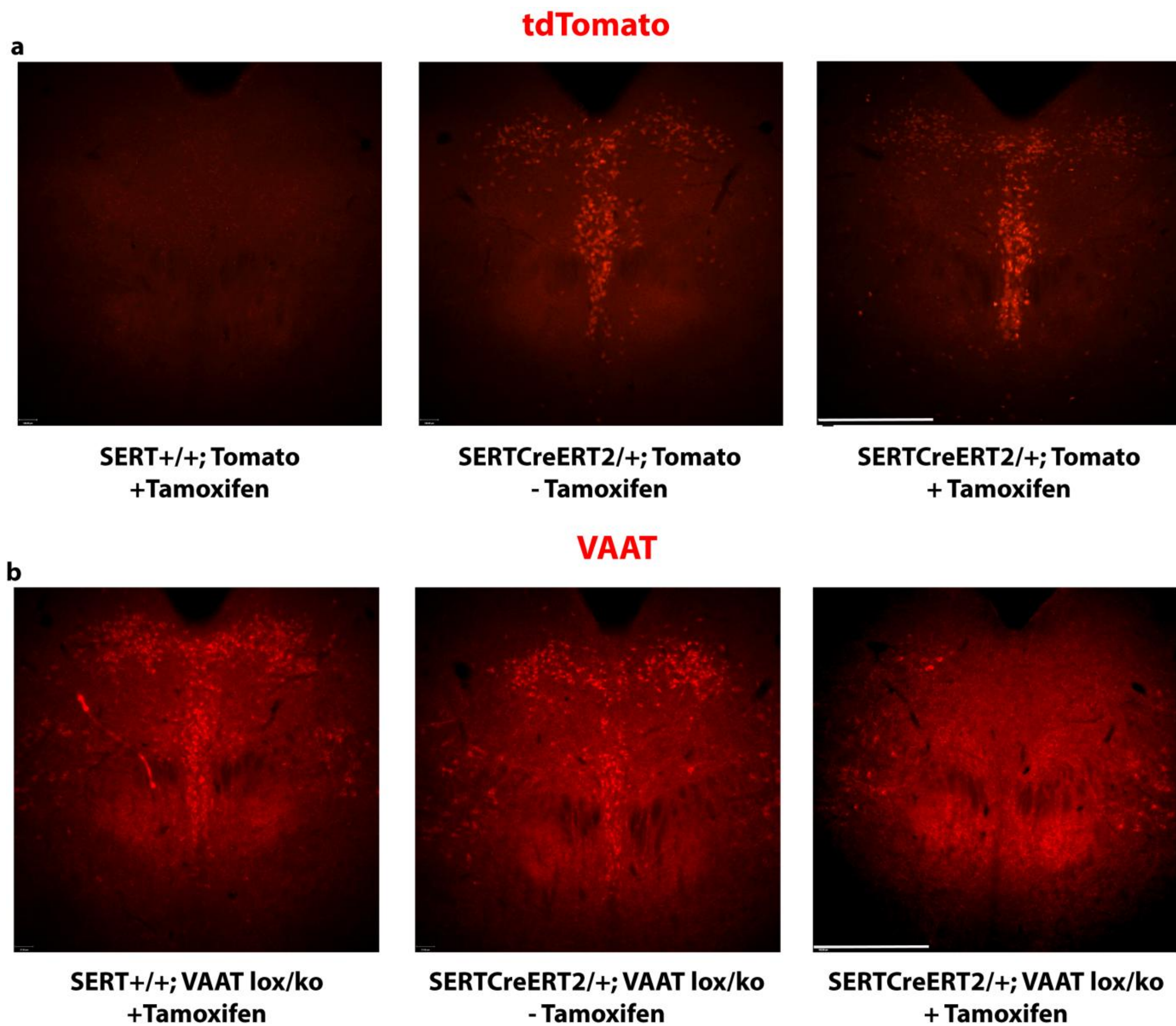


Figure 14: Inducibility of SERTCreERT2. (a) Tomato reporter expression irrespective of tamoxifen administration. **(b)** VAAT immunostaining demonstrating that the complete deletion of VAAT happens only in the presence of tamoxifen. Scale bar: 600µm

Quantification of the inducibility of VAAT deletion

As the leaky translocation of CreERT2 to the nucleus was evident from the Tomato expression in the absence of tamoxifen, a detailed quantification of VAAT deletion was carried out. The ratio of VAAT positive cells relative to the number of NeuN positive cells within caudal (- 5.34 to-4.96mm), intermediate (4.84 and 4.72mm) and rostral (4.60 and 4.48mm) raphe regions of $SERT^{+/+}$;$VAAT^{lox/ko}$ (control) was compared to that of $SERT^{CreERT2/+}$; $VAAT^{lox/ko}$ (experiment) mice, either with or without tamoxifen administration (one animal per group). Throughout the entire

raphe nuclei, not even a single VAAT positive neuron was observed in the experimental animals that received tamoxifen treatment. Two - way ANOVA revealed significant influence of the genotype on the relative cell count ($P<0.0001$), but no influence of the rostro-caudal bregma value ($P= 0.1048$). Moreover, dunnett's posthoc test revealed a significant reduction in the relative cell count of VAAT positive neurons in the experimental animals with and without tamoxifen compared to that of control within the different subdivisions of raphe nuclei (n=5 per subdivision) (**Fig 15.a**). Furthermore, this relative cell count was used to calculate the percentage of knockout within each sub region. The leaky activity of CreERT2 resulted in 35.3% knockout in experimental animals before tamoxifen, while complete recombination after tamoxifen administration resulted in 100% knockout of VAAT throughout raphe nuclei, thus generating the VAAT inducible and conditional knockout (VAATicKO) (unpaired students t test, $P<0.0001$) (**Fig 15.b**). This quantitative analysis demonstrates that even with the background activity of $SERT^{CreERT2}$, 64.7% of the cells still retain VAAT expression, confirming that VAAT deletion is partially inducible in $SERT^{CreERT2/+}$; $VAAT^{lox/ko}$.

Henceforth the tamoxifen administered $SERT^{CreERT2/+}$; $VAAT^{lox/ko}$ is referred to as VAATicKO mice.

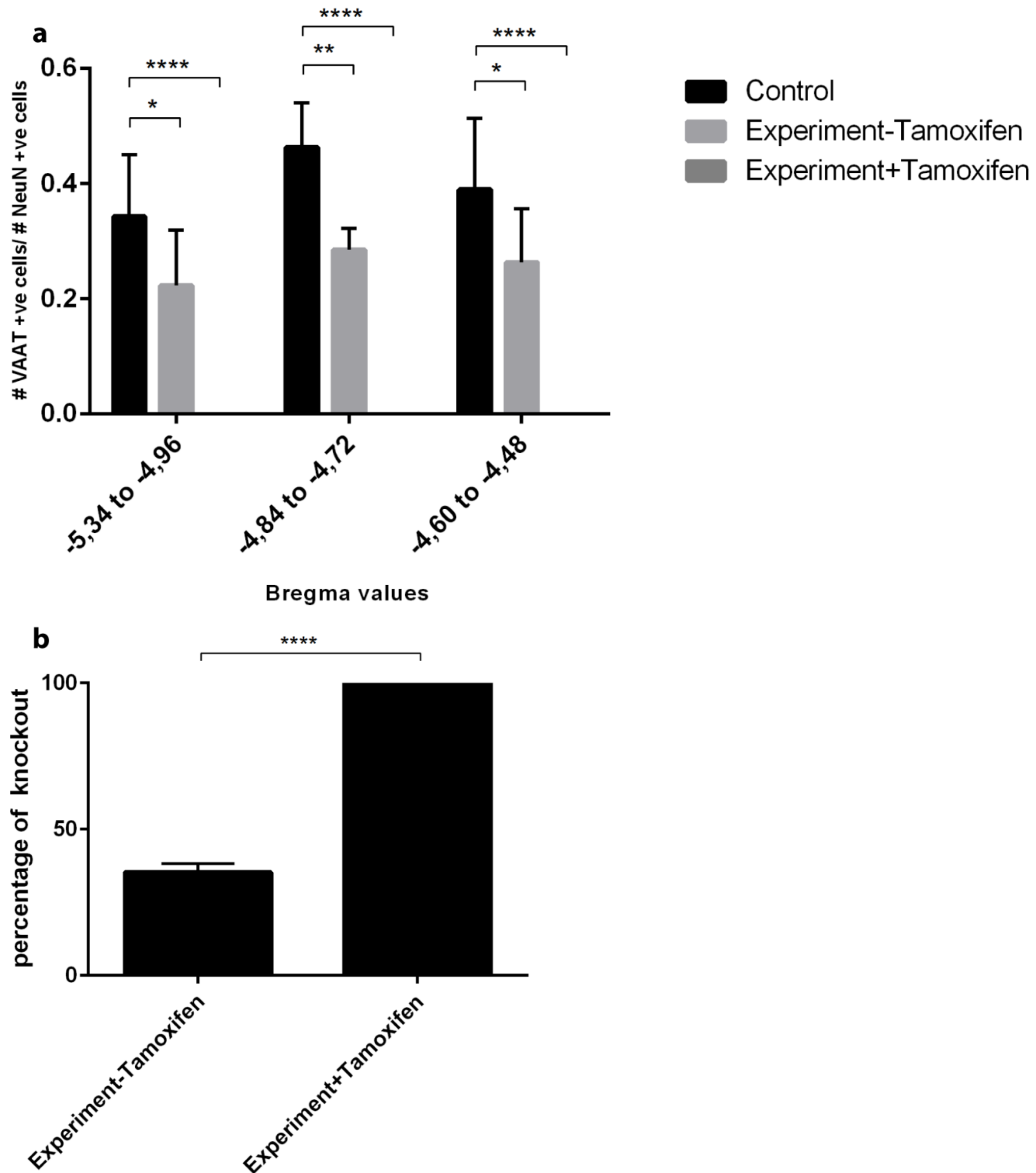


Figure 15: Quantification of the inducibility of VAAT deletion (a) Quantification of no: of VAAT positive neurons relative to the no: of NeuN positive neurons in different sub regions of dorsal raphe nuclei under different tamoxifen treatments. Graph shows average VAAT/NeuN ratio + SD. Two way ANOVA with Dunnet's multiple comparison test; n=5 for all the genotypes within each bregma range; P values obtained from Dunnet's multiple comparison test- *P<0.05, **P<0.01, ****P<0.001. **(b)** Percentage of knockout in experimental animals in the presence and absence of tamoxifen. Graph shows average of the % knockout in the three defined sub regions of dorsal raphe nuclei + SD. Two tailed, unpaired students t test; n=3; ****P<0.0001.

Neuronal effects of VAATicKO

As mentioned before, previous studies published using VAAT constitutive knockout mice have shown compensatory changes in gene expression and altered central monoamine levels, which might counterbalance the defective monoamine homeostasis (Patra, K. et al., 2015, Larhammar, M. et al., 2015). The main goal of the inducible and conditional knockout of VAAT used in the present study was to avoid the compensatory changes, enabling an evident behavioural phenotype highlighting the contribution and significance of VAAT in serotonergic system. Recently published reports suggest that stressors alter the activity of raphe and its downstream targets (Jasinska, A. J. et al., 2012). Hence the effects of VAATicKO were monitored under normal and stressful conditions. For the induction of stress a behavioural scheme was implemented in which the mice were subjected to repeated behavioural tests (See Methods). The different behavioural tests used in this scheme are the current models of depression that are used to monitor animal's 'depression-related' responses upon acute stress (Krishnan, V. and Nestler, E. J., 2008). The behavioural scheme implemented to induce stressful conditions is based on the hypothesis that the repetition of these tasks over several weeks will induce conditions mimicking chronic mild stress.

Effect on serotonergic gene expression: The mRNA levels of genes essential to serotonergic signalling such as Serotonin transporter (Sert), Vesicular monoaminergic transporter 2 (Vmat2) and Serotonin receptors 1A and 2A ($5\text{-}HT_{1A}$ and $5\text{-}HT_{2A}$) in the raphe nuclei of VAATicKO were monitored under the presence and absence of stress. Kruskal-Wallis test followed by Dunnet's posthoc analysis revealed no significant difference in relative gene expression ($P>0.65$ for all the genes analysed) between VAATicKO and control ($SERT^{+/+}$; $VAAT^{lox/ko}$ or $SERT^{CreERT2/+}$; $VAAT^{+/ko}$) mice under normal (control: n=3; experiment: n=3) and stressful situation (control: n=2; experiment: n=4) for all the serotonergic genes analysed (**Fig 16**). This demonstrates that VAATicKO does not lead to compensatory gene expression changes.

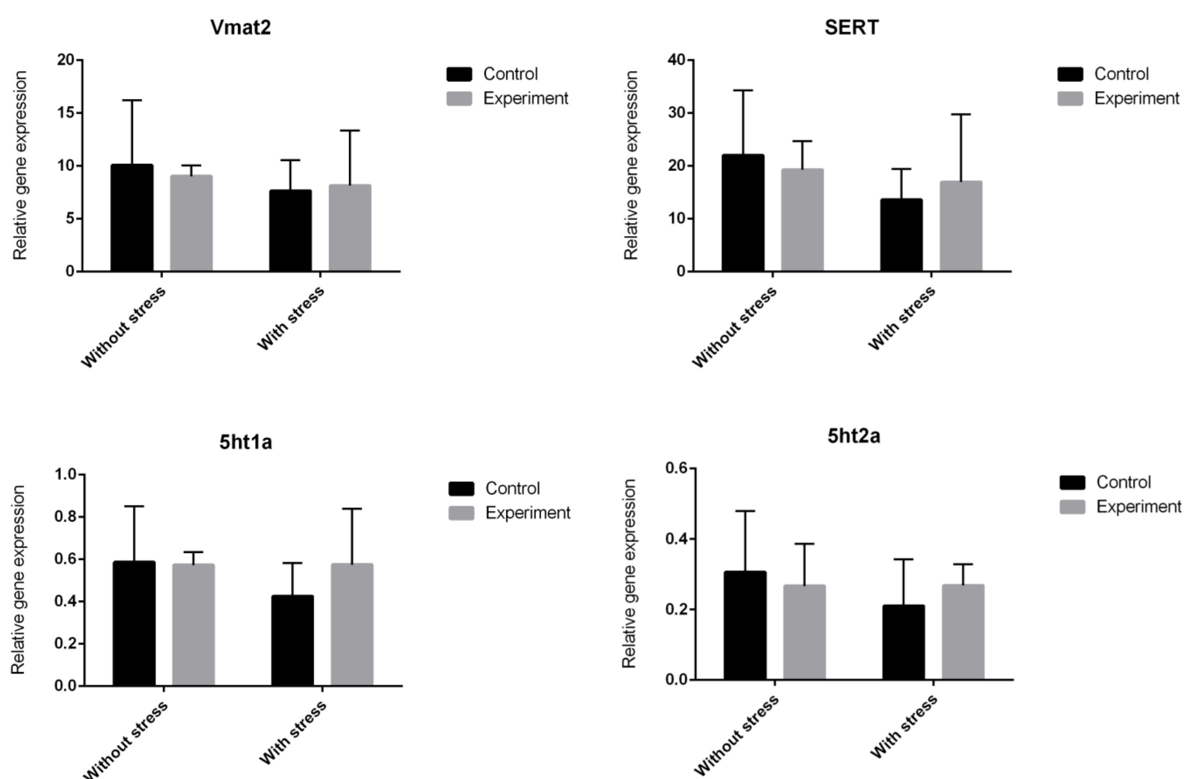


Figure 16: Effect of VAAT deletion on serotonergic gene expression Relative gene expression of vmat2, sert, 5-ht1a and 5-ht2a in the raphe nuclei of control and experiment (VAATicKO) animals, in the presence and absence of stress showed no significant difference. Graphs show average relative gene expression + SD. Kruskal-Wallis test; without stress- control & experiment n=3; with stress- control n=2 & experiment n=3; not significant.

Effect on central serotonin levels: Rostral cluster of raphe nuclei, especially dorsal raphe, extensively project to the forebrain region where higher cognitive abilities are monitored (Witteveen, J.S. et al., 2013). Moreover the raphe-prefrontal cortex network is necessary for the adaptive behavior under stressful situation (Robbins, T.W. et al., 2005). Hence the serotonin released in the forebrain region was quantified using ELISA (**Fig 17.a**), to check for any alterations following VAATicKO under normal and stressed situations. Without stress, serotonin levels in VAATicKO mice (n=3) were equivalent to that of control (n=3) (unpaired students t-test P=0.820). Unpaired students t-test with two tailed distribution showed VAATicKO (n=4) has significantly increased serotonin levels compared to control (n=3) under stress with 90% confidence level (P=0.0926) (**Fig 17.b**). This indicates that VAAT might be involved in the modulation of serotonin levels under stress.

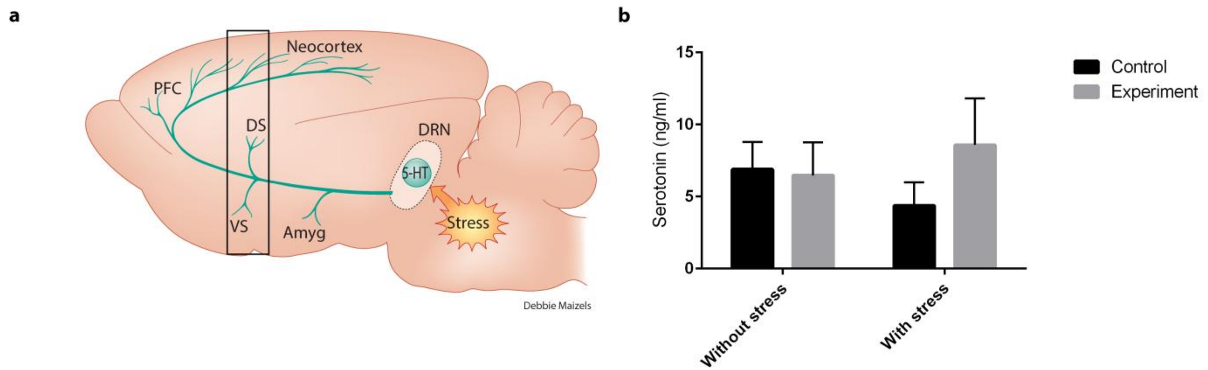


Figure 17: Effect of VAAT deletion on central serotonin levels. **(a)** Schematic diagram depicting the serotonergic projections to different forebrain regions (dorsal - DS and ventral – VS striatum, Amygdala – Amyg) necessary for the adaptive behavior under stressful situations (modified from Robbins, T.W. et al., 2005). Rectangle shows the region used for serotonin ELISA. **(b)**Quantification of serotonin in control and experiment (VAATicKO) animals under normal and stressful situations. Under stress, higher serotonin levels were observed in VAATicKO mice compared to that of control (unpaired students t-test with two tailed distribution; P=0.0926; Experiment n=4, control n=3). Graph shows average serotonin level (ng/ml) +SD.

Discussion

In this study, a conditional allele of VAAT was generated and characterised for the first time. This genetic tool can now be combined with different Cre lines to dissect the function of VAAT in individual aminergic nuclei and its role in higher cognitive functions. Furthermore, this study also provides the baseline characterisation for the serotonergic nuclei specific conditional study of VAAT using SERT CreERT2. Here, we demonstrate that the SERTCreERT2 has a very specific expression pattern in the serotonergic raphe nuclei, but provides only a limited control over the temporal onset of recombinase activity. Madison L. et al., 2010 argue that the leaky CreERT2 activity can be dependent on the way in which the transgenic line is created. In this study they show that the CreERT2 line which was generated by random integration into the genome has background activity, while the one which is in frame to the coding sequence has none. The background activity observed in SERT CreERT2 can be attributed as a transgenic artefact which can be possibly overcome by careful generation of Cre lines and strictly regulated Cre expression levels.

The difference in the CreERT2 recombinase activity observed between the TomatoAi9 and VAAT lox constructs illustrate the caveat of depending on recombinase activity in reporter lines to access the knock in/out of gene of interest. This observation also highlights the importance of direct visualisation of the protein of interest in order to verify the efficiency of knock in/out by different Cre driver lines. The possible reason for this variation can be the different levels of sensitivity of loxP sites based on their genetic location and the local chromatin structure or the difference in the distance between loxP sites at different target sequence (Vooijs, M. et al.,2001, Tronche, F. et al., 2002). Nevertheless, the detailed quantitative analysis has shown that the SERT CreERT2 offers partial inducibility despite its leaky activity.

In addition to the rigorous characterization of the VAATicKO in serotonergic neurons, a preliminary evaluation on the neuronal effects of VAAT deletion was also shown in this study. The major caveat in this analysis is the small sample size. Increasing the sample size might also increase the significance of these results. However, our experimental design is by itself interesting, as it accesses the effect of stress induced by the behavioral tasks used to study depression tendencies in rodents. If the trend

observed in this baseline study is biologically relevant, it would indicate a possible role of VAAT in regulating the serotonin availability upon stress. Such a discovery will create a new milestone in the treatment of depression, with the addition of VAAT as a promising drug target.

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