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Abstract

SLC10A4 (Solute carrier family 10 member 4) is a member of the sodium/bile acid co-transporter protein family. Previous studies indicated that this protein is a molecular link between two subpopulations of neurons i.e the monoaminergic and cholinergic. These neural subpopulations are widespread in the central nervous system (CNS) and are involved in most processes of our behaviour related to motivation, perseverance, reward system as well as all of the motor output. In this research study the focus has been on using *Slc10a4* wild type (WT), knockout (KO) and *Slc10a4* over expressing mouse, to characterize the function of a Vesicular Aminergic Associated transporter (VAAT) in cholinergic and dopaminergic neurons in the brain. Increased motor activity and increased drug-induced hyperactivity have been observed in KO mice. At subcellular level VAAT co-localizes with Vesicular Acetylcholine Transporter (VAChT) and Vesicular monoamine transporter (Vmat-2) on small synaptic vesicles (SV) at axon-termini in the brain. Experiments were performed to understand the significance of VAAT in cholinergic and dopaminergic systems, investigate VAChT distribution in neuromuscular junctions (NMJ) in VAAT mutant mice, study dopamine (DA) uptake efficiency in vesicles, investigate the epileptic link of VAAT and understand motor behavior of mutant mice. It was observed that VAAT colocalizes with VAChT in NMJ's of diaphragm and gastrocnemius mouse muscles. The protocol for vesicular uptake studies was optimized under different conditions. Results illustrated that VAAT over-expressing mice show higher DA uptake compared to WT, while KO mice show less vesicular uptake. RNA expression analysis of muscarinic, nicotinic and dopaminergic receptors in striatum and hippocampus brain regions was performed. RT-qPCR results indicated no significant difference in expression levels; in both control and KO VAAT samples.

Keywords: VAAT, VAChT, cholinergic, dopaminergic, synaptic vesicles, neuromuscular junctions, *Slc10a4*, dopamine uptake, striatum, hippocampus

Contents

1. Introduction

It has been a challenging task for researchers to comprehend the intricate complexities of human brain. Neuronal circuits responsible for various body functions are an integral part of CNS. They are believed to play a key role in locomotory behavior, although its genetic basis still remains uncharacterized. Witnessing quite complex human behaviours in everyday life has instigated neuroscientists to ponder over several questions. Why do people respond in a certain fashion in distinct social situations? How does CNS coordinate with the brain through neural circuits in a fraction of seconds and instantly directs the body to act in a certain way? What makes people to promptly remove their hands from a hot surface, when they feel a burning sensation? What causes this instantaneous response? Why do animals including humans feel pain, fear and stress? What stimulates these explicit behavioural manifestations? Several simple and spontaneous behavioural responses still remain complex puzzles for scientists; they stand clueless about the fundamental underlying mechanism in which the CNS coordinates these behaviors. As researchers move closer to understand the principles of neuromodulatory systems in which human brain and CNS operate, it seems like a never-ending mirage. The unsolved mystery behind this continues to fascinate neuroscientists, prompting them to dwelve deeper to demonstrate enough substantial evidences to support their findings. Over the years significant advances in genetics have brought a revolution in the field of neuroscience, simplifying and expediting complex experiments to understand the neuronal connections. Several organisms like nematode worms, frogs, zebra fish, snails, fruitflies, rats, humans and mice have been used by scientists in different laboratories around the world, to study the dynamics of neural circuits. Although each promising breakthrough in neuroscience brings with it a ray of hope, the scientific community continues to raise more questions. This research study is an attempt to understand the everlasting enigma of CNS and neuronal circuits.

1.1 Background

The focus of research in the lab was on central pattern generators (CPG) in the CNS using mouse model. CPG's are the neuronal circuits that control rhythmic movements, like breathing, chewing, feeding, swimming and walking. They have a significant role in locomotion and co-ordinating body movements *(1).* It has been observed that neuromodulators including neurotransmitters like DA, Serotonin, ACh, and Histamine, directly affect the output of physiological system by acting on CPG's. These neuromuscular modulators act from neural inputs to develop contractions that result in muscular outputs *(2).* Multiple approaches were used to understand the role of neurotransmitters in distinct neuronal sub-populations. The identification of novel spinal cholinergic markers was a significant breakthrough, and a major turning point in itself. Gradually the research focus shifted to identifying subtype-specific genes for cholinergic neurons in the mouse spinal cord. Several methods were used, and 159 such genes, specific for cholinergic neurons were screened. A small subset of genes was shortlisted among these 159 genes, for detailed analysis. Microarray and *in situ* hybridisation (ISH) experiments were performed to identify exclusive cholinergic marker genes, *Slc10a4* (Solute carrier family 10, member 4) was found to be one of those genes *(3).* Later *Slc10a4* was also found to be present in dopaminergic neural subpopulations. Along with the identification of this orphan transporter protein, research interest switched further to investigating rudimentary function of *Slc10a4* in different neuronal systems. Due to its exclusive vesicular location, this protein was named as Vesicular aminergic associated transporter or VAAT *(4).* The current study involves identifying function of *Slc10a4*, the fourth member of *Slc10* family, in cholinergic and dopaminergic systems using mouse models.

1.2 Past studies on *Slc10a4*

SLC10A4 belongs to the solute carrier super family consisting of more than 360 member proteins *(5)*. Earlier Solute carrier family 10 (*Slc10*) was regarded to consist of only two sodium-dependent bile acid transporters SLC10A1 (also referred as Na+/taurocholate co-transporting polypeptide or NTCP) and SLC10A2 (also known as apical sodium-dependent bile acid transporter or ASBT). Another four members SLC10A3, SLC10A4, SLC10A5 and SLC10A6 (known as Sodium dependent organic anion transporter or SOAT) were later included in the SLC10 family *(6)*. SLC10A4 was originally discovered in 2004, as a hypothetical protein *(7)*. The expression of *Slc10a4* was found to be pre-eminent in nervous tissue; it was identified in different regions in humans, rats and mice. In *Homo sapiens* it was found in brain, eye, soft tissues and neuroblastomas, while in rodents it was present specifically in adrenal glands; in mouse it was identified in vascular system, ganglia, stomach, diencephalons, cortex, brain stem, eyeball and sympathetic ganglion. Although it shares highest phylogenetic relationship with sodium dependent bile acid transporter SLC10A1, it showed no transport activity for bile acids in the presence of sodium *(8)*. *Slc10a4* was cloned from the Rat adrenal gland *(9)*. Immuno-fluorescence and co-localization studies with cholinergic marker protein VAChT and monoaminergic marker protein VMAT2 revealed that SLC10A4 was found in both cholinergic and monoaminergic neurons in rat CNS and peripheral nervous system *(10)*. Western blot (WB) studies also illustrated the presence of SLC10A4 in brain vesicles *(9)*.

1.3 VAAT as a molecular link between two distinct neuronal subpopulations

Due to its expression pattern in the neuronal and non-neuronal cholinergic systems, it was hypothesised that SLC10A4 has a specialized function in regulation, release and storage of neuronal components like neurotransmitters, neuropeptides, or in exocytosis and retrieval of vesicles and granules *(4)*. It was also assumed that both cholinergic and monoaminergic neurons share common functions in modulating behaviour. But to this date no genetic or biochemical discoveries linked these systems together. We proposed that VAAT could be considered as a significant molecular link between these two neuronal subpopulations. These widespread neural subpopulations in the CNS are implicated in behaviour related to motivation, perseverance, reward system as well as all of the motor output. Several independent studies investigated dopaminergic, cholinergic, adrenergic, and epileptic links of VAAT in mouse models at subcellular, vesicular, molecular and behaviour levels. ISH experiments were done to study the m-RNA expression pattern of VAAT in different neuronal populations and regions in brain and spinal cord. Electron Microscopy (EM) experiment was conducted to study terminal localization of *Slc10a4*. Stimulated Emission Depletion Microscopy (STED) was performed to find out whether VAAT colocalizes with VAChT or VMAT2 on same vesicles. In-situ proximity ligation assays (PLA) on enriched SV were performed to investigate vesicular co-localization of VMAT2 and VAAT with VAChT. Different behavior tests were conducted in VAAT WT and KO mouse to study neuromodulatory systems like Hanging wire test to analyse muscular strength, Elevated plus maze test to evaluate anxious behavior, Multivariate concentric square field test to analyze risk taking behavior, Eight-armed radial arm maze test to check learning and memory, and Locobox maze tests to measure locomotory behavior. High Performance Liquid Chromatography (HPLC) analysis was performed to determine concentrations of DA and its metabolites in Striatal brain tissues. DA turnover was calculated by evaluating synthesis, release, reuptake and metabolism of DA; and estimating the ratios between DOPAC (3, 4-Dihydroxy phenyl acetic acid), HVA (Homovanillic acid) and DA *(4)*.

Electrophysiology experiments were conducted to identify the epileptic link of VAAT. The results obtained from these parallel studies were promising and supported our original hypothesis. The current project was designed to establish and connect the missing links on VAAT and further strengthen our presumptions, that VAAT has a specialised function in regulation and release of neuronal components.

In this study the focus has been on using a WT, KO and VAAT over-expressing mice to understand VAAT functionality in cholinergic and dopaminergic neurons in the brain, and investigate its epileptic link. *Slc10a4*$^{+/-}$ mice were obtained from Texas A&M Institute, USA, after series of heterozygote breedings *Slc10a4*$^{-/-}$ mice were produced. Transgenic NSE-*Slc10a4* mice were developed by incorporation of *Slc10a4* c-DNA into mouse genome using genetic recombination tools. Experiments were performed to understand the significance of VAAT in cholinergic and dopaminergic systems, investigate VAChT distribution in NMJ's in VAAT mutant mice, study DA uptake efficiency in SV, and study m-RNA expression analysis in striatal and hippocampus tissue.

Dopaminergic link

The neurotransmitter DA plays a significant role in brain functions that modulate behavior, movement, cognition, attention, learning, emotional response, pain and pleasure. DA containing neurons accumulate in the midbrain region, which is known as substantia nigra *(11).* Substantia nigra performs a key role in vital functions like reward, addiction and movement. Especially, pars compacta region supplies DA to the striatum, hence dopaminergic synapses can be found in the striatum *(12).* Membrane and vesicular transporter proteins for DA modulate neuronal activity. Dopamine active transporter (DAT) is responsible for reuptake of DA from synaptic cleft for recycling *(13).* Reuptake is necessary to inactivate DA action at postsynaptic targets. DAT has a significant function in DA homeostasis as observed in DAT mutant mice. It showed five times increase in extracellular DA content, while 95% decrease in Striatal DA content *(14)*. Vesicular monoamine transporters (VMAT) 1 and 2 are vesicular proteins; VMAT2 specifically transports monoaminergic neurotransmitters into SV *(15)*. Previous studies on VMAT2 null mutant mice exhibit abnormal storage and vesicular release properties in the animals. Aberrant DA activity has been associated with various neurological disorders *(16).*

To investigate the role of VAAT in modulating DA homeostasis, SV purification and DA uptake studies were performed. The presence, absence and over expression of VAAT in respective animals were verified by vesicle enrichment and WB technique. Several modifications were done to the previous, established SV purification and uptake procedures. After conducting series of experiments using different uptake buffers, an exclusive protocol was standardized for DA uptake experiments.

Epileptic link

Several genetic studies in animals suggest that aberrations in synaptic proteins can result in different kinds of neurological disorders; Epilepsy is believed to be one of them. Previous studies established the presence of VAAT in cholinergic terminals. Another study also indicated altered locomotor activity and greater drug-induced hyperactivity in null mutant mice *(4).* Electrophysiology experiments revealed that carbachol; a cholinergic agonist impels gamma oscillations in hippocampal slices. These oscillations were observed in VAAT KO mice before treatment, and after injecting carbachol agonist, pilocarpine the

epileptic seizures grew rapidly. It was assumed that absence of VAAT gives rise to a greater response to cholinergic impetus *(17)*. To investigate the epileptic link of VAAT further, and whether absence or presence of VAAT at receptor level significantly influences or gives rise to epileptic activity; we studied mRNA expression pattern in VAAT WT and KO mice. RT-qPCR experiments were done to check relative expression levels of various receptors in hippocampus and transporters in striatum regions of mice brain.

Cholinergic link

Cholinergic neurons are widespread in the peripheral and central nervous system, and play significant role in behavior modulation *(18)*. NMJ is the synapse where the axon terminal of a motor neuron meets the motor endplate, the highly excitable region of muscle fiber plasma membrane responsible for initiating action potentials across the muscle's surface, ultimately causing the muscle to contract. The signal passes through the NMJ via the neurotransmitter acetylcholine. Terminal branches expand outward from the motor nerve and emerge from their myelin sheath at the muscle to form terminals. These terminals are filled with synaptic vesicles, mitochondria, and tubules from smooth endoplasmic reticula. Synaptic terminals permit the necessary communication between motor neurons and their target muscles for muscle contraction *(19)*. The motor end-plate is densely populated by acetylcholine receptors. This part of the research project involved co-localization studies of VAAT with VAChT in pure cholinergic synapses and analysis of VAChT distribution in NMJ's of VAAT WT and KO mice using immunofluorescence. Diaphragm and gastrocnemius muscles of mice were selected for this study.

2. Aims of the project

The main purpose of this research study was to understand the rudimentary function and biological significance of this recently discovered orphan transporter protein *Slc10a4*. It is found to be present in both cholinergic and dopaminergic neural sub-populations widespread in the CNS. It is widely known that both dopaminergic and cholinergic neuronal systems share common functions in modulating behavior, but no genetic discoveries link these systems together. It was assumed that VAAT could be considered as a significant molecular link between these two neuronal systems. It was also hypothesised that VAAT has a significant role in regulation, release, and storage of synaptic vesicles and neurotransmitters, like DA and ACh. Aberrations in synaptic proteins and neurotransmitter signalling are also often associated with neurological disorders, like epilepsy. This research project was thus designed to connect the missing links on VAAT by investigating the dopaminergic, cholinergic and epileptic links of VAAT in WT, KO and NSE-VAAT mouse models at subcellular, vesicular and molecular levels. Also, further analysis was required to provide additional insights on the potential role of VAAT and treatment possibilities in aminergic systems.

1) Investigate Dopaminergic link – Dopaminergic signalling in the brain has a significant impact on our behavior related to motivation, reward, mood, attention, learning and motor behavior. It was hypothesized that VAAT has a significant role in modulating DA homeostasis and could reveal unexplored targets for curing neurological disorders. Thus, the purpose of this part of the research study was to elucidate the possible role of VAAT in pre-synaptic vesicles and its influence on DA signalling. To investigate further how the VAAT levels in synaptic vesicles affect DA uptake, a comparative analysis was performed by carrying out Synaptic vesicle assay studies in different vesicle samples extracted from the striatum regions of the brains of VAAT WT, KO and NSE-VAAT mice.

2) Investigate Epileptic link - Epilepsy is believed to be one of the neurological disorders caused due to abnormality in synaptic proteins. Previous studies established the presence of VAAT in cholinergic terminals; they also indicated altered locomotor activity and higher drug-induced hyperactivity in null mutant mice. Electrophysiology experiments revealed that carbachol; a cholinergic agonist impels gamma oscillations in hippocampal slices. These oscillations were observed in VAAT KO mice before treatment, and after injecting carbachol agonist, pilocarpine the epileptic seizures grew rapidly. It was hypothesized that absence of VAAT gives rise to a greater response to cholinergic impetus. To further investigate and get a deeper insight of the epileptic link of VAAT, we studied mRNA expression pattern in VAAT WT and KO mice. RT-qPCR experiments were done to find out whether presence or absence of VAAT significantly affects epileptic activity.

3) Investigate Cholinergic link – The axon terminal of a motor neuron meets the motor endplate at NMJ, the most excitable region of muscle fiber plasma membrane responsible for causing action potentials across the muscle's surface, leading to muscular contraction. The signal moves through the NMJ via neurotransmitter acetylcholine. Acetylcholine significantly affects our Behavior and influences locomotion and coordination of body movements. Cholinergic link of VAAT was thus investigated to identify co-expression of VAAT with VAChT, in NMJ's of diaphragm and gastrocnemius muscles of VAAT WT and KO mice. Co-localization studies of VAAT with VAChT in pure cholinergic synapses were performed and VAChT distribution was analyzed in NMJ's using immunofluorescence.

3. Materials and Methods:

3.1 SV purification, dopamine uptake and Western Blot studies

VAAT WT, KO and NSE-VAAT Mice

Slc10a4 WT, KO and Transgenic NSE-VAAT animals were used for DA uptake experiments. *Slc10a4*$^{+/-}$ mice (strain 129/SvEvBrd) were obtained from Texas A&M Institute for Genomic Medicine (College Station, TX, USA). Heterozygote breedings were setup to generate adult male and female homozygous mice and their WT littermate controls. Transgenic NSE-*Slc10a4* (NSE-VAAT) mice were developed using genetic tools. The full length *Slc10a4*-cDNA (RIKEN clone E130304D01, Source Bioscience, Nottingham, UK) was inserted downstream of the rat neuron specific enolase (NSE, enolase 2) promoter, as depicted in *Fig.1*. HindIII linkers were attached to *Slc10a4* c-DNA by Polymerase Chain Reactions (PCR) and cloned into the HindIII site of Pnse-Ex4 vector. Correct insert orientation was verified by PCR using primers: 5'-CTCTTGTCCACCCAAGGAGA and 5'-GACCGGGACTAGAGGTGACA. Linearized NSE-*Slc10a4* construct was purified and introduced to the genome by pronuclear injection at Uppsala University Transgenic Facility (UUTF). Founders were identified by PCR, and analyzed by ISH, Immunohistochemistry (IHC) and WB experiments. NSE-*Slc10a4* was here referred to as NSE-VAAT animal. Guidelines by Swedish regulation and European Union Legislation were followed in keeping the mice. All animal experiments were conducted as approved by relevant animal ethics committee in Uppsala. Male and female mice between 7 and 24 weeks were used for vesicular experiments. *Slc10a4*$^{-/-}$ (VAAT mutant) mice were investigated with sibling WT mice as controls. Mice were killed by cervical dislocation and decapitation. Specific brain regions were dissected and rinsed in ice-cold homogenisation buffer.

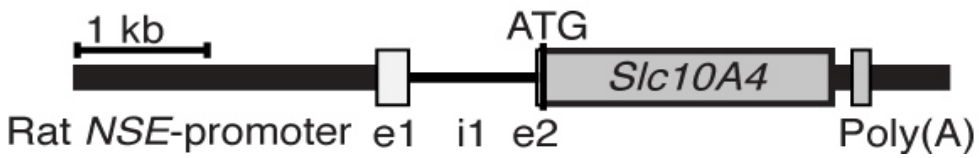

Fig.1 Transgenic NSE-VAAT model

Primers

For Genotyping *Slc10a4* mice, following primers were used:
WT_forward 5'-GGAAAGACATGGCTGACTCTG,
WT_reverse 5'-CAGGTAAAGGGACCACAGG,
KO_forward 5'-CAGGTAAAGGGACCACAGG,
KO_reverse 5'-ACACCGGCCTTGTATTTGTAGC
For TgNSE- *Slc10a4* animals, following primers were used:
forward 5'-CTCTTGTCCACCCAAGGAGA,
reverse 5'-GACCGGGACTAGAGGTGACA.
Maxima™ Hot Start Taq DNA Polymerase enzyme and PCR reagents were used for genotyping *Slc10a4* animals.

Antibodies and Reagents

Antibodies for WB were used in the following dilutions: Rabbit anti-*Slc10a4* (Sigma Aldrich) 1:500, Guinea pig anti-Vacht (Millipore, MA, USA) 1:500, Synaptophysin 1:1000, VAAT 1:1000, Rpt-4 1:1000, HRP conjugated protein-A 1:5000,
Loading dye (LD): 95% 4X Loading Buffer (containing 16% Sodium Do-decyl Sulphate content) 5% Beta-mercaptoethanol (Beta-m)
Luminol Reagent (LR): LR A (5µl), LR B (1000µl), 3% Hydrogen peroxide (5µl)

Genotyping

Tail Prep: To extract genomic DNA, tail biopsies were first incubated in Tail-Prep Buffer 1, (volume of buffer added was according to the tail size). After 20 minutes at 95ºC, 450 RPM the samples were placed at room temperature for 5min. Subsequently equal amount of Tail-Prep Buffer 2 was added. PCR was performed according to the protocol and program mentioned in *Tables 2* and *3* incuded in the *Appendix*. PCR products were detected on 1% agarose gel, and mice genotypes were determined.

Synaptic Vesicle Purification

Standardization of Synaptic vesicle isolation and purification process was accomplished after extensive studies and modifications of previous protocols used by various researchers for SV purification in different animal models. Pioneering work on SV purification by *Whittaker et al (20)* from cerebral cortex in guinea-pigs; by *Huttner et al (21)* from Rat cerebral cortex*;* and by *Jahn et al* from Rat brains *(22,23)* was reviewed. Later protocols used by *Teng et al (24)*, *Takamori et al (25)* and by *Nickell JR et al (26)* were also studied, and modified to design the final protocol. Biophysical principles, homogenization procedures, buffers and fractionation protocols used to isolate and purify vesicles were analysed. DA uptake efficiency in different uptake buffers like Jahn uptake buffer, Teng's buffer and Kreb's ringer buffer was compared. These buffers were originally tested in SV uptake experiments on other animal models, but in this current experiment they were being used on mouse models. Kreb's ringer was identified as an ideal uptake buffer and used for further experiments.

Three brains (excluding cerebellum and prefrontal cortex) were pooled and homogenized in 15ml of ice-cold 0.32 M sucrose solution at 900 RPM with 10 up and down strokes of tight fitting Teflon homogenizer. Brain Lysate samples were separated and labelled as P1. The whole process was carried out at 4ºC. Cell debris, intact cells and nuclei separated in the form of pellet, when homogenate was centrifuged at 2.000 x g for 10 min. Supernatant was transferred to a separate tube and centrifuged at 15.000 x g for 15 min. Buffy coat pellet was dissolved in 2 ml of ice-cold 0.32 M sucrose and poured into a glass Teflon homogenizer containing 7 ml of deionized water to provide an osmotic shock. The suspension was homogenized within a minute at 2.000 RPM with 3 up and down strokes. Osmolarity was restored by adding 90 µl 1M HEPES (*4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid*) pH 7.4 (KOH), 900 µl 1M potassium tartrate (pH 7.4) (KOH) and protease inhibitor (PI) cocktail (Roche Diagnostics Scandinavia). Pellet containing Synaptosomal fraction was separated in a tube and labelled as P2. A second centrifugation at 20.000 x g for 20 min was carried out to remove intact synaptosomes and other big membrane pieces. Intact vesicles and some contaminating organelles were obtained in the supernatant. The resulting supernatant was centrifuged at 55.000 x g for 60 min to remove contaminating membrane pieces and bigger vesicular structures. Pellet containing large myelin membrane pieces of mitochondria and leftover intact synaptosomes was separated and labelled as P3. To the supernatant (100 µl) 0.01 M $MgSO_4$, 0.25 M HEPES pH 7.4 (KOH), 1M Potassium tartrate pH 7.4 (KOH) was added and centrifuged at

100.000 x g for 120 min. The final pellet containing small synaptic vesicles was dissolved in 300 µl Krebs-Ringer buffer (in mM, NaCl 140, KCl 5, $MgCl_2$ 2, $CaCl_2$ 2, HEPES 10, sucrose 6, glucose 10, pH 7.4). Final vesicular fraction containing small synaptic vesicles was separated in a tube and labelled as P4. Protein content was quantified using Bradford assay *(Appendix 10.2.2)* and BSA as standard.

Dopamine Uptake Process

DA Uptake was initiated by incubating 15 µg protein equivalents of freshly prepared vesicles in 100 µl assay buffer (Krebs Ringer buffer with 50 nM [3H] DA (Perkin Elmer, Massachusetts, USA)) and 2 mM ATP-Mg final concentration at 32ºC for different time points. Triplicate samples were measured for different mouse samples, at every repeat. Uptake was stopped by dilution in 3 ml of wash buffer (in mM: 10 MOPS, 100 Potassium gluconate, 2 $MgCl_2$). Non-specific uptake was measured after incubating SV in reserpine (200 nM) for 5 min at 32ºC before adding assay buffer, and by uptake performed on ice. Samples were filtered through Whatman GF/F filters (Whatman, Maidstone, UK) and washed with 5 ml of ice-cold wash buffer. Scintillation counter was used for counting of [3H] DA retained in the filters. Non-specific uptake was subtracted for each time point and genotype. Data counts for each sample were represented as percent of WT control counts after 16 min incubation period.

Western Blot Studies

WB experiments were performed to establish presence of VAAT in SV samples of NSE-VAAT, VAAT WT & KO samples. Presence of vesicular protein Synaptophysin and RPT4 was investigated in vesicle precipitates collected at different stages during SV purification process in different mice genotypes. These brain lysate samples were labelled as P1 (Brain Lysate samples), P2 (Synaptosomal fraction), P3 (Large myelin membrane pieces of mitochondria and left over intact synaptosomes) and P4 (Final enriched vesicle fraction containing small synaptic vesicles). Samples were prepared by adding loading dye (Composition mentioned in *Section 4.1 Antibodies and Reagents*), as it provides uniform charge to the protein; $1/4^{th}$ volume (LD) of the total volume was added to the sample. Beta-mercaptoethanol present in it, acts as a reducing agent, so the protein stays in a reduced form due to its presence. Samples were separated on 4-15% mini-PROTEAN TGX precast gel (Bio-Rad Laboratories AB, Sweden) at 121V for 1h. Subsequently, samples present in the gel were transferred on to a nitrocellulose membrane (Bio-Rad Laboratories AB, Sweden), at 15V for 30 min in a semi-dry electrophoretic transfer cell (Bio-Rad Laboratories AB, Sweden). Nitrocellulose membrane was blocked with 3% BSA for 1 h and incubated with primary antibody in blocking solution for 2 h at RT, followed by four washes in TBST (50 mM Tris pH 8, 150 mM NaCl, 0.05% Tween20). Membrane was incubated with protein A-HRP conjugates (Bio-Rad Laboratories AB, Sweden) for 1h at RT and washed in TBST. Then the membrane was treated with Luminol reagent, and the film was developed and printed in the dark room.

3.2 Transcription studies

VAAT WT and KO Mice

Slc10a4 WT (+/+) and KO (-/-) animals were used for RNA expression studies. Striatum and Hippocampus tissues were specifically analysed in this experiment.

Primers

Primers for muscarinic receptors (M1, M2, M3, M4 and M5); nicotinic receptor (Chrna7), and dopaminergic receptors (D1 and D2) included in *Table.1* in the *Appendix*; were used to study the m-RNA expression in WT and KO mice.

Tissue Preparation and Homogenization

Striatum and Hippocampus tissues were dissected from mouse brains. Tissue samples were homogenized in Tri-Reagent solution, using a pestle manually. Homogenate was incubated for 5 min at RT. It was centrifuged at 12.000 x g for 10 min at 4ºC, and supernatant was transferred to a fresh tube.

RNA extraction

Bromo-chloro-phenol (or BCP) (100µl/1ml of Tri-Reagent) was added to the tube containing supernatant and incubated at RT for 5-15 min. The mix was then centrifuged at 12.000 x g for 10-15 min at 4ºC. Aqueous phase was then transferred to a new tube.

RNA Precipitation and Wash

Iso-propanol (500µl/1ml of Tri-reagent) was added to the tube containing aqueous solution, vortexed for 10 sec. and incubated at RT for 10 minutes. Tubes were then centrifuged at 12.000 x g for 8 minutes at 4-25ºC, and supernatant was discarded. After discarding supernatant, 75% ethanol (1ml alcohol/1ml of tri-reagent solution) was added to the tube, and centrifuged at 75.000 x g for 5 min. Ethanol was removed and RNA pellet was air-dried briefly. Nuclease free water was then added to the tube containing RNA pellet.

RNA quality check

Agarose gel electrophoresis was performed to judge the integrity and overall quality of RNA samples by checking the 28S and 18S rRNA bands. The secondary structure of RNA alters its migration pattern in gels so that it will not migrate according to its true size. Bands are generally not as sharp as in denaturating gels, and a single RNA species may migrate as multiple bands representing different structures. RNA samples (1 µg) were loaded directly on 0.7 % agarose gels in TBE (89 mM Tris-HCl pH 7.8, 89 mM borate, 2 mM EDTA) with 0.5 µg/ml ethidium bromide added to the buffer. Same amount of ethidium bromide was added in all the samples (including the size marker) because ethidium bromide concentration affects RNA migration in agarose gels. An aliquot of intact RNA was run as a positive control to rule out unusual results. The gel was run at 5-6 V/cm measured between the electrodes.

Genomic DNA contamination check and removal of genomic DNA

After RNA quality check, the presence of contaminating gDNA was tested in the RNA samples. Housekeeping gene, Tubb5 primers were used to amplify RNA samples by running a PCR *(Appendix Table 4* and *5).* The quality of the starting template and its integrity can be determined by amplifying a housekeeping gene. The target gene(s) as well as housekeeping gene are co-amplified in the same reaction, eliminating the well-to-well variability that would occur if separate amplification reactions were carried out. These genes do not vary in expression between different samples. PCR products were then run on a gel to test the presence of contaminating gDNA in these RNA samples. Subsequently, RNA samples contaminated with gDNA were treated with DNase1, Protocol for DNase1 treatment is provided in the *Appendix* in *Table.6*. Post DNase treatment another gel was run to check presence of gDNA in these samples.

cDNA Synthesis

cDNA was synthesised using reagents and protocols suggested in Invitrogen kit. Primers, d-NTP's and water were added to the extracted RNA samples. Mix was heated to 65ºC for 5 min and quickly chilled on ice. Subsequently Buffer, DTT and RNase out reagent were added to the tube, mixed and incubated at 37ºC for 2 min. Reverse transcriptase was added, and the mix was incubated at 25ºC for 10 min. Tubes were further incubated for 50 min at 37ºC, heat inactivated at 70ºC for 15 min, stored at -20ºC, and cDNA samples were diluted.

Primer quality check

Synthesized cDNA samples of hippocampus were used as templates to perform PCR on five muscarinic receptors, one nicotinic receptor and two dopaminergic receptors. Subsequently, the PCR products were run on an agarose gel to evaluate primer efficacy.

Quantitative PCR experiments

QPCR- SYBR Green (SG) mix, primers, water and cDNA were added to the PCR plate, using KAPA SYBR FAST qPCR Kit. The PCR program used for these experiments can be found in *Table 7*. Q-PCR Data was analysed using iQ5 software.

3.3 Co-localization studies in Neuromuscular junctions

VAAT WT and KO mice

Slc10a4 WT (+/+) and KO (-/-) mice were used for co-localization studies of VAAT with Vacht in NMJ's of Diaphragm and Gastrocnemius muscles. Two day old baby mice and adult mice were used for IHC experiments.

Antibodies

Primary antibodies used for IHC experiments were Guinea Pig Vacht (1:200), and Rabbit VAAT (1:500), while secondary antibodies used were Goat anti Guinea pig Cy3 (1:500), Donkey anti rabbit Alexa 647 (1:400), and Anti Btx (1:5000).

Tissue preparation

Chat-Tom-Cre, Slc10a4 WT and KO mice were anesthetized with Isofluorane; according to body weight 100 µl/ 10 g sedative was administered to each mice. Phosphate buffered saline (PBS) solution with 4% formaldehyde was perfused by cardiac puncture in the heart. Once blood flow stopped, mice were dissected. Diaphragm and gastrocnemius muscles were removed and placed in 4% formaldehyde solution in PBS overnight (O/N). Prior to cryo-sectioning, tissue was cryo-protected in 30% sucrose in PBS O/N, and embedded in tissue tek.

Cryo-sectioning

20 µm sections were collected in a cryostat (Microme HS, 560) on glass slides (Thermo Scientific, Superfrost plus) and stored at -20ºC until use.

Immunohistochemistry

IHC protocol was standardised by conducting test runs, PBStx and TBStx solutions were used separately to compare their individual efficiency. Slides processed using TBStx solution had superior results, compared to the ones processed using PBStx solution. So TBStx solution was used for further IHC experiments. Prior to Immuno-staining, slides were kept at room temperature for 30 min. Tissue tek was removed by placing the slides in TBStx (0.5% TritonX in TBS) for 10 min. Blocking solution (TBStx + 1% Bovine Serum Albumin (BSA) or Donkey serum) was applied for 1h to reduce unspecific antibody binding. Post blocking, slides were incubated with primary antibody diluted in blocking solution O/N at 4ºC. Unbound antibody was removed by TBStx washing. Slides were incubated with secondary antibody in TBStx for 45 min in dark, at RT. Secondary antibody was removed by TBStx washing. Triton-X was removed by TBS washing. Extra liquid was dabbed off from the slides, and they were mounted with Mowiol. Slides were kept in dark and cold. Fluorescence microscopy was used to view the immuno-staining in these sections.

Image analysis

Immuno-stained NMJ images captured using fluorescence microscope, were analysed using Image-J software. The degree of overlap or co-localization co-efficient of VAChT with VAAT in WT immuno-stained NMJ images was characterized *(27)*.

4. Results

4.1 Synaptic Vesicle Purification, Dopamine Uptake and Western Blot Studies

WB studies were performed to investigate vesicle enrichment of vesicular protein Synaptophysin and non-vesicular protein RPT4. The SV enrichment results are shown in *Fig.2.* Blot indicates that Synaptophysin was present in different amounts from P1 to P4 fraction, while RPT4 was present in almost equal amount from P1 to P3 fraction, while it was present in least amount in final vesicular fraction P4.

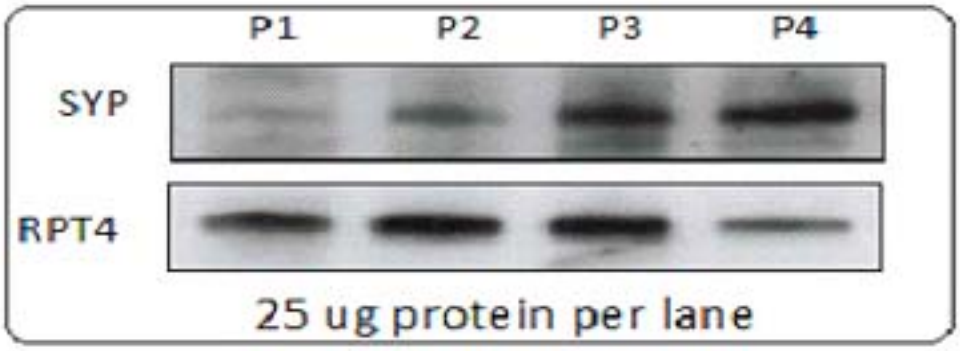

Fig.2. Western blot of Vesicular fractions P1, P2, P3 and P4 for detecting Vesicular protein Synaptophysin (SYP) and Non-vesicular protein RPT4. P1: Brain Lysate samples, P2: Synaptosomal fraction, P3: Large myelin membrane pieces of mitochondria left over intact synaptosomes, P4: Final enriched vesicle fraction containing small synaptic vesicles.

The Presence of VAAT and Synaptophysin was determined (as 72 KDa and 38 KDa proteins) and detected in P4 vesicular precipitate (containing small synaptic vesicles) of VAAT WT, KO and NSE-VAAT mice using WB studies. As shown in *Fig.3,* VAAT was present in WT and NSE-VAAT samples in different amounts, while it was absent in KO sample. Synaptophysin was present in equal amounts in all three mouse genotypes.

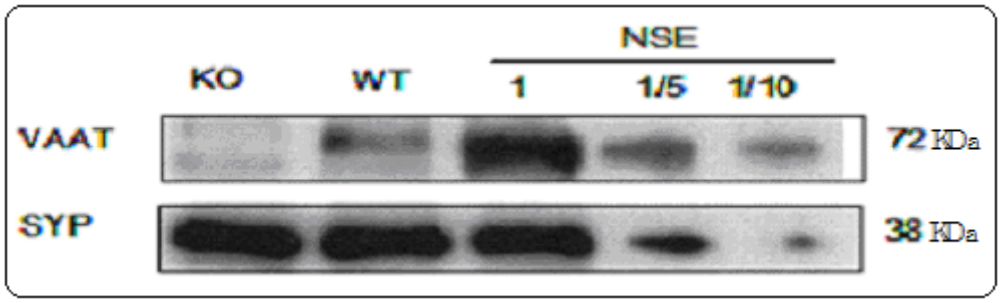

Fig.3. Presence of VAAT (as 72 KDa mol. weight protein) and Synaptophysin (SYP as 38 KDa protein) in protein extracts from Knockout (KO), Wild type (WT), and NSE-VAAT (NSE) mice. For NSE mice, several dilutions (1, 1/5, and 1/10) were applied. Detection of the Proteins were performed by Western Blot using specific antibodies.

DA uptake efficiency in Jahn uptake buffer, Teng's buffer and Kreb's ringer buffer was compared. The results are illustrated in *Fig.4* and *5*.

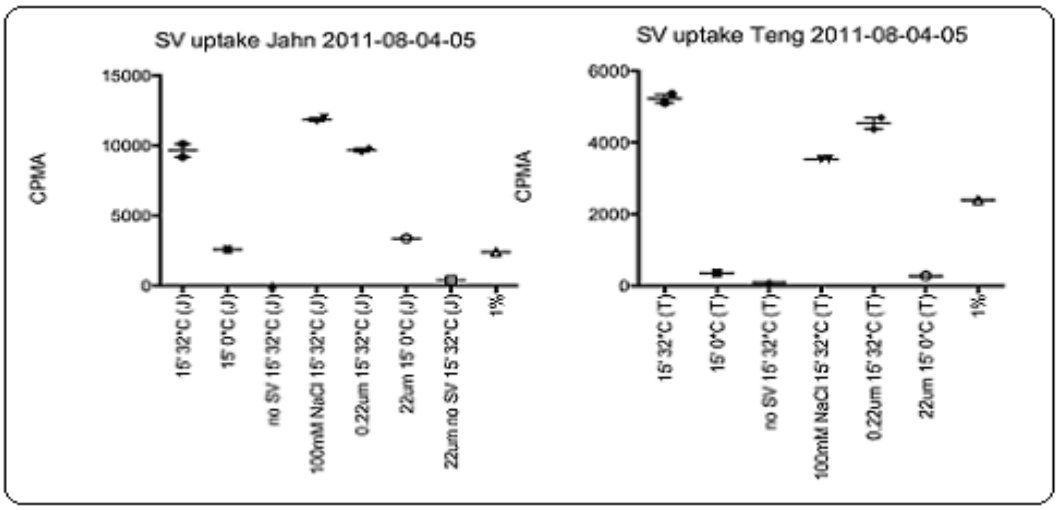

Fig.4. Comparison of DA uptake using (a) Jahn Buffer (b) Teng Buffer in different samples at different time points and temperatures.

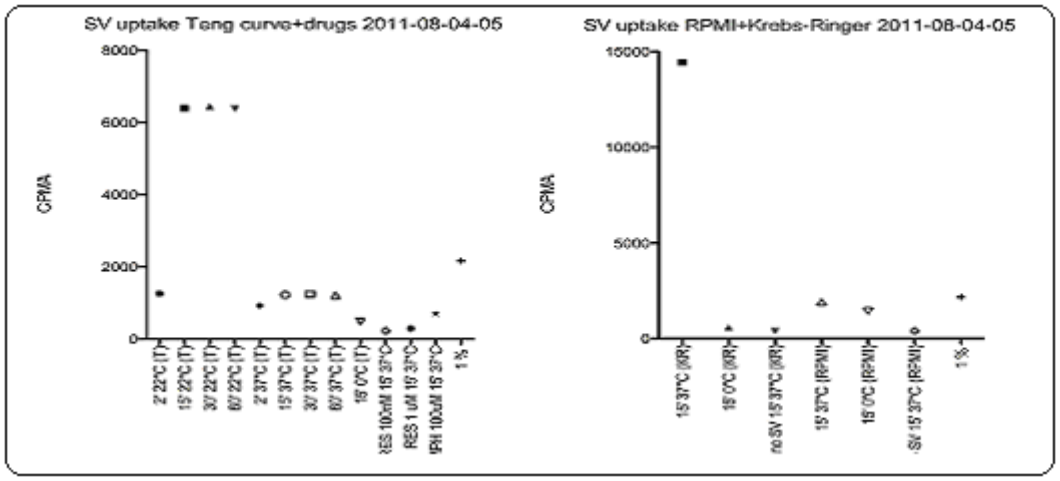

Fig.5. Comparison of DA uptake using (a) Teng Buffer (b) Kreb's ringer Buffer in different samples at different time points and temperatures.

DA uptake in vesicles from NSE-VAAT, WT and KO animals at different time points is illustrated in the form of a graph in *Fig.6*. Non-specific uptake determined in the presence of reserpine was deducted at each time point for all three genotypes. DA uptake was quantified and normalized according to control samples after removal of uptake in the presence of reserpine. Vesicles from VAAT null mice show less DA uptake while vesicles from NSE-VAAT mice show an increased uptake compared to vesicles from WT mice. No significant differences in DA uptake of different genotypes were seen at 8 min, because a saturation level was reached.

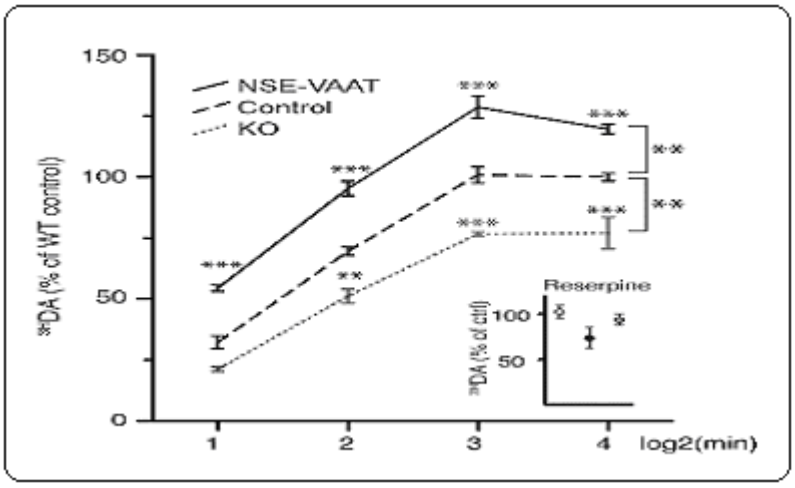

Fig.6. Comparison of DA Uptake by NSE-VAAT, WT and KO samples. Solid line indicates uptake by NSE-VAAT samples, dashed line indicates uptake by WT samples, while dotted line indicates uptake by KO samples. Reserpine plot represents uptake shown by control samples of all three genotypes in the presence of reserpine.

4.2 Transcription studies Results

The relative m-RNA expression levels of transporters and receptors in Hippocampus and Striatum regions were checked in VAAT WT and KO animals. The m-RNA quality and contamination in RNA samples was checked at every step during the experiment. Primer efficiency of different primers was verified by running PCR, and Q-PCR experiments using those primers. The gel picture in *Fig.7* corresponds to samples from different brain regions. In our actual experiment we took 7 WT and 7 KO samples (hippocampus or striatum) and did the same quality checks and gel runs.

RNA prepared by Tri-reagent protocol was quality checked on an agarose gel. Quality of extracted RNA samples can be determined by the appearance of 28S and 18S discrete bands on an agarose gel. Crisp bands are indicative of intact m-RNAs the majority of which are between 2 Kb to 6 Kb in length, whereas smears trailing from 28S or/and 18S bands are indicative of degradation. RNA isolated in our case was intact as shown in *Fig.7*.

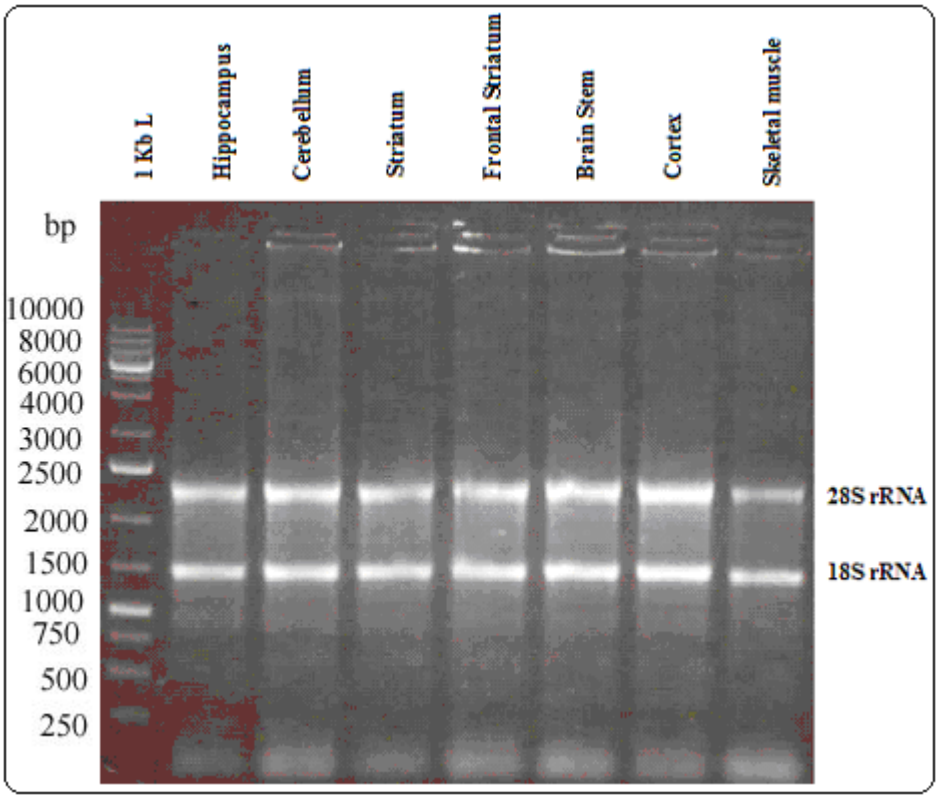

Fig.7. RNA samples isolated from different tissues. The upper band represents the 28S rRNA and the lower band the 18S rRNA. Samples were separated by gel electrophoresis as described in Materials and Methods.

Contamination with genomic DNA is a common problem while isolating RNA. Traces of gDNA can cause skewed quantitation of mRNA by RT-qPCR. RNA samples were used as templates to amplify Tubb5, a housekeeping gene to check gDNA contamination. The quality of the starting template and its integrity was determined in this experiment, by amplifying Tubb5. The target RNA as well as housekeeping gene is co-amplified in the same reaction, eliminating the well-to-well differences that would occur if separate amplification reactions were carried out. From all samples excluding skeletal muscle sample amplification products were obtained (*Fig. 8)*, which indicate contamination in those RNA samples. Genomic DNA from a tail biopsy was used as a positive control.

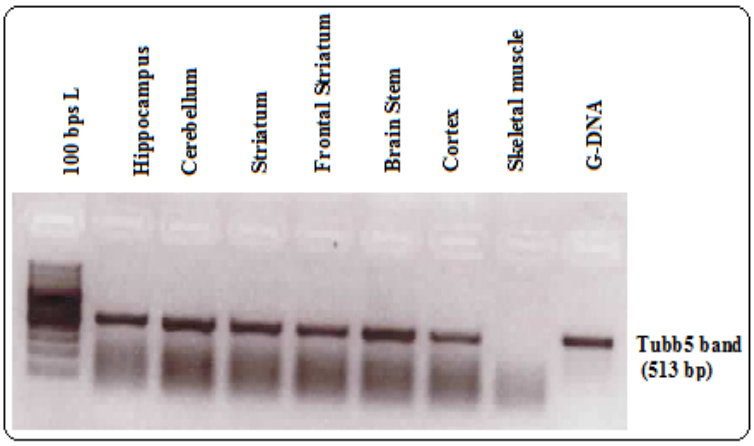

Fig.8. Genomic DNA contamination check in different RNA samples. Presence of gDNA confirmed in the form of band in all samples, except skeletal muscle sample.

To remove genomic DNA contamination from RNA samples, DNase treatment was given to the contaminated samples as described in *section 4.2*. Post-DNase treatment a gel was again run to check the quality of RNA. The gel picture in *Fig.9* shows smears in all the samples. This indicates that total RNA lost integrity during the DNase I treatment process. However, it was suggested that this degradation of total RNA should not create a problem because the amplicon size for the RT-qPCR primer pairs used varied between 80 bps to 200 bps whereas the smear range was between 500 bps to 4000 bps.

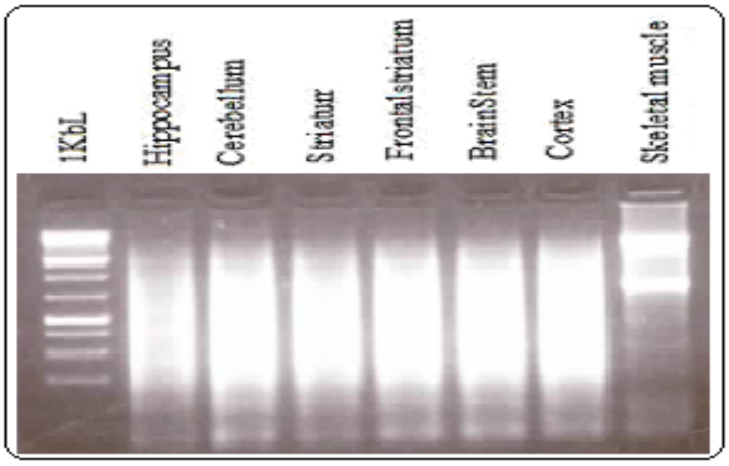

Fig.9. Post DNase treatment, quality of different RNA samples checked; Smears observed on gel.

After DNase treatment m-RNA samples were subjected to DNA contamination check as mentioned above; m-RNA samples were used as a template to amplify from Tubb5 primers and checked on the gel to monitor contamination. Gel picture in *Fig.10* shows no bands in any of the RNA samples, which suggests that there was no contamination in the samples anymore.

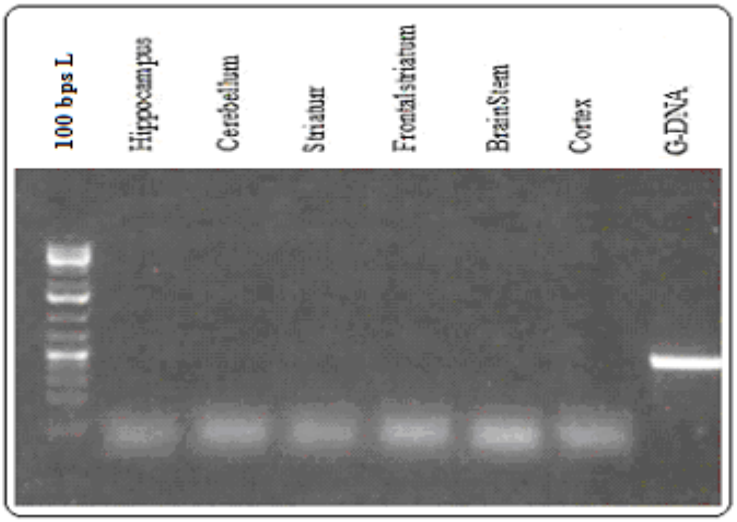

Fig.10. Genomic DNA contamination check of different RNA samples using Tubb5 primers, post DNase treatment. No gDNA bands in RNA samples confirm absence of gDNA after DNase treatment.

All the primer pairs underwent efficiency check by running a PCR using same cDNA sample. Some size differences were observed for different amplicons (*Fig.11).* The amplification was considered to be efficient while no band was observed in the last well where Water sample was run, and primer pairs were used for Q-PCR experiments.

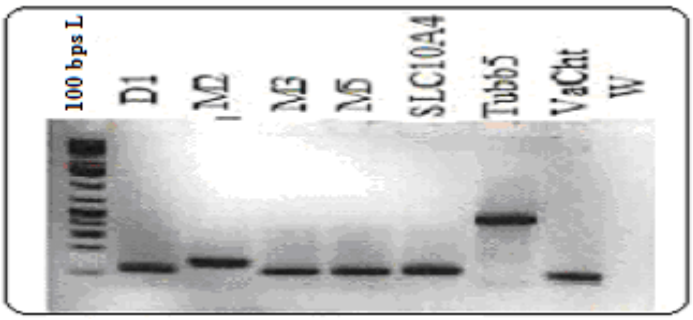

Fig.11. Primer efficiency check of Dopaminergic receptor (D1), Muscarinic receptors (M2, M3, M5), Slc10a4 (VAAT), housekeeping gene Tubb5, and Vesicular acetylcholine transporter (VAChT).

Primers for different muscarinic receptors M1, M2, M3, M4 and M5; nicotinic receptor Chrna7, and dopaminergic transporters D1 and D2; were used to study the m-RNA expression in WT and KO mice. Q-PCR experiments were performed and the data obtained was further analysed using iQ5 software. Relative m-RNA expression levels of different receptors are shown in *Fig. 12*.

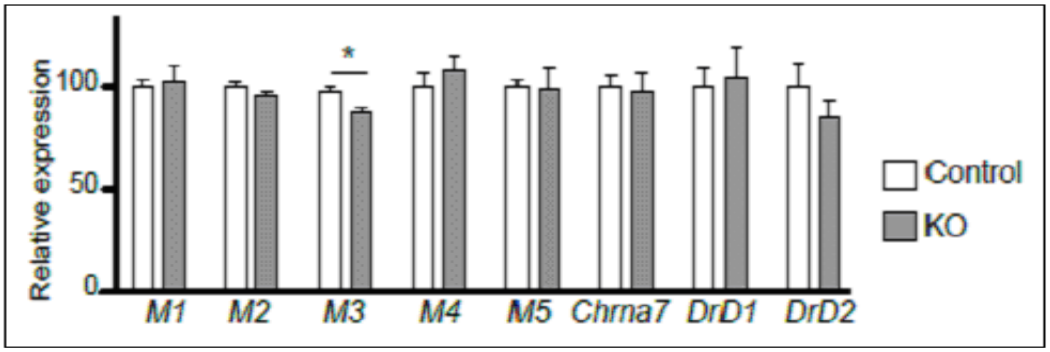

Fig.12. Relative expression levels of Muscarinic (M1, M2, M3, M4, M5), and Nicotinic (Chrna7) receptors in Hippocampus and Dopaminergic (D1 and D2) transporters in striatum region of VAAT WT and KO mice.

4.3 Immunohistochemistry Results

By performing IHC experiments, the extent of distribution of vesicular transporter protein, VAChT was studied in 25 WT and KO NMJ images. Co-localization studies of VAChT with VAAT were done in pure cholinergic synapses and sections were immuno-stained for anti-bungarotoxin (Btx) (Green), anti-VAChT (Golden) and anti-VAAT (Red). Microscopic images of NMJ's at different resolutions (400X, 630X and 200X respectively) were captured. Confocal images representing co-localization in gastrocnemius and diaphragm muscle sections of VAAT WT and KO mice; are illustrated in *Fig. 13, 14 and 15.* After analysis of 25 WT immuno-stained NMJ images using Image-J software, Pearsson's co-localization coefficient of 0.86 was obtained indicating 86% co-localization of VAChT with VAAT in WT NMJ's.

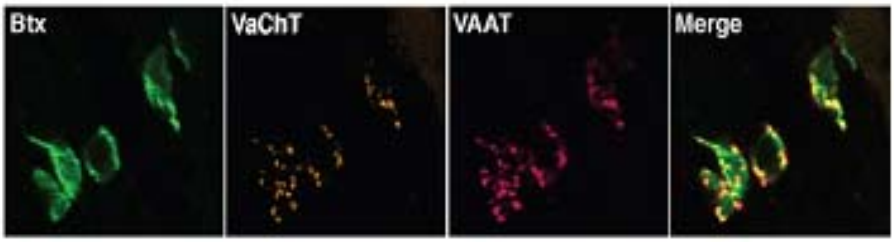

Fig.13. Two day-old gastrocnemius muscle NMJ's (400X). First panel shows anti-Btx stained image, second panel illustrates anti-VaChT staining, third panel shows anti-VAAT stained image, while section in the fourth panel is a merged image of triple stained section, which indicates co-localisation of VaChT with VAAT.

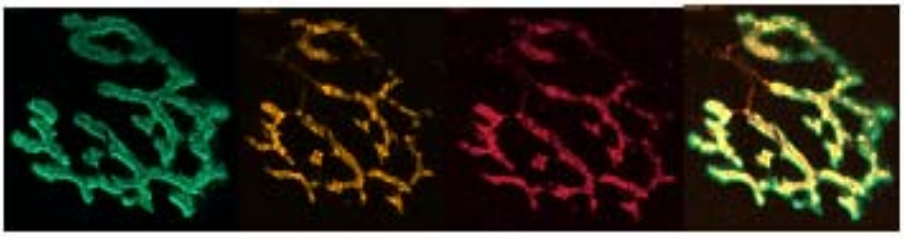

Fig.14. Adult gastrocnemius muscle NMJ's (630X). First panel shows anti-Btx stained image, second panel illustrates anti-VAChT staining, third panel shows anti-VAAT stained image, while section in the fourth panel is a merged image of triple stained section, which indicates co-localisation of VAChT with VAAT.

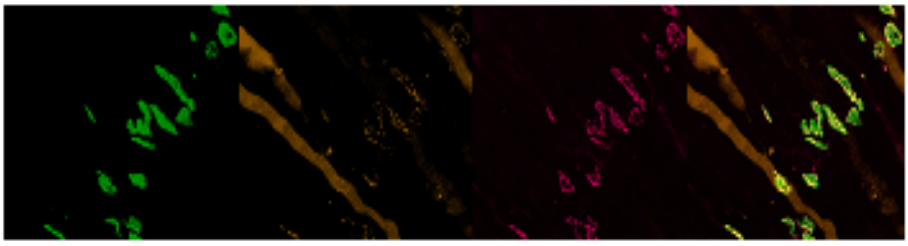

Fig.15. Adult Diaphragm muscle NMJ's (200X). First panel shows anti-Btx stained image, second panel illustrates anti-VAChT staining, third panel shows anti-VAAT stained image, while section in the fourth panel is a merged image of triple stained section, which indicates co-localisation of VAChT with VAAT.

5. Discussion and Conclusion

Vesicular-, RNA- and co-localization studies supported our hypothesis that VAAT is a novel transporter protein present in presynaptic vesicles of monoaminergic neurons, and is an important molecular link between dopaminergic and cholinergic neuronal systems.

Vesicular studies strengthened the assumptions on the role of VAAT in DA homeostasis. These studies revealed that VAAT is present in pre-synaptic vesicles and fractions of pre-synaptic vesicles. WB experiments confirmed the presence of VAAT, a 72 KDa protein in NSE and WT vesicle samples, while as expected, it was absent in VAAT KO samples. The Presence of Synaptophysin, a major vesicular protein was also confirmed in all three genotypes, in almost equal amount. Another WB depicted enrichment of Synaptophysin from P1 to P4 vesicular precipitate fractions. This suggests that the last vesicular precipitate fraction, P4 contains highest vesicular content, which implies that P4 also contains highest amount of VAAT.

Remarkable difference was observed in DA uptake in different mouse genotypes at various time points. The transport of DA into synaptic vesicles was also affected by VAAT, where vesicles lacking VAAT showed a decreased vesicular DA uptake whereas over-expression of VAAT resulted in an increased uptake compared to controls. Thus, highest uptake was found in NSE-VAAT mice followed by WT samples, which show intermediate uptake, and least uptake was observed in VAAT KO samples. No significant difference in uptake was seen for 8min samples. These data suggest that mice lacking VAAT were found to have decreased striatal levels of DA. Thus loss of VAAT leads to less efficient DA vesicle uptake and decreased clearance of exogenously applied DA in vivo. The *in-vivo* functional analyses also suggested that the presence of VAAT enhances the efficiency of DA reuptake and to some degree also affects its release. These effects explain the higher DA concentrations which remain extracellular; and are in concert with the observed hyperactivity in VAAT mutant mice during behavior studies *(4)*.

It was also assumed that disturbed vesicular uptake could possibly occur due to several reasons. Change in pH could be attributed as one of them, to further investigate, Acridine orange experiment was performed by other researchers. Synaptic vesicles isolated from brains of NSE-VAAT displayed greater fluorescent quenching compared to vesicles from WT mice. No significant difference between VAAT null mice and WT were observed. Together these findings indicate that VAAT affects vesicular neurotransmitter filling properties, possibly through a greater ΔpH over the membrane.

It has been observed in earlier electrophysiology studies that cholinergic system has important function in epileptic seizures. It was also observed during some parallel e-phys studies, that absence of VAAT increases the reaction to a cholinergic stimulus *(17)*. But RNA expression studies of muscarinic, nicotinic receptor and dopaminergic receptors; in striatum and hippocampus regions, indicate no significant difference in relative m-RNA expression levels of VAAT KO and WT samples. The immunohistological analysis of gastrocnemius and diaphragm muscle sections, of post-natal and adult mice, provides evidence of co-expression of VAAT with VaCht. Co-localization of VAChT and VAAT was observed in NMJ's; Subsequently Pearsson's co-localization coefficient of VaCht with VAAT was found to be 0.86. Distribution of VaCht in WT and KO NMJ's was studied and significant difference was not observed among them. This justifies the fact that no muscle related impairment or abnormality was observed in VAAT KO animals *(4)*.

With these findings, it is concluded that VAAT has a significant molecular link between different aminergic neural subpopulations. VAAT is a vesicular transporter shared by the cholinergic and dopaminergic systems and adds functionality to dopaminergic neurons through a mechanism involving DA uptake into synaptic vesicles. It demonstrates a novel functional role for this unique transporter shared by aminergic neurons, through a mechanism likely to include the vesicular location of SLC10A4. The localisation of VAAT suggests analogous effects on other aminergic neurons. Immuno analysis suggests that VAAT colocalizes with VAChT in pure cholinergic synapses, at NMJ's. Both monoaminergic and cholinergic neurons are involved in multiple nervous system diseases and the identified functional role of VAAT provides a novel target to modify the activity of dopaminergic neurons, and quite probable, all aminergic neurons. Moreover, the existence of VAAT raises the possibility of a commonly transported, and so far, unidentified molecule, which potentially is co-released with acetylcholine and monoamines. Mental and neurological diseases in humans are often associated with an imbalance between monoaminergic and cholinergic transmitters and further analysis of VAAT may thus offer additional insights into aberrant signalling in these two systems. The presence of VAAT protein was confirmed in mouse brain tissue, both by western blot analysis and in brain tissue sections. Thus, the function of VAAT described here could potentially be preserved in humans, indicating that the discovered role of this transporter is of relevance for normal human behaviour.

6. Future work

Future objective is to study electrical properties, pattern, amplitude and frequency of action potentials of acetylcholine synapses at neuro-muscular junctions, spinal cord and brain. Study acetylcholine uptake efficiency in synaptic vesicles. Perform RNA expression analysis of nicotinic receptors in different regions of brain, in VAAT KO and control samples. And perform detailed co-localization studies of VAChT with VAAT in WT NMJ's.

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9. Appendix

9.1 Abbreviations

1. ASBT - Apical sodium-dependent bile acid transporter
2. ACh - Acetylcholine
3. BCP – Bromo-Chloro-Phenol
4. BL – Brain Lysate
5. BSA – Bovine serum albumin
6. Btx - Bungarotoxin
7. Chat – Choline transporter
8. cDNA – Complementary DNA
9. CNS – Central Nervous System
10. CPG – Central Pattern Generator
11. Cy3 – Cyanine-3
12. DA – Dopamine
13. DAT – Dopamine active transporter
14. DNA – De-oxy Ribonucleic acid
15. DOPAC – 3, 4 Di-hydroxy phenyl acetic acid
16. EM – Electron Microscopy
17. HEPES - (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
18. HPLC – High Performance Liquid Chromatography
19. HVA – Homovanillic acid
20. IHC – Immunohistochemistry
21. ISH – Insitu hybridisation
22. KO – Knock out
23. LD- Loading dye
24. m-RNA – Messenger Ribonucleic acid
25. NTCP - Na+/taurocholate co-transporting polypeptide
26. RNA – Ribonucleic acid
27. NMJ – Neuromuscular junction
28. NSE – Non specific enolase
29. PBS – Phosphate buffered saline
30. PCR – Polymerase Chain Reaction
31. PLA - Proximity Ligation Assays
32. RNA – Ribonucleic acid
33. RPM – Revolutions per minute
34. RT – Room temperature
35. RT-PCR – Real time Polymerase Chain Reaction
36. SDS – Sodium Do-decyl Sulphate
37. SG – Sybr Green
38. SOAT - Sodium dependent organic anion transporter
39. STED – Stimulated emission depletion microscopy
40. SV – Synaptic Vesicle
41. TBS – Tris buffered saline solution
42. TBStx – Tris buffered saline solution with triton-X
43. UUTF - Uppsala University Transgenic Facility
44. VAAT- Vesicular aminergic associated transporter protein
45. VAChT – Vesicular acetylcholine transporter protein
46. VMAT-2 – Vesicular monoaminergic associated transporter protein
47. WB – Western Blot
48. Wt- Wild type

9.2 Reagent Recipes

9.2.1 Genotyping reagents

Tail-Prep Buffer Recipe:
10x Buffer 1:
250 mM NaOH
2 mM EDTA

10x Buffer 2:
400 mM Tris-HCl pH 8.0
Adjust pH 8 by adding HCl

9.2.2 SV Uptake Buffer Recipe

Gluconate or Jan Buffer:
Mops (10mM) – 2.09 g (10ml is added to the 1M solution prepared)
K-Gluconate (100mM) – 23.42 g
MgCl2 (2mM) – 0.4 g
pH 7.3 (adjusted using KOH)

Kreb's Ringer-KCl solution:
KCl 140 mM
$MgCl_2$ 2 mM
$CaCl_2$ 100 µl
HEPES 10 mM
Sucrose 6 mM
Glucose 10 mM
MQ water
Adjust pH to 7.38 with KOH

Bradford reagent:
1 ml. Protein assay kept at 4ºC
4 ml. Milli-Q water

Assay Buffer:
3HDA 8.35 µl
ATP 16.5 µl
Buffer A/B 1630 µl

9.2.3 IHC Solutions

TBS 10X (Concentrated Tris-buffered Saline, 1 liter)
Tris base 24g
NaCl 88g
Dissolve in 900 ml DW
Adjust pH to 7.6 with 12 N HCl
Add DW to make a final volume of 1 liter

TBS 1X
1 part of TBS 10X
9 parts DW
pH 7.6
Final molar concentration of 1X solution is 20 mM Tris and 150 mM NaCl

TBST (Tris-buffered saline with 0.1% Tween-20)
100 ml TBS 10X
900 ml DW
1 ml Tween-20

TBS 0.5% TritonX-100
TritonX-100 500 µl
pH 7.6 to 7.8

Primary antibody diluent
Na-azide 20 µl
TBStx 49.5 ml
1% BSA 0.5 ml

9.2.4 Reagents used in Immuno-staining:

Primary antibodies:
Guinnea Pig-VAChT 1:200
Rabbit-VAAT 1:500

Secondary antibodies:
Goat-Guinnea Pig Cy3 1:500 (Ex 554, Em 566)
Donkey-Rabbit Alexa 647 1:400 (Ex 631, Em 647)
Anti-Btx 1:5000 Alexa 488 (Ex 430, Em 450)

Blocking solution:
2% Na-azide (1:100) 500 µl
5% Donkey serum 2.5 ml
5% Goat serum 2.5 ml
TBStx 44.5 ml

9.3 Tables

Table 1. Primer pairs:

M1	forward GCACAGGCACCCACCAAGCAG reverse AGAGCAGCAGCAGGCGGAACG
M2	forward GGCAAGCAAGAGTAGAATAAA reverse GCCAACAGGATAGCCAAGATT
M3	forward GTCTGGCTTGGGTCATCTCCT reverse GCTGCTGCTGTGGTCTTGGTC
M4	forward AGTGCTTCATCCAGTTCTTGTCCA reverse CACATTCATTGCCTGTCTGCTTTG
M5	forward CTCATCATTGGCATCTTCTCCA reverse GGTCCTTGGTTCGCTTCTCTGT
Chrna7	forward GCAGATCATGGATGTGGATG reverse CAAGACGTTGGTGTGGAATG
D1	forward CAGTCCACGCCAAGAATTGCC reverse ATTGCACTCCTTGGAGATGGAGCC
D2	forward GCAGCCGAGCTTTCAGGGCC reverse GCAGCCGAGCTTTCAGGGCC

Table 2. PCR Program followed for Genotyping:

STEP 1	95ºC	2 min
STEP 2	95ºC	30 sec
STEP 3	62ºC	40 sec
STEP 4	72ºC	40 sec
STEP 5	Go to STEP 2	32 times
STEP 6	72ºC	5 min
STEP 7	4ºC	∞

Table 3. PCR - mix (1x)

Constituents	Final Concentration	Volume (20 µl reaction)
10xBuffer	1x	2 µl
(25 mM) $MgCl_2$	1.5 mM	1.2 µl
(10 µM) Fwd. Primer	0.5 µM	1 µl
(10 µM) Rev. Primer	0.5 µM	1 µl
(10 mM) d-NTP's	0.25 mM	0.5 µl
Enzyme (5 U/µl)	0.05 U/µl	0.2 µl
DNA	100 ng/µl	1 µl
Water		13.10 µl

Table 4. PCR program followed for RNA amplification:

STEP 1	95ºC	3 min
STEP 2	95ºC	30 sec
STEP 3	60ºC	30 sec
STEP 4	72ºC	45 sec
STEP 5	Go to STEP 2	35 times
STEP 6	72ºC	5 min
STEP 7	10ºC	∞

Table 5. PCR – mix (1x)

Constituents	Final concentration	Volume (20 µl reaction)
10x Buffer	1x	2 µl
(25 mM) MgCl2	1.5 mM	1.2 µl
(10 µM) Fwd. Primer	0.2 µM	0.4 µl
(10 µM) Rev. Primer	0.2 µM	0.4 µl
(10 mM) d-NTP's	0.2 mM	0.4 µl
Enzyme (5 U/µl)	0.05 U/µl	0.2 µl
RNA	200 ng/µl	2 µl
Water		13.8 µl

Table 6. DNase treatment protocol:

Constituents	Volume
RNA	4.5 g
10x Buffer	2.5 µl
2 units DNaseI	0.2 µl
Water	17.3 µl

- Mix RNA sample with Buffer and incubate at 75ºC for 10 min. Subsequently add enzyme and incubate the reaction-mix at 37ºC for 2 hrs. Deactivate the reaction by keeping the mix at 75ºC for 15 min. Incubate mix at 37ºC for 20 min. Stop DNase treatment by Trizol extraction.

Table 7. QPCR program

STEP 1	95ºC	3 min
STEP 2	95ºC	3 min
STEP 3	60ºC	20 sec
STEP 4	Repeat 40 cycles	
STEP 5	60ºC	1 min
STEP 6	65ºC	8 sec