THE ROLE OF VGLUT3 IN BEHAVIOUR, WITH A FOCUS ON VGLUT3 POSITIVE NEURONS OF THE MEDIAN RAPHE REGION

PhD thesis in Neuroscience

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Introduction

In the central nervous system (CNS) neurons are classified based on the neurotransmitters they express. To accomplish this, transporter proteins play a pivotal role by encapsulating neurotransmitters within vesicles. Subsequently, these molecules are released into the synaptic cleft. One of the most abundant types of neurons in the CNS is the glutamatergic cells, which exert excitation via the release of glutamate (Glu). Neurons utilising Glu as a neurotransmitter express vesicular glutamate transporter (VGLUTs). Three isoforms of vesicular glutamate transporters (VGLUTs) have been identified: VGLUT1, 2 and 3. While VGLUT3 was identified in 2002, its characterisation, especially its role in behaviour is still being studied.

VGLUT3, compared to the other two isoforms, is less abundant but can be found in key brain areas such as the cortex, striatum, hippocampus (HC), or the raphe nuclei, among many others. Another interesting characteristic of VGLUT3 is its co-expression with other molecules that are traditionally considered as main neurotransmitters, such as acetylcholine in the striatum, ɤ-aminobutyric acid (GABA) in the HC, or serotonin in the raphe nuclei. VGLUT3 and therefore the uploaded Glu not only play a role in the facilitation of vesicular filling of the main neurotransmitter (a process named vesicular synergy), but Glu can also be a main neurotransmitter within the same neuron. By utilising mouse strains with complete gene knockout (KO), it has been shown that VGLUT3 plays a role in hearing, touch sensation, the stress axis and potentially even insulin secretion in the peripheral system. Behaviourally, KO mice show enhanced anxiety-like behaviour and altered locomotor activity compared to their wild type (WT) littermates. However, despite its presence in key brain areas such as the prefrontal cortex (PFC) or HC, not much is known about VGLUT3 contribution to social behaviour, learning, or memory formation.

As previously mentioned, VGLUT3 is expressed in some serotonergic neurons of the raphe nuclei. Moreover, in the midbrain median raphe region (MRR) numerous glutamatergic neurons are purely VGLUT3+ and project to forebrain areas such as the PFC, medial septum or HC. As a serotonergic nucleus, MRR is known for its role in emotional behaviour, especially in anxiety. Previous studies showed that the MRR is involved in social behaviour and releases not only serotonin, but also Glu in the PFC. On the other hand, numerous electrophysiological data suggests that MRR regulates - either

directly or indirectly - HC oscillations such as ripples and theta, which are major contributors to memory formation. However, the behavioural implications of this anatomically and electrophysiologically described connection are not yet fully understood.

While KO strains offer insights into the possible physiological roles of a protein, the lifelong absence of the protein could affect the whole body and potentially influence other neurotransmitter systems as well. Therefore, to investigate this question, it is essential to employ manipulations that are specific to time, brain regions, and contextual factors. With the help of adenoassociated viral vectors (AAVs), exogen gene expression can be induced in *in vivo* animals. In chemogenetics, designer receptors exclusively activated by designer drugs (DREADDs) are artificial receptors, which can be coded in AAVs with a reporter red fluorescent protein (RFP). They manipulate neuronal activity upon binding their artificial ligand, the clozapine-N-oxide (CNO), and induce G-protein coupled pathways (e.g.: G_q and G_i). Alternatively, with optogenetics AAVs encode light sensitive ion channels, which can be activated by implanted laser fibre probes. Contrary to chemogenetics, these channels induce excitation via the influx of cations (Channelrhodopsin). Although the two technics ultimately have the same effect – that is, excite or inhibit neuronal activity-, their temporal resolutions are fundamentally different. While chemogenetics provide a less invasive way to affect neurons, its effect lasts as long as CNO remains in the body. In contrast, optogenetics involves chronic probe implantation, but enables controlled neuronal activity manipulation through programmed laser stimulation. Thus, by harnessing both techniques, more precise and refine results can be attained.

Thanks to advances in technology, mouse strains that express the so called ’causes recombination’ (Cre) protein under a specific promoter are viable for research. By utilising these animals, and double floxing genes in the AAVs, cell type specific expression of the encoded proteins can be achieved. This approach enables us to investigate whether the overall effects found in VGLUT3 KO mice can be attributed specifically to the MRR-VGLUT3+ subpopulation.

Objectives

Our objective was to enhance the existing literature on VGLUT3’s role in social behaviour, learning and memory, with a specific emphasis on the VGLUT3+ neuron

subpopulation of the MRR. To achieve this, we conducted comprehensive behavioural test batteries, cell, and brain area specific manipulations, as well as molecular techniques for validation in both mouse and human models. This led us to the execution of the following projects:

Project 1: Behavioural characterisation of the VGLUT3 KO mice, with a focus on:

a) Social behaviour (*paper under review 1*);
b) Learning and memory (Fazekas et al., 2019).

Project 2: Anatomical study of VGLUT3 in the mouse and human MRR:

a) In mice by fluorescent *in situ* hybridization (sdFISH), with 5-HT co-expression on mRNA level;
b) In human by real time quantitative polymerase chain reaction (RT-qPCR) (*paper under review 1*).

Project 3: Investigation of the role of MRR-VGLUT3 neurons:

a) Validation of chemogenetics:
    1. Confirmation of AAV-transmitted RFP expression in MRR-VGLUT3 cells of VGLUT3-Cre mice (*paper under review 1*);
    2. Chemogenetic-induced neurotransmitter release in HC by microdialysis (Fazekas et al., 2021);
    3. Confirmation of CNO-induced neuronal activation by c-Fos immunohistochemistry (*paper under review 1*);
b) Social behaviour by chemogenetic interventions (*paper under review 1*);
c) Learning and memory formation after chemo- and optogenetic interventions (*paper under review 2*).

Materials and methods

Project 1. Adult male VGLUT3 WT (N=10) and KO mice (N=11) (14-15-week-old, C57BL/6J background) were compared in different behavioural paradigms during their active period. In the first test battery, social behaviours (sociability, social interaction test [SIT], resident intruder test [RIT]) and its possible confounding factors, such as locomotion (open field [OF]) and anxiety-like behaviour (elevated-plus maze [EPM]) were investigated along with exploration-based memory tasks (social discrimination test [SDT], Y-maze). In another set of animals (VGLUT3 WT: N=8; KO: N=8), the known

HC based spatial learning and memory paradigm, the Morris watermaze (MWM) was used. It consisted of a 6-day long learning phase and a 3-day long cognitive flexibility phase, each with a separate test day.

Project 2. C57BL/6NRj mice (4 males and 4 females, 7-week-old) were acquired from Janvier Laboratories (France) were sacrificed and their brains were fresh frozen, then sectioned in cryostat. Fluorescein and digoxigenin conjugated antisense riboprobes were hybridized to VGLUT3 and Tph2 mRNA, respectively, and revealed with Cy2-tyramide and Cy3-tyramide, respectively. After scanning (NanoZoomer 2.0-HT, 20x resolution, Hamamatsu Photonics), VGLUT3+ and VGLUT3+/Tph2+ co-expressing cells throughout the MRR (from Bregma -4.04 mm to -5.02 mm) were manually counted using QuPath software for Windows. Similarly, to show the expression of VGLUT3 on RNA levels, reverse transcription quantitative polymerase chain reaction (RT-qPCR) was done on healthy human brainstem samples which included midbrain and pontine raphe nuclei, lateral parabrachial nucleus, and pontine reticular formation.

Project 3. Validation of chemogenetics was done in three separate ways. In the MRR of C57BL/6J mice AAV (serotype 8) coded RFP (control, N=3) and excitatory DREADD+RFP (N=4) sequences were injected, and four weeks later the neurotransmitter release in a known MRR target area, the HC (AP: -2.4 mm; L: -2.2 mm; DV: 2.5 mm from Bregma), was measured via microdialysis (EICOM CX-I Brain Probe). Samples were collected both before and after intraperitoneal (ip.) CNO injection (1 mg/10 mL/kg solved in saline, Tocris) and were analysed by high-performance liquid chromatography (HPLC) system (Shimadzu LC-20 AD Analytical & Measuring Instruments System, Agilent 1100 Series Variable Wavelength Detector). Cell specific AAV expression was checked in VGLUT3-Cre mice (8-week-old, Jackson Laboratories) after injection of Cre-dependent AAV containing RFP sequences (20 nl; Addgene; #50459) into the MRR (AP: -4.1 mm; L: 0 mm; DV: 4.6 mm from Bregma) and labelled by double fluorescent immunohistochemistry against VGLUT3 and the AAV-coded RFP, evaluated by C2 confocal laser-scanning microscope (Nikon Europe; 20× objective). Finally, excitatory DREADD (N=3) functionality was checked by c-Fos immunohistochemistry in control (N=6) after 30 mins of ip. CNO injection + 90 mins c-

Fos expression waiting time, scanned by Pannoramic MIDI II Slidescanner (3DHISTECH, Budapest, Hungary)

Cell and brain site specific manipulation during behaviour was done by the previously validated chemogenetic techniques. VGLUT3-Cre male mice were injected with double floxed AAVs encoding control (N=8), excitatory (N=13) or inhibitory (N=15) DREADD sequences. After recovery, the same behavioural paradigms as described in Project 1 were conducted, except for the MWM, where a more sensitive protocol was employed. All animals were ip. injected with CNO 30 minutes before the start of the experiment. Viral infection and expression were later validated with immunohistochemistry. Regarding the MWM, the study was conducted through a collaborative French-Hungarian PhD program, aiming to reproduce, compare and complement experiments. Thus, the chemogenetic experiments were done in France (control N=6♀7♂; excitatory N=6♀6♂), with a replication in Hungary (control N=7♀7♂; excitatory N=8♀5♂), whereas the optogenetic experiments were done in Hungary (control N=5♀6♂; excitatory N=6♀7♂). MWM was divided into three phases: cue task (3 days), spatial reference memory (5 days, daily excitation) and memory probes (10 mins and 72 hours). The procedures and experimental protocols were identical in both countries.

For the statistical analysis StatSoft 13.4 (Tulsa, USA) and GraphPad Prism version 8.0.0 for Windows softwares were used. Data were expressed as mean±standard error of mean (SEM) and $p<0.05$ was considered statistically significant, while $0.1<p<0.05$ was accepted as a marginal difference.

All tests were approved by the local committees of animal health and care (France: Ministère de l'Agriculture et de la Forêt, Service Vétérinaire de la Santé et de la Protection Animale, authorization #01482.01 from ethics committee Darwin #5; Hungary: Animal Welfare Committee of the Institute of Experimental Medicine, Budapest, Hungary and Government Office for Pest County, PEI/001/33-4/2013, PE/EA/254-7/2019) and performed according to the European Communities Council Directive recommendations for the care and use of laboratory animals (2010/63/EU).

The study was approved by the Hungarian Medical Research Council – Scientific and Research Ethical Committee (Egészségügyi Tudományos Tanács – Tudományos és Kutatásetikai Bizottság, #40197-2/2019/EKU), in accordance with the Ethical Rules for

Using Human Tissues for Medical Research in Hungary (HM 34/1999) and the Code of Ethics of the World Medical Association (Declaration of Helsinki). *Post-mortem* brain samples were obtained from the Human Brain Tissue Bank, Semmelweis University (Budapest, Hungary).

Results

Project 1: **Social behaviour, learning and memory formation of VGLUT3 KO mice.** As potential confounding factors, first locomotion and anxiety-like behaviour were also investigated. OF confirmed the circadian rhythm dependent locomotor activity differences in VGLUT3 KO mice: as opposed to our previously experienced hypolocomotion during inactive period under light, animals during their active period, in dark apparatus showed no differences in locomotion compared to WT littermates. However, the elevated baseline anxiety-like behaviour was still detectable, as KO mice spent less time in the centrum of the OF, in the open arm of EPM, and showed less risk assessment behaviour. In sociability test, VGLUT3 KO mice showed increased interest towards the social stimulus. However, this proved to be anxiety dependent, since in the anxiogenic SIT inadequate aggression appeared, while in RIT they behaved normally, as also indicated by literature. SDT 24-hour later showed once again an increase in social interest in VGLUT3 KO mice, but also a strong preference for the old, already familiar conspecific. Interestingly, short-term working memory in Y-maze did not show impairment, as opposed to our previous results. Hippocampus based learning and memory in MWM showed no major deficits, only a lack of quadrant preference during cognitive flexibility phase, test day in VGLUT3 KO mice.

Project 2: Confirmation of the presence of VGLUT3 in the MRR. On the mRNA level, we counted that 19.329%±1.641% of MRR neurons were co-expressing the serotonergic Tph2 marker (95±8571 VGLUT3+/Tph2+ co-positive cells out of 496.875±28.934 VGLUT3+ cells). Moreover, healthy human brainstem samples showed detectable VGLUT3 mRNA levels not only in the midbrain raphe nuclei and pontine raphe nucleus, but also in the lateral parabrachial nucleus and pontine reticular formation (RT-qPCR).

Project 3: Validatation of chemogenetics. Microdialysis measurements showed that after the chemogenetic excitation of the whole MRR, there is a transient increase in

the extracellular Glu levels in the HC, which is a major projection area for the MRR and its VGLUT3+ neurons. Specifically targeting of VGLUT3+ neurons in the MRR were done in VGLUT3-Cre mice that expressed AAVs encoding target genes double floxed. The reporter protein, RFP, was expressed exclusively in VGLUT3+ neurons, and the ip. administration of the synthetic ligand of excitatory DREADD, CNO, induced c-Fos expression.

Effects of MRR-VGLUT3+ neurons on behaviour. Chemogenetic inhibition marginally decreased distance moved only in the OF, while increased social interest only 24 hours after manipulation. Interestingly, it had a context specific effect on friendly social behaviour: in SIT it marginally increased, while in RIT it decreased friendly social behaviour. Conversely, chemogenetic excitation had an anxiolytic effect in the EPM. However, these manipulations did not affect social or short-term working memories.
While excitation of the MRR-VGLUT3+ neurons did not affect learning in MWM, extended manipulation through chemogenetics led to an increased performance only in the long-term memory probe. This result was confirmed in the two laboratories, on two distinct VGLUT3-Cre mouse strains. However, short-term excitation through optogenetics did not affect either the learning curve or the spatial memory.

Conclusions

Altogether, these projects successfully expanded the literature on the role of VGLUT3+ neurons in behaviour. Moreover, human relevance of these studies was confirmed by the RT-qPCR detection of VGLUT3 mRNA in human raphe nuclei. Although direct parallels were not found between the VGLUT3 KO mice and MRR-VGLUT3 manipulated animals, it remains conceivable that the reduced activity of MRR-VGLUT3 neurons could contribute, at least in part, to the observed hypolocomotion and increased social interest in VGLUT3 KO mice. Conversely, the highly anxious behavioural phenotype could be due to the lack of activity from the MRR in KO mice, as excitation of VGLUT3+ neurons was anxiolytic. However, the role of VGLUT3 in learning and memory formation is much more complex: while VGLUT3 KO mice showed only long-term social memory deficits and minor spatial memory impairments, the excitation of MRR-VGLUT3+ neurons had a long-term improvement effect in spatial

memory formation. Nonetheless, the ineffectiveness of optogenetic manipulations could indicate that this effect probably takes place during the dormant period(s) of the animals.

In summary, this PhD thesis presents evidence on the role VGLUT3 positive neurons in social behaviour and cognitive processes. Moreover, by focusing on a specific subpopulation, we also showed that MRR -VGLUT3 neurons could potentially regulate numerous behaviours, with a profound effect on hippocampus dependent functions.

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