VGLUT3+ Neurons in Hippocampal Activity and Behaviour

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Neurons using glutamate as a neurotransmitter can be characterised by vesicular glutamate transporters (VGLUTs). Among the three subtypes, VGLUT3 is often co-localise with other “classical” neurotransmitters and can modulate their release. Its contribution to sensory processes (including seeing, hearing, and mechanosensation) is well characterised. However, its involvement in learning and memory can only be assumed based on its prominent hippocampal presence. Beside local VGLUT3 positive network hippocampus gets innervation from the median raphe. This hippocampal glutamatergic network plays a pivotal role in several important processes (e.g., learning and memory, emotions, epilepsy, cardiovascular regulation).

vesicular glutamate transporter hippocampus sensory processes learning and memory emotions

1. Introduction

In the central nervous system (CNS), neurons are classified based on the neurotransmitters they express. While Dale’s principle originally stated that one neuron utilises one neurotransmitter, researchers now know that a cell can express multiple different molecules to communicate [1]. However, even today, it is still regarded such that neurons have one main “classical” neurotransmitter type (e.g., excitatory glutamate (Glu) or inhibitory gamma aminobutyric acid (GABA)) and express numerous other secondary ones, mainly peptides. As these “classical” neurotransmitters are small molecules, they are often intermediates of the metabolism and thus detectable in all cells. Therefore, neuron classification is based mainly on the transporter proteins that pack the neurotransmitters into vesicles, from which the molecules are later released into the synaptic cleft [2]. One of the most abundant types of neurons in the CNS is the glutamatergic cells, which exert excitation in most cases via the release of Glu. Two distinct protein families transport Glu through membranes: the excitatory amino acid transporters (EAATs) and the vesicular glutamate transporters (VGLUTs). EAATs, being responsible for the termination of the synaptic signal, can be found in the plasma membrane of pre- and postsynaptic neurons, as well as in glial cells, and thus cannot be used to characterise glutamatergic neurons [3].

On the contrary, VGLUTs are expressed on neuronal synaptic vesicles’ membrane and thought to be characteristic to neurons only. They belong to the solute carrier family 17, which is a sodium-dependent phosphate transporter family. To maintain balance of charge, pH, and ions, glutamatergic synaptic vesicle membranes contain V-ATPases (proton pumps) as well, which establish acidic pH inside the vesicles. VGLUTs themselves carry not only Glu in its

anionic form but also require Cl^- and a cation (preferably H^+ or K^+) to work [4][5][6]. According to Preobraschenski's model, in the first conformation state, VGLUTs bind a Glu^- and a K^+ molecule from the lumen, while a Cl^- ion is constantly bound due to the high affinity [4]. After changing conformation, in the second state, the transporter lets go of the Glu^- and K^+ inside and instead gains high affinity to Cl^- and H^+, which are transported to the cytosol to restart the cycle.

2. Characterisation of VGLUT3

2.1. Anatomical Distribution of VGLUT3 in the Central Nervous System

The DNA sequence of VGLUT3 is over 70% identical to the other isoforms, and it utilises the same molecular mechanism to load vesicles with Glu [5][6][7][8][9]. Moreover, its presence is enough to induce the glutamatergic phenotype, as Glu release was detected in GABAergic striatal primary cultures infected with VGLUT3-expressing lentivirus. After 14 days, Glu release-induced EPSCs were detected in the infected cells, whereas no activation was observed in the control GABAergic cells [7].

However, VGLUT3 also shows numerous distinctive characteristics. Firstly, its anatomical distribution is unique: while VGLUT1 and 2 show complementary localisation, VGLUT3 appears intermingled with other transporters, appearing mainly, but not exclusively in subcortical structures. On the mRNA level, it has been shown in neurons of the cortex (layers II, III, and VI), caude putamen, amygdala, hippocampus, hypothalamus, nucleus accumbens, habenula, bed nucleus of stria terminalis (BNST), striatum, ventral tegmental area (VTA), substantia nigra pars compacta, and midbrain raphe nuclei [5][6][10][11][8][12][13][14][15][16], with controversial results in the cerebellum (in the granular layer, molecular layer, Purkinje cells reported in [5], but not found by others [10][8]).

Immunohistochemistry on protein level strengthened the mRNA findings: cortical neurons indeed express VGLUT3 alongside the mRNA [14]. Inhibitory interneurons and pyramidal cells expressing VGLUT3 proteins are also present in layers II and III of the cortex as well as boutons, representing VGLUT3+ synapses in layers II, III, V, and VI [17]. In the hippocampus, pyramidal cell bodies and their dendrites are innervated by VGLUT3+ synapses, while the stratum radiatum somas were also VGLUT3-positive [5][6][10][8]. Similar results were shown in the neurons of olfactory bulb, caudoputamen, nucleus accumbens, striatum, hypothalamus, VTA, substantia nigra pars compacta, and raphe nuclei [5][6][11][8][18][19][20][21]. Moreover, VGLUT3 is not exclusively expressed in the nerve terminals or cell bodies but can also be found in dendrites [5][11]. Interestingly, astrocytes [5][22] and ependymal cell [11][8] were also VGLUT3 positive; however, in situ hybridization did not confirm this on the mRNA level [6][13][23].

VGLUT3 is also detectable in the spinal cord. Numerous VGLUT3+ axon terminals can be found in its intermediolateral cell column, where they form both excitatory (asymmetric) and inhibitory (symmetric) synapses, putatively having a role in thermoregulation [16][24][25]. The retrotrapezoid nucleus, responsible for chemoreception, is also innervated by VGLUT3+ projections [26]. However, VGLUT3 mRNA-positive somas were not detected in the spinal cord [25]. Interestingly, in rat, pulpal blood flow was regulated by VGLUT3+ nerve terminals [27], suggesting the possibility of an even more peripheral projection. Moreover, VGLUT3 immunoreactivity was detected in the

heart, liver, and kidney but not in intestinal or lung tissue [28]. However, a specific VGLUT3 isoform is characteristic to the CNS.

2.2. Glutamate as a Secondary Neurotransmitter in VGLUT3+ Neurons

Another interesting characteristic of the VGLUT3 is the fact it is co-expressed with other molecules that are considered as traditional main neurotransmitters. Controversially, less is known about VGLUT3 co-expression with non-classical, peptide neurotransmitters.

VGLUT3 is often found in symmetric, thus, inhibitory nerve terminals, especially in the hippocampus and the cortex [5][8][14][15][29]. A small portion of cortical GABAergic interneurons that are projecting locally are VGLUT3 positive, and they also co-express neurokinin B and cholecystokinin (CCK) markers. These neurons form basket-like arborisations around other, putatively neurokinin B positive interneurons [14]. In the hippocampus, glutamate decarboxylase positive (GAD+), GABAergic neurons also express VGLUT3, indicating that inhibitory interneurons also release Glu [5][29][30][31].

Around ≈7% of the GABAergic neurons in the BNST are positive for VGLUT3 mRNA, and part of them project to the VTA [15][32]. In the basal nucleus of the amygdala, a subset of CCK+ GABAergic interneurons also express VGLUT3, along with cannabinoid receptor type 1 (CB_1R) in their axon terminals [21][33]. Interestingly, these neurons show little electrophysiological and no morphological differences compared to their calbindin positive counterparts [21], but they form an interesting invagination type of synapse into the cell bodies of pyramidal neurons [33].

In the striatum, virtually all cholinergic cells co-express the vesicular acetylcholine transporter (VAChT) and VGLUT3 [5][6][8]. In the basal forebrain (horizontal diagonal band of Broca), cholinergic neurons also co-express VGLUT3, however, in a more restricted way [12][29][34][35]. Some of these cells project to the internal plexiform layer of the main olfactory bulb, although electrophysiological measurements showed that postsynaptic currents are derived from nicotinic and GABAergic activation rather than glutamatergic [34]. Other cells from the basal forebrain project to the basolateral amygdala and express both choline acetyltransferase (ChAT) and VGLUT3 [35]. Interestingly, in the amygdala, some CCK and CB_1R-positive interneurons also express VGLUT3 [21]. In the striatum, VGLUT3 plays a crucial role in the vesicular loading of ACh [36][37] and excites local fast-spiking interneurons via both α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and N-methyl-D-aspartate (NMDA) (both are ionotropic glutamatergic) receptors. It is thought that this co-release of Glu and ACh plays a role in the regulation of locomotor activity [38]. Similar results were found in basal forebrain nuclei such as the medial septum, diagonal bands, and nucleus basalis [12].

Midbrain raphe nuclei are mostly known for their 5-HT content, which is marked by serotonin transporters (SERT). Interestingly, in these cell groups, SERT+ and VGLUT3+ markers are often co-expressed, but they can also be found separately [5][8][13][14][39][40][41][42][43][44]. Terminals originating from serotonergic neurons often co-express VGLUT3 in the cortex, especially in layers II/III [14]. The source of these terminals is mainly in the dorsal raphe (DR) [13][41][43][44][45][46], which also projects to the striatum [43]. Interestingly, these axons form varicosities that are

morphologically larger than others [43]. Other projections in the VTA and nucleus accumbens play a role in reward signalling [46][47][48]. In VTA, both 5-HT and Glu originating from VGLUT3+ axon terminals are released, contributing to social stress susceptibility: their inhibition facilitated social avoidance after subthreshold social defeat stress [47]. However, it is unknown whether the VGLUT3+ subpopulation plays a role in this. In another study, it was shown that the VGLUT3+/5-HT+ DR projections to VTA dopaminergic neurons were excitatory and induced DA release in the nucleus accumbens, positively driving conditioned place preference [48]. Similarly, 5-HT and VGLUT3 co-localisation was detected in the DR-basal amygdala projections, possibly contributing to fear memory [49]. In the basal amygdala, the axon terminals either release 5-HT or Glu based on the frequency of firing [50]. Other than these areas, DR VGLUT3+ neurons also project to the substantia nigra pars compacta, different thalamic and hypothalamic nuclei, where they do not necessarily co-express 5-HT, and their somatas are mainly located in the shell region of the DR [13]. Additionally, there is a subset of VGLUT3+ cells in the superior colliculus that also project to substantia nigra pars compacta and form asymmetric and thus excitatory synapses on local dopaminergic neurons [51].

In another known serotonergic nucleus, the median raphe (MR), Glu released from VGLUT3+ vesicles can be the main neurotransmitter, but it can also be found in serotonergic as well as—in small percentage—in GABAergic neurons [13][17][52]. Interestingly, primary raphe cell cultures from VGLUT3 KO mice show vulnerability and are less likely to survive in vitro compared to cells isolated from wild-type animals [40]. However, there seems to be topological heterogeneity in the neurochemical characteristics of differently projecting serotonergic and VGLUT3+ axon varicosities. For example, in the cortex, hippocampus, nucleus accumbens, and striatum, most varicosities expressed both SERT and VGLUT3 markers [43][53]. On the other hand, Voisin et al. [40] showed the opposite results: in the septum, striatum, and hippocampus, these two markers were barely co-expressed in the same varicosities. Similarly, in the hippocampus, second rhombomere (R2)-derived, Pet1+ (transcription factor known to represent serotonergic cells [54]) boutons were mostly VGLUT3+ but not 5-HT+ [55]. However, serotonergic neurons originating from other rhombomeres co-expressed VGLUT3+ and 5-HT in their terminals. As of now, it is unknown whether this is a technical difference (antibody, different animal strains) or physiologically important observation related to functionality. It is important to note that while this segregation (i.e., 5-HT+, GLUT3+, or co-expressed) in MR-hippocampus projections was confirmed [56], it was also highlighted that VGLUT3 may be co-expressed in vesicular monoamine transporter 2 positive (VMAT2+) and 5-HT+ terminals even if they were negative for the SERT marker. As of now, it is believed that this co-expression facilitates the vesicular filling of the main neurotransmitter (so-called vesicular synergy) [36][56][57]. However, in the case of GABAergic co-expression, both pro [58] and contra [59][60] arguments have been published, leaving the question open. A most probable explanation is that the same projection may have different subtypes based upon their co-expression profile, and different authors found different populations in their samples by chance. The co-expression of “classical” neurotransmitters may be further coloured by an array of peptide co-transmitters [61][62].

Similar to the midbrain raphe nuclei, VGLUT3 can be found alone or co-expressed in a subset of putatively GABAergic and/or aminergic cells in the medullary raphe nuclei, such as the raphe pallidus, raphe magnus, raphe obscurus, and parapyramidal area. They send projections to the spinal cord (see earlier) [16][24]. It has also been

suggested that VGLUT3 is also co-expressed with VGLUT1 and 2, but this seems to be brain area [9] and species specific (might be different even between rats and mice) [9][63].

Interestingly, VGLUT3 has the ability to signal retrogradely [5][64]. Crepel et al. showed that cerebellar principle cells utilise Glu released from VGLUT3 containing vesicles to retrogradely signal and regulate incoming signals. In the cortex, VGLUT3 is also present in the dendrites of layer II principal cells and may negatively control the input from local interneurons [18].

2.3. Electrophysiological Characteristics of VGLUT3

Lentiviruses containing the sequences of the three VGLUT isoforms were used in primary autopathic cultures from VGLUT1 KO hippocampal and VGLUT2 KO thalamic tissue for direct comparison. All three types of VGLUT expression rescued the deficit in EPSC peaks and charges and showed no significant differences from hippocampal VGLUT1 wild-type (WT) neurons or from each other. Thus, it was concluded that all 3 isoforms perform the basic function in a similar manner. However, compared to WT VGLUT1+ and lentiviral-rescued VGLUT1 cells, VGLUT2 and VGLUT3-expressing neurons showed significantly greater release probability indicated by increased paired-pulse depression [7].

Table 1 shows some representative VGLUT3+ neuron populations in comparison to general GABAergic interneurons and their electrophysiological characteristics. Even though they are located anatomically differently, their major characteristics do not vary in great length.

Table 1. Electrophysiological characteristics of different VGLUT3 containing and non-containing interneurons in the central nervous system.

	GABAergic Interneurons in the Cortex	GABAergic Interneurons in the Hippocampus	VGLUT3+ Interneurons in the Amygdala	VGLUT3+ Interneurons in the Hippocampus
Resting membrane potential	−57.48–−49.40 mV	NA	NA	−59.00–−56.90 mV
Input resistance	219.77–419.61 MΩ	107.89 MΩ	168.10 MΩ	149.70–158.50 MΩ
Action potential threshold	−32.67–−27.82 mV	−42.81 mV	−38.80 mV	−41.90–−39.86 mV
Action potential amplitude	71.30–86.11 mV	74.27 mV	71.60 mV	55.70–57.40 mV
Firing frequency	19.34–52.48 Hz (2×)	15.00 Hz (steady trace)	31.50 Hz (2×)	31.30–34.90 Hz (2×)
Amplitude of after-hyperpolarisation	8.60–17.63 mV	12.68 mV (new method)	14.70 mV	−11.80–−10.30 mV

	GABAergic Interneurons in the Cortex	GABAergic Interneurons in the Hippocampus	VGLUT3+ Interneurons in the Amygdala	VGLUT3+ Interneurons in the Hippocampus
Co-transmitters	CCK	CCK	CCK, GABA	CCK, GABA
Reference	[65], all subtypes displayed	[66]	[21]	[59], both subtypes displayed

The expression of VGLUT3 does not change the main properties of the interneurons. For detailed information and results, please refer to each original research article. CCK: cholecystokinin; NA: not available; VGLUT3: vesicular glutamate transporter type 3.

3. Implications of VGLUT3 in Physiology

As a recently discovered protein, the exact role of VGLUT3 is not completely understood. Scarce information are available both from the CNS and the periphery.

First of all, VGLUT3 is highly implicated in sensory processes. In the retina, VGLUT3 plays a crucial role in the physiology of a new subtype of putatively excitatory amacrine cells [67][68][69]. VGLUT3 is also expressed in the inner hair cells and spiral ganglion cells of the cochlea [59][70] as well as in the inhibitory sound localisation pathway [71]. They are needed for auditory coding, as without properly functioning proteins, the stereocilary bundle structure and synaptic organisation was lost [72]. As a result, VGLUT3 KO mice are deaf [70][73][74]. VGLUT3-mediated glutamatergic neurotransmission is also responsible for noise-induced threshold shifts [75]. Lastly, low threshold mechanosensitive cells in the hairy skin that project to the dorsal horn of the spinal cord with C-type fibres also utilise VGLUT3 to signal pleasant touch information [63][76][77]. However, unpleasant sensory information of an electric foot-shock was not influenced by the lack of VGLUT3 in KO mice [78].

The participation of VGLUT3 in endocrine regulation was confirmed by several studies. Firstly, glutamate may influence the stress response at several points [79], and VGLUT3 might be especially involved in catecholaminergic regulation, as around 25% of the chromaffin cells in the adrenal medulla also express VGLUT3 [80]. The expression of the hypothalamic regulator of the so-called stress axis (hypothalamic–pituitary adrenocortical axis, HPA), the corticotropin-releasing hormone (CRH) was increased in VGLUT3 KO mice and the stressor-induced corticosterone (end-hormone of the HPA axis) elevation was higher in them compared to their WT littermates [78][79][81]. However, researchers might assume that the role of VGLUT3 might be on remote brain areas influencing the perception/interpretation of the stressor, rather than a direct effect on the HPA axis as its presence (both in the somas as well as in afferent fibres) was not confirmed so far on its elements. Moreover, an array of stressors decreases the number of VGLUT3+ neurons in the DR, where inputs from the central amygdala might play a significant role [82].

As for the endocrine regulation, VGLUT3 may play an important role in insulin secretion directly in the ß-cells of the Langerhans islets of the pancreas [83].

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4. Characteristics of the VGLUT3 KO Mice

Among the three subtypes, VGLUT1 and 2 seem to be utmost important as their lack is fatal: VGLUT1 KO mice die around weaning, while VGLUT2 KO mice die at birth [90][91][92][93].

VGLUT1 KO animals have progressive neurological symptoms, including blindness and incoordination, supporting its role from an early stage of development [94]. At birth, VGLUT1 KO and WT animals are indistinguishable. However, after birth, VGLUT1 expression increases, accounting for 50% of Glu transmission in 3–5-day-old wild-type mice. In VGLUT1 KO animals during the following 2 weeks, a sharp decrease in residual activity was detected, and in mice older than 2 months, the excitatory transmission was essentially eliminated, leading to death.

VGLUT2 KO mice die due to a complete loss of stable autonomous respiratory rhythm, which is generated by the pre-Bötzinger complex [95]. Similar to VGLUT1-KO, the heterozygous VGLUT2 +/− mice are not obviously different from their WT littermates, despite expressing 50% less VGLUT2 protein [93]. However, various behavioural tests presented well that the partial loss of VGLUT2 significantly affected thalamic function. Among other things, conditioned taste aversion and defensive marble burying were impaired, while motor function, learning and memory, acute nociception, and inflammatory pain remained intact. The same study reported a nearly 50% reduction in the amplitude of spontaneous release events in VGLUT1 null hippocampal and VGLUT2 null thalamic cell cultures [93].

Contrary to the other two isoforms, both heterozygous and homozygous VGLUT3 KO mice are viable and reach adulthood without any need for intervention [79]. It is logical to assume that the elevation of VGLUT1 and 2 compensate the absence of VGLUT3 in the KO mice; however, no VGLUT1 and 2 alterations (both at the mRNA and protein level) were found in their brain [36]. Moreover, in adult VGLUT3 KO mice, the expression of other biochemical markers related to cholinergic, dopaminergic, GABAergic, or neuropeptidergic (substance P, enkephalin) regulation were equal to the WT.

VGLUT3 KO mice show no major macroscopic anatomical discrepancies in the brain compared to their WT littermates [19]. Although in vitro raphe primary cell cultures that lack VGLUT3 are less likely to survive [40], in vivo VGLUT3 KO mice do not show reduced serotonergic cell number in their midbrain raphe nuclei. However, in the

striatum and dorsal hippocampus of KO mice, the number of serotoninergic varicosities was decreased, while in the ventral hippocampus it was increased [40]. On a molecular level, VGLUT3 KO mice show decreased 5-HT [19][56] and ACh turnover [36].

Since the main VGLUT isoform expressed in the striatum, an area that has an important role in the regulation of movement, is VGLUT3, locomotor alteration in VGLUT3 KO mice could be supposed. However, their motor coordination is normal [79]. Interestingly, during short observations (e.g., 5–10 min open field), a reduced locomotion was detected [78][79], while during more prolonged observations (up to 5 h), even a hyperlocomotion was observed [36], especially during the dark, active phase [96]. The reduced locomotion seems to be due to enhanced anxiety [56][78], leading to a more cautious behaviour in a new environment, while the hyperlocomotion was connected to their altered DA levels [36][96], suggesting its possible role in Parkinson disease.

In line with altered HPA axis activity mentioned earlier [78][79], VGLUT3 KO mice were repeatedly reported to be more anxious [56][78][81][97]. This anxiety is innate and can be detected already during the early postnatal period (in 8-day-old mice) by enhanced maternal separation-induced ultrasound vocalisation (at 40–90 kHz) [56][81]. Furthermore, increased anxiety-like behaviour is still detectable in adult mice on numerous behavioural tests such as the elevated plus maze [78][97], or in marble burying, and novelty suppressed feeding paradigms [56].

In line with the described hyperlocomotion, sensitivity to pharmacological treatments was also altered in the VGLUT3 KO mice [36][96][97][98][99]. Cocaine-induced locomotor activity was exaggerated in them [36][97], and their L-DOPA-induced dyskinesia was reduced [96][99]. Additionally, amphetamine-induced locomotion was also decreased after complete deletion of the VGLUT3 gene [100].

In relation to the above-mentioned drug-induced motor disturbances, addictive behaviour was also altered in VGLUT3 KO mice. These animals proved to be more sensitive, as they responded to smaller amount of cocaine in a conditioned place preference test than their WT littermates [101] and were more responsive when it was used as a reward [98]. This might have a human relevance, as variations in the VGLUT3 gene in patients also correlated with severe addiction [98].

The previously mentioned contribution of VGLUT3 to hearing was confirmed by the loss of auditory brainstem responses in VGLUT3 KO animals [70][73][74]. Interestingly, the p.A211V point mutation of the VGLUT3 also results in progressive deafness in humans [70]. However, in contrast to the progressive deafness, no major behavioural phenotype was observed due to the same point mutation, only an in vitro decreased VGLUT3 expression was detected [102].

As numerous brainstem areas involved in respiration and thermogenesis also contain VGLUT3+ neurons (see earlier), researchers might assume alteration in these systems as well. Indeed, despite preserved structure, the respiratory rhythm generator neurons of the brainstem in VGLUT3 KO mice fired with decreased amplitude and duration in response to hypoxic stress, which was probably due to an altered 5-HT turnover [19]. Moreover, their thermoregulation was also disrupted [19].

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Learning and memory formation in VGLUT3 KO mice was also investigated, and no major disruption was found [103]. Indeed, an earlier study suggested the role of VGLUT1 and 2 rather than 3 in the measured parameters [104]. However, further studies might be needed to reveal the role of local VGLUT3 positive neurons and terminals in the processes leading to spatial, emotional, and other types of memories.

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