

1 **Leveraging VGLUT3 functions to untangle brain dysfunctions**

2 Mathieu Favier¹, Nicolas Pietrancosta^{2,3}, Salah El Mestikawy^{1,2}, Giuseppe Gangarossa⁴

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6 ¹Douglas Mental Health University Institute, Department of Psychiatry, McGill University,
7 Montreal, QC, H4H 1R3, Canada

8 ²Sorbonne Université, INSERM, CNRS, Neurosciences Paris Seine - Institut de Biologie
9 Paris Seine (NPS - IBPS), 75005, Paris, France

10 ³Laboratoire des Biomolécules, LBM, Département de chimie, École Normale Supérieure,
11 PSL University, Sorbonne Université, CNRS, 75005, Paris, France.

12 ⁴Université de Paris, BFA, UMR 8251, CNRS, F-75013 Paris, France

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14 Correspondence to: giuseppe.gangarossa@u-paris.fr (GG, @PeppeGanga)

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19 **Abstract**

20 Vesicular glutamate transporters (VGLUTs) were long thought to be specific markers of
21 glutamatergic excitatory transmission. The discovery, two decades ago, of the atypical
22 VGLUT3 has thoroughly modified this oversimplified view. VGLUT3 is strategically
23 expressed in discrete populations of glutamatergic, cholinergic, serotonergic and even
24 GABAergic neurons. Recent reports show the subtle, but critical, implications of the
25 VGLUT3-dependent glutamate co-transmission and its roles in the regulation of diverse brain
26 functions and dysfunctions. Progresses in the neuropharmacology of VGLUT3 could lead to
27 decisive breakthroughs in the treatment of Parkinson's disease, addiction, eating disorders,
28 anxiety, presbycusis or pain. This review summarizes recent findings on VGLUT3 and its
29 vesicular underpinnings as well as on possible ways to target this atypical transporter for
30 future therapeutic strategies.

31 **Beyond the “canonical” glutamate transmission**

32 Neurotransmission is a key form of cellular communication. Mechanisms underlying
 33 synaptic transmission between neurons largely rely on postsynaptic responses (activation,
 34 inhibition, modulation). These responses depend on chemical messengers or neurotransmitters
 35 actively released by presynaptic terminals. Before their release through the complex steps of
 36 exocytosis, neurotransmitters must be packaged into synaptic vesicles by specific types of
 37 vesicular transporters. Therefore, **vesicular neurotransmitter transporters** (see glossary),
 38 which must not be confused with **plasma membrane neurotransmitter transporters** [1], are
 39 pivotal for neurotransmission and synaptic communication.

40 The so called “Dale’s principle” formulated as “*neurons release only a single type of*
 41 *neurotransmitter at all of their synapses*” is incorrect or simply misunderstood. This notion
 42 was in fact formulated by Eccles at a time when only two neurotransmitters were known:
 43 acetylcholine (ACh) and norepinephrine (NE). Nowadays, this assumption has been widely
 44 refuted since several neuronal populations express two vesicular transporters, thus
 45 highlighting **their** capability to **co-exist and** co-release two distinct canonical
 46 neurotransmitters [2,3]. Despite **this** game-changing evidence, neuronal populations **are still**
 47 **often classified** according to their initially discovered neurochemical **signatures**: e.g.
 48 glutamatergic, GABAergic, cholinergic, dopaminergic, noradrenergic or serotonergic
 49 neurons.

50 At the beginning of this millennium, three vesicular glutamate transporters (VGLUT1,
 51 VGLUT2 and VGLUT3) were identified and characterized [4–13]. These structurally related
 52 isoforms mediate **the uptake of** glutamate into synaptic vesicles and are driven by a proton
 53 **electrochemical gradient** generated by the vacuolar H⁺-ATPase. **VGLUT1 and VGLUT2**
 54 **were initially considered as** “canonical” cortical and subcortical vesicular glutamate
 55 transporters. In contrast, VGLUT3 was mainly found in discrete populations of “non-
 56 glutamatergic” neurons and only in scattered glutamatergic neurons [2] (Figure 1). VGLUT3
 57 is expressed by **subgroups** of glutamatergic neurons in the cerebral cortex, raphe nuclei [7,14]
 58 and **by inner hear cells** of the cochlea [15,16] (Figure 1). In addition, central VGLUT3 is
 59 detected in **cholinergic interneurons** (ChIs) of the dorsal and ventral striatum [7,17,18],
 60 GABAergic cortical and hippocampal interneurons [19–21], serotonergic raphe neurons
 61 [7,14] and **GABAergic neurons in the olfactory bulb** [22] (Figure 1). Therefore, VGLUT3-
 62 positive neurons act as both local interneurons (VGLUT3-interneurons in the cortex,
 63 hippocampus and striatum) and/or as projecting principal neurons (VGLUT3-neurons in the

64 raphe). In addition, VGLUT3 is also present outside the brain, notably in the spinal cord [23–
65 25] and in the retina [26–28].

66 This unconventional topography reveals that VGLUT3-positive cells form a small neuronal
67 subset with highly heterogeneous neurochemical functions.

68 The main objectives of this review are (i) to summarize the most recent discoveries related to
69 the specific role of VGLUT3 and (ii) to question whether VGLUT3-positive neurons and/or
70 VGLUT3 itself may represent an innovative and promising therapeutic target to more
71 efficiently treat brain disorders.

72

73 **Neurochemical features of VGLUT3-neurons and glutamate transmission**

74 The presence of VGLUT3 in some ACh-, 5-HT- and GABA-neurons raises several
75 key questions: (i) Can glutamate influence the vesicular loading of other transmitters? (ii)
76 What is the releasing *modus operandi* of two distinct neurotransmitters from the same
77 presynaptic varicosity? (iii) Have these two neurotransmitters similar or different effects? (iv)
78 Which receptors are targeted by VGLUT3-dependent glutamate?

79 Tackling these fundamental questions is essential to understand and efficiently target
80 VGLUT3 networks. It is important to mention that subsets of non-glutamatergic neurons also
81 co-express VGLUT2 or VGLUT1 with other classical transmitters [29–33]. However, here
82 we will specifically focus on the therapeutic windows opened by VGLUT3-operated co-
83 transmission.

84

85 *Vesicular synergy in ACh, 5-HT and GABA neurons*

86 Beyond co-transmission, it is now well-established that the co-loading of glutamate with
87 ACh, 5-HT or GABA modifies both their vesicular contents as well as the quantal size of
88 released neurotransmitters [2,3,17,19,34]. This mechanism, named **vesicular synergy** [17],
89 was initially observed in striatal cholinergic interneurons and then extended to 5-HT- and
90 GABA-neurons (through VGLUT3) and to DA-neurons (through VGLUT2) [3,35].

91 It is currently hypothesized that the biophysical mechanisms underlying vesicular synergy are
92 related to the fact that the negatively charged neurotransmitter glutamate increases the pH
93 gradient across vesicular membranes [2,35] (Figure 2). This increased $\Delta\mu\text{H}^+$, generated by a
94 vacuolar-type H^+ pump (V-ATPase), triggers a driving force that promotes the vesicular
95 uptake into the vesicular lumen of positively charged neurotransmitters such as ACh or 5-HT
96 [17,34,36] as well as of the zwitterionic GABA [19,32] (Figure 2). Vesicular synergy is a
97 quite powerful mechanism since it may increase ACh transmission by as much as +200%

98 [17]. Whether VGLUT3 also contributes to the regulation of GABA vesicular accumulation is
 99 still matter of debate [37–39]. Indeed, reduced evoked and spontaneous GABA-dependent
 100 miniature inhibitory postsynaptic currents (mIPSCs) were detected in VGLUT3-KO or
 101 GABA-restricted VGLUT3-cKO (VIAAT^{Cre/+::VGLUT3^{flox/flox}}) mice [19], suggesting the
 102 possibility of a GABA/glutamate vesicular synergy.

103 Overall, vesicular synergy operated by VGLUT3-positive terminals allows discrete neuronal
 104 populations to scale their signaling intensity. This represents a novel form of communication
 105 that may be pharmacologically targeted to hack dysfunctional circuits.

106

107 *Insights into glutamate co-release: simultaneous or differential release?*

108 Theoretically, many varicosities from the VGLUT3-positive network can release glutamate in
 109 addition to their “canonical” neurotransmitters (ACh, 5-HT or GABA). However, there is still
 110 a limited understanding of the signaling modalities by which VGLUT3-positive neurons
 111 control or scale the activity of other neuronal populations. In 2011, taking advantage of
 112 optogenetics, Higley and colleagues provided the first electrophysiological evidence that the
 113 presence of VGLUT3 in striatal ACh-neurons allowed ChIs to generate glutamatergic
 114 excitatory postsynaptic potentials (EPSPs) in neighboring striatal medium spiny neurons
 115 (MSNs) [40]. In this experimental setting, a single 4 ms light-pulse elicited a VGLUT3-
 116 dependent NMDA response. Similarly, a few years later, optogenetic activation of ChIs was
 117 shown to induce monosynaptic nicotinic and glutamatergic AMPA and NMDA receptors-
 118 mediated currents in striatal fast-spiking interneurons (FSIs) [41]. These responses were
 119 evoked by a train of 5 ms pulses of blue light (470 nm) at 5 Hz and their glutamatergic
 120 component tightly relied on VGLUT3. These reports elegantly showed that single light pulse
 121 stimulations of ChIs result in the release of ACh and glutamate. Interestingly, in absence of
 122 VGLUT3 a strong attenuation of nicotinic signaling was observed on FSIs [41], an effect
 123 mostly attributed to the loss of vesicular synergy. Taken together, these findings suggest that
 124 within cholinergic varicosities, ACh and glutamate are stored in the same presynaptic vesicles
 125 and co-released (Figure 2).

126 In addition to these mechanistic advances, recent reports on a VGLUT1-operating circuit
 127 complement previous findings and highlight a much more complex and dynamic scenario.
 128 Cholinergic neurons from the ventral area of the medial habenula (vMHb) project to the
 129 central part of the interpeduncular nucleus (IPN) and are involved in addiction and mood
 130 disorders [42]. “Cholinergic” varicosities from the central IPN co-express VGLUT1 and
 131 VAcHT [43,44]. Ren and colleagues reported that brief (5 ms) optogenetic stimulation of

132 cholinergic fibers from the IPN evoked fast glutamatergic responses, whereas stronger light
 133 pulses (such as continuous 20 sec at 20-50 Hz) evoked a nicotinic current [33]. Interestingly,
 134 these tetanic opto-stimulations seem to release both ACh and glutamate. Within this
 135 **VGLUT1-positive** network, ACh was found to potentiate glutamate vesicular accumulation in
 136 a form of “reverse” vesicular synergy [43]. Furthermore, VGLUT1 colocalizes with VACHT
 137 in a significant number of synaptic vesicles (but not all) [33,43]. Taken together these reports
 138 suggest that vMHB-to-IPN projections have at least 2 **pools** of synaptic vesicles: *i*) purely
 139 VGLUT1-positive vesicles (**pool #1**) and *ii*) mixt VGLUT1/VACHT-positive vesicles (**pool**
 140 **#2**). At low frequencies these terminals preferentially release glutamate **from pool #1**,
 141 whereas at more sustained neuronal activity mixt ACh/glutamate vesicles are mobilized **from**
 142 **pool #2**.

143 This **overview** suggests an unsuspected level of complexity of ACh/glutamate co-
 144 transmission operated by VGLUT1 and potentially also by VGLUT3 (Figure 2). A **key**
 145 question emerging from these **reports** is whether this complexity also applies to 5-
 146 HT/glutamate or GABA/glutamate VGLUT3-**dependent** co-transmission.

147 **Serotonergic fibers from the dorsal raphe nucleus (DRN) co-express VGLUT3 and VMAT2.**
 148 **Therefore, vesicular synergy and co-transmission may also occur in a subpopulation of 5-HT**
 149 **fibers [34].** Interestingly, low frequency opto-stimulation (<1 Hz) of these fibers induced a
 150 fast glutamatergic response **in interneurons of the basal amygdala (BA)**. In contrast, higher
 151 frequency stimulation (10-20 Hz) induced a slow 5-HT-dependent excitation [45,46].
 152 Therefore, it is tempting to propose that VGLUT3-positive 5-HT varicosities could possess
 153 synaptic vesicles that, **depending on neuronal activity (low vs high frequency bursts), allow**
 154 **either the release of glutamate alone or the co-release of 5-HT/glutamate.** In the near future, it
 155 will be essential to determine the functions of this tonic glutamatergic efflux and to precisely
 156 decipher how each transmitter released **from serotonergic terminals impacts** mood-associated
 157 functions and dysfunctions.

158 Overall, these pioneering reports suggest that we are far from grasping the full
 159 complexity accompanying VGLUT3- (or VGLUT1-) dependent co-transmission at the
 160 vesicular level. Once these points clarified, they will undeniably offer subtle ways to leverage
 161 VGLUT3 and treat several brain disorders.

162

163 **Involvement of VGLUT3 in brain disorders**

164 The **heterogenous distribution** of VGLUT3 raises the **interesting** possibility that
 165 VGLUT3 may actively contribute **to several brain functions and dysfunctions.** Indeed, **this**

166 [view](#) is supported by recent reports clearly establishing that VGLUT3 represents a key player
167 in shaping brain [and sensory](#) networks. Here, we provide [a brief](#) overview of some recent
168 breakthroughs related [to the involvement of VGLUT3](#) in severe and debilitating diseases.

169

170 *VGLUT3, fear and mood disorders*

171 Mood disorders, such as anxiety, exacerbated stress and excessive fear, are disabling
172 psychiatric disorders with relevant clinical and socio-economic burden. [VGLUT3-associated](#)
173 [neuronal circuitries regulating mood disorders have not been fully identified yet. However,](#)
174 [the constitutive deletion of VGLUT3 results in an anxiety-like phenotype \[34\]. This is further](#)
175 supported by a study showing that the differential expression of *Slc17a8* (VGLUT3) mRNA
176 in several mouse strains correlates with anxiogenic traits [47]. In addition, VGLUT3-KO mice
177 show enhanced innate fear and compromised stress axis [48,49].

178 Raphe neurons are centrally involved in controlling emotional behaviors and mood disorders
179 [50–52]. [Interestingly, VGLUT3-positive 5-HT projections \[9,34\] widely project to key brain](#)
180 [regions involved in the regulation of](#) emotional states [53]. Recently, it has been reported that
181 [VGLUT3-positive serotonergic](#) neurons, by releasing both 5-HT and glutamate, are pivotal
182 regulatory elements in the control of mood-like disorders [45,46,54]. Indeed, optogenetic
183 activation of [VGLUT3/5-HT fibers projecting](#) in the ventral tegmental area (VTA) [was](#)
184 sufficient to promote resilience [of](#) susceptible mice in [the](#) chronic social defeat stress model
185 [54].

186 The basal amygdala (BA) is another important regulatory node of emotional circuits [55].
187 Interestingly, within the BA, 5-HT, ACh and local GABAergic fibers co-express VGLUT3
188 [37,45,46,56,57]. However, functional implications of VGLUT3 in BA cholinergic or
189 GABAergic fibers remain poorly understood. In contrast, it has been shown that optogenetic
190 stimulation of 5-HT fibers evoked glutamate co-release and excitatory postsynaptic currents
191 (EPSCs) in BA neurons and powerfully mediated fear-related memory [45,46]. However, a
192 specific mechanistic role of VGLUT3-dependent glutamate release from 5-HT-neurons in
193 mood-like functions and dysfunctions remains to be fully established.

194

195 *VGLUT3, Parkinson's disease and dyskinesia*

196 Parkinson's disease (PD) [is](#) a severe neurodegenerative disorder characterized by the
197 [progressive](#) loss of dopamine (DA) neurons in the substantia nigra *pars compacta* (SNc). The
198 reduction of DA transmission is known to trigger profound maladaptive modifications in DA-
199 sensitive brain structures, notably in the dorsal striatum. Among these PD-related

200 modifications, the **increased** striatal cholinergic tone is a central feature since ChIs powerfully
201 modulate local **striatal microcircuits** [58–61]. This has led to intense investigations aimed at
202 establishing **anti-cholinergic** treatments for PD symptoms. However, manipulation of striatal
203 ChIs also directly results in concomitant alterations of local glutamate striatal transmission. In
204 fact, the key neurochemical feature of ChIs-mediated glutamate release cannot be neglected
205 since VGLUT3 is highly expressed in the striatum (**Figure 1 and 3A, B**). Furthermore,
206 VGLUT3-dependent glutamate transmission influences the activity of GABA **MSNs** and
207 GABA fast-spiking interneurons (FSIs) [40,41]. Interestingly, genetic deletion of VGLUT3
208 ameliorates motor symptoms in PD mouse models [62]. In line with this evidence, DA
209 depletion **in 6-OHDA-lesioned mice** increases the striatal expression of VAcHT and
210 VGLUT3 [63]. Indeed, cell type-specific inhibition of ChIs and/or pharmacological blockade
211 of cholinergic striatal transmission alleviates PD-like motor symptoms [64,65].

212 Current therapeutic strategies for PD are mainly based on the pharmacological
213 replacement of DA through its precursor L-DOPA. However, chronic administration of L-
214 DOPA leads to the development of abnormal and involuntary movements, also known as L-
215 DOPA-induced dyskinesia (LID). Cell type-specific chemogenetic activation or short pulse
216 optogenetic stimulation of striatal ChIs **aggravates** LID [66–68]. These mechanisms have
217 been usually **described as ACh-dependent** since pharmacological agents targeting nicotinic
218 (nAChR) and muscarinic (mAChR) receptors modulate LID [58,69–73]. Importantly, genetic
219 deletion of VGLUT3 dramatically dampens LID and its associated molecular adaptations in
220 the striatum [62,63]. This suggests that, beyond **the inhibition of striatal cholinergic**
221 **transmission**, inhibition/blockade of VGLUT3 represents a promising therapeutic strategy to
222 ameliorate PD and LID.

223

224 *VGLUT3, reward and addiction*

225 Addiction is a chronic neuropsychiatric disorder characterized by the compulsive and
226 uncontrollable use of drugs [74]. **Addiction is clearly** related to the dysregulation of midbrain
227 DA-neurons, which ultimately leads to long-term maladaptive changes in several brain
228 circuits [74]. The strategic **position** of VGLUT3 within the striatal network (**Figure 3A, B**)
229 suggests that VGLUT3-dependent signaling may represent a major player in modulating
230 reward and addictive behaviors. In line with this hypothesis, it is important to mention the
231 relatively high frequency of rare variants of SLC17A8 (the gene encoding **for** VGLUT3) in
232 patients with severe drug addiction [18]. **The coding sequence of VGLUT3 was analyzed in**
233 **healthy controls and in subjects with severe cocaine and/or opiate addiction** [18]. Seven

234 mutations predicting an amino acid change in the VGLUT3 protein sequence and two
235 synonymous variations were detected in patients with drugs addiction, whereas only one
236 missense mutation was found in healthy controls [18]. Indeed, genetic silencing of VGLUT3
237 (VGLUT3-KO mice) results in a stark increase of cocaine-triggered positive reinforcement
238 [18]. This behavior is associated with an increased DA release and enhanced DA signaling in
239 the nucleus accumbens (NAc) of VGLUT3-KO mice [18]. According to this report, glutamate
240 co-released by ChIs binds to mGluRs located on DA terminals and consequently inhibits DA
241 release in the NAc (Figure 3A). Conversely, optogenetic short pulses that generate only one
242 action potential in ChIs elicit DA release in the ventral striatum [75,76]. In both cases, this
243 glutamate/ACh-mediated regulation is independent from the firing activity of SNc/VTA DA-
244 neurons. In fact, it is operated by a panel of nicotinic (nAChRs) and muscarinic (mAChRs)
245 acetylcholine receptors as well as by metabotropic glutamate receptors (mGluRs) located on
246 dopaminergic fibers (Figure 3A) [18,76]. As illustrated in Figure 3A, because of these
247 presynaptic receptors, ACh and glutamate released by ChIs exert opposite effects on DA
248 efflux in the NAc.

249 Throughout striatal and accumbal territories [77,78], dopaminoceptive MSNs are
250 anatomically and functionally segregated into D1- or D2-positive neurons (Figure 3B) [79].
251 Upon release, DA activates D1-MSNs and inhibits D2-MSNs [79]. Therefore, increased
252 release of DA results in an overall modulation of striatum-associated behaviors. In agreement
253 with this classic model, the enhanced release of DA caused by VGLUT3 deletion leads to a
254 constitutive molecular, morphological and behavioral activation of the D1-MSNs [18].
255 Consequently, VGLUT3 deletion exacerbates cocaine self-administration [18]. In contrast,
256 specific deletion of VACHT in the striatum, which decreases DA efflux [18,80], has only
257 limited effects on the stimulant properties of cocaine [81]. However, it is important to
258 mention that D1- and D2-MSNs also differentially express mAChRs (Figure 3A, B). In fact,
259 both cell types express Gq-coupled muscarinic M1 receptors (M1AChRs), whereas Gi-
260 coupled muscarinic M4 receptors (M4AChRs) are exclusively found in D1-MSNs [82]
261 (Figure 3B). Therefore, variations in the acetylcholine/glutamate/dopamine balance can
262 trigger complex dynamics by scaling presynaptic and postsynaptic functions at the
263 microcircuit level (Figure 3B).

264 Interestingly, silencing of VGLUT3 enhanced locomotor activity induced by
265 psychostimulants while it dampened chronic amphetamine-induced stereotypies with a
266 concomitant reduction in stereotypies-evoked Δ FosB striatal activation [83]. Therefore, the

267 presence of VGLUT3 in ChIs provides these interneurons with complex and subtle regulatory
 268 properties over reward-guided behaviors.

269 In addition to its action within the striatum, recent reports have shown that VGLUT3
 270 expressed by 5-HT-neurons is also able to regulate reward-like behaviors [at the level of](#)
 271 [midbrain DA-neurons](#) [84,85]. [Optogenetic activation of VGLUT3-positive serotonergic](#)
 272 [varicosities within the VTA evoked AMPA-mediated excitatory responses in DA-neurons,](#)
 273 [ultimately leading to increased DA release in the NAc](#) as well as to enhanced positive
 274 reinforcement (conditioned-place preference) [85]. Overall, these recent discoveries call for
 275 integrative and cooperative effects of several VGLUT3-positive pathways which converge on
 276 [several brain areas](#) to modulate reward behaviors.

277

278 *Dual regulation of eating disorders by ChIs-mediated ACh/glutamate transmission*

279 Behavioral choices are governed by a yet unidentified neurochemical arbitration between
 280 **goal-directed behavior** (GDB) and **habit formation**. While GDB dynamically evaluates
 281 optimal choices and is flexible, habit values efficiency and is inflexible [86]. The balance
 282 between GDB and habit strategies, which involves the DA and basal ganglia systems [87,88],
 283 is compromised in eating disorders [89,90].

284 Recently, the respective roles of ACh and glutamate released by ChIs on the transition
 285 between GDB to habits in eating disorders was investigated using cell type-specific deletion
 286 of either VGLUT3 (VGLUT3-cKO mice) or VAcHT (VAcHT-cKO mice) [80]. VGLUT3-
 287 cKO mice showed increased tendency to use GDB. Conversely, VAcHT-cKO mice were
 288 characterized by decreased behavioral flexibility and facilitation of habit formation.
 289 Importantly, tendency to habits in VAcHT-cKO animals predicted vulnerability to develop
 290 compulsive eating disorders (binge eating and self-starvation) [80].

291 These opposite effects of ChIs-driven ACh/glutamate co-release in the GDB/habits balance
 292 and the concomitant establishment of maladaptive eating disorders are mirrored by a
 293 dichotomic regulation of DA release in the NAc and in the dorsal striatum (as described
 294 above and in Figure 3). Therefore, VAcHT-cKO mice have a deficit of both ACh and DA
 295 transmission in the striatum which leads to major maladaptive behaviors [80]. Interestingly,
 296 administration of L-DOPA (to enhance DA transmission) or donepezil (to enhance ACh
 297 transmission) to VAcHT-cKO mice reduced self-starvation of these animals in the **activity-**
 298 **based anorexia** (ABA) model [80]. These results indicate that pharmacological and/or bio-
 299 engineering interventions aimed at correcting the striatal ACh/glutamate balance may be of
 300 interest for long-term maladaptive dysfunctions, notably compulsive disorders.

301

302 *VGLUT3 and sensory (dys)functions*

303 As mentioned above, VGLUT3 is also expressed in the inner hair cells (IHCs) of the cochlea
304 [16] and in peripheral sensory neurons [25]. Consequently, VGLUT3 participates to key
305 sensory dysfunctions such as deafness and pain.

306 Deafness – Deafness is one of the most common sensory deficits. Moreover, around 50% of
307 individuals above 75 years suffer from presbycusis. Pioneering studies have shown that loss
308 of VGLUT3 results in sensorineural deafness [15,16] and that a missense mutation in the
309 VGLUT3-encoding human gene SLC17A8 may underlie hearing loss [15]. In fact,
310 constitutive VGLUT3-KO mice exhibit abnormally thin and elongated ribbons in IHC
311 synapses, loss of vesicular glutamate uptake in IHCs and lack of synaptically evoked
312 glutamate currents at the IHC-afferent nerve synapse [15,16]. Importantly, virally mediated
313 restoration of VGLUT3 in IHCs through an adeno-associated viral strategy (AAV1-
314 VGLUT3) promoted hearing recovery in VGLUT3-KO mice [91]. This suggests that not only
315 VGLUT3 is necessary and sufficient for hearing functions but also that VGLUT3-targeted
316 gene therapy may represent an innovative tool for congenital deafness.

317 Pain – During development VGLUT3 is transiently expressed in peripheral C-fibers and in
318 excitatory interneurons of the dorsal horns [24,92]. In adult animals VGLUT3 is almost
319 exclusively expressed in unmyelinated C-low threshold mechanoreceptors (C-LTMRs) [25].
320 Genetic deletion of VGLUT3 triggers deficits in acute and persistent mechanical pain as well
321 as in inflammatory pain, while sparing other somatosensory behaviors [25,93]. Interestingly,
322 taking advantage of different Cre-restricted cell type-specific approaches, deletion of
323 VGLUT3 in C-LTMRs did not recapitulate the mechanical sensory deficits observed in
324 VGLUT3-KO mice [24,92]. Accordingly, the critical role of VGLUT3 in mechanical pain
325 may mainly arise from its transient expression in dorsal horn interneurons [24] and/or from
326 VGLUT3-expressing central brain regions. However, the latter hypothesis has not been
327 investigated yet.

328 These sensory functions of VGLUT3 need to be considered when designing VGLUT3-
329 specific targeting strategies to alleviate dysfunctions such as fear, mood disorders,
330 Parkinson's disease, dyskinesia, reward and addiction. For example, VGLUT3-specific
331 inhibitors, although interesting for ameliorating abnormal movements or eating disorders
332 [63,80], may also decrease audition, or increase mechanical and/or inflammatory pain
333 [15,16,25,93].

334

335 **Different modalities for targeting VGLUT3: when how and where matter**

336 *Indirectly: receptors targeted by VGLUT3-dependent glutamate*

337 As depicted above, VGLUT3 provides neurons with a complex and sophisticated signaling
 338 machinery. This is particularly well-illustrated in the case of the regulation of DA striatal
 339 efflux by ChIs and ACh/glutamate co-transmission (Figure 3A, B). In subregions of the
 340 striatum, such as the NAc or the dorsomedial striatum, glutamate released from ChIs binds to
 341 mGluRs and inhibits DA release [80]. Yet a different type of subtle organization is observed
 342 with CCK-basket cells in the hippocampus. These inhibitory interneurons express both
 343 VIAAT and VGLUT3 and paradoxically release both GABA (inhibitory neurotransmitter)
 344 and glutamate (excitatory neurotransmitter) (Figure 1 and 3C). GABA released by CCK-
 345 basket cells binds to GABA_A and inhibits pyramidal cells, whereas glutamate released by the
 346 same interneurons binds to type III mGluR “hetero-autoreceptors” located on the membrane
 347 of CCK-baskets cells, thus leading to the auto-inhibition of GABA release (Figure 3C, [19]).
 348 This unforeseen “hetero-autoregulation” of hippocampal basket cells have important
 349 regulatory effects on CA1 network properties such as oscillations and plasticity [19]. The
 350 above-described reports clearly indicate that the precise identification of VGLUT3-associated
 351 networks and receptors will probably open the door to a refined control of brain areas
 352 involved in major pathologies such as Parkinson’s disease, addiction, eating disorders,
 353 epilepsy and mood disorders.

354

355 *Directly: shRNA, virus, antibodies, nanobodies and VGLUT3 pharmacology*

356 To date, pharmacological tools that specifically and selectively inhibit VGLUT3 are not
 357 available. One alternative to control VGLUT3 activity would therefore be to use subtype-
 358 specific short hairpin RNA (shRNA) or antisera. Indeed, virally mediated shRNA (lentivirus
 359 or adenovirus) are already commercialized and could be directly injected in strategic brain
 360 regions to specifically silence VGLUT3. However, such approach requires several weeks
 361 following viral infusion before obtaining a reduction of VGLUT3 expression (El Mestikawy,
 362 personal communication). Although not tested yet, shRNA-mediated silencing of VGLUT3 in
 363 the striatum could be a potential strategy to reduce PD and LID symptoms.

364 Remarkably, an adeno-associated viral strategy (AAV1-VGLUT3) to restore VGLUT3 in
 365 inner hair cells (IHCs) promoted hearing recovery in VGLUT3 KO deaf mice [91], thus
 366 revealing that VGLUT3-targeted gene therapy may represent an innovative strategy for other
 367 VGLUT3-related dysfunctions.

368 Specific antisera could also be used to inhibit VGLUT3. However, the main limitation of
369 antibodies is their large size and low penetration into tissues, therefore representing a
370 suboptimal strategy as VGLUT3 resides on the membrane of synaptic vesicles (intracellular
371 compartment). Smaller in size, nanobodies are a new class of antigen-binding fragments
372 derived from single-domain camelid immunoglobulins [94] and they have been successfully
373 used to target mGluR2 both *in vitro* and *in vivo* [95]. Thus, nanobodies represent potential
374 candidates for the development of VGLUTs-specific ligands with interesting pharmacological
375 properties (small size, strong affinity, solubility). In line with this research strategy, an anti-
376 VGLUT1 nanobody with an inhibitory activity and that blocks glutamate vesicular
377 accumulation has recently been described *in vitro* [96]. However, targeting VGLUT3 (or
378 other VGLUTs) in specific brain areas through nanobodies remains a major challenge because
379 of their poor bioavailability, their rapid clearance from the plasma [97], and the
380 intracellular/vesicular location of VGLUTs.

381 The best way to control the activity of VGLUT3 would then be to develop small chemicals
382 that inhibit (or activate) VGLUT3 without altering the activity of VGLUT1 and VGLUT2. In
383 term of chemical compounds, only few dyes such as trypan blue, brilliant yellow and
384 synthetic analogues efficiently target VGLUT1-3 indistinctly [98] (Box 1). These compounds
385 modulate glutamatergic transmission *in vitro* and *in vivo*, although their efficacy and affinity
386 drastically decrease in intact tissues [99]. This is mostly due to the fact that the bioavailability
387 of current ligands in the brain is still critical.

388 The limited development of VGLUTs neuropharmacology is due to several major bottlenecks,
389 including the intracellular localization of VGLUT3 and the weakness of medicinal chemistry
390 pipelines to identify new scaffolds. First, unlike receptors or reuptake sites, a vesicular
391 transporter like VGLUT3 is an intracellular target. The few available VGLUTs inhibitors
392 have proven to be useful when used *in vitro*, with synaptic vesicles preparations, for the
393 characterization of [³H]-glutamate uptake [98]. Only few attempts have been made in brain
394 slices or *in vivo* [99,100]. On hippocampal slices or in the cochlea, several orders of
395 magnitude are necessary to impair VGLUTs functions [99,100]. Therefore, in order to be used
396 *in vivo*, future VGLUT3-specific drugs should be designed to cross the blood brain barrier
397 and the neuronal plasma membrane.

398 Second, the three VGLUTs show a high level of structural homology (~ 60-70%, Box 1,
399 Figure IA) and particularly in the central region of the three transporters which harbors the
400 transmembrane domains and the glutamate pore. However, a higher variability is observed in
401 the N- and C-terminus (Box 1, Figure IA). Because of this high level of identity, VGLUTs

402 share kinetic and pharmacological profiles. As a consequence, compounds specifically
403 targeting VGLUT3 (or any given VGLUTs) are not yet available. The recent resolution of the
404 SLC17 family structures [101] allows the design of 3D models and virtual screening based on
405 sequence differences between the three subtypes of VGLUTs (Figure IB-E). Alignment of
406 protein sequences of VGLUT1-3 show the presence of several amino acids specific to
407 VGLUT3 (Box 1, Figure IA). A large number of these VGLUT3-specific amino acids are
408 located in the N- and C-terminus which cannot be modeled in 3D. Interestingly, 3D
409 homology models of VGLUT3 reveal that some of these residues (G294, A295, N296, V298,
410 S299, L300, S301, K302 and F303) are strategically located inside the pore and in loop 6
411 which share only 20% of identity with VGLUT1 and VGLUT2. Some of these VGLUT3-
412 specific amino acids are located on the surface and within the glutamate binding pocket in the
413 outward conformation (Box 1, Figure IA, C). This 3D structural information opens the way to
414 the design of small chemical compounds that could specifically interact with VGLUT3-
415 specific residues, thereby allowing the generation of VGLUT3-specific ligands (Box 1, Figure
416 IF, G). These putative subtype-specific ligands could establish hydrophilic interactions with
417 side chains of polar residues (G294, S299, S301 or K302) and/or hydrophobic interactions
418 with non-polar amino acids (A295, V298, or F303) that are present only in VGLUT3 (Figure
419 IG). According to virtual docking models, these ligands could be able to bind to VGLUT3
420 with an affinity 10-50 times higher than for VGLUT1 or VGLUT2. However, it is worth to
421 mention that VGLUT1 and VGLUT2 largely outnumber VGLUT3 in the brain (>80%,
422 [102]). Therefore, in order to avoid dramatic side effects on VGLUT1 and VGLUT2,
423 VGLUT3-specific drugs should demonstrate a 100 to 1000 higher affinity for VGLUT3.
424 Nonetheless, once available, these VGLUT3-specific compounds may pave the way to the
425 pharmacological targeting of specific subsystems and above-mentioned pathologies. For
426 example, a specific inhibition of VGLUT3 could be beneficial to alleviate L-DOPA-induced
427 dyskinesia [63], whereas specific activation of VGLUT3 could help to reduce DA efflux in
428 the nucleus accumbens and the dorsolateral striatum [18,80] and therefore decrease the
429 reinforcing properties of psychostimulant drugs.

430 Conclusions and future perspectives

431 In this review, we have collected, highlighted and discussed the most recent discoveries
432 related to VGLUT3, from its functions at the vesicular level to its key roles in gating brain
433 functions and dysfunctions. Indeed, recent breakthroughs in the field of VGLUTs open
434 fruitful future perspectives at both fundamental and therapeutic levels. Overall, these findings
435 illustrate the urge to further explore the role of this atypical vesicular transporter in brain
436 disorders and to massively invest in optimizing novel strategies to modulate its mechanistic
437 functions. In fact, future challenges and promising directions of the VGLUT3-related research
438 field now aim at conceiving therapeutic tools (nanobodies, virally mediated gene therapy,
439 chemical or engineered ligands) to either boost or dampen the VGLUT3 machinery.
440 Pharmacological compounds specifically targeting VGLUT3 are now within reach thanks to
441 the recent elucidation of the 3D structure of VGLUT3 (Box 1, Figure I). Importantly, it is
442 reasonable to assume that, because of its role in gating the quantal co-release of other
443 neurotransmitters, VGLUT3 targeting actions may also be beneficial (i) in scaling
444 pharmacological treatments aimed at restoring ACh, GABA and 5-HT levels in several brain
445 disorders and (ii) in reducing the secondary side effects of current therapeutic strategies.
446 However, such promising directions also raise new exciting questions that need to be tackled
447 to maximize efforts and optimize the therapeutic potential of targeting VGLUT3 (see
448 Outstanding Questions).

449 Box 1: Current ligands and chemical difficulties to target VGLUTs

450 Although essential in ensuring the excitatory glutamate transmission, the pharmacology of
451 VGLUTs remains still elusive. In fact, only few competitive and non-competitive ligands
452 have been successfully tested in *ex vivo* brain preparations for their efficacy in modulating
453 glutamate transmission [99,100,103]. Unfortunately, because of the high degree of homology
454 among VGLUTs amino acids sequences (Figure IA), selective compounds targeting distinct
455 VGLUTs are not currently available. However, current knowledge on chemical properties of
456 VGLUTs ligands coupled to recent 3D models (Box 1, Figure IB-E) may open new venues
457 for further investigations.

458 Substrate analogs. Cyclic and non-cyclic glutamate analogs, such as (2S, 4R)-4-methyl-
459 glutamate (IC₅₀ = 0.7 mM), L-trans-ACPD (IC₅₀ = 0.23 mM), and DL-trans-ACHD (IC₅₀
460 = 0.8 mM) are among the most potent competitive modulators of glutamate uptake [104,105].

461 Substituted quinolines and quinoxaline analogs. Although also possibly binding to iGluRs
462 and mGluRs, kynurenate and its analogs, xanthurenate, 7-chloro-kynurenate, and 2-
463 quinoxaline carboxylate are potent competitive modulators of glutamate transport
464 [103,104,106–109].

465 Azoic Dyes. These molecules are characterized by two regions, one polar and the other
466 hydrophobic, interconnected by an azo bond (-N=N-). To date, azo dyes that successfully
467 inhibit VGLUTs with high affinity are: Bright Yellow (IC₅₀ = 15 nM), Chrysophenine
468 (IC₅₀ = 27 nM), Trypan blue (IC₅₀ = 50 nM), Evans blue (IC₅₀ = 90 nM), and Violet Direct
469 51 (IC₅₀ = 148 nM) [110–113]. Structure-activity relationships (SAR) studies have revealed
470 that the hydrophobic aryl body is critical to ensure affinity to VGLUTs. Moreover, the affinity
471 of azoic dyes to VGLUTs also depends on: (i) the capacity to form a hydrogen bond with
472 Tyr195 of VGLUTs, thereby preventing glutamate binding, (ii) the distance between the azo
473 bond and the negatively charged sulfonate group (N-O distance), and (iii) the negative
474 charges of the naphthyl-disulphonic motif [110,111]. Recently, a derivative of Trypan blue
475 (LSP5-2157) has been shown to be efficient in modulating VGLUTs functions *ex vivo* and *in*
476 *vivo* [99].

477 Non-competitive ligands. Among these ligands, the fluorescein analog Rose Bengal possesses
478 the highest affinity for VGLUTs (IC₅₀ = 25 nM) [114,115] and it may act as an allosteric
479 modulator of VGLUTs [116,117]. However, Rose Bengal can also inhibit other vesicular
480 carriers than VGLUTs [114].

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488

489 **Disclaimer Statement**

490 [The authors declare no conflicts of interest.](#)

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763

764 **Figure legends**

765

766 **Figure 1. Distribution of VGLUT3 mRNA and protein in different neuronal**
767 **populations.**

768 VGLUT3 (*Slc17a8*) mRNA is expressed by principal projecting neurons as well as local
769 interneurons distributed in different brain regions (horizontal brain section). In the cochlea,
770 VGLUT3 is abundantly present in inner ear cells (IHCs). In the brain, VGLUT3 is mostly
771 localized in scattered glutamatergic (green), GABAergic (red), cholinergic (yellow) and
772 serotonergic (blue) cell types. Coronal mouse brain sections show the expression of VGLUT3
773 protein in the hippocampus, striatum, nucleus accumbens, and raphe nuclei (highlighted).

774

775 **Figure 2. Vesicular synergy, release, co-release and differential release operated by**
776 **VGLUT3.**

777 Synaptic communication can occur as mono-transmission and/or co-transmission (see
778 glossary). VGLUT3-positive synaptic vesicles in cholinergic, serotonergic or GABAergic
779 varicosities upload negatively charged glutamate allowing an increased accumulation of H⁺
780 ions. This increased Δ pH permits higher accumulation of ACh, 5-HT or GABA in a process
781 named vesicular synergy. This putative explanation of vesicular synergy implies that two
782 classic neurotransmitters can co-exist into the same subpopulation of synaptic vesicles and
783 hence be co-released. In addition to this co-releasing modality, recent reports also point to the
784 existence of a differential release at some glutamate synapses [33,45]. This suggests the
785 presence, within the same terminal, of separate pools of vesicles expressing distinct vesicular
786 transporters (VGLUT3, VACHT, VMAT2 or VIAAT) and hence storing distinct
787 neurotransmitters (glutamate, ACh, 5-HT or GABA). Following their release from VGLUT3-
788 positive synapses (either by co-release and/or by differential release), the various
789 neurotransmitters will bind to metabotropic and/or ionotropic receptors to trigger postsynaptic
790 signaling cascades.

791

792 **Figure 3. Simplified representation of circuits and microcircuits involving VGLUT3 in**
793 **the striatum and the hippocampus.**

794 (A) In the striatum, glutamatergic cortico-striatal fibers regulate the activity of D1- and D2-
795 containing medium spiny neurons (MSNs). This flow of information is filtered by dopamine
796 D1 or D2 receptors located on MSNs dendritic spines. DA fibers from the substantia nigra

797 *pars compacta* (SNc)/ventral tegmental area (VTA) are under the dual control of
 798 acetylcholine (ACh) and glutamate released from cholinergic fibers (ChIs). Importantly, ACh
 799 stimulates DA efflux through nicotinic receptors (nAChR), whereas VGLUT3-dependent
 800 glutamate binds to metabotropic receptors (mGluR) and inhibits DA release.

801 (B) In addition to the modulation of presynaptic DA release, co-released ACh/glutamate from
 802 ChIs can directly modulate the activity of D1- and D2-positive MSNs as these two cell types
 803 express a panel of cholinergic and glutamatergic receptors (M1AChR, M4AChR, AMPA and
 804 NMDA receptors). Overall, the ACh/glutamate co-transmission from ChIs will regulate the
 805 balance between the GO (D1-MSNs) and NO GO (D2-MSNs) pathways resulting in
 806 physiological and/or pathological behaviors.

807 (C) In the hippocampus, a subgroup of CCK-positive GABAergic basket cells expresses
 808 VGLUT3. These VGLUT3-positive interneurons densely synapse on pyramidal neurons and
 809 release GABA that binds to inhibitory GABA_A receptors. In addition, these interneurons, via
 810 the presence of VGLUT3, co-release glutamate and consequently inhibit GABA efflux
 811 through inhibitory “hetero-autoreceptors” mGluRs (green arrows).

812

813 **Figure I. *In silico* pharmacological screening approach to specifically target VGLUT3.**

814 (A) Alignment of VGLUT1-3 protein sequences show that the three transporters are highly
 815 conserved (residues boxed in green). However, some amino acids (boxed in purple) are found
 816 exclusively in VGLUT3. Importantly, while most of these VGLUT3-specific residues are
 817 found in the N- and C-terminus, some of them are also present in central parts of the
 818 transporter. (B and D) Homology model of VGLUT3 based on E. coli D-galactonate (pdb
 819 code: [6E9N](https://www.rcsb.org/structure/6E9N) <https://www.rcsb.org/structure/6E9N>) in its open conformation in membrane (B)
 820 and cytosolic (D) view. (C, E) Homology model of the VGLUT3 closed conformation based
 821 on rat-VGLUT2 crystal structure (pdb code: [6V4D](https://www.rcsb.org/structure/6V4D) <https://www.rcsb.org/structure/6V4D>) in a
 822 membrane (C) and cytosolic (E) view [101,118]. Specific residues of VGLUT3 are depicted
 823 in purple and the central glutamate channel, common to VGLUT1-3, is highlighted in green.
 824 (F) The putative specific binding region of VGLUT3 is highlighted in purple and a putative
 825 VGLUT3-specific ligand (in yellow) is docked into this specific binding region. (G) Typical
 826 interactions expected for a selective ligand able to bind to this VGLUT3-specific region.

827

828 **Glossary**

829

830 **Activity-based anorexia:** animal model of anorexia nervosa in rodents. In this model,
831 animals are housed with running wheels while daily access to food is progressively restricted.
832 This creates a conflict between “eating” and “running”. During the restriction period mice
833 show paradoxical hypophagia and hyperactivity leading to self-starvation.

834

835 **Cholinergic interneurons (ChIs):** also called tonically active neurons (TANs), cholinergic
836 interneurons, constitute 1-3% of all striatal neurons and represent the main source of
837 acetylcholine within the striatum. ChIs possess the remarkable capacity to signal with both
838 acetylcholine and glutamate.

839

840 **Co-release:** modality of releasing two or more neurotransmitters from a single population of
841 synaptic vesicles.

842

843 **Co-transmission:** broad term to define the release of different neurotransmitters from the
844 same terminals or varicosities. Co-transmission can occur by co-release (same vesicles) or
845 differential release (distinct vesicles)

846

847 **Differential release:** modality of releasing two or more neurotransmitters from distinct
848 populations of synaptic vesicles.

849

850 **Electrochemical gradient:** driving force generated by a vesicular proton pump (H^+ -ATPase)
851 which allows the accumulation of charged neurotransmitters into the vesicular lumen.

852

853 **Goal-directed behavior (GDB):** observed when an individual is engaged in reaching a
854 specific objective. Action selection is governed by an association between the response and
855 the outcome. Goal-directed behaviors are characterized by active deliberation of future
856 consequences and adaptive flexibility to changing environments. They are sensitive to reward
857 (outcome) devaluation.

858

859 **Habit:** a behavioral adaptation emerging from goal-directed learning when behaviors become
860 automated due to repetition. Habits are driven by environmental stimuli and are independent

861 from the outcome. They are shaped by past experience and are characterized by efficiency
862 and inflexibility. Habits are resistant to reward devaluation.

863

864 **Medium spiny neurons (MSNs):** striatal GABAergic principal neurons. They constitute
865 around 95% of all striatal neurons and represent the main outputs of the striatum. Classically,
866 they are functionally and anatomically segregated in two populations depending on the
867 expression of dopamine D1 and/or D2 receptors.

868

869 **Plasma membrane neurotransmitter transporters:** proteins located in the neuronal plasma
870 membrane and responsible for the reuptake of neurotransmitters from the extracellular space.

871

872 **Striatal microcircuits:** local circuits within striatal territories consisting in functional
873 interactions between afferents (glutamate and DA fibers), local interneurons and principal
874 neurons. Striatal microcircuits tune the spontaneous and evoked activity of principal
875 projecting neurons (MSNs).

876

877 **Synaptic vesicles:** microstructures located inside the varicosities of presynaptic terminals and
878 containing one or multiple neurotransmitters. In response to increased intracellular Ca^{2+}
879 levels, synaptic vesicles fuse with the presynaptic neuronal membrane and release their
880 content into the synaptic cleft.

881

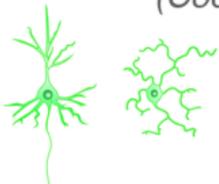
882 **Vesicular synergy:** the capacity of one vesicular transporter to influence the efficiency of
883 another vesicular transporter located in the same synaptic vesicle. This is exemplified here by
884 the functional relationship between VGLUT3 (glutamate) and VACHT (acetylcholine).

885

886 **Vesicular neurotransmitter transporters:** proteins located in the membrane of synaptic
887 vesicles. They concentrate neurotransmitters into synaptic vesicles. Three vesicular isoforms
888 exist for the accumulation of glutamate (VGLUT1-2-3).

VGLUT3 cell types

 Inner ear cells
(Cochlea)



Glutamatergic neurons



GABAergic interneurons
(CCK-positive basket cells)

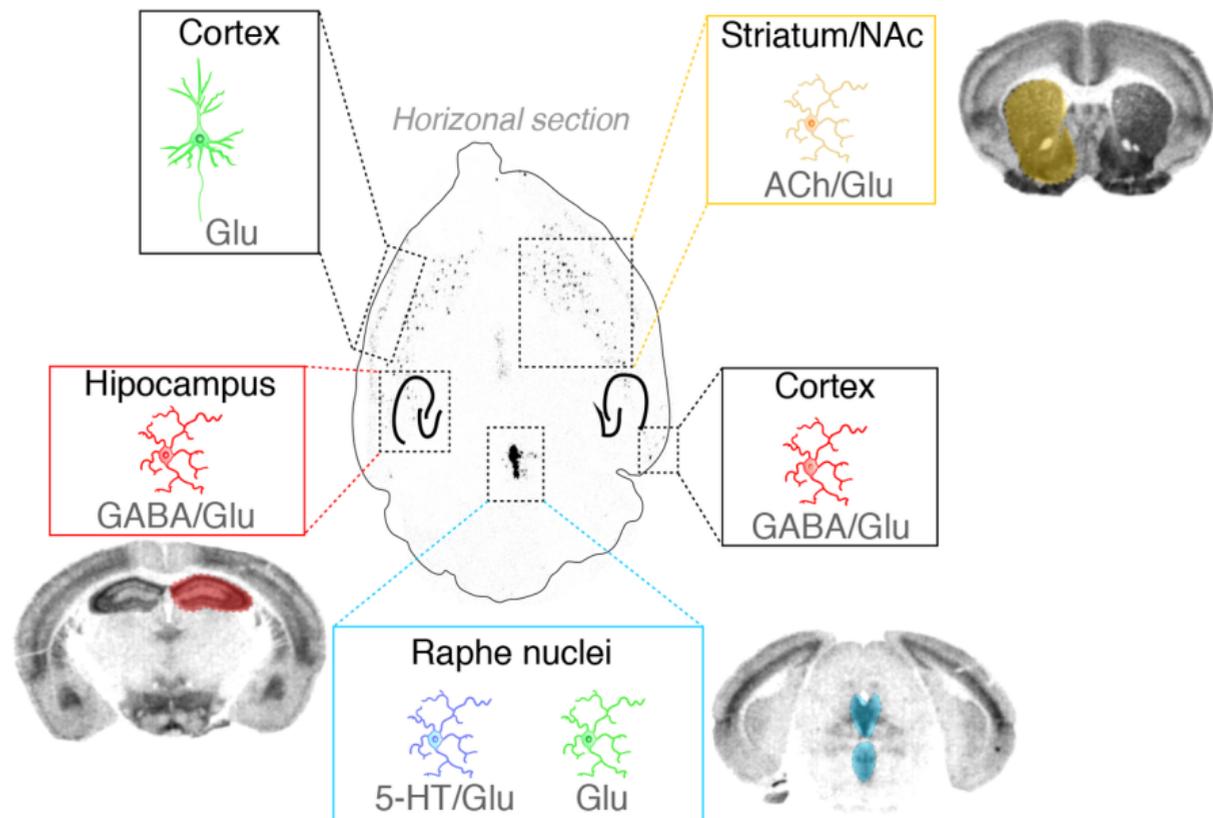


Cholinergic interneurons

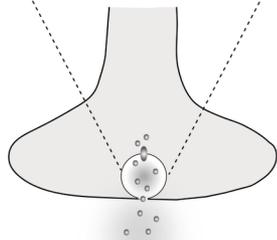
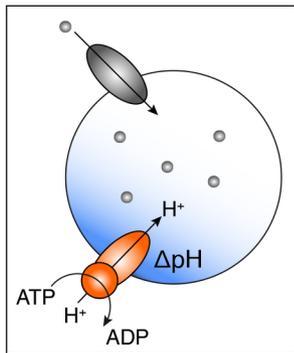


Serotonergic neurons

VGLUT3 mRNA and protein expression in the mouse brain



Mono-transmission

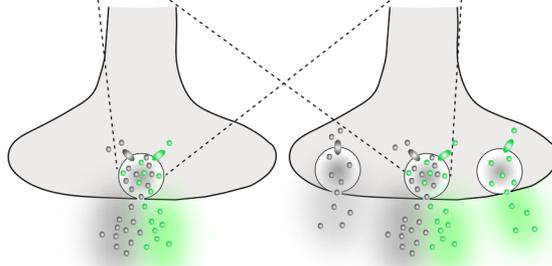
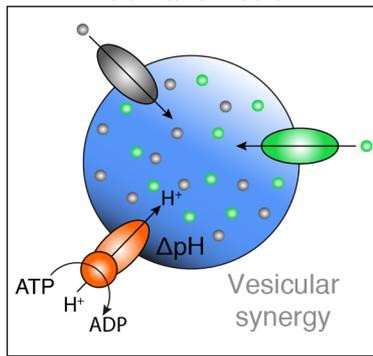


Mono-release

- Neurotransmitters (ACh, 5-HT or GABA)
- Vesicular transporters (VACHT, VMAT2 or VIAAT)
- Metabotropic receptors (ACh, 5-HT or GABA)
- Ionotropic receptors (ACh, 5-HT or GABA)

 V-ATPase

Co-transmission



Co-release

or ?

Differential release

In presence of VGLUT3

 Glutamate

 VGLUT3

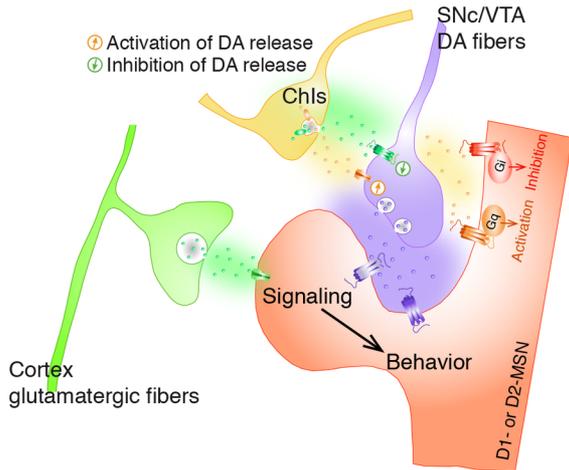
 mGluRs

 iGluRs

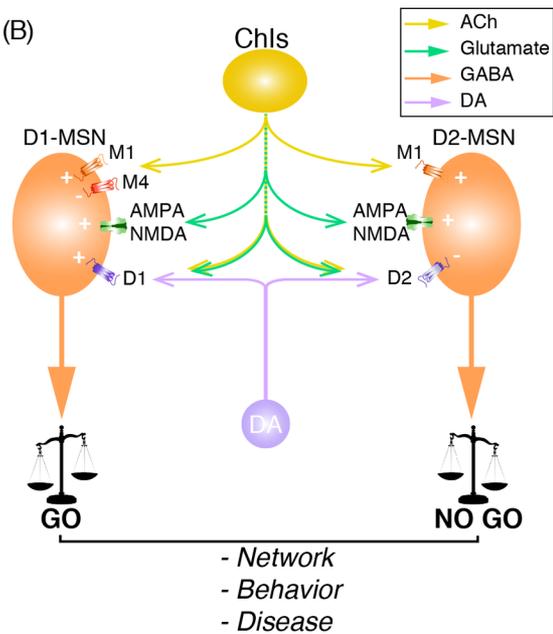
Signaling events

Dendritic spine

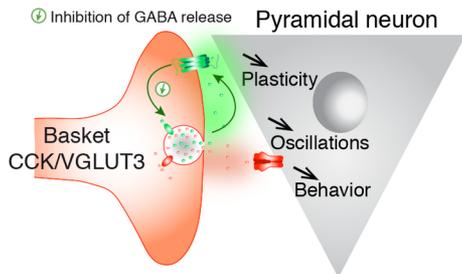
(A) Striatum



(B)



(C) Hippocampus



Potential targets:

Transmitters	Vesicular transporters	Receptors
GABA ●	VIAAT ●	GABA _A ▶
Glutamate ●	VGLUT3 ●	iGluR ▶ mGluR ▶
ACh ●	VACht ●	nAChR ▶ M1AChR ▶ M4AChR ▶
DA ●		D1R ▶ D2R ▶