

Neuronal Excitability

VGLUT3 Ablation Differentially Modulates Glutamate Receptor Densities in Mouse Brain

Karim S. Ibrahim,^{1,2,3}  Salah El Mestikawy,^{4,5}  Khaled S. Abd-Elrahman,^{1,2,3,*} and Stephen S.G. Ferguson^{1,2,*}

<https://doi.org/10.1523/ENEURO.0041-22.2022>

¹Brain and Mind Research Institute, University of Ottawa, Ottawa, Ontario K1H 8M5, Canada, ²Department of Cellular and Molecular Medicine, University of Ottawa, Ottawa, Ontario K1H 8M5, Canada, ³Department of Pharmacology and Toxicology, Faculty of Pharmacy, Alexandria University, Alexandria 21521, Egypt, ⁴Neuroscience Paris Seine–Institut de Biologie Paris Seine (NPS–IBPS) INSERM, CNRS, Sorbonne Université, 75006 Paris, France, and ⁵Department of Psychiatry, Douglas Hospital Research Centre, McGill University, Montreal, Quebec H4H 1R3, Canada

Abstract

Type 3 vesicular glutamate transporter (VGLUT3) represents a unique modulator of glutamate release from both non-glutamatergic and glutamatergic varicosities within the brain. Despite its limited abundance, VGLUT3 is vital for the regulation of glutamate signaling and, therefore, modulates the activity of various brain microcircuits. However, little is known about how glutamate receptors are regulated by VGLUT3 across different brain regions. Here, we used VGLUT3 constitutive knock-out (VGLUT3^{-/-}) mice and explored how VGLUT3 deletion influences total and cell surface expression of different ionotropic and metabotropic glutamate receptors. VGLUT3 deletion upregulated the overall expression of metabotropic glutamate receptors mGluR5 and mGluR2/3 in the cerebral cortex. In contrast, no change in the total expression of ionotropic NMDAR glutamate receptors were observed in the cerebral cortex of VGLUT3^{-/-} mice. We noted significant reduction in cell surface levels of mGluR5, NMDAR2A, NMDAR2B, as well as reductions in dopaminergic D₁ receptors and muscarinic M1 acetylcholine receptors in the hippocampus of VGLUT3^{-/-} mice. Furthermore, mGluR2/3 total expression and mGluR5 cell surface levels were elevated in the striatum of VGLUT3^{-/-} mice. Last, AMPAR subunit GluA1 was significantly upregulated throughout cortical, hippocampal, and striatal brain regions of VGLUT3^{-/-} mice. Together, these findings complement and further support the evidence that VGLUT3 dynamically regulates glutamate receptor densities in several brain regions. These results suggest that VGLUT3 may play an intricate role in shaping glutamatergic signaling and plasticity in several brain areas.

Key words: brain; glutamate; GPCR; synaptic plasticity; transporter

Significance Statement

Type 3 vesicular glutamate transporter (VGLUT3) is an atypical vesicular glutamate transporter that is discretely expressed by subpopulations of nonglutamatergic neurons within the brain. Through these neurons, VGLUT3 regulates mood, movement coordination, rewarding behavior, and cognition. Despite extensive research on VGLUT3 neurotransmission in nonglutamatergic neurons, little is known on how glutamate receptors are regulated by VGLUT3 signaling in the brain. Using VGLUT3 knockout in mice, we show that VGLUT3 differentially regulates glutamate receptor densities in various brain regions, further supporting its critical role in regulating synaptic function and plasticity in brain circuits.

Received January 25, 2022; accepted April 10, 2022; First published April 20, 2022.

Author contributions: K.S.I., K.S.A.-E., and S.S.G.F. designed research; K.S.I. performed research; S.E.M. contributed reagents/analytic tools; K.S.I. and K.S.A.-E. analyzed data; K.S.I., S.E.M., K.S.A.-E., and S.S.G.F. wrote the paper.

S.S.G.F. holds a Tier I Canada Research Chair in Brain and Mind. K.S.A.-E. holds clinician postdoctoral fellowships from Alberta Innovates Health Solutions and the Canadian Institutes for Health Research. The authors declare no other competing financial interests.

Introduction

The atypical type 3 vesicular glutamate transporter (VGLUT3) is a unique member of the Solute Carrier 17a transporter family that mediates vesicular packaging of glutamate in primarily nonglutamatergic varicosities, as well as in subpopulations of glutamatergic neurons in the brain (Freneau et al., 2004; El Mestikawy et al., 2011). VGLUT3 is expressed by discrete populations of neurons and interneurons in the cerebral cortex, raphe nuclei, hippocampus, striatum, olfactory bulb, and inner hair cells, and is transiently expressed in the cerebellum (Gras et al., 2002; Herzog et al., 2004; El Mestikawy et al., 2011). In addition to its well characterized vesicular glutamate transport functions, VGLUT3 modulates acetylcholine, serotonin, and GABA transmission (Gras et al., 2002; Somogyi et al., 2004; Trudeau and El Mestikawy, 2018). Functionally, VGLUT3 plays an important role in the neuronal networks involved in motor coordination, reward processing, and cognition (Ibrahim et al., 2020).

Evidence shows that VGLUT3 can modify glutamate release and mediate tonic excitatory postsynaptic inputs via both ionotropic glutamate receptor (iGluR) and metabotropic GluR (mGluR; Nelson et al., 2014; Sakae et al., 2015; Fasano et al., 2017). For instance, genetic deletion of VGLUT3 diminishes excitatory currents from tonically active interneurons that is mediated via AMPARs and NMDARs on the medium spiny neurons and GABAergic interneurons of the striatum (Higley et al., 2011; Nelson et al., 2014). Similarly, VGLUT3 regulates dopamine and GABA efflux via mGluR-dependent mechanisms in the nucleus accumbens and in the hippocampus (Sakae et al., 2015; Fasano et al., 2017). The overlapping distribution of VGLUT3 with different members of mGluRs strongly suggests that VGLUT3/mGluR cross talk is vital for synaptic plasticity modulation in various brain microcircuits (Fasano et al., 2017; Ibrahim et al., 2020). However, it remains unclear how GluRs can be regulated by VGLUT3 neurotransmission across different brain regions. In this study, we used VGLUT3 knock-out mice (VGLUT3^{-/-}) to assess whether the loss of VGLUT3 alters total and cell surface expression of GluRs in the brain.

We report that the loss of VGLUT3 in mice upregulates the total expression of mGluR5 and mGluR2/3, while it downregulates NMDA2B and dopaminergic D₁ (D1R) cell surface expression in the cerebral cortex. Furthermore, significant reductions in cell surface expression of mGluR5, NMDAR2A/B, D1R, and muscarinic M1 acetylcholine

receptor (M1 mAChR) are observed in the hippocampus of VGLUT3^{-/-} mice. In addition, mGluR2/3 total expression and mGluR5 cell surface levels are higher in the striatum of VGLUT3^{-/-} mice. Finally, AMPAR subunit GluA1 total expression is upregulated throughout cortical, hippocampal, and striatal brain regions of VGLUT3^{-/-} mice. Together, these results provide evidence that GluR densities are dynamically regulated by VGLUT3 in several brain areas, highlighting the key role of this atypical transporter in the regulation of glutamatergic signaling, and potentially synaptic plasticity in these circuits.

Materials and Methods

Reagents

NeutrAvidin agarose beads (catalog #29200), goat anti-rabbit (catalog #G-21234) IgG (H+L) cross-adsorbed horseradish peroxidase secondary antibody, and rabbit anti- β -actin (catalog #PA1-183) were from Thermo Fisher Scientific. Rabbit anti-mGluR 2/3 (catalog #ab6438) and anti-vinculin (catalog #ab129002) were from Abcam. Rabbit anti-mGluR5 (catalog #AB5675), anti-NMDAR2A (catalog #AB1555), anti-NMDAR2B (catalog #AB1557), anti-AMPA1 (catalog #AB1504), and anti-M1 mAChR (catalog #M9808) were from Sigma-Aldrich. Reagents used for Western blotting were from BIO-RAD, and all other biochemical reagents were from Sigma-Aldrich.

Animals

All experimental protocols were approved by the Institutional Animal Care Committee and in compliance with the Canadian Council of Animal Care guidelines. Animals were group housed under a constant 12 h light/dark cycle and given food and water *ad libitum*. Heterozygous knock-out VGLUT3 mice (VGLUT3^{+/-}) were crossed with C57BL/6 mice to establish a mouse colony. Six-month-old wild-type (WT) and homozygous VGLUT3^{-/-} male littermates were used for experiments.

Cell surface biotinylation and immunoblotting

Cell surface biotinylation and immunoblotting experiments were performed as previously reported (Abd-Elrahman et al., 2018; Ibrahim et al., 2021). Mice were euthanized by live cervical dislocation followed by dissection of the cerebral cortex, hippocampus, and striatum. Tissues were cross-chopped with a McIlwain tissue chopper with a slice thickness of 300 μ m and were recovered for 40 min at 37°C in ACSF solution (127 mM NaCl, 2 mM KCl, 1 mM CaCl₂, 1 mM MgSO₄, 10 mM glucose, 1.2 mM KH₂PO₄, 26 mM NaH₂CO₃, pH 7.4) gassed with 95% O₂/5% CO₂. Samples were transferred to tubes and incubated with 1 mg/ml sulfo-NHS-SS-biotin in ACSF for 45 min on ice, and then washed three times with ACSF. Biotinylation was then quenched with 100 mM glycine in ACSF for 70 min on ice. Following three washes in ACSF, samples were homogenized and lysed in RIPA buffer (1% Nonidet P-40 substitute, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl, 150 mM NaCl, and 5 mM EDTA, pH 7.2) containing protease inhibitors (100 μ M 4-benzenesulfonyl fluoride hydrochloride, 80 nM aprotinin, 2 μ M leupeptin, 5 μ M bestatin, 1.5 μ M E-64, and 1 μ M pepstatin A). Protein

This study was supported by Canadian Institutes for Health Research Grants PJT-148656, PJT-165967, and PJT-178060; and funding from the Krembil Foundation to S.S.G.F.

Acknowledgment: We thank Shaunessy Hutchinson for breeding the mouse colony.

*K.S.A.-E. and S.S.G.F. contributed equally to this work as senior authors.

Correspondence should be addressed to Stephen S.G. Ferguson at serguso@uottawa.ca or Khaled S. Abd-Elrahman at kshabdel@ucalgary.ca.
<https://doi.org/10.1523/ENEURO.0041-22.2022>

Copyright © 2022 Ibrahim et al.

This is an open-access article distributed under the terms of the Creative Commons Attribution 4.0 International license, which permits unrestricted use, distribution and reproduction in any medium provided that the original work is properly attributed.

lysates were centrifuged at $18,000 \times g$ at 4°C for 10 min. The supernatant was then collected, and total protein concentrations were quantified using a DC Protein Assay Kit (BIO-RAD). Biotinylated fractions in $500\ \mu\text{g}$ samples were captured with NeutrAvidin agarose beads and were eluted in β -mercaptoethanol containing $3\times$ SDS sample buffer ($187.5\ \text{mM}$ Tris-HCl, 6% SDS, 30% glycerol, 0.006% Bromophenol Blue). Total protein lysates were also aliquoted and diluted to $1\ \mu\text{g}/\mu\text{l}$ in a mixture of lysis buffer and β -mercaptoethanol containing $3\times$ SDS sample buffer and boiled for 10 min at 90°C .

Aliquots containing a total of $30\text{--}40\ \mu\text{g}$ of proteins were resolved by electrophoresis on 6% and 7.5% SDS-PAGE and transferred onto nitrocellulose membranes. Blots were blocked in Tris-buffered saline, pH 7.6, containing 0.05% Tween 20 (TBST) and 5% nonfat dry milk for 1 h at room temperature. Blots were then incubated overnight at 4°C with primary antibodies diluted (1:1000) in TBST containing 1% nonfat dry milk. Membranes were washed three times in TBST and incubated with secondary antibodies (anti-rabbit) diluted (1:5000) in TBST containing 1% nonfat dry milk for 1 h. Blots were imaged using the BIO-RAD Chemidoc Gel Imaging System. Band densities were quantified using Image Lab Software and normalized to the loading control.

Statistical analysis

Mean \pm SEM values and sample sizes for each independent experiment are shown in the figure legends. Data were analyzed with GraphPad Prism 9 for normality and statistical significance. A two-tailed unpaired Student's t test was used for comparisons. The significance level was set at $p < 0.05$.

Results

VGLUT3 deletion differentially alters glutamatergic and nonglutamatergic receptor densities in the mouse cerebral cortex

VGLUT3 is localized in cortical layers II and VI as well as in subpopulations of interneurons within the cerebral cortex (Schäfer et al., 2002; Herzog et al., 2004). Therefore, we assessed the cell surface densities of mGluR5 and mGluR2/3, as they actively modulate synaptic plasticity responses in cortical circuits and are potentially regulated by VGLUT3-expressing fibers (Ballester-Rosado et al., 2010; Joffe et al., 2020; Abd-Elrahman and Ferguson, 2022). We found that VGLUT3 deletion significantly elevated mGluR5 and mGluR2/3 expression levels in the cerebral cortex of VGLUT3^{-/-} male mice compared with WT mice (Fig. 1A,B). Both NMDAR and AMPAR mediate VGLUT3 signaling in different brain areas (Higley et al., 2011; Nelson et al., 2014; Wang et al., 2019). We therefore assessed the expression of iGluR members NMDARs and AMPARs via probing for their essential regulatory subunits NMDAR2A/2B and GluA1, respectively (Paoletti et al., 2013; Henley and Wilkinson, 2016). We detected no change in the total expression levels of NMDAR2A/2B subunits in the cortex of VGLUT3^{-/-} mice compared with WT mice (Fig. 1C,D). However, cell surface expression of

NMDAR2B, but not NMDAR2A, was significantly reduced in the cortex of VGLUT3^{-/-} mice (Fig. 1C,D). On the contrary, the loss of VGLUT3 significantly upregulated cell surface and total expression levels of AMPAR subunit GluA1 (Fig. 1E).

Given that VGLUT3⁺ neurons actively regulate dopaminergic and cholinergic neurotransmission (Trudeau and El Mestikawy, 2018), we probed for dopaminergic D1R and muscarinic M1 mAChR that are enriched in cortical GABAergic interneurons and pyramidal neurons, respectively (Levey et al., 1991; Anagnostaras et al., 2003; Puig et al., 2014). VGLUT3 ablation did not alter the total expression of either D1R or M1 mAChR in the cortex of VGLUT3^{-/-} mice (Fig. 1F,G). However, cell surface D1R level, but not M1 mAChR level, was significantly reduced in the cortex of VGLUT3^{-/-} mice (Fig. 1F,G).

Deletion of VGLUT3 reduces cell surface levels of mGluR5, NMDAR, D1R, and M1 mAChR in the hippocampus

Within the hippocampus, mGluRs and iGluRs are expressed in both excitatory glutamatergic and inhibitory GABAergic interneurons, with distinct patterns for each receptor subtype (Reiner and Levitz, 2018). Hippocampal VGLUT3 is found in subpopulations of GABAergic interneurons, cholecystokinin-positive (CCK⁺) basket cells, as well as in subsets of VGLUT3-expressing serotonergic varicosities (Somogyi et al., 2004; Amilhon et al., 2010). In the hippocampus of VGLUT3^{-/-} mice, we found that cell surface mGluR5 levels were significantly reduced, with no change in their total expression levels (Fig. 2A). In addition, both cell surface and total expression levels of mGluR2/3 remained unaffected by VGLUT3 deletion in mice (Fig. 2B). However, cell surface fractions, but not total expression, of NMDAR2A and NMDAR2B were significantly reduced in the hippocampus of VGLUT3^{-/-} mice (Fig. 2C,D). Furthermore, total but not cell surface GluA1 levels were elevated in the hippocampus of VGLUT3^{-/-} mice (Fig. 2E). The loss of VGLUT3 reduced the cell surface levels of D1R and M1 mAChR without altering their total expression in the hippocampus (Fig. 2F,G).

VGLUT3 loss increases the total expression of mGluR2/3, AMPARs, and cell surface mGluR5 levels in the striatum

Striatal VGLUT3 signaling is chiefly mediated via cholinergic interneurons (Higley et al., 2011; Gonzales and Smith, 2015). These interneurons use both acetylcholine and glutamate to fine-tune dopaminergic inputs onto the medium spiny neurons, ultimately regulating striatal network activities (Gras et al., 2008; Higley et al., 2011; Sakae et al., 2015). We found that the loss of VGLUT3 increased cell surface mGluR5 levels in the striatum, with no detectable changes in cell surface mGluR2/3 levels (Fig. 3A,B). However, total mGluR2/3, but not mGluR5, levels were significantly elevated in the striatum of VGLUT3^{-/-} mice (Fig. 3A,B). We also detected no changes in the total or cell surface expression of NMDAR2A and NMDAR2B in the striatum

Cerebral cortex

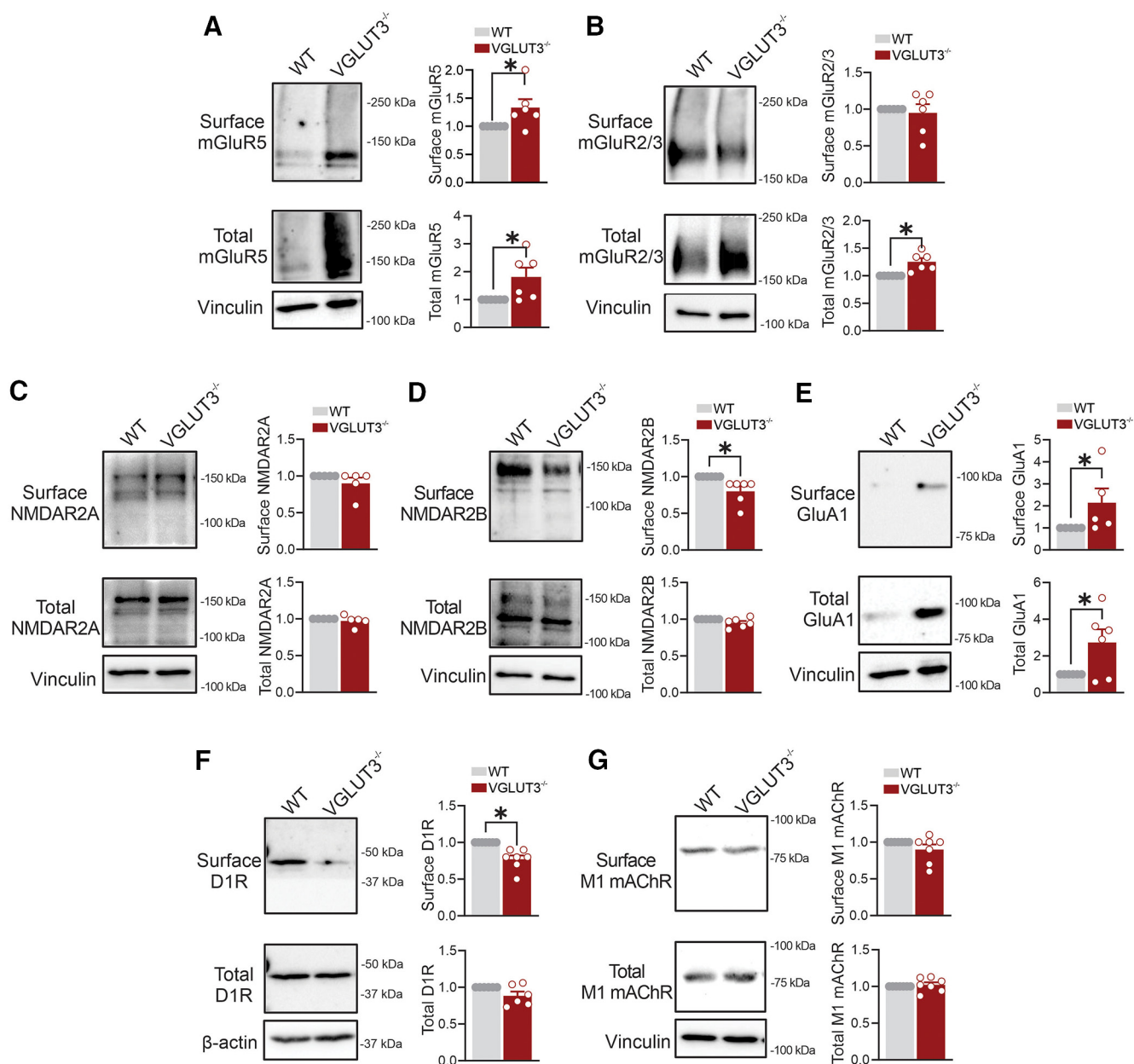


Figure 1. The effect of VGLUT3 deletion on glutamate and nonglutamate receptor expression in the mouse cerebral cortex. **A–G**, Representative immunoblots and quantification of cell surface and total levels of mGluR5 (**A**), mGluR2/3 (**B**), NMDAR2A (**C**), NMDAR2B (**D**), GluA1 (**E**), D1R (**F**), and M1 mAChR (**G**) in cortical lysates from VGLUT3^{-/-} and C57BL/6 (WT) male mice. Data are the mean \pm SEM ($n = 5$ –6 mice/group), normalized to the respective surface or total WT values. * $p < 0.05$ assessed by unpaired Student's t test.

of VGLUT3^{-/-} mice compared with WT mice (Fig. 3C,D). Yet, VGLUT3 loss elevated both cell surface and total expression levels of GluA1 subunits (Fig. 3E). The expression of D1R and M1 mAChR in the striatum remained unaffected by VGLUT3 deletion (Fig. 3F,G).

Discussion

Despite the robust evidence supporting the role of VGLUT3 in regulating nonglutamatergic neurotransmission

(for review, see Trudeau and El Mestikawy, 2018; Ibrahim et al., 2020), little is known about how VGLUT3-associated networks regulate glutamatergic neurotransmission. As summarized in Table 1, we show that genetic deletion of VGLUT3 differentially alters the expression of glutamate and nonglutamate receptors in several brain areas. Specifically, VGLUT3 ablation upregulates the expression of mGluR5 and mGluR2/3, and reduces cell surface NMDA2B and D1R levels in the cerebral cortex. In the hippocampus, VGLUT3 loss reduces cell surface levels of mGluR5, NMDAR2A/2B, as well as

Hippocampus

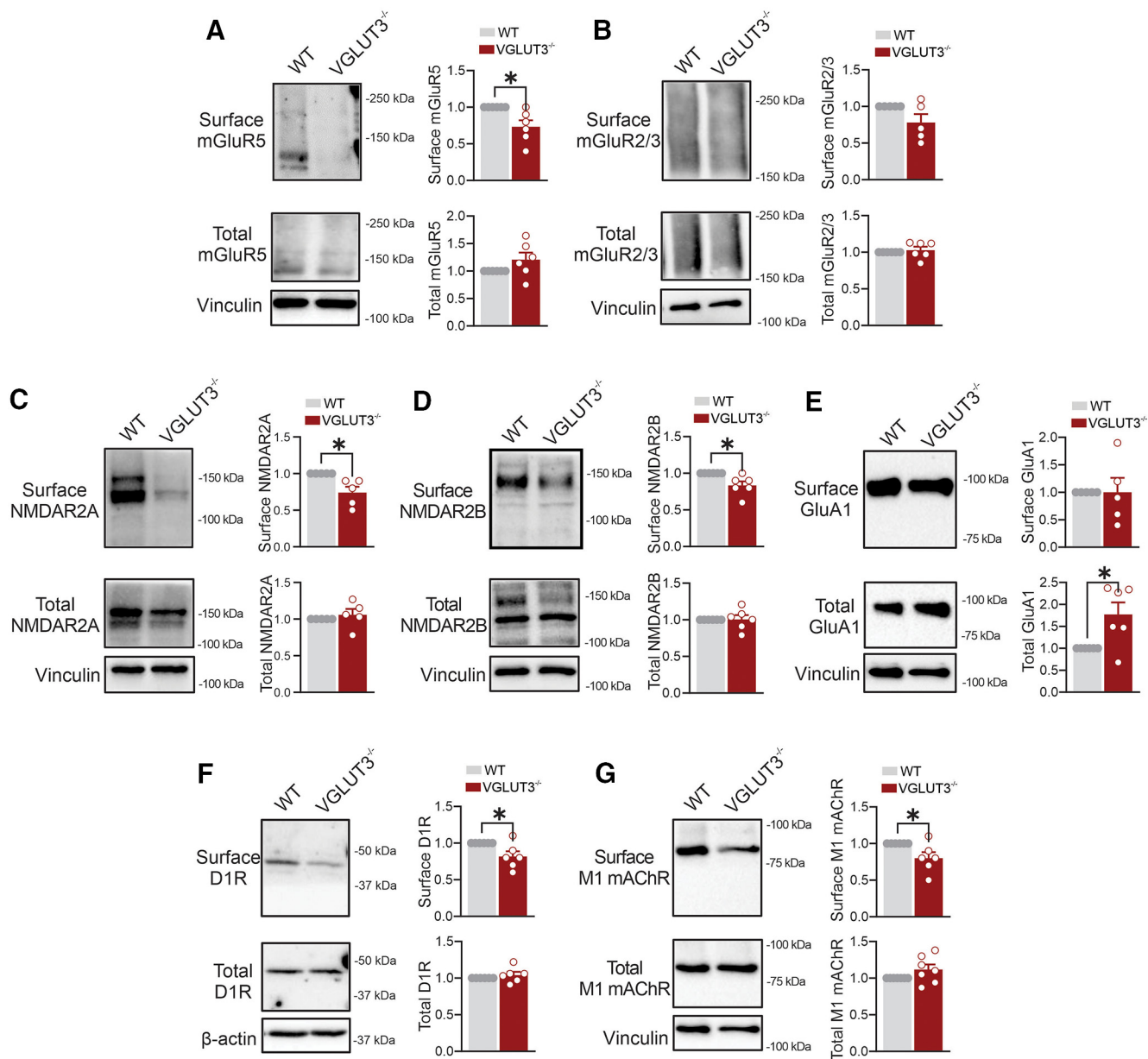


Figure 2. The effect of VGLUT3 deletion on glutamate and nonglutamate receptors expression in the mouse hippocampus. **A–G**, Representative immunoblots and quantification of cell surface and total levels of mGluR5 (**A**), mGluR2/3 (**B**), NMDAR2A (**C**), NMDAR2B (**D**), GluA1 (**E**), D1R (**F**), and M1 mAChR (**G**) in hippocampal lysates from VGLUT3^{-/-} and C57BL/6 (WT) male mice. Data are the mean ± SEM (*n* = 5–6 mice/group), normalized to the respective surface or total WT values. **p* < 0.05 assessed by unpaired Student’s *t* test.

D1Rs and M1 mAChRs. Cell surface mGluR5 and total mGluR2/3 densities are increased in the striatum of VGLUT3^{-/-} mice. Last, the loss of VGLUT3 upregulates AMPAR subunit GluA1 in the cortex, the hippocampus, and the striatum. Together, these findings complement and provide further evidence that VGLUT3, despite its low abundance, is a key modulator of GluR densities across the brain, highlighting its role in glutamatergic transmission, synaptic plasticity, and brain circuit homeostasis.

Evidence indicates that VGLUT3 modulates cortical and hippocampal microcircuits via CCK⁺ basket cells (Herzog et al., 2004; Somogyi et al., 2004; Nguyen et al., 2020). These interneurons use VGLUT3-dependent glutamate to dampen GABAergic currents onto pyramidal neurons via feedback activation of presynaptic mGluR autoreceptors (Fasano et al., 2017). VGLUT3 transmission also mediates excitatory inputs onto pyramidal neurons as well as interneurons via activation of AMPARs and

Striatum

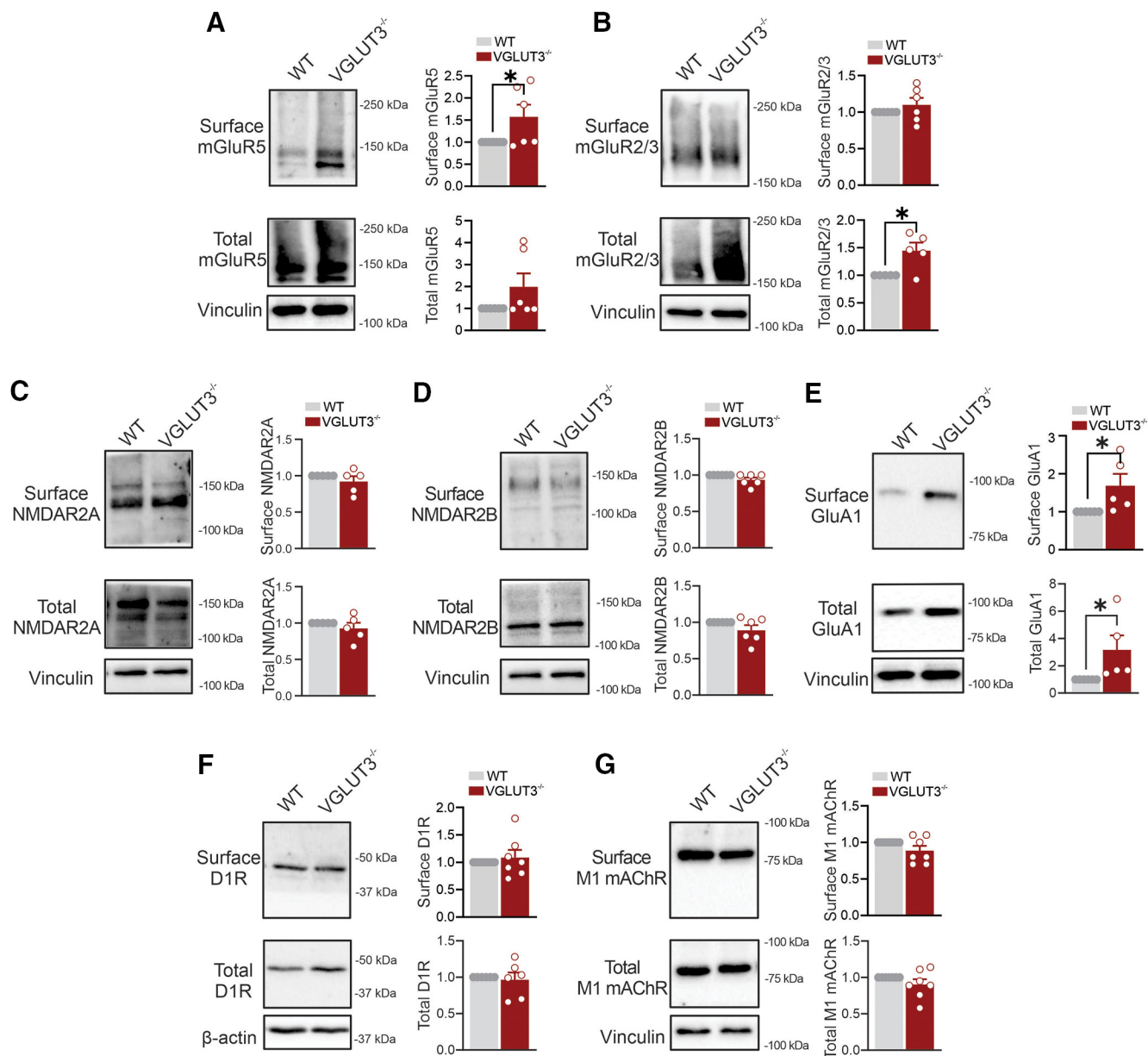


Figure 3. The effect of VGLUT3 deletion on glutamate and nonglutamate receptor expression in the mouse striatum. **A–G**, Representative immunoblots and quantification of cell surface and total levels of mGluR5 (**A**), mGluR2/3 (**B**), NMDAR2A (**C**), NMDAR2B (**D**), GluA1 (**E**), D1R (**F**), and M1 mAChR (**G**) in striatal lysates from VGLUT3^{-/-} and C57BL/6 (WT) male mice. Data are the mean ± SEM (*n* = 5–6 mice/group), normalized to the respective surface or total WT values. **p* < 0.05 assessed by unpaired Student’s *t* test.

Table 1: Summary of changes in cell surface and total levels of receptors in the different brain regions of VGLUT3^{-/-} mice

Brain regions	mGluR5		mGluR2/3		NMDAR2A		NMDAR2B		GluA1		D1R		M1 mAChR	
	Surface	Total	Surface	Total	Surface	Total	Surface	Total	Surface	Total	Surface	Total	Surface	Total
Cerebral cortex	↑	↑	-	↑	-	-	↓	-	↑	↑	-	-	-	-
Hippocampus	↓	-	-	-	↓	-	↓	-	-	↓	-	-	↓	-
Striatum	↑	-	-	↑	-	-	-	-	↑	↑	-	-	-	-

(-) denotes no change.

NMDARs (Nguyen et al., 2020). Moreover, VGLUT3-expressing basket cells form synaptic contacts with mGluR5 on pyramidal neurons in the cortical and cortex-like amygdaloid regions (Omiya et al., 2015). Consistent with these reports, we observed upregulation in both mGluR5 and AMPAR subunit GluA1 in the cortex upon deletion of VGLUT3. Yet, cell surface levels of NMDAR2B, but not NMDAR2A, were decreased in the cortex of VGLUT3^{-/-} mice. This intriguing observation suggests that VGLUT3 exerts sophisticated regulation of some NMDAR subpopulations in cortical neurons. Interestingly, NMDAR2B-containing receptors exhibit slower deactivation kinetics and facilitated surface mobility compared with NMDAR2A-containing receptors (Vicini et al., 1998; Groc et al., 2006), ultimately affecting the nature of the plasticity response (Paoletti et al., 2013). Thus, the reduction in NMDAR2B cell surface levels in VGLUT3^{-/-} cortex suggests that VGLUT3 may alter the induction thresholds of long-term potentiation (LTP) and long-term depression (LTD) in the cortical neurons. This agrees with the observed decreases in cell surface D1R levels and upregulation in mGluR2/3 levels in VGLUT3^{-/-} cortex: both actively modulate LTP and LTD induction/maintenance in the cortex (Otani et al., 1999; Huang et al., 2004; Joffe et al., 2020, 2021). Together, our findings further support the role of VGLUT3 in modulating the balance between LTP and LTD plasticity responses in corticohippocampal networks (Fasano et al., 2017).

The observed reductions in mGluR5 and NMDAR membrane recruitment in VGLUT3^{-/-} hippocampus suggest an adaptive reduction in glutamatergic transmission strength in the hippocampal circuits. Both group I mGluRs and NMDARs represent key players in regulating synaptic metaplasticity in hippocampal neurons (Reiner and Levitz, 2018). Similarly, inputs from VGLUT3⁺ interneurons are reported to modulate metaplasticity in glutamatergic synapses within the regional circuits of the hippocampus (del Pino et al., 2017; Fasano et al., 2017). Therefore, it is possible that the reduction in mGluR5 and NMDAR cell surface levels may underlie altered metaplasticity in the ventral hippocampus of VGLUT3^{-/-} mice (Fasano et al., 2017). We also noted a decrease in cell surface densities of D1R and M1 mAChR, both of which are expressed in VGLUT3⁺ interneurons and regulate synaptic plasticity mechanisms and memory-processing functions in the hippocampus (Cea-del Rio et al., 2010; Furini et al., 2014; Dennis et al., 2016; Puighermanal et al., 2017). Collectively, these changes in glutamatergic and nonglutamatergic receptor topographies might explain the impaired working memory and cognitive flexibility previously reported in VGLUT3^{-/-} mice (Fazekas et al., 2019).

VGLUT3 signaling plays an important role in striatal microcircuits involved in reward-guided behaviors (Ibrahim et al., 2020). We show here that VGLUT3 loss triggers an increase in cell surface mGluR5, as well total mGluR2/3 levels in the striatum. Both receptors play a well characterized role in the striatal circuits modulating drug reward and motor function (Kenny and Markou, 2004; Conn et al., 2005; Paterson and Markou, 2005; Peters and Kalivas, 2006). Thus, our findings indicate that VGLUT3, via its dynamic regulation of striatal mGluR densities, can play a

critical role in the neuropathophysiology of drug addiction and movement disorders (Sakae et al., 2015; Areal et al., 2019; Ibrahim et al., 2020).

An intriguing aspect of our findings is the persistent upregulation of AMPAR subunit GluA1 across cortical, hippocampal, and striatal regions of VGLUT3^{-/-} mice. This suggests that AMPAR activation might be regulated by VGLUT3 neurotransmission, independent of NMDARs. Previous reports have shown that VGLUT3-expressing interneurons mediate excitatory inputs via AMPAR activation in the prefrontal cortex (Nguyen et al., 2020), the hippocampus (Pelkey et al., 2020), and the striatum (Nelson et al., 2014). Furthermore, synaptic scaling of AMPARs is directly implicated in central neural circuits that control spatial working memory functions (Bannerman et al., 2004; van Vugt et al., 2020) and reward behavior (Sutton et al., 2003). Thus, the observed AMPAR alterations could potentially be implicated in the impaired working memory and exacerbated cocaine hedonic effects reported in VGLUT3^{-/-} mice (Sakae et al., 2015; Fazekas et al., 2019) which warrants further investigation.

It is worth noting that our findings are subject to at least two limitations. First, while cell surface biotinylation is efficient in yielding highly purified plasma membrane proteins, the technique cannot provide spatial information on precise distribution of the receptors in different synaptic domains. Second, we focused on male VGLUT3^{-/-} mice as prior studies did not report major differences between male and female knock-out mice, in either their phenotype or network activities (Amilhon et al., 2010; Higley et al., 2011; Divito et al., 2015; Ramet et al., 2017). Nonetheless, we cannot rule out sex-dependent changes in GluR densities in VGLUT3^{-/-} mice.

In summary, we provide evidence that VGLUT3, despite its low abundance, can be a key regulator of mGluR and iGluR densities in different brain regions, highlighting the role of VGLUT3 in synaptic function and plasticity. Moreover, because VGLUT3 regulates specialized brain circuitries involved in reward processing, emotional states, and motor coordination, this neuronal adaption of GluR densities might provide a more comprehensive understanding of VGLUT3-dependent mechanisms underlying various neurologic and neuropsychiatric disorders such as anxiety (Amilhon et al., 2010), drug addiction (Sakae et al., 2015), or motor disorders such as Parkinson's disease (Divito et al., 2015; Ribeiro et al., 2017).

References

- Abd-Elrahman KS, Hamilton A, Vasefi M, Ferguson SSG (2018) Autophagy is increased following either pharmacological or genetic silencing of mGluR5 signaling in Alzheimer's disease mouse models. *Mol Brain* 11:19.
- Abd-Elrahman KS, Ferguson SSG (2022) Noncanonical metabotropic glutamate receptor 5 signaling in Alzheimer's disease. *Annu Rev Pharmacol Toxicol* 62:235–254.
- Amilhon B, Lepicard E, Renoir T, Mongeau R, Popa D, Poirel O, Miot S, Gras C, Gardier AM, Gallego J, Hamon M, Lanfumey L, Gasnier B, Giros B, El Mestikawy S (2010) VGLUT3 (vesicular glutamate transporter type 3) contribution to the regulation of serotonergic transmission and anxiety. *J Neurosci* 30:2198–2210.

- Anagnostaras SG, Murphy GG, Hamilton SE, Mitchell SL, Rahnama NP, Nathanson NM, Silva AJ (2003) Selective cognitive dysfunction in acetylcholine M1 muscarinic receptor mutant mice. *Nat Neurosci* 6:51–58.
- Areal LB, Hamilton A, Martins-Silva C, Pires RGW, Ferguson SSG (2019) Neuronal scaffolding protein spinophilin is integral for cocaine-induced behavioral sensitization and ERK1/2 activation. *Mol Brain* 12:15.
- Ballester-Rosado CJ, Albright MJ, Wu C-S, Liao C-C, Zhu J, Xu J, Lee L-J, Lu H-C (2010) mGluR5 in cortical excitatory neurons exerts both cell-autonomous and -nonautonomous influences on cortical somatosensory circuit formation. *J Neurosci* 30:16896–16909.
- Bannerman DM, Deacon RMJ, Brady S, Bruce A, Sprengel R, Seeburg PH, Rawlins JNP (2004) A comparison of GluR-A-deficient and wild-type mice on a test battery assessing sensorimotor, affective, and cognitive behaviors. *Behav Neurosci* 118:643–647.
- Cea-del Rio CA, Lawrence JJ, Tricoire L, Erdelyi F, Szabo G, McBain CJ (2010) M3 muscarinic acetylcholine receptor expression confers differential cholinergic modulation to neurochemically distinct hippocampal basket cell subtypes. *J Neurosci* 30:6011–6024.
- Conn PJ, Battaglia G, Marino MJ, Nicoletti F (2005) Metabotropic glutamate receptors in the basal ganglia motor circuit. *Nat Rev Neurosci* 6:787–798.
- del Pino I, Brotons-Mas JR, Marques-Smith A, Marighetto A, Frick A, Marin O, Rico B (2017) Abnormal wiring of CCK+ basket cells disrupts spatial information coding. *Nat Neurosci* 20:784–792.
- Dennis SH, Pasqui F, Colvin EM, Sanger H, Mogg AJ, Felder CC, Broad LM, Fitzjohn SM, Isaac JTR, Mellor JR (2016) Activation of muscarinic M1 acetylcholine receptors induces long-term potentiation in the hippocampus. *Cereb Cortex* 26:414–426.
- Divito CB, Steece-Collier K, Case DT, Williams S-PG, Stancati JA, Zhi L, Rubio ME, Sortwell CE, Collier TJ, Sulzer D, Edwards RH, Zhang H, Seal RP (2015) Loss of VGLUT3 produces circadian-dependent hyperdopaminergia and ameliorates motor dysfunction and L-Dopa-mediated dyskinesias in a model of Parkinson's disease. *J Neurosci* 35:14983–14999.
- El Mestikawy S, Wallén-Mackenzie Å, Fortin GM, Descarries L, Trudeau L-E (2011) From glutamate co-release to vesicular synergy: vesicular glutamate transporters. *Nat Rev Neurosci* 12:204–216.
- Fasano C, Rocchetti J, Pietrajtis K, Zander J-F, Manseau F, Sakae DY, Marcus-Sells M, Ramet L, Morel LJ, Carrel D, Dumas S, Bolte S, Bernard V, Vigneault E, Goutagny R, Ahnert-Hilger G, Giros B, Dumas S, Williams S, El Mestikawy S (2017) Regulation of the hippocampal network by VGLUT3-positive CCK-GABAergic basket cells. *Front Cell Neurosci* 11:140.
- Fazekas CL, Balázsfi D, Horváth HR, Balogh Z, Aliczki M, Puhova A, Balagova L, Chmelova M, Jezova D, Haller J, Zelena D (2019) Consequences of VGLUT3 deficiency on learning and memory in mice. *Physiol Behav* 212:112688.
- Fremeau RT, Voglmaier S, Seal RP, Edwards RH (2004) VGLUTs define subsets of excitatory neurons and suggest novel roles for glutamate. *Trends Neurosci* 27:98–103.
- Furini CRG, Myskiw JC, Schmidt BE, Marcondes LA, Izquierdo I (2014) D1 and D5 dopamine receptors participate on the consolidation of two different memories. *Behav Brain Res* 271:212–217.
- Gonzales KK, Smith Y (2015) Cholinergic interneurons in the dorsal and ventral striatum: anatomical and functional considerations in normal and diseased conditions. *Ann N Y Acad Sci* 1349:1–45.
- Gras C, Herzog E, Belenchi GC, Bernard VRV, Ravassard P, Pohl M, Gasnier B, Giros B, El Mestikawy S, El Mestikawy S (2002) A third vesicular glutamate transporter expressed by cholinergic and serotonergic neurons. *J Neurosci* 22:5442–5451.
- Gras C, Amilhon B, Lepicard ÈM, Poirel O, Vinatier J, Herbin M, Dumas S, Tzavara ET, Wade MR, Nomikos GG, Hanoun N, Saurini F, Kemel M-LL, Gasnier B, Giros B, Mestikawy SE (2008) The vesicular glutamate transporter VGLUT3 synergizes striatal acetylcholine tone. *Nat Neurosci* 11:292–300.
- Groc L, Heine M, Cousins SL, Stephenson FA, Lounis B, Cognet L, Choquet D (2006) NMDA receptor surface mobility depends on NR2A-2B subunits. *Proc Natl Acad Sci U S A* 103:18769–18774.
- Henley JM, Wilkinson KA (2016) Synaptic AMPA receptor composition in development, plasticity and disease. *Nat Rev Neurosci* 17:337–350.
- Herzog E, Gilchrist J, Gras C, Muzerelle A, Ravassard P, Giros B, Gaspar P, El Mestikawy S (2004) Localization of VGLUT3, the vesicular glutamate transporter type 3, in the rat brain. *Neuroscience* 123:983–1002.
- Higley MJ, Gittis AH, Oldenburg IA, Balthasar N, Seal RP, Edwards RH, Lowell BB, Kreitzer AC, Sabatini BL (2011) Cholinergic interneurons mediate fast VGLUT3-dependent glutamatergic transmission in the striatum. *PLoS One* 6:e19155.
- Huang Y-Y, Simpson E, Kellendonk C, Kandel ER (2004) Genetic evidence for the bidirectional modulation of synaptic plasticity in the prefrontal cortex by D1 receptors. *Proc Natl Acad Sci U S A* 101:3236–3241.
- Ibrahim KS, McLaren CJ, Abd-Elrahman KS, Ferguson SSG (2021) Optineurin deletion disrupts metabotropic glutamate receptor 5-mediated regulation of ERK1/2, GSK3 β /ZBTB16, mTOR/ULK1 signaling in autophagy. *Biochem Pharmacol* 185:114427.
- Ibrahim KS, Abd-Elrahman KS, El Mestikawy S, Ferguson SSG (2020) Targeting vesicular glutamate transporter machinery: implications on metabotropic glutamate receptor 5 signaling and behavior. *Mol Pharmacol* 98:314–327.
- Joffe ME, Santiago CI, Oliver KH, Maksymetz J, Harris NA, Engers JL, Lindsley CW, Winder DG, Conn PJ (2020) mGlu2 and mGlu3 negative allosteric modulators divergently enhance thalamocortical transmission and exert rapid antidepressant-like effects. *Neuron* 105:46–59.e3.
- Joffe ME, Santiago CI, Vermudez SAD, Fisher NM, Dogra S, Niswender CM, Jeffrey Conn P (2021) Frontal cortex genetic ablation of metabotropic glutamate receptor subtype 3 (mGlu3) impairs postsynaptic plasticity and modulates affective behaviors. *Neuropsychopharmacology* 46:2148–2157.
- Kenny PJ, Markou A (2004) The ups and downs of addiction: role of metabotropic glutamate receptors. *Trends Pharmacol Sci* 25:265–272.
- Levey AI, Kitt CA, Simonds WF, Price DL, Brann MR (1991) Identification and localization of muscarinic acetylcholine receptor proteins in brain with subtype-specific antibodies. *J Neurosci* 11:3218–3226.
- Nelson AB, Bussert TG, Kreitzer AC, Seal RP (2014) Striatal cholinergic neurotransmission requires VGLUT3. *J Neurosci* 34:8772–8777.
- Nguyen R, Venkatesan S, Binko M, Bang JY, Cajanding JD, Briggs C, Sargin D, Imayoshi I, Lambe EK, Kim JC (2020) Cholecystokinin-expressing interneurons of the medial prefrontal cortex mediate working memory retrieval. *J Neurosci* 40:2314–2331.
- Omiya Y, Uchigashima M, Konno K, Yamasaki M, Miyazaki T, Yoshida T, Kusumi I, Watanabe M (2015) VGLUT3-expressing CCK-positive basket cells construct invaginating synapses enriched with endocannabinoid signaling proteins in particular cortical and cortex-like amygdaloid regions of mouse brains. *J Neurosci* 35:4215–4228.
- Otani S, Auclair N, Desce J-M, Roisin M-P, Crépel F (1999) Dopamine receptors and groups I and II mGluRs cooperate for long-term depression induction in rat prefrontal cortex through converging postsynaptic activation of MAP kinases. *J Neurosci* 19:9788–9802.
- Paoletti P, Bellone C, Zhou Q (2013) NMDA receptor subunit diversity: impact on receptor properties, synaptic plasticity and disease. *Nat Rev Neurosci* 14:383–400.
- Paterson NE, Markou A (2005) The metabotropic glutamate receptor 5 antagonist MPEP decreased break points for nicotine, cocaine and food in rats. *Psychopharmacology (Berl)* 179:255–261.
- Pelkey KA, Calvigioni D, Fang C, Vargish G, Ekins T, Auville K, Wester JC, Lai M, Mackenzie-Gray Scott C, Yuan X, Hunt S,

- Abebe D, Xu Q, Dimidschstein J, Fishell G, Chittajallu R, McBain CJ (2020) Paradoxical network excitation by glutamate release from VGLUT3⁺ GABAergic interneurons. *Elife* 9:e51996.
- Peters J, Kalivas PW (2006) The group II metabotropic glutamate receptor agonist, LY379268, inhibits both cocaine- and food-seeking behavior in rats. *Psychopharmacology (Berl)* 186:143–149.
- Puig MV, Rose J, Schmidt R, Freund N (2014) Dopamine modulation of learning and memory in the prefrontal cortex: insights from studies in primates, rodents, and birds. *Front Neural Circuits* 8:93.
- Puighermanal E, Cutando L, Boubaker-Vitre J, Honoré E, Longueville S, Hervé D, Valjent E (2017) Anatomical and molecular characterization of dopamine D1 receptor-expressing neurons of the mouse CA1 dorsal hippocampus. *Brain Struct Funct* 222:1897–1911.
- Ramet L, Zimmermann J, Bersot T, Poirel O, De Gois S, Silm K, Sakae DY, Mansouri-Guilani N, Bourque M-J, Trudeau L-E, Pietrancosta N, Daumas S, Bernard V, Rosenmund C, El Mestikawy S (2017) Characterization of a human point mutation of VGLUT3 (p.A211V) in the rodent brain suggests a nonuniform distribution of the transporter in synaptic vesicles. *J Neurosci* 37:4181–4199.
- Reiner A, Levitz J (2018) Glutamatergic signaling in the central nervous system: ionotropic and metabotropic receptors in concert. *Neuron* 98:1080–1098.
- Ribeiro FM, Vieira LB, Pires RGW, Olmo RP, Ferguson SSG (2017) Metabotropic glutamate receptors and neurodegenerative diseases. *Pharmacol Res* 115:179–191.
- Sakae DY, et al. (2015) The absence of VGLUT3 predisposes to cocaine abuse by increasing dopamine and glutamate signaling in the nucleus accumbens. *Mol Psychiatry* 20:1448–1459.
- Schäfer MKH, Varoqui H, Defamie N, Weihe E, Erickson JD (2002) Molecular cloning and functional identification of mouse vesicular glutamate transporter 3 and its expression in subsets of novel excitatory neurons. *J Biol Chem* 277:50734–50748.
- Somogyi J, Baude A, Omori Y, Shimizu H, El Mestikawy S, Fukaya M, Shigemoto R, Watanabe M, Somogyi P (2004) GABAergic basket cells expressing cholecystokinin contain vesicular glutamate transporter type 3 (VGLUT3) in their synaptic terminals in hippocampus and isocortex of the rat. *Eur J Neurosci* 19:552–569.
- Sutton MA, Schmidt EF, Choi K-H, Schad CA, Whisler K, Simmons D, Karanian DA, Monteggia LM, Neve RL, Self DW (2003) Extinction-induced upregulation in AMPA receptors reduces cocaine-seeking behaviour. *Nature* 421:70–75.
- Trudeau L-E, El Mestikawy S (2018) Glutamate cotransmission in cholinergic, GABAergic and monoamine systems: contrasts and commonalities. *Front Neural Circuits* 12:113.
- van Vugt B, van Kerkoerle T, Vartak D, Roelfsema PR (2020) The contribution of AMPA and NMDA receptors to persistent firing in the dorsolateral prefrontal cortex in working memory. *J Neurosci* 40:2458–2470.
- Vicini S, Wang JF, Li JH, Zhu WJ, Wang YH, Luo JH, Wolfe BB, Grayson DR (1998) Functional and pharmacological differences between recombinant *N*-methyl-D-aspartate receptors. *J Neurophysiol* 79:555–566.
- Wang H-L, Zhang S, Qi J, Wang H, Cachepe R, Mejias-Aponte CA, Gomez JA, Mateo-Semidey GE, Beaudoin GMJ, Paladini CA, Cheer JF, Morales M (2019) Dorsal raphe dual serotonin-glutamate neurons drive reward by establishing excitatory synapses on VTA mesoaccumbens dopamine neurons. *Cell Rep* 26:1128–1142.e7.