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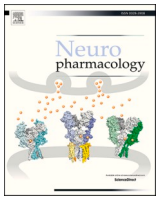

Evidence for low affinity of GABA at the vesicular monoamine transporter VMAT2 – Implications for transmitter co-release from dopamine neurons

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ABSTRACT

Midbrain dopamine (DA) neurons comprise a heterogeneous population of cells. For instance, some DA neurons express the vesicular glutamate transporter VGLUT2 allowing these cells to co-release DA and glutamate. Additionally, GABA may be co-released from DA neurons. However, most cells do not express the canonical machinery to synthesize GABA or the vesicular GABA transporter VGAT. Instead, GABA seems to be taken up into DA neurons by a plasmalemmal GABA transporter (GAT1) and stored in synaptic vesicles via the vesicular monoamine transporter VMAT2. Yet, it remains unclear whether GABA indeed interacts with VMAT2.

Here, we used radiotracer flux measurements in VMAT2 expressing HEK-293 cells and synaptic vesicles from male and female mice to determine whether GABA qualifies as substrate at VMAT2. We found that GABA reduced uptake of VMAT2 substrates in mouse synaptic vesicle preparations from striatum and cerebellum at millimolar concentrations but had no effect in VMAT2-expressing HEK-293 cells. Interestingly, while the closely related amino acid glycine did not affect substrate uptake at VMAT2 in mouse synaptic vesicles, the amino sulfonic acid taurine reduced uptake similar to GABA. Lastly, we discovered that the majority of mouse and human midbrain DA neurons in the substantia nigra of either sex expressed VMAT2 and GAT1 suggesting that most of them could be capable of co-releasing DA and GABA. Together, our findings suggest that GABA is a low-affinity substrate at VMAT2 with potential implications for basal ganglia physiology and disease.

1. Introduction

The majority of dopamine (DA) neurons in the central nervous system are located in the midbrain. Midbrain DA neurons can grossly be subdivided into substantia nigra *pars compacta* (SNc) and ventral tegmental area (VTA) (Dahlstroem and Fuxe, 1964). Though the overall number of DA neurons in these regions is comparatively small (about 500,000 DA neurons in the human SNc and VTA and about 25,000 DA neurons in mouse SNc and VTA) (Damier et al., 1999; German et al., 1996; Hirsch et al., 1989; Nelson et al., 1996), the DA system is of particular interest to neuropharmacology because of many neuropsychiatric diseases associated with DA system dysfunction, most notably Parkinson's disease (PD), schizophrenia, substance use disorders and attention-deficit hyperactivity disorder (Carlsson, 2001; Costa and Schoenbaum, 2022; Hornykiewicz, 2006; Iversen and Iversen, 2007).

For a long time, DA neurons were thought to be a rather homogenous population of cells that despite innervating different brain structures and involvement with many neurologic and psychiatric diseases, are largely similar due to their synthesis and release of the neurotransmitter DA. The complex nature and heterogeneity of the DA system was only slowly recognized in the last 30 years and became widely appreciated with advances in single-cell profiling (for summary see (Poulin et al., 2020)).

One feature that underlines the complexity of midbrain DA neurons

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results from co-release of multiple neurotransmitters. For instance, subsets of DA neurons located in the medial VTA and dorsolateral SNc express the vesicular glutamate transporter VGLUT2 and co-release glutamate, the major excitatory neurotransmitter in the central nervous system (Dal Bo et al., 2004; Kawano et al., 2006; Yamaguchi et al., 2007). Expression of VGLUT2 is both necessary and sufficient to allow DA neurons to store and release glutamate (Hnasko et al., 2010; Steinkellner et al., 2018; Yamaguchi et al., 2011), because glutamate is presumably present in all neurons at millimolar concentrations (Featherstone, 2010).

It was also shown that some DA neurons co-release the major inhibitory neurotransmitter ɣ-aminobutyric acid (GABA) (Tritsch et al., 2012) but GABA co-release is anatomically and functionally less well defined because DA neurons do not widely express the classical enzymes required for GABA synthesis (glutamic acid decarboxylase 1 and 2; GAD1/2) and/or the vesicular GABA transporter (VGAT), which is generally required for vesicular sequestration of GABA into synaptic vesicles (McIntire et al., 1997).

Rather, the vesicular transporter for DA, VMAT2, seems to be required for the transport of GABA into synaptic vesicles (Tritsch et al., 2012). This finding was rather surprising because GABA is a zwitterionic amino acid and structurally very different from classical VMAT2 substrates that have an aromatic ring and a positive charge (Peter et al., 1994; Yelin and Schuldiner, 1995; Zheng et al., 2006). In contrast to glutamate, which is presumably present in all cells with active gene expression, GABA is typically produced through decarboxylation of glutamate in cells containing GAD1 or GAD2, which are little expressed in DA neurons (Azcorra et al., 2023; Gaertner et al., 2024; Tritsch et al., 2014). But how do DA neurons get GABA in the first place?

It seems that GABA is largely taken up into DA neurons from outside via the plasmalemmal GABA transporters GAT1 (Tritsch et al., 2016). Alternatively, GABA may be synthesized *de novo* in DA neurons through aldehyde dehydrogenase 1a1 (ALDH1a1) (Kim et al., 2015), an enzyme widely expressed in SNc DA neurons. Importantly, there is now also evidence for a physiological role of GABA release from DA neurons wherein GABA was shown to reduce phasic DA release via an inhibitory autofeedback mechanism (Patel et al., 2024) and to be important for the maintenance of DA synapses (Kim et al., 2023)

Together, though there is strong electrophysiological and genetic evidence that GABA is released from DA neurons in a VMAT2-dependent manner, the relative affinity of GABA at VMAT2 remains unknown. Using radiotracer flux measurements, we show that GABA weakly competes with monoamine uptake at VMAT2 that is considerably lower than that of the cognate substrates DA or serotonin (5-HT). Finally, we provide evidence for widespread expression of GAT1 not only in mouse but also in human midbrain DA neurons suggesting that mechanisms subserving GABA co-release may be conserved across mice and humans.

2. Materials and methods

2.1. Materials

3,4-[Ring-2,5,6-^{3}H]-dihydroxyphenylethylamine (dopamine), 1 mCi/mL and 5-[1,2–^{3}H(N)]-hydroxytryptamine creatinine sulfate (serotonin; 5-HT), 1 mCi/mL were purchased from Revvity. (±)-alpha-[2–^{3}H]-dihydrotetrabenazine, 10–20 Ci/mmol was purchased from American Radiolabeled Chemicals. Scintillation fluid (Rotiszint eco plus) was purchased from Carl Roth GmbH (Karlsruhe, Germany). Tetrabenazine was purchased from Eubio. Rabbit GFP polyclonal antiserum (A-11122; RRID AB_221569) was from Thermo Fisher Scientific. RNAscope Multiplex Fluorescent Reagent Kit v2, RNAscope 2.5 HD Duplex Reagent Kit, mouse RNAscope probes against VMAT2 (Mm-Slc18a2-C3; # 425331-C3), GAT1 (Mm-Slc6a1-C2; # 444071-C2) and VGLUT2 (Mm-Slc17a6; # 319171-C1), and human probes (Hs-TH-C2, #441651-C2; Hs-Slc6A1, # 545121; Hs-Slc17A6, #415671) were purchased from ACDBio/Bio-Techne Ireland Limited. All other chemicals were purchased from Sigma-Aldrich (Vienna, Austria).

2.2. Animals

We used adult (2–6 months old) male and female C57BL/6J mice and adult (3–4 months old) female Sprague-Dawley rats (note that only female rats were used because they were specifically ordered from our Animal Breeding Facility as pregnant dams to harvest pups for primary neuronal cell culture needed for other studies). All animals were bred in the Animal Breeding Facility of the Medical University of Vienna in Himberg and housed at the Institute of Pharmacology at least one week prior to the experiments.

C57BL/6J wildtype mice were derived from the breeding of heterozygous DAT^{Cre} (Jackson stock 006660; RRID:IMSR_JAX:006660), heterozygous Vglut2^{Cre} (Jackson stock 016963; RRID: IMSR_JAX:016963) or heterozygous ChAT^{Cre} (Jackson stock 006410; RRID:IMSR_JAX:006410) mice generated for a previous study (Garcia Moreno et al., 2024). Mice and rats were group-housed and maintained on a 12:12-h light: dark cycle with food and water available *ad libitum*.

All animals were used in accordance with protocols approved by the Animal Welfare Committee of the Medical University of Vienna and the Austrian Federal Ministry of Science and Research (BMBWF licenses 2021-0.373.073 and 2023-0.515.074).

2.2.1. Human tissue

De-identified formalin-fixed paraffin-embedded sections (FFPE) from the midbrain including segments of the substantia nigra at the level of the emergence of the third cranial nerve of two adult patients (one male, 61 years and one female, 76 years) without pathological changes in the substantia nigra were obtained from the neuro biobank of the Division of Neuropathology and Neurochemistry/Department of Neurology at the Medical University of Vienna.

2.2.2. Cell culture

HEK-293 cells were purchased from ATCC (CRL-1573, ATCC, Manassas, VA, USA;

RRID: CVCL_0045) and transfected with human VMAT2-eGFP in pcDNA6.2 using jetPRIME (Polyplus). A polyclonal cell line was generated through blasticidin S (Thermo Fisher Scientific; 10 μg/mL) selection, *i.e.* not a single clone, but a mixture of clones resistant to blasticidin S were expanded and maintained in Dulbecco's modified eagle medium (DMEM; Sigma-Aldrich) containing 10 % fetal bovine serum (Sigma-Aldrich), penicillin (100 IU/mL, Sigma-Aldrich), streptomycin (100 μg/mL, Sigma-Aldrich) and blasticidin S (6 μg/mL). Cells were maintained in a humidified 5 % CO_2 atmosphere at 37 °C.

2.3. Fluorescent microscopy

Live VMAT2-eGFP polyclonal cells were imaged at a Nikon confocal microscope using a 100x objective. Cells were incubated with 0.05 % trypan blue in phosphate-buffered saline (PBS) for 10 min prior to imaging.

2.4. Immunoblotting

VMAT2-eGFP cells were grown in a 10 cm plastic dish, washed two times in PBS and lysed in RIPA buffer containing (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS and 1% deoxycholate supplemented with protease inhibitors) and incubated at 4 °C for 30 min on a rotator followed by centrifugation at 12,600×*g* for 10 min.

Proteins were separated on a 10 % SDS-PAGE and electrotransferred onto nitrocellulose (Amersham) before blocking and incubation with a polyclonal GFP IgG antibody (A-11122; Thermo Fisher Scientific; RRID: AB_221569) overnight as described (Garcia Moreno et al., 2024). The secondary antibody used was IRDye 680RD goat anti-rabbit IgG (P/N 926–68071, LI-COR; RRID AB_10956166) at a concentration of 1:5000

in blocking buffer and visualized using the LI-COR Odyssey CLx infrared imaging system.

2.5. Synaptic vesicle preparation

Vesicles were prepared as described previously (Pifl et al., 2014) with minor modifications. Briefly, mice were deeply anaesthetized with pentobarbital (100 mg/kg i.p.; Exagon® 500 mg/mL, Richter Pharma) before decapitation. Rats were euthanized by CO_2 asphyxiation and then decapitated. Mouse and rat brains were rapidly removed, and striatum and cerebellum were dissected. Rodent striatum and cerebellum were homogenized in ice-cold 0.3 M sucrose containing 25 mM Tris (pH = 7.4) using a glass Teflon Potter-type homogenizer (10–15 strokes). Homogenates were centrifuged for 15 min at 1000×*g* at 4 °C. The supernatant was centrifuged at 20,000×*g* for 30 min at 4 °C. The resulting 'P2' pellet was osmotically shocked through resuspension in 2 mL ice-cold H_2O and additional 5–10 strokes in a glass Teflon Potter-type homogenizer, the supernatant ('SN2') was kept on ice. The aqueous P2 suspension was centrifuged at 22,000×*g* for 15 min and osmolarity of SN re-adjusted by addition of 1.3 M potassium phosphate buffer (pH = 7.4) in 1/10 of the volume. SN2 (from above) was centrifuged at 100,000×*g* for 30 min at 4 °C and resuspended in the hyposomotically shocked P2 suspension. The combined suspension was centrifuged at 100,000×*g* for 60 min at 4 °C and the resulting 'LP2' pellet was resuspended in 0.13 M potassium phosphate (KP) buffer and kept on ice or stored at −80 °C until the start of the experiment.

2.6. Radioligand uptake

Polyclonal HEK-293-VMAT2:eGFP cells were grown in a 48-well plate coated with poly-D-lysine (100 μg/mL). Prior to uptake, cells were washed with 200 μL NMDG buffer (in mM: 150 N-methyl-D-glucamine, 10 HEPES, 2 magnesium sulfate, 2 potassium chloride, 10 potassium gluconate; pH = 7.3) and permeabilized in 50 μM digitonin in NMDG buffer for 15 min at room temperature. Preincubation with inhibitors (reserpine, tetrabenazine) or substrates (dopamine) was performed for 5 min in NMDG buffer followed by incubation containing 100 nM [^{3}H]dopamine (40 Ci/mmol; PerkinElmer) in addition to unlabeled substrates or inhibitors. Uptake was terminated after 30 min at room temperature by addition of ice-cold NMDG buffer. After two additional washes with ice-cold NMDG buffer, cells were lysed in 1 % SDS, transferred to scintillation vials containing scintillation cocktail and radioactivity was measured in a liquid scintillation counter. Each cell uptake was performed in triplicate determinations.

Uptake in rodent or human synaptic vesicles was performed in KP buffer containing 2 mM ATP-Mg and 2 mM potassium chloride in a final volume of 200 μL and in the presence of [^{3}H]5-HT (100 nM). Uptake was started after addition of 10 μL vesicles (roughly 2 μg of protein) to ^{3}H solution and incubation in a water bath at 30 °C for 5 min. Ice-cold KP buffer was added to tubes to terminate the uptake followed by rapid filtration of suspension through polyethyleneimine-coated (1 % w/v) GF/B glass fiber filters (Whatman) and two additional washes with 2 mL kP buffer. Filters were then transferred to scintillation vials, vortexed and measured in a liquid scintillation counter (PerkinElmer/Revvity). Each vesicle uptake was performed in duplicate determinations.

2.7. Radioligand binding

Binding of [^{3}H]-dihydrotetrabenazine was performed as described (Pifl et al., 2014). Briefly, striatal synaptic vesicles were added to binding buffer (25 mM sodium phosphate; pH = 7.4) containing 20 nM [^{3}H]-dihydrotetrabenazine and different concentrations of unlabeled GABA. The reaction was incubated for 90 min at 30 °C. Non-specific binding was determined in the presence of 10 μM tetrabenazine. Binding was stopped by adding ice-cold binding buffer and samples were filtered onto GF/B filters presoaked in 1 % polyethylenimine using an automated cell harvester filtration device (Skatron Instruments AS). The radioactivity bound to filters was measured by liquid scintillation counting. Binding reactions were always determined in duplicates.

2.8. Fluorescent mRNA in situ hybridization in mouse brain

Male mice were anaesthetized with pentobarbital and killed by decapitation. Brains were extracted rapidly, frozen in chilled isopentane and stored at −80 °C. Sections were serially cut (20 μm) on a cryostat and mounted directly onto Epredia Superfrost Plus glass slides (Epredia Netherlands B.V.). Slides were stored at −80 °C until starting the multiplex fluorescent RNAscope assay v2 (Advanced Cell Diagnostics). Briefly, sections were fixed with 4% PFA for 1 h at 4 °C followed by dehydration in increasing ethanol concentrations and protease IV treatment. RNA hybridization probes included antisense probes against VMAT2 (Mm-Slc18a2-C3; # 425331-C3), GAT1 (Mm-Slc6a1-C2; # 444071-C2) and VGLUT2 (Mm-Slc17a6; # 319171-C1).

Slides were counterstained with DAPI and coverslipped using Fluoromount-G mounting medium. Images were taken at 20× magnification using a Vectra Polaris slide-scanner (Akoya) at the imaging core facility of the Medical University of Vienna.

2.9. Chromogenic mRNA in situ hybridization in human brain

Slides containing human FFPE midbrain sections were baked at 60 °C for 1 h in HybEZ II oven (ACDBio) and deparaffinized in xylenes followed by washes in 100% ethanol and air dried for 15 min. Next, tissue was pretreated with H_2O_2, boiled in target retrieval solution and incubated with protease plus before probe hybridization (Hs-TH-C2, #441651-C2; Hs-Slc6A1, # 545121; Hs-Slc17A6, #415671) and amplification according to the RNAscope 2.5 HD Duplex Assay (ACD-Bio). Slides were counterstained with Mayer's hematoxylin and coverslipped using VectaMount media. Images were taken at 40× magnification using a Vectra Polaris slide-scanner (Akoya) at the imaging core facility of the Medical University of Vienna.

2.10. Data analysis and statistics

Vesicle uptake and binding experiments were performed in duplicate determinations for each biological replicate (sample size 'n' corresponds to individual mice or rats used for brain extractions) and analyzed as follows: The average of the uptake or binding counts (counts per minute; cpm) obtained in the presence of vehicle (control) was set to 100 %. Counts in the presence of test compounds (GABA, reserpine, glycine, taurine) are expressed as percentage of control. Normalization was performed due to variabilities in baselines.

For data acquisition, we made sure that the order of sample incubations with different compounds and subsequent filtration of vesicle samples were varied to reduce potential positional biases.

Uptakes in HEK-293 cells were performed in duplicate or triplicate determinations for each biological replicate and analyzed the same way as the rodent vesicle uptakes. Positions of compound incubations in the well plates were varied to reduce potential positional biases.

GraphPad Prism 10 (GraphPad Software Inc., San Diego, CA; RRID: SCR_002798) was used to analyze data and create graphs. All data are expressed as mean ± SEM. All 'n' reported (number of observations) refer to biological replicates.

To determine statistical differences, *i.e.* whether different concentrations of a compound under investigation (GABA, glycine, taurine) affected substrate uptake, we used one-way ANOVA followed by Dunnett's multiple comparisons test; for the latter, the lowest inhibitor concentration was compared to all other concentrations. Statistical analyses were only performed with group sizes equal to or greater than n = 5. Post-hoc tests were only performed if one-way ANOVA was significant ($p < 0.05$).

Cell counting: For RNAscope, a neuron was deemed positive for a

given mRNA if at least 4 puncta were present in close proximity to a DAPI- (mice) or hematoxylin- (humans) labeled nucleus. 4 sections covering the mouse midbrain (spaced approximately 100–150 μm) from 3 male animals were counted. For human RNAscope, one section containing both hemispheres from one male and one female donor was counted. Cell fractions were averaged across the different mice (n = 3) or humans (n = 2) and expressed as percentage of total cell counts.

3. Results

3.1. GABA does not inhibit [^{3}H]dopamine uptake in HEK-293 cells stably expressing human VMAT2

To test whether GABA would interact with VMAT2 we created a stable polyclonal HEK-293 cell line expressing human VMAT2 with a C-terminal eGFP tag (HEK-293-VMAT2:eGFP). Live cell imaging shows that VMAT2 is diffusely expressed in intracellular compartments consistent with previous reports (Erickson et al., 1992) (Fig. 1A). Immunoblotting confirms expression of VMAT2:eGFP at around 80–90 kD (Fig. 1B).

We generated this line to perform vesicular uptake assay in adherent cells. To do so we utilized the steroidal saponin detergent digitonin to permeabilize the plasma membrane in order for VMAT2 substrates to reach the intracellular compartments. Our uptake inhibition experiments demonstrate that uptake of the VMAT2-substrate [^{3}H]dopamine (DA) into digitonin-permeabilized HEK-293-VMAT2:eGFP cells is potently inhibited by the competitive VMAT inhibitor reserpine (IC_{50} = 25.2 nM; 95% CI: 20.4–31.2 nM; n = 2), the non-competitive inhibitor tetrabenazine (IC_{50} = 28.8 nM; 95% CI: 21.4–38.7 nM; n = 2) as well as DA itself (IC_{50} = 4.9 μM; 95% CI: 4.4–5.6 μM; n = 2) (Fig. 1C). These affinities are well in line with published values (Erickson et al., 1992; Kanner et al., 1979; Liu et al., 1992) and support the robustness of this assay to investigate VMAT2 function in a heterologous expression system.

Accordingly, we used these cells to test whether GABA would reduce uptake of [^{3}H]DA into HEK-293-VMAT2:eGFP cells as would be expected if it were a VMAT2 substrate. However, GABA did not affect [^{3}H]DA uptake (Fig. 1D).

3.2. GABA weakly inhibits uptake of [^{3}H]5-HT in rodent striatal synaptic vesicles

HEK-293 cells are non-neuronal cells and do not have synaptic vesicles. Expression of VMAT2:eGFP in intracellular compartments of HEK-293 cells may therefore not well mimic the native environment of VMAT2 present in neuronal cells such as DA neurons. We therefore tested effects of GABA at VMAT2 in isolated synaptic vesicles from rodent brains. To measure VMAT2 uptake in vesicles we relied on [^{3}H]serotonin (=5-hydroxytryptamine; 5-HT) as a substrate rather than [^{3}H]DA because of its superior signal-to-noise ratio, which may be related to 5-HT's slightly higher affinity at VMAT2 (Erickson et al., 1992; Liu et al., 1992). In our preparation, 5-HT shows saturable uptake kinetics with an apparent Michaelis-Menten constant (K_M) of about 200 nM (Fig. 2A).

In contrast to what we observed in HEK293-VMAT2:eGFP cells, GABA significantly reduced uptake of [^{3}H]5-HT into both, mouse and rat striatal synaptic vesicles at millimolar concentrations (Fig. 2B and Suppl. Figure 2A). This suggests that GABA may indeed be a low-affinity substrate at VMAT2.

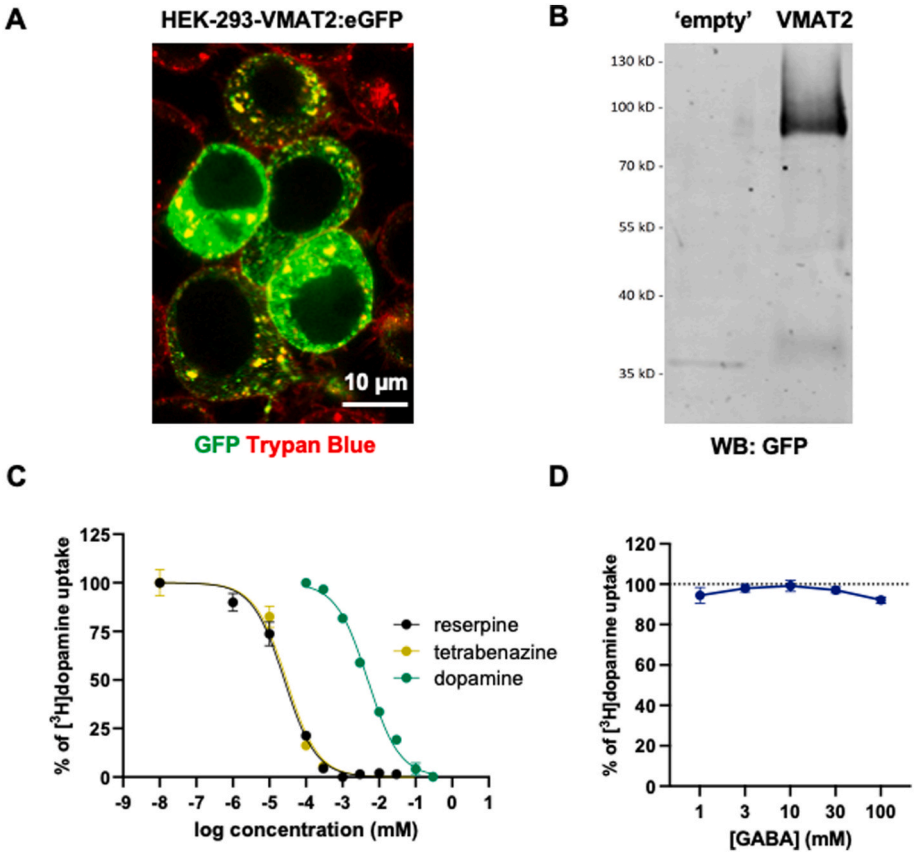

Fig. 1. GABA did not affect dopamine uptake at VMAT2 in polyclonal HEK-293 cells. (A) Trypan blue labeling indicates that VMAT2:eGFP is expressed in intracellular compartments of a polyclonal HEK-293 cell line. **(B)** Detection of VMAT2:eGFP at 80–90 kD by immunoblotting using an anti-GFP antiserum. **(C, D)** Inhibition uptake of [^{3}H]dopamine in digitonin-permeabilized HEK-293 VMAT2:eGFP cells with increasing concentrations of **(C)** reserpine (n = 2), tetrabenazine (n = 2) and dopamine (n = 2) and **(D)** GABA (n = 8/concentration); one-way ANOVA followed by Dunnett's multiple comparisons test, F(3,35) = 1.306, P = 0.2867.

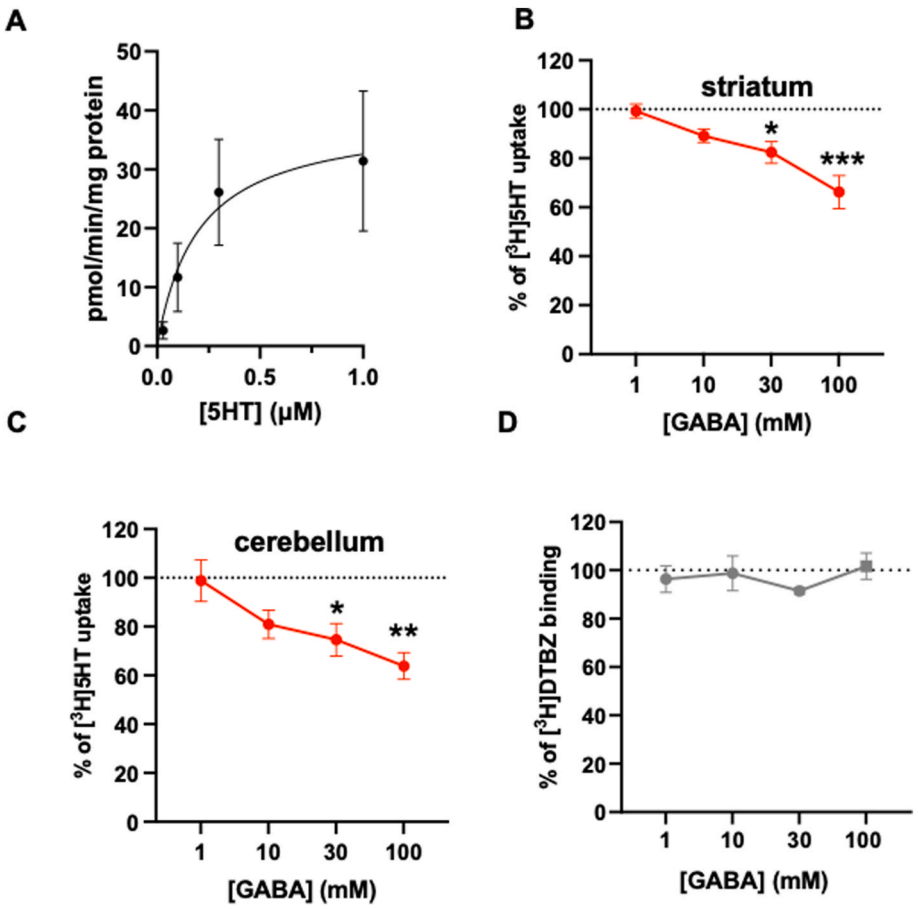

Fig. 2. GABA decreased uptake of 5-HT in mouse synaptic vesicles. (A) Saturation uptake of [^{3}H]5-HT in mouse striatal synaptic vesicles (n = 5). **(B)** GABA inhibition uptake of [^{3}H]5-HT in mouse striatum (pooled data: n = 5 for 1 mM, n = 8 for 10 mM, n = 9 for 30 mM, n = 6 for 100 mM); one-way ANOVA followed by Dunnett's multiple comparisons test: F(3,24) = 7.866, P = 0.0008; 1 mM vs. 10 mM: P = 0.3074, 1 mM vs. 30 mM: *P = 0.0424, 1 mM vs. 100 mM: ***P = 0.0003. **(C)** GABA inhibition uptake of [^{3}H]5-HT in mouse cerebellum (n = 7); one-way ANOVA followed by Dunnett's multiple comparisons test: F(3,28) = 4.802, P = 0.0080; 1 mM vs. 10 mM: P = 0.1658, *1 mM vs. 30 mM: P = 0.0419, 1 mM vs. 100 mM: **P = 0.0026. **(D)** Binding of [^{3}H]dihydrotetrabenazine is not affected by GABA in striatal synaptic vesicles (n = 5); one-way ANOVA followed by Dunnett's multiple comparisons test: F(4,21) = 0.7467, P = 0.5711.

The rodent striatum receives very dense axonal input from midbrain DA neurons, and comparatively fewer input from serotonergic or noradrenergic fibers suggesting that the preponderance of VMAT2 measured in striatal synaptic vesicles is derived from DA neurons, although a separation between the different vesicles is of course impossible. Based on neurochemical measurements of tissue monoamine levels by high-performance liquid chromatography, there is about 10-fold more DA than 5-HT, and about 10-fold more 5-HT than norepinephrine (NE) (Peneder et al., 2011).

Nonetheless, we wondered whether VMAT2 would also be GABA-sensitive in a brain area where VMAT2 derives primarily from serotonergic and noradrenergic innervation and less so from DA neurons. Hence, we also prepared synaptic vesicles from mouse cerebellum, which has 10–40 fold lower levels of DA compared to NE (Glaser et al., 2006) and again measured [^{3}H]5-HT uptake in the presence of increasing concentrations of GABA. Comparable to our findings in mouse and rat striatal vesicles, cerebellar [^{3}H]5-HT uptake was decreased in the presence of millimolar concentrations of GABA (Fig. 2C).

Next, we wondered whether GABA would displace the high-affinity non-competitive antagonist [^{3}H]dihydrotetrabenazine (DTBZ) from binding to VMAT2. Given that GABA may interact with VMAT2 differently than monoamines, we speculated that it might interfere with DTBZ binding. The apparent dissociation constant (K_D) of [^{3}H]DTBZ in mouse striatal synaptic vesicles was determined at around 5 nM (Supplemental Fig. 1). Striatal synaptic vesicles were incubated with a saturating concentration of [^{3}H]DTBZ (20 nM) and increasing concentrations of unlabeled GABA. However, we did not observe any [^{3}H]DTBZ displacement even at very high millimolar GABA concentrations (100 mM) in both mouse (Fig. 2D) and rat striatal vesicles (Supplemental Fig. 2B). These findings suggest that GABA and DTBZ bind to distinct, non-overlapping sites on VMAT2.

3.3. *Effects of glycine and taurine on VMAT2*

It is possible that not GABA but another inhibitory transmitter is packaged into vesicles by VMAT2 and responsible for the activation of postsynaptic striatal $GABA_A$ receptors that was reported after optogenetic stimulation of DA terminals (Melani and Tritsch, 2022; Patel et al., 2024; Tritsch et al., 2012, 2014). We tested two such potential compounds in the mouse vesicle uptake, glycine and taurine, both of which are structural analogues of GABA that have been reported to activate $GABA_A$ receptors, though presumably with much weaker affinity (Jonas et al., 1998).

While glycine did not affect uptake of [^{3}H]5-HT into mouse striatal synaptic vesicles even at high millimolar concentrations (Fig. 3A), taurine weakly reduced [^{3}H]5-HT uptake into mouse striatal vesicles at 10 and 30 mM (Fig. 3B) suggesting that it may also be packaged into vesicles by VMAT2. Importantly, the lack of any inhibitory effects of glycine even at the high concentration of 100 mM argues against simple unspecific osmotic effects inhibiting substrate uptake at VMAT2 in our assay.

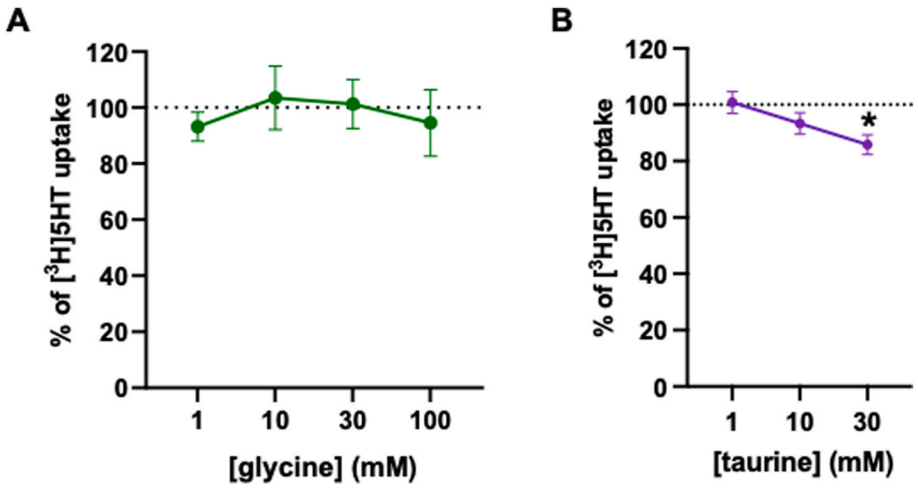

Fig. 3. Taurine but not glycine reduces 5-HT uptake in mouse synaptic vesicles. Effects of **(A)** glycine (n = 5) and **(B)** taurine (n = 8) on [^{3}H]5-HT uptake in mouse striatal synaptic vesicles. Glycine: one-way ANOVA: F(3,16) = 0.2715, P = 0.8450; taurine: one-way ANOVA followed by Dunnett's multiple comparisons test: F(2,21) = 4.058, P = 0.0324; 1 mM vs. 10 mM: P = 0.2869, 1 mM vs. 30 mM: *P = 0.0179.

3.4. Gat1 mRNA is expressed in most SNc and VTA DA neurons

GAT1 was proposed to be mainly responsible for GABA uptake into DA neurons and found to be expressed in almost 90 % of VMAT2$^+$ DA neurons in the mouse SNc (Tritsch et al., 2014).

If VMAT2 transports GABA, all GAT1$^+$ DA cells are theoretically equipped with the machinery to store and release GABA. We wondered whether GAT1 was only expressed in SNc or also in VTA DA neurons, and whether GAT1$^+$ DA neurons would be distinct from the VGLUT2$^+$ DA neuron population.

To investigate this, we performed multiplex fluorescent *in situ* hybridization in mouse midbrain sections targeting *Slc18a2* (*VMAT2*), *Slc6a1* (*GAT1*) and *Slc17a6* (*VGLUT2*) mRNAs (Fig. 4A and B). We determined that about 60 % of SNc DA neurons were positive for *VMAT2* and *GAT1* mRNAs (Fig. 4C), which is lower than previously reported (Tritsch et al., 2014). In contrast, only about 30 % of VTA DA neurons were co-expressing *VMAT2* and *GAT1* mRNAs (Fig. 4D). In line with previous results, about 9 % of SNc and about 14 % of VTA DA neurons were positive for *VMAT2* and *VGLUT2* (Fig. 4) (Conrad et al., 2024; Steinkellner et al., 2018). Interestingly, about half of these *VMAT2*$^+$/*VGLUT2*$^+$ cells in both, SNc and VTA also expressed *GAT1* (4.8 %) (Fig. 4C) suggesting that these neurons may co-release DA, glutamate and GABA.

3.5. Human DA neurons express Gat1 mRNA and may co-release GABA

Human DA neurons share many molecular features with their mouse counterparts. For instance, the expression pattern of VGLUT2 in DA neurons seems similar in mice and humans (Buck et al., 2021; Root et al., 2016; Steinkellner et al., 2022) suggesting that human DA neurons are capable of glutamate co-release. But it is not known whether human midbrain DA neurons express GAT1, or whether they may co-release GABA. We used postmortem human midbrain sections from one male (61 years) and one female (76 years) donor and performed chromogenic duplex mRNA *in situ* hybridization to detect human *tyrosine hydroxylase* (*TH*), a catecholaminergic marker, and *GAT1* mRNAs (Fig. 5). Similar to our observations in mice, about 66 % of human SNc (Fig. 5C) and about 55 % of human VTA (Fig. 5D) DA neurons were positive for *GAT1* mRNA. This suggests that human SNc and VTA DA neurons may co-release GABA.

4. Discussion

The heterogeneity of midbrain DA neurons is increasingly recognized and may help better explain and understand the manifold diseases associated with DA dysfunction such as Parkinson's disease, schizophrenia or substance use disorders.

One interesting feature that highlights the complexity of midbrain DA neurons is their capability to co-release transmitters other than DA. The old idea that one neuron releases just one neurotransmitter ('Dale's principle') turns out to be the exception rather than the rule within the DA system (Trudeau et al., 2014).

For instance, it is now well established that the excitatory neurotransmitter glutamate is co-released from subpopulations of VTA and SNc DA neurons, because these neurons express VGLUT2, which is both necessary and sufficient to store and release glutamate (Adrover et al., 2014; Dal Bo et al., 2004; Hnasko et al., 2010; Silm et al., 2019; Stuber et al., 2010; Sulzer et al., 1998). In fact, the only requirement for any neuron including DA neurons to co-release glutamate seems to be the expression of a vesicular glutamate transporter (Steinkellner et al., 2018; Takamori et al., 2000). Importantly, relevance of glutamate co-release from DA neurons has also been linked to Parkinson's disease (Dal Bo et al., 2008; Shen et al., 2018; Steinkellner et al., 2018, 2022), motivated behaviors and responses to psychostimulant drugs such as amphetamine or cocaine (Alsio et al., 2011; Birgner et al., 2010; Hnasko et al., 2010).

In contrast to glutamate, co-release of GABA from DA neurons is less well understood. Though there seem to be small subsets of DA neurons expressing GAD1/2 (Azcorra et al., 2023; Gaertner et al., 2024) and even VGAT (Conrad et al., 2024) the majority does not. Rather, it was suggested that GABA is taken up from outside by the plasmalemmal GABA transporter GAT1 (Melani and Tritsch, 2022; Tritsch et al., 2014) and/or synthesized *de novo* by ALDH1a1 (Kim et al., 2015) prior to being loaded into vesicles by VMAT2 (Melani and Tritsch, 2022; Tritsch et al., 2012).

VMAT2 is the major VMAT isoform expressed in the CNS and responsible to transport monoamines (DA, 5-HT, NE, epinephrine, histamine) into synaptic vesicles (Eiden and Weihe, 2011). In contrast to monoamines, GABA is an amino acid that occurs as a zwitterion at physiological pH, and thus does not resemble the classical VMAT2 substrates that have an aromatic ring and a positively charged amino group (Peter et al., 1994; Yelin and Schuldiner, 1995; Zheng et al., 2006).

It is therefore not surprising that GABA has never been recognized as a substrate at VMAT2. There is however now compelling pharmacological and genetic evidence that GABA can be loaded into vesicles by VMAT2. GABA co-release was reduced in the presence of VMAT2 inhibitors, and ectopic expression of VMAT2 in non-dopamine GABA neurons in which VGAT was genetically deleted was sufficient to rescue

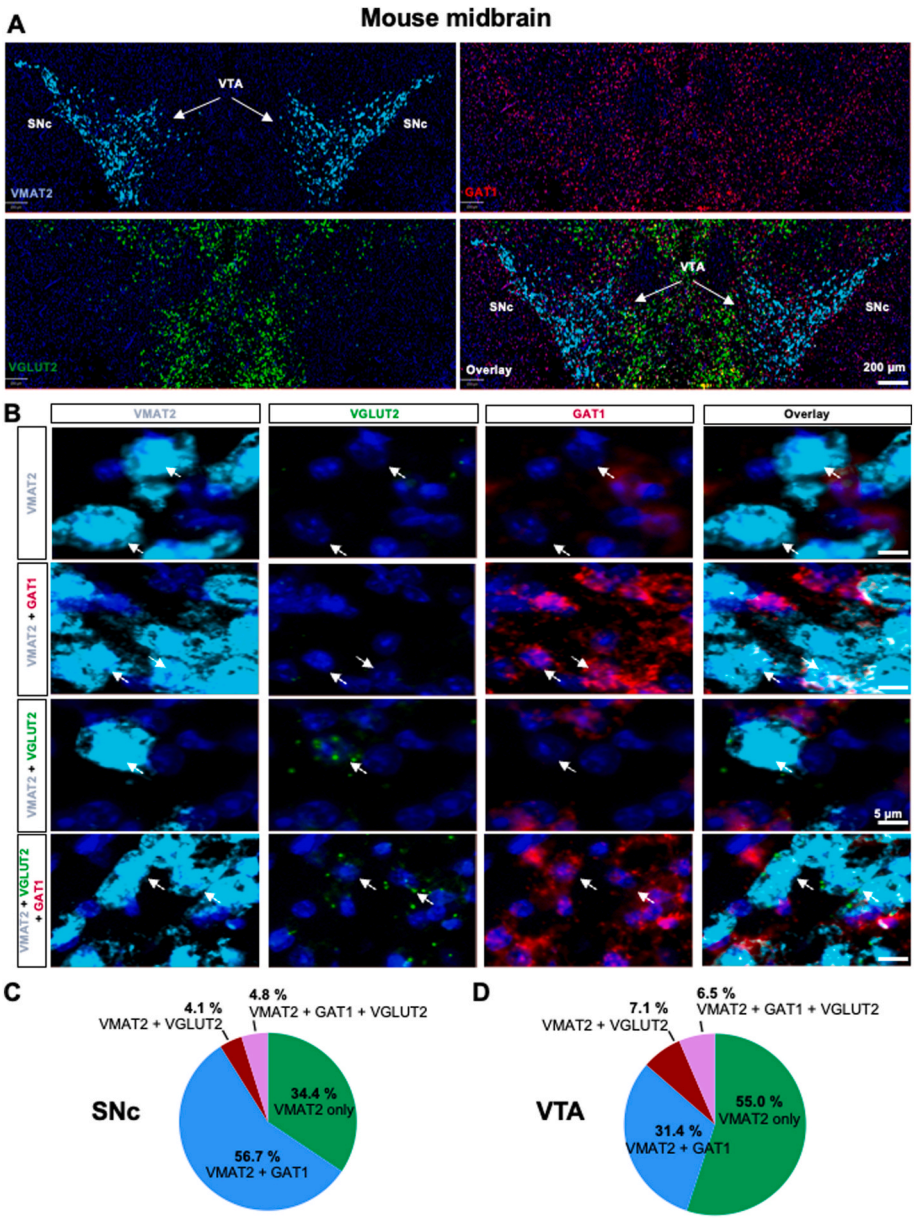

Fig. 4. Expression of VMAT2, GAT1 and VGLUT2 mRNAs in mouse ventral midbrain dopamine neurons. (A) Example *in situ* hybridization images in coronal sections through the mouse ventral midbrain; mRNA encoding VMAT2 (light blue), GAT1 (red) and VGLUT2 (green) with DAPI counter-stain (dark blue). **(B)** Higher power images of different cell-types in the SNc. White arrows indicate cell-types highlighted on the left. **(C, D)** Pie charts quantifying the relative percentages of single, double or triple positive cells in SNc **(C)** and VTA **(D)**; n = 3.

GABA release (Tritsch et al., 2012).

In the current study, we aimed to determine the relative affinity of GABA at VMAT2 and found that GABA may indeed qualify as a weak substrate at VMAT2: millimolar GABA concentrations were required to partially block uptake of the endogenous VMAT2 substrate 5-HT in rodent synaptic vesicle preparations. But does such a low affinity make sense physiologically?

The cognate vesicular transporter for GABA, VGAT, transports the inhibitory neurotransmitters GABA and glycine. Interestingly, the affinity of GABA for VGAT turned out to be surprisingly low (apparent K_M around 5 mM) (Burger et al., 1991; Hell et al., 1988; McIntire et al., 1997); and the affinity of glycine at VGAT was suggested to be even lower because it could not be measured experimentally (Edwards, 2007; McIntire et al., 1997). The affinity of GABA at VGAT is therefore considerably lower than the affinity of monoamines at VMAT2 (K_M values in the sub-micromolar range) (Liu et al., 1992; Yelin and Schuldiner, 1995), but not so different from the affinity of glutamate at VGLUTs, which is also in the low millimolar range (Edwards, 2007; Maycox et al., 1988; Tabb and Ueda, 1991).

Besides the affinity of a substrate to its transporter, the ambient concentration of the substrate determines whether it will be transported and concentrated within a compartment.

Cytosolic DA concentrations are estimated to be in the low micromolar range in adrenal chromaffin cells (Mosharov et al., 2003), and even lower in DA neurons (Mosharov et al., 2009) explaining why a sub-micromolar K_M of DA for VMAT2 allows for efficient sequestration and concentration of monoamines in synaptic vesicles at millimolar concentrations.

In contrast, cytosolic GABA concentrations remain unknown, but are presumably much higher than cytosolic monoamines (Edwards, 2007) and likely comparable to the estimated millimolar levels of glutamate (Featherstone, 2010; Ishikawa et al., 2002). Importantly, cytosolic

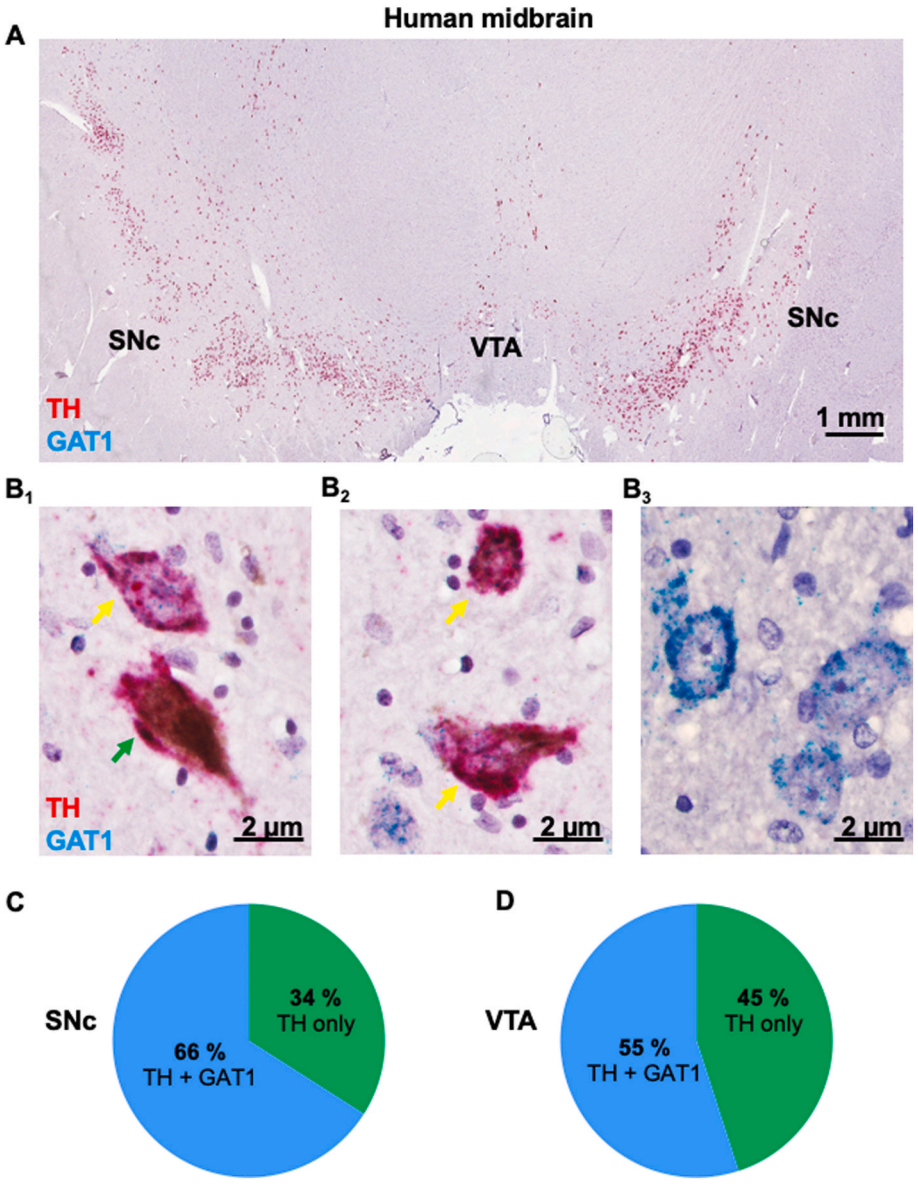

Fig. 5. GAT1 is expressed in human SNc and VTA DA neurons. (A) Widefield view of transverse section containing SNc from a human brain donor with chromogenic labeling for mRNAs encoding TH (diffuse magenta) and GAT1 (blue puncta) counter-stained with hematoxylin; brown signal is neuromelanin. **(B)** Higher magnification images of SNc DA neurons (B_1 and B_2) expressing TH with (yellow arrow) or without (green arrow) GAT1, or putative GABA neurons expressing GABA without TH in the SNc (B_3). **(C, D)** Pie charts quantifying the relative percentages of single, double or triple positive cells in SNc **(C)** and VTA **(D)**; n = 2 (1 male, 1 female).

GABA concentrations would need to be several-fold above the suggested K_M of 5 mM, to enable sufficient synaptic loading. If these concentrations are also reached within DA neurons (*e.g.* locally at uptake sites), GABA could be transported despite its apparent low affinity at VMAT2.

But is it really GABA that is transported by VMAT2 and released from DA neurons?

There is compelling genetic and physiological evidence, that GABA can be taken up and released in a VMAT2-dependent manner (*e.g.* (Melani and Tritsch, 2022; Tritsch et al., 2012), while our evidence for GABA being a substrate at VMAT2 is indirect: we showed that increasing concentrations of GABA reduced the uptake of the VMAT2 substrate 5-HT into rodent synaptic vesicles. Although we attempted to directly measure the uptake of [^{3}H]GABA, we were unable to detect any reserpine- or tetrabenazine-sensitive VMAT2-mediated uptake of [^{3}H]GABA into either rodent synaptic vesicles or VMAT2-expressing HEK-293 cells. However, we also could not confirm VGAT-mediated [^{3}H]GABA uptake in either heterologously expressing cells or synaptic vesicles, as excess unlabeled GABA failed to block VGAT. Consequently, these findings remain difficult to interpret.

There are several possibilities that may explain these negative findings: *i)* GABA is not actually transported by VMAT2; *ii)* our radioisotope dilution method was not sensitive enough to measure direct uptake of a substrate with a low apparent affinity such as GABA at VMAT2 and VGAT; *iii)* GABA may not be directly taken up by VMAT2 but rather acts as a negative allosteric modulator of monoamine transport at VMAT2; *iv)* under physiological conditions, not GABA, but another inhibitory transmitter is taken up by VMAT2.

In fact, since $GABA_A$ receptors are promiscuous ionotropic receptors that bind many different endogenous and exogenous ligands capable of activating or modulating the receptor, we tested two potential agonists that endogenously occur in mammals in high concentrations and have been shown to activate $GABA_A$ receptors: glycine and taurine (Jonas et al., 1998). Interestingly, while glycine did not affect 5-HT uptake at VMAT2 in mouse synaptic vesicles, taurine weakly reduced 5-HT

uptake, suggesting that it may also be taken up and released *in vivo*. Of course, additional requirements for synaptic taurine release would need to be fulfilled such as either adequate biosynthesis of taurine in these cells and/or uptake of extracellular taurine via a transporter.

Our finding that GABA did not affect [^{3}H]DA uptake in human VMAT2:eGFP-expressing HEK-293 cells could be explained by several factors: *i)* there could be species differences (mouse and human VMAT2 share 91 % of the amino acid sequence; mouse and rat VMAT2 share 95 %); *ii)* the C-terminal eGFP moiety may prevent the interaction of GABA and VMAT2; *iii)* the non-neuronal environment of HEK-293 cells lacks other important components (*e.g.* interacting proteins) only present in neuronal preparations.

Hence, we moved to synaptic vesicle preparations from rodent brains to determine whether GABA would reduce VMAT2 uptake and indeed found that GABA weakly reduced it. However, it is also important to stress that our synaptic vesicle preparation did not contain many of the additional features present in intact nerve cells including the surrounding boutons and local microdomains that may impact on transport. For instance, it is conceivable that *in vivo*, VMAT2 transport is further influenced by many additional factors such as the local concentrations of GABA, interacting proteins and differences in the local pH: the local concentration of GABA within specific boutons or presynaptic terminals could vary and therefore efficient uptake of GABA into vesicles may be enhanced, when local GABA concentrations are higher. VMAT2 and GAT1 may functionally couple as has been demonstrated for VGAT and GAD1/2 (Jin et al., 2003), or VMAT2 and the plasmalemmal dopamine transporter (DAT) (Cartier et al., 2010). Finally, the local pH may play an important role in affecting the ionization of GABA (Krishek et al., 1996; Takeuchi and Takeuchi, 1967). For instance, if the local pH is lower than the average cytosolic pH of 7.2, the relative stoichiometry of zwitterionic over cationic GABA may decrease, and affect uptake by VMAT2, which typically transports cations.

Lastly, our study demonstrates for the first time that not only mouse but also human midbrain DA neurons express GAT1 in about 50–60 % of the cells indicating that GABA co-release from DA neurons is evolutionarily conserved. In fact, the potential for GABA co-release from DA neurons may be far more prevalent than the co-release of glutamate which is restricted to only few DA neurons in SNc and VTA. Glutamate co-release from DA neurons has been implicated to play important roles in motivated behaviors and may also play a role in Parkinson's disease (Hnasko et al., 2010; Shen et al., 2018; Steinkellner et al., 2018, 2022). In contrast, the potential role of GABA co-release from DA neurons for physiology and disease is largely unknown though some evidence for a role in ethanol drinking and reward-based behaviors have been reported (Kim et al., 2015). Interestingly, we also found a subpopulation of DA neurons that was positive for VMAT2, GAT1 and VGLUT2, suggesting that these cells may be able to co-release three transmitters.

In conclusion, we provide pharmacological support that GABA could be a low-affinity atypical substrate at VMAT2, that seems to be taken up by GAT1 into the majority of SNc and many VTA DA neurons in mice and possibly also in humans.

CRediT authorship contribution statement

Sivakumar Srinivasan: Methodology, Formal analysis, Data curation. **Fabian Limani:** Methodology, Formal analysis, Data curation. **Michaela Hanzlova:** Methodology, Formal analysis, Data curation. **Ségolène La Batide-Alanore:** Methodology, Formal analysis, Data curation. **Sigrid Klotz:** Resources. **Thomas S. Hnasko:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Conceptualization. **Thomas Steinkellner:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Ethics approval and consent to participate

All mice were used in accordance with protocols approved by the Animal Welfare.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuropharm.2025.110367.

Data availability

Data will be made available on request.

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List of abbreviations

5HT: 5-hydroxytryptamine
DA: dopamine

DAT: dopamine transporter
DTBZ: dihydrotetrabenazine
GAD1/2: glutamic acid decarboxylase 1/2
GAT1: GABA transporter 1
NE: norepinephrine
SNc: substantia nigra pars compacta
TBZ: tetrabenazine
TH: tyrosine hydroxylase
VGAT: vesicular GABA transporter
VGLUT2: vesicular glutamate transporter 2
VMAT2: vesicular monoamine transporter 2
VTA: ventral tegmental area