Interaction of betaine with the γ-aminobutyric acid (GABA) transporters

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Laboratory of Cellular and Molecular Physiology

Department of Biotechnology and Lifesciences (DBSV)

Author: Manan Bhatt

Matriculation No.745779

Supervisor: Prof. Dr. Elena Bossi

Coordinator: Prof. Dr. Mauro Fasano

NeuroTrans
NEUROtransmitter TRANSporters:
From Single Molecules to Human Pathologies

MARIE CURIE ACTIONS

Preface

Our brain propagates information by alternating electrical signals, the action potential, and chemical signals by releasing neurotransmitters. It is essential to rapidly remove neurotransmitters, failing to which can lead to neuronal imbalance and consequently to different neuronal conditions. The sodium-dependent neurotransmitter symporter (NSS) is a family of proteins responsible for the removal of classical neurotransmitters like glycine, dopamine, serotonin, and γ-aminobutyric acid (GABA). Several drugs have been developed that target these transporters, however, most of these drugs have been associated with different unwanted side-effects. Betaine, a natural endogenous molecule, has been proposed as a novel therapeutic in treating neurological diseases like AD, PD, and schizophrenia. The cellular and molecular mechanism related to its effects are little understood and its action in these neuropathology seems related to a positive effect in maintaining the excitatory/inhibitory balance. In this research work the interaction of betaine with GABAergic pathways was studied investigating GABA transporter 1 (GAT1) describing the possible transport mechanism and the peculiar relationship between GABA and Betaine. Under the umbrella of NeuroTrans (from molecule to malady), a Marie- Skłodowska Curie Actions network (Grant agreement no. 860954), this project investigated the betaine GAT1 relationship using multidisciplinary methods.

The doctoral thesis introduction is based on two published peer-reviewed papers that constitute one of the goals of the Neurotrans project, followed by detailed description of methodologies used for the original results. The findings on the interaction of betaine with GATs have been described in detail for which the research activities were regularly monitored and reviewed by the supervisory team of Dr. Elena Bossi (University of

Insubria), Dr. Harald Sitte (Medical University of Vienna), Dr. Christine Ziegler (University of Regensburg), and Dr. Andre Bazzone (Nanion Technologies GmbH).

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Introduction

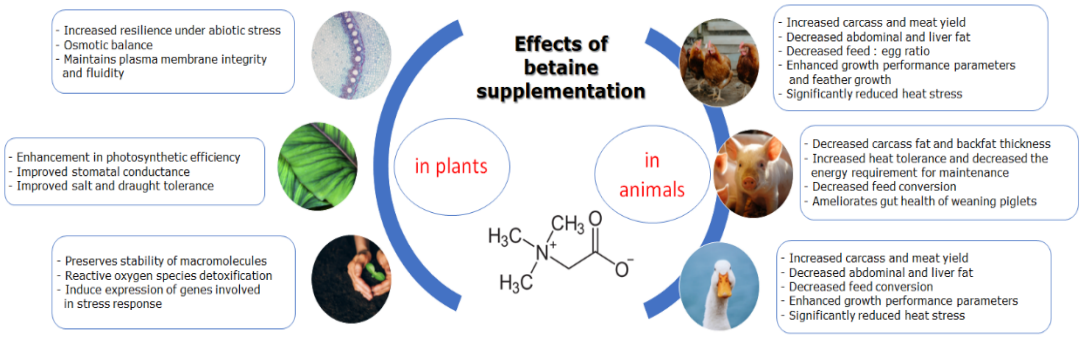
Chapter 1 Betaine

A natural dietary nutrient

Betaine, also known as N, N, N trimethyl glycine, is a naturally occurring zwitterionic osmolyte that is widely distributed in animals, plants, and microorganisms. It derives the name from its discovery in 1869 by Carl Scheibler, when he isolated betaine from sugar extracted from *beta vulgaris* (Scheibler 1869). Later this molecule was found at high concentration in other dietary sources such as wheat bran, spinach, and seafoods. While humans absorb and accumulate betaine from dietary uptake, it can also be synthesized in small amounts from the spare choline in the mitochondria (Craig 2004).

The exogenous betaine supplements benefits plants and poultry animals

The role of betaine has been studied well in plants and bacteria. The synthesis and accumulation of betaine occur in several plants in response to environmental constraints like abiotic stress (Rhodes and Hanson 1993). In Poaceae, betaine is one of the main compatible osmolyte that contains nitrogen, its exogenous application has been shown to induce the expression of genes involved in oxidative stress response (Annunziata, Ciarmiello et al. 2019). In plants, the exogenous application of betaine facilitates the growth and survival of the plants. In animals, betaine is widely used in cultivating livestock production. Betaine is shown to decrease fat accumulation in laying hens, pigs, broilers, ducks, while promoting growth in pigs and rabbits (Figueroa-Soto and Valenzuela-Soto 2018). It can also facilitate transfer of salmon from sea water to fresh water and stimulate appetite in fish and prawns (Figueroa-Soto and Valenzuela-Soto 2018).



<u>Figure 1 Effects of betaine supplementation in plants and animals:</u> The systematic supplementation of betaine has been reported to be beneficial in both plants and animals (livestock) (Eklund, Bauer et al. 2005, Ratriyanto, Mosenthin et al. 2010, Mendoza, Boyd et al. 2017, Kumari, Kapoor et al. 2019, Zulfiqar, Akram et al. 2019, Francesca, Raimondi et al. 2020, Ghorai, Patsa et al. 2021, Hamani, Li et al. 2021, Abd El-Ghany and Babazadeh 2022).

Role of betaine in metabolism

Betaine is extensively studied as an osmolyte in plants, bacteria, and also animal cells. Due to its ability to stabilize protein expressions, it is also known as osmoprotector (Figueroa-Soto and Valenzuela-Soto 2018). In humans, betaine with its three methyl groups, plays an effective role in cellular metabolism of homocysteine (Hcy) to methionine especially in kidney and liver (Figueroa-Soto and Valenzuela-Soto 2018). Many early studies have shown beneficial effects of betaine on Hcy metabolism, and it is effectively used in diseases involving high serum and/or plasma Hcy levels. In liver and adipose tissue, betaine also plays an important role in metabolism of alcohol, lipids, and carbohydrate.

The ethanol metabolism, in liver, decreases methionine synthase and methionine adenosyl transferase, resulting in increased Hcy concentrations. Betaine can counteract this effect via increased methionine synthesis by inducing increased betaine homocysteine methyl transferase (BHMT) expression and S-adenosyl methionine (SAM) concentration. Using rat models, it has been shown that betaine protects against the development of alcohol-induced hepatic steatosis (Arumugam,

Paal et al. 2021). Betaine also protects liver against the oxidative stress (Kim, Jung et al. 2008, Oliva, Bardag-Gorce et al. 2011) and lipid peroxidation (Varatharajalu, Garige et al. 2010) caused by high ethanol consumption. Betaine can also regulate lipid metabolism by stimulating oxidation of fatty acids and inhibition of lipogenesis. Apart from that, betaine also induces changes in expression of enzymes involved in oxidative stress, sugars and lipid metabolism, and Hcy/methionine cycle. Hence, betaine is a natural molecule with wide variety of roles in cellular metabolism.

Chapter 2 Betaine - the Dark Knight of the brain

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Manan Bhatt[1,2], Angela Di Iacovo[1,2], Tiziana Romanazzi[1,2], Cristina Roseti[1,3], Elena Bossi[1,3]

[1]Laboratory of Cellular and Molecular Physiology, Department of Biotechnology and Life Sciences, University of Insubria, Varese, Italy.
[2]PhD Schools in Experimental and translational Medicine, University of Insubria, Varese, Italy.
[3]Centre for Neuroscience- University of Insubria, Varese, Italy.

Abstract

The role of betaine in the liver and kidney has been well documented, even from the cellular and molecular point of view. Despite literature reporting positive effects of betaine supplementation in Alzheimer's, Parkinson's and schizophrenia, the role and function of betaine in the brain are little studied and reviewed. Beneficial effects of betaine in neurodegeneration, excitatory and inhibitory imbalance and against oxidative stress in the central nervous system (CNS) have been collected and analysed to understand the main role of betaine in the brain. There are many 'dark' aspects needed to complete the picture. The understanding of how this osmolyte is transported across neuron and glial cells is also controversial, as the expression levels and functioning of the known protein capable to transport betaine expressed in the brain, betaine-GABA transporter 1 (BGT-1), is itself not well clarified. The reported actions of betaine beyond BGT-1 related to neuronal degeneration and memory impairment are the focus of this work. With this review, we underline the scarcity of detailed molecular and cellular information about betaine action. Consequently, the requirement of detailed focus on and study of the interaction of this molecule with CNS components to sustain the therapeutic use of betaine.

1. Introduction

The solute carrier (SLC) 6 family of secondary active transporters includes neurotransmitter sodium symporters responsible for transporting neurotransmitters such as dopamine transporters, serotonin transporter and γ-aminobutyric acid (GABA) transporters. One of the GABA transporters is betaine-GABA transporter 1, BGT-1 (SLC6A12), which is expressed in the liver, kidney, and brain.[1] It has a unique ability to translocate both GABA and betaine (an osmolyte found in animals, plants, and microorganisms) across the membrane.[2] While the roles of betaine and BGT-1 in liver and kidney have been studied well, they are very ambiguous and understudied in the brain. At the same time, there is a remarkable surge in the literature on betaine, demonstrating its positive role and therapeutic potential in neurological and neurodegenerative diseases, which demands focused attention from the scientific community. A review discussing and presenting contemporary information on betaine and its transporter in the brain could help in pushing the frontiers. With this aim, we investigate literature evidence regarding possible mechanisms of action of betaine in the brain and review its beneficial role in the brain, and how this molecule can be accumulated and transported in this organ.

Betaine, also known as glycine betaine and as N, N, N trimethyl glycine, is a zwitterionic amino acid derivative that can be endogenously produced by the oxidation of choline[3] and exogenously absorbed as a dietary nutrient. The name betaine comes from its discovery from Beta vulgaris (beets) in the 1860s, but later, it was found at high concentration in other dietary sources like wheat bran, spinach and seafood.[4–6] In mammals, from the physiological point of view, betaine serves primarily two roles: as one of the major osmolytes accumulated in the tissues for cell volume regulation, mainly in the kidney, and as a methyl donor for the toxic

metabolite, homocysteine (Hcy), to convert it into methionine.[3,6] The daily betaine uptake in the human diet ranges from 1 to 2.5 g/day, based on individual consumption. The study on red blood cell physiology at high betaine doses showed mild perturbance and suggested that safe daily betaine intake was 9–15 g/day.[4] The active absorption of betaine in cells happens via different carriers and transporters (see Figure 1). Apart from BGT-1, the betaine could also be translocated via proton-coupled amino acid transport, PAT1 (SLC36A1), and also passive sodium independent in the epithelia.[3,4,7,8] Also, other transporters, such as proline transporter SIT1/XT3s1 (SLC6A20) that arises in the embryo post-fertilization, could act as the regulator of the oocytes-derived betaine levels.[9,10] Another SLC transporter in neurons, called SNAT2 (SLC38A2), is shown to interact with betaine, where it functions as a protective layer in the placenta against osmotic changes in maternal or foetal plasma.[11] Using these transport systems, betaine could be adsorbed and distributed across the human body. Rapid adsorption and distribution up to 1–3 mM within 1–2 h of intake have been reported in human studies about betaine supplementation.[4,12] However, it should be noted that the tissue concentration for an osmolyte would be higher than the plasma concentration.[13]

Apart from diet and supplementation, betaine can be synthesized via a two-step irreversible process using choline in mitochondria (see Figure 1). Firstly, the enzyme choline dehydrogenase oxidates choline into betaine aldehyde. And then, betaine aldehyde is converted to betaine by the same enzyme in the presence of nicotinamide adenine dinucleotide (NAD+). This betaine is catabolized via transmethylation reactions involved in vital biological processes.[4] This transmethylation is catalysed by betaine-homocysteine methyltransferase (BHMT), which detoxifies Hcy by converting it into methionine and producing S-adenosylmethionine (SAM).

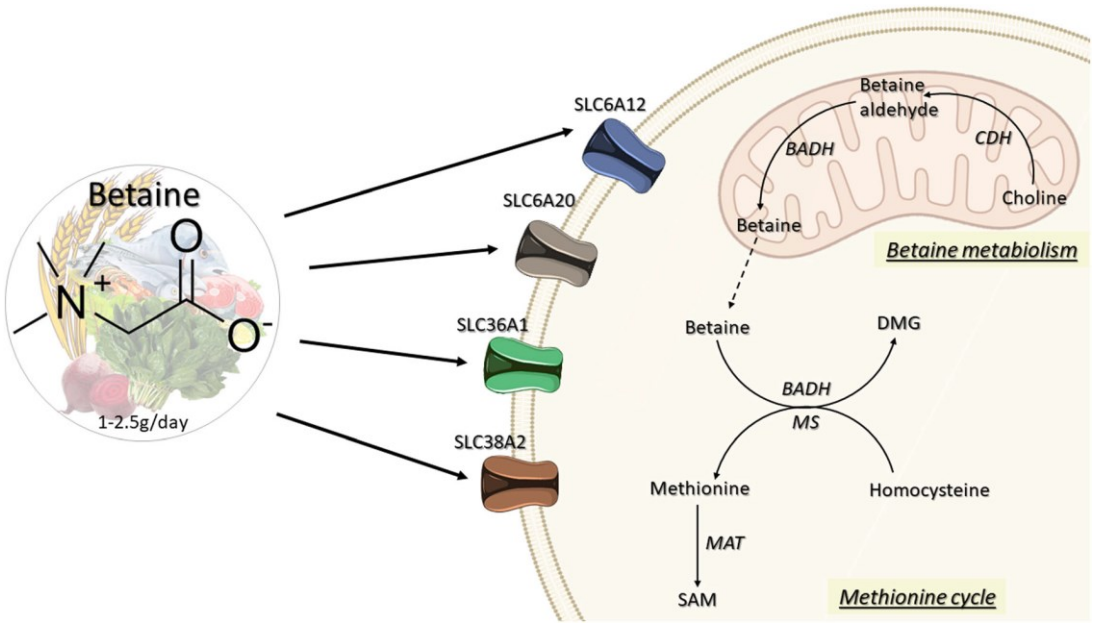

Figure 1 Cellular metabolism of betaine. Betaine is found in abundance in beets, wheat bran, seafood, and spinach. Once it enters the cell through betaine/GABA transporter 1 (BGT-1 - SLC6A12), IMINO transporter (SIT-1/XT3s1 - SLC6A20), proton/amino acid transporters (PAT-1 - SLC36A1), or sodium-dependent neutral amino acid transporter-2 (SNAT-2 - SLC38A2) it is metabolized according to the methionine cycle to synthesize methionine from homocysteine. Also, in the mitochondria, betaine can be directly synthesized from choline in small quantities. All the pathway enzymes refer human metabolism: choline dehydrogenase (CDH); betaine-aldehyde dehydrogenase (BADH); betaine-homocysteine methyltransferase (BHMT); methionine synthase (MS); methionine adenosyl transferase (MAT).

1.1 The dual role and distribution

Betaine helps maintain the intracellular osmotic pressure, as it binds little to nothing with protein surfaces and enables cellular control of water surface tension. Thus, it stabilizes protein structure and function, while protecting cells, proteins, and enzymes from osmotic stress. This role is relevant, especially in the kidney, where betaine can be present in extraordinary concentrations (>100 mM).[3] Other than the kidney, betaine is also found in the human liver and brain. However, the role of osmolyte and methyl donor has been studied in the liver and kidney, and much less in the nervous system. Moreover, recently, it was shown that BHMT, one of the enzymes involved in the detoxification of homocysteine, is present not only in the liver and kidneys but is also expressed in the intestine and white adipose tissue, suggesting a role of betaine also in these tissues.[6,14,15] Betaine is a potential therapeutic against alcohol-induced and metabolic-associated diseases and heavy-metal toxicity in the liver.[6,16] Betaine supplementation was also shown to have a role in muscle strength and power.[17] It helps to improve body composition in both males and females, but improvement of muscular performance only in males has been reported.[18] Betaine also helps against heat tolerance and increases resilience against thermal stressors.[19] Recently, an increasing number of studies show beneficial effects of betaine in cognition, early-stage neuronal development and in reducing neurodegeneration and memory impairment, suggesting an important role of betaine in human (neuro)physiology.[12]

1.2 Betaine: A therapeutic nutrient

Traditional eastern medicines have effectively used herbs and food ingredients as therapeutics for several different diseases. The primary advantage of such substances over modern medicine would be the absence of any severe side effects. One approach by modern

pharmacologists has been to integrate these herbs and nutrients with currently effective drug administration and develop new therapies. Betaine is one such stable, natural, and nontoxic substance that has shown beneficial effects in several diseases.

Homocystinuria, a sulphur metabolism pathway disorder characterized by increased accumulation of Hcy in cells and plasma, can cause osteoporosis, arteriosclerosis, dislocated eye lenses, intellectual disability and neurodegenerative pathologies such as Alzheimer's, Parkinson's and dementia.20 As a treatment, betaine therapy (6–9 g/day of oral administration) is used to decrease Hcy levels by converting it to methionine and thus increasing the flux through the re-methylation pathway. Since 2020, betaine is an FDA-approved drug marketed as Cystadane®. This treatment has not been reported to cause any severe side effects except mild body odour and a rare possibility of cerebral oedema due to hypermethioninemia.[20,21] Apart from homocystinuria, betaine supplementation also shows beneficial effects on diseases such as alcohol-induced liver diseases, hepatic steatosis, heart disease, dehydration and heat tolerance.[3,4,6,17,19,22] As said above, an increasing number of papers and studies demonstrate the beneficial role of betaine in the brain as well. However, the positive effects in the CNS are very little understood and investigated. In this work, we present an overview of papers that highlight the potential therapeutic role of betaine in neuronal disease and disorders. The cellular or molecular mechanism involved are also point out when they were known and clearly reported by the literature considered.

1.3 The presence of betaine in the brain

The reported betaine concentration in the vertebrate brain is lower than in the liver and kidney.[1,13] The SLC6 transporter BGT-1 is thought to be the primary regulator of betaine in the brain. In literature, BGT-1 localization

has been reported in the cerebral cortex, cerebellum, brainstem, hippocampus, micro-vessels (i.e., blood-brain barrier BBB) and leptomeninges.[23–25] The expression and localization of BGT-1 in the brain has been debatable, due to the lack of controls for specificities of the antibodies used and/or for the use of cell cultures instead of intact brains.[1] While some reports indicate isolated expression of BGT-1 on the surface of the neocortex and rule out the role of BGT-1 in regulation of GABA in rodent CNS,[1,24,26] other reports are suggesting astrocytic and neuronal expressions of BGT-1 in hippocampal slices of mice and support the clinical and behavioural studies showing beneficial neuroprotective effects of betaine against oxidative and osmotic stress.[12,27,28]

Since the relationship between blood plasma concentration and tissue accumulation of betaine is not very related, there are some anomalies in the reported betaine blood plasma concentration (1–3 mM).[13,29–31] Knight and collaborators have shown time, dose and osmolarity dependent betaine accumulation in the hippocampal tissues of mice.[12] They showed that the active betaine accumulation also affects the accumulation of other osmolytes in nervous tissues. Under isosmotic conditions, betaine significantly reduces the accumulation of creatine, taurine, and myo-inositol but not glutamate. On contrary, under hyperosmotic conditions, betaine increases the accumulation of glycine and glutamate. Also, it is to be noted that the betaine intracellular accumulation reaches a peak (8 h after first exposure) around 12 mM, which is four times higher than the given extracellular concentration (3 mM). This work suggests that apart from being an osmolyte and serving as a methyl donor, betaine could also influence GABA production/recycling and GABAergic pathways, which resonates with the findings of Kunisawa et al. that showed mediation of GABAergic pathways by betaine.[32]

Interestingly, the suggested role of betaine in the brain has not been limited to its osmolyte characteristic only. The recent work on nematodes *Caenorhabditis elegans* involving the betaine transporter SNF-3 (a BGT-1 ortholog that can only transport betaine) and receptor ACR-23, expressed in neurons and body muscles, suggests a possible signalling role of betaine for locomotion.[33,34] Peden and collaborators show that SNF-3 clears the extracellular betaine, which could be toxic for betaine-gated ACR-23, using the signalling pathway.[33] When seen with the work of Senesi and collaborators where they observe muscle fibre promotion by betaine activating insulin gain factor IGF-1 using signal pathway, it could be hypothesized that betaine might play a signalling role in the brain.[1,35]

1.4 Effects of betaine in neurological diseases and disorders

To have healthy physiological functioning and stable control of neuronal circuits, the excitatory/inhibitory (E/I) balance of the brain must be maintained. Since the E/I ratio is essential to maintain and regulate signalling transmission, the inhibitory system represents a key point to re-stabilize the neural network function when a predominance of excitation over inhibition rises, as in brain disorders.[36] GABA is the primary inhibitory neurotransmitter in the adult CNS, and the imbalance in its levels can be related to many neurodevelopmental and neurodegenerative diseases such as autism spectrum disorder (ADS), schizophrenia, epilepsy, depression, Parkinson's and Alzheimer's disease (AD) (Figure 2).[37]

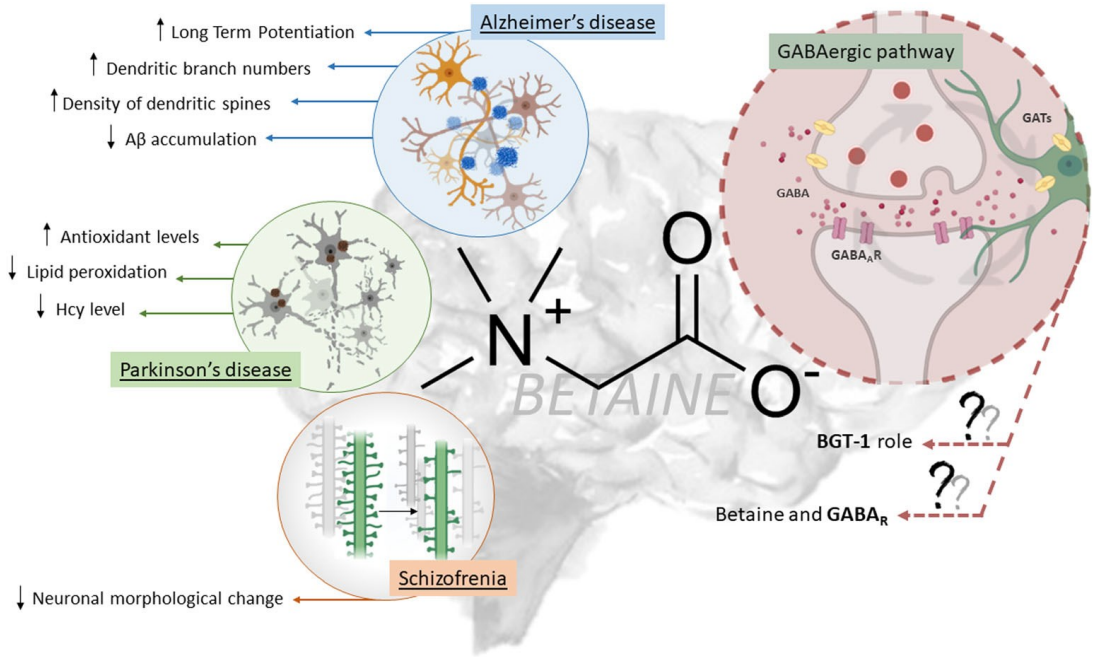

Figure 2: Schematic for the role of betaine in the brain. Betaine plays an active role in neuroprotection and against oxidative stress in cells. In neurodegenerative disease such as Alzheimer's, Parkinson's and schizophrenia, betaine can be a possible therapeutic. Although betaine is actively regulated by BGT-1 in GABAergic pathways, the complete mechanism behind is to be determined.

1.4.1 Role in epilepsy

The GABA transporters regulate GABA synaptic concentration; the majority of GABA uptake in the brain is done by GABA transporter 1 (GAT1). It is a pharmaceutical target to treat disorders related to neuronal E/I imbalance; consequently, it is often the target for antiepileptic and anticonvulsant drugs.[38] The FDA-approved antiepileptic drug, Tiagabine, is a selective inhibitor of GAT1.[39,40] The synergic anticonvulsant effect of tiagabine with the selective inhibitor EF1502 on both GAT1 and BGT-1 raised a functional role of BGT-1 in regulating diffused GABA from synaptic regions.41,42 While the controversies around the localization of BGT-1 failed to justify if it could play any effective role in the clearance of GABA, the development of BGT-1 knockout (KO) mice by Lehre and collaborators shed some light on this.[1] The seizure threshold experiments on BGT-1 KO mice showed no alteration and ruled out a role of BGT-1 in seizure

susceptibility.[26] This work suggests that EF1502 is an anticonvulsant because it inhibits GAT1; the inhibition of BGT-1 is irrelevant in controlling the seizure threshold. However, this study fails to address the compensatory mechanism from other GATs, which could have masked the effects arising from the deletion of BGT-1. Although this work implies, based on BGT-1 KO mice, that the synergic anticonvulsant effect of tiagabine and EF1502 should be due to GAT1 and not BGT-1, it requires conclusive evidence where such synergic anticonvulsant effect would be recorded using GAT1 KO mice and/or GAT1-BGT-1 KO mice.[26] Also, in 2020, Lie et al.[43] studied healthy adult mice and not samples from epileptic patients, limiting the direct correlation of these results with seizure-controlling therapies. Hence, BGT-1 still might be playing a role in epileptic seizures and GABAergic imbalance in CNS, which demands the development of BGT-1-specific brain permeating inhibitors.

1.4.2 Role in stress related disorders

The stress-induced psychiatric disorders like depression, anxiety, and post-traumatic stress disorder (PTSD) are associated with abnormalities in GABAergic neurotransmission functioning.[37,44,45] The study of water-immersion restraint strain (WIRS) induced stress (in mice) resulting in memory impairments showed amelioration by betaine.[32] This improvement could be inhibited by antagonists of BGT-1, GABAA and GABAB receptors. Also, the betaine treatment post-WIRS significantly decreased the expression of GABA transaminase (GABA-T), the enzyme responsible for breaking down GABA when not needed. GAT1, GAT3 and BGT-1 expressed in astrocytes regulate GABA levels,[46] and inhibition of GABA-T also increases GABA levels in the synaptic cleft.[47] As betaine is transported by BGT-1 and decreases GABA-T expression, betaine could be asserting its positive effects by changing GABA levels in the CNS. Thus,

betaine does not work only as a substrate of BGT-1, but it could also interact largely with the entire GABAergic system.

In psychology, social defeat is seen as a form of stress that could cause depression, anxiety, PTSD and so forth.[48] The resilience against such stress can be mediated by adaptive changes in neural circuits of neurotransmitters (like GABA) and molecular pathways.[49] Anhedonia is one of the main symptoms of stress-related disorders. While studying the role of the brain-gut-microbiota axis in such disorders, Qu and collaborators found that the mice subjected to chronic social defeat stress (CSDS), when given betaine supplementation, showed resilience to anhedonia via anti-inflammation action.[50] This study reports amelioration of the abnormal diversity and composition of the microbiota in the host's gut after CSDS by betaine supplementation; however, the specificity of the microbiome or conclusive evidence of CSDS is the reason behind the abnormal composition of gut microbiota. But overall, the study implicates the gut-brain axis playing a role in susceptibility to stress-related disorders, and betaine supplementation could be a prophylactic nutrient to prevent or minimize the relapse by stress in patients of such psychiatric disorders.[50]

1.4.3 Neuroprotective role against Alzheimer's and dementia

AD is the most prevalent neurodegenerative disease that is characterized by progressive impairment of cognition, memory, and intellectual functions. So far, there have not been many approved agents that ameliorate cognition and the overall function of AD patients, and different therapeutical approaches have been developed.

The damage caused to cellular proteins, lipids, and DNA, due to the excessive production of reactive oxygen species and reactive nitrogen species, is collectively called oxidative stress. The brain is more susceptible to oxidative damage, which has been correlated with the pathogenesis of

diseases like AD and vascular dementia (VaD).[51] The amyloid-ß (Aß) aggregation is hypothesized to play a pivotal role in the onset and progression of AD; hence, it could be an important biomarker in AD-like pathologies.[52] Aß is generated by amyloid precursor protein (APP) via sequential cleavage from ß- and γ-secretases. The generation and deposition of Aß have been associated with altered oxidative stress, inflammation, tau phosphorylation and synaptic dysfunction, contributing to the progression of the disease and eventually leading to death. Also, the increased levels of Hcy have been associated with the onset of AD, along with hyper-homocysteinemia.[53]

As betaine is an effective methyl donor to convert Hcy to methionine, a therapeutic approach is being developed to use betaine supplementation to target increased Hcy and reduce AD progression. Chai and collaborators report that betaine supplementation ameliorates AD-like Hcy induced memory deficits, enhances long-term potentiation, and increases dendritic branch numbers and density of dendritic spines.[54] It also attenuated the tau phosphorylation and Aß accumulation by altering APP processing.[54,55] In a study, Sun and collaborators demonstrated that subjects treated with betaine supplementation showed amelioration in cognitive deficit resulting in better recall of words, improved visual–spatial capacity and so forth.[56] Their results showed betaine supplementation (200 µg/kg for a month) reverses Aß accumulation and stimulates the regulation of memory-related protein (NR1, NR2A and NR2B); however, their small sample size and absence of molecular mechanism description need to be considered. Overall, betaine could be an effective therapeutic tool to treat AD but the mechanism of action of this molecule in AD needs to be investigated.

VaD is the other most common type of dementia in aged people after AD and lacks effective therapy. Chronic cerebral hypoperfusion (CCH) is thought to be the primary reason behind cognitive impairment in VaD

patients. The rats underwent the surgery of applied permanent bilateral occlusion of common carotid arteries; to produce a condition of CCH in humans, Nie and collaborators report that betaine administration, in rats, could mitigate the memory deficits induced by CCH.[51] They studied the CCH-induced decline of synaptic proteins PSD93, PSD95 and MAP 2, which are critical for learning, memory and synaptic plasticity. This investigation on spatial learning memory retention was done using the Morris water maze examination that revealed that betaine treatment restores the expression of these synaptic proteins. They proposed that CCH induces loss of postsynaptic expression of PSD93, PSD95 and MAP 2 via increased oxidative damage, which betaine treatment prevents and ameliorates memory deficits. However, they do not suggest any possible mechanism that using betaine suppresses oxidative stress. Nevertheless, this evidence furthers the therapeutical role of betaine in neurodegenerative disease.[51]

1.4.4 Protective role against Parkinson's

Parkinson's disease (PD) would be the second most prevalent neurodegenerative disease (only after AD) characterized by uncontrolled muscular activity, an increase in the metabolic concentrations of sulphate and nitrate compounds, a decline in dopamine levels due to neuronal degeneration and sleep disturbance. In PD patients, the oxidative stress in the brain has been suggested to give rise to the processes leading to dopaminergic neuronal degeneration[57,58] While the modelled motor aspects of PD in animals have highlighted the molecular pathways and mechanisms by which oxidative stress could yield progression of PD, the clinical studies have produced inconsistent results.[57,59] Interestingly, the inhibitory effects of betaine on the neurotoxic nitric oxide (NO), one of the free radicals causing oxidative damage in microglial cells, show that betaine could be used as an antioxidant in PD treatment.[60]

Unfortunately, this oxidative stress in PD patients does not arise only due to the imbalance between the production of free radicals (like NO and H_2O_2) and the antioxidant mechanism of the body for detoxification. The administration of the prominently prescribed dopaminergic drug for PD called laevo-3-4-dihydroxyphenylalanine (Levodopa, L-DOPA) increases plasma levels of Hcy.61 The high concentration of L-DOPA could produce excessive Hcy, which in turn increases oxidative stress that would assert toxic effects on dopaminergic neurons. The L-DOPA toxicity could be prevented by the addition of antioxidants at physiological concentrations or by soluble factors generated by glial cells.[62] Moreover, to cross the BBB and avoid peripheral toxicity, L-DOPA is often administered with a dopa decarboxylase inhibitor such as benserazide, which by catechol-o-methyltransferase elevates Hcy levels further. The clinical and experimental trials in PD patients show that a high accumulation of Hcy could contribute to accelerated neurodegeneration and the onset of atherosclerotic and neuropsychiatric symptoms.[63,64] Hence, such treatment overall poses a risk of hyperhomocysteinemia in PD patients, leading them towards other neurodegenerative diseases such as AD and dementia.[65] Alirezaei and collaborators studied the effects of betaine administration (in rats) on oxidative stress and increased Hcy levels induced by L-DOPA/benserazide treatment.[66] They demonstrated the neuroprotective qualities of betaine against L-DOPA-induced oxidative stress in the brain tissues of rats. They propose that, by continuous generation of SAM, betaine could decrease excessive Hcy levels generated by L-DOPA/benserazide, elevate antioxidant levels, and decrease lipid peroxidation. However, this study requires supportive evidence of physiological parameters like the behavioural test in PD animal model.

Rotenone is an inhibitor of mitochondrial complex I, breaks ATP production and enhances the production of mitochondrial ROS causing apoptosis and

inducing neurotoxicity. It is widely used as a model of the pathogenesis of PD. Neuronal cell death is one of the major factors behind cognitive decline in AD and PD. It was demonstrated that betaine performs neuroprotective effects against rotenone-induced neurotoxicity in PC12 cells.[67] The increasing oxidative stress and inflammation in the brain can cause brain ischemia and ischemic stroke. The betaine treatment of PC12 cells (with oxidative stress induced by H_2O_2) resulted in decreased proinflammatory cytokine production and reduced oxidative stress.[68] Betaine also increased the expression of antioxidative enzymes and non-enzymatic genes. These results showcase how betaine can and should be considered as a protector against oxidative stress and neurodegeneration.

1.4.5 A therapeutic agent for schizophrenia

Schizophrenia has always had sleep dysfunction as one of its primary descriptions.[69] The sleep pressure, the driving force of the homeostatic process, builds up during wakefulness and dissipates when asleep. Sleep pressure and sleep disturbance are associated with onsets for patients with psychosis. It is shown that with increasing high sleep pressure, specific metabolomic alterations occur like decreased levels of betaine in the whole brain.[70] Hence, betaine is proposed as one of the biomarkers for the diseases and treatments associated with sleep deprivation.

The neurons in schizophrenic brains tend to undergo gross morphological changes. The neuronal morphogenesis-related traits are significantly alleviated by a high-betaine diet, suggesting that betaine, through a neuroprotective mechanism, could be effective for refractory schizophrenia patients.[71] In CHDH- (a gene for betaine synthesis) deficient mice, schizophrenia-related molecular perturbations in the brain were recorded. It was shown that betaine supplementation induced improvements in cognitive performance dependent on genetic background.[72] These observations suggest that betaine could fit as

treatment for patients with schizophrenia, as an atypical drug at initial low doses, under observation of the response to treatment and side effects;[73] even if also in these conditions, the cellular and molecular mechanism is not understood.

2. Discussion

Given its dual role, betaine has always been considered an important nutrient for human physiology. But the evident growing literature highlights a possible third role, which is the positive effects of betaine in CNS disorders. The betaine in the liver and kidney has been well studied, reviewed, and understood. Despite the reported benefits of betaine supplementation in improving brain conditions, the mechanism of action at the cellular and molecular level is not yet clear.

In this review, we highlight the positive effects and therapeutic potential of betaine in brain-associated diseases like AD, PD, dementia, schizophrenia, depression, PTSD, epilepsy, and anhedonia reported in recent literature. Although BGT-1 can actively uptake betaine, the little expression in the brain and lack of understanding around its role and the role of betaine in the CNS raise questions over the mechanism behind accumulation and its impact on the brain. The work of Zhou and collaborators localizes BGT-1 in leptomeninges, which is supported by the BGT-1 KO mice model of Lehre and collaborators, ruling out any significant role of BGT-1 in controlling levels of GABA (in mice) and suggesting it as a facilitator to the probable signalling role of betaine.[1,24,26,33–35] On the other hand, the work of Knight in mouse hippocampal slices (containing little meninges) show active uptake and accumulation of betaine and correlate this with reported astrocytic and neuronal expression of BGT-1.[12,28,74] Whereas this conclusion fails to address the issue of uncertainty around immunochemistry data (due to the absence of BGT-1 KO then as a negative control),[1] it is certainly supported by the reported beneficial

effects of betaine supplementation in neurological and neurodegenerative diseases. Another problem with their conclusion is that they simply credit the reported rapid astrocytic and neuronal uptake of betaine by BGT-1 without considering or falsifying the involvement of any other transport mechanism in the brain. For example, Nishimura and collaborators showed that the osmo-sensitive system A transporter SNAT2, expressed in central neurons, can transport betaine, and could be involved in its regulation.[11] Moreover, Kunisawa and collaborators also highlight this issue by showing that the interaction of betaine with the GABAergic pathway could not be limited to just BGT-1.[32] Their results indicate a possible betaine interaction with $GABA_A$ and $GABA_B$ receptors. The work from Ibi and collaborators supports this hypothesis by showing that the prevention of cognitive impairment by betaine is mediated by BGT-1 but not its antioxidant effects.[75] Thus, the mechanism behind the reported uptake and accumulation of betaine in hippocampal slices[12] could not be limited to just BGT-1. Hence, it is critically important to explore any possible involvement of other transport mechanisms for betaine in the brain, to satisfactorily demystify its beneficial effects.

With such ambiguities over BGT-1 localization, one could simply question the presence of betaine in the brain itself. The recent work of Wang and collaborators on cylindrical polymer brushes (CPB) in vivo answers this question by modification of CPB using betaine.[76] The macromolecule CPB cannot cross the BBB by itself, but its modification with betaine (poly-carboxyl) allows it to successfully cross BBB via BGT-1 expressed on capillaries.[76] Thus, BGT-1 can not only transport betaine across BBB, but it can also help in the development of nano-drugs modified with betaine.[76,77]

Knight and collaborators also showed that, under hyperosmotic conditions, betaine significantly influences the uptake of glycine and glutamine.[12]

Since glycine is the precursor to GABA and glutamine to glutamate, a possible role for betaine in maintaining the balance between inhibitory and excitatory neurotransmission cannot be denied. Also, the effects of betaine against stress-induced diseases and memory loss indicate a connection with the GABAergic pathway in CNS.[32,50,51,54–56,66,71,72]

Betaine exhibits neuroprotective properties that could prevent the progression of neurodegenerative diseases like AD, PD and dementia.[51,54–56,60,66] Along with choline, folic acid, vitamin B6 and B12, betaine in the maternal diet is correlated with early neuronal development and attenuation of cognitive function at the later stage of life.[78] One way betaine helps is by reducing the Hcy levels in the neurons and promoting the expression of memory-related proteins.[54,55] Also, it can convert Hcy to methionine and increase SAM, which protects the brain against a variety of toxic agents causing oxidative stress.[79]

As a therapeutic, betaine is already in use as an FDA approved drug to treat homocystinuria. The reported side effects (for 6–9 g/day) are relatively mild such as gastrointestinal illness, mild body odour, increased urination, feeling dry mouth and preference for salty food.[80] The excess of betaine has been associated with cardiovascular disease and pulmonary hypertension.[81,82] While there are very few side effects recorded for betaine,[83,84] there is still not enough information on the long-term effects of regular betaine supplementation.

3. Conclusion

Although betaine is not considered an essential osmolyte, its beneficial and therapeutic roles in human physiology make it important in the diet. The role of betaine in diseases related to the liver and kidney is better understood but not so for the brain. The reported effects of betaine in the past two decades confirm its neuroprotective and antioxidative qualities with a positive correlation with the GABAergic pathway, but very little is

known about the mechanisms that using betaine asserts these effects. With emerging results showing the involvement of betaine with neurological pathways, it is critically important to explore the contribution of all possible transport mechanisms for betaine in the brain, to satisfactorily demystify its beneficial effects. Despite having such a protective role against several neurological diseases, very little is known about its molecular targets, implying betaine simply as the dark knight of the human brain.

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Chapter 3 Neurotransmitter transporters

In the nervous system, cells communicate mainly through the presynaptic release of neurotransmitters followed by the diffusion across the synapse cleft and the postsynaptic activation of receptors. The released neurotransmitters are then removed from the extracellular space and transported back to the neuron or in the surrounding glial cells by neurotransmitter transporters (NTT). These proteins play an essential role in the central nervous system (CNS), as the prolonged presence of high concentrations of neurotransmitters can be detrimental to the stability and functionality of the CNS (Kristensen, Andersen et al. 2011).

This work focuses on the primary inhibitory neurotransmitter of the CNS: γ-aminobutyric acid (GABA). The NTTs responsible for GABA re-uptake are called GABA transporters (GATs), which belong to the solute carrier superfamily (SLC) 6. SLC transporters encompass more than 450 transporters distributed across 66 canonical and several noncanonical families (Dvorak and Superti-Furga 2023). Apart from GATs, the NTTs responsible for the re-uptake of neurotransmitters such as serotonin, dopamine, glycine, and norepinephrine also belong to SLC6 (Kristensen, Andersen et al. 2011). While this work focuses on the interaction of betaine with GATs, especially GAT1, the only known GAT able to transport betaine is BGT-1 called betaine-GABA transporter-1. It is considered the transporter responsible for the translocation of betaine across the blood brain barrier and it is part of the GABA group of the SLC6 family. Among all GATs, GAT1 is the most studied, characterized and, well-understood transporter, whereas BGT-1 is the less investigated as its expression and role in the brain is still questionable (Lehre, Rowley et al. 2011, Kempson, Zhou et al. 2014, Bhatt, Di Iacovo et al. 2023).

As a part of the early stage research, an extensive literature study was done on both GAT1 and BGT-1, with the goal to understand their similarities and differences to design a well-directional research path. Hence, a detailed comparative literature review of GAT1 and BGT-1 was undertaken, and the peer-reviewed publication was achieved, presented in the next chapter.

Chapter 4 A comparative review on the well-studied GAT1 and the understudied BGT-1 in the brain

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Manan Bhatt[1,2†], Laure Gauthier-Manuel[3†], Erika Lazzarin[4†], Rocco Zerlotti[3,5†], Christine Ziegler[3], Andre Bazzone[5], Thomas Stockner[4], and Elena Bossi[1,2]

[1]Department of Biotechnology and Life Sciences, University of Insubria, Varese, Italy
[2]Centre for Neuroscience—University of Insubria, Varese, Italy
[3]Department of Biophysics II/Structural Biology, University of Regensburg, Regensburg, Germany
[4]Center for Physiology and Pharmacology, Institute of Pharmacology, Medical University of Vienna, Waehringerstr, Vienna
[5]Nanion Technologies GmbH, Munich, Germany

[†]These authors have contributed equally to this work and share first authorship

Abstract

γ-aminobutyric acid (GABA) is the primary inhibitory neurotransmitter in the central nervous system (CNS). Its homeostasis is maintained by neuronal and glial GABA transporters (GATs). The four GATs identified in humans are GAT1 (SLC6A1), GAT2 (SLC6A13), GAT3 (SLC6A11), and betaine/GABA transporter-1 BGT-1 (SLC6A12) which are all members of the solute carrier 6 (SLC6) family of sodium-dependent transporters. While GAT1 has been investigated extensively, the other GABA transporters are less studied and their role in CNS is not clearly defined. Altered GABAergic neurotransmission is involved in different diseases, but the importance of the different transporters remained understudied and limits drug targeting. In this review, the well-studied GABA transporter GAT1 is compared with the less-studied BGT-1 with the aim to leverage the knowledge on GAT1 to shed new light on the open questions concerning BGT-1. The most recent knowledge on transporter structure, functions, expression, and localization is discussed along with their specific

role as drug targets for neurological and neurodegenerative disorders. We review and discuss data on the binding sites for Na^+, Cl^-, substrates, and inhibitors by building on the recent cryo-EM structure of GAT1 to highlight specific molecular determinants of transporter functions. The role of the two proteins in GABA homeostasis is investigated by looking at the transport coupling mechanism, as well as structural and kinetic transport models. Furthermore, we review information on selective inhibitors together with the pharmacophore hypothesis of transporter substrates.

1 Introduction

The primary inhibitory neurotransmitter in the mammalian central nervous system (CNS) is γ-aminobutyric acid (GABA). Its presence in the brain was discovered independently by three groups in the year 1950 (Awapara et al., 1950; Roberts and Frankel, 1950; Udenfriend, 1950). GABA is estimated to be the neurotransmitter in 60%–75% of the synapses in the CNS (Fagg and Foster, 1983), and in about 30% of synapses in the brain (Durkin et al., 1995). In the cerebral cortex, GABA is involved in the control of the balance between excitation and inhibition, and consequently in network synchronization, information processing, and neuronal plasticity and excitability (Krnjević and Schwartz, 1967; Blatow et al., 2003; Conti et al., 2004; Imbrosci and Mittmann, 2011; Brady et al., 2018; Bi et al., 2020; Xu et al., 2020). Alteration of GABA homeostasis plays a significant role in several neuropathological diseases including epileptic seizures, Alzheimer's, Parkinson's, Huntington's disease, and multiple sclerosis (Wojtowicz et al., 2013; Cawley et al., 2015; Rozycka and Liguz-Lecznar, 2017; Zafar and Jabeen, 2018).

The four human GABA transporters GAT1 (SLC6A1), BGT-1 (SLC6A12), GAT2 (SLC6A13), and GAT3 (SLC6A11) are part of the solute carrier 6 (SLC6) family. The GATs are expressed in both presynaptic neurons and

surrounding glial cells: GAT1 is the most abundantly expressed GABA transporter, while the expression of GAT2 is the lowest in the CNS (Conti et al., 1999). To terminate GABAergic signaling GABA is either transported back into the releasing neuron (80%) or into the surrounding glial cells (20%), where it is metabolized further into glutamine (Zafar and Jabeen, 2018).

The selective inhibition of GABA uptake into astrocytes would lead to an increase in neuronal levels by elevated neuronal uptake. This indirect potentiation of neuronal GABA uptake has therapeutic importance as it ameliorates conditions that are caused by low GABA concentration in the GABAergic synapse (Schousboe et al., 2014; Patel et al., 2015). Multiple selective GABA inhibitors have been developed and are used as therapeutics (Kickinger et al., 2021). Among GATs, GAT1 has been investigated extensively, while BGT-1 is understudied and requires well-directional additional research to obtain a fair understanding of its structure, function, and role in human physiology. Using the research model of GAT1, several questions for BGT-1 could be inferred or answered. Here, we present a comprehensive review that focuses on GAT1 and BGT-1 with the aim to help the community inspire research in BGT-1 by inference from GAT1 data. We cover recent knowledge of GAT1 and BGT-1 on brain distribution, structure-function, and their roles in treating neurological and neurodegenerative disorders. We focus on the binding sites for Na^+, Cl^-, and substrates, and highlight specific molecular determinants of transporter functions and consequences for GABA homeostasis. We discuss the transport coupling mechanism, computational structural modelling, the recent cryo-EM structure (Motiwala et al., 2022; Kanner and Dayan-Alon, 2023), and define the structure of the binding site for GAT1 (Joseph et al., 2022). We also

summarize information on GAT1 and BGT-1 specific inhibitors as well as the pharmacophore hypothesis of transporter substrates.

2 Historical perspective of GABA transporters

The first GAT was purified from rat brain in 1986 and cloned in 1990 (Radian et al., 1986; Guastella et al., 1990). It consists of 599 amino acids, twelve transmembrane helices, and has an apparent $K_{0.5}$ in the μM range. The human GAT1 was also cloned in the same year (Nelson et al., 1990). Thereafter GAT2 and GAT3 were cloned from rat and human brain (Borden et al., 1992; Lopez-Corcuera et al., 1992; Borden et al., 1994). And lastly, the fourth GABA transporter was cloned from Madin-Darby canine kidney (MDCK) cells. This transporter is regulated by hypertonicity and was shown to transport the osmolyte betaine in addition to GABA, thus was called betaine/GABA transporter 1 (BGT-1) (Yamauchi et al., 1992). The human orthologue of BGT-1 was also cloned 2 years later from brain and kidney (Borden et al., 1994; Rasola et al., 1995). Like other SLC6 transporters, the transport function of GATs is energized by the transmembrane gradient of Na^+ (Guastella et al., 1990).

The four GABA transporters were also identified and cloned in mice and named sequentially as GAT1, GAT2, GAT3, and GAT4 (Liu et al., 1992; Liu et al., 1993). This differentiation of nomenclature (See Table 1) can be confusing and was only resolved by the introduction to the systematic nomenclature of the SLC6 transporter family (Hediger et al., 2004). To avoid any misinterpretation caused by this ambiguity, in this work we will use nomenclature as per the human paralogues.

3 Expression and localization

GATs are localized both in astrocytes and neurons (See Figure 1); in which they are present at the synaptic level and/or extra synaptically. Their tissue expression is reported in Table 1. GAT1 shows the highest

expression rate among all GATs in the CNS, predominantly in the adult frontal cortex (Conti et al., 2004). GAT1 is expressed in GABAergic axon terminals, astrocytes, oligodendrocytes, and microglia (Minelli et al., 1995; Melone et al., 2015; Fattorini et al., 2020a). Expression of GATs changes with life (Sundman-Eriksson and Allard, 2006; Banuelos et al., 2014; Pandya et al., 2019; Khozhai, 2020). In the adult CNS the main localization of GAT-1 is neuronal and of GAT3 on the glial cells, whereas in the neonatal mammalian brain a larger abundance of GAT-1 was reported in astrocytes and of GAT3 in neurons. The early expression of GATs is most likely related to the high cytoplasmic Cl− concentration at the initial brain developmental stages and the unusual roles of GABA in some brain regions, where it occasionally excites immature neurons. The function of GABA becomes inhibitory with the increased expression of the potassium chloride exporter KCC2 (Borden, 1996a; Yan et al., 1997; Rivera et al., 1999; Minelli et al., 2003; Conti et al., 2004; Ben-Ari et al., 2012; Kilb, 2012). The distribution of BGT-1 in the human tissues differs from the expression pattern of the other GABA transporters, which is linked to its unique ability to transport the osmolyte betaine. The localization of BGT-1 in the brain is not well defined. Based on data from cultured astrocytes and astrocytoma lines, the expression of BGT-1 was reported at the blood-brain barrier and brain endothelium (Takenaka et al., 1995; Bitoun and Tappaz, 2000; Ruiz-Tachiquin et al., 2002). Interestingly, BGT-1 was also found in dendritic spines of glutamatergic synapses, showing a possible localization at extra-synaptic regions (Zhu and Ong, 2004a; Zhu and Ong, 2004b). The importance of BGT-1 for regulating brain GABA levels might be limited, because of the low BGT-1 expression, the slow turnover rates, and the low affinity for GABA in comparison to GAT1. Hence, suggesting that BGT-1 might not be able to functionally replace GAT1 for the maintenance of physiological GABA levels, therefore pointing to a role of

BGT-1 in brain tissues that differs from regulating GABA levels which might be linked to its ability to transport betaine and to a protective mechanism against osmotic stress. In addition, it was reported that a polymorphism in SLC6A12 can increase the risk of temporal lobe epilepsy (Sacher et al., 2002; Gonzalez-Burgos and Lewis, 2008; Kempson et al., 2014; Li et al., 2015).

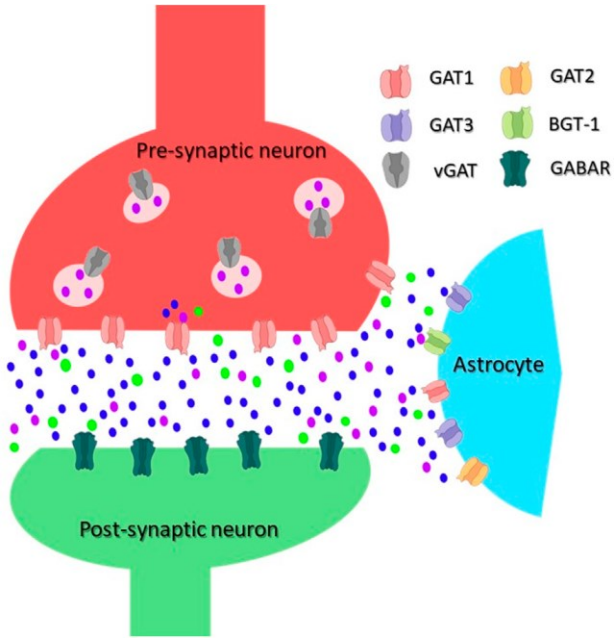

Figure 1 Scheme of an inhibitory synapse showing the distribution of GATs. In the pre-synaptic terminal of GABAergic neurons, GABA is synthesized from glutamate and packaged into vesicles by vesicular GAT (vGAT). GABA is released into the synaptic cleft upon depolarization of the plasma membrane. An inhibitory signal can be generated in the post-synaptic neuron by its binding to and opening of the ionotropic GABA receptors ($GABA_R$). Released GABA is removed from the synaptic cleft by GATs, the transport is energized by cotransport of Na^+/Cl^-. The main GABA transporter in neurons is GAT1, whereas in astrocytes, mainly GAT3 but

also GAT2 and BGT-1 are responsible for the GABA transport. GABA molecules are shown as purple dot, Na^+ ions as blue dot, and Cl^- as green dot.

SLC6 gene	Species	Currently accepted name	Known uptake substrates	Known uptake inhibitors	Tissue expression	References
SLC6a1	Human	GAT1	GABA, nipecotic acid, guvacine, ACHC	Nipecotic acid, L-DABA, guvacine, SKF-89976a, tiagabine, ACHC, THPO, Cl-966, NO-711, EF1502, LU-32-176B, EGYT-3886	Cerebellar cortex, medulla, hippocampus, thalamus, hypothalamus, olfactory bulb, basal ganglia, cerebellum, interpeduncular nucleus, substantia nigra, basal forebrain, retina.	Augood et al. (1995), Yasumi et al. (1997), Eulenburg and Gomeza (2010), Jin et al. (2011), Ghirardini et al. (2018), Fattorini et al. (2020b)
	Rat	GAT1				
	Mouse	GAT1				
SLC6a12	Human	BGT-1	GABA, betaine	EGYT-3886, EF1502, bicyclo-GABA, NNC 05-2045, NNC05-2090	Liver, kidney, cerebral cortex, cerebellum, brainstem, hippocampus, leptomeninges.	Lopez-Corcuera et al. (1992), Yamauchi et al. (1992), Borden, 1996b, Jin et al. (2011), Zhou and Danbolt (2013), Kempson et al. (2014), Lie et al. (2020)
	Rat	BGT-1				
	Mouse	GAT2				
SLC6a13	Human	GAT2	GABA, nipecotic acid, ß-alanine	Nipecotic acid, L-DABA, guvacine, EGYT-3886	Liver, kidney, leptomeninges, pia mater, arachnoid complex.	Zhou et al. (2012), Zhou and Danbolt (2013)
	Rat	GAT2				
	Mouse	GAT3				
SLC6a11	Human	GAT3	GABA, nipecotic acid, ß-alanine	Nipecotic acid, L-DABA, guvacine, EGYT-3886, SNAP-5114, NNC-05-2045	Brainstem, cerebellum, cortex frontalis, cortex occipitalis, hippocampus, olfactory bulb, retina, hypothalamus, midline thalamus, medulla oblongata, basal forebrain, striatum.	Durkin et al. (1995), Jin et al. (2011), Ghirardini et al. (2018)
	Rat	GAT3				
	Mouse	GAT4				

Table 1 Nomenclature, uptake substrates, inhibitors, and tissue expression of GABA transporters.

4 Role of ions and substrates in transport function

4.1 Ion dependence

GAT1 and BGT-1 are both secondary active transporters, which exploit the inward-directed Na+ electrochemical gradient to energize the uphill transport of their substrate (Riggs et al., 1958; Kanner, 1978; Matskevitch et al., 1999; Hertz et al., 2013). In addition, Na^+ ions also play a role in stabilizing the bound substrate inside the binding pocket of SLC6, leading to binding cooperativity between the co-transported ions and the organic substrate (Rosenberg and Kanner, 2008; Piscitelli et al., 2010). Replacing Na+ with other cations such as K^+, Cs^+, Li^+, ammonium, and choline does not trigger GABA transport in GAT1, but L^i+, and to some extent Cs^+, can permeate the transporter in an uncoupled manner, leading to the leak-currents (Mager et al., 1996; Grossman and Nelson, 2003). GABA transport by GAT1 and BGT-1 is electrogenic, i.e., substrate uptake by both

transporters leads to the net translocation of elementary positive charge(s) into the cell. As a consequence, the transport is voltage-dependent (Guastella et al., 1990; Matskevitch et al., 1999). Moreover, it has been observed that Cl− ions are required for transport by GAT1 (Lu and Hilgemann, 1999a; Lu and Hilgemann, 1999b; Loo et al., 2000; Bossi et al., 2002; Giovannardi et al., 2003; Zomot et al., 2007) and BGT-1 (Yamauchi et al., 1992). The role of Cl^- ions remains incompletely understood since the equilibrium potential of Cl^- is close to the membrane resting potential, which means that the energetic contribution of co-transporting Cl^- does not contribute to the driving forces of the transport reaction (Loo et al., 2000). On the other hand, the presence of a negative charge in the binding site helps in stabilizing the Na^+-bound conformation of the protein, facilitating transport (Zomot et al., 2007; Rosenberg and Kanner, 2008).

4.2 Kinetic parameters

For defining kinetic parameters of the transport reactions, two parameters are particularly important: the concentration of substrate (or co-substrate) necessary to reach the half maximum transport rate (in this work defined as $K_{0.5}$), and the maximum turnover V_{max}. Data on the apparent affinities of GAT1 and BGT-1 for their substrates have been collected using electrophysiological measurements and radioactive uptake assays (See Table 2). It is interesting to note that the $K_{0.5}$ of GAT1 for GABA, which is in the low μM range, shows a two-fold difference when switching the membrane potential from −90 mV to −10 mV, while the same membrane potential modulation exerts a larger effect on $K_{0.5}$ for Na^+ and Cl^-. For BGT-1, conversely, $K_{0.5}$ of the organic substrates (μM range for GABA and low mM range for betaine) increases around four-folds, while the $K_{0.5}$ for Na^+ and Cl^- seems to be less affected by voltage changes. The V_{max} for GAT1 in the forward transport mode (GABA influx) is between 6 s^{-1} and 13 s^{-1}

at voltages between −60 mV and −80 mV, at room temperature (between 20°C and 25°C), and can be influenced by modifying the temperature in *Xenopus laevis* oocytes; at 37°C and −50 mV or −90 mV the V_{max} is 73 s^{-1} and 93 s^{-1}, respectively (Binda et al., 2002; Fesce et al., 2002; Bicho and Grewer, 2005; Gonzales et al., 2007). In the reverse transport mode (GABA efflux), the V_{max} is 3 s^{-1} at −120 mV and 60 s^{-1} at +120 mV (Lu and Hilgemann, 1999a).

Transporter	Substrate	Technique	Condition	$K_{0.5}$	References
rGAT1	GABA	[^{3}H]GABA uptake in *X. laevis* oocytes	96 mM NaCl, 22.2 mCi [^{3}H]GABA	7 μM	Guastella et al. (1990)
hGAT1	Na^+	TEVC on *X. laevis* oocytes	−80 mV, 120 mM Cl^-, 0.3 mM GABA	44 mM	Mager et al. (1996)
hGAT1	GABA	TEVC on *X. laevis* oocytes	−90 mV, 21°C, 100 mM Na^+, 106 mM Cl^-	34 μM	Gonzales et al. (2007)
			−10 mV, 21°C, 100 mM Na^+, 106 mM Cl^-	17 μM	
	Na^+	TEVC on *X. laevis* oocytes	−90 mV, 21°C, 5 mM GABA, 106 mM Cl^-	33 mM	
			−10 mV, 21°C, 5 mM GABA, 106 mM Cl^-	108 mM	
	Cl^-	TEVC on *X. laevis* oocytes	−90 mV, 21°C, 5 mM GABA, 100 mM Na^+	9 mM	
			−10 mV, 21°C, 5 mM GABA, 100 mM Na^+	88 mM	
mGAT2[a]	GABA	[^{3}H]GABA uptake in *X. laevis* oocytes	100 mM NaCl, 0.1 μCi [^{3}H]GABA	80 μM	Lopez-Corcuera et al. (1992)
hBGT-1	GABA	[^{3}H]GABA uptake in HEK293 cells	140 mM NaCl, 2.76 mCi [^{3}H]GABA	18 μM	Kvist et al. (2009)
cBGT-1	GABA	TEVC on *X. laevis* oocytes	−100 mV, 96 mM Na^+, 103.6 mM Cl^-	9 μM	Matskevitch et al. (1999)
			−30 mV, 96 mM Na^+, 103.6 mM Cl^-	31 μM	
	betaine	TEVC on *X. laevis* oocytes	−80 mV, 96 mM Na^+, 103.6 mM Cl^-	500 μM	
			−30 mV, 96 mM Na^+, 103.6 mM Cl^-	2 mM	
	Na^+	TEVC on *X. laevis* oocytes	−90 mV, 1 mM GABA, 100 mM Cl^-	66 mM	
			−50 mV, 1 mM GABA, 100 mM Cl^-	89 mM	
	Cl^-	TEVC on *X. laevis* oocytes	−90 mV, 1 mM GABA, 96 mM Na^+	78 mM	
			−50 mV, 1 mM GABA, 96 mM Na^+	114 mM	

[a]mGAT2 is the mouse homologous of BGT-1 in dog, rat, and human (see Table 1).

Table 2 Apparent affinities of the transporters GAT1 and BGT-1 for the respective substrates with different techniques, samples, and conditions.

4.2 Ion cooperativity

Cooperativity is an effect of the modulation of the binding affinity for a substrate by the binding of other co-substrates. Since the binding affinity (K_d) partially describes the kinetic $K_{0.5}$, it is only natural to find that different concentrations of one substrate can exert an effect over the $K_{0.5}$ of the other substrates. For GAT1, Loo

and collaborators used two-electrode voltage clamp (TEVC) on *X. laevis* oocytes to study the cooperativity between Na^+, Cl^-, and GABA at −110 mV (Loo et al., 2000). The authors studied the cooperativity among substrates by considering the effect that a specific substrate extracellular concentration has on a kinetic parameter dependent on another substrate or voltage. The parameters considered are either the $K_{0.5}$, or the V_{max} (proportional to the maximum current amplitude in TEVC, I_{max}). From this study emerges that the apparent affinity for GABA is highly dependent on both external Na^+ and Cl^- concentrations. The half-maximal GABA concentration ($K_{0.5,GABA}$) decreases several folds when increasing external Na^+ and Cl^- concentrations, while it increases dramatically when holding the voltage to a more positive value (See Figure 2A). This is expected since, as stated in Section 4.1, Na^+ is needed to energize the transport and it is also possible that it can help stabilize the substrate in the binding pocket. Moving on Cl^-, the half-maximal Cl^- concentration ($K_{0.5,Cl-}$) increases upon increasing the extracellular GABA concentration, while no significant differences have been noted when altering external Na^+ concentrations (See Figure 2B). Concerning Na^+, the half-maximal Na^+ concentration ($K_{0.5,Na+}$) shows a slight increase when increasing extracellular GABA and a slight reduction when increasing extracellular Cl^- (See Figure 2C). The maximal current driven by saturating GABA ($I_{max,\ GABA}$) seems not to be significantly altered by external Cl^- concentrations, while it increases significantly when increasing the extracellular Na^+ concentration (See Figure 2D). Transport currents in presence of saturating external Cl^- ($I_{max,\ Cl-}$) increase when increasing both

external GABA and Na^+ (See Figure 2E), while the transport currents in presence of saturating extracellular Na^+ ($I_{max, Na+}$) are unaffected by changes in extracellular Cl^- concentrations but increase dramatically when increasing external GABA (See Figure 2F).

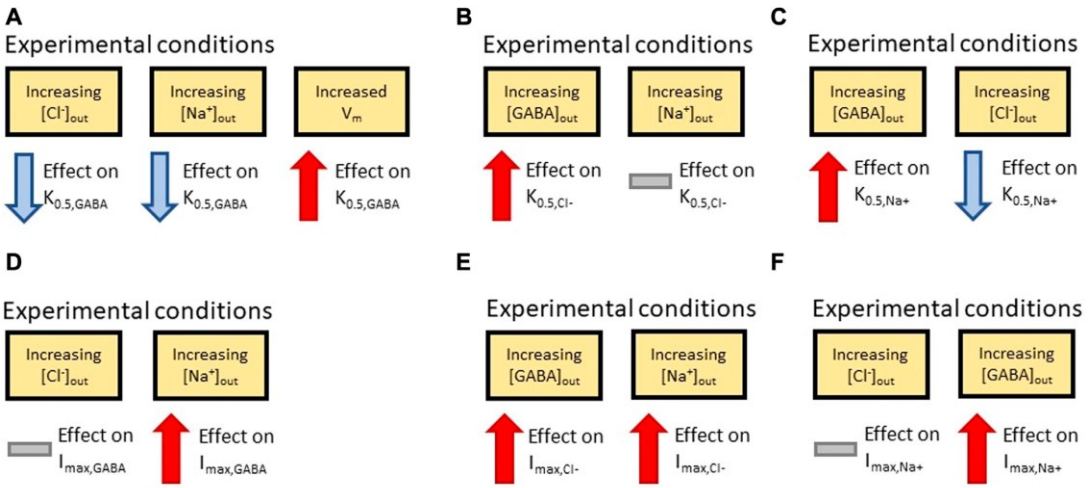

Figure 2 Changes in $K_{0.5}$ or I_{max} as a consequence of the variation of experimental conditions; Figure is adapted from Loo et al. (2000). From (A–F): The orange boxes represent changes in the experimental conditions, their effects on the parameter $K_{0.5}$ or I_{max} are indicated below. A red upward arrow indicates an increase of the respective parameter, a blue downward arrow indicates a decrease, while a grey rectangle indicates no significant change.

4.4 Effect of Cl^- ions on the transport activity

When changing the membrane potential, the GABA-evoked current in the absence of Cl^- is greatly reduced when the holding potential is set to 0 mV, while at −150 mV the current is half of the amplitude of that obtained in the presence of 106 mM Cl^- (Loo et al., 2000). This is consistent with the idea that the main role of Cl^- is to attract positive charges in the binding site of GAT1, thus facilitating transport. Consistently, it is easier for positive charges (Na^+) to access to the substrate and ion binding site in the center of the protein at highly negative membrane potential compensating for the absent Cl^- (Rosenberg and Kanner, 2008). A study conducted on the mutants S331E and S331D (Zomot et al., 2007)

supports this hypothesis. Residue S331, which is involved in chloride binding, is substituted by these mutations with a negatively charged amino acid. The presence of this negative charge can, to some extent, substitute for the negative charge of the chloride ion, and the mutants partially restore transport in the complete absence of chloride anions. Similar to GAT1, also for BGT-1 the transport of betaine and GABA is dependent on Na^+, and only partially dependent on Cl^-. As for GAT1, hyperpolarization of the membrane increases affinity for Na^+ in BGT-1. The reduction of external Na^+ implies a decrease in both the maximal current and the affinity of the transporter for GABA. The dependence is mutual: decreasing the concentration of GABA below its $K_{0.5}$ leads to a shift in $K_{0.5}$ of Na^+ from 50 mM to 120 mM. As for GAT1, GABA transport is partially dependent on Cl^-, with currents decreasing by 80% at −90 mV and by 90% at −50 mV in the absence of Cl^-. This implies that the Cl^- dependence is related to membrane polarization. Reducing the Cl^- concentration from 150 mM to 50 mM brought a slight decrease in GABA affinity. The affinity of Na^+ and Cl^- is also mutually dependent: decreasing Cl^- brings a slight reduction in Na^+ affinity, while the same reduction in Na^+ leads to a non-saturable Cl^- concentration dependence (Matskevitch et al., 1999).

4.5 The reverse transport mode

The reverse transport phenomenon triggers the release of GABA in the synaptic cleft through GAT1 or BGT-1, as well as the release of betaine through BGT-1. The reverse transport remains understudied in both transporters, while the role of substrate concentration in regulating the ratio between reverse and forward transport remains largely unknown due to the difficulties in controlling cytoplasmic concentrations. For GAT1, a number of interesting results have been described. The reported ratio between the forward and the reverse transport mode is tuned by membrane potential, if no specific conditions are applied to intra- and

extra-cellular substrates concentrations: at negative potentials, forward transport is favored, while the equilibrium is shifted towards reverse transport with membrane depolarization (Wu et al., 2001). This observation has biological relevance as the membrane potential reaches a positive value when the neuron is firing at an action potential. The work of Loo and collaborators show that altering the Na^+ gradient can regulate the influx or efflux of GABA (Wu et al.,2006). If Na^+ is present only inside the cell, this results in a net, albeit small outward-directed GABA-evoked current. The results were more complex, when extracellular GABA was removed: a GABA evoked outward-directed current was recorded followed by an inward-directed current that was attributed to GABA being reabsorbed by the cell. The effect, though, is stronger when altering the Na^+ gradient as compared than changing the GABA gradient due to the stoichiometry of the transport reaction. This indicates a possible regulatory role in a physiological environment: once a neuron has fired a series of action potentials with high frequency the internal sodium concentration may become sufficiently high to allow for limited release of GABA to the synaptic cleft (Wu et al., 2006) or to reduce the uptake of synaptically released sodium. Similarly reducing the cytoplasmic concentration of Cl^- favors GABA uptake, while high intracellular Cl^- promotes reverse transport (Grossman and Nelson, 2003). Consistently, Bertram and collaborators showed that in the presence of internal GABA, the depletion of internal Cl^- in *X. laevis* oocytes expressing GAT1 resulted in a significant decrease of reverse transport activity (Bertram et al., 2011).

4.6 Effect of Na^+ and Cl^- on pre-steady state current

In electrophysiology, the continuous transport activity by an electrogenic transporter is registered as the elicitation of a steady state current. The electrophysiological measurements, apart from the steady-state currents,

also let to record an exponentially decaying transient current, known as the pre-steady state current. They represent the intramembrane charge movement seen as a transient current in response to voltage or Na^+ concentration jumps and can be recorded by voltage clamp in *X. laevis* oocytes (Bhatt et al., 2022) and in HEK cells (Bicho and Grewer, 2005). These currents are associated with the initially synchronized out of equilibrium events such as binding and release of ions, conformational rearrangements, and charge movements across the membrane electric fields (Wadiche et al., 1995; Peres et al., 2004). For the GABA transporter rGAT1, the charge movement is related to the displacement of ions (Na^+) between the extracellular space and a cavity in the transporter. The analysis of these currents provides kinetic parameters such as rate constants and charge dislocation during the pre-steady state event (Holmgren and Rakowski, 1994). Using voltage clamp protocols in *X. laevis* oocytes expressing transport protein it was possible to isolate pre-steady state currents elicited by GAT1 and BGT-1 (Mager et al., 1993; Mager et al., 1996; Bossi et al., 2002; Grossman and Nelson, 2003). These transient currents in GAT1 and BGT-1 are strictly Na^+-dependent and reflect the movement of Na^+ in the transporter vestibule inside the membrane electric field for the initial step of the transport. For GAT1, this charge relocation accounts for most of the total charge transferred per transport cycle (Bicho and Grewer, 2005; Lopez-Redondo et al., 2018). Interestingly, the pre-steady state currents in BGT-1 and GAT1 are different. In GAT1, the Na^+ induced transient currents are symmetric at both positive and negative membrane potentials. Whereas in BGT-1 the Na^+-induced pre-steady state currents are only detectable at negative membrane potentials. This behavior can be modified by modulating external Na^+ concentrations for both BGT-1 and GAT1: if extracellular Na^+ is lower than its $K_{0.5}$ for the respective transporter, the pre-steady state currents are detected only at

negative membrane potentials, while if extracellular Na^+ is several folds higher than its $K_{0.5}$, these currents were detected only at positive potentials. These results suggest that the pre-steady state current results from the exposure and the occlusion of charges on the transporter surface (Forlani et al., 2001; Binda et al., 2002; Grossman and Nelson, 2003). For GAT1, reducing the concentration of external Na^+ and/or Cl^- would shift the charge versus voltage relation of the transient currents towards more negative potentials, where the effect of Cl^- is less than Na^+. In GAT1, the transient currents are still observable in the absence of external Cl^- indicating an only partial dependence of transport on extracellular Cl^- (Bossi et al., 2002).

4.7 pH dependence

Another factor that influences transport is pH. In BGT-1, the current elicited by GABA decreases with decreasing pH (at pH 5.5, the current reduces to 20% with respect to the current at physiological pH) and strongly increases at pH 8.5. In contrast, for GAT1 the GABA induced currents decrease only slightly at acidic pH, while no change was observed at alkaline pH (Forlani et al., 2001). For both transporters, acidification combined with a decrease in Na^+ reduces currents much stronger than by the sole decrease in Na^+. In BGT-1, the change in pH does not significantly alter the $K_{0.5}$ for GABA and Na^+. In contrast, the Cl^- $K_{0.5}$ is highly affected by pH: it is 50 mM at pH 8.5, but seemingly non-saturable at pH 7.0 (Matskevitch et al., 1999). The pre-steady state currents of GAT1 are affected by alkaline pH: while present at both positive and negative potentials with similar absolute amplitudes, at alkaline pH the currents are measurable almost exclusively at positive potentials (Grossman and Nelson, 2003). No pre-steady state current is detectable for BGT-1 at acidic pH, while at pH 8.5 they can be observed at positive potentials (Matskevitch et al., 1999). An interpretation of these observations could

be that H^+ could compete with Na^+ for the sodium binding sites in both GAT1 and BGT-1, but not facilitate a substrate transport (Matskevitch et al., 1999; Forlani et al., 2001; Binda et al., 2002). In GAT1 it has been reported that one of the determinants of the pH dependence is located in external loop 5 (EL5) (Forlani et al., 2001).

4.8 Substrate stoichiometry

There are still controversies regarding the stoichiometry of the transport reaction for GAT1. Initial studies on membrane vesicles and proteoliposomes from rat brain slices pointed to a substrate stoichiometry of 2 Na^+: 1 Cl^-: 1 GABA, which was confirmed by the measurements of reversal potential in *X. laevis* oocytes heterologously expressing GAT1 (Radian and Kanner, 1983; Keynan and Kanner, 1988; Lu and Hilgemann, 1999b). Other authors observed a charge translocation that exceeds the before mentioned predicted stoichiometry and attributed the observation to a "channel-mode" behavior of GAT1 (Krause and Schwarz, 2005). This has been contested by others, who could not observe the same behavior in the same cell types (Matthews et al., 2009). Lately, a stoichiometry of 3 Na^+: 1 Cl^-: 1 GABA has been proposed from substrate uptake data and reversal potential measurements in *X. laevis* oocytes, because the reported data accounted by modelling of the experimentally observed elementary charges translocation could be explained better by the modified stoichiometry (Mager et al., 1996; Omoto et al., 2012; Willford et al., 2015; Eskandari et al., 2017). The importance of Cl^- for substrate transport is well documented (See Section 4.4 and Section 4.6) but its role remains debated. Some authors suggested a Cl^-/Cl^- exchange mechanism for GAT1, suggesting no occurrence of the net Cl^- transport, and proposed that the net stoichiometry for the transport cycle should be 2 Na^+: 1 GABA (Loo et al., 2000; Bicho and Grewer, 2005). For BGT-1 only one transport stoichiometry has been reported. Matskevitch and collaborators measured

radioligand uptake in *X. laevis* oocytes and reported that two ratios would be possible: 3 Na^+: 1 or 2 Cl^-: 1 GABA/betaine (Matskevitch et al., 1999).

5 GABA transporter structure

5.1 Early studies of structural elements

Initially, structures of bacterial homologues of the human SLC6 family, like LeuT in Aquifex aeolicus or MhsT transporter in Bacillus halodurans were resolved by crystallography followed by the first structures of transporter from eukaryotic organism: the biogenic amine transporter, the Drosophila melanogaster dopamine transporter (dDAT), and the human serotonin transporter (hSERT) (Yamashita et al., 2005; Penmatsa et al., 2013; Coleman et al., 2016). More recently, using cryo-electron microscopy (cryo-EM), a number of mammalian transporters from the SLC6 family have been determined, including the substrate-bound SERT, the glycine transporter 1 GlyT1, the neutral amino acid transporter B^0AT1 and the GABA transporter 1 GAT1 (Coleman et al., 2019; Shahsavar et al., 2021; Joseph et al., 2022; Motiwala et al., 2022). While the structure of BGT-1 is still not known, we can obtain insights from these existing structural models, especially GAT1 given that they belong to the same sub-family.

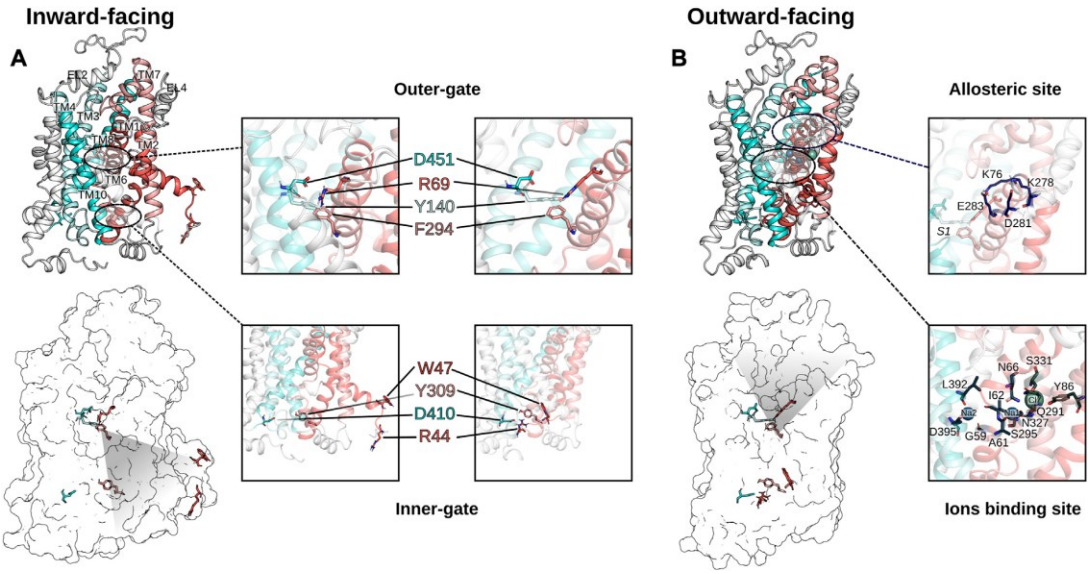

Figure 3 Comparison of the inward-facing conformation (A) and the outward facing conformation (B) of GAT1. The inward-open and the outward-open conformations of GAT1 are shown in secondary structure rendering to visualize the TM helices (scaffold domain in cyan colors, bundle domain using red colors) and as transparent surface rendering to highlight the residues of the extracellular gate and the intracellular gate. The inserts zoom-in on the outer and the inner gate to highlight the structural differences of the two conformations. On the right of (B) are shown two zoom-in inserted on the allosteric site S2 and on the ion binding residues, respectively.

Despite the low sequence homology between LeuT and the human homologues (20%–25%), the fold (LeuT fold), the transport mechanism, and several residues essential for function are conserved. The LeuT fold displays an inverted pseudo-twofold symmetry comprising 12 transmembrane helices (TM), two sodium binding sites (Na1 and Na2), the substrate binding site S1, and an allosteric site S2 (Yamashita et al., 2005; Forrest et al., 2007) as indicated in Figure 3. The scaffold domain is composed of TM3, 4, 8, 9, and the first intracellular loop. The SLC6 transporters have two dominant conformations, which strikingly differ in their accessibility to the substrate binding site S1 that is located halfway through the membrane. In the outward-facing conformation, the S1 can be reached only from the extracellular side through the open extracellular vestibule, while in the inward facing conformation, the outer vestibule is

sealed and the S1 can be reached by the open intracellular vestibule. The transport cycle of SLC6 transporters can be described by the alternating access model (Jardetzky, 1966), where the transporter cycles between the outward-facing conformation that is ready for binding substrate from the extracellular side and the inward-facing conformation that releases substrate to the cytosol. These structural changes are accomplished by motions of the bundle domain. It comprises TM1, 2, 6, 7, and the connecting intracellular (IL) and extracellular loops (EL). TM1 and 6 have an unwound section halfway across the membrane (Forrest et al., 2007). These two discontinuous helices (TM1a/b and TM6a/b) expose backbone atoms involved in ion and substrate binding to create each a dipole that contributes to tight binding of the substrate (Yamashita et al., 2005; Gradisch et al., 2022). Therefore, the centrally located TM1 and 6 form the core of the transporters and prominently contribute to the substrate and sodium binding sites. While the non-transmembrane N and C-terminals are not essential for function, the ELs and ILs are crucial structural elements for structural and functional integrity (Pantanowitz et al., 1993; Kanner et al., 1994; Tamura et al., 1995; Zomot and Kanner, 2003; Rosenberg and Kanner, 2008; Ben-Yona and Kanner, 2009; Dayan et al., 2017; Dayan-Alon and Kanner, 2019).

Several GAT1 mutations in TM1, 6, 10, and EL4 and 5 were assessed for their impact on transporter function. The ELs were initially studied by residue deletions from EL4 and EL5, which resulted in less than 2% of GAT1 wild-type activity (Kanner et al., 1994). Also, the chemical modification of the GAT1 EL4 cysteine mutant (A364C) by sulfhydryl reagents affected transport function: no steady-state current in the presence of Na^+ and GABA could be measured, suggesting that the chemical modification of A364C blocks the conformational changes required during GABA transport. In contrast, pre steady-state current in

the presence of Na^+ remained unchanged, showing that Na^+ and GABA binding were unaffected (Zomot and Kanner, 2003). It was observed that the chemical modification of A364C increased the accessibility of residues in the inner vestibule (C399, E402, T406, and D410), supporting the notion of affecting the conformational equilibrium and stabilizing an inward-facing conformation by opening the cytoplasmic pathway to the S1 (Ben-Yona and Kanner, 2009). The S1 can be accessible from the extracellular side or the intracellular side through an open passage, but at least one of the two passages is closed at any time by a gate, each supported by an intramolecular salt bridge across the passage. The extracellular gate involves residues from TM1b, 6a, 10, and EL4, 5. Sequence alignments showed that TM10 of GABA transporters carries an additional residue as compared to the other SLC6 transporters, which might form a π-helix element and represent a flexing point that is important for the conformational changes of the transport cycle (see Figure 4). Individual deletion of one residue (ΔS456, ΔG457, and ΔM458) from this π-helix element in TM10 resulted in loss of function mutants and constitutive leak currents (Dayan et al., 2017). TM1 was extensively studied as it is a versatile transmembrane helix involved in intracellular and extracellular gating and is important for the switch from a leaking state to a transport-competent conformation (Yu et al., 1998; Zhou et al., 2004; Ben-Yona and Kanner, 2013). The Y60C mutant induces leak currents in the presence of Na^+, suggesting that Y60 is involved in sodium affinity or in closing of the inner gate (Kanner, 2003). The modification of the G63C mutant with sulfhydryl reagent blocked transient currents typically induced by lithium. The conserved tryptophan (W68 in GAT1) in TM1 is essential for structural activity (Zhou et al., 2004). Its mutation leads to a loss of expression or function in all SLC6 transporters. In GAT1, conservative substitution of W68 with another aromatic residue resulted

in a loss of surface expression and function (Kleinberger- Doron and Kanner, 1994). Residue R69 (TM1) is part of the salt bridge with D451 (TM10) of the outer gate and one of the five charged residues located in the transmembrane region. A conservative R69K mutation or the double mutant R69K and D451E blocked transport activity (Pantanowitz et al., 1993; Ben Yona and Kanner, 2013). At the intracellular gate, the symmetry related salt bridge of the inner gate is formed by R44 (TM1) and D410 (TM8). The rGAT1 mutants R44H and D410E retained GABA transport activity, but their function was impaired, while single mutant R44K showed a reduced affinity for sodium, thereby suggesting that these mutants altered conformational coupling and/or the conformational equilibrium (Bennett et al., 2000; Ben-Yona and Kanner, 2013; Dayan-Alon and Kanner, 2019). Consistent with this interpretation, a double mutant of both aspartate residues (inner gate D410E and outer gate D451E) to glutamate restored transport activity of the respective transport deficient single mutants of GAT1 (Dayan-Alon and Kanner, 2019). Similar to TM1, early data on the reaction of the cysteine mutants of W285C, L286C, D287C, S295C on TM6a with sulfhydryl reagent indicated that these residues should line the access path from the extracellular space to the S1 as the presence of GABA decreased their reactivity with sulfhydryl reagents (Rosenberg and Kanner, 2008).

5.2 Na^+ and Cl^- binding sites

Most human SLC6 are chloride dependent, with the exception of B^0AT1 and B^0AT2 (Broer et al., 2006; Margheritis et al., 2013), while also LeuT is chloride independent. The chloride binding site was independently identified by Zomot and collaborators by comparing LeuT with GAT1 (Zomot et al., 2007) and by Forrest and collaborators (Forrest et al., 2007) in SERT. In GAT1, Zomot and collaborators identified residues Y86, Q291, S295, N327, S328 and S331 to constitute the putative chloride binding

site. In designing chloride-independent mutants they established that the S331E mutant functionally replaces the chloride in GAT1, while in LeuT, the corresponding residue E290 is essential for chloride independent transport (Ben-Yona et al., 2011). Similarly, by a comparison with LeuT, Forrest and collaborators (Forrest et al., 2007) identified the chloride binding site in SERT by substituting S372 with D or E, thereby rendering transport in SERT chloride independent (Forrest et al., 2008). This chloride binding site could later be confirmed by structure determination of the dDAT (Penmatsa et al., 2013). These results established that the negative charge next to the Na1 binding site is essential for transport. While in the chloride independent SLC6 transporters the charge is introduced by a glutamate in TM7 in the position corresponding to residue E290 of LeuT, in the human SLC6 transporters, this position is in most cases a serine (i.e., S331 in GAT1 or S372 in SERT) and predisposes the transporter for chloride binding. The exception is NTT5, where the serine is a leucine (see Figure 4). Interestingly, B^0AT1 and B^0AT2 also harbor a serine in this position. It was shown that these two transporters have a higher chloride affinity, and it was therefore suggested that chloride remains continuously bound (Broer et al., 2006).

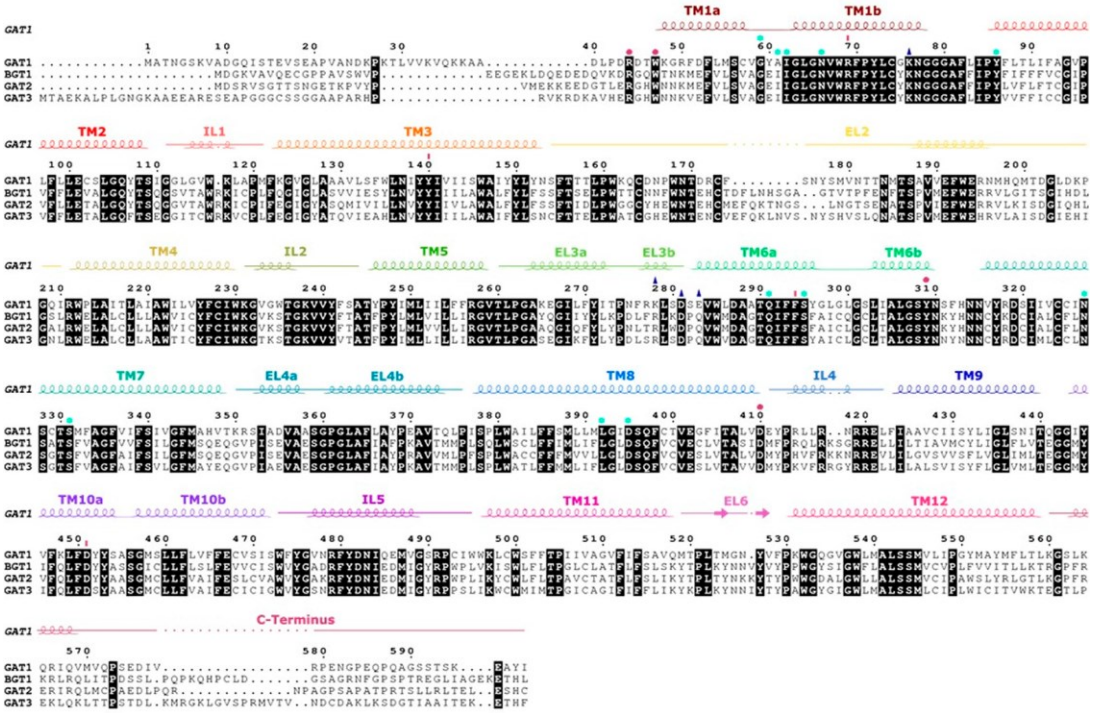

Figure 4 Sequence alignment and secondary structure of the four human GATs. Strictly conserved residues are highlighted in black. The α-helices and β- strands in hGAT1 are shown as coils and arrows, respectively, above the alignment as present in the structure of human GAT1 [PDB ID: 7SK2 (Motiwala et al., 2022)]. Intracellular and extracellular regions are indicated by a strike-through line. Amino acids involved in the inner and outer gates are symbolized by pink dots and lines, residues interacting with Na^+ and Cl^- are represented by light blue stars. The amino acids of the allosteric site are shown with a dark blue triangle.

Q291 in GAT1 is a residue that is highly conserved in the eukaryotic NSS transporter and the bacterial homologues. It is located in TM6 and is in direct contact with the chloride ion. Replacing Q291 in GAT1 with smaller or acidic side chains, it was shown that this residue plays an important role in ion binding, substrate transport and currents (Mari et al., 2006; Ben-Yona et al., 2011). The importance of this residue for the transport cycle through its role in coordinating the sodium-stabilizing chloride ion is highlighted by the complete loss of detectable GABA uptake activity and the strongly altered voltage dependence of the GABA-induced steady state current in the Q291S and Q291N mutants (Ben-Yona et al., 2011). While it has been established that Q291 is involved in chloride coordination, Ben-

Yona et al. (2011) inferred a second role for Q291 from the similarities in the increased apparent sodium affinity of Q291N and of R69K, suggesting that R69 and Q291 directly interact during the transport cycle. This suggestion was confirmed by the recent cryo-EM structure of GAT1 (Motiwala et al., 2022).

Cation binding to the Na1 and Na2 of GAT1 and their kinetics have been studied in detail and are described in Section 4 (MacAulay et al., 2002; Mari et al., 2006; Zhou et al., 2006; Meinild and Forster, 2012). Sodium binding to GAT1 was shown to precede GABA translocation (Radian and Kanner, 1983; Fesce et al., 2002; Bicho and Grewer, 2005). Kinetic modeling of GABA transport derived from electrophysiological data indicated that the sodium affinities to Na1 and Na2 of GAT1 differ ~100 fold, with a K_d of 10 mM for the high affinity side and 920 mM for the low affinity side (Hilgemann and Lu, 1999). In contrast to sodium, lithium does not support transport activity or induce pre-steady state currents. It has been suggested that the lithium-bound state is structurally distinct from the sodium-bound state (MacAulay et al., 2002), as leak currents were detected in the presence of lithium, which can be blocked by low concentrations of Na^+. This is not due to a competition between the two cations, but because of a switch from a leak current state to the sodium-stabilized outward-open conformation (Zhou et al., 2006). The structure of LeuT allowed to identify the residues contributing to the Na1 and Na2 sites in GAT1 through sequence comparison: residues N66, N327 and S295 coordinate sodium in the Na1 site, while D395 and S396 coordinate sodium in the Na2 site (Zhou et al., 2006). These predictions through modelling were experimentally verified. In the presence of 150 mM sodium, the transport velocity of the N327A and N327C variants was reduced by 20 to 80 folds. Transport of GABA could still be observed, but required higher concentrations of sodium (Zhou et al., 2006), showing that

the GAT1 N327 variants remained sensitive to sodium despite mutation of the Na1 site. The Na2 site is considered to be more promiscuous, because a D395T or D395C mutant maintained coupled GABA currents and interactions with lithium (Zhou et al., 2006). Another residue in GAT1 that was studied for its role in sodium binding and GABA transport is E101 (Keshet et al., 1995). This residue is conserved in the SLC6 family and corresponds to E62 in LeuT, E66 in MhsT, and E136 in SERT (See Figures 3, 4). In GAT1, even the conservative mutant E101D reduced transport activity to 1% (Keshet et al., 1995), while its mutation in SERT (E136D) reduces transport to a lesser extent, while any other mutant abolished transport (Korkhov et al., 2006). Structures of SLC6 transporters showed later that E101 does not directly interact with sodium ions, it is not exposed to the substrate binding site S1 or the permeation path through the transporter. This conserved glutamate residue can be considered a structurally and mechanistically essential residue that is associated with maintaining structural integrity, most importantly of the unwound region of TM6.

5.3 Substrate binding sites

Sodium binding is the first step of the transport cycle, because the high extracellular NaCl concentration leads to fast binding. The outward-facing state is stabilized by sodium binding and the affinity of the substrate increases strongly with the presence of Na^+ (Keshet et al., 1995; Mager et al., 1996; Lu and Hilgemann, 1999a; Lu and Hilgemann, 1999b; Kanner, 2003; Szollosi and Stockner, 2021; Szollosi and Stockner, 2022).

Sequence comparison revealed that the composition of the six ELs in GAT1 are significantly different compared to the three other GABA transporters (See Figure 4). Respective amino acid substitutions resulted in a change in affinity towards GABA, β-alanine, or taurine (Tamura et al., 1995). In fact, the ELs interact with inhibitors and maintain a conformation that

allows the substrate to access the substrate binding site. Inhibitors were found to bind to the orthosteric substrate binding site S1 or to the allosteric binding site S2 site located in the outer vestibule (See Figure 4). Crystallization of LeuT with tricyclic antidepressant drugs (TCA) showed that D401 (EL5) interacted with the nitrogen atom of the drug through a salt bridge (Singh et al., 2007; Zhou et al., 2007; Wang et al., 2013). This is supported by the impact of TCAs on the net currents of the GAT1 K448E mutant, which is equivalent to the residue of D401 in LeuT and emphasizes the role of this residue in GAT1 for the interaction with inhibitors (Cherubino et al., 2009). Consistently, several inhibitors of SERT and LeuT were found to bind to S2 (Singh et al., 2007; Quick et al., 2009; Wang et al., 2013; Coleman et al., 2016; Erlendsson et al., 2017).

The recent structure of GAT1 (Motiwala et al., 2022) showed that G63 (TM1 unwounded region) and S295 (TM6a) coordinate the carboxyl group of the inhibitor, and suggested coordinating also the carboxyl group of GABA, confirming earlier results indicating that G63 and S295 are involved in GABA binding and transport (Kanner, 2003; Rosenberg and Kanner, 2008). Three other residues predicted in earlier studies to interact with GABA are G65 and L64 on TM1b and Y140 on TM3 could be confirmed (Bismuth et al., 1997; Mari et al., 2006). Interestingly, the saturation uptake assay of GAT1 in the presence of tiagabine suggested a two steps mechanism leading to GAT1 inhibition, whereby the first step is consistent with a competitive inhibition followed by a transition to a noncompetitive inhibition. If confirmed and generalizable, the inhibition mechanism would resemble the binding of ibogaine (Coleman et al., 2019) at the monoamine transporters and indicate that also other non-competitive inhibitors might use this mode of binding (Cherubino et al., 2009). For the monoamine transporters, this mode of binding has been observed for ligands that were shown to have the potential for rescuing folding deficient transporter

mutants (Freissmuth et al., 2018). It raises the importance of pharmacology research on GABA transporters to identify compounds able to rescue disease-causing folding deficient GATs.

5.4 Oligomeric state

SLC6 transporters can form oligomers, whereby the dimerization interface is limited to the scaffold domain, but not conserved: for example, in LeuT it involves TM9 and the structurally non-conserved TM12, in the bacterial Na^+/H^+ exchanger NhaA it consists of most TM helices of the scaffold domain and is stabilized by a non-conserved additional β-sheet structure (Yamashita et al., 2005; Lee et al., 2014). The oligomeric state and its role for human SLC6 transporters is still under discussion, while the functional unit seems to be the transporter monomer. GlyT1 and GlyT2 are monomeric, whereas hSERT shows a range of oligomeric states (monomer to hexamers) that is in part controlled by the concentration of the signaling lipid phosphatidylinositol-4,5- biphosphate (PIP2) (Horiuchi et al., 2001; Anderluh et al., 2017). Similarly, DAT and NET show PIP2 dependent formation of dimers (Das et al., 2019). On SDS-PAGE, GAT1 is visible as a dimer and more broadly an oligomer, but similar to the monoamine transporters its functional unit is the monomer (Farhan et al., 2004; Soragna et al., 2005; Mari et al., 2006). The oligomeric state of the NSS is important for surface expression, but it seems to play no direct role in the function of cellular substrate uptake (Mari et al., 2006; Anderluh et al., 2017; Jayaraman et al., 2018; Das et al., 2019; Jayaraman et al., 2021). Oligomerization simulations of the DAT have shown that the interface between SLC6 transporter in the oligomers is confined to the scaffold domain, while sparing the transport domain, as no interactions involving this domain were observed (Jayaraman et al., 2018). This can be rationalized by the fact that the bundle domain needs to move during the

transport cycle. Any interaction involving this domain would therefore at least decelerate, if not completely arrest the transport cycle.

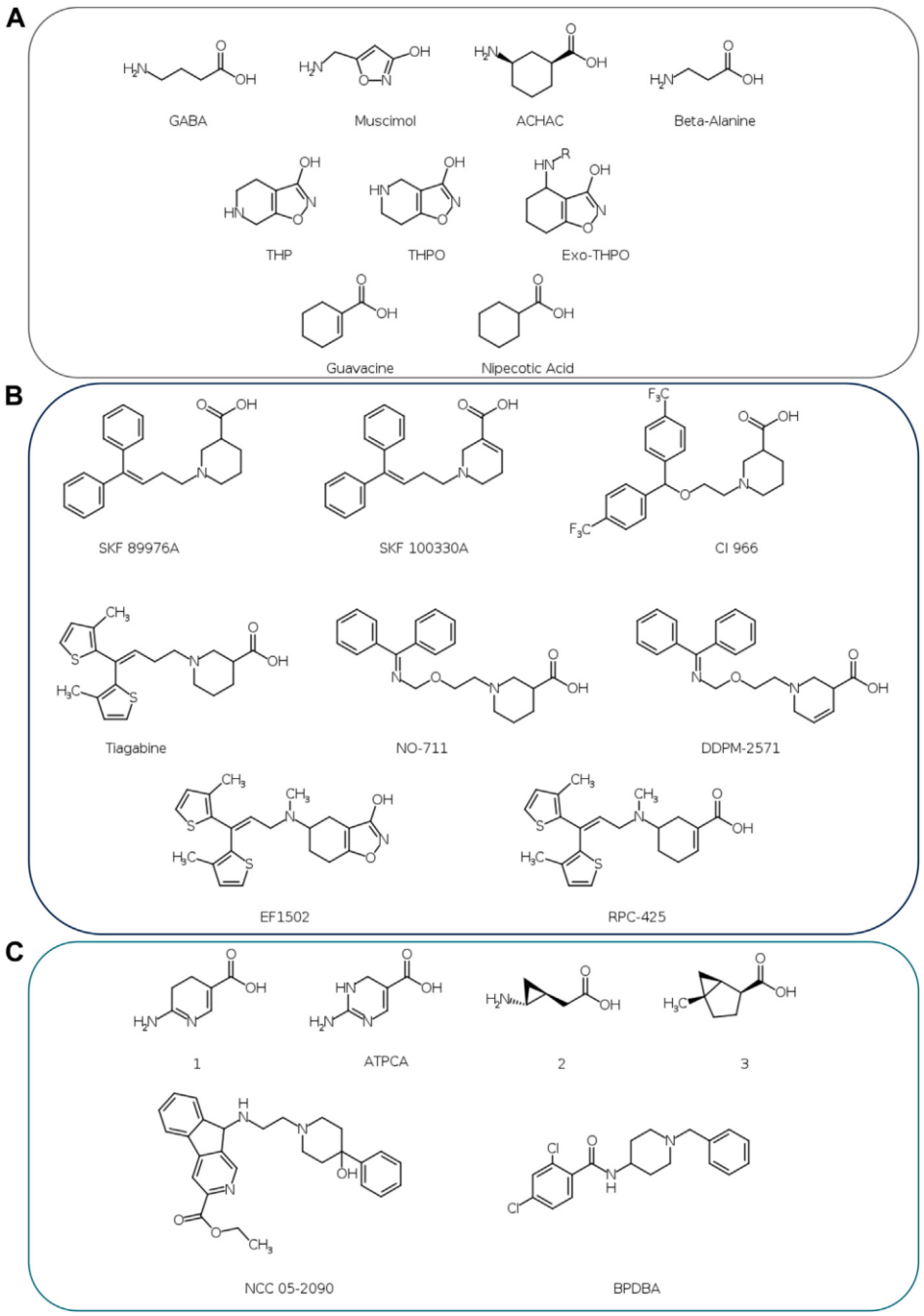

Figure 5 (A) Chemical structures of GABA and its analogues. These analogues show affinity to GATs and were used as starting points for developing competitive inhibitors. (B) Molecular structures of potent GAT1 inhibitors. (C) Molecular structures BGT-1 inhibitors.

5.5 Pharmacology of GATs

GAT1 is a well-recognized therapeutic target for the treatment of neurological disorders that are linked to a dysregulation of GABA homeostasis. The GABAergic system is known for its inhibitory role in the CNS and the periphery (Kanner, 2003). The physiological levels of GABA, and therefore brain homeostasis, are maintained by GATs through the rapid reuptake of the synaptically released GABA into the presynaptic neuron and glial cells. The pharmacological purpose of targeting GATs by inhibition is to bring excitatory/inhibitory balance in the CNS by increasing the GABA concentration in the synaptic cleft providing an increased inhibitory neurotransmission. To date, GAT inhibitors are used for the treatment of epilepsy, depression, and anxiety (Schousboe et al., 2004; Schousboe et al., 2017; Schousboe and Madsen, 2017). The first step towards the characterization of the structure-activity relationship for GATs started with the discovery of Muscimol, a natural alkaloid, present in the mushroom Amanita muscaria, that could act as a potent GABAA agonist and uptake inhibitor (Krogsgaard-Larsen et al., 1979; Krogsgaard-Larsen et al., 1981; Fariello and Ticku, 1983). Since the discovery of GABA transporters, it became clear that GABA could be accumulated in different cell types (neuronal and glial cells), suggesting the presence of different transport systems (Iversen and Kelly, 1975; Johnston, 1978; Hyden et al., 1986). To investigate GABA uptake inhibition, several analogues have been generated by systematic modification of the endogenous structure (See Figure 5A). Some of the GABA analogues are used as neuronal markers (ACHAC, ß-alanine) (Iversen and Kelly, 1975; Krogsgaard-Larsen et al., 1981). This process led to the rational design of a rigid scaffold. An important step was the synthesis of 4,5,6,7-tetrahydroisoxazolo(5,4-c)pyridin-3-ol (THIP) and tetrahydroisoxazolo[4,5-c]pyridin-3-ol (THPO). The former compound has a high affinity for GABA receptor, and the latter

is a potent inhibitor of the GATs but has no affinity for the GABA receptors (Schousboe et al., 1981). These compounds allowed for the first time to isolate the effects mediated by the receptor from those originating from transporter activity. The activity of various transporter systems determines the balance of uptake into glial vs. neuronal cells. The availability of exo-THPO, which showed selectivity for glia cell transporters, allowed to differentiate GABA uptake into glial over neuronal uptake (Schousboe et al., 1979; Falch et al., 1999). In further developments, the conformationally restricted GABA analogue THPO becomes the scaffold for designing potent and selective GAT1 (neuronal) inhibitors, whereas exo-THPO led the design of GAT2 (hBGT-1) inhibitors (Clausen et al., 2005).

The first available inhibitors nipecotic acid and guvacine, synthesized through bio-isostere substitution of the isoxazole to carboxylic acid of the THPO scaffold, failed to cross the blood brain barrier (BBB) (Schousboe et al., 2004; Schousboe et al., 2014; Schousboe et al., 2017). Both compounds are potent and selective in vitro inhibitors of GAT1, and share the common feature of being zwitterionic, which retains a similarity to the endogenic zwitterionic GABA, but also makes them poorly permeable through the BBB because of the simultaneous presence of a positive and a negative charge (Johnston et al., 1975; Johnston et al., 1976a). To increase brain penetration, the molecules were modified through esterification of the carboxylic acid, creating mono and diesters prodrugs (Frey et al., 1979; Croucher et al., 1983; Falch et al., 1987). The resulting molecules were effective on seizure modulations (in rats), but showed limited potency and non-specific side effects mediated by activity on muscarinic receptors, thereby limited their applicability (Frey et al., 1979; Falch et al., 1987). In an attempt to increase the lipophilicity of the active molecules (See Figure 5B), a diphenylbut-3-en-1-yl (DPB) group was introduced on the secondary amino group of guvacine, (R)-nipecotic acid,

and THPO: the diaromatic chain-substituent analogues resulted in the highly potent and orally active GABA uptake inhibitors SKF89976-a and SKF100330-a (Bolvig et al., 1983; White et al., 1983; Ali et al., 1985). Moreover, the inhibition kinetics associated with these compounds were different from the previously reported competitive inhibitors, because instead these molecules were not translocated as substrate, but rather acted as blockers (Johnston et al., 1976b; Larsson et al., 1988). This discovery started the development of several lipophilic analogues that eventually resulted in selective GAT1 vs. GAT2-3 and BGT-1 inhibitors (Dhar et al., 1994). Further developments of these hGAT1 selective compounds increased the BBB permeability and resulted in vivo anticonvulsive activity of CI-966(([1-[2-[bis-4-(trifluoromethyl)-phenyl]methoxy]ethyl]-1,2,5,6-tetrahydro-3-pyridinecarboxylic acid)), which entered phase 1 clinical trial, but the compound suffered from severe side effects such as myoclonus and catatonic-like states (Sedman et al., 1990; Taylor et al., 1990). Further developments were achieved by introducing an oxime in the compound NNC-711 (1-(2-(((diphenylmethylene)amino)oxy)ethyl)-l,2,4,6-tetrahydro-3-pyridinecarboxylic acid hydrochloride (Smith et al., 1995). Finally, tiagabine, an isoxazole bio-isoster of the aromatic moiety, emerged as a potent and selective GABA uptake inhibitor and became the first approved drug for the treatment of epilepsy (Braestrup et al., 1990; Nielsen et al., 1991; Halonen et al., 1996; Meldrum, 1996; Kern and Wanner, 2015). Currently, the lipophilic analogs tiagabine (NO-328) and NNC-711 (1-(2-(((diphenylmethylene)amino)oxy)ethyl)-1,2,5,6-tetrahydro-3-pyridinecarboxylic acid hydrochloride) are approved as the selective antiepileptic drugs, but the severe cognitive side effects (confusion, abnormal mood swings, dizziness, tremor, fatigue, nervousness) caused by the tiagabine limits their applicability and raises the need for alternative

medication (Krogsgaard-Larsen et al., 1979; Krogsgaard-Larsen et al., 1981; Fariello and Ticku, 1983; Schousboe et al., 2004; Schousboe and Madsen, 2017).

In 2014, a series of N-substituted guvacine derivatives have been screened as novel GAT1 inhibitors, which lead to the identification of DDPM-2571, which is a di-chloro-phenyl derivative that showed a four-time higher potency (Kern and Wanner, 2015). Screening o5-substituted hydrazones of nipecotic acid resulted in the first allosteric modulator of GAT1 (Hauke et al., 2018). Selective pharmacology towards BGT-1 started when EF1502 was developed from N-Me-exo-THPO and tiagabine. It showed equal inhibition efficacy for GAT1 and BGT-1, but not for GAT2 and GAT3, whereas its S-enantiomer was the first BGT-1 selective inhibitor with moderate affinity. This S-enantiomer of EF1502 showed anticonvulsive effects in-vivo experiments (Smith et al., 2008). If combined with tiagabine, a synergistic effect could be observed, suggesting that BGT-1 has a functional role in the CNS and supporting the notion of the importance of BGT-1 as a target for anticonvulsant drugs (White et al., 2005; Smith et al., 2008). To further increase the selectivity for BGT-1, the lipophilic side chain of tiagabine was combined with different amino-acidic groups, which led to the conformationally restricted analogue RPC-425, selectivity towards BGT-1; in combination with tiagabine the effect is not synergistic but additive, indicating different pharmacological effect or apparent mechanism (Vogensen et al., 2013).

In a different approach aiming at gaining affinity and selectivity, the conformation of GABA was restricted with a cyclopropane ring (Roberts and Frankel, 1950) (Nakada et al., 2013) (See Figure 5C). It was observed that when the cyclopropyl and carboxyl group were on the same side (syn conformation), the compound (Udenfriend, 1950) has the highest biological activity, resulting in the currently most potent BGT-1 inhibitor

(Kobayashi et al., 2014). Based on nipecotic acid as a template, the bioisosteric substitution of the amino group with guanidine resulted in the selective BGT-1 inhibitor 2-amino- 1,4,5,6-tetrahydropyrimidine-5-carboxylic acid (ATPCA) (Al- Khawaja et al., 2018). Interestingly, the pyridine derivative (Awapara et al., 1950) decreased the potency, indicating the importance of maintaining the position of the guanidinium N3 and carboxylic acid, respectively, in an orientation that is similar to ß-alanine. In regards of non-amino acid scaffolds, substitution of the GABA carboxyl group with a hydroxyl group led to the synthesis of the BGT-1 selective compound 1-(3-(9H-carbazol-9-yl)-1-propyl)-4-(2-methoxyphenyl)-4-piperidinol (NNC 05-2090) that showed 10-fold selectivity towards BGT-1 and K_i = 1.4±0.3 µM, thereby proving that the amino acid scaffold was not necessary to target and inhibit BGT-1 (Thomsen et al., 1997). Derived from the scaffold of NCC 05- 2090, N-(1-benzyl-4-piperidinyl)-2,4-dichlorobenzamide (BPDBA) was then identified as the first non-competitive inhibitor of BGT-1, suggesting a possible allosteric mode of interaction (Kragholm et al., 2013; Kickinger et al., 2020). More recently, selectivity of inhibitors towards BGT-1 were explored by developing analogues of ATPCA and bicyclic N-methylated GABA, which showed increased selectivity for BGT-1 at the expense of affinity (Kickinger et al., 2020; Kickinger et al., 2021).

6 Computational studies in hGAT1

In 2005, the first crystal structure of LeuT from A. aeolicus was resolved in an outward-facing occluded conformation, in which the substrate binding site S1 was occupied by the substrate (leucine) and shielded by the hydrophobic gate residues (V104, Y108, and F253) from the extracellular environment (Yamashita et al., 2005). The LeuT structures served as the template for the generation of GAT1 homology models, all owing for investigating the binding mode of GABA and analogues (Pallo et

al., 2007; Skovstrup et al., 2010; Skovstrup et al., 2012; Zafar and Jabeen, 2018; Zafar et al., 2019; Latka et al., 2020). The endogenous ligand (GABA) was docked in the S1 and showed favorable h-bonding interaction with TM1 (Y60) and TM8 (S396)) (Pallo et al., 2007). The amino and carboxyl groups of GABA overlapped with the respective groups of leucine as observed in LeuT, whereby the carboxyl group of GABA completed the octahedral coordination of the sodium ion. A more extended conformation of GABA was proposed later (Wein and Wanner, 2010). In this pose, the amino group interacts with side chains of T400 and S396 on TM8, while interacting with the backbone of Y60 of S396. The hydroxyl group of Y140, the nitrogen of G65, and the backbone oxygen of F294 contributed a stabilizing network of hydrogen bonds, whereas T400 and G297 were proposed to be major contributors to GABA selectivity (Wein and Wanner, 2010). Skovstrup et al. (2010) described the S1 site in the occluded conformation as a pocket, in which the backbone of A61 and Y296 (I53 and F295 in BGT-1), the side chains of Y60, G297, L300, T400 build the lower floor, while the residues G63, G65, L136, Y140, F294, S295, S396 complete the S1. Interestingly, the latter group of residues is fully conserved within the GAT subfamily, whereas the floor of the pocket presents some difference that could be key in the transporter selectivity. The recent structure of GAT1 in the inward-facing conformation and the AlphaFold model in the outward-facing conformation confirmed the prediction for most residues (See Figure 6). In particular, Y60 of GAT1 (E52 in BGT-1) is proposed to contribute to substrate specificity, together with T400, as both residues differ within the GAT subfamily (Melamed and Kanner, 2004; Baglo et al., 2013).

A later docking study using a GAT1 homology model based on the X-ray crystal structure of the open-to-out conformation of the dDAT showed a GABA binding mode that agreed with the earlier studies, in which the

carboxyl group completes the coordination of Na^+ in Na1, while the amino group is mainly stabilized by interactions with Y140 and Zafar and collaborators reported that the presence of the co-transported Na^+ and Cl^- ions increase binding strength, in particular Na^+ bound to the Na1 site (Wang et al., 2015; Zafar et al., 2019).

Consistent with the binding pose of GABA, flexible docking of the short inhibitors (nipecotic acid, guvacine, 4-amino-isocrotonic, taurine, and 4-amino-2-hydroxubutanoic-acid) to the S1 binding site of GAT1 models derived from the substrate-bound outward occluded conformation of LeuT showed the same polar contacts (Wein and Wanner, 2010). When comparing the binding of nipecotic acid with the binding pose of tiagabine, an additional stabilizing interaction formed with the backbone of F294 that adopts a different orientation in respect of the occluded conformation, suggesting that larger inhibitors act as blockers by stabilizing the outward-open conformation and preventing occlusion (Skovstrup et al., 2010).
Studies of the translocation pathway of substrates in GAT1 were performed by steered molecular dynamics simulation, in which a pulling force was applied on the substrate to bias it towards leaving the S1 towards both the extracellular and the intracellular side (Skovstrup et al., 2012). The study recapitulated the S2 in the extracellular vestibule in a similar position as first identified in LeuT (Shi et al., 2008). The charged residues K76, K278, D281, E283, D287 appear to attract and guide the substrate towards the S2: related mutants show a loss in substrate transport supporting the role of these residues in substrate association (Keshet et al., 1995; Rosenberg and Kanner, 2008). E101 was suggested to play a role in the translocation of substrate from the S1 to the intracellular side (Keshet et al., 1995). For the inhibitor nipecotic acid, a similar dissociation path as for GABA was observed, whereas tiagabine showed stronger hydrophobic interactions with TM1 and TM6, most extensively with

residues between the S1 and S2 sites (Skovstrup et al., 2012). Docking studies of nipecotic acid to GAT1 showed poses that place the amino nitrogen towards the intracellular side forming a hydrogen bond with the F294 backbone (Pallo et al., 2007; Wein and Wanner, 2010). Docking of tiagabine also showed a similar ligand-amine to F294 interaction, but the large tiagabine could not be fitted into the too-small binding site of the occluded-state and its thiophene rings are positioned towards the extracellular side (Rosenberg and Kanner, 2008; Skovstrup et al., 2010). This size dependence suggested that the inhibitory kinetics should depend on the binding mode, as smaller inhibitors fit perfectly into the closed site S1 and their nitrogen forms a hydrogen bond with the oxygen atoms of Y60 located at the floor of the S1 (Wein et al., 2016). The binding mode of tiagabine was also investigated through experiment-guided docking and molecular dynamics simulation by Jurik et al. (2015). The orthosteric S1 binding site was described as a space divided into two hydrophobic cavities by S1-exposed residues: the sub-pockets are confined by the side chains of I143 and Y140 and by W68, F294, A358, whereby each sub-pocket accommodates an aromatic moiety of the inhibitor. This description of the binding site is in accordance with the results of ligand-based drug design studies, in which a polar atom in the linking region between the two aromatic moieties of inhibitors resulted in increased binding affinity (Knutsen et al., 1999).

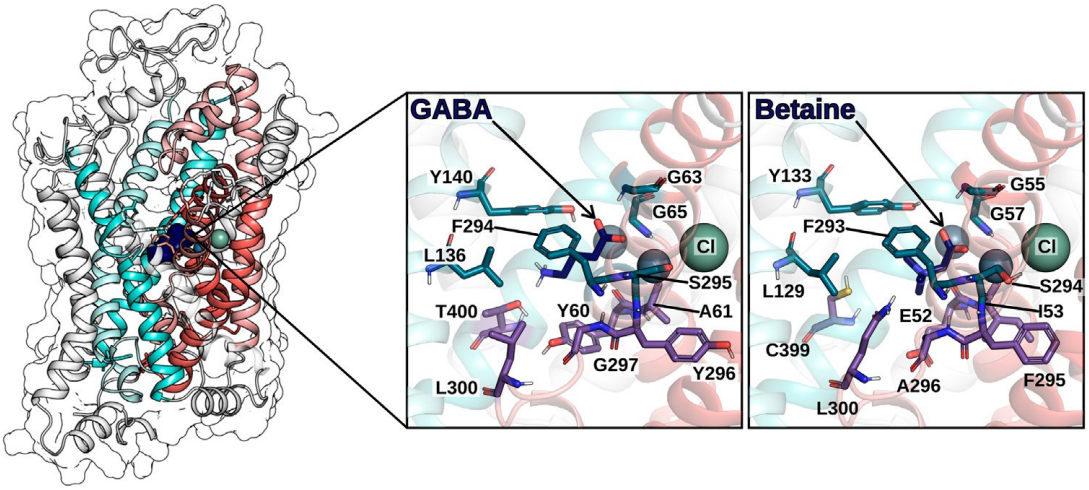

Figure 6 hGAT1 is shown in ribbon representation and in transparent surface rendering. The colouring scheme follows Figure 4: TM helices of the scaffold domain are shown in cyan colours, the TM helices of the bundle domain in red colours. GABA (in the S1) is shown by dark blue van der Waals rendering. The zoom-in panels highlight the residues on the surface of the S1 for the GABA bound GAT1 and the betaine bound BGT-1, respectively. Spheres indicate the sodium ions (grey-blue) and the chloride ion (grey-green).

Recently, the first cryo-EM structure of full-length wild-type hGAT1 in complex with tiagabine was resolved (Motiwala et al., 2022). The electron density of tiagabine in the inward-open conformation adopts an orientation that differs from the predicted conformations, as the aromatic moieties are oriented towards the bottom of the binding pocket and interact with Y60, L303 and L306, whereas the nipecotic acid moiety is located between the two sodium-binding sites Na1 and Na2.

7 Energetics of the transport cycle

7.1 Energy coupling/third binding site

The switching from the inward-facing to the outward-facing state requires transporter isomerization in the absence of the substrate and the co-transported ions, which seems to be the rate-limiting step and accordingly defines the overall transport velocity. The transport cycle of GATs is energized by the transmembrane gradient of Na^+ and utilized for the co-transport of the substrate, thus accelerating the transition of the outward

facing state to the inward-facing state in the presence of substrate and ions. This allows cellular accumulation of substrate by GATs as long as the transmembrane electrochemical gradient of sodium is present. The stoichiometry for the GATs remains disputed between 2 or 3 sodium ions per transported GABA as described in Section 4.8 (Radian and Kanner, 1983; Keynan and Kanner, 1988; Mager et al., 1996; Matskevitch et al., 1999; Matthews et al., 2009; Omoto et al., 2012; Willford et al., 2015; Eskandari et al., 2017). According to the Nernst equation, the accumulating power depends on the stoichiometry of transport, which enters as an exponent to the equation. A stoichiometry of 3 Na^+ per GABA vs. 2 Na^+ per GABA leads to an energy difference in the concentrative power of ~14 kJ/mol at physiological concentrations. Similar to the GABA transporters, also the glycine transporters (GlyT1 and GlyT2) have different stoichiometries: GlyT1 (expressed in glia cells) has a stoichiometry of 2 Na^+: 1 Cl^-: 1 glycine, while GlyT2 (located on pre-synaptic neurons) shows a stoichiometry of 3 Na^+: 1 Cl^-: 1 glycine for GlyT2. The different stoichiometry of GAT1 and BGT-1 could implicate difference of roles they play in CNS, especially in terms of substrate accumulation.

7.2 Proposed kinetic models of transport

A useful tool in the description of the secondary active transport is the kinetic model, a scheme that tries to allocate individual reaction steps within the transport cycle, and by estimating the rates for each step it can predict the experimental results (Burtscher et al., 2019). For secondary active transporters, the most generally accepted hypothesis explaining the stoichiometric transport of substrates is the “alternating access” model, stating that substrates can bind at each time from one side of the membrane only, and accessibility alternates depending on the conformational state of the transporter (Jardetzky, 1966).

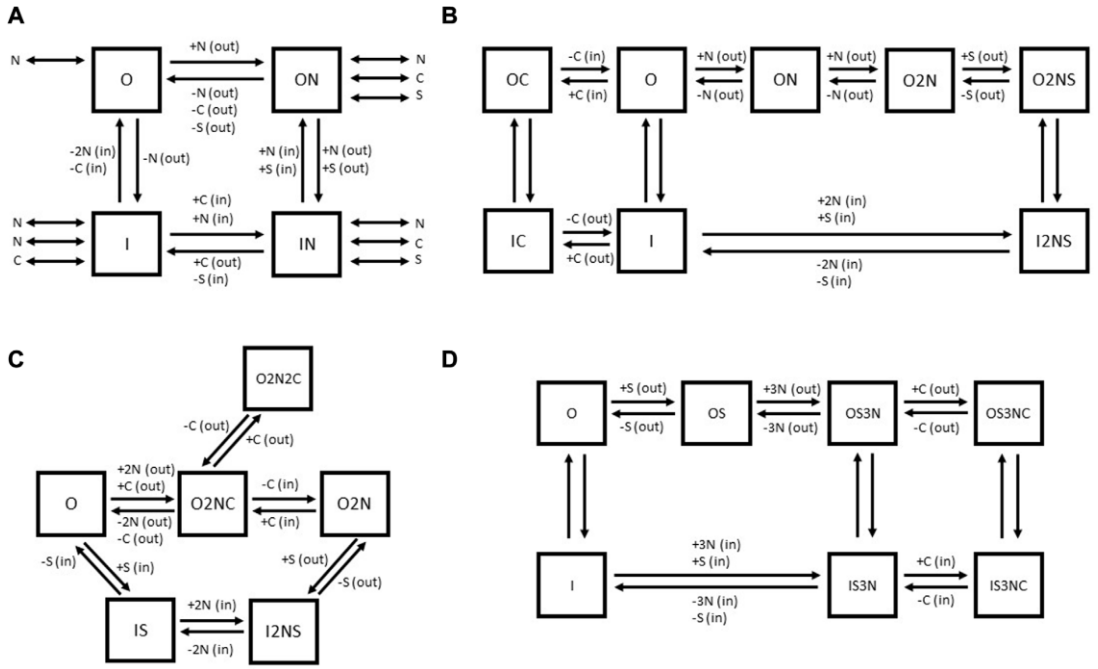

Figure 7 Comparison of published kinetic schemes for GAT1 (A–C) and BGT-1 (D). The outward-facing states of transporters are indicated by the letter "O," while the inward-facing states are indicated by the letter "I." "N" indicates Na^+, "C" indicates Cl^- and "S" indicates the substrate GABA (for both GAT1 and BGT-1) or betaine (for BGT-1). In parenthesis, (out) or (in) indicates the extracellular or intracellular localization of the respective substrate. For the schemes (B–D), the (+) and (−) signs alongside the substrates near the individual reaction arrows indicate the binding (+) or unbinding (−) of the respective substrate. The kinetic scheme (A) is adapted for GAT1 from Hilgemann and collaborators (Hilgemann and Lu, 1999), (B) is based on the kinetic scheme for GAT1 proposed by Bicho and collaborators (Bicho and Grewer, 2005). The kinetic scheme (C) for GAT1 was originally proposed by Cherubino et al. (2012). (D) The kinetic scheme for BGT-1 was proposed by Matskevitch et al. (1999).

For GAT1, a certain number of models have been proposed (See Figure 7). Based on the impossibility to detect leak currents in *X. laevis* oocytes, an early model by Hilgemann and Lu (1999) proposed a four state kinetic model (See Figure 7A). This model assumes that the empty carrier facing the cytoplasmic side and the outward-facing carrier with one bound Na^+ ion resemble the stable states of the cycle, whereas the empty carrier facing the extracellular side and the inward-facing carrier with one Na^+ bound are assumed as "metastable" states capable of accumulation during

the reverse transport. The (+) or (−) signs alongside the substrates near the individual reaction arrows indicate whether the respective substrate must bind to the carrier for the reaction to occur (+) or if it must be released from the carrier for the reaction to proceed (−) (See Figure 7A). The substrates indicated by the double headed arrows are considered in a dissociation equilibrium with the carrier. It is important to note that each double-headed arrow represents an intermediate state not directly shown in the model. The authors suggested ordered binding, while the different conformational transitions were expected to conclude only when a particular combination of ions is bound. For further details, we refer to the original publication (Hilgemann and Lu, 1999). The authors attributed a high electrogenicity to the conformational change because the alternating exposure of the Na^+ binding site to either side of the membrane implies that charges associated with the Na^+ binding site cross the electric field of the membrane during the conformational isomerization. This isomerization reaction depends on the extracellular Na^+ concentration; Na^+ binding to the empty carrier in the outward facing conformation shifts the equilibrium within the two empty carrier states, i.e., reduces the amount of inward-facing empty carriers that undergo the conformational transition. The negative charge moved across the membrane during this transition was predicted to account for the majority of the charge transferred during the whole transport cycle, because it is not neutralized by bound Na^+ ions as in the substrate bound carrier. This model was able to predict accurately the data from *X. laevis* oocytes but failed to explain uncoupled currents observed by others (Fesce et al., 2002; Krause and Schwarz, 2005).

Bicho and Grewer (2005) tried to refine the kinetic model of GABA transport and to include the role of Cl^- by exploiting experimental information coming from rapid substrate jumps (generated by pulsed-laser photolytic release of caged GABA) in combination with patch clamp

measurements. The major electrogenicity in this model was associated with the reaction "O" to "ON" (the binding of the first Na^+ ion to the empty, outward-facing carrier) (See Figure 7B). The authors were able to resolve two fast reactions, which are weakly electrogenic and associated with conformational rearrangements of GAT1 upon GABA binding, suggesting that the change in exposure of the S1 from extracellular to intracellular moves a small amount of charge across the membrane electric field. This kinetic model proposes that the Na^+ ions bind sequentially and thereby enhance the affinity of GAT1 for GABA. Then GABA binds in a non-electrogenic reaction, a step followed by GAT1 orienting to the inward-facing state through a weakly electrogenic passage and successively by the release of the substrates. At this point, external Cl^- is predicted to bind to the inward-facing transporter, allowing it to switch to the outward-facing conformation. In the absence of external Cl^-, only a slow (rate-limiting) reorientation of the empty carrier can take place. In this model Cl^- transport is considered independent from GABA translocation. The effect of Cl^- would be to accelerate GAT1 reorientation from the inward-facing to the outward-facing state, thereby enhancing apparent affinity of GAT1 for Na^+ and GABA and accelerating the overall transport rate (See Figure 7B). The first model after solving the structure of the SLC6 transporter family aimed to propose a kinetic scheme that accounts for interactions of intracellular substrates and included experimental data observed with different intracellular conditions on pre-steady state currents and reverse transport reaction employing TEVC on *X. laevis* oocytes (See Figure 7C) (Cherubino et al., 2012). Cherubino et al. (2012) noticed that an increase in cytoplasmic Cl^- can accelerate the rate constant of outward Na^+-induced pre-steady state currents, while the same rate constant is decreased by increasing extracellular Cl^-. To include this phenomenon in the kinetic model, the authors proposed the existence of

a state outside the transport cycle in which GAT1 is sequestered by a second external Cl^- anion after the inward movement of charges. This would decrease the outward rate of the charge movement and the reverse transport current. In this scheme, the electrogenic step is a single step in which all the co-substrates bind to the transporter in the outward-facing conformation ("O" to "O2NC"). The out-of-the-cycle state "O2N2C" that binds two Cl^- is necessary to explain the decrease of outward Na^+-induced transient, while the step "O2NC" to "O2N" is required to explain the opposite effect of intracellular Cl^- on the outward Na^+-induced transient.

For BGT-1, Matskevitch et al. (1999) draw three important conclusions from their data collection: (Awapara et al., 1950): the maximum velocity of GABA uptake decreases with decreasing external Na^+ and Cl^-, (Roberts and Frankel, 1950), the maximum velocity can still be reached at the sub-saturating GABA concentrations, (Udenfriend, 1950), there is still significant transport in the absence of Cl^-. These considerations would be consistent with a model in which GABA or betaine binds first to a 2 Na^+ bound BGT-1, followed by the binding of third Na^+. At this point, the transporter can orient in the inward-facing state even in absence of Cl^-, through a slow reaction. The presence of Cl^- consents to the binding at a faster rate, augmenting the maximum velocity of the transporter (See Figure 7D).

8 Conclusion

Since its discovery, GAT1 has been extensively studied and targeted for treating neuropathological disorders, while BGT-1 remains understudied. In contrast to GAT1, BGT-1 is not localized in GABAergic synapses and its GABA transport efficiency is lower than GAT1, hence its function in the brain may be mainly related to its ability to transport betaine. While the potent inhibitors of GAT1 are used to treat epileptic seizures, the in vivo studies demonstrating the ability of BGT-1 to impact the severity of

epileptic seizures, raised a possibility of functional similarities between GAT1 and BGT-1. The first class of GABA transporter inhibitors (guvacine and nipecotic acid) are also transporter substrates, while tiagabine acts as an inhibitor, a behaviour that was predicted by computational studies and supported by experimental data: the drug stabilizes the outward-open conformation by stretching from the S1 to the S2. The first cryo-EM structure of tiagabine-bound GAT1 suggests a different mode of interaction, as tiagabine was found to bind the inward-facing state of GAT1. Resolving this discrepancy is important for understanding transporter inhibition and for further improving currently available medication targeting the GABA transporters. The recent developments of BGT-1 selective inhibitors based on the GAT1 inhibitors (N-Me-exo-THPO and tiagabine) show that using GAT1 as a research model could effectively further the study of BGT-1.

A deeper understanding of the ion dependence and their specific role in the transport reaction in GAT1 and BGT-1 may prove important in better defining the interaction of inhibitors and help designing new drugs. Both proteins are Na^+ dependent, and substituting Na^+ with other cations (e.g., K^+) either blocks transport or activates a leaking state of the carrier (e.g., Li^+). The role of Cl^- remains unclear, but undoubtedly Cl^- is important for achieving maximal transport functionality. It is postulated that Cl^- could have a compensating effect on the charges carried by the Na^+ ions, but such a role could also be fulfilled by a generic negative charge. Evidence for this resides in the fact that some transport functions remain in absence of Cl^- at highly negative membrane potentials, and more specifically in the GAT1 mutant S331E, which can transport GABA in the absence of Cl^-. In this GAT1 variant, the serine residue that is part of the Cl^- binding site has been substituted with glutamate, thereby effectively placing a negative charge into the space which Cl^- would occupy. The reason for the

importance of Cl^- may lie in the protein structure as a significant number of residues involved in Cl^- binding are also involved in Na^+ binding.
Another source of debate is the stoichiometry of transport. For BGT-1 a stoichiometry of 2 or 3 (Na^+): 1 or 2 (Cl^-): 1 (GABA/betaine) has been proposed, while for GAT1, it is unclear if 2 or 3 Na^+ ions are co-transported with one GABA molecule and some authors propose a Cl^-/Cl^- exchange mechanism. The order of ion and GABA binding remains unresolved from kinetic modelling studies and models also suggest that there could be a difference between GAT1 and BGT-1. The potential difference between GAT1 and BGT-1 is intriguing and could be exploited to better understand the origin of transporter selectivity. Therefore, there is a big need for more basic knowledge on BGT-1 and for expanding the existing knowledge on GAT1 to clarify these uncertainties to better understand their fundamental roles and to improve medication for patient treatment that suffers from transporter related pathologies.

Author contributions

MB, RZ, EL, and LG-M. drafted the manuscript under the guidance of EB. The figures and table were designed by MB, RZ, and RZ, while the bibliography was prepared by MB. The final review of the manuscript was done by EB, TS, and AB.

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Conflict of interest

AB and RZ were employed by Nanion technologies GmbH. The remaining authors declare that the research was conducted in the absence of any

commercial or financial relationships that could be construed as a potential conflict of interest.

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Materials and Methodology

cRNA preparation

The recombinant plasmids (*pAMV* rGAT1, *pAMV* cBGT-1, *pEXP* hGAT2, and *pEXP* hGAT3) were introduced into JM109 strain of *E. coli* using heat shock procedure. To do so, the cells were incubated with DNA on ice for 20 minutes and then immediately transferred to 42°C for two minutes followed by two minutes of transfer on again ice, thus inflicting a heat shock. Post this transformation, the bacteria were grown by incubation for an hour in Luria-Bertani (LB) medium and centrifuged (4000g for 10 minutes). These transformed bacteria were collected on Petri dishes containing LB-agar with ampicillin 50mg/ml and left overnight at 37°C to grow. Single colonies were picked from the Petri dish and inoculated in LB-agar with ampicillin 50mg/ml at 37°C under overnight agitation. Post inoculation, the plasmid DNAs were extracted using Wizard Plus SV Miniprep (Promega Italia, Italy) by following supplier instructions and eluted in 50µl of nuclease free water. The DNA plasmids were linearized using NotI, and *in vitro* cRNA synthesis was done in the presence of Cap Analog 10mM and T7 RNA polymerase 200U (Promega Italia, Italy). The synthesis was controlled using electrophoresis on agarose gel then the samples were quantified using nanodrop and stored at -80°C.

Xenopus laevis oocytes model for the heterologous expression of proteins

The oocytes of *Xenopus laevis*, west African clawed frogs, contain a very efficient biosynthetic apparatus capable of performing the needed post-translational modification for protein function and targeting (Bhatt, Di Iacovo et al. 2022). For the heterologous expression of the GABA transporters, oocytes were micro-injected with cRNA encoding the target protein (12.5ng/50nl). The oocytes were collected from adult *X. laevis* females by performing laparotomy on abdomen (using antiseptic agent

Providone-Iodine 10%) to remove portions of the ovary. Before the surgery the frog was immersed in anaesthetic solution made of 0.1% (W/V) tricaine methane sulphonate (MS222, Merck, Italy) in tap water and pH adjusted to 7.6 using sodium bicarbonate. The collected oocytes were treated with 1.5 mg/ml collagenase (collagenase type IA from Clostridium histolyticum, C0130 from Merck, Italy) in ND96 (NaCl 96mM, KCl 2mM, $CaCl_2$ 1.8mM, $MgCl_2$ 1mM, HEPES 5mM, pH7.6) for about 40-50 minutes on a shaker at 18°C. Healthy and fully grown oocytes (cycle III-IV) were selected and stored in an incubator at 18°C in NDE (ND96 plus 2.5mM pyruvate, 50µg/ml gentamycin sulphate, penicillin streptomycin solution, 10U/ml) (Bhatt, Di Iacovo et al. 2022). Based on the expression time taken by each transporter (on an average 72h), the oocytes were stored in a Petri dish containing NDE at 18°C in the incubator before experimental usage. The oocytes used in the experiments of this work were directly collected from the collaborators, who followed the experimental protocol approved locally by the Committee of the "Organismo Preposto al Benessere Animale" of the University of Insubria, Varese, Italy, and nationally by Ministero della Salute (n. 449/2021-PR), Italy and used in agreement with the Art.18 of decreto legislativo 4 marzo 2014, n. 26 and Art. 26 in directive 2010/63/eu of the European parliament and of the council of 22 September 2010.

Electrophysiological study using the two-electrode voltage clamp technique

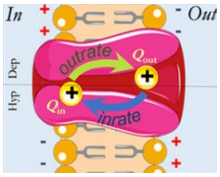
The two-electrode voltage clamp technique has been historically used to perform functional study of the electrogenic protein expressed in *X. laevis* oocytes. Its feature of membrane voltage control allows the collection of data about the ionic current in defined experimental conditions. Typically, a TEVC experiment records the inward or outward ion flux (Bhatt, Di Iacovo et al. 2022).

Using this technique, we collected the steady-state transport current and pre-steady state transport currents for GATs. The transport current is observed when the oocyte is exposed to the substrate, whereas the pre-steady state reflects the movement of ions between the extracellular space and a cavity in the transporter (Mager, Naeve et al. 1993, Peres, Pisani et al. 2004) within the membrane electric field. For all the electrophysiological measurements using TEVC in this work, the holding potential was kept at -60mV. To obtain pre-steady state responses, the voltage-step experiments were performed, where the oocytes were exposed to square voltage pulses (0.8s long) ranging from -120mV to +20mV (with a jump of 20mV).

It has been reported that for GAT1, the addition of the blocker SKF89976a supresses the transport activity blocking the Na^+ interaction and consequently any charge movement inside the transporter responsible of the classical slow transient (pre-steady state currents) (Mager, Naeve et al. 1993). To isolate and analyse the pre-steady state currents, the obtained signal in the presence of the Na^+ buffer solution or in the presence of the non-saturating substrates was subtracted from the signal obtained in the presence of the inhibitor, eliminating the passive oocyte resistance and capacitance (Mager, Naeve et al. 1993). Hence, the fitting with the single exponentials provided the time constant of signal decay, and their integration resulted in the amount of displaced charge within the membrane electric field.

The resultant τ-V and Q-V relationships of the pre-steady state currents provided information about the rate and amount of charge dislocation to and from the transporter cavities. The Q-V curves plotted in this work were obtained by Boltzmann equation fitting to the sigmoidal curve (Eq. 1), which also provided maximal displaceable charge Q_{max} (Fesce, Giovannardi et al. 2002, Vacca, Gomes et al. 2022). The further derivation of τ-V and

Q-V curves yielded the unidirectional rate constants inrate (α) and outrate (β) (Eq. 1).



$$Q_{in} = \frac{Q_{max}}{1 + \exp\left[\frac{-(V - V_{0.5})}{\sigma}\right]} \qquad inrate = \frac{1}{\tau}\frac{Q_{in}}{Q_{max}} \quad outrate = \frac{1}{\tau}\left(1 - \frac{Q_{in}}{Q_{max}}\right)$$

Equation 1: The visual representation of inrate and outrate of charge dislocation associated with pre-steady state of the transporter (left). The Boltzmann equation (centre) quantifies Q_{in}, total displaced charge. Where Q_{max} is the maximal displaceable charge, $V_{0.5}$ is the voltage at which half maximal charge is displaced (i.e., midpoint of the sigmoidal), σ represents the slope factor. The inrate and outrate constants provide the unidirectional constants α and β respectively (Vacca, Gomes et al. 2022).

The kinetic parameters such as I_{max} (maximal transport associated current) and $K_{0.5}$ (half-maximal transport concentration) were obtained from the dose response experiment data fitted using logistic equation using Origin 8.0 (OriginLab, USA) and Graphpad Prism8 (Graphpad, USA). The number of samples and batches, and the method for testing significance were reported in each figure.

Substrate detection in Xenopus laevis oocytes using liquid chromatography mass spectroscopy (LCMS-MS)

The high-performance liquid chromatography (HPLC) as a method of quantifying substrate uptake was not applicable to measure GABA and betaine translocated inside the oocytes, due to the peculiar characteristics of the cytoplasm of this cell, particularly rich of lipoprotein, and to the small amount of substrate present. In this work, we developed a novel method using LCMS-MS based approach to detect the substrates taken up by rGAT1 heterologously expressed in *Xenopus laevis* oocytes (see figure).

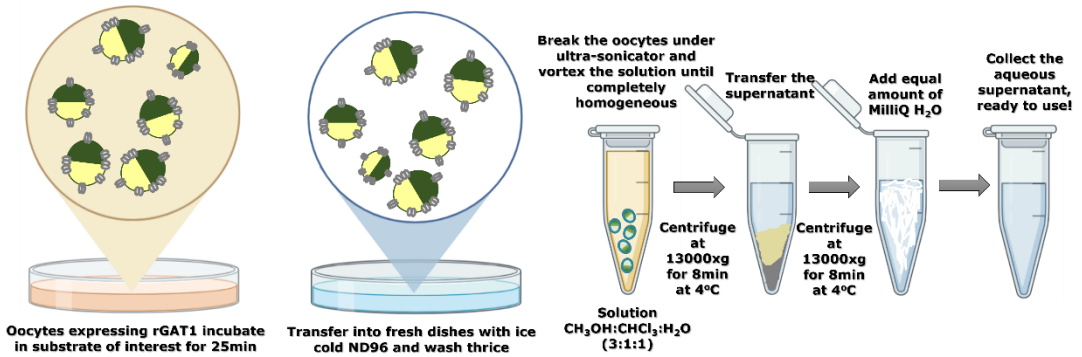


Figure 2 Sample preparation protocol for Xenopus laevis oocytes to be analysed using LCMS-MS technique: The oocytes expressing rGAT1 were incubated in substrate of interest for 25min and then washed multiple times with ice-cold ND96, and then transferred to Eppendorf containing $CH_3OH:CHCl_3:H_2O$. These oocytes were broken using ultra-sonicator and the cytosol contents were extracted and transferred to a fresh Eppendorf to be used immediately or stored at -80°C (up to 3 months).

In order to uptake the substrate, the oocytes expressing protein of interest were incubated in a fresh Petri dish containing the substrate+ND98 for 25 minutes in the incubator at 18°C. Then the oocytes were transferred to a fresh Petri containing ice-cold ND96 and were washed carefully on an agitator for two minutes, three times. In this way the extracellular presence of the substrate was controlled. At the end of the washing, the oocytes (in the group of 5 or 10) were transferred in a fresh Eppendorf containing $CH_3OH:CHCl_3:H_2O$ (MilliQ, Merck, Italy) with proportion of 3:1:1 v/v. The ratio of the oocytes to the solvent was maintained at 1:100 by considering the volume of the oocyte as 1µl. Using water ultra-sonicator the oocytes were broken, and the centrifuged at 13,000xg for 7-8 minutes at 4°C. The supernatant from these samples were removed, and the pallet containing cytosol content with oocyte debris were diluted in H_2O (MilliQ, Merck, Italy) to extract the hydrophilic compounds. These samples were centrifuged at 13,000xg for 7-8 minutes at 4°C and the aqueous top part of the resulting sample (containing cytosol content) was transferred carefully to a fresh Eppendorf, which were used immediately for LCMS-MS detection or were stored at -80°C to use later (up to 3 months).

The mass spectroscopy analysis was performed using a QExactive mass spectrometer coupled to a nano EasyLC 1000 (Thermo Fisher Scientific Inc., USA). For channel A the solvent composition was 0.1% formic acid with LCMS grade water (Merck, Italy) and for channel B was 0.1% formic acid with acetonitrile (Merck, Italy). Post drying of each sample, it was dissolved in 0.1% formic acid with LCMS grade water (Merck, Italy); and 10µl of the dissolved sample was injected into the reverse-phase C18 column (75µm × 150mm, ReproSil-Pur 120 C18-AQ, 1.9µm; Dr. Maisch GmbH, Germany).

Radiolabelled release assay to detect substrate induced efflux

The cloning of neurotransmitter transporters has enabled their study in cellular systems containing only the protein of interest using uptake and release experiments in cells transfected with the respective cDNA (Sitte, Scholze et al. 2000). In this work, the release efflux assay experiments were performed using [^{3}H]GABA on HEK293 cells expressing YrGAT1(Sitte, Singer et al. 2002); in the laboratory of Prof. Dr. Harald Sitte at Medical University of Vienna, Austria.

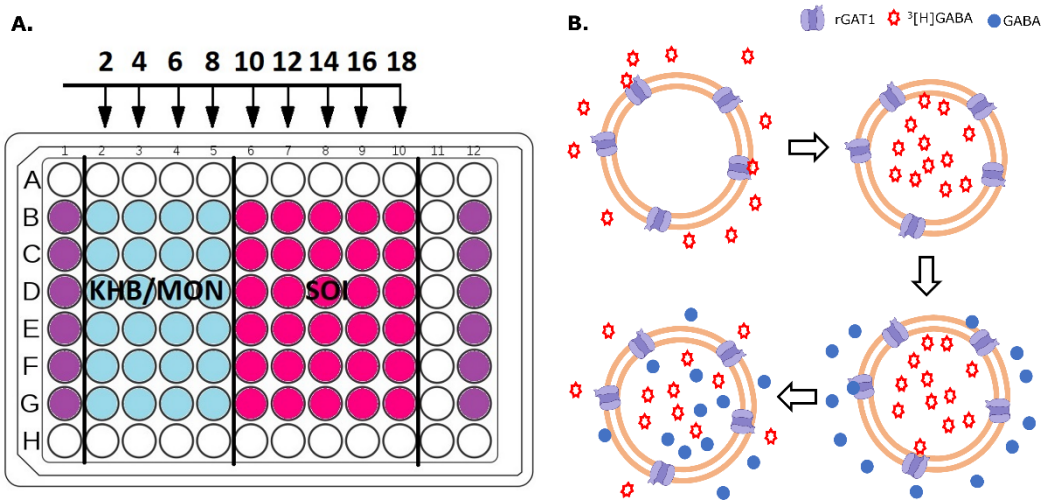


Figure 3 Release assay experiment in HEK293 cells: A) the layout of coating the wells with poly-D-lysine on 96-well plate. B) The cells expressing protein of interest is loaded with radiolabelled substrate, and post incubation when the cells

are exposed to unlabelled substrate at different concentrations, there will be a detectable efflux of radiolabelled compound generated to preserve the cellular volume.

The stable rGAT-1 transfectants were grown in Dulbecco's minimal essential medium (DMEM) containing L-analyl-L-glutamine (L-glutamax I™, Gibco life technologies, USA), gentamicin 50mg/l, 500µg/ml geneticin, and 10% heat-inactivated foetal bovine serum on 100mm-diameter cell culture dishes at 37°C in an atmosphere of 5% CO_2 and 95% air. The transfected cells were grown overnight onto poly-D-lysine coated 96-well plates at 4×10^4 cells/well (in the formation shown in figure 3 A). Prior to the release assay, DMEM was removed, and the cells were loaded with 0.01µM [^{3}H]GABA (0.24Ci/mM; 1.2µCi/well) in 100µl of KBH buffer (Krebs-Ringer HEPES buffer; (mm): HEPES 10, NaCl 120, KCl 3, $CaCl_2$ 2, $MgCl_2$ 2, glucose 20, pH 7.3) for 20 min at 30°C. Post incubation, the cells were washed using 100µl of KHB to establish a stable baseline three times at two minutes fraction. Then these loaded cells were exposed to the substrate of the interest (100µl) for two minutes. After every two minutes the supernatant of the wells was transferred to another empty lysine coated well (at two minutes fraction), where the released radiolabelled GABA would have been collected (see figure 3 B). These experiments were also performed using KHB buffer containing the Monensine 10µM, i.e., an ionophore which selectively complexes and transports sodium cation across lipid membranes (Mollenhauer HH, Morré DJ et al. 1990).

Molecular docking simulations

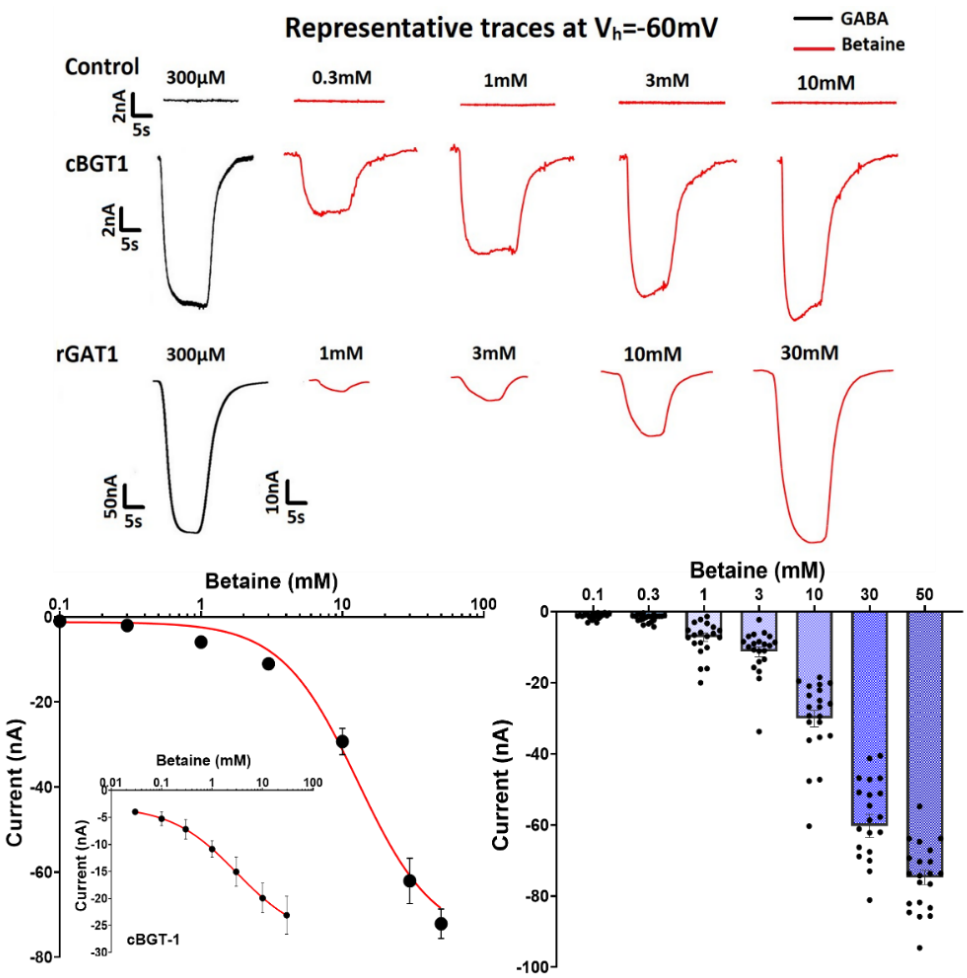
In the absence of a crystal or cryo-EM structure of hGAT1 in the outward-facing conformation, we selected the Alphafold homology model to analyse the stability of the bound GABA and betaine (Jumper, Evans et al. 2021, Varadi, Anyango et al. 2022). The bound-ions were added to the hGAT1 model using as a reference the outward-open human SERT crystal

structure (PDB ID: 5I71) (Coleman, Green et al. 2016). The docking experiments were performed using GOLDscore scoring function on GOLD molecular docking software (The Cambridge Crystallographic Data Centre, UK) (Jones, Willett et al. 1997). The experiments were conducted under the guidance of Prof. Doc. Thomas Stockner at Medical University of Vienna, Austria.

Results

Betaine induces inward transport currents in Xenopus oocyte expressing rGAT1, similar to cBGT-1.

The dose response experiment on oocytes expressing cBGT-1 was performed using betaine 0.3, 1, 3, 10, and 30mM. As expected, betaine perfused inward transport current with maximal transport current I_{max}=-23.01±1.2nA and half-maximal transport coefficient $K_{0.5}$=1.55±0.3mM (see figure 4). The same experiment was performed on oocytes expressing rGAT1 using betaine 1, 3, 10, 30, and 50mM. At these concentrations, betaine induced inward transport current with I_{max}=-76.16±1.1nA and $K_{0.5}$=11.57±1.3mM (see figure 4). As a negative control, the non-injected oocytes were also perfused with the same betaine concentration.



Kinetic parameters of betaine dose response		
	I_{max} (nA)	$K_{0.5}$ (mM)
cBGT-1	-23.01±1.2	1.55±0.3
rGAT1	-76.16±1.1	11.57±1.3

Figure 4 Betaine dose response in Xenopus laevis oocytes heterologously expressing rGAT1 and cBGT1 (V_h=-60mV): The representative traces of currents recorded for GABA 300µM and increasing betaine concentration on oocytes expressing cBGT1 and rGAT1 show induction of inward transport current (non-injected oocyte used as control) The histogram shows betaine-induced dose dependent inward transport current (n=18). The kinetic parameters were calculated by the transport current measured at V_h=-60mV analysed using logistic fitting model for both rGAT1 and cBGT-1 (inset), values showed in the tabular form. Data are means (SEM) of 8-16 oocytes from 1-5 batches.

The betaine transport by rGAT1 is sodium-dependent and can be blocked by selective inhibitors.

Given that GAT1 is a sodium dependent symporter, any transport activity via GAT1 should not occur in the absence of Na^+ (Guastella, Nelson et al. 1990), when Na^+ was replaced with trimethylammonium (TMA^+), as happened for GABA the inward transport currents induced by betaine in the oocyte heterologously expressing rGAT1 disappeared. Hence, the betaine transport current is sodium dependent (see figure 5). Based on the secondary substrate of GAT1 called nipecotic acid, many molecular derivatives have been synthetized to block transport activities of GAT1 effectively e.g., tiagabine, SKF89976a, NO711 (Bhatt, Gauthier-Manuel et al. 2023). We tested the effect of SKF89976a 30µM on the transport current induced by GABA and betaine on the oocyte expressing rGAT1 (see figure 2). The current induced by GABA 300 µM was strongly inhibited, whereas the current induced by betaine 10mM was completely blocked (see figure 5). Similar effect was observed for tiagabine and NO-711 as well (data not shown), confirming the betaine induced currents are mediated by rGAT1.

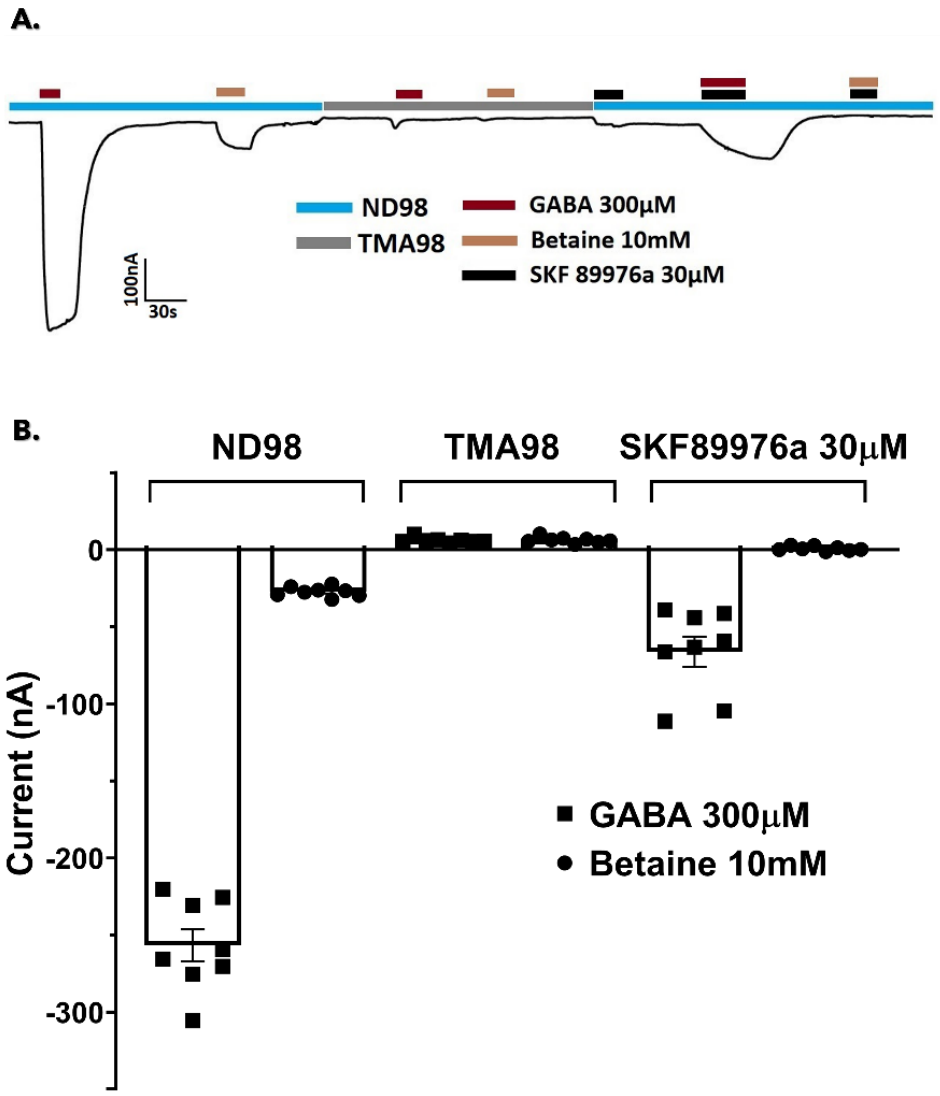


Figure 5 Sodium dependence and SKF89976a induced inhibition of GABA and betaine transport in rGAT1: A representative trace for current response of rGAT1 to GABA 300µM and betaine 10mM, in presence of ND98, TMA98, and SKF89976a 30µM, shows that both these substrates require sodium to be translocated and can be inhibited by SKF89976a (A). When Na^+ in the buffer solution is replaced by trimethylamine (TMA^+), the inward transport of GABA and betaine both vanish (B). The perfusion of SKF89976a 30µM strongly inhibits the large inward current for GABA 300µM, whereas transport current induced by betaine 10mM is almost undetectable. All recordings were performed at V_h=-60mV and data are means (SEM) of 8-12 oocytes from 1-3 batches.

The voltage-dependent transport of betaine by rGAT1 shows that it is a slower substrate than GABA.

rGAT1 is a voltage dependent transporter and a voltage-jump experiment on *X. laevis* oocytes expressing rGAT1 will provide pre-steady state current, steady state current, and leak current (Mager, Kleinberger-Doron et al. 1996, Bossi, Giovannardi et al. 2002, Kanner 2003). The pre-steady state currents in GAT1 are due to the binding and unbinding of Na^+ into the transporter cavities inside the membrane electric field and disappear in the presence of a saturating substrate (Bossi, Giovannardi et al. 2002). The voltage-jump experiments were performed on oocytes expressing rGAT1 in the presence of betaine (1, 3, 10, 30, 50mM). In rGAT1, the betaine similarly to GABA, induces currents that are voltage-dependent (see figure 6 and 7 A).

The pre-steady state currents in rGAT1 can be observed in the presence of ND98 and disappear completely in the presence of saturating GABA (300μM). The SKF89976a 30μM (see figure 6, first row) instead blocks the access of Na^+ to the GAT1 substrate vestibule (Motiwala, Aduri et al. 2022). The voltage step recorded in the presence of the inhibitors shows only the fast relaxation due to the membrane capacitance of oocytes. Even in the presence of increasing betaine concentrations (even high concentration like 30mM), we observe the persistent presence of slow transients (see figure 6, middle and last row) larger from what expected if compared to no saturating GABA concentrations.

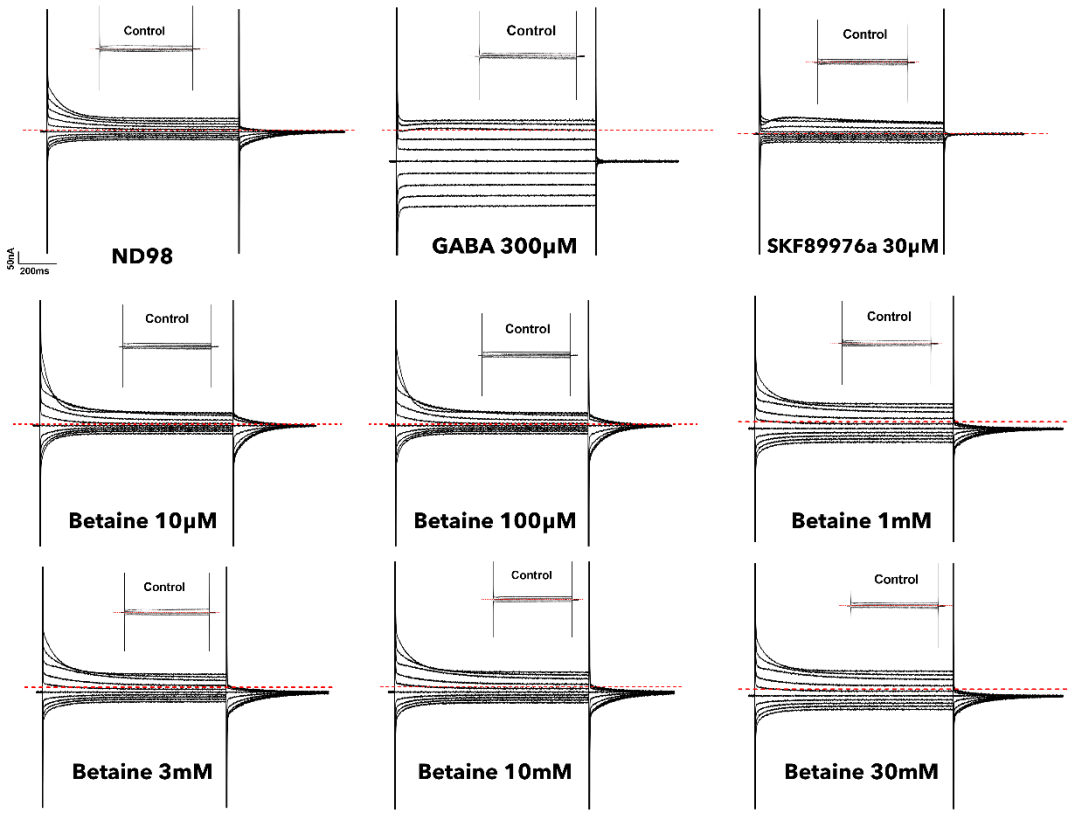


Figure 6 Representative traces of the voltage step response for betaine in X. laevis oocytes expressing rGAT1: The oocytes were clamped at V_h=-60mV, and voltage jumps of +20mV for t=800ms were given from -120mV to +20mV. For the buffer solution ND98, the large transient currents due to Na^+ binding were evident, which disappear in the presence of the substrate GABA 300µM and the inhibitor SKF89976a 30µM (first row). Although betaine induces inward transport current, the large transients with slow relaxation were persistent even at its high concentration of 30mM (middle and last row). The red line indicates holding current at V_h=-60mV and inset for each shows the response from a non-injected oocyte for the given concentration. The scale indicated for ND98 is the same for the rest of the traces.

To analyse the transients current in the presence of betaine the subtraction of the traces recorded in the presence of the blocker were subtracted as described in methods (Mager, Naeve et al. 1993, Bhatt, Di Iacovo et al. 2022). These subtracted traces were integrated to obtain the relationship between dislocated charge (Q) and voltage; and fitted with single exponential for relaxation time constant (τ) and voltage. The τ-V relationship of these data provided the rate of transport measured at each

potential, whereas the Q-V relationship corresponded to the total charge moved in the membrane electric field. The data were further analysed to calculate outrate α and inrate β that indicates rate of charge moving to and from the transporter cavity respectively. All of the Q-V, τ-V, and rate constant analysis were done for betaine 0.1, 0.3, 1, 3, 10, 30, and 50mM. To avoid overcrowding and graphically complicating the figures, the figures were presented using betaine 0.1, 1, 10, and 50mM betaine.

The Q-V relationship showed that betaine at low concentrations (0.1-1mM) has charge displacement similar to ND98 alone. With further increase in extracellular betaine (10 and 50mM) the induced charge displacement decreased but did not disappear like for saturating GABA (see figure 7 B). The τ-V relationship provided similar result, where for low betaine concentration (0.1 and 1mM) total decay time was similar to ND98 alone. With increase in betaine concentration, the relaxation time constant value decreased, indication accelerated transport rate in the presence of betaine (see figure 7 C). The further analysis of inrate and outrate showed that like ND98 at low betaine concentrations the charge entered the transporter cavities much faster than it could leave (β>α); the rate of the β in the presence of betaine 0.1mM is curiously slower than in the presence of Na-buffer alone in particular at voltages lower than -80 mV (see figure 7 D).

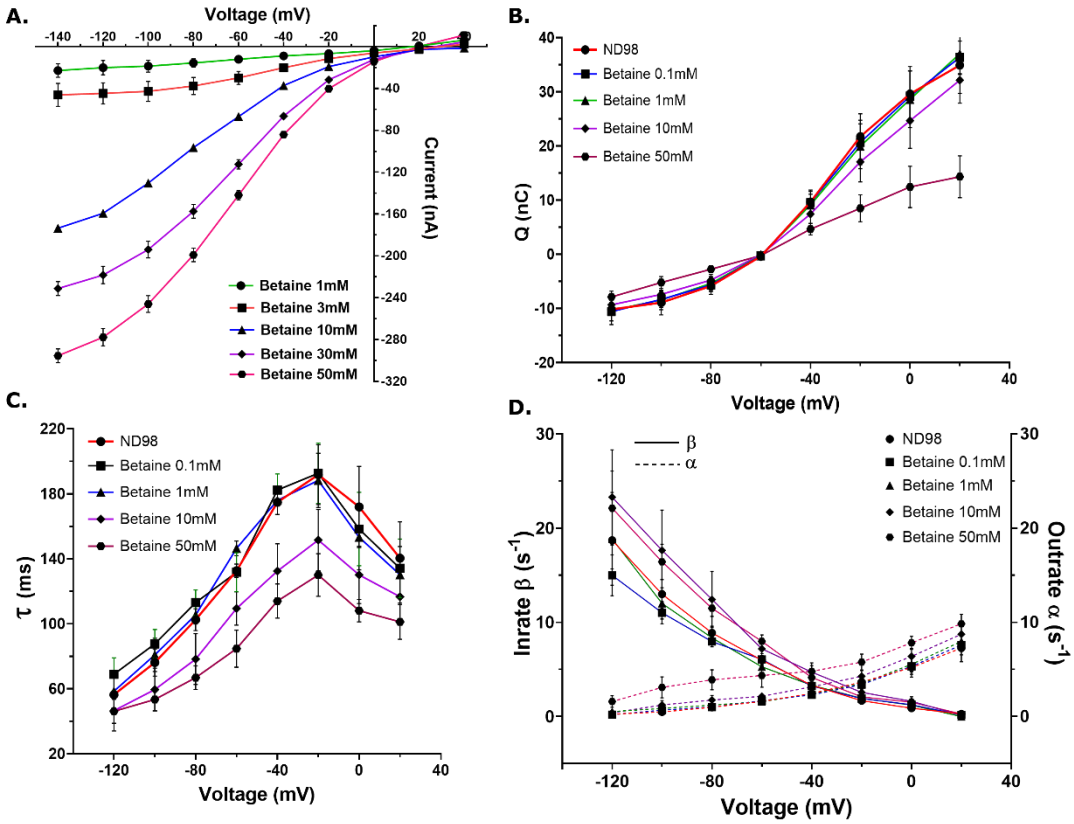


Figure 7: Pre-steady state and steady state currents, and transporter efficiency for betaine in rGAT1: The I-V relationship of betaine induced steady state current show voltage-dependent transport (A). The Q-V and τ-V relationship of intramembrane charge movement induced by betaine indicate large charge dislocation (B), high time decay (C), and faster inrate of charge than outrate (β>α) (D), especially at low betaine concentration. The oocytes were clamped at V_h=-60mV and voltage jumps of +20mV for t=800ms were given from -120mV to +20mV. Data are means (SEM) of 8-14 oocytes from 1-3 batches.

Like GABA, betaine also induces efflux of [^{3}H]GABA in release assay experiment in rGAT1 expressing HEK293 cells.

The effects of GAT1 substrates (GABA, nipecotic acid, guvacine, L-DABA, DL-DABA) and uptake inhibitors (SKF89976a, tiagabine, NO-711, Cl-966) on efflux of [^{3}H]-GABA in HEK193 cells stably expressing rGAT1 have been in detail reported by Sitte et al.(Sitte, Singer et al. 2002). Like other rGAT1 substrates, the addition of betaine to the HEK293 cells, pre-loaded with 0.01μM [^{3}H]-GABA at 30°C for 20 minutes, resulted in a time- and dose-dependent increase in [^{3}H]-GABA efflux. The basal [^{3}H]-GABA efflux of

these pre-loaded cells was 0.12±0.02% min^{-1} (basal efflux defined as the mean of the three two-minute fractions before addition of the substrate of interest)(Sitte, Singer et al. 2002). Betaine induced the concentration dependent efflux was observed in the samples with and without the ionophore Monensine 10μM (figure 8 C and D). Also, as a positive control the pre-loaded HEK293 cells stably expressing rGAT1 were exposed to GABA as well and the resulting time- and dose-dependent efflux data matched with that reported in the literature (Sitte, Singer et al. 2002). The kinetic analysis of the GABA and betaine induced efflux per minute (with Monensine 10μM, n=3) was performed using the logistic fitting model. The drug-induced efflux was calculated as the mean efflux of the fraction where the value started plateauing divided by the length of the fraction (=2 min). The negative control was performed with tiagabine as the uptake inhibitor of rGAT1 (data not shown). The kinetic analysis of the data provided $K_{0.5,GABA}$ of 36.28±7.3μM and $K_{0.5,betaine}$ of 6.73±2.2mM in KBH; and in the solutions with KHB and Monensine 10μM $K_{0.5,GABA}$ of 36.48±2.5μM and $K_{0.5,betaine}$ of 7.71±2.1mM. These values are close to the values obtained from the electrophysiological experiments of betaine dose-response in *X. laevis* oocytes heterologously expressing rGAT1.

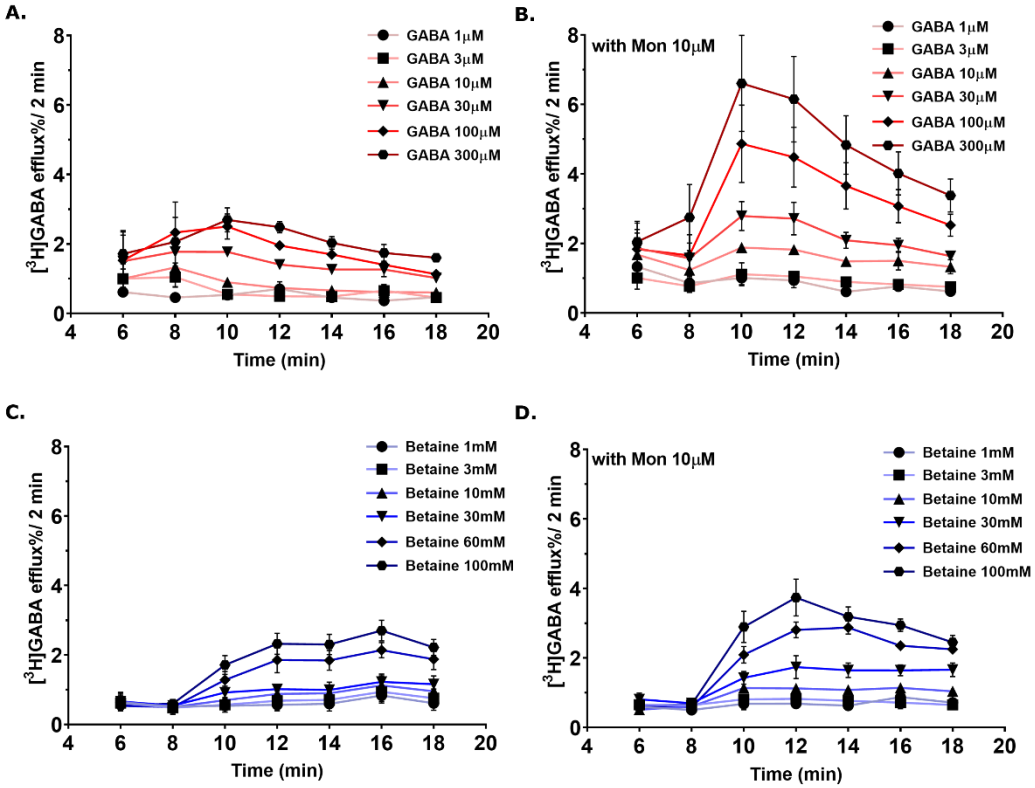
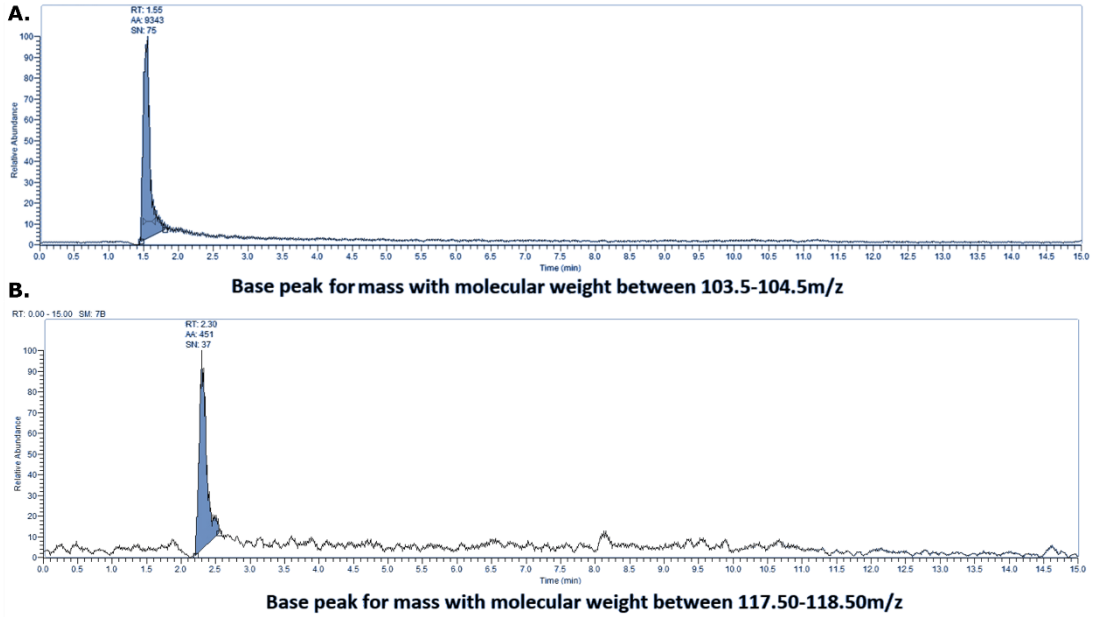


Figure 8 Effect of GABA and betaine on efflux of [^{3}H]-GABA in HEK-293 cells stably expressing rGAT-1. After four fractions of the basal efflux (2-8 min), the buffer was switched with different concentrations of GABA (A) or betaine (C) alone or respectively with Monensine 10μM (B, D). Data shown as fractional efflux, defined as the percentage of radioactivity present in the cell at the beginning of that fraction, from n=3 performed in duplicate with ±SEM.

Detection of GABA and betaine using LCMS-MS protocol on X. laevis oocytes expressing rGAT1.

The *X. laevis* oocytes heterologously expressing rGAT1 were incubated in different concentrations of GABA (1, 3, 10, 30, 100, 300μM) and betaine (0.1, 0.3, 1, 3, 10, 30mM) for 25 minutes in the group of five oocytes per sample. Each sample was prepared as per the novel protocol mentioned in the methodology. The control standards were developed by diluting GABA and betaine in LCMS grade H_2O, and the collision energy required to detect primary and production ion of the target molecule for GABA was 23eV and for betaine 30eV. The detection time (in 15min long protocol)

and production ion for GABA was 1’ 55” with 87m/z and for betaine 2’ 30” with 59.58m/z respectively (see table in figure 9). The detection for the uptake of both GABA and betaine was concentration dependent (see figure 10).



Condition	Pre-cursor ion (m/z)	Collision energy (eV)	Retention time (min)	Production ion (m/z)
GABA	104.18	23	1’ 55”	87
Betaine	118.17	30	2’ 30”	59.58

Figure 9 Representative traces of GABA and betaine uptake by X. laevis oocytes expressing rGAT1. A) Representative GABA (104.18m/z) peak detection at 1’55” with collision energy 23eV and production ion of 87m/z in the oocytes incubated in GABA (1mM) for 20min. B) Representative betaine (118.17) peak detection at 2’30” with collision energy of 30eV and production ion of 59.58m/z in the oocytes incubated in betaine (10mM). Both traces were obtained from sample of 5 oocytes incubated for 20min.

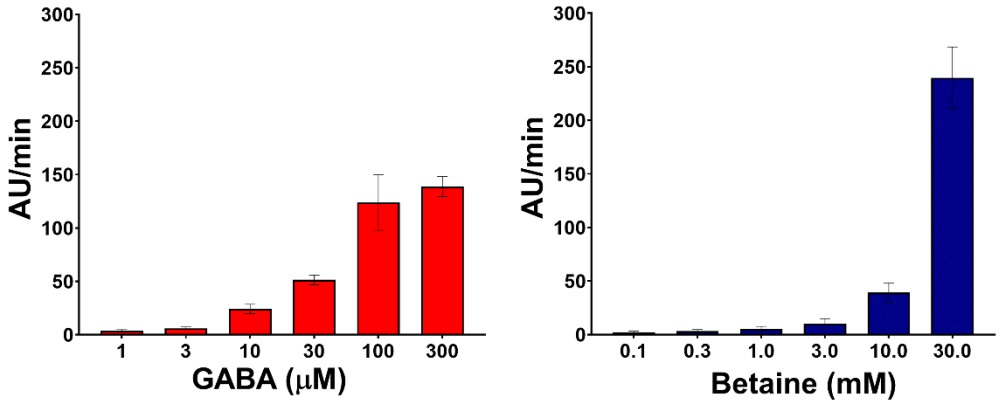


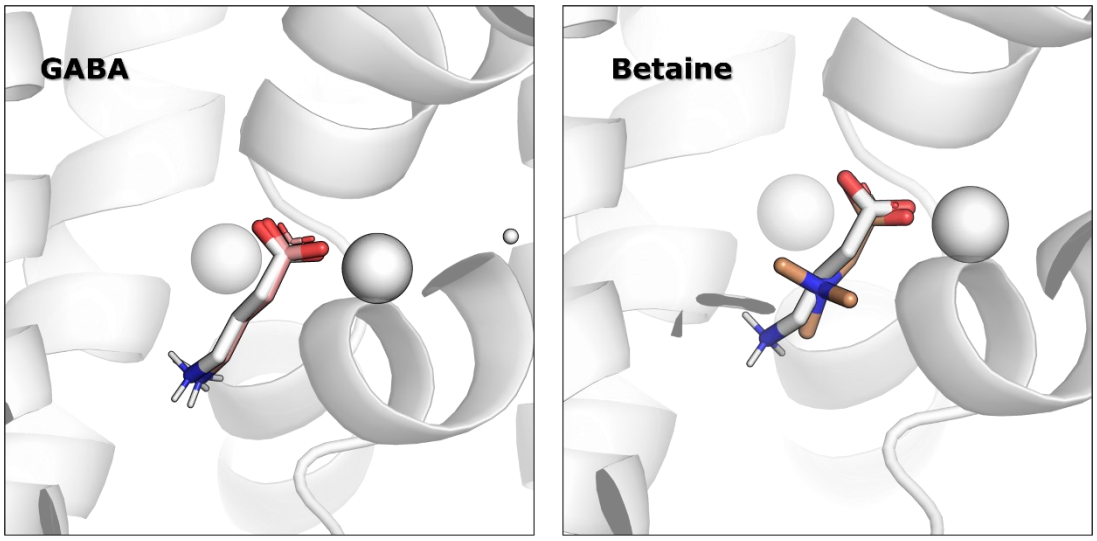
Figure 10 Qualitative detection of GABA and betaine in X. laevis oocytes expressing rGAT1. The uptake of both GABA and betaine in concentration-dependent manner was detected in oocytes incubated in different concentrations. Data are mean (SEM) of 10-15 oocytes distributed in 1-3 batches.

Betaine docks in the binding pocket of hGAT1 successfully.

Using Alphafold model of hGAT1 in outward open confirmation, GABA was docked into the binding pocket with two Na^+ and Cl^- as explained in the methodology. Using the docked GABA as a reference molecule, the transporter structure was given betaine molecule to dock with centre of the mass of docked GABA as the binding sphere, since GOLD has the docking sphere 15Å around it (Feinstein and Brylinski 2015).

The docking was performed using GOLDscore function, as it is a better docking function than ChemScore or ChemPLP (Verdonk, Cole et al. 2003). The default setting of number of the dockings to be performed on each ligand was accepted (i.e., ten), and the best three fitness scores within 1.5Å of each other were taken as the final results. The docking of betaine was successful with scoring function of 42.24, whereas the docking of GABA yielded scoring function of 50.52 (see the table in figure 11). By proximity analysis of docked GABA and betaine, the polar and non-polar contacts withing hGAT1 binding pocket were also identified (see the table in figure 11). The charged tail of GABA molecule allows it to have more polar contacts than betaine with three methyl groups as its tail (see figure

10). Using the docking results, the molecular dynamics simulation was also performed for betaine in hGAT1 for 50ns that yielded a stable binding density for betaine in the GAT1 binging pocket (data not shown).



Ligand	Docking fitness score	Polar contacts	Non-polar contacts
GABA	50.52	Y60, G65, Y140, S396, T400	L64, F294, S295
Betaine	42.24	L64, G65, Y140	G63, S295, S396

Figure 11 Molecular docking of GABA and betaine in hGAT1 Alphafold model in outward open confirmation. The reference GABA molecule is in white covering the resultant docked GABA in pink (left) and betaine in copper (right), in the presence of two Na^+ and one Cl-. The docking fitness score for both GABA and betaine indicate successful docking using GOLDscore function, and the polar and non-polar contacts were obtained with proximity function (within 5Å radius).

Relationship of GABA and betaine in interaction with rGAT1 depends heavily on their extracellular concentrations.

The data collected show that betaine is allow affinity secondary substrate of rGAT1. To better investigate the role of betaine, it is important to understand the relationship with the primary substrate GABA. The relationship between GABA and betaine was investigated using TEVC and LCMS-MS. The electrophysiological experiments on TEVC were performed using GABA (1-300μM) with betaine (1μM-50mM) following a perfusion protocol shown in figure 12.

Buffer Solution	GABA [X]M	GABA [X]M + Betaine [Y]M	GABA [X]M	Buffer Solution

Figure 12: Competitive assay for GABA-betaine relationship in X. laevis oocytes expressing rGAT1 on TEVC at holding potential V_h=-60mV. The GABA [X]M ranges from 1-300μM and betaine [Y]M ranges from (0.001-50mM). Same experiments were also performed with buffer solution followed by betaine [Y]M.

The GABA-betaine relationship is starkly dependent on their individual concentration (see figure 13 A). With GABA concentration below 10 μM and betaine less than 10mM (≈$K_{0.5,betaine}$), we observed blocking of transport current. With betaine 10mM and above, the blocking effect disappeared, and a collective larger inward transport current was observed. However, this kind of dual effect of betaine on GABA transport vanished when the extracellular GABA concentration was 30, 100, and 300 μM i.e., larger than $K_{0.5,GABA}$. This behaviour can be visualized the best at GABA 10μM concentration with different betaine concentrations (figure 13 D), when GABA 10μM is perfused with betaine 1-300μM, a dose dependent inhibition of GABA 10μM transport current was observed (IC_{50}=7.84±3.91μM). At the same GABA concentration, the perfusion with betaine 1-50mM yielded in a dose-dependent transport current increase resulting in increased $K_{0.5,betaine+GABA10\mu M}$ to 15.24±7.71mM, if compared to $K_{0.5,betaine}$ 11.57±1.3mM recorded in its absence; and $I_{max,betaine+GABA10\mu M}$ to -151.15±29.32nA nearly doubled than $I_{max,betaine}$ alone -76.16±1.1nA and $I_{GABA10\mu M}$ alone -72.73±3.1nA. The collective representation of this dataset for GABA (1-300μM) and betaine (10^{-3}-50mM) is shown as a heat map (figure 13 B), where the concentration dependent dual behaviour of betaine on GABA transport by rGAT1 can be seen clearly.

Since, the detection time for GABA and betaine uptake by rGAT1 in our LCMS-MS protocol are different with distinct production-ions, it was possible to perform GABA and betaine competition in the same oocyte. As the electrophysiological findings showed that betaine blocked GABA

transport the most around 0.1mM, we incubated oocytes expressing rGAT1 with GABA 3, 10, and 30μM with betaine 0.1mM for 20 minutes (n=3, five oocytes per sample). The cytosolic content detection by LCMS-MS showed that in the presence of betaine 0.1mM: at 3μM no GABA was taken up, at 10μM the uptake was reduced significantly, and at 30μM the uptake was slightly increased but not significantly (see figure 13 C). This result agrees with the electrophysiological findings and provides direct supporting evidence to the modulation of the extracellular GABA by rGAT1 using betaine.

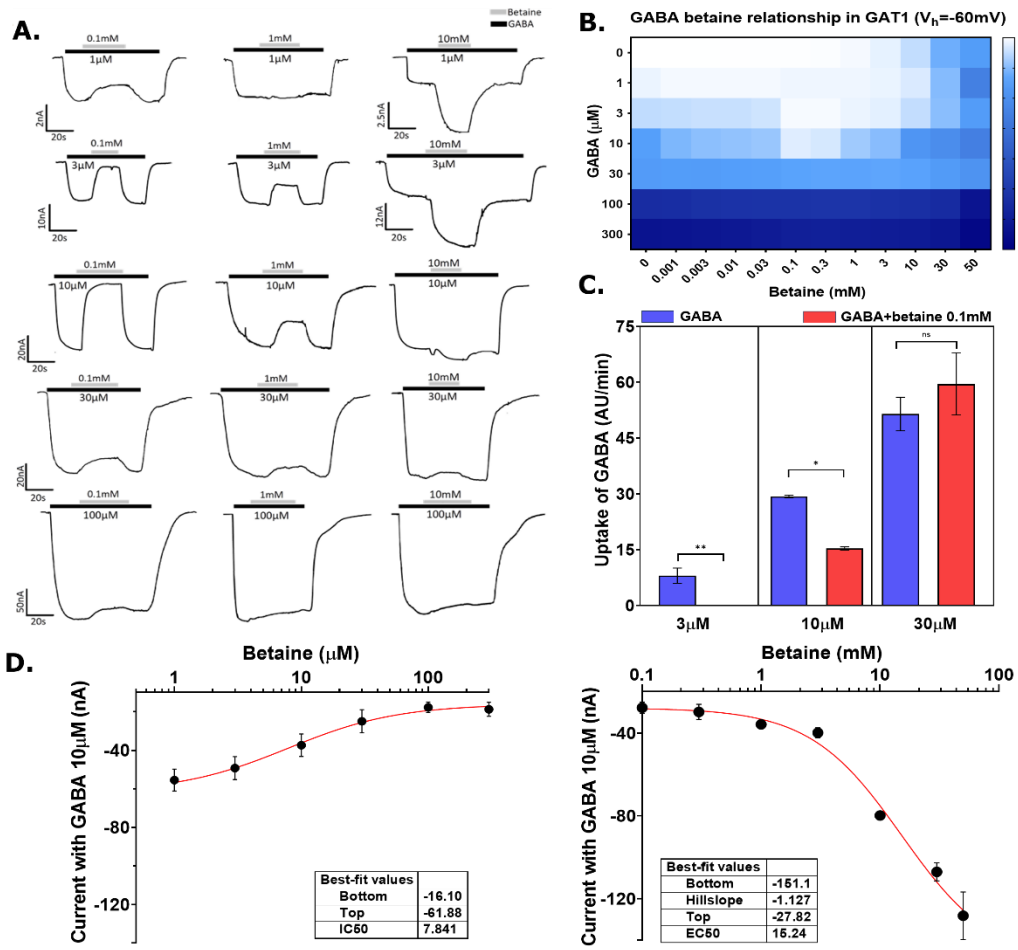


Figure 13: Dual behaviour of betaine in rGAT1. A) Representative traces of GABA betaine assay in X. laevis oocytes expressing rGAT1 at holding potential V_h=-60mV, where the oocyte was perfused with different GABA concentration (1, 3, 10, 30, 100µM) with betaine 0.1, 1, and 10mM. B) Heatmap of GABA betaine assay currents in oocytes expressing rGAT1 at V_h=-60mV, n=6, for combination of GABA (1-300µM) with betaine (10^{-3}-50mM). D) Dual behaviour of betaine at GABA 10µM in oocytes expressing rGAT1 at V_h=-60mV with error bars representing SEM for n=6. At µM concentration betaine blocked GABA 10µM induced current up to 75% with IC_{50}=7.84±3.91µM. With extracellular betaine at mM, the half maximal for betaine changes to $K_{0.5,\ betaine}$= 15.24±7.71mM and $I_{max,\ betaine}$= -151.15±29.32nA. Data was fitted using logistic fitting model. C) LCMS-MS detection of betaine and GABA in the oocytes, expressing rGAT1, incubated in GABA 3, 10, and 30µM with and without betaine 0.1mM for 20min. The qualitative analysis of GABA and betaine taken up by the oocytes is represented in this bar plot with uptake values (arbitrary units) of each oocyte per minute, data shown with SEM and obtained from n=3 with five oocytes in each sample. The p values were obtained by ordinary one-way ANOVA method followed by Bonferroni's multiple comparisons test, with a single pooled variance.

Betaine slows down rGAT1 transport cycle disallowing binding of GABA that results in inhibition of GABA uptake by GAT1.

The competitive assay experiment of GABA and betaine on oocytes expressing rGAT1 was also performed with voltage jump of -20mV (each for t=0.8s from -120mV to +20mV) to study steady and pre-steady state transport currents. The I-V relationship of the GABA 10μM current in the absence and in presence of betaine 0.1mM (figure 14 A) clearly showed the blocking effect of betaine. Observing the representative traces in the presence of GABA 10 μM with or without betaine 0.1mM it is clear the peculiar action of betaine, it reduces the current and increase the transient in responses to the voltages jumps increasing the relaxation time as clearly visible . In fact, the τ-V relationship for betaine 0.1mM shows similar overall decay constant as ND98. For GABA 10μM the decay constant τ was much faster than for ND98 and betaine 0.1mM and increases strongly in the presence of betaine 0.1mM (see figure 14 C).

The Q-V relationship shows that the total charge displaced in the presence of betaine 0.1mM does not differ from that of ND98 alone. Interestingly the charge displacement in the presence of GABA 10μM was increased strongly when betaine 0.1mM was added to the solution (Figure 14 B). Similarly, the decay constant, outrate α and inrate β calculated for GABA 10μM with and without betaine 0.1mM, shows a reduction of both inrate and outrate in the presence of betaine 0.1mM (see figure 14 D). Overall, it can be seen that in the presence of betaine the transport rate of GAT1 decreased.

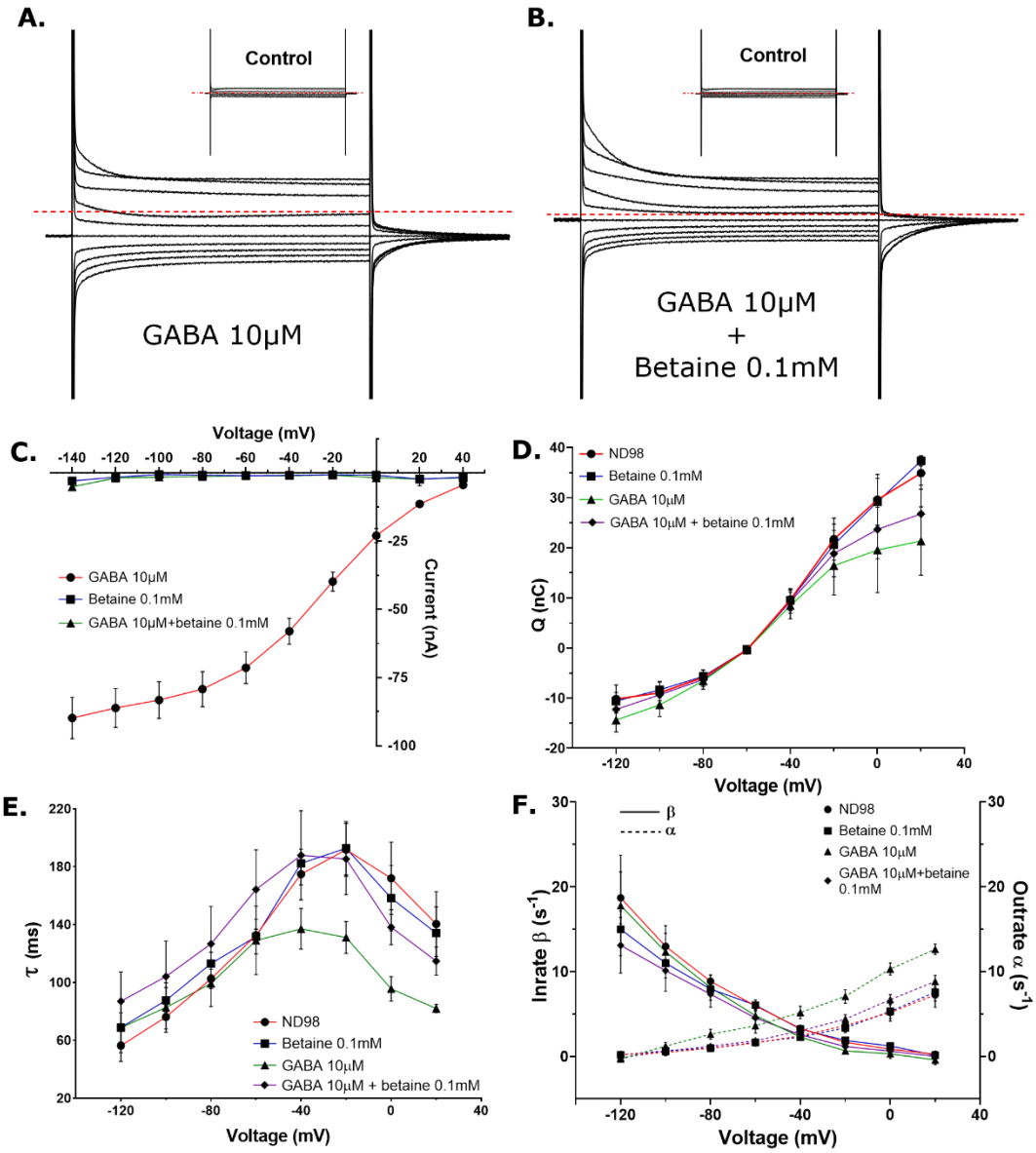


Figure 14: Pre-steady state analysis of GABA betaine competitive assay. Representative traces of voltage step protocols in the presence of GABA 10μM alone (A) and with 0.1 mM betaine (B), where the dashed redline indicates the baseline for holding potential. The I-V relationship (C) of transport current by GABA 10µM alone and with betaine 0.1mM. The Q-V (D) and τ-V (E) relationship and the unidirectional rate constant (F). The oocytes were clamped at holding potential V_h=-60mV and voltage jumps of +20mV for t=800ms were given from -120mV to +20mV. Data are means (SEM) of 5-7 oocytes from 1-3 batches.

Discussion

While the multiple publication showed that the systematic betaine supplementation can be beneficial for neuronal diseases and disorders, the cellular mechanism involved in translocation betaine across the neuronal membrane had remained a puzzle (Bhatt, Di Iacovo et al. 2023). With BGT-1 being able to transport betaine, it was hypothesized that betaine may interact with GABAergic pathways in CNS (Kunisawa, Kido et al. 2017, Bhatt, Di Iacovo et al. 2023). In this work, we have shown that GAT1, the most expressed neuronal GABA transporter, can not only transport betaine, but also can modulate extracellular GABA levels.

The primary source of betaine accumulation in humans is its oral uptake from the dietary sources. Since betaine is an excellent osmolyte, it tends to get accumulated in cells and tissues. This tendency also results in underestimation of the betaine absorption using the traditional blood plasma assays (Awwad, Kirsch et al. 2014, Knight, Piibe et al. 2017). The pharmacokinetics of this accumulation is dose-dependent with peak betaine concentrations reaching from 1-3mM within hours (Matthews, Johnson et al. 2002, Schwahn, Hafner et al. 2003). In liver and kidney, the concentration levels of betaine can reach up to ~30mM and ~100mM respectively (Wehner, Olsen et al. 2003, Bhatt, Di Iacovo et al. 2023). Recently, Knight et al showed that neurons from hippocampal tissues of mice, can uptake and accumulate betaine up to 12mM (Knight, Piibe et al. 2017). While the reported betaine level in the mice brain is relatively low and unclear (Schwahn, Laryea et al. 2004, Kempson, Zhou et al. 2014), from the findings of Knight et al. it was evident that the interaction of neurons with betaine should be at mM levels. With this argument along with the fact that affinity of betaine for cBGT-1 also ranges towards mM (Matskevitch, Wagner et al. 1999), we used betaine at mM levels to study its interaction with GATs. The electrophysiological experiments using TEVC

in *X. laevis* oocytes heterologously expressing rGAT1 resulted in betaine induced dose-, voltage-, and sodium dependent transport currents with $K_{0.5}$ ~11mM, which can be inhibited by GAT1 potential inhibitors such as SKF89976a, tiagabine, and NO711 (see figure 4-7). The transport of betaine by GAT1 was also confirmed in HEK293 cells using radiolabelled efflux assay (see figure 8) and patch-clamp technique (not shown here). The LCMS-MS analysis of cytosol contents of *X. laevis* oocytes incubated in different betaine concentration gave a direct confirmation of the uptake by GAT1 (see figure 9, 10). Interestingly, the molecular docking of betaine in GAT1 showed that it forms polar contacts with three residues L64, G65, and Y140, which is two residues less than GABA (see table in figure 11). It would imply that the transport would require more energy to translocate betaine than GABA, explaining the lower affinity and being a "slow" substrate.

The detailed analysis of pre-steady state currents gives information about the initial steps of the transporter cycle and the interaction of transporter with substrate and ions (Bhatt, Gauthier-Manuel et al. 2023). By breaking down the decay rate constant of the large transient currents lasting in the presence of betaine in rGAT1, into inrate β and outrate α showed that in the presence of betaine the transport rate of rGAT1 decreased. It seems that betaine binds in the vestibule, but it is not able to induce the fast conformational changes to be translocated, as the inrate β for betaine is larger than its outrate α, when compared with rate constants for GABA (see figure 7 and 14). These slow rate constants allow to hypothesize that the presence of pre-steady state also when betaine is perfused at high concentrations could be related to fact that the betaine traps sodium ions and requires a third "interaction" to be properly transported as in BGT-1 (Matskevitch, Wagner et al. 1999). The hypothesis is further supported by the Q-V relationship for betaine associated transients in GAT1, where the

curve is shifted to more positive voltage than for GABA without significant changes in Q_{max} (maximum moveable charge) or σ (slope factor of the curve) (see figure 7 C, 14 C and table 1). Similar shift in the $V_{0.5}$ (voltage where half of the charge is moved) occurs when the extracellular Na^+ concentration is changed (Mager, Kleinberger-Doron et al. 1996). Since no such changes have been made during our experimental conditions, this shift can be related to either an apparent change in Na^+ concentration near the transporter, or a decrease in Na^+ dissociation constant (Forlani, Bossi et al. 2001).

	ND98	Betaine 0.1mM	GABA 10μM	GABA 10μM+ betaine 0.1mM
τ_{max} (ms)	191.72±18.3	192.67±18.5	137.01±14.2	187.83±30.8
Q_{max} (nC)	38.14±1.0	43.59±2.3	25.74±3.3	30.29±2.1
$V_{0.5}$ (mV)	-32.86±1.1	-27.16±2.6	-43.19±3.4	-39.18±2.5
σ (mV)	20.36±0.7	23.49±1.6	25.21±3.5	21.81±1.3

Table 1: The maximum value of decay time constant (τ_{max}) and the fitting parameters from Q-V relationship in rGAT1 obtained using the Boltzmann distribution equation. Q_{max} is the maximum moveable charge, $V_{0.5}$ is the voltage where half of the charge can be moved, and σ is the slope factor of the sigmoidal curve. Data are means (SEM) of 5-7 oocytes from 1-3 batches.

The GABA-betaine competitive assay experiments clearly showed that their relationship is heavily concentration dependent (see figure 12). At high GABA concentrations, the secondary substrate betaine does not show any impact on the transport by GAT1. Whereas, given that the extracellular GABA concentration is lower than its $K_{0.5}$ (~15μM), betaine at lower concentrations can effectively inhibit GABA transport by GAT1. This selective inhibition of GABA transport by betaine could be associated with the slowing down of GAT1 transport rate in the presence of betaine (see figure 14 C D). In the presence of low extracellular betaine, both inrate and outrate of GABA reduced starkly (see table 2). As the time required for betaine to be moved inside the cell is larger than GABA, the betaine

bound to the GAT1 vestibule will not allow transport of GABA (when $<K_{0.5,GABA}$).

	Inrate β (s^{-1})		Outrate α (s^{-1})	
Voltage (mV)	**GABA 10μM**	**GABA 10μM+ betaine 0.1mM**	**GABA 10μM**	**GABA 10μM+ betaine 0.1mM**
-120	17.78±5.9	13.11±3.3	-0.30±0.2	-0.16±0.1
-100	12.33±3.1	10.12±2.4	1.22±0.4	0.61±0.1
-80	8.12±1.5	7.34±1.5	2.61±0.6	1.21±0.1
-60	4.82±1.1	4.55±0.8	3.62±0.9	1.86±0.1
-40	2.28±1.5	2.56±0.4	5.18±0.7	3.03±0.4
-20	0.66±0.1	1.17±0.1	7.08±0.8	4.40±0.5
0	0.31±0.5	0.64±0.1	10.32±0.7	6.70±0.6
20	-0.41±0.5	0.01±0.1	12.63±0.6	8.84±0.7

Table 2: Unidirectional rate constants outrate α (s^{-1}) and inrate β (s^{-1}) for voltages -120mV to +20mV. Data are means (SEM) of 5-7 oocytes from 1-3 batches.

Thus, the low extracellular betaine concentration causes a large number of transporters to fail in completing their cycle, allowing the binding-unbinding of Na^+ or directly trapping the Na^+ ions before the confirmational change is triggered. This behaviour indicates that at low concentration betaine can modulate the uptake of extracellular GABA by GAT1, given that GABA is present at concentration near its $K_{0.5}$. When the concentration of GABA increases, betaine is replaced by GABA in the vestibule and the transporters can function properly. While at high concentrations ($>K_{0.5,\ betaine}$) in the same condition, betaine itself behaves like a regular secondary substrate as the higher chemical gradient allows its slower intracellular translocation by GAT1. This inhibitory role of betaine for low GABA concentrations was also confirmed by LCMS-MS technique, where the uptake of extracellular GABA, when $<K_{0.5,GABA}$, was significantly blocked by betaine 0.1mM (see figure 13 C). Such a selective inhibition of GABA reuptake will allow the CNS to maintain the excitatory/inhibitory (E/I) balance in particular in critical conditions and preserving a healthy environment for the neurons. This could be one of the molecular

explanation of the betaine supplementation has shown beneficiary results in E/I balance related neurological diseases.

Recently it was shown Hardege et al that the *Caenorhabditis elegans* nervous system can synthesize betaine, specifically in the interneurons, and use it as a modulator of different behavioural states (Hardege, Morud et al. 2022). They also showed that the vesicular transporter CAT-1 in the interneurons could transport betaine, suggesting nematode neurons can load betaine in the synaptic vesicles. Also, the work from Kunisawa et al showed that the lack of betaine in rats could affect the GABAergic transmission and memory formations. They also showed that these effects of betaine are regulated not solely by BGT-1 and mediated in part by modulating GABAergic system (Kunisawa, Kido et al. 2017). Knight et al showed that hippocampal neurons can uptake and accumulate betaine up to four times extracellular in time-, dose-, and osmolality dependent manner (Knight, Piibe et al. 2017). In hippocampal slices under isometric conditions, the betaine uptake also significantly influenced the uptake of other osmolytes taurine, creatine, and myo-inositol. Whereas under hyperosmotic conditions, betaine uptake was preferred over the uptake of glycine and glutamine, suggesting a modulatory role of betaine in the inhibitory neurotransmission. These results along with our own, strongly suggest that the betaine should have a specific role in the CNS (possibly a neuromodulator) apart from being an osmoregulator and a methyl donor.

Lastly, as we have shown here that betaine is a substrate of BGT-1 and GAT1 both, it is natural to raise the question whether other two GATs could also interact with betaine. We performed electrophysiological experiments on *X. laevis* oocytes heterologously expressing hGAT2 and hGAT3 and observed the inward transport current induced by betaine 10mM (see figure 15). This preliminary result warrants a detailed investigation of the

betaine uptake by GAT2 and GAT3, in particular regarding the capability of blocking the GABA reuptake.

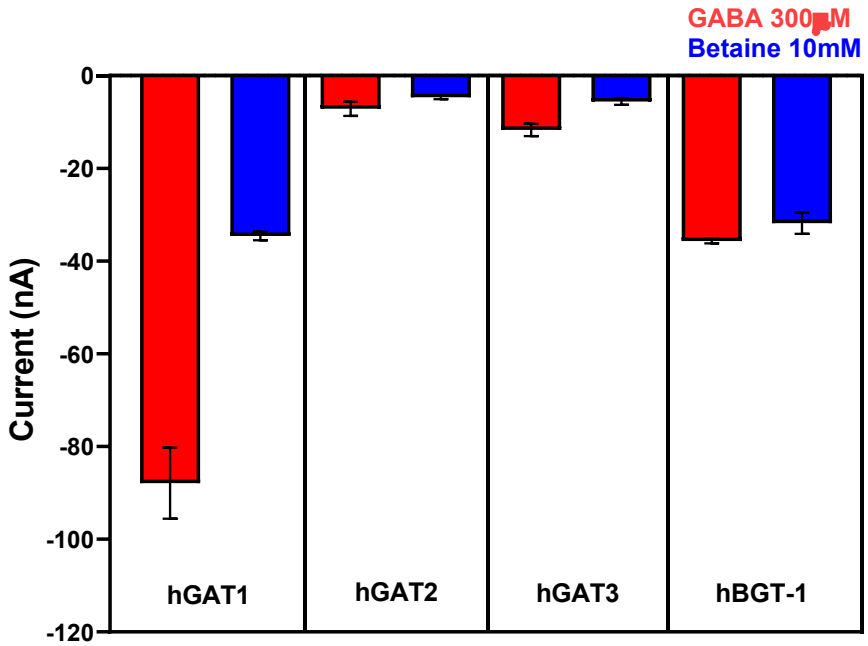


Figure 15 Interaction of betaine with GATs. The X. laevis oocytes respectively expressing hGAT1, hGAT2, hGAT3, and hBGT-1 were perfused with GABA 300μM and betaine 10mM at holding potential V_h=-60mV. Both GABA 300μM and betaine 10mM induced inward transport current in all GATs. Data are means (SEM) of 3-7 oocytes from 1-3 batches.

Conclusion

Betaine, an excellent natural osmolyte, is an effective therapeutic in diseases related to not only liver and kidney, but also neurodegenerative and neurological diseases. The role of betaine in the brain and the cellular mechanisms behind any neuronal interactions has remained a mystery. The current literature has attributed the beneficial effects of betaine supplementation in neuronal diseases to its osmotic characteristics that reduced oxidative stress and ability to get rid of the toxic Hcy (Bhatt, Di Iacovo et al. 2023). In order to do so, betaine has to enter the neurons and our results suggest GAT1 to be a possible transport mechanism responsible for that.

The findings of this research work also suggest a new and very important action of betaine: its capability of selectively blocking GABA reuptake by GAT1, given there is low extracellular GABA concentration. Betaine, as a secondary substrate, provokes such a modulation of GABA uptake by slowing down the transport rate of GAT1. This characteristic allows GAT1 to be blocked by low betaine concentrations, reducing the uptake of extracellular GABA when the GABA concentration is $<K_{0.5,GABA}$. This characteristic of betaine will enable the increase of GABA in the neuronal environment resulting in the modulation of the E/I balance in the CNS.

In summary, our findings highlight that betaine can interact with GABAergic pathways beyond BGT-1 and can modulate GABA concentration in the CNS via GAT1.

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