Research Description.

My laboratory is involved in a research program on the structural and mechanistic studies of integral membrane transporters in diverse niches of neurotransmitter transport and multi-drug efflux. Integral membrane transporters comprise a large set of specialized proteins capable of transporting polar substrates across the hydrophobic bilayer with the aid of ion-gradients or ATP hydrolysis and are extensively employed by prokaryotic and eukaryotic cells to transport diverse substrates and toxins including sugars, aminoacids, ions, metabolites, neurotransmitters and antibiotics. Most transporters are also vital drug targets that can be blocked to control metabolite entry or efflux across cell membranes and therefore affect disease outcomes(Cesar-Razquin et al., 2015). Over the last eight years, my lab has worked towards achieving our research goals through a three-pronged approach to

- **Delve into fundamental mechanisms of ion-coupled transport**.
- **Explore pharmacology of transport inhibition** for deciphering antidepressant /antinociceptive mechanisms
- **Develop novel technologies** to effectively use for biomedical applications.

Using the above as guiding principles and a highly interdisciplinary approach, we performed the following research to answer questions in the specialized niches of **I. Neurotransmitter transport and release** and **II. Multi-drug efflux** (Fig. 1), with substantial implications particularly for drug/pharmacological research. For the Sun Pharma research award, the studies under focus would the research on noradrenaline and GABA uptake that we performed in the recent years.

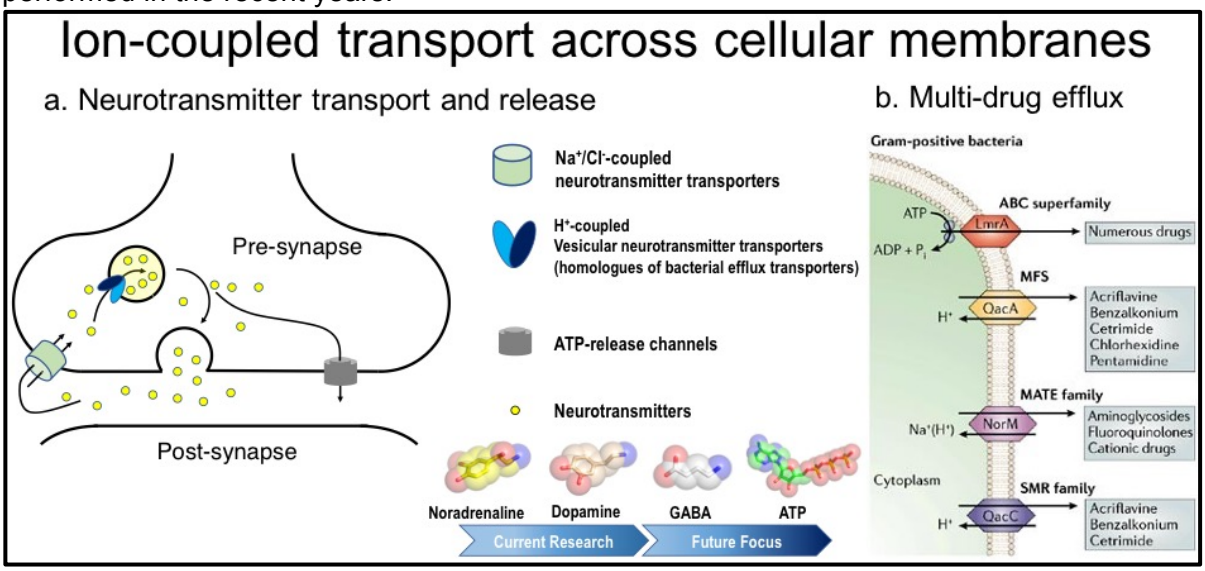

Figure 1. Ion-coupled transport research in my lab is focused on a, neurotransmitter transport and release and b, multi-drug efflux.

Neurotransmitter transport at the neural synapse.

A. Norepinephrine recognition and transport inhibition. Communication between neurons happens at junctions referred to as synapses where neurotransmitters are released into the neural synapse in response to the action potential driven Ca^{2+}-channel activation(Jessell and Kandel, 1993). The released neurotransmitters activate ligand-gated ion channels and G-protein coupled receptors to propagate the post-synaptic potential/signals induced by neurotransmitter release. Spatiotemporal control of neurotransmitter levels is enforced by Na^{+}/Cl^{-}-coupled transporters for a majority of neurotransmitters including dopamine (DA), serotonin (5-HT), noradrenaline/norepinephrine (NE), glycine and γ-aminobutyric acid (GABA)(Iversen, 2006). In the past few years, we have worked to decipher the mechanism of noradrenaline recognition using the *Drosophila* dopamine transporter (dDAT) as a surrogate for the human norepinephrine/noradrenaline transporter (hNET)(Pidathala et al., 2021). We established baculoviral mammalian cell expression system and optimized insect cell expression of antibody fragments to generate

the antibody to crystallize and study neurotransmitter transporters(Goehring et al., 2014). X-ray structures of the dDAT bound to a synthetic antibody fragment allowed us to decipher the distinct conformation in which norepinephrine interacts with the transporter in comparison to dopamine. We have also elucidated the structure of dDAT in complex with multiple inhibitors of chronic pain and fibromyalgia including duloxetine (Cymbalta), milnacipran (Savella) and tramadol (Ultracet) (Fig. 2). These inhibitors block noradrenaline uptake in the dorsal horn of the spinal cord that facilitates inhibition of descending pain pathways to suppress chronic pain. Tramadol in particular acts as an agonist of μ-opioid receptors, which allows it to provide pain relief by a dual mechanism. It is also a drug of abuse when consumed in high doses.

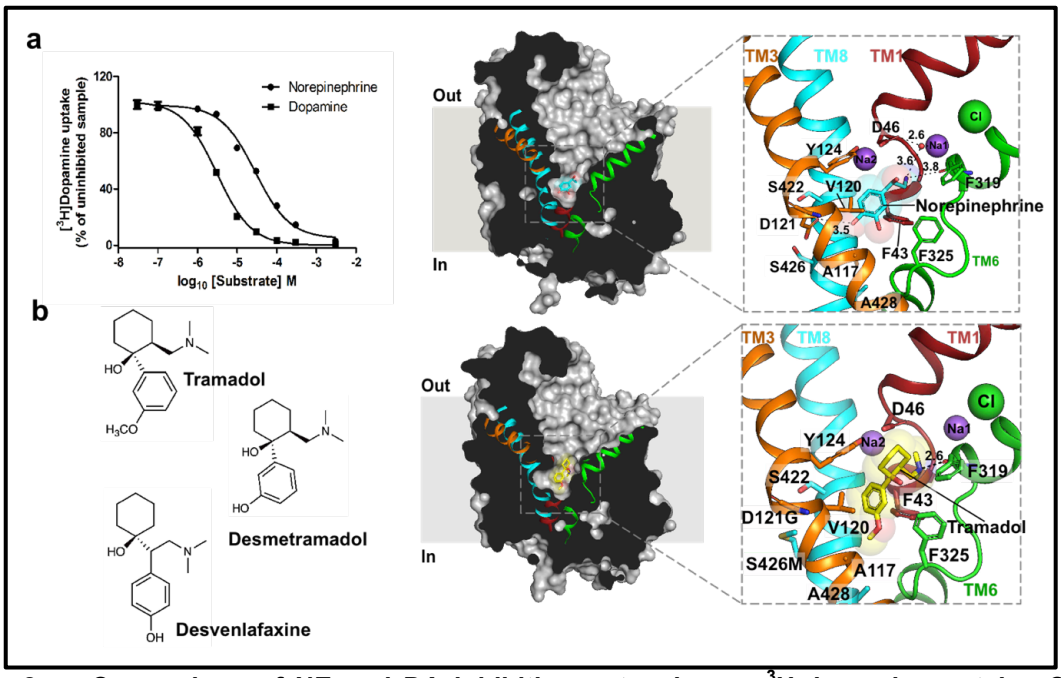

Figure 2. a, Comparison of NE and DA inhibition potencies on ^{3}H-dopamine uptake. Cross-section of the transporter displays norepinephrine bound within the substrate binding pocket. b, Tramadol and its closely related analogues that inhibit catecholamine uptake. Cross section of binding pocket displays the interactions of tramadol in the vicinity of subsite C.

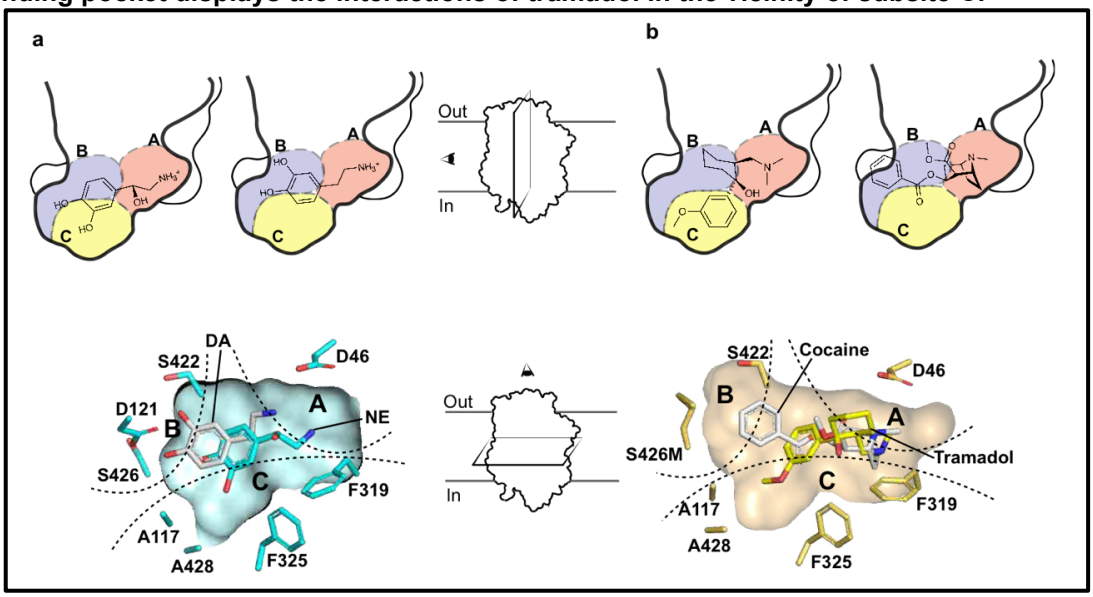

Figure 3. Norepinephrine and chronic pain inhibitor recognition. A, dotted line indicates the buried binding site. B, the binding pocket can be subdivided to A, B and C and displays indicate that the noradrenaline reuptake inhibitors specifically interact with subsite C.

The three inhibitors bind in the primary binding site and have overlapping interactions within the binding pocket particularly with regions that specifically interact with noradrenaline (Fig. 2, 3). The study represents the first atomic-resolution evidence of noradrenaline and its reuptake inhibitors interacting with its cognate transporter. The study also highlights the specific regions within the binding pocket that are responsible for governing specificity of reuptake inhibitors and affinity determinants for inhibitors that can interact with dopamine and norepinephrine transporters (Fig. 3).

Publication: Shabareesh, P., Mallela, A.K., Joseph, D. & Penmatsa, A. Structural basis of norepinephrine recognition and transport inhibition in neurotransmitter transporters. *Nat Commun.* 12, 2199 (2021).

B. Insights into GABA transport inhibition by antiepileptic drugs. GABA (γ-aminobutyric acid) is the predominant inhibitory neurotransmitter in the central nervous system. A decarboxylated form of L-glutamate, GABA release at synapses balances the excitatory and inhibitory neurotransmission at most pathways. Levels of synaptic GABA are controlled by four isoforms of the GABA transporter (GAT). GABA activates its downstream receptors $GABA_A$ and $GABA_B$ that elicit inhibitory post synaptic potentials and signaling cascades in the GABAergic pathways and interneurons. Controlling synaptic GABA inputs becomes vital in excitotoxic pathologies like epilepsies(Treiman, 2001). A proven strategy for GABA control is to inhibit GABA reuptake by GATs. Therefore, antiepileptic drugs like tiagabine and NO711 are known to inhibit GAT1 and enhance synaptic levels of GABA. Tiagabine and NO711 are structural analogues and differ significantly from antidepressants that block monoamine uptake despite being competitive inhibitors(Borden, 1996). In this study we resorted to engineer the binding site of GAT in a thermostabilized dDAT ($dDAT_{GAT}$) (Fig. 4). The structure of $dDAT_{GAT}$ reveals an altered binding site that is now capable of binding GAT1 inhibitors and displays remarkable differences in the organization of the binding pocket. Instead of subsites A, B, and C the binding pocket in GAT displays subsites A and C' (Fig.4). A widened gap is formed near the non-helical linker within TM6 in the GAT-like binding pocket in comparison to monoamine transporter. The results from the study highlight the specificity of anti-epileptic drugs against the GABA transporter and the unique pharmacology displayed by GAT1 in comparison to other neurotransmitter transporters(Joseph et al., 2022).

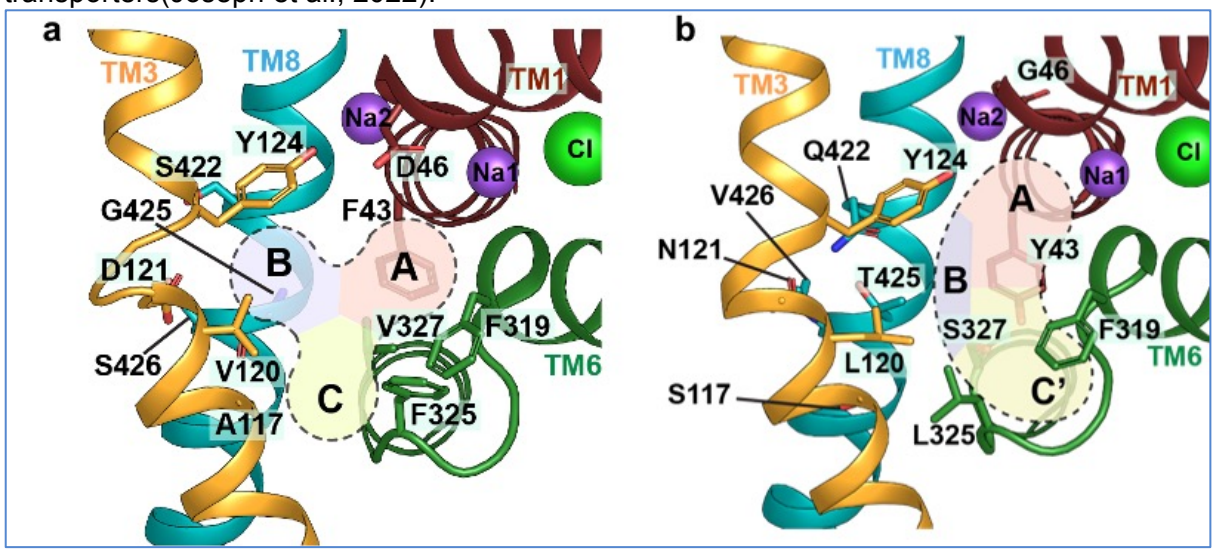

Figure 4: Comparison of subsite architectures in a, dDAT and b, $dDAT_{GAT}$ X-ray structures. The typical trilobed A, B, C subsite organization in monoamine transporters is disrupted in GATs to have a pronounced subsite C'.

Publication: Joseph, D., Nayak, S.R., & Penmatsa, A.* Structural insights into GABA transport inhibition using an engineered neurotransmitter transporter. *EMBO J.* (2022). 41:e110735.

C. CryoEM structure of GAT1 provides mechanistic insights into GABA reuptake machinery.

We decided to study the GABA transporter directly upon studying the GABA reuptake inhibitors using an engineered neurotransmitter transporter (described above). Among GATs the isoform GAT1 is the most prevalent and is the target of antiepileptic medications(Jurik et al., 2015). In order to study its structure we decided to transfer the epitope for a Fab from dopamine transporter to the GABA transporter. This allowed us to use an available antibody as a fiducial marker to image the transporter and reconstruct its structure at a high resolution using cryoEM despite the small size of the transporter. The structure of GAT1 revealed a substrate (GABA) bound and ion bound transporter in the cytosol facing state. This conformation is the prerelease state of the transporter and aids in understanding the mechanism of GABA uptake (Fig. 5)(Nayak et al., 2023). The strategy of epitope transfer can be applied to related transporters to study their structures and further studies on GAT1 would be focused on understanding the mechanism of inhibitor interactions in alternate conformations.

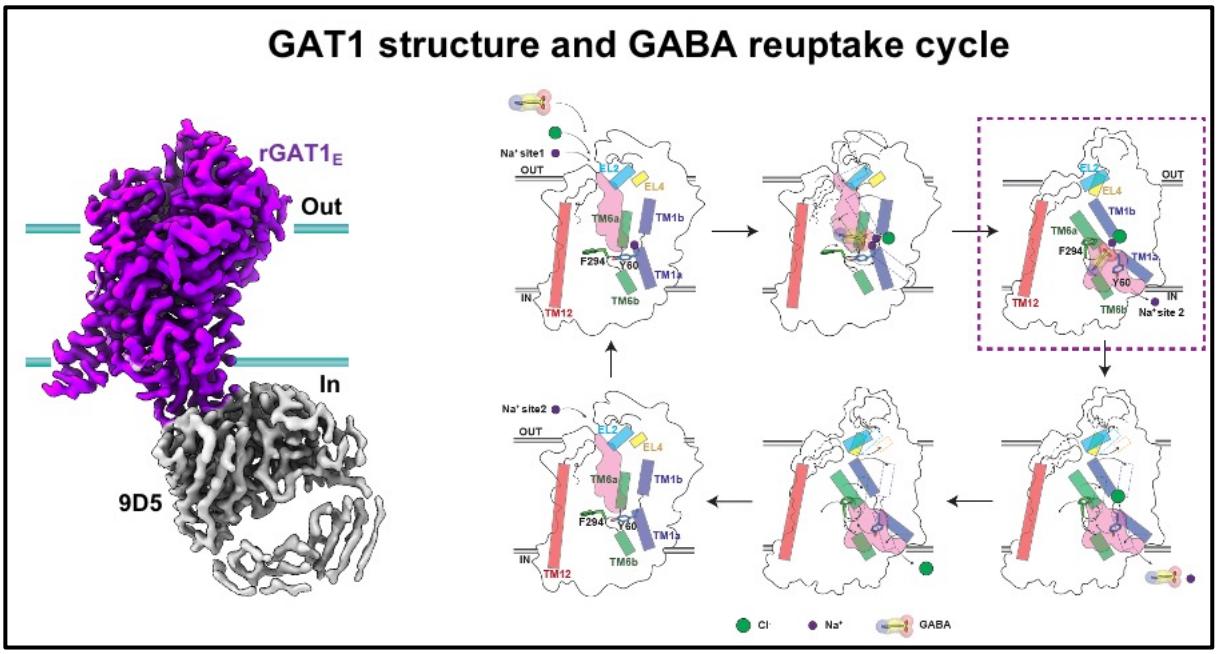

Figure 5. CryoEM structure of GAT1 done through epitope transfer of a Fab from dopamine to GABA transporter. The right panel depicts the reuptake cycle of GABA and the boxed step indiciates the intermediate step in which the current structure was obtained.

Publication. Nayak, S.R., Joesph, D., Hoefner, G., Dakua, A., Athreya, A., Wanner, K.T., Kanner, B.,& Penmatsa, A.* CryoEM structure of GABA transporter 1 reveals substrate recognition and transport mechanism. *Nat. Struct. Mol. Biol.* (2023). 30, 1023–1032.

Summary. The research accomplishments described above are the primary published studies being put forth for consideration for the Sun Pharma Research awards for 2023 in Pharmaceutical sciences. In addition to this part of the work our research also focuses on transporters involved in multi-drug efflux and developing single-domain camelid antibodies as tools to study membrane proteins. A majority of the research was performed in my laboratory through graduate students and postdocs. I further certify that the research projects described were not considered for awards elsewhere.

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Aravind Penmatsa, PhD
Associate Professor,
DBT/Wellcome Trust India Alliance Senior Fellow &
EMBO Global Investigator,
Molecular Biophysics Unit,
Indian Institute of Science,
Bangalore, 560012,
Karnataka, India.
Phone. +91-80-2293 2458; off: 3552
e.mail: penmatsa@iisc.ac.in
web: aplabmbu.weebly.com