4-phenylbutyrate rescue of SLC6A1 mutation-mediated disorders in patient derived cell and mouse models

Jing-Qiong (Katty) Kang, MD, PhD
Department of Neurology & Pharmacology
Vanderbilt Brain Institute
Vanderbilt University Kennedy Center of Human Development
Vanderbilt University Medical Center
Nashville, TN, 37232
2011
My VICTR award “4-phenylbutyrate as a novel treatment option for epilepsy”

Gene Liau
Terry Jo Bichell
Tom Davis
Amber Freed
Zachery Grinspan, then Scott, Kim....
The story about Maxwell
I got SLC6A1(S295L) mutation, let’s fight!!!
Mutations in genes directly or indirectly affecting GABAergic pathway and the associated epilepsy syndromes, autism and intellectual disability.

- **SLC6A1**
- IGE, CAE, JME, IAE, MAE, LGS, ID, autism
- *Gene will be studied*

**Table**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAE</td>
<td>Childhood absence epilepsy</td>
</tr>
<tr>
<td>IAE</td>
<td>Idiopathic absence epilepsy</td>
</tr>
<tr>
<td>ID</td>
<td>Intellectual disability</td>
</tr>
<tr>
<td>LGS</td>
<td>Lennox-Gastaut syndrome</td>
</tr>
<tr>
<td>JME</td>
<td>Juvenile myoclonic epilepsy</td>
</tr>
<tr>
<td>IAE</td>
<td>Idiopathic absence epilepsy</td>
</tr>
<tr>
<td>MAE</td>
<td>Myoclonic astatic epilepsy</td>
</tr>
</tbody>
</table>

**Figure 1**

- GABR, GABRA1,3,5,6, GABRB1,2,3, GABRG2, GABRD
- SCN, SCN1A, 2A, 3A, 8A, SCN1B
- STXBP1
- Others
- GRIN2A-B
- SLC12A5 (KCC2)
- SLC6A1 (GAT-1) *

~80 established epilepsy genes and ~1000 epilepsy associated genes
What does it mean if a child carries a mutation in \textit{SLC6A1}?

Loss of function?

Gain of function?

Altered function?
How do we study the impact of a mutation?

**Insights, tools and protocols from GABAA receptors**

We have studied the trafficking and function of mutant GAT-1 in HEK293T, iPSCs, mouse neurons and astrocytes.
We have extensively studied GABA<sub>A</sub> receptor subunits (~100 mutations)
Signal peptide to mature peptide, from transmembrane domain to intracellular loop

β3(P11S; S15F) CAE
β3(P11S) Autism (multiple Pedigrees)

γ2(Q40X) SMEI (Dravet syndrome)
γ2(R136X) GEFS+, Autism
γ2(R82Q) CAE & FS
γ2(R289M) FS, GEFS+
γ2(K289M) FS, GEFS+

α1(A322D) JME
α1(S326fs328X) CAE

δ(E177A) FS, GEFS+
δ(R220H, R220C) JME, GEFS+

Noncoding region mutations:
γ2(IVS6+2T G) CAE & FS
Mutations in β3 promotor region: CAE

γ2 (Q390X) FS, SMEI (Dravet syndrome)

GABA<sub>A</sub> receptor subunit gene mutations associated with epilepsies

In collaboration with Dr. Macdonald
R44Q#: epilepsy with myoclonic-atactic seizures
R44W: developmental disorder
L73F: epilepsy
G75R#*: generalized epilepsy, intellectual disability
Y140C#: epilepsy with myoclonic-atactic seizures, mild to moderate intellectual disability
S145F#: mild intellectual disability
L151RS*35#: intellectual disability
W193X#: epilepsy with myoclonic-atactic seizures
G232V#: epilepsy with myoclonic-atactic seizures, mild to moderate intellectual disability
G234S: Lennox-Gastaut Syndrome
A288V#: autism spectrum disorder
S295L: SLC6A1-related disorder, hypotonia
G297R#: epilepsy with myoclonic-atactic seizures
G299V: autism spectrum disorder
A305T: developmental disorder
A305V: myoclonic atonic epilepsy
A334P#: epilepsy with myoclonic-atactic seizures
A357V#: Rett-like syndrome
P361T: autism spectrum disorder, epilepsy with absence and atonic seizures
F385L#: epilepsy with myoclonic-atactic seizures, mild to moderate intellectual disability
G457Hfs*10#: epilepsy with myoclonic-atactic seizures
V511M#: Generalized epilepsy, mild intellectual disability
G550R#: autism spectrum disorder

Cai .. Kang Exp Neuro 2019
Wang .. Kang Mol Brain 2020
Poliquin .. Kang Exp Neuro 2021
Mermer .. Kang Brain 2021
Nwosu .. Kang Brain Comm 2022
Mermer .. Kang Neurobiology of Disease 2022
Most of the SLC6A1 mutations, if not all, are partial or complete loss of function.

- **R44Q**: epilepsy with myoclonic-atonic seizures
- **R44W**: developmental disorder
- **L73F**: epilepsy
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- **G550R**: autism spectrum disorder

**B** % ^3^H GABA uptake of wt (pmol/µg/min)

**C** % ^3^H GABA uptake of wt (pmol/µg/min)
What can cause the mutant transporters to lose function?

insights gained from collaboration with two experts from deep learning

Prof. Dong Xu
Chair/Distinguished Professor
Univ of Missouri

Dr. Juexin Wang
Assistant professor
Indiana Univ-Purdue Univ

Expert in Computer Science/
Bioinformatics/protein structural biology/
Single cell RNAseq/AlphaFold
AI tools predict the mutations (two MAE patients in a Chinese cohort) to have reduced protein stability

Machine learning tools
1. SDM
2. mCSM
3. DUET
4. Dynamut
5. INPS-MD(sequence only)
6. INPS-MD(with structure)
7. MAESTROweb

Mermer et al., 2022
Neurobiology of Disease
Machine learning and structure modeling suggest the GAT-1(V288) protein is more hydrophobic but less stable compared with the wildtype.

Wildtype GAT-1(A288)  mutant GAT-1(V288)

Mutation from Alanine to Valine at residue 288 adds a side chain isopropyl group, making the residue much more hydrophobic.
where are the mutant GAT-1 transporters?
Maxwell’s mutant protein is retained inside ER

**wildtype**

**mutant (S295L)**

**Live Astrocyte**

**Live neuron**
Impaired trafficking: the mutant transporters may interfere with the wildtype

Red = wildtype endogenous GAT-1
Green = recombinant GAT-1

wildtype
mutant
mutant

astrocytes
When will the cells start to generate the mutant protein? 

**human iPSC cells**

When will the cells start to generate the mutant protein? From conception on?

<table>
<thead>
<tr>
<th>overlay</th>
<th>oct4</th>
<th>GAT-1</th>
<th>TO-PRO-3</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="overlay" alt="Overlay Image" /></td>
<td><img src="oct4" alt="oct4 Image" /></td>
<td><img src="GAT-1" alt="GAT-1 Image" /></td>
<td><img src="TO-PRO-3" alt="TO-PRO-3 Image" /></td>
</tr>
</tbody>
</table>

A low level of GAT-1 was detected in human iPSCs
We then tested PBA effect in various cell models: Human iPSC derived NPC, neurons and astrocytes.
iPSC derived

Neurons

Enlarged

astrocytes
Maxwell’s neurons show reduced GAT-1 at synapse
Maxwell’s astrocytes show reduced and ER-retained GAT-1

A overlay corrected

GABA

GAT-1

TO-PRO-3

B Total GAT-1 fluorescence in human astrocytes

Raw values

Corrected patient

C live astrocytes

Corrected

Corrected

Maxwell

Maxwell

D Ratio of fluorescence periphery over center

1.50

1.25

1.00

0.75

0.50

0.25

0

0.00

0.25

0.50

0.75

1.00

1.25

1.50

wt S295L
High throughput flow cytometry identified that the mutant GAT-1 had reduced cell surface expression (Retained inside cells).
PBA restored GABA uptake in all 8 tested mutations
In “heterozygous” condition

mixed cDNAs
Patient condition
t wt mut

Ratio of $^3$H GABA uptake PBA vs DMSO
“heterozygous”
PBA 2mM
24 hrs.

wt   mut
966  711
E16* V125M A288V S295L G362R D410E L460R W495X

0.0 0.2 0.4 0.6 0.8 1.0 1.2 1.4 1.6
Ratio to DMSO

wt
In heterozygous patients, which allele is rescued by PBA?
The wildtype GAT-1 protein expression is upregulated across mutations.
PBA effect on mutant GAT-1 is mutation-dependent

Normalized protein IDVs of GAT-1 with PBA

Ratio to DMSO treated

DMSO=1

HA=wildtype allele

YFP(GFP)=mutant allele
PBA effect is allele-dependent
wildtype vs mutant allele

Good news for heterozygous patients that wildtype in all mutations is upregulated!!

Normalized protein IDVs of GAT-1 with PBA

- wt
- E16X
- G362R
- L460R
- W495X

Ratio to DMSO treated

DMSO=1

Kang unpublished
Ongoing work is testing PBA for 30 mutations. We differentiate iPSCs to NPCs to Astrocytes or to inhibitory neurons.
Two knockin mouse models

Focused on two representative mutations
A288V and S295L

Slc6a1+/A288V
Charlie
Partial loss of function

Slc6a1+/S295L
Maxwell
Complete loss of function
Robust GAT-1 expression in all major brain regions shown by $[^3\text{H}]$Tiagabine (5.2 nM) binding

Wildtype

High binding:
- Superior colliculus
- Substantia nigra
- Lateral hypothalamic area
- VP: ventral pallidum

Low binding: Striatum (caudate, putamen ventral striatum)
~50% lower [3H]Tiagabine binding (GAT-1 expression) in Slc6a1+/A288V Het vs. WT littermates

Data generated in collaboration with Roche by Michael Honer’s lab-Neuroscience and Rare Diseases, Roche Pharma Research and Early Development
50% lower [3H]Tiagabine binding (GAT-1 expression) in Slc6a1+/S295L Het vs. WT littermates

Data generated in collaboration with Roche by Michael Honer’s lab-Neuroscience and Rare Diseases, Roche Pharma Research and Early Development
Both mouse models display absence seizures

\( \text{Slc6a1}^{+/A288V} \)
Baseline  \textit{Slc6a1^{+/S295L}}

A

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure_a}
\caption{Baseline and \textit{Slc6a1^{+/S295L}} audiogenic seizure activity over time.}
\end{figure}

B

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure_b}
\caption{\textit{Slc6a1^{+/S295L}} audiogenic seizure activity over time.}
\end{figure}
GAT-1 is abundantly expressed in all major brain regions and is reduced globally in both mouse models

<table>
<thead>
<tr>
<th>A</th>
<th>wt</th>
<th>SLC6A1(^{+/S295L})</th>
<th>SLC6A1(^{+/A288V})</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cor</td>
<td>cb</td>
<td>hip</td>
<td>thal</td>
</tr>
<tr>
<td>GAT-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATPase</td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

C

Protein IDVs normalized to wildtype cortex

D

Protein IDVs normalized to the wildtype cortex
PBA increased GAT-1 expression in the cortex, hippocampus and thalamus in both mouse models.

**GAT-1**

**ATPase**

**Protein IDVs normalized to vehicle treated**

**Ratio to vehicle**

E  

F  

G  

H  

Slc6a1+/S295L  

Slc6a1+/A288V
PBA alone (acute treatment) reduced seizures (>70%) in the heterozygous mutant mice

Nwosu et al., 2022, Brain Commun
We have a large program on SLC6A1 mutations

Our goal: **Find a CURE!**

Provide proof-of-principle guidance for treatment development from pre-clinical patient derived cell (iPSCs) and mouse models
Partial or complete loss of function is common across SLC6A1 mutations regardless of clinical phenotypes.

The mutant GAT-1 is retained inside endoplasmic reticulum in both neurons and astrocytes.

PBA alone can restore GABA uptake and mitigate seizures.

PBA can be beneficial for most of patients if not all.

PBA can upregulate the function of the wildtype allele.

The effect of PBA on the mutant allele is mutation-dependent.
Acknowledgements

Everyone in Kang lab (previous trainees Felicia, Marshall)

SLC6A1 Connect

Many patients & parents

Industry partners: UCB, Roche, BioMarin
Taysha gene Therapies

Many physicians: Drs. Zachery Grinspan,
Kim Goodspeed,
Scott Demarest,
Robert Carson,
Inna Hughes
Emma Grace,
Many Chinese physicians

National Institute of Neurological Disorders and Stroke
Reducing the burden of neurological disease...
Do not forget the original intention

Bu wang chu xin

You all must work hard to find a better drug!
Welcome to Music City!