Cation Chloride Cotransporter Modulation of the Seizure Phenotype in Rat Cortical MEA

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1. Abstract

The Cation chloride cotransporters (CCCs) mediate neuronal intracellular chloride levels and are therefore involved in regulating inhibitory tone in CNS. Modulators of CCCs are also reported to affect $GABA_A$-R-induced inhibition. Mutations of CNS-specific subtypes KCC2 (extrudes Cl^-) and NKCC1 (pumps in Cl^-) are reported in epileptic disorders. The present study was conducted therefore to test if VU0240551 (KCC2 inhibitor) affected drug-induced seizure endpoints in our rat cortical phenotypic Multi Electrode Array (MEA) assay, and to correspondingly confirm the lack of direct action of VU0240551 on $GABA_A$-Rs using whole cell patch clamp. In CNS MEA assay, picrotoxin ($GABA_A$-R antagonist) application primarily altered the spontaneous electrical activity for network burst frequency, organization and synchrony metric endpoints. The study was conducted by culturing cryopreserved rat brain cortex cells (RCX-500, Lonza) on MEA plates (48-well Accuspot, Axion) for two weeks and treated either with DMSO (0.1%; n = 12 wells), VU0240551 (0.12-10 μM; n = 3 wells/concentration) or picrotoxin (3 μM, n = 6 wells) on Day 15. Thirteen endpoints (firing/burst rate and connectivity/organization/synchrony endpoints) were analyzed to compare the treatments. The activity of VU0240551 against respective controls in agonist/antagonist/PAM modes were examined on $hGABA_A$-Rs ($\alpha1\beta3\gamma2$) stably expressed in HEK293 cells using IonFlux automated patch clamp platform (n ≥ 3). In MEA assay, VU0240551 decreased both firing rate and synchrony (10 μM), thus showing anti-seizurogenic effects. When co-applied, VU0240551 mitigated picrotoxin-induced seizurogenic endpoints compared to picrotoxin application alone. In IonFlux assay, VU0240551 showed no activity in all three pharmacological modes on $hGABA_A$-Rs within the concentration range tested; (IC_{50} or EC_{50} > 100 μM). In conclusion, the data suggested that VU0240551 produced an anti-seizurogenic effect that is mediated via KCC2 inhibition. In general, $GABA_A$-R-mediated effects in a phenotypic seizure-liability MEA assay can be influenced by a mechanism involving cation chloride cotransporter modulation.

4. CNS MEA

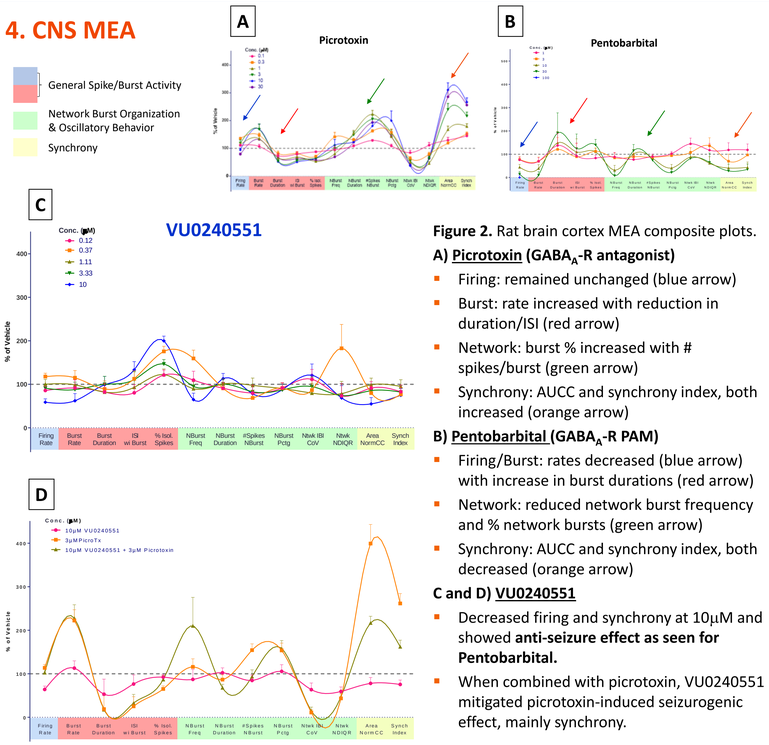

Figure 2. Rat brain cortex MEA composite plots.

A) Picrotoxin ($GABA_A$-R antagonist)

- Firing: remained unchanged (blue arrow)
- Burst: rate increased with reduction in duration/ISI (red arrow)
- Network: burst % increased with # spikes/burst (green arrow)
- Synchrony: AUCC and synchrony index, both increased (orange arrow)

B) Pentobarbital ($GABA_A$-R PAM)

- Firing/Burst: rates decreased (blue arrow) with increase in burst durations (red arrow)
- Network: reduced network burst frequency and % network bursts (green arrow)
- Synchrony: AUCC and synchrony index, both decreased (orange arrow)

C and D) VU0240551

- Decreased firing and synchrony at 10μM and showed **anti-seizure effect as seen for Pentobarbital.**
- When combined with picrotoxin, VU0240551 mitigated picrotoxin-induced seizurogenic effect, mainly synchrony.

2. Background

- Many clinically reported undesired effects are associated with CNS.
- These (Table 1) are among the effects least studied/predicted preclinically.
- MEA platform combined with primary cells and iPSC-derived neuron/astrocytes has been an evolving technique to address the Seizure liability.
- Voltage-gated Na^+, K^+ or Ca^{++} ion channels, ligand-gated NMDA and $GABA_A$ channels are not always enough to explain observed seizurogenic effects.
- Increasing evidence on Cation-Chloride Cotransporters (CCCs) suggest their role in seizurogenic activity.
- 7 out of 9 CCCs are plasmalemmal ion transporters: 2 NKCCs; 1 NCC and 4 KCCs.
- All CCCs, except for NKCC2 and NCC are expressed in specific cell type, brain region, developmental stages, or pathophysiological condition (epilepsy).
- **NKCC1**: two isoforms, a and b; a is expressed primarily in the brain; **pumps Chloride ion into the cell.**
- **KCC2**: two isoforms, a and b; expressed in the plasms membrane of somata and dendrites on pyramidal neurons and interneurons from the hippocampus and neocortex; **pumps Chloride ion out of the cell.**

Table 1. Major adverse effects associated with the clinical use of drugs

GI tract: Hepatitis and/or hepatocellular damage, Constipation, Diarrhea, Nausea and/or vomiting, Ulceration, Pancreatitis, Dry mouth	Pericarditis, Cardiomyopathy	Sexual disfunction, Gynecomastia, Addison syndrome, Galactorrhea
Hematology: Agranulocytosis, Hemolytic anemia, Pancytopenia, Thrombocytopenia, Megaloblastic anemia, Clotting and/or bleeding, Eosinophilia	**Metabolic:** Hyperglycemia, Hypoglycemia, Hyperkalemia, Hypokalemia, Metabolic acidosis, Hyperuricemia, Hyponatremia	**Neurological:** Seizures, Tremor, Sleep disorders, Peripheral neuropathy, Headache, Extrapyramidal effects
Dermatology: Erythemas, Hyperpigmentation, Photodermatitis, Eczema, Urticaria, Acne, Alopecia	**Respiratory:** Airway obstruction, Pulmonary infiltrates, Pulmonary edema, Respiratory depression, Nasal congestion	**Psychiatric:** Delirium, confusion, Depression, Hallucination, Drowsiness, Schizophrenic and/or paranoid reactions, Sleep disturbances
Cardiovascular: Arrhythmias, Hypotension, Hypertension, Congestive heart failure, Angina and/or chest pain	**Musculoskeletal:** Myalgia and/or myopathy, Rhabdomyolysis, Osteoporosis	**Ophthalmic:** Disturbed color vision, Cataract, Optic neuritis, Retinopathy, Glaucoma, Corneal opacity
	Renal: Nephritis, Nephrosis, Tubular necrosis, Renal dysfunction, Bladder dysfunction, Nephrolythiasis	**Otological:** Deafness, Vestibular disorders
	Endocrine: Thyroid dysfunction	

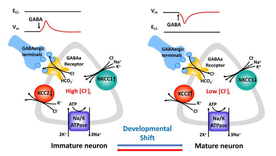

Figure 1. Chloride concentration regulatory mechanisms underlying $GABA_A$ receptor-mediated responses in immature and mature CNS neurons. The relative activity of NKCC1 and KCC2 and their contrasting effects on intracellular chloride determines the value of E^{Cl-} relative to the membrane potential (V_m) (Liu et al 2020).

5. IonFlux

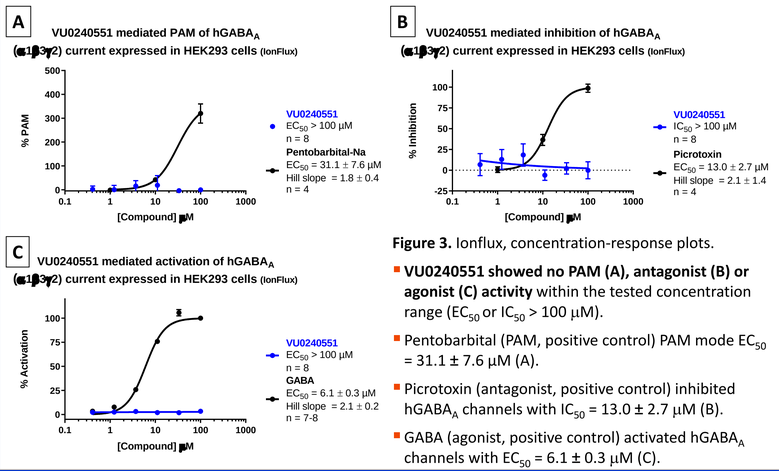

Figure 3. Ionflux, concentration-response plots.

- **VU0240551 showed no PAM (A), antagonist (B) or agonist (C) activity** within the tested concentration range (EC_{50} or IC_{50} > 100 μM).
- Pentobarbital (PAM, positive control) PAM mode EC_{50} = 31.1 ± 7.6 μM (A).
- Picrotoxin (antagonist, positive control) inhibited $hGABA_A$ channels with IC_{50} = 13.0 ± 2.7 μM (B).
- GABA (agonist, positive control) activated $hGABA_A$ channels with EC_{50} = 6.1 ± 0.3 μM (C).

3. Materials and Methods

Chemicals

Picrotoxin and γ-amino butyric acid (Sigma Aldrich); VU020551 (Tocris); Pentobarbital Na (Oak Pharmaceuticals). All chemicals were dissolved and serial diluted in 100% dimethyl sulfoxide (DMSO) at 1000x of the treatment concentration. Final dilutions were made in media (MEA) or assay buffer (IonFlux). Final assay concentrations: DMSO, 0.1%.; VU0240551 (0.12-10 μM, MEA and 0.41-100 μM, IonFlux); picrotoxin (3μM, MEA and 1-100 μM, IonFlux); Pentobarbital-Na (1-100 μM, MEA and IonFlux); GABA (0.41-100 μM, IonFlux).

CNS MEA:

- Rat cortical neurons (E18.5; QBM Biosciences).
- Maestro Multi-electrode Array System (Axion BioSystems; 48-well plate).
- Assay performed after cells are maintained 14-17 days in serum-free culture medium.
- 15 min recording of neuronal network activity taken immediately prior to and at 1 hr. post compound addition .
- Statistical analysis (t-test) on average % steady-state changes. Comparisons made for each test concentration normalized to separate vehicle control group. (n = 3-6 wells / treatment).

Endpoint Measures (Bradley et al 2018)

- Single-channel-level spike and burst activity parameters
- Network burst characteristics
- Synchrony indicators

IonFlux Automated Patch Clamp:

- HEK cell line stably expressing $hGABA_A$ ($\alpha1\beta3\gamma2$).
- 10 μM GABA (control GABA response, antagonist mode).
- 3 μM GABA (control, PAM mode).
- IonFlux platform optimized for ligand-gated ion channel assays.

Compound	MoA	IonFlux results
GABA	Agonist	EC_{50}=5.0μM
Muscimol	Agonist	EC_{50}=1.5μM
Isoguvacine	Agonist	EC_{50}=22.7μM
Gabazine	Antagonist	IC_{50}=0.6μM
Bicuculline	Antagonist	IC_{50}=1.8μM
Picrotoxin	Antagonist	IC_{50}=2.3μM
Etomidate	PAM	EC_{50}=2.4μM
Pentobarbital	PAM	EC_{50}=20.9μM

6. Summary and Conclusions

- In the rat brain cortex MEA assay, VU0240551 decreased firing and synchrony at 10μM and showed anti-seizure effect as seen for Pentobarbital.
- Picrotoxin ($GABA_A$-R antagonist) application primarily altered the spontaneous electrical activity for network burst frequency, organization and synchrony metric endpoints.
- When combined with picrotoxin, VU0240551 mitigated picrotoxin-induced seizurogenic effect, mainly the synchrony metric endpoints.
- In the IonFlux assays, VU0240551 showed no activity in agonist, PAM and antagonist modes within the tested concentration range (EC_{50} or IC_{50} > 100 μM).
- Combined together, the data suggested that VU0240551 produced anti-seizurogenic effect is mediated via KCC2 inhibition.

Acknowledgements

- Lindsey Talecki, Patricia Valentine, Shuya Wang and Tom Hudzik

References

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