

EAAT3 INVESTIGATED USING SSM-BASED ELECTROPHYSIOLOGY

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EAAT3

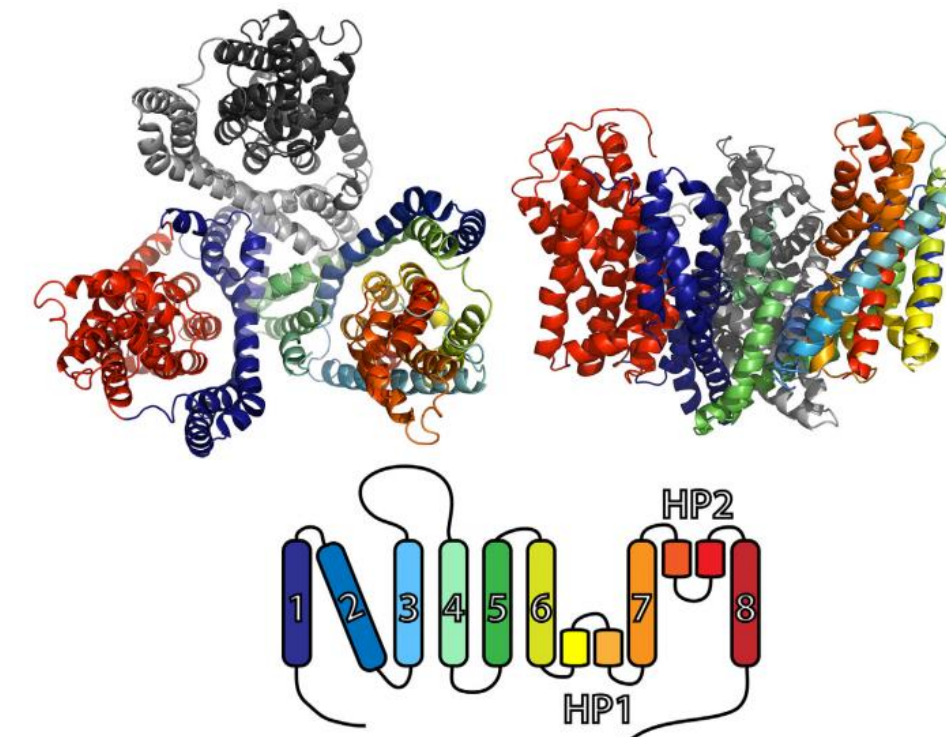
The excitatory amino acid transporter 3 (EAAT3, EAAC1) is an electrogenic secondary active cotransporter of the SLC-family (SLC1A1). Structure and function of this transporter are comparatively well characterized.

A crystal structure and other data from the glutamate transporter homologue GlTph revealed, that the protein is a homo trimer, with each monomer consisting of 7 TMDs and 2 hairpins. Driven by the concentration gradients of sodium and potassium it transports glutamate, aspartate and cysteine into the cell lumen. It is expressed throughout the CNS, but also in many organs such as intestines, kidney and liver, as well as in cardiac and skeletal muscle.

EAAT3 is involved in the neuronal re-uptake and the homeostasis of glutamate. It plays a central role in the regulation of excitatory neurotransmission and synaptic plasticity. It also transports cysteine, necessary for the synthesis of glutathione and GABA, and seems to provide the main pathway for aspartate in some tissues.

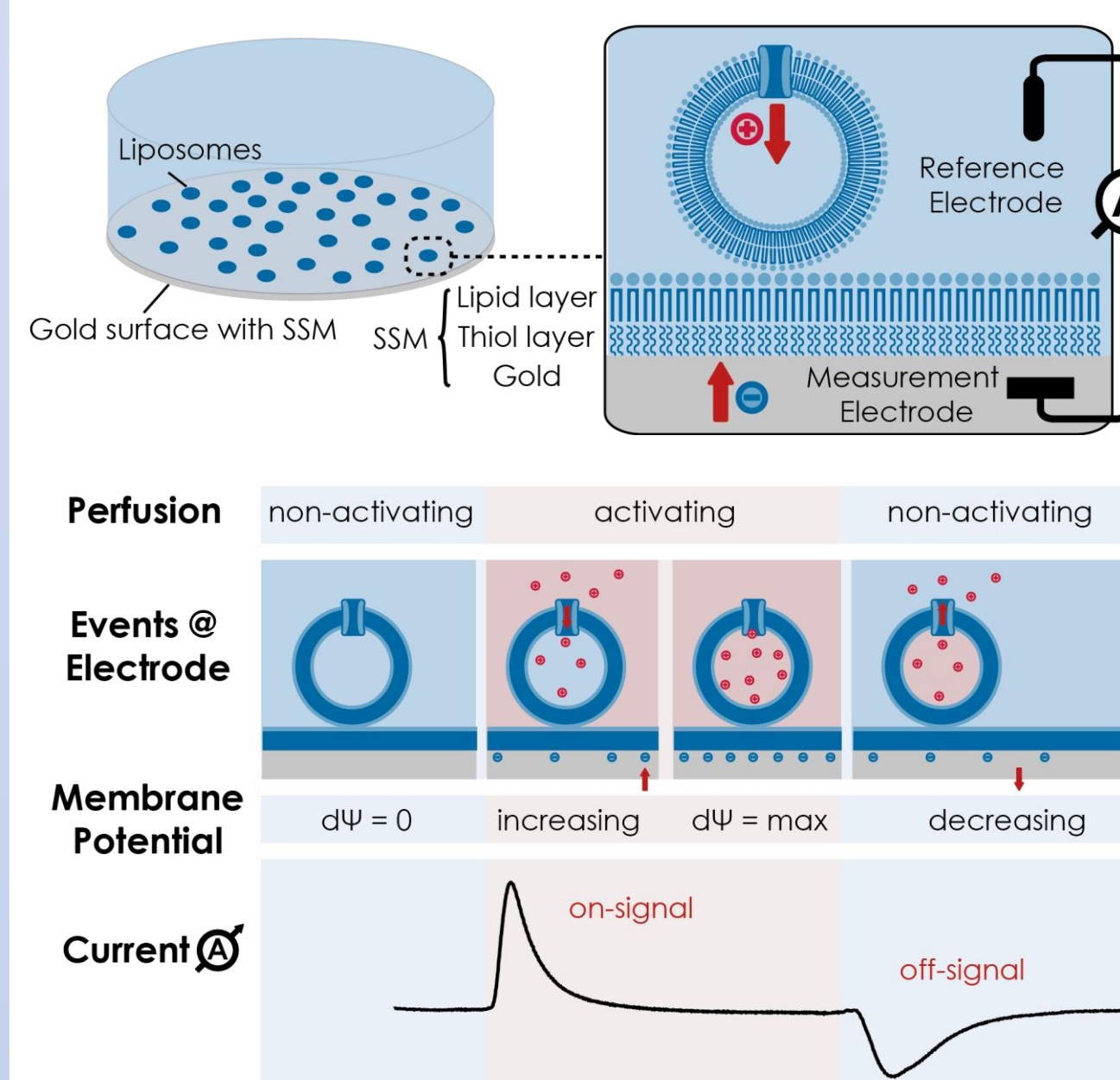
Linked to this physiological functions several connections of EAAT3 to severe neuronal disorders like Huntington's and Alzheimer's disease, epilepsy and schizophrenia have been described, as well as metabolic disturbances concerning the maintenance of aspartate and cysteine levels.

Given all this implications of EAAT3, pharmacological modulation of this transporter is of interest. The unusually detailed characterization of the physiological and biophysical properties qualify this protein as a potential drug target. However, no selective inhibitors of EAAT3 are known today. To facilitate the search for inhibitors, we developed an functional assay using SSM-based electrophysiology with high throughput and robustness.



The Importance of the Excitatory Amino Acid Transporter 3 (EAAT3). W Björn-Yoshimoto and S M Underhill. Neurochem. Int. 2016 Sep; 98: 4-18.

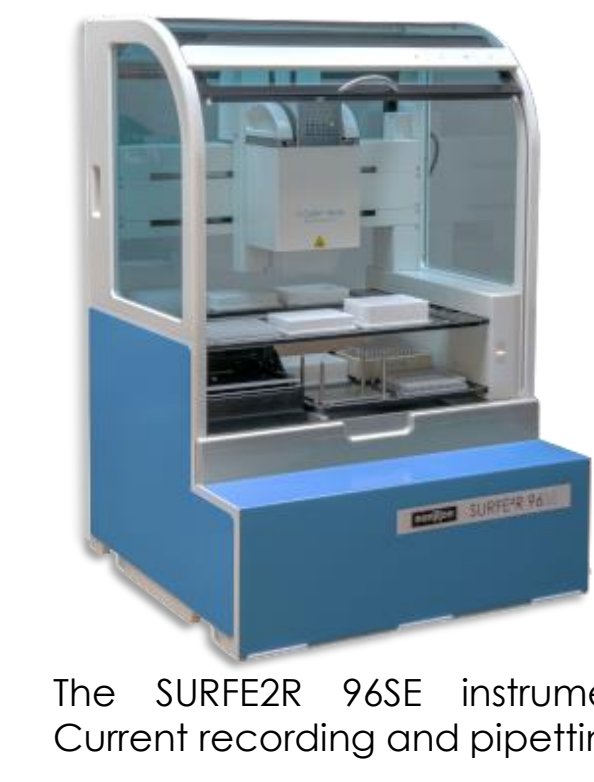
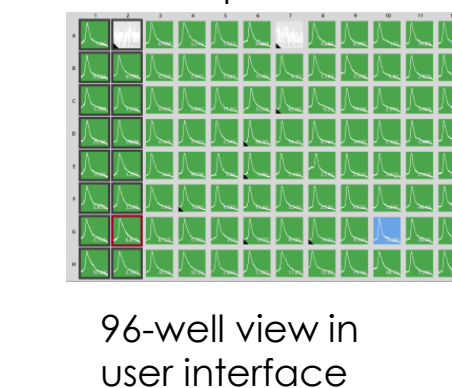
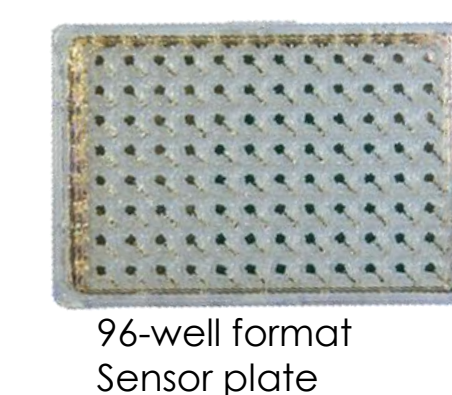
SSM-based electrophysiology



↑ The method in detail. Top: Structure of a SSM sensor. The 3 mm diameter, thin layer gold electrode is coated with a SSM consisting of a thiol and a lipid monolayer. On top liposomes or membrane vesicles containing the target protein are adsorbed. Bottom: Protocol of one activation cycle. The sensor is perfused with a passive non activating solution followed by an activating solution, containing a transported substrate or ATP. During this step a transient current occurs, since the active transporter moves charge into the vesicles what generates a membrane potential.

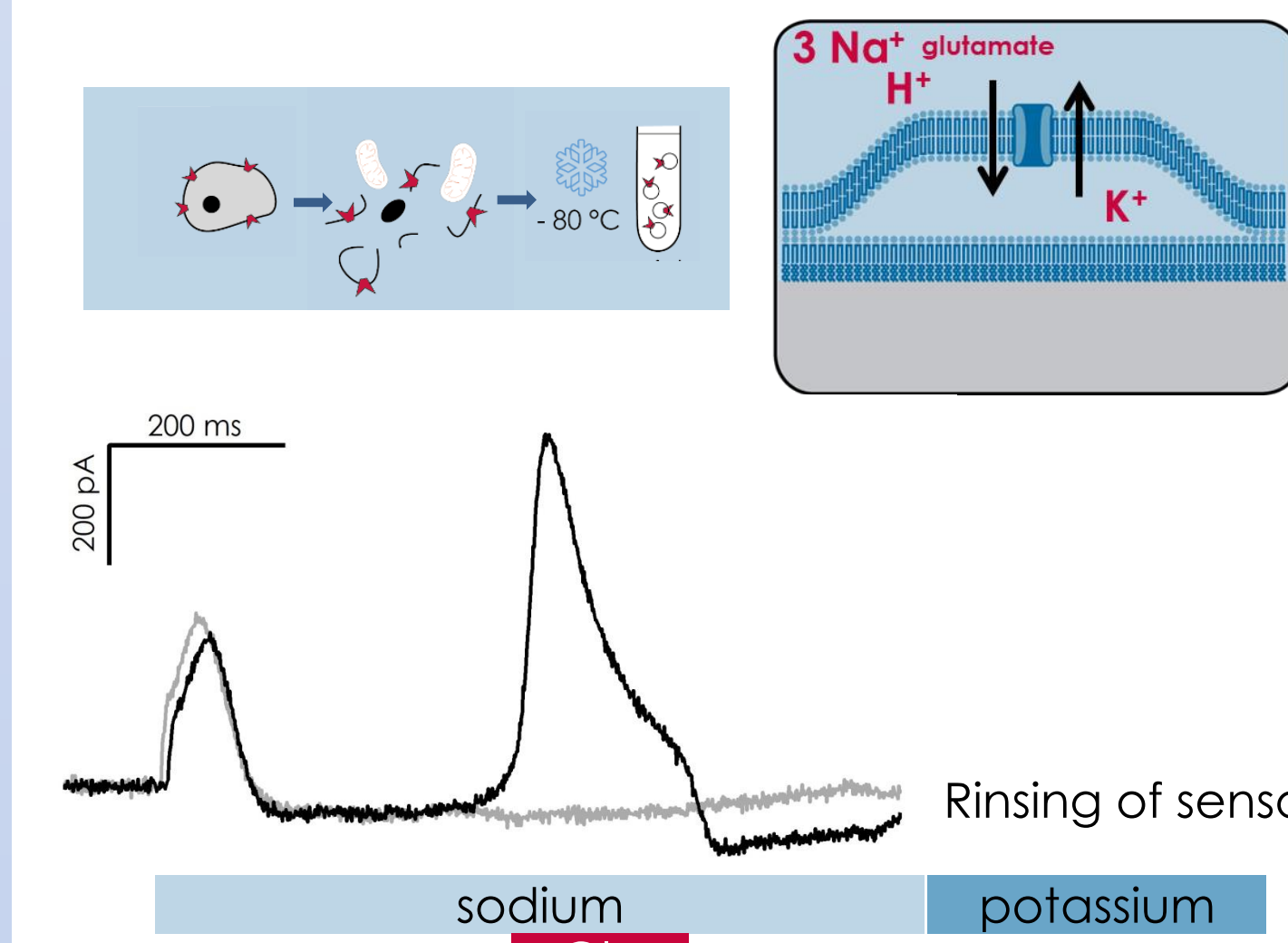
→ Up scaling: Right: The SURFE²R 96SE is a platform for automated recording of 96 SSM sensors in parallel. Upper left: 96 sensors in a well plate. Lower left: interface view of 96 measurements.

"Solid Supported Membrane (SSM) based electrophysiology" is a method, which allows to resolve low amplitude electric events in biological membranes. This is why it enables real-time activity measurement of electrogenic membrane proteins for which conventional electrophysiology is unsuitable, like many membrane transporters and pumps, or targets localized in intracellular or bacterial membranes. Unlike in cell-based electrophysiology a large sensor coated with purified membrane vesicles or proteoliposomes is used, leading to a high sum current signal. The method is established since the 90ies and was up scaled to 96-well format recently.



The SURFE²R 96SE instrument: Current recording and pipetting

Assay development



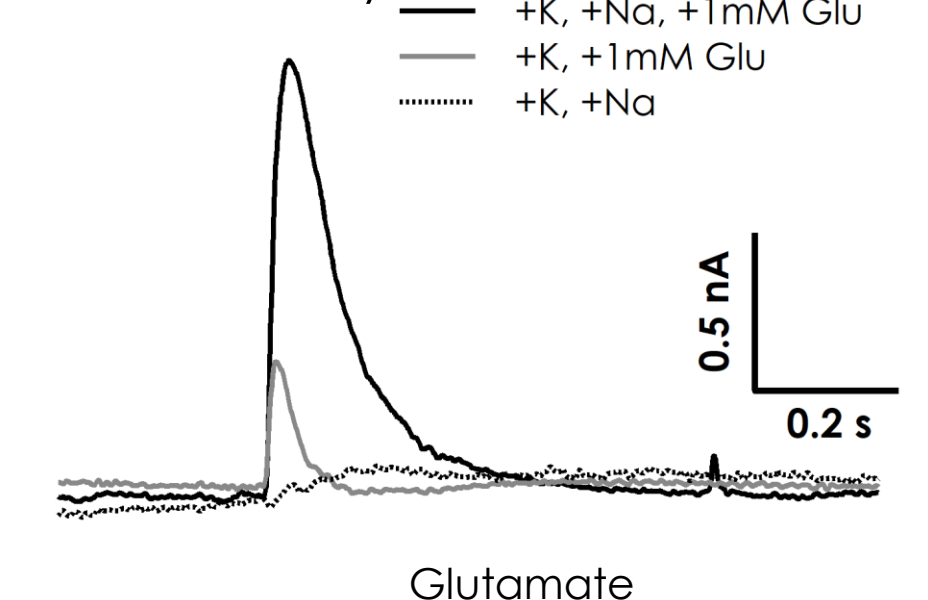
↑ Top left: EAAT3 is expressed in CHO cells and the membrane is purified and stored. Top right: The measurement principle: EAAT3 transports glutamate into the cells driven by the physiological gradients of sodium and potassium. To simulate this, the membrane vesicles were loaded with potassium. After immobilization of the vesicles on the SSM the external buffer was exchanged to a sodium based solution. Under these conditions glutamate was added to the vesicles triggering EAAT3 activity. Bottom: The resulting current response and the perfusion protocol.

→ Control experiment: If the vesicles are perfused with sodium a big transient current occurs, indicating the net inward current. Are the vesicles perfused with potassium, glutamate causes only a small peak indicating the binding of glutamate. In absence of glutamate no current signal occurs.

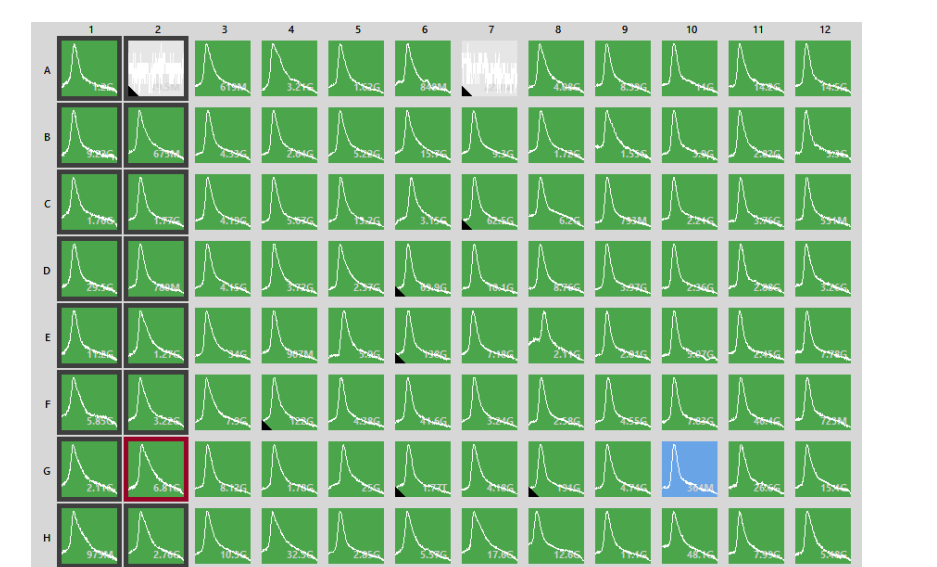
EAAT3 was overexpressed in CHO cells. The cells were grown, harvested, lysed and the plasma membrane isolated by partial centrifugation and a density gradient centrifugation. The resulting membrane vesicles were frozen.

At the day of the experiment an 10 µl aliquot of the membrane vesicles was unfrozen, diluted and adsorbed to a 96-well SSM-sensor. During this process the vesicles were loaded with potassium.

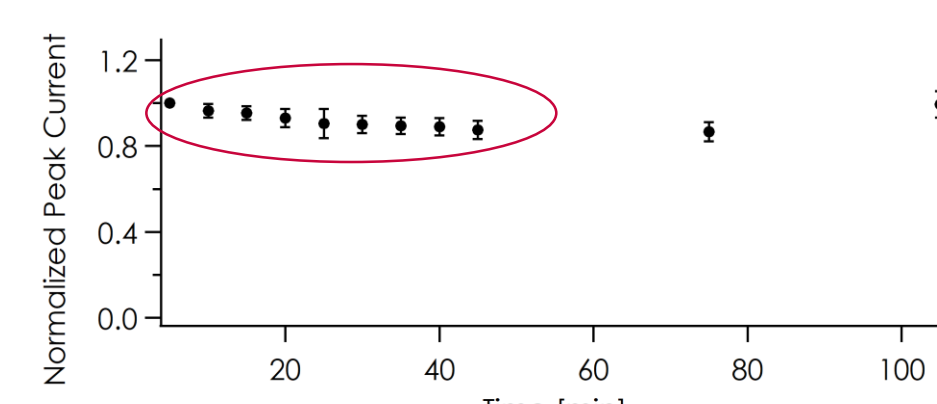
During the experiment the sensor is perfused with a sodium buffer, leading to an inward directing sodium gradient. When adding glutamate a transient current occurs. This current is caused by sodium and proton inward current while potassium moves out of the vesicles. The sensor is rinsed in potassium containing buffer to restore the ion gradients before the next activation cycle.



Validation



High sensor quality **Intermediate sensor quality**
Failed well (no contact, no signal or SSM not sufficient)
Representative view of one 96 well plate with current traces. The color coding indicates the quality of the sensor



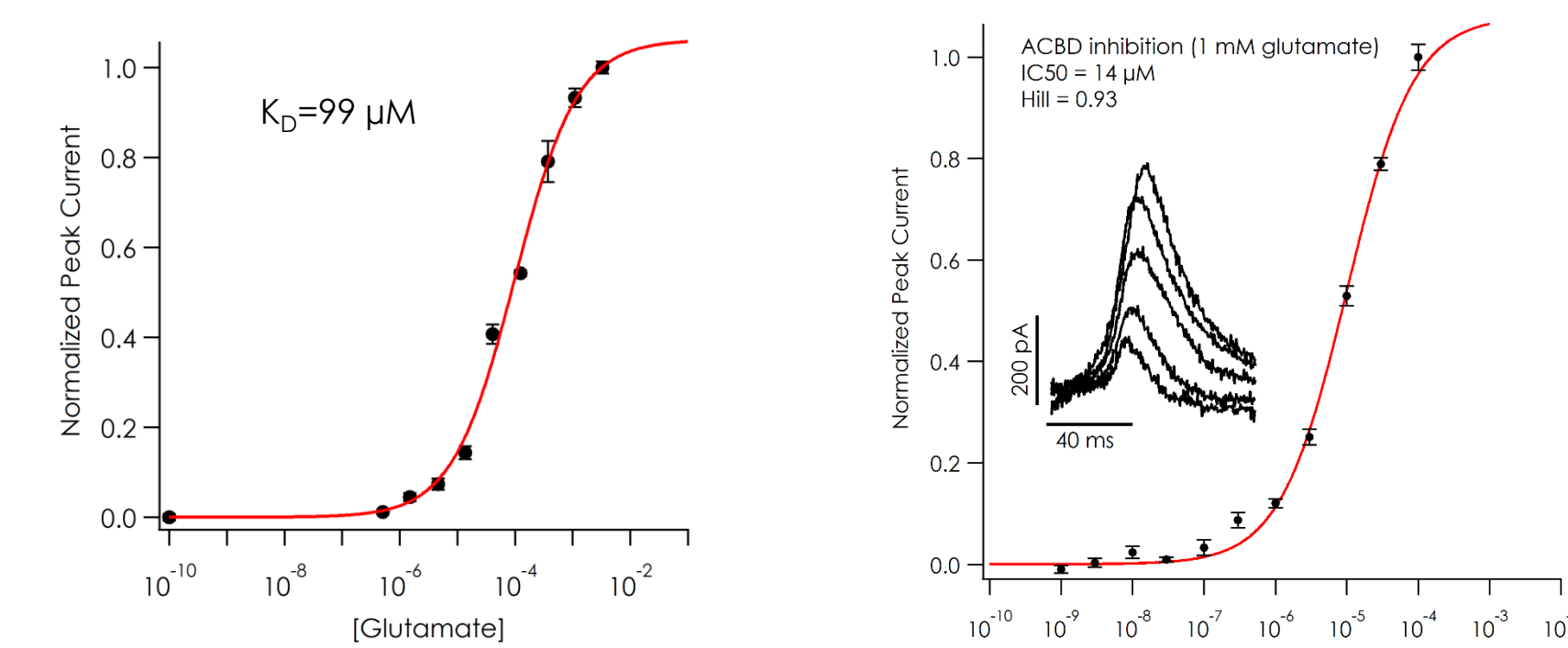
Signal stability: The activation cycle described above was repeated every 5 minutes for 45 minutes, followed by a reduced repetition frequency (30 min, later 2 hours). After 12 hours the sensor still generated a current response of the initial amplitude, demonstrating a remarkable stability of the assay setup. A typical inhibitor screening assay takes about 15-25 min.

Time course of the mean transporter peak current of 94 wells normalized to initial amplitude over 12 hours. The average assay time is marked in red. Errors indicate the standard deviation, N=94.

Success rate: The conductance, capacitance and current response in each well were analyzed. A conductance below 200 MΩ or a capacitance below 15 nF indicates a leaky SSM and the respective wells were rejected. This resulted in a total success rate of 97±3 % (mean value of 960 individual sensors).

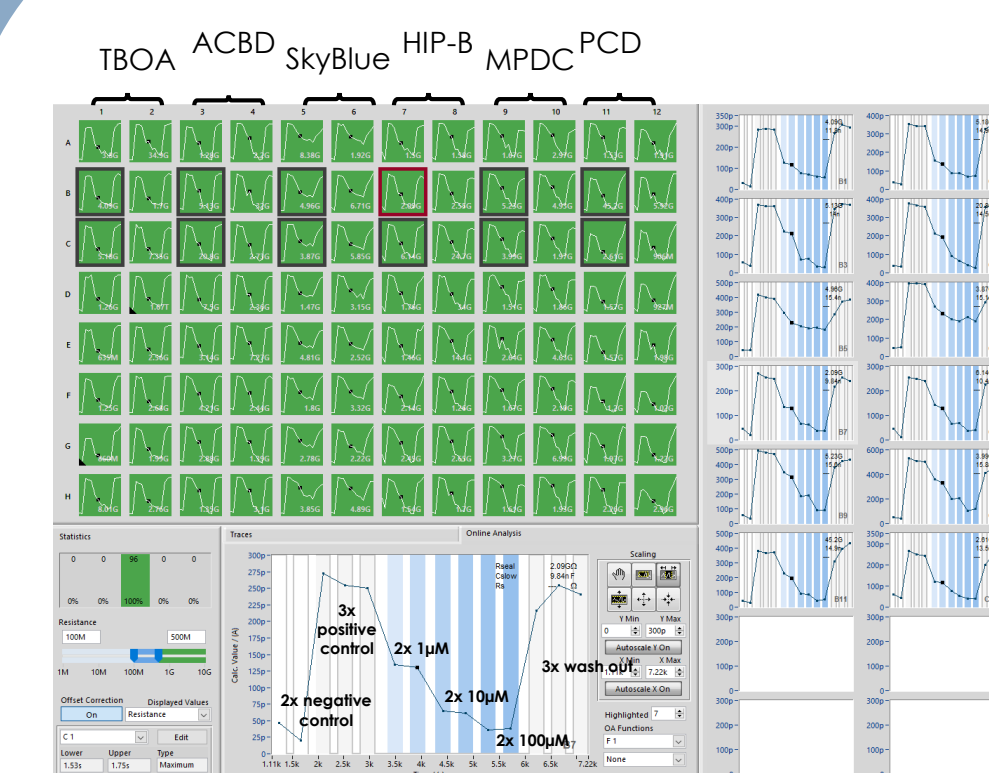
Comparison with other assay techniques:

EAAT3 has been investigated in the past using two-electrode voltage clamp with oocytes, patch clamp and uptake assays with radiolabeled substrates. To confirm validity of the SSM-based assay, IC₅₀ values of 6 compounds were determined and compared to published values. Although the inhibitor potency is influenced by the glutamate concentration and the assay conditions, the IC₅₀s correlate well.

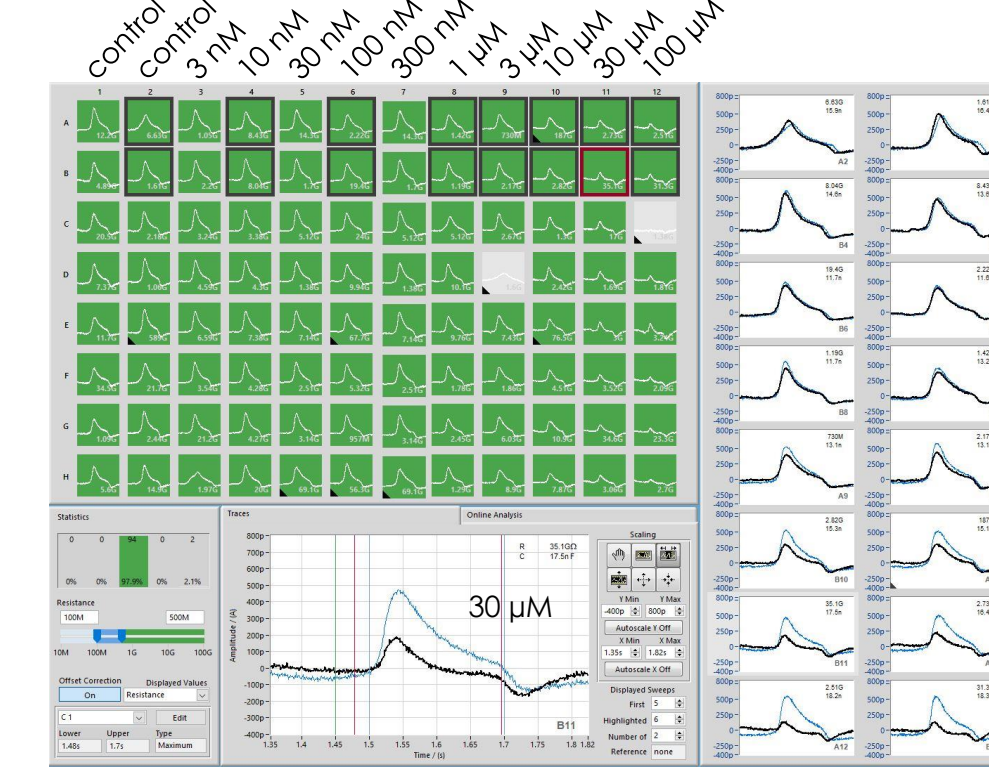


	IC ₅₀ SURFE ² R	IC ₅₀ Literature
HIP-B	1.18 µM	10 µM (Voltage Clamp)
MPDC	5.1 µM	5 µM (uptake, radio)
ACBD	14 µM	30 µM
Skyblue	8.5 µM	0.8 µM
PDC	0.92 µM	1.5 µM
L-TBOA	0.29 µM	2 µM

Assay modes



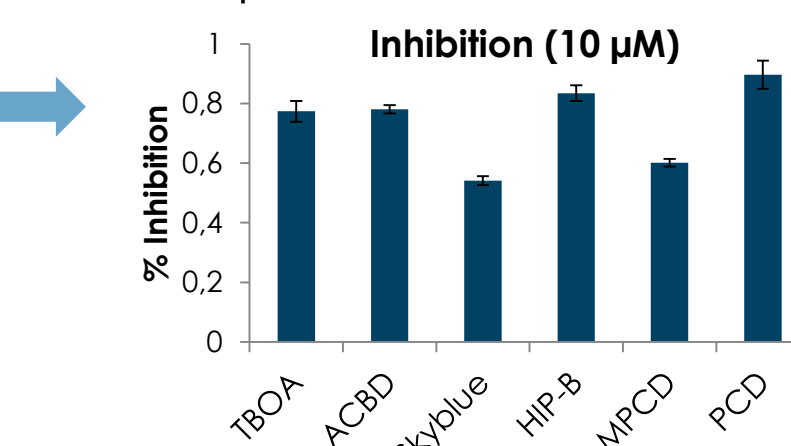
Example of an experiment setup to test several compounds in one plate. Here 16 wells are used per compound. The figure displays the user interface during the measurement. The points in the graphs reflect the peak amplitude during the time course of the experiment. One well is enlarged at the bottom and 12 wells at the right side.



Example of an experiment setup to determine an IC₅₀. 8 wells/one column is used per concentration. The figure displays the user interface during the measurement, showing the current response in each well and several enlarged views.

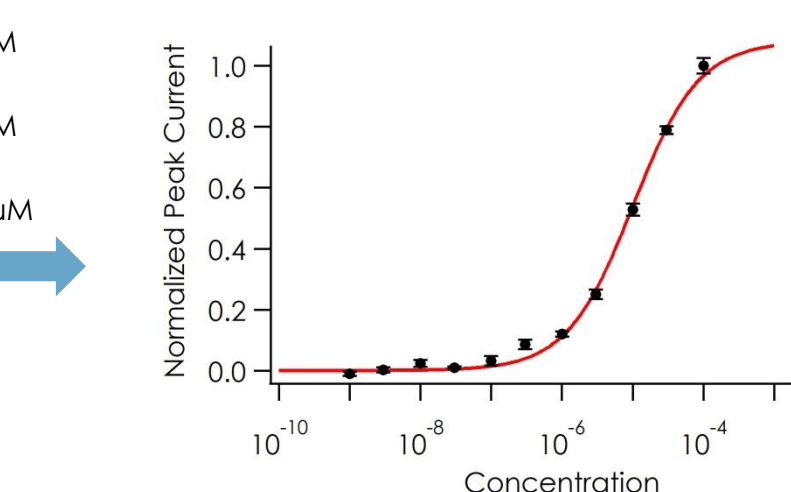
Screening mode:

A reduced number of concentrations is used, instead the number of compounds per plate is increased. It is possible to test up to 96 compounds, each corresponding to one well. In addition of the compound injection at least one negative control and two positive controls (full activation with glutamate) should be performed. This experiment takes 20 minutes.



Characterization mode:

A high number of concentrations of one or two compounds is tested to determine an IC₅₀ or EC₅₀. In the beginning a negative control and a full activation is performed. In total this experiment takes 15 minutes.



Conclusion

- We developed an assay to measure the activity of EAAT3 direct and in real time using SSM-based electrophysiology.
- We tested for the affinity of glutamate, determined IC₅₀s for several known inhibitors and compared them with values derived from other techniques (TEVC, uptake), which showed good correlation.
- The automated SSM-based assay has a throughput of about 2000 data points during an 8 hour day (negative and positive control excluded), which is sufficient for an secondary screening assay.
- The method has some relevant advantages: In contrast to other assaying techniques no running cell culture is required. The read-out techniques is direct and does not require radioactive labelling.
- We expect, that the developed EAAT3 assay can be transferred to other glutamate transporters without modification.

Literature:

The Importance of the Excitatory Amino Acid Transporter 3 (EAAT3). W Björn-Yoshimoto and S M Underhill. Neurochem. Int. 2016 Sep; 98: 4-18.
An automatic electrophysiological assay for the neuronal glutamate transporter mEAAC1. R Krause. Journal of Neuroscience Methods 177 (2009) 131-141
The substrate specificity of a neuronal glutamate transporter is determined by the nature of the coupling ion. D Menaker. Journal of Neurochemistry. 2006. 99. 20-28
Syntheses of optically pure beta-hydroxyaspartate derivatives as glutamate transporter blockers. K Shimamoto. Bioorg. Med. Chem. Lett., 10 (21): 2407-10.