**SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)**

# Astrocyte-mediated and direct neuronal neuromodulatory actions on murine and human brain slices

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# Introduction

## Astrocytes

Astrocytes are involved in many brain processes, such as metabolic support of neurons, supporting neuron survival, differentiation and maintenance of synaptogenesis, ion and water homeostasis, neurotransmitter secretion and excretion, as well as degradation and the blood-brain barrier. They are also involved in learning, memory, sleep and wakefulness, as well as neuroendocrine regulation. The processes of a single astrocyte are found in a certain area of the central nervous system which is known as astrocytic domain. Each domain contains approximately 300-600 dendrites and $10^5$ synapses, but in the case of human astrocytes these numbers can be orders of magnitude higher.

Regardless astrocytes are non-excitable cells, they can communicate with surrounding cells by calcium waves or oscillations. Calcium waves are not only intracellular but intercellular signals between cells, i.e. the calcium can spread from one cell to another. Besides producing calcium signals, they can release various neuroactive substances known as gliotransmitters (glutamate, ATP, tumor necrosis factor α (TNFα) and D-serine).

Astrocytes express both ionotropic and metabotropic glutamate receptors on their membranes, which are associated with the increase of the ic. $Ca^{2+}$ concentration. Among the ionotropic receptors, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and N-methyl-D-aspartate (NMDA) receptors are the most abundant, while the most

common subtype of metabotropic receptors is mGluR5, which releases calcium via the IP3 pathway.

Astrocytes not only ensure the uptake and recycling of glutamate released by neurons but can also release glutamate itself as a gliotransmitter. One of the main functions of astrocytes is to remove glutamate via transporters from the extracellular space diffused out of the synaptic cleft. To accomplish this, astrocytes express specific glutamate transporters in their plasma membrane. Among the five subtypes, the excitatory amino acid transporter type 1 (EAAT1) and the excitatory amino acid transporter type 2 (EAAT2) are the most important. The neuronal glutamate increases the intracellular concentration of calcium in astrocytes. In response to the calcium signal, the astrocyte can release glutamate itself via vesicular exocytosis, the cystine-glutamate antiporter, purinergic receptors or hemichannels, or by reverse transport through the glutamate transporter, opening anion channels due to cell swelling. The most common form of glutamate release is via vesicles.

**Glutamate receptors**

While the concentration of glutamate is almost as high as mol/l (~0.1 M) inside the vesicles, it diffuses into the synaptic cleft at only 6-7 mM. This further diffuses into the extracellular space and reduces the concentration of glutamate to about 0.18-2.12 μM (between nM and μM range depending on the measurement method).

*NMDA receptors*

NMDA receptors are ligand-gated, heteromeric ion channels which can be activated by glutamate, or D-serine and glycine as coactivators.

After ligand binding, the channel becomes permeable to sodium, potassium and calcium. Under physiological conditions, the ligand alone cannot open the pore, membrane depolarization is also required. NMDA receptors are blocked by $Mg^{2+}$ and a prerequisite for receptor activation is that this blockade is removed. The heteromeric channels are formed by GluN1, GluN2A-D, GluN3A-B subunits in di- or triheteromeric forms. A GluN1 subunit is always involved in the structure of the channels and is essential for the channel function. The GluN2A subunit is typically a component of NMDA receptors in synapses, whereas GluN2B is mostly located extrasynaptically. NMDA receptors are not uniformly distributed across the membrane, but in cluster. They are located along dendrites and in perisynaptic regions.

**Synaptic plasticity**

A decrease or increase in synaptic activity affects the strength of synaptic responses. These activity-dependent changes in synaptic transmission are called synaptic plasticity. Depending on the duration of the mechanism, short- and long-term plasticity can be distinguished. Short-term plasticity is a change from milliseconds to minutes, which may increase or decrease the probability of neurotransmitter release. Long-term plasticity can range from a change several hours to months or years.

*Long-term potentiation (LTP)*

In experiments, LTP can be triggered in several ways: by stimulation or chemically. Brief but high frequency stimulation (~100 Hz; HFS) increased the amplitude of synaptic potentials. Chemically, both

activation of mGluRs and NMDARs can induce LTP. The activation of presynaptic metabotropic glutamate receptors and presynaptic NMDA receptors by astrocytic glutamate release can alter the probability of neurotransmitter release for hours. In order to maintain potentiation for such a long time, postsynaptic and astrocytic stimulation is required in addition to presynaptic stimulation. While the presynaptic and postsynaptic cells are activated synchronously in time, the presynaptic cell releases glutamate, and the postsynaptic cell depolarizes, NMDA receptors are released from the magnesium blockade and open. The activation of NMDA receptors leads to an increase in intracellular $Ca^{2+}$ concentration in the postsynaptic cell, which triggers a signaling cascade. Additional AMPA receptors are located at the end of the cascade. The composition of NMDA receptors, the identity of the brain area and age also influence LTPs.

*Long-term depression (LTD)*

Long-term depression can be induced by low-frequency stimulation (LFS), where stimulation between 0.5 and 5 Hz for 5-15 minutes induces LTD. Like LTP, LTD can be induced chemically, requiring activation of mGluRs and/or NMDA receptors. Several theories have been put forward regarding the development of LTD. One theory is that activation of both mGluRs and NMDA receptors can induce LTD, while Fujii et al (2004) found that LTD induced by mGluRs can be suppressed by NMDA-dependent LTP, i.e. the identity of the receptor determines whether LTP or LTD is formed. Another hypothesis is that whether LTP or LTD is formed depends on the rate at which NMDA receptors are opened and

the magnitude of change in $Ca^{2+}$ concentration in the postsynaptic cell. If the calcium signal is small and prolonged, LTD develops, if it is large and transient, LTP is formed. The composition of NMDA receptors is also important for LTD: in young animals, the GluN2B subunit dominates.

*Spike timing-dependent plasticity*

McNaughton et al. (1978) were probably the first to recognize that the timing and order of pre- and postsynaptic action potentials (spikes) relative to each other can affect synaptic plasticity. In the classical Hebbian STDP, LTP is formed if the postsynaptic spike is preceded by presynaptic activity by about 0-20 ms. When the postsynaptic spike comes first, followed by the presynaptic spike in the time frame of 0 and 20-100 ms, LTD is formed. In cortical cells, timing-dependent LTP (t-LTP) requires postsynaptic NMDA receptors, whereas timing-dependent LTD (t-LTD) requires presynaptic ones.

Transmitters released from the presynaptic cell activate NMDA receptors in the postsynaptic cell, which opens and $Ca^{2+}$ enters the cell. Like the classical form, the amount of $Ca^{2+}$ influx will determine whether LTP or LTD develops.

STDP does not synchronize the neurons, but leads to a balanced yet irregular firing state, where pre- and postsynaptic action potentials are causally related. STDP may vary with age. While t-LTD was present during the first and second postnatal weeks of juvenile rats, it disappeared by adulthood, in contrast to t-LTP. The presence of both Hebbian and anti-Hebbian STDP has also been demonstrated in young adults (21-38

years). STDP has also been observed in surgical hippocampal samples collected from human patients aged 20-66 years.

**Slow inward currents (SIC)**

The phenomenon of slow inward currents (SICs) was first described by Alfonso Araque et al. in 1998. It was found that SICs require a calcium signal in astrocytes and astrocytic glutamate release. SIC can be clearly distinguished from EPSC in terms of its parameters and origin. Although the amplitude of SICs may be similar to EPSCs, the rise time and the decay time are clearly different and therefore perfectly distinct. While EPSCs are consequences of neuronal activation, astrocytes significantly contribute to formation of SICs. As a hallmark of astrocytic activation, $Ca^{2+}$ concentrations increases in them, leading to an enhancement in the frequency of SICs on the neighboring neurons.

Neuronal synaptic events are not involved in the formation of SICs, as SICs can be measured on neurons even when action potentials are inhibited, so they are insensitive to TTX. This suggests that SICs are triggered by the astrocytic gliotransmitter release. Glutamate is released from astrocytes, which in turn activates the neuronal extrasynaptic NMDA receptors.

SICs have been observed in several brain areas, such as hippocampus, thalamus, nucleus accumbens, olfactory bulb, visual cortex, spinal cord, medial nucleus of the trapezoid body, and pedunculopontine nucleus. Over the years, several theories have been proposed about the function and role of SICs. Although the SICs have been investigated extensively

and evidence for their existence has been found, their physiological role remains unclear.

**Food-intake and arcuate nucleus**

Under physiological conditions, the arcuate nucleus (ARC) regulates food and energy intake. The orexigenic and anorexigenic neuropeptides are produced and secreted in the arcuate nucleus of the hypothalamus. The orexigenic peptides are agouti-related protein (AGRP) and neuropeptide Y (NPY), while the anorexigenic peptides are proopiomelanocortin (POMC) and cocaine-amphetamine-coupled transcript (CART). The orexigenic peptides increase food intake and decrease energy expenditure, while the anorexigenic peptides behave in the opposite way - they decrease food intake and increase energy utilization. The synthesis and secretion of these neuropeptides are regulated by negative feedback, and disruption of these mechanisms can cause metabolic disturbances and eating disorders. Food intake is well regulated in short- and long-term. Short-term regulation is limited to the beginning and end of a meal, while long-term regulation affects energy storage and consumption.

*Astaxanthin*

Astaxanthin (3′3-dihydroxy β-β ′-carotene-4,4′-dione, ASX) is a lipophilic marine xanthophyll carotenoid produced by marine algae species, but also by bacteria and yeasts. These are consumed by smaller shrimps. Species that consume algae or shrimps containing astaxanthin will themselves turn pink. The astaxanthin content of krill oil is between 0.1 and 1.5 mg/ml. The hydrophobic structure of ASX allows it to diffuse

easily across the cell membrane. This ability helps it to provide excellent protection against oxidative stress - both reactive oxygen species and free radical oxidation - on both sides of the cell membrane. ASX is also marketed as a dietary supplement for human consumption in the USA, Japan and the European Union.

Consumption of ASX as a dietary supplement has been shown to improve blood flow and have beneficial for erythrocytes, but researchers have also found promising results for its anti-inflammatory, immunostimulatory, antioxidant, anti-cancer and anti-diabetic, cardiovascular-, ocular-, skin- and neuroprotective effects. Due to its lipophilic nature, ASX is also able to cross the blood-brain barrier, and its effects in various neurodegenerative diseases have been studied.

## Objectives

Based on the above, our objectives for this thesis were to examine the following:

1. In what parameters the SICs in mouse and human neocortical pyramidal cells differ from each other?
2. How do SICs affect synaptic plasticity?
3. How does aging affect SICs and synaptic plasticity influenced by them?
4. How does chronic astaxanthin consumption affect the excitability and synaptic currents of neurons in arcuate nucleus?
5. How does acute astaxanthin treatment affect the excitability and synaptic currents of neurons in arcuate nucleus?

## Materials and methods

### Solutions and chemicals

In our experiments, we used artificial cerebrospinal fluid (aCSF) as a general buffer for patch-clamp and calcium imaging experiments. We used modified low sodium aCSF to prepare brain slices, and prepared magnesium-free aCSF for SIC experiments.

### Human samples

We examined samples of 13 patients in our experiments. The patients were both females (6) and males (7) aged between 38 and 74 years. All patients suffered from malignant tumors involving the neocortex (5 glioblastoma multiforme, 8 carcinoma metastasis). The slicing and recording protocols were same for human and mouse samples.

### Animals

For the SIC experiments, we used 9-585 day-old mice of both sexes, which included control mice (lox-tdTomato; n = 71) and mice that expressed fluorescent protein tdTomato in a glial fibrillary acidic protein (GFAP)- dependent manner (n=12). Young adult (9-12 week old animals of both sexes) lox-tdTomato mice were injected by stereotaxic injection with ready-to-use viruses carrying plasmids encoding the chemogenetic activator hM3D(Gq) and mCherry fluorescent marker expressed under GFAP promoter or mCherry marker alone as control. Mice were anesthetized by intraperitoneal injection of ketamin (100 mg/kg) and xylazine (10 mg/kg). 100-100 nl of viruses were injected into the cortical surface and parietal cortex at 200 and 400 μm depths using Hamilton syringe and microinjector.

In the astaxanthin experiments, young adult (3-month-old) wild-type C57BL6 mice (n=16) were used (8 fed ASX-contained food and 8 fed normal food) in chronic 4-week ASX feeding experiments. The special rodent pellet was prepared by adding 4 g/kg of AstaReal A1010 (dissolved in 100% ethanol) to the standard rodent pellet – final ASX concentration was 0.02%. Mice (n=8 in both groups) expressed proopiomelanocortin (POMC)-dependent or glutamate decarboxylase type 2 (GAD2)-dependent tdTomato fluorescent protein. 3 mice expressed the genetically encoded calcium indicator GCaMP6f in type 2 vesicular gamma amino-butyric acid transporter (VGAT2)-dependent manner were used for the acute experiments.

**Preparation of slices**

200 μm thick slices were prepared in coronal plane at the level of the parietal cortex and from the area including the hypothalamus. Preparation was performed in ice-cold (approximately 0- -2 $^{o}$C), low sodium aCSF using a Microm HM 650 V vibratome. Prior to recording, the slices were incubated in normal aCSF for 1 hour at 37 $^{o}$C.

**Electrophysiology**

Patch-clamp recordings were performed on neocortical pyramidal cells in layer III-IV and on POMC-and GAD-positive cells of the hypothalamic arcuate nucleus. The resistance of the patch pipettes was 6-8 MΩ. The pipette solution contained 8 mM biocytin. Whole cell patch clamp recordings were performed at room temperature (24-26$^{o}$C) using Axopatch 200A amplifier. All data were recorded using Clampex 10.0

software and Clampfit 10.0 and Synaptosoft MiniAnalysis software were used for data analysis.

In current clamp experiments, 1-s-long square current pulses, ranging from −30 pA to +120 pA with 10 pA increments were applied on human pyramidal neurons to test the viability of the neurons. For ASX experiments, we used the same protocol to record action potentials. The resting membrane potential was set to -60 mV. The spontaneous firing frequency was recorded in current clamp.

Measuring SICs, tonic currents, spontaneous EPSCs and IPSCs were recorded in gap-free at -60 mV holding potential, in voltage clamp configuration. Our elicited EPSCs were also recorded in voltage clamp configuration. In these experiments, the presynaptic fibers innervating the neuron were stimulated with a tungsten bipolar electrode approximately 50-100 μm from the soma of the neuron. Each stimulus was applied every 20 seconds.

The measurements were performed in normal and magnesium-free aCSF. In NMDA receptor subunit composition experiments, 500 nM PPPA (GluN2A-specific inhibitor), 5 μM ifenprodil (GluN2B-specific inhibitor) and 10 μM D-AP5 (non-specific NMDA inhibitor) were used. In other experiments, the EAAT1 and 2 glutamate transporters were inhibited. We used UCPH101 at 10 and 25 μM (specific inhibitor of EAAT1), WAY213613 at 10 and 100 μM (the specific inhibitor of EAAT2), and DL-TBOA at 100 μM (non-specific EAAT inhibitor).

Electrical stimulation was combined with chemogenetic activation of astrocytes. In these experiments, viral vectors carrying the plasmids were

injected into mice. After control recordings, 10 µM clozapine-N-oxide (CNO) was applied. The control recordings were performed in normal aCSF and 0.1% DMSO (since CNO stock solution was dissolved in DMSO), supplemented with 1 µM strichnine and 10 µM bicuculline. In another experimental set-up, 30 µM MNI-caged glutamate was applied to the recording chamber and the whole chamber was illuminated with UV and near-UV (wavelength below 395 nm) light using a Rapp lamp. In the next series of recordings, a previously recorded SIC was used as a voltage command in combination with presynaptic electrical stimulation.

For our astaxanthin experiments, 2.5 µM ASX was dissolved in normal aCSF containing 0.1 % ethanol.

**Calcium imaging**

For calcium imaging experiments, samples from VGAT2-GCaMP6 mice were prepared as described for electrophysiological experiments. Frames with a resolution of 344x260 pixels were acquired at a frame rate of 10 Hz.

## Results

### Murine SICs are astrocyte- and GluN2B subunit containing NMDA receptor-dependent events

Gap-free recordings were performed on mouse neocortical pyramidal cells in voltage-clamp. Control recordings were performed in normal aCSF solution, which was replaced with magnesium-free aCSF. In the magnesium-free condition, not only the activity of SICs was increased, but also the amplitude, rise time, and decay time of EPSCs, resulting in an increase in the charge transfer of EPSCs, as it was expected beforehand. The amplitude, rise time, decay time and charge transfer (SIC "area") of SICs were also significantly larger than the same parameters of the EPSCs. The rise time served as an appropriate cut-off value for the separation of SICs and EPSCs, and it was set at 20 ms for both human and murine samples.

To investigate the composition of NMDA receptors, we used GluN2A- and Glun2B subunit selective inhibitors and a non-selective NMDA inhibitor. We found that SIC activity was unchanged by 500 nM PPPA (GluN2A subunit inhibitor) compared to control but 5 μM ifenprodil (GluN2B subunit inhibitor) significantly abolished SIC activity, and 10 μM D-AP5 (non-selective NMDA inhibitor) completely abolished SICs.

Astrocytes were chemogenetically activated. The SIC activity was significantly increased by CNO. These changed were not observed in mCherry control.

**Human SICs are astrocyte-and GluN2B subunit containing NMDA receptor-dependent events as well**

Gap-free recordings were performed in human neocortical pyramidal cells in voltage clamp. The holding potential was -60 mV. The amplitude, rise time, decay time and charge transfer (SIC "area") of SICs were significantly larger than the same parameters of EPSCs, so SICs and EPSCs were easily distinguishable in human samples. SICs were slower events as in murine samples.

We also examined the composition of NMDA receptors in human samples. The SIC activity was almost completely abolished by ifenprodil and D-AP5 compared to the control. WAY 2131613 (EAAT2 specific glutamate transporter inhibitor) was used as a substitute for chemo-or optogenetic measurements. SIC activity was increased significantly by 100 μM WAY in human pyramidal neurons.

**SICs work as electrical signals to trigger timing-dependent synaptic plasticity (STDP)**

The amplitude and frequency of spontaneous EPSCs were compared before and after the first SIC appearance. In some cases both frequency and amplitude was increased, while in other measurements we observed a decrease. There were also cases where no change was observed.

Astrocytes expressing the hM3D chemogenetic actuator were activated by CNO. Simultaneously, the excitatory synaptic input of pyramidal cells were electrically stimulated. When the peak of the SIC and the EPSC elicited by electrical stimulation occurred within 1 s, we observed a change in EPSC amplitude. In half of the cases the amplitude

of EPSCs changed in long term (n=18). If SIC preceded the elicited EPSC (negative shift) or occurred at the same time (zero shift), we observed an increase in EPSC amplitude of 21-137%. If SIC followed the EPSC (positive shift), we found a 22-33 % decrease in amplitude of EPSCs (n=3).These changes were maintained throughout the measurement time (40 min).

Summarizing our results, negative or zero shift led a long-term increase in amplitude of EPSCs, while the positive shift showed a decrease. Based on these results, we concluded that SIC induced by chemogenetic activation of astrocytes affects plasticity in timing-dependent manner.

In order to exclude the influence of other factors and to provide evidence that glutamate release from astrocytes is the cause of changes, we used flash photolysis to release MNI-caged glutamate. EPSCs were induced by stimulating the excitatory input of the pyramidal cell and the flash-light-induced glutamate release was timed as described above. In more than half of the cases (58%), a long-term shift of EPSCs was found (n=17). When the current induced by glutamate release preceded the EPSC (negative shift) or appeared at the same time (zero shift), the amplitude increased by 17-116%. When the glutamate-induced current appeared after the EPSC (positive shift), the amplitude of EPSCs decreased (21-26%). As in the previous experiment, SIC induced a weak STDP.

To investigate whether SICs as postsynaptic electrical signals are capable of altering synaptic strength, we converted a previously recorded

SIC into a voltage command and applied it with a timing similar to previous experiments. These artificial SICs induced weak STPD-like plasticity. In 44% of cases, EPSC amplitudes changed in response to SIC commands (n=36). The negative and zero shift between SIC command and EPSC elicited by electrical stimulation caused EPSC amplitude enhancement of 27-117% in 35 % of the cases and decay of 26-30% in 10 % of the cases. The positive shift caused a 28-45% decrease in EPSC amplitude.

**Effect of SICs on synaptic plasticity in human samples**

Gap-free recordings were performed according to the criteria described for mouse experiments in magnesium-free solution. We found that the amplitude and frequency of EPSCs increased after the first SIC and showed a long-term change. The EPSC amplitudes showed an increase of 10-42% in 83% of cases (n=10). In 66% of all cases, the EPSC frequencies also increased.

**Aging of mouse and human SICs and their effects on synaptic plasticity**

Next, we aimed to investigate how aging affects SICs and their impact on synaptic plasticity. We examined SICs in recordings from 9-547-day-old mice where we found that charge transfer was not changed by age, but amplitude, rise time and decay time showed age-related fluctuations. In addition, in mouse samples, the frequency of SICs had a moderate but significant decrease with age. In consequence, the activity of SICs also showed weak decline.

In contrast to results in mice, we observed a drastic decline in charge transfer and activity of human SIC with age. The most interesting difference is that SICs disappeared completely in human samples after 70 year-old.

In the middle-aged populations, the amplitude of SICs in human samples was significantly smaller, whereas decay time and charge transfer were significantly larger than in mice. These results suggest that SICs in human samples had slower kinetics.

The change in SIC activity and the ability of SICs to influence synaptic plasticity are partly due to changes in the expression of the GluN2B subunit. In synaptophysin- and GFAP-colocalized GluN2B immunostainings of human and mouse samples from the middle-aged and elderly groups, we observed a decrease in fluorescence intensity in elderly.

In summary, the parameters of individual SICs were not changed with age in mice, but a significant decrease in charge transfer was found in human samples. Aging affected the responsiveness to SICs in mice, whereas in human samples the SIC activity itself was found to be age-dependent.

**Effect of chronic astaxanthin feeding on hypothalamus**

First, we examined changes in excitability by astaxanthine. The maximal action potential firing frequency was significantly larger than the control. The average firing frequency also showed a tendency to increase after astaxanthin feeding.

As the next step of experiment, spontaneous excitatory and inhibitory postsynaptic currents (EPSC and IPSC) were investigated. The results showed that ASX did not alter the frequency and amplitude of EPSCs. Interestingly, the IPSC frequency was significantly increased in ASX-fed population, but the amplitude was not changed.

To summarize the findings of this chapter, long-term ASX feeding increased the excitability of neurons in arcuate nucleus and increased the frequency of inhibitory synaptic currents.

**Acute effects of astaxanthin on hypothalamic neurons**

Similar to the chronic ASX-feeding experiment, we examined the effect of acute treatment of ASX on changes of synaptic currents. The frequency and amplitude of EPSCs were not changed, but the IPSC frequency numerically increased with ASX treatment, however, this change was not significant.

Next, we investigated the effect of ASX on GABAergic neurons and synapses. Using cells expressing GCaMP6 calcium indicator under VGAT2 promoter, we found a total of 22 ROIs that were shown exhibit calcium transients. Acute application of ASX increased the frequency of calcium transients or induced transients in 77.3% of ROIs. It had no effect on cell activity in 9.1% and reduced or abolished calcium transients in the remaining 13.6%. Correspondingly, smaller fluorescent dots (likely axon terminals of GABAergic neurons) also showed a similar response when ASX was applied. The average calcium transient frequency was $2.42 \pm 0.78$ Hz, which increased to $3.73 \pm 0.75$ Hz after ASX (an overall increase of $3.27 \pm 1.57$ times).

The spontaneous action potential firing rate of GABAergic neurons was 0.57 ±0.13 Hz, which increased to 1.12 ± 0.24 Hz with ASX. We also analyzed synaptic currents and their changes and found no significant differences in the EPSC frequency and amplitude.

In summary, the suppression of POMC neurons and the increased activity of GABAergic neurons described in this chapter are consistent with increased food intake under the influence of astaxanthin.

## Discussion

In this thesis we have demonstrated that – as found in the mouse brain – SICs are present in the human brain as well. We showed that these events are distinct from SICs found in mouse samples. In contrast to mice the charge transfer of human SICs is larger, which was mainly observed in the middle-aged population, where the highest difference in kinetic parameters between the two species were observed. We also found that SICs were able to affect the strength of the excitatory synapses in a timing-dependent manner in about half of the cases. This was seen in mouse samples as well as in human, and shown to be age-dependent. In mice, the magnitude and kinetics of SICs were not decreased with age, whereas the frequency and the capability to alter synaptic strength decreased with age. In human, the area and kinetics of SIC and their frequency also were decreased with age. Most interestingly, after 70, SICs were disappeared completely.

### Human and mouse SICs are different

Human SICs had a significantly greater charge transfer compared to mouse SICs, but the amplitude was typically smaller than in mouse samples. In human, SIC parameters showed a strong age dependence, so we performed a comparative analysis between the middle-aged mouse and human population, and while SICs in mice showed faster kinetics, SIC amplitudes in human samples were found to be significantly smaller. The possibility cannot be excluded that we found lower amplitudes of human SICs due to different tissue transport and treatment, but the kinetic parameters clearly showed a species heterogeneity.

**The ability of SICs to influence synaptic strength**

Spike timing dependent plasticity (STDP) in an extensively studied process of long-term plasticity, where the experimental protocol is to trigger presynaptic input and postsynaptic action potentials in a time-dependent manner and to apply this repeatedly (10-300 times) over time. Comparing the hippocampus of young rodents and human temporal cortex, an inverse relationship was found between timing and time-shifting of presynaptic stimulation and postsynaptic spikes in human samples. When the postsynaptic spikes preceded the presynaptic signal, LTP was formed, whereas the reverse was observed for LTD. The intracellular calcium signals can regulate the occurrence of LTP and LTD, more specifically, the temporal length and timing of the calcium signal in comparison to other events. Intracellular calcium changes depend on several mechanisms: it is influenced by activity of metabotropic glutamate and NMDA receptors, as well as the voltage-gated calcium channels. Long-term synaptic plasticity may also be altered by inhibition of glial cell function. Chemical inhibition of glial cells may influence long-term synaptic plasticity, and thus glial cells (including astrocytes) play an important role in development of synaptic plasticity.

NMDA-dependent LTD may be modified by alterations in glutamate uptake by astrocytes in mouse hippocampus. Astrocytes can modulate LTP by releasing glutamate and by activating NMDA receptors containing GluN2A subunit. In the mouse striatum, inhibition of the glutamate transporter EAAT2 caused a change in the induced STDP, with

an irregular pattern of plasticity due to activation of extrasynaptic NMDA receptors.

In this work, we showed that SICs can induce STDP in roughly half of the synapses that correspond to the anti-Hebbian rules. It is assumed that our result do not follow the classical Hebbian rules (i.e. when presynaptic excitatory spike precedes postsynaptic action potentials, LTP is induced) because the protocol we used is not one of the conventional protocols. Therefore, it is suggested that SIC arriving at the same time as presynaptic stimulation is associated with an increase in intracellular calcium levels, which may lead to LTP if SIC precedes or occurs simultaneously with EPSC. When SICs are formed after the induced EPSC, there is presumably only an initially small and slower-forming increase in calcium levels in the STDP time window. The slight rise in calcium concentration leads to LTD.

Our experiments on human samples suggest that SICs can also induce synaptic plasticity in humans. Human SICs have greater charge transfer, but slower kinetics, which may have implications for their role in regulating synaptic plasticity by inducing a longer and slower increase in somatodendritic intracellular calcium concentration over time.

**Aging affects SICs and SIC-dependent changes in synaptic strength.**

Astrocytes undergo several morphological and functional changes as their aging. In mouse and primate hippocampus, astrocyte branch length and terminal complexity increase in juvenile, culminate in adulthood, and

decline in elderly. The spatial size of astrocyte processes decreases, so the domain of astrocytes also declines with age.

The ability of astrocytes to influence synaptic function also alters with age. Some factors need to be considered in aging SICs. These factors include a.) calcium excitability and the number of astrocytes that possess calcium waves, b.) glutamate uptake and release by astrocytes, c.) diffusion of glutamate from astrocytes to extrasynaptic NMDA receptors, and d.) the number of extrasynaptic NMDA receptors which can be activated.

We have mostly confirmed previous literature data on mouse aging that individual SICs are not affected by aging, and although SICs in rodents did not disappear with age, the frequency was decreased. In humans, however, the changes in parameters of SICs fundamentally differ from those found in mice. Charge transfer and kinetic parameters were significantly reduced in human samples and SIC activity was completely abolished at the age of 70. One possible explanation could be the age-dependent changes in glutamate levels related to astrocytes. Changes in glutamate uptake and release, as well as changes in the expression, function, and composition of extrasynaptic NMDA receptors in neurons may all be responsible for the disappearance of SIC.

Synaptic plasticity was affected differently by SICs in the two species. In mice, synaptic plasticity is affected by SICs in an age-dependent way. There are several possible reasons behind this finding, such as decrease in mGluR5 signaling or age-dependent changes in NMDA receptors. However in humans, not only synaptic plasticity but also the SIC was

affected itself. Several factors may be involved in it, such as age-related changes in glutamate uptake or release in astrocytes and age-related changes in the composition of neuronal NMDA receptors. We have shown that the density of the GluN2B subunit is slightly but significantly reduced, which may have implications for the sensitivity of synaptic plasticity to SICs and their activity.

**Effects of astaxanthin on hypothalamic neurons**

As part of the hypothalamus, the arcuate nucleus plays an important role in the uptake of nutrients. The two main groups of neurons found here regulate appetite and food intake in opposite ways: anorexinergic POMC cells decrease food intake, whereas orexinergic NPY and AgRP-positive cells increase food intake. Depending on the current energetic state of the body, GABAergic inputs can influence the function of POMC cells. GABAergic inhibitory inputs rise from the dorsomedial part of the hypothalamus and from orexinergic AgRP cells. In fibers innervating POMC neurons, the frequency of spontaneous IPSCs increases during caloric deficit.

While the oleic acid induced a tonic current, contrary to our expectations, ASX showed no such effect on excitability. However, similar to starvation, the frequency of spontaneous IPSCs was increased in POMC neurons. It is probably due to pleiotropic effects on GABAergic neurons, as the spontaneous firing frequency was also increased. This could be partly due to the increase in excitatory inputs. An increase in GABAergic inhibition could promote increased consumption of astaxanthin-containing food in mice, as inhibition of

POMC neurons increases food intake. Although the coronal slices we prepared contained the arcuate nucleus and the dorsomedial hypothalamus, which are major sources of GABAergic inputs and affect POMC neurons by inhibitory synapses, unfortunately, some synaptic connections may be lost during preparation of brain slices, and thus we may have underestimated the effect of inhibitory inputs on IPSCs.

## Summary

In this work we aimed to investigate the origin of SICs and the differences in their parameters in mouse and human cortical samples. SICs are distinct from EPSCs, and the amplitude, rise time, decay time and charge transfer of SICs are significantly greater than the same parameters of EPSCs, in both species. According to the literature, GluN2B-containing extrasynaptic NMDA receptors are required for the formation of SICs. We investigated the role of astrocytic activation and glutamate release in the development of SICs using a chemogenetic actuator in mice. Using the hM3D actuator and its astrocyte-specific activation, SIC activity showed a significant increase compared to the control. In human samples (in the absence of the possibility of chemogenetic experiments), WAY213613 was used to model glutamate release by inhibition of the EAAT2 glutamate transporter. WAY significantly increased the activity of SICs. The only significant difference between the two species was found in charge transfer, which was greater in human. We also investigated the influence of SICs on synaptic plasticity and found that by timing SICs to evoked EPSCs, using chemogenetic activation of astrocytes, releasing glutamate by flash photolysis and using SICs as a voltage command, SICs were able to influence synaptic plasticity in a timing-dependent way in a long term. Examining the relationship between SICs and aging, we found that there was a greater difference between mice and human samples. In mice the charge transfer of individual SICs does not change with age, but SIC activity was moderately but significantly reduced. In human, in contrast,

SIC activity and charge transfer was decreased, and SICs were completely disappeared after the age of 70. In addition, the capability of SICs to elicit synaptic plasticity was affected by age in mice.

Long-term feeding of astaxanthin in mice increased the excitability of the hypothalamic arcuate nucleus neurons and the frequency of IPSCs. In the arcuate nucleus, acute astaxanthin application on POMC-positive neurons did not affect IPSC frequency significantly. However, it significantly increased calcium transient frequency and firing rate in GABAergic neurons of arcuate nucleus.

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Registry number: DEENK/531/2023.PL
Subject: PhD Publication List

Candidate: Andrea Csemer
Doctoral School: Doctoral School of Molecular Medicine
MTMT ID: 10071339

## List of publications related to the dissertation

1. **Csemer, A.**, Kovács, A., Maamrah, B., Pocsai, K., Korpás, K. L., Klekner, Á., Szűcs, P., Nánási, P. P., Pál, B.: Astrocyte- and NMDA receptor-dependent slow inward currents differently contribute to synaptic plasticity in an age-dependent manner in mouse and human neocortex.
*Aging Cell. 22* (9), e13939, 2023.
DOI: http://dx.doi.org/10.1111/acel.13939
IF: 7.8 (2022)

2. Gönczi, M.*, **Csemer, A.***, Szabó, L., Sztretye, M., Fodor, J., Pocsai, K., Szenthe, K., Keller-Pintér, A., Köhler, Z. M., Nánási, P. P., Szentandrássy, N., Pál, B., Csernoch, L.: Astaxanthin Exerts Anabolic Effects via Pleiotropic Modulation of the Excitable Tissue.
*Int. J. Mol. Sci. 23* (2), 917, 2022.
DOI: http://dx.doi.org/10.3390/ijms23020917
* These authors contributed equally to this work.
IF: 5.6

## List of other publications

3. Maamrah, B., Pocsai, K., Bayasgalan, T., **Csemer, A.**, Pál, B.: KCNQ4 potassium channel subunit deletion leads to exaggerated acoustic startle reflex in mice.
*Neuroreport. 34* (4), 232-237, 2023.
DOI: http://dx.doi.org/10.1097/WNR.0000000000001883
IF: 1.7 (2022)

4. Bayasgalan, T., Stupniki, S., Kovács, A., **Csemer, A.**, Szentesi, P., Pocsai, K., Dionisio, L., Spitzmaul, G., Pál, B.: Alteration of mesopontine cholinergic function by the lack of KCNQ4 subunit.
*Front. Cell. Neurosci. 15*, 707789, 2021.
DOI: http://dx.doi.org/10.3389/fncel.2021.707789
IF: 6.147

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5. Bayasgalan, T., **Csemer, A.**, Kovács, A., Pocsai, K., Pál, B.: Topographical Organization of M-Current on Dorsal and Median Raphe Serotonergic Neurons.
   *Front. Cell. Neurosci. 15*, 614947, 2021.
   DOI: http://dx.doi.org/10.3389/fncel.2021.614947
   IF: 6.147

6. Kovács, A., Baksa, B., Bayasgalan, T., Szentesi, P., **Csemer, A.**, Pál, B.: Orexinergic actions modify occurrence of slow inward currents on neurons in the pedunculopontine nucleus.
   *Neuroreport. 30* (14), 933-938, 2019.
   DOI: http://dx.doi.org/10.1097/WNR.0000000000001298
   IF: 1.394

**Total IF of journals (all publications): 28,788**
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The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

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