Monitoring extracellular glutamate in the rat brain by microdialysis and microsensors
van de Zeyden, Miranda

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Chapter 8
Evaluation of hydrogel-coated glutamate microsensors in acute hippocampal slices

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Abstract

Acute hippocampal slices are used extensively and has permitted the study of physiology and pharmacology of the brain in more detail. However the application of a real time glutamate microsensor in combination with in vitro slice preparation is scarce. In this study the hydrogel coated glutamate microsensor was used (A) to further understand and explore the performance of the glutamate microsensor in acute hippocampal slices (B) to determine changes in extracellular glutamate concentration between rats of different ages, (C) to determine pharmacological induced changes in the presents of high potassium (D) to establish possible effects of electrical stimulation on extracellular glutamate. The studies have shown little significant data. It is concluded that the high potassium-approach in combination with the slow response time of the hydrogel coated glutamate microsensor may have been too rudimentary to measure the subtle pharmacological changes of glutamate in the acute slices.
1. INTRODUCTION
The development of microsensors for real-time monitoring of glutamate in the mammalian brain is important to understanding neuronal events at the molecular level. It is a promising approach in elucidating the dynamics of glutamate action during physiological and pathological states in the CNS. Significant efforts are being devoted towards the development of glutamate microsensors with rapid, sensitive and selective detection. Improvements of the temporal and spatial resolution are important, because the neuronal activity that releases and metabolizes glutamate is a very fast event and hence the concentration of glutamate detected by the microsensor depends on the location and distance of the sensor from the neurons.

We have previously demonstrated that glutamate release can be detected using the second generation hydrogel-coated glutamate microsensor in vitro and in vivo (Oldenziel et al. 2007; Oldenziel et al. 2006c). While this offers a proof of concept for the type of microsensor, the biological significance of monitoring glutamate in brain tissue is limited by the properties of the microsensor. To further evaluate the properties of the glutamate microsensors, we investigated the use of the glutamate microsensor in acute hippocampal slices.

Brain slices are one of the most common methods to study neurotransmitter modulations in brain tissue, and has proven to be very important for experimenting with perfusion of drugs under controlled conditions. In vitro slice preparations have permitted the study of the physiology and pharmacology of the brain in a relatively well controlled experimental environment (Robert et al. 1997).

Recent discoveries have revealed that the physiology of glutamatergic neurotransmission in the CNS is mediated by an intimate partnership between neurons and astrocytes (Nedergaard et al. 2002). To further explore/understand these physiological processes; the use of glutamate microsensors may play an important role. To that end we have studied various properties of the hydrogel-coated glutamate sensor. In this study (A) the performance of the glutamate microsensor in acute hippocampal slices was evaluated (B) rats of different age groups were used to make acute hippocampal slices, in order to determine whether changes in glutamate concentration can be detected (C) various pharmacological agents were administered with a S1S2 stimulus (D) acute slices were electrically stimulated to induce changes in extracellular glutamate basal levels.
2. MATERIALS AND METHODS

2.1 Animals and acute hippocampal slices preparation

Male albino rats of a Wistar-derived strain (275-320 g; Harlan, Zeist, The Netherlands) were used for the experiments. The rats were decapitate (550020; Havard Apparatus, Holliston, MA, USA) and the brain was rapidly removed and stored in ice-cold, continuously gassed (mixture of 95% O₂ /5% CO₂), artificial cerebrospinal fluid (aCSF). After several minutes, transverse slices (400 µm) of the hippocampus were prepared with a tissue chopper (UO600; Proscitech, Kirwan, Australia). The slices were stored on a nylon mesh within a beaker, which contained continuously circulating, oxygenate (95% O₂ / 5% CO₂) aCSF at room temperature. The slices were stored at least 1 hour before use. The composition of the aCSF solution was (in mM): 124 NaCl, 3.5 KCl, 1.25 NaH₂PO₄, 1.5 MgSO₄, 2.0 CaCl₂, 2.5 NaHCO₃ and 10 glucose; pH 7.4 adjusted with sodium hydroxide. The aCSF solution was made in ultra-purified water (U.P.; elgastat maxima, Salm and Kipp).

2.2 Microsensors recording in slices

A single slice was transferred to a recording chamber (21052; Fine Science Tools, Heidelberg, Germany) where they were fully immersed in carbonated artificial cerebrospinal fluid (aCSF) of the following composition (in mM): NaCl, 124; KCl, 3.5; NaH₂PO₄, 1.25; MgSO₄, 1.5; CaCl₂, 2.0; NaHCO₃, 2.5 and D-glucose, 10; pH 7.4 adjusted with sodium hydroxide. The aCSF was bubbled with 95% O₂ -5% CO₂ and flowing at 1.5 ml/min (31-32°C). The microsensor recordings were performed by implanting both a glutamate and background microsensor in close proximity (<200 µm; V-shaped form) in the CA1 area of the hippocampal slice. The implantation procedure was visually controlled with a stereo microscope (Exacta-Optech, Diever, The Netherlands). The reference electrode (Ag/AgCl) was placed in the bathing solution of the recording chamber. The current output of both microsensors was monitored and after stabilization (±20 minutes) different pharmacological agents were administered to the slice. Administration was performed by applying the drugs to the aCSF reservoir and perfusing the slice with this solution. The drug TTX was not washed in –small amounts are expensive-, but was administered directly in the bathing solution of the recording chamber at the opposite site from the slice where suction takes place, to guarantee that TTX perfuses through the slice. During this approach the rate of aCSF perfusion was also decreased to about 1 ml/min and after a short stabilization period (±5min) at this particular flow rate TTX was applied. It was observed that the distribution of TTX was more difficult to control with this approach.
2.3 Microsensors

Microsensors (glutamate (GLUS) and background sensors (BGS)) were constructed and selected for in vitro recordings as previously reported (Oldenziel et al. 2004; Oldenziel and Westerink 2005). Briefly, a single carbon fibre (P-55s, Thornel carbon fibre’s, Amoco; 10 µm diameters) was sealed with epoxy into a pulled glass capillary (TW100F-3, World Prevision Instruments, Sarasota, FL, USA) and the exposed fibre was trimmed to a length of 300-400 µm. The microsensors were prepared by immobilization of a five-component redox-hydrogel, in which l-glutamate oxidase (GluOx) (0.0065 Units/µl; 10 µl), horseradish peroxidase (HRP) (0.711 Units/µl; 10 µl), and ascorbate oxidase (AAOx) (1.5 Units/µl; 10 µl) were wired via poly(ethylene glycol) diglycidyl ether (PEDGE) to an osmium containing redox polymer abbreviated as POs-EA. The redox polymer POs-EA (1 mg/ml; 20 µl) and cross-linker PEDGE (3 mg/ml; 4 µl) were dissolved in ultra-pure water. Enzymes solutions were made in HEPES buffer (pH 8.00) prepared by the addition of the HEPES sodium salt to a 10 mM solution of the acid. The coating procedure was mechanized by using an automated dipcoater. After coating, the sensors were cured for 1 hr at 37°C, followed by a 10 minute dip in ultra pure water and 2 hrs drying in ambient air. The sensors are affected by non-specific interference from electroactive compounds, mainly ascorbate (AA) and possible 4-hydroxy-3-methoxyphenylacetic acid (DOPAC), 5-hydroxyindolacetic acid and urate. Application of a thin Nafion coating therefore completed the fabrication. Nafion repels anions such as 3,4 dihydroxyphenylacetic acid (DOPAC) and ascorbic acid (AA). The Nafion coating was performed by dipping the hydrogel-coated microsensors repetitively 5 times for 10 sec, with 20 sec intervals, in a 0.5% Nafion solution (1:10 dilution in isopropanol p.a). All the sensors were coated with Nafion, unless stated otherwise. The sensors were cured overnight in refrigerator before calibration the next day. The sentinel or background sensors were prepared similarly as the glutamate microsensors, only GluOx was not incorporated into the hydrogel.

Electrochemical detection of glutamate by the sensor occurs as follows. The glutamate is converted by GluOx to H$_2$O$_2$, α-ketoglutarate and NH$_3$. Next, this H$_2$O$_2$ is converted by HRP, which in turn communicates with the Pos-EA. Finally, the Pos-EA mediates the electron transfer to the CFE surface. AAOx is incorporated to prevent from interference by AA. For a more detailed description of the performance of the sensor reference is made to previous reports (Oldenziel and Westerink 2005; Oldenziel et al. 2006b). The experiments were carried out by amperometrically operating the microsensor at a constant potential of ~150 mV (vs. Ag/AgCl). Before the microsensors were used in vitro, they were calibrated in a flow-injection analysis system (FIA). Several microsensor
properties were determined during this calibration, as described before (Oldenziel and Westerink 2005): shape and height of the CV (nA; determined as the sum of the cathodic \( I_{pc} \) and anodic peak \( I_{pa} \) current), sensitivity (\( \text{pA}/\mu\text{M} \)), interference (%), linearity (R2), detection limit (\( \mu\text{M} \)); 3 times signal to noise ratio and response time (sec); time required for the signal to increase from 10 to 90 %. Only the sensors that could not detect the oxidative current of ascorbate up to 200\( \mu \text{M} \) were used.

After the \textit{in vitro} experiment was performed, the microsensors were post-calibrated. The sensitivity of the microsensors for glutamate were calculated in the conditions described previously (Oldenziel \textit{et al.} 2006b). This sensitivity was used to correlate the detected current (\( \text{pA} \)) to final extracellular glutamate concentrations. In addition, compound used in slice experiments were also injected during the calibration and induced no effect.

\textbf{2.4 Electric stimulation}

In the equilibration period of 15 min, a bipolar stainless steel stimulus electrode (60 \( \mu \text{m} \) diameter, insulated except for the tip) was placed in the Schaffer collaterals. Nervous tissue microstimulation requires a selective and controlled activation of a population of neurons therefore the most effective way to stimulate a cell using extracellular electrodes is to have a highly concentrated change in the extracellular voltage gradient localized within distances of about one length constant (200 \( \mu \text{m} \)). Accordingly the recording glutamate microsensor was position in close proximity in the CA1. Biphasic stimuli (250 \( \mu \text{s} \)) were applied through a Neurolog stimulus isolator (NL 800) driven by a home made software program. Current evoked in CA1 neurons were made at holding potential by increasing stimulus intensities from 10 to 500 \( \mu \text{A} \), given when pressed.

\textbf{2.5 Reagents/ Chemicals}

Glutamate oxidase (GluOx; G-0400; 6.5 units/mg) was purchased from USBiological (Swampscott, MA, USA). Horseradish peroxidase type II (HRP; P-8250; 158 units/mg), [4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid] (HEPES), HEPES sodium salt, l-glutamate, l-ascorbic acid, poly(ethylene glycol) diglycidyl ether (PEDGE), silver chloride and Nafion (5\% Nafion solution,1100 equivalent weight), tetrodotoxine (TTX) and all other chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA), unless stated otherwise. DL-threo-\( \beta \)-benzyloxyaspartate (DL-TBOA) and (S)-4-carboxyphenylglycine (CPG) were obtained from Tocris Bioscience (Bristol, UK). D-glucose, salts, acetone p.a. and 2-propanol p.a. were obtained from Merck (Darmstadt, Germany). AAOx was initially obtained from Seravac (Cape Town, South Africa; Product nr. 011530), but was purified prior to its use, (Oldenziel \textit{et al.} 2006a). aCSF was used for the calibration procedures.
and for the dissolution of drugs. It had the following composition: 145 mM Na+, 1.2 mM Ca2+, 2.7 mM K+, 1.0 mM Mg2+, 152 mM Cl- and 2.0 mM phosphate; pH 7.4 adjusted with sodium hydroxide. aCSF solutions were made in ultra-purified water (U.P.; Elgastat maxima, Salm en Kipp).

2.6 Statistics
Data are presented as mean ± SEM. The recordings of the microsensors (in pA) were averaged and represented as a black line (mean) with a grey area (SEM). Each experiment (n) represents the number of slices used. Each acute slice was obtained from a different animal. Each slice was investigated with a different set of microsensors. SPSS14.0 for windows was used to calculate statistics. Statistical analysis was performed using One Way Anova with repeated measures followed by a Dunnett’s T3 posthoc test. The level of significance was set at p <0.05. For statistical analysis the data (each second, a data point) were averaged to sections of minutes and the output of both microsensors at the minute prior to treatment was compared to the different post-treatment minutes.
3. RESULTS AND DISCUSSION

SECTION A: Basic properties of the glutamate microsensor applied to acute hippocampal slices

The experiments in the present study were conducted by inserting both a glutamate (GLUS) and background microsensor (BGS) in close proximity (±200 µm) in the CA1 area of acute hippocampal slice. The sensors were implanted through the whole thickness of the slice (<300 µm) to insure optimal amount of contact. As the basal extracellular concentration are low and close to the detection limit we have chosen to stimulate glutamate release by applying a high potassium concentration to the slice. All drugs were studied in the presence of the potassium stimulation. Two stimuli (called S1 and S2) of 120 mM KCl were applied to the aCSF which was washed into the flow chamber where the slices were present (Figure 1). Several pharmacological agents with specific pharmacological properties were applied in combination with 120 mM KCl in the second stimuli (S2). The current output of both microsensors were monitored and the change in current represented the extracellular glutamate concentration ([Glu]₀). The difference in current output from the glutamate and background microsensor after drug administration compared to the difference prior to administration was examined. For reasons of clarity, data are given as column graphs representing the area under the curve of the stimulated glutamate. In addition figures are given in which the S2 is given as a percentage of stimulus of S1 which is set to 100%.

I Calibration of the glutamate microsensor

The output of the microsensors is expressed in current (pA). In order to determine glutamate concentrations (in µM) measured in the slices, the sensors were calibrated in a flow injection system in order to determine sensitivity and selectivity.

From the pre-calibration (i.e. pre-\textit{in vitro}) of the microsensors used in acute hippocampal slices, a total set of 48 microsensors (GLUS and BGS) used, a sensitivity of 13.7 ± 1.45 pA/µM (mean ± SEM) for glutamate was obtained. The sensitivity of the sensors are known to be decreased by interferent agents such as ascorbate (AA), uric acid, electroactive metabolites such as 3,4-dihydroxyphenyl acetic acid (DOPAC), 4-hydroxy-3-methoxyphenyl acetic acid (HVA) and 5-hydroxyindolacetic acid (5-HIAA) as well as neurotransmitters such as dopamine (DA), norepinephrine (NE), serotonin (5-HT) (Hu \textit{et al.} 1994).
II Basal extracellular glutamate levels
Basal extracellular glutamate concentrations present in the acute slices from an average of 48 experiments showed that the difference between the current output of the glutamate and background sensor was 13.80 pA ± 5.0 pA (mean ± SEM). When the microsensors were post-calibrated at the conditions encountered in the slice, a sensitivity of 1.25 ± 0.25 µM (mean ± SEM) was calculated.

III Stability of glutamate microsensors in acute slices: role of Nafion
In general the hydrogel glutamate microsensor displays a limited stability. Both decrease in absolute sensitivity and biofouling might contribute to a decreased sensitivity. In the various figures the second potassium stimulation is consistently lower than the first. This lower response can be explained by a decreasing sensitivity of the sensor but also by depletion of glutamate stores. The diffusion of glutamate from the slice interior into a bath solution inevitably occurs (Oka et al. 2009; Herman and Jahr 2007). Likewise a study by Patterson et al (1995) indicated that the amount of glutamate in various areas of brain slices decreased when a second KCl stimulus was given (Patterson et al. 1995). Another microsensor study also showed a significant decrease of the response between the first and second KCl exposure (Walker et al. 2007). In the present approach the S1/S2 ratio of control slices corrects for the observed decreases in glutamate output.

A possibility to decrease biofouling is the use of a Nafion layer. Nafion is commonly applied as an outer layer of a sensor to improve the biocompatibility and to diminish biofouling. These properties are mostly influenced by the thickness of the layer applied on top of the hydrogel coated enzymes. A thick Nafion layer protects better against biofouling and excludes reducing agents such as ascorbate and dopamine to a large extent, but has also shown to decrease sensitivity for glutamate. The opposite is true for a too thin layer. Applying an ideal quantity of layer is therefore tricky.

In acute slices, microsensors coated with Nafion and without Nafion were tested by measuring glutamate response to two stimuli (S1 and S2) of 120mM KCl (Figure 1). The Nafion coated sensors showed a tendency to decrease the sensitivity of the sensors, however Nafion also decreased biofouling in the slice experiments as the glutamate concentration of the second stimulus (S2) is higher in experiments were sensors were coated with Nafion. With Nafion the percentage biofouling is 3-16% while without it is 12-28%. We therefore decided to use Nafion in the following experiments.
Figure 1: Difference of a control experiment with sensors coated with and without Nafion

The microsensors coated with Nafion were also photographed with a scanning electron microscope. In Figure 2, six scanning electron microscopic micrographs (SEMMs) of the glutamate microsensors are shown. The microsensors show a mean diameter of 14 ± 3 µm. In the SEMMs photos of Oldenziel dissertation (page 21), the CF, the hydrogel and the Nafion layer are all separately visible; this is not the case in these more recent photos. Several cracks are visible in coating of the microsensors in some of the photos. These cracks could be induced by the vacuum conditions of the electron microscope (Garguilo and Michael 1994), but is more likely induced by the drying of the Nafion.

Figure 2: Scanning electronic microscope micrographs (SEMMs) of the six glutamate microsensor coated with nafion.
IV Reproducibility between batches

The reproducibility of the glutamate microsensor has been a battle, as all components of the five component hydrogel needs to in balance with one another and with enzymes in the active state, otherwise the sensitivity and selectivity of the microsensors are compromise (Heller 1992). It is also possible that in one’s laboratory the compounds can degrade over a period of time, for example glutamate oxidase may become less active over a period of weeks. The difference between three batches of glutamate microsensors prepared a few months apart were compared and evaluated with a control experiment of two potassium stimulus (S1S2). The first batch of sensors is the most sensitive to glutamate (Figure 3), and shows the least biofouling over time making this the best batch of the three.

![Graph](image)

**Figure 3:** Difference between batches of sensors coated with Nafion. Data is given as S1 and S2 where both are given as a % of control/baseline. First batch (n=8), second batch (n=7) and third batch (n=9).

SECTION B: Acute sliced of different age groups exposed to a first and second stimulus (S1 and S2) of 120mM KCl

With age synapses and neuronal organization changes, however relatively few studies have been conducted to study the relationship between glutamate and the aging of the brain. Only two *in vivo* microdialysis studies of basal glutamate in the hippocampus during aging have been reported. These two studies where however contradicting. (Massieu and Tapia 1997) found an increase in extracellular glutamate levels in aged as compared to young Wistar rats, and (Zhang *et al.* 1991) found a decrease in extracellular
glutamate levels in aged compared to young F344 rats. A study with a microsensor in the rat hippocampus has reported that resting levels of glutamate in the extracellular space were unchanged with aging and that there are subregional specific alterations in stimulus-evoked glutamate release and clearance capacity during aging Michelle et. al. (2009)

In this study we tried to determine if it was possible to measure with the glutamate microsensor difference in glutamate concentrations between various age groups of rats. Rats of different age groups were used to make acute hippocampal slices. Age groups were divided according to weight, thus group A: 130-160 g, group B: 230-260 g and group C: 310-360 g. Two stimuli (S1S2) of 60 mM or 120 mM KCl were given in order to determine if the glutamate concentration in the acute hippocampal slices increased with age (weight) (Figure 4).

The glutamate microsensors did detect in all age groups a significant increase in extracellular glutamate concentrations when an elevated [K+] (60mM or 120 mM KCl) was perfused through the slice (Table 1).

A significant increase in glutamate was seen in the acute hippocampal slices with an increase in age (weight) group F (2,131)= 4.032 p=0.020. The highest basal glutamate concentrations was seen in the oldest group C (310-360 g). It is possible that the lower glutamate concentration measured in the “younger slices” is due to tighter regulation of glutamate in the synapse of these acute slices, by glutamate transporters (Saransaari and Oja 1995). Similar results were found in a vitro study were an increase in the release of glutamate induced by K+ up to 50% in aged versus young mice was seen (Saransaari and Oja 1995).
Table 1: Posthoc Dunett T3 test for all compounds individually tested, p values are given.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Baseline &amp; S1 (%)</th>
<th>Baseline &amp; S2 (%)</th>
<th>S1 &amp; S2 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>130-160g 60mM</td>
<td>(21.06% ↑)</td>
<td>(28.38% ↑)</td>
<td>(6.05% ↑)</td>
</tr>
<tr>
<td></td>
<td>(F(2, 419=8.557 p=0.000))</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>130-160g 120mM</td>
<td>(53.19% ↑)</td>
<td>(94.46% ↑)</td>
<td>(26.94% ↑)</td>
</tr>
<tr>
<td></td>
<td>(F(2, 531=46.417 p=0.000))</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>230-260g 60mM</td>
<td>(30.82% ↑)</td>
<td>(94.46% ↑)</td>
<td>(-3.01% ↓)</td>
</tr>
<tr>
<td></td>
<td>(F(2, 419=7.602 p=0.001))</td>
<td>0.028</td>
<td>0.788</td>
</tr>
<tr>
<td>230-260g 120mM</td>
<td>(66.39% ↑)</td>
<td>(49.37% ↑)</td>
<td>(-10.23% ↓)</td>
</tr>
<tr>
<td></td>
<td>(F(2, 419=10.051 p=0.000))</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>310-360g 60mM</td>
<td>(9.85% ↑)</td>
<td>(0.65% ↑)</td>
<td>(-8.37% ↓)</td>
</tr>
<tr>
<td></td>
<td>(F(2, 701=9.366 p=0.000))</td>
<td>0.001</td>
<td>0.000</td>
</tr>
<tr>
<td>310-360g 120mM</td>
<td>(65.10% ↑)</td>
<td>(51.39% ↑)</td>
<td>(-8.30% ↓)</td>
</tr>
<tr>
<td></td>
<td>(F(2, 639=10.340 p=0.000))</td>
<td>0.000</td>
<td>0.000</td>
</tr>
</tbody>
</table>

**Figure 4:** Comparison between baseline, S1 and S2. Expressed as surface area under the curve of experiment.
SECTION C: Influence of several pharmacological agents

Acute sliced were exposed to a first stimulus (S1) of 120mM KCl and to a second stimulus (S2) of 120mM KCl combined with a pharmacological compound, to determine the degree of modulation of glutamate release induced by the pharmacological compound. As expected, in all the experiment the second stimulus (S2) induced a smaller increase than the first stimulus (S1) (Figure 5).

Table 2: Posthoc Dunett T3 test for all compounds individually tested, p values are given.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Baseline &amp; S1</th>
<th>Baseline &amp; S2</th>
<th>S1 &amp; S2</th>
</tr>
</thead>
<tbody>
<tr>
<td>TTX</td>
<td>(F(2.41)=2.916 p=0.066)</td>
<td>0.003</td>
<td>0.008</td>
</tr>
<tr>
<td>AP4</td>
<td>(F(2.41)=3.268 p=0.308)</td>
<td>0.006</td>
<td>0.018</td>
</tr>
<tr>
<td>Cd²⁺</td>
<td>(F(2.41)=2.301 p=0.114)</td>
<td>0.002</td>
<td>0.002</td>
</tr>
<tr>
<td>CPG</td>
<td>(F(2,41)=2.984 p=0.062)</td>
<td>0.010</td>
<td>0.029</td>
</tr>
<tr>
<td>HCA</td>
<td>(F(2.41)=4.045 p=0.025)</td>
<td>0.003</td>
<td>0.008</td>
</tr>
<tr>
<td>MSO</td>
<td>(F(2.41)=2.166 p=0.128)</td>
<td>0.011</td>
<td>0.002</td>
</tr>
</tbody>
</table>

Table 3: Comparison between S2 of compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>% decrease of S2 from S1</th>
<th>% difference between S2 of KCl and S2 of compound</th>
<th>Significance between S2 of KCl and S2 of compounds</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCl (control)</td>
<td>42%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TTX</td>
<td>35%</td>
<td>7% increase</td>
<td>0.993</td>
</tr>
<tr>
<td>AP4</td>
<td>39%</td>
<td>3% increase</td>
<td>1.000</td>
</tr>
<tr>
<td>Cd²⁺</td>
<td>7%</td>
<td>35% increase</td>
<td>0.975</td>
</tr>
<tr>
<td>CPG</td>
<td>41%</td>
<td>1% increase</td>
<td>1.000</td>
</tr>
<tr>
<td>HCA</td>
<td>50%</td>
<td>8% decrease</td>
<td>0.999</td>
</tr>
<tr>
<td>MSO</td>
<td>33%</td>
<td>9% increase</td>
<td>1.000</td>
</tr>
</tbody>
</table>

Glutamate derived from synaptic sources?

In an attempt to explore whether the detected glutamate is directly derived from synaptic events TTX, 4AP and Cd²⁺ were applied. TTX is a sodium channel blocker. It is the most commonly used pharmaceutical compound to determine whether the basal extracellular glutamate levels are derived from neuronal origin. 4-aminopyridine (4-AP) is one of the most commonly used potassium channel inhibitors, blocking voltage-dependent potassium and sodium activated K⁺ channels. The general effect of 4-AP on neuronal tissue is therefore to enhance neurotransmitter release at both inhibitory and excitatory
synapses. **Cadmium (Cd\textsuperscript{2+})** a non-specific blocker of voltage dependent calcium channels mediating vesicular release.

We observed no significant difference between S1 and S2 for these 3 compounds (Table 2). Where a decrease in glutamate concentration was expected after exposure to TTX and Cd\textsuperscript{2+} a non-significant increase was seen, and where a significant increase was expected after 4-AP exposure, no change was observed (Figure 5). When the S1 values of the experiments were set as 100%, again no significant differences were apparent (Figure 6). This indicates that the potassium induced glutamate as detected by the microsensors does not display the properties of synaptic release. It is generally accepted that various pools of glutamate might be present in neurons as well as in astrocytes. It is possible that glial pools of glutamate were simultaneously stimulated by high potassium. However it remains surprising that none of these stimulated pools were displayed any sensitivity to TTX, 4-AP or Cd\textsuperscript{2+}.

A possible explanation for the absence of effects of TTX might be the way it was administered to the slice. TTX is expensive in comparison to other pharmacological agents, for this reason TTX was locally applied by pipetting directly onto the surface of the slice instead of washed in. It cannot be excluded that TTX did not reach the sensor surface.

Literature data suggested similar results. In a study using acute slices where TTX and KCl were combined, the effect of KCl (30 mM) was not prevented by an addition of TTX (1 µM) (Benavides et al. 1988). In a more recent hippocampal organotypic slice study glutamate release evoked by depolarization of nerve terminals with high KCl (30 mM) was unaffected by TTX or TEA (Godino Mdel.C et al. 2007). A previous study in our laboratory showed that the basal release of glutamate in acute slices were not affected by the sodium-channel blocker TTX (Oldenziel et al. 2007).

In an early slice study, 4-AP was capable of stimulating the spontaneous release of endogenous glutamate from cerebellar slices only at a higher concentration of 1 mM (Barnes et al. 1989). Furthermore 4-AP induced a glutamate increase only in cerebellum slices but not in hippocampal slices (Patterson et al. 1995). Contrary it is now well established that 4-AP induces changes of synaptic transmission in the CA1 area of rat hippocampus on both presynaptic terminals and postsynaptic receptors of glutamate (Gu et al. 2004; Tapia and Sitges 1982). The reason why no effect was seen in glutamate levels in this study may be due to the fact that both potassium and 4-AP are depolarizing...
agents. The most potent of the two agents; the potassium stimulus (Patterson et al. 1995) may have reached a ceiling in the increase in glutamate levels in the acute hippocampal slice.

In previous studies Cd\(^{2+}\) reduced glutamate release in the hippocampal slices and culture pyramidal cells (Wu and Saggau 1994; Jabaudon et al. 1999). Oldenziel et al. (2007) found by using microsensors in acute slices, a small not significant decrease in glutamate release during Cd\(^{2+}\) application (Oldenziel et al. 2007). Contrary to expectation, in this study Cd\(^{2+}\) during high potassium application increased extracellular glutamate concentration (35%; although not statistically significant) in comparison to the control experiment.

II Glutamate derived from astrocytic sources?

The cystine-glutamate antiporter represents a nonvesicular release mechanism of glutamate which role in glutamate neurotransmission has been debated in recent years (Baker et al. 2002; Cavelier and Attwell 2005). The antiporter is a plasma membrane bound, Na\(^+\) dependent, anionic amino acid transporter that exchanges extracellular cystine for intracellular glutamate. Baker et al. (2002) demonstrated that inhibition of the cystine-glutamate exchanger decreased extracellular levels of glutamate in a microdialysis experiment in vivo. The cystine-glutamate exchange blocker (S)-4-carboxyphenylcycligine (CPG) and homocysteic acid (HCA) induced a non-significant increase (1%) and a slight but significant decrease (8%) respectively in the extracellular glutamate levels when comparing the S2 stimulus to the S2 stimulus of the control (Table 2 and 3). The interpretation of the absence of effects of CPG is hampered by the fact that CPG is also an antagonist of mGluR1 and mGluR5, and an agonist of mGluR2.

Glutamine synthetase maintains a low glutamate concentration in astrocytes by converting glutamate to glutamine (Ottersen et al. 1992; Jabaudon et al. 1999). By blocking this enzyme with L-methionine sulfoximine (MSO), a decrease in extracellular glutamine and glutamate concentrations is found in microdialysis experiments (Rothstein and Tabakoff 1984; Paulsen and Fonnum 1989), which was confirmed in Chapter 6. The decrease in glutamine obviously reflects the drug effect (i.e., inhibition of glutamine synthetase) and the decrease in glutamate may be explained by a direct MSO effect or the lack of glutamine as precursor for the resynthesis of glutamate at later stages. We assessed whether decreasing glial cell glutamate concentrations with MSO would influence the extent of glutamate released from astrocytes. However in this study MSO
induced no change in extracellular glutamate concentrations in comparison to the control experiment S2.

**Figure 5:** Comparison between S1 and S2 as percentage of control (baseline)

**Figure 6:** Comparison between S1 and S2 where S2 is a percentage of 100% S1
SECTION D: Electric stimulation

Previous studies have shown that intermittent electrical field stimulation increases the release of glutamate in the CA1 region of acute hippocampal slices. The microsensors used in these studies were multi-array electrode microsensors (MAE’s) (Table 4). In the present study electric stimulation of the CA1 region did not induce any change in glutamate concentrations as measured with the hydrogel coated glutamate microsensors (Figure 7). Furthermore local application of the high affinity transport blocker TBOA in combination with electrical stimulation did not induce the expected depolarization processes in the slice either (data not shown). The lack of change in basal glutamate levels is more likely due to the properties of the glutamate microsensor. The response time (9 sec) due to the thick hydrogel layer of the microsensor, may be too slow to detect dynamic changes in glutamate induced by electric stimulation. However it is disappointing that even with continuous electric stimulation no change in glutamate was observed during this experiment, which has been shown to be detectable in dialysates in other experiments.

Interesting is that the sensors were more sensitive and selective in the post-calibration (after the electric stimulation experiments) than in the pre-calibration. The sensors became “super sensors” as the sensors could be used again the next day for proceeding experiments after a pre-calibration, which is usually not the case (data not shown).

Figure 7: Electric stimulation of acute hippocampal slices measured with the hydrogel coated glutamate microsensor.
### Table 4: Various methods used to measure glutamate release induced by electric stimulation

<table>
<thead>
<tr>
<th>Method</th>
<th>Material</th>
<th>Effect seen</th>
<th>Author</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEA biosensor</td>
<td>Acute slices</td>
<td>Significant effect</td>
<td>(Lante et al. 2008)</td>
</tr>
<tr>
<td>Planar electrode array/</td>
<td>Acute slices</td>
<td>Significant effect</td>
<td>(Kasai et al. 2002)</td>
</tr>
<tr>
<td>glutamate sensor array (MEA biosensor)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 min microdialysis samples</td>
<td>Acute slices</td>
<td>No effect</td>
<td>(Robert et al. 1997)</td>
</tr>
<tr>
<td>20 sec microdialysis samples</td>
<td>Acute slices</td>
<td>Significant effect</td>
<td>(Robert et al. 1997)</td>
</tr>
<tr>
<td>MEA biosensor</td>
<td>Anaesthetised rat</td>
<td>Significant effect</td>
<td>(Wassum et al. 2008)</td>
</tr>
<tr>
<td>Dialysate electrode with 5 sec resolution</td>
<td>Anaesthetised rat</td>
<td>Significant effect</td>
<td>(Walker et al. 1995)</td>
</tr>
<tr>
<td>time</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutamate pt needle sensor</td>
<td>Anaesthetised rat</td>
<td>Significant effect</td>
<td>(Hu et al. 1994)</td>
</tr>
</tbody>
</table>
### Table 5: Real time monitoring in brain slices

<table>
<thead>
<tr>
<th>Group</th>
<th>Article</th>
<th>Slice</th>
<th>Brain area</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>(France)</td>
<td>(Lante et al. 2008)</td>
<td>Acute slices</td>
<td>Hippocampal</td>
<td>MEA biosensor</td>
</tr>
<tr>
<td>Westerink</td>
<td>(Oldenziel et al. 2007)</td>
<td>Acute slice Wistar Rats</td>
<td>Hippocampal</td>
<td>Second generation hydrogel coated microsensor</td>
</tr>
<tr>
<td>(Japan)</td>
<td>(Oka et al. 2007)</td>
<td>Acute slice Mice</td>
<td>Hippocampal</td>
<td>Glass capillary based enzyme electrode</td>
</tr>
<tr>
<td>Sugawara</td>
<td>(Nakamura et al. 2005)</td>
<td>Acute slice ddY Mice</td>
<td>Hippocampal</td>
<td>Glass capillary based enzyme electrode</td>
</tr>
<tr>
<td>Sugawara</td>
<td>(Nakajima et al. 2003)</td>
<td>Acute slice ddY Mice</td>
<td>Hippocampal</td>
<td>Glass capillary based enzyme electrode</td>
</tr>
<tr>
<td>Torimitsu</td>
<td>(Kasai et al. 2002)</td>
<td>Acute slice Wistar Rats</td>
<td>Hippocampal</td>
<td>Planar electrode array/ glutamate sensor array</td>
</tr>
<tr>
<td>Torimitsu</td>
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<td>Acute slice Wistar Rats</td>
<td>Hippocampal</td>
<td>Planar electrode array/ glutamate sensor array</td>
</tr>
</tbody>
</table>
4. CONCLUSION
Acute hippocampal slices are an important research tool in this process of evaluating the glutamate microsensor properties. In contrast to in vivo experiments, brain slices allow a closer examination of cellular activity while experimental conditions can be better controlled and manipulated. Not many studies (7 studies up to now Table 5) have been reported using a biosensor or microsensor for measuring glutamate in slices. This is probably due to the fact that there are technical difficulties in the construction and practical difficulties in the application of a miniaturized sensor to slices. The methods for obtaining real-time measurements are still limited.

The microsensors enable us to monitor basal changes in glutamate levels in the acute hippocampal slices. In this study it was found that Nafion does prevent biofouling to some extent. We studied whether the potassium-induced increase in glutamate release varied among rats of different age. We could detect a differences between the various age groups; aged rats responded more pronounced than elevated potassium than younger ones.

Oldenziel reported by using the hydrogel glutamate sensor, changes in glutamate concentrations in acute slices after various pharmacological conditions (Oldenziel et al. 2007). In this study we have repeated these experiments during high-potassium infusions. We could however not detect any pharmacological effect of TTX, 4AP, Cd$^{2+}$, HCA, CPG and MSO in the presence of high-potassium. Elevated glutamate concentrations due to electric stimuli in the acute hippocampal slices were not detected by the hydrogel coated glutamate microsensor. The lack in detection of electric stimuli in the slices might be due to the long response time of the glutamate microsensor. The thick hydrogel layer and Nafion layer increases the diffusion time of glutamate and subsequently the response time of the sensor.

It is concluded that the high potassium-approach in combination with the slow response time of the hydrogel coated glutamate sensor may have been too rudimentary to measure the subtle pharmacological changes of glutamate in the acute slices.