Application of a glutamate microsensor to brain tissue

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Chapter 7

In vivo monitoring of extracellular glutamate in the brain with a microsensor

This chapter is based on the following paper:

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Abstract

Recent discoveries have revealed that glutamatergic neurotransmission in the central nervous system is mediated by a dynamic interplay between neurons and astrocytes. To enhance our understanding of this process, the study of extracellular glutamate is crucial. At present, microdialysis is the most frequently used analytical technique to monitor extracellular glutamate levels directly in the brain. However, the neuronal and physiological origin of glutamate detected by microdialysis is questioned, as it does not fulfil the classical release criteria for exocytotic release, such as calcium dependency or response to the sodium-channel blocker tetrodotoxine (TTX). It is hypothesized that an analytical technique with a higher spatial and temporal resolution is required. Glutamate microsensors provide a promising analytical solution to meet this requirement. In the present study we applied a 10 µm diameter hydrogel-coated glutamate microsensor to monitor extracellular glutamate levels in the striatum of anesthesized rats. To explore the potential of the microsensor, different pharmacological agents were injected in the vicinity of the sensor at an approximate distance of 100 µm. It was observed that KCl, exogenous glutamate, kainate and the reuptake inhibitor $_{DL}$-threo-β-benzyloxyaspartate ($_{DL}$-TBOA) increased the extracellular glutamate levels significantly. TTX decreased the basal extracellular glutamate levels approximately 90 %, which indicates that the microsensor is capable of detecting neuronally derived glutamate. This is one of the first studies in which a microsensor is applied in vivo on a routine base. It is concluded that the microsensor can contribute significantly to improve our understanding of the physiology of glutamatergic neurotransmission in the brain.

7.1 Introduction

l-Glutamate is the predominant excitatory neurotransmitter in the central nervous system (CNS) and is involved in most aspects of normal brain functioning, such as cognitive processes, the formation of memory and the development and plasticity of the CNS. Consequently, glutamate is involved in the pathophysiology of many neurological, neurodegenerative and psychiatric disorders, such as epilepsy, Parkinson's disease, Alzheimer's disease, amyotrophic lateral sclerosis, depression, stroke and schizophrenia (Danbolt, 2001; Coyle et al., 2002; Javitt, 2004).

Recent discoveries have revealed that the physiology of glutamatergic neurotransmission in the CNS is mediated by an intimate partnership between neurons and astrocytes (Haydon 2001; Nedergaard et al., 2002; Volterra and Meldolesi, 2005). To further improve our understanding of these processes, the assessment of extracellular glutamate in the brain is crucial. Several analytical techniques are currently being applied to monitor glutamate in the brain, e.g. microdialysis (Ungerstedt, 1991), push-pull perfusion (Gaddum, 1961), or direct sampling by fused silica tubing in combination with capillary electrophoresis (Kennedy et al., 2002). Microdialysis is by far the most frequently used of these techniques. However, the physiological origin of glutamate in dialysate is uncertain (Westerink et al., 1987; Miele et al., 1996; Timmerman and Westerink, 1997; Drew et al., 2004). It appears that glutamate detected by microdialysis does not fulfil the classical release criteria for exocytotic release, as it does not show calcium dependency or response to sodium-channel blockade. Detection of extracellular glutamate with a higher spatial- and temporal resolution is apparently required to facilitate sampling of the synaptic pool (Rossel et al., 2003; Drew et al., 2004).

Evidence is provided that microsensors may give a closer approach to the glutamatergic physiology in the brain due to their analytical properties (Hu et al., 1994; Kulagina et al., 1999; Burmeister and Gerhardt., 2001; Rahmann et al., 2005; Day et al., 2006). However, until now glutamate microsensors are hardly used on a routine base. It appears that the use of microsensors is often hampered by technical difficulties in their construction and application.

To overcome these technical difficulties, we gave much attention to the construction and characterization of a 10 µm diameter hydrogel-coated glutamate microsensor (chapters 2-4). In addition, we have evaluated the microsensor both *in*

vivo and *in vitro* (chapter 5 and 6). In the present study the glutamate microsensor is applied on a routine base for monitoring extracellular glutamate in the striatum of anesthesized rats. The influence of different pharmacological agents, which are known to affect the release of glutamate, was investigated.

7.2 Experimental section

Animals and *in vivo* experiments

Male albino rats of a Wistar-derived strain (275-320 gr; Harlan, Zeist, The Netherlands) were used for the *in vivo* experiments. Rats were anaesthetized with Equitensine (Bo et al., 2003), placed in a stereotaxic frame (Kopf, Tujunga, CA, USA) and kept unconscious with additional doses of Equitensine. The body temperature was monitored and maintained at 37°C with a homeothermic blanket (Temperature controller CMA 150; CMA, Solna, Sweden). Small holes were drilled in the skull for insertion of both microsensors (a glutamate and background microsensor) and the micropipette. Both microsensors were placed in close proximity (< 200 µm) in a V-shaped form. The microsensors were implanted in the striatum at an angle of 25 ° at the following coordinates, AP: + 0.9, ML: - 6.1 and VD: - 7.5 mm from bregma point and dura respectively (Paxinos and Watson, 1986).

Drugs were applied via intracranial injection with a micropipette. Micropipettes were prepared by pulling a glass capillary (0.8 mm i.d., 1 mm o.d.; TW100F-3, World Precision Instruments, Sarasota, FL) with a capillary puller (PN-3, Narishige, Tokyo, Japan). The tip of the pulled glass capillary was bumped to a diameter of 10-20 µm and the shank was also pulled as small as possible to minimize tissue trauma. Before an experiment, the micropipette was filled with a solution of interest and was connected to the picospritzer (Picospritzer III, Intracell, Herts, UK). The micropipette was implanted at an approximate distance of 100 µm from the microsensors at the following coordinates AP: + 0.9, ML: -3.0 and VD: - 6.0 mm. The implantation of the microsensors and micropipette was controlled with a stereomicroscope (Exacta-Optech, Diever, the Netherlands). The Ag/AgCl reference electrode was coated with Nafion in a similar way as the microsensors to prevent biofouling (Moussy and Harrison, 1994), and was placed in the prefrontal cortex. After implantation of the microsensors and micropipette, a stabilization period of minimally 1 hr was maintained. Different volumes (200 nl, 500 nl, 1 µl, or 2-3 µl) of drugs were injected intracranially via the picospritzer (general valve operation: 50 psi; 0.1-1.5 sec). All the drugs were dissolved in Artificial Cerebrospinal Fluid (aCSF; see below). Note, because many drugs diffuse poorly in the brain, relatively high concentrations were applied (Burmeister and Gerhardt, 2001; Westerink and de Vries, 2001).

After the *in vivo* experiment was finished, the brain was removed and the placement of the microsensors and micropipette was retrospectively verified by histological inspection. Slices of approximately 400 µm were prepared from the brain with a cryostat (CM3050S, Leica Microsystems, Germany) and were investigated under a microscope. Slices were prepared in a semi-coronal way (oblique) to examine the track of microsensors and micropipette as good as possible. The distance between the microsensors and micropipette was inspected in particular. No staining procedures were used. Experiments were approved by the Animal Care Committee of the College of Mathematics and Natural Science of the University of Groningen.

Microsensors

Microsensors (glutamate- and background sensors) were constructed as reported in the previous chapters. Briefly, carbon fiber electrodes (CFEs) with a diameter of 10 µm were trimmed to a length of 300-500 µm. The microsensors were prepared by coating the CFE with a five-component redox-hydrogel, in which l-glutamate oxidase, horseradish peroxidase and ascorbate oxidase were wired via poly(ethyleneglycol) diglycidyl ether to an osmium containing redox polymer (abbreviated as POs-EA). A thin Nafion coating completed the construction. Note, background microsensors were prepared in a similar way as glutamate microsensors, only Glu-ox was not incorporated into the hydrogel. The *in vivo* experiments were carried out by amperometrically operating the microsensor at a constant potential of – 150 mV versus an Ag/AgCl reference electrode [0.15 M NaCl]. Before the microsensors were used *in vivo*, they were calibrated in a flow-injection analysis system (FIA). Several microsensor properties were determined during this calibration, as described in before chapter in 3: shape and height of the CV (nA; determined as the absolute value of the anodic (I_{pa}) minus the cathodic (I_{pc}) peak current), sensitivity (pA/µM), interference (%), linearity (R^2), detection limit (µM; 3 times signal to noise ratio) and response time (sec; time required for the signal to increase from 10 to 90 %).

After the *in vivo* experiment was finished, the microsensors were calibrated again at conditions mimicking the *in vivo* situation most closely: the sensitivity for glutamate in the presence of 400 µM ascorbic acid and 100 µM uric acid at oxygen deprived conditions (~ 2 %), as indicated in chapter 5. This sensitivity was used to correlate the detected current (pA) to final extracellular glutamate concentrations. In

addition, the drugs that were applied in the *in vivo* experiments were also injected during the calibration. None of the investigated drugs induced an effect.

Reagents

Glutamate oxidase (Glu-ox; 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 (PEGDGE), silver chloride and Nafion (5 % Nafion solution,1100 equivalent weight) and all other chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA). Salts, acetone p.a. and 2-propanol p.a. were obtained from Merck (Darmstadt, Germany). $_{DL}$-TBOA was obtained from Tocris Bioscience (Bristol, UK). Ascorbate oxidase was initially obtained form Seravac (Cape Town, South Africa; Product nr. 011530), but was purified prior to its use, as indicated in chapter 4. aCSF was used for the calibration procedures and for the dissolution of drugs. It had the following composition: 145 mM Na^+, 1.2 mM Ca^{2+}, 2.7 mM K^+ , 1.0 mM Mg^{2+}, 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). Enzyme solutions were made in HEPES buffer. This buffer was prepared by adding the salt form of HEPES to a 10 mM solution of the acid form, until pH 8.

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 gray area (SEM). Each experiment (n) represents a different animal investigated with a separate set of microsensors. Sigmastat 3.0 was used to calculate statistics. Statistical analysis was performed using One Way Anova with repeated measures followed by a Student-Newman-Keuls posthoc test. 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.

7.3 Results

Microsensor recordings in the striatum.

The experiments in the present study were conducted by placing both a glutamate and background microsensor together with a micropipette in the striatum of anesthesized rats. The current output of both microsensors was monitored and the difference in current was considered to represent the extracellular glutamate concentration ($[Glu]_0$). Different pharmacological agents were applied via a micropipette and their influence on the microsensor recordings was monitored.

The difference in current output between the glutamate and background microsensor after drug administration was divided by the difference prior to administration (set at 100 %) and expressed as percentage (%).

The placement of the microsensor in the brain was investigated retrospectively by histological examination. A typical example of a histological section is presented in Fig 1. Visualized are the implantation sites of the micropipette and of a microsensor in the striatum. Only one microsensor is visible as the other one was situated more anterior. To follow the track of both the micropipette and sensor as good as possible, slices were prepared in a semi-coronal way (oblique).

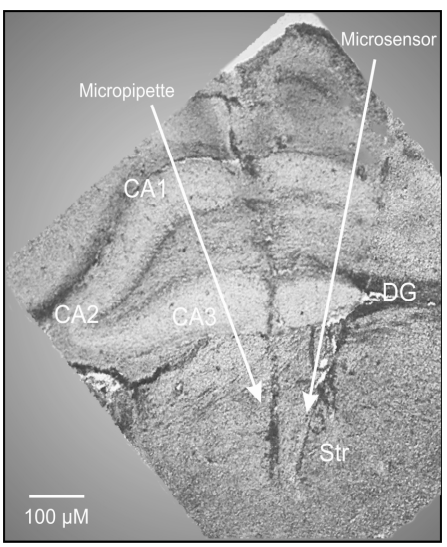

Figure 1: An example of a histological section. Visualized are the track of the micropipette and of one microsensor. Note: the slice was prepared in a semi-coronal way (oblique) to follow the track of the microsensor and micropipette.

Basal extracellular glutamate levels

In a total of 85 experiments the average current output of the glutamate microsensor was 53.9 ± 5.1 pA (mean ± SEM), and of the background sensor 41.3 ± 4.1 pA. When the difference in current between the glutamate and background microsensor for each individual experiment was examined, a statistically significant difference of 10.1 ± 5.3 pA was observed ($p < 0.05$; Mann-Whitney Rank Sum Test). To correlate this current to final glutamate concentrations, the microsensor needs to be calibrated at conditions that are encountered *in vivo*. The average sensitivity of the microsensor under these conditions was approximately 0.55 pA/µM (chapter 5), which indicates that the difference between both sensors represents a $[Glu]_0$ of 18.2 µM ± 9.3 µM. This sensitivity is displayed as a footnote in the figures.

During the experiments we observed that the depth of anaesthesia affected the current output of the microsensors, i.e. the deeper the level of anaesthesia, the lower the current output of the microsensors. For example, when the anaesthetic tended to wear off, which was monitored by hind-limb compression, an increase in the current output of the microsensors was observed (results not shown). Throughout this study the anaesthetic equitensine was used, as this compound is known to have lesser effect on the extracellular glutamate concentrations in comparison to other anaesthetics (Lada et al., 1998; Rozza et al., 2000; Bo et al., 2003). In addition, a dramatic decrease of the microsensor recordings was observed when the rat was euthanized with an overdose of pentobarbital (chapter 5).

Influence of different pharmacological agents.

At first, the influence of a saline injection was investigated (Fig. 2). Different volumes of saline (aCSF) were injected, respectively 200 nl, 500 nl, 1 µl and 2-3 µl, which did not affect the microsensor recordings. The distance between the microsensor and the micropipette was approximately 100 µm. In some experiments a dilution or pressure artifact was observed, in particular when the micropipette was situated closer (< 100 µm) to the microsensors (observed by retrospective histological examination).

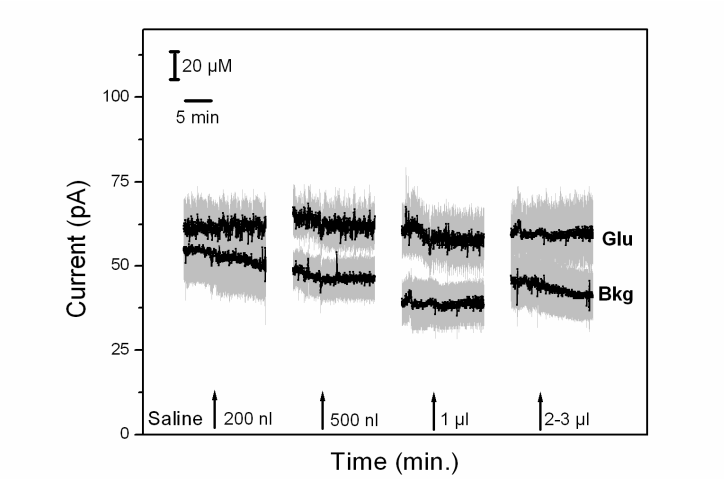

Figure 2: Influence of saline injections on the extracellular glutamate levels. A subsequent range of 200 nl (n = 4), 500 nl (n = 4),1 µl (n = 4) and 2-3 µl (n = 4) of saline was applied and the glutamate levels detected by the glutamate (Glu) and background (Bkg) microsensors were monitored.

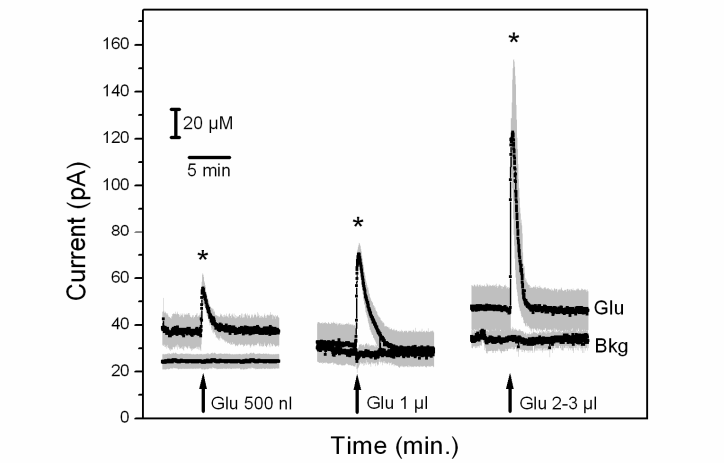

*Figure 3: Influence of exogenous glutamate administration on the glutamate detection properties of the microsensors. A subsequent range of 500 nl (n = 6), 1 µl (n = 8) and 2-3 µl (n = 6) of 1 mM glutamate was applied and the glutamate levels detected by the glutamate (Glu) and background (Bkg) microsensors were monitored. * Denotes a statistical significant difference (p < 0.001; One Way Anova followed by a Student-Newman-Keuls posthoc test).*

To investigate whether the microsensors were capable of detecting dynamic changes of glutamate in the brain, the influence of exogenous glutamate administration was investigated. In Fig. 3 a subsequent range of different volumes (500 nl, 1 µl and 2-3 µl) of 1 mM glutamate was applied, which increased the detected glutamate levels dose-dependently 260 % [$F(10,65) = 11.003$, $p < 0.001$], 500 % [$F(10,87) = 26.596$, $p < 0.001$] and 750 % [$F(10,65) = 8.148$, $p < 0.001$] respectively. The following dynamics were observed: the response time was approximately 6 sec and after 15-20 sec the glutamate levels started to decline. Finally, after approximately 2 minutes the detected glutamate returned to basal values.

To further explore the potential of the microsensor, the influence of a subsequent range of different volumes (500 nl, 1 µl and 2-3 µl) of 120 mM KCl was examined (Fig. 4). The different volumes induced a dose-dependent increase in $[Glu]_0$ of 150 % [$F(10,76) = 5.547$, $p < 0.001$], 230 % [$F(10,65) = 10.375$, $p < 0.001$] and 400 % [$F(10,65) = 8.342$, $p < 0.001$] respectively. Again, short lasting elevations of $[Glu]_0$ were observed with the same dynamics as observed with exogenous glutamate application.

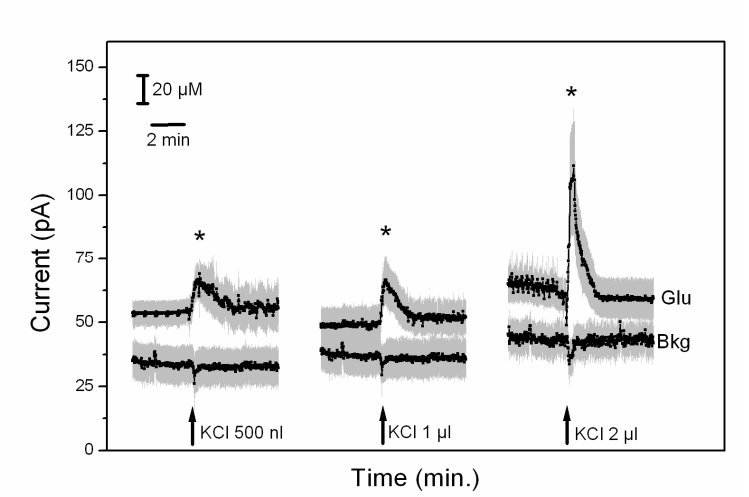

*Figure 4: Influence of KCl on the extracellular glutamate levels. A subsequent range of 500 nl (n = 7), 1 µl (n = 6) and 2-3 µl (n = 6) of 120 mM KCl was applied and the glutamate levels detected by the glutamate (Glu) and background (Bkg) microsensors were monitored. * Denotes a statistical significant difference (p < 0.001; One Way Anova followed by a Student-Newman-Keuls posthoc test).*

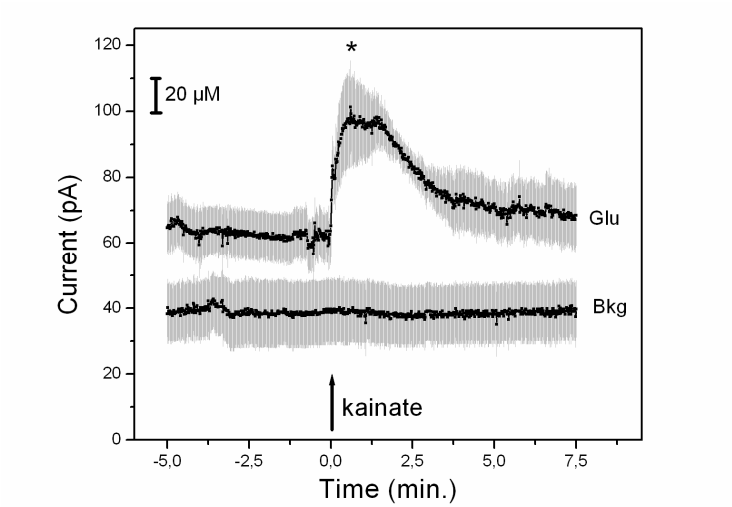

*Figure 5: Influence of kainate on the extracellular glutamate levels. 500 nl of 1 mM kainate (n = 5) was applied and the glutamate levels detected by the glutamate (Glu) and background (Bkg) microsensors were monitored. * Denotes a statistical significant difference (p < 0.001; One Way Anova followed by a Student-Newman-Keuls posthoc test).*

The influence of the excitatory amino acid kainate was investigated in Fig. 5. Injection of kainate (1 mM, 500 nl) increased the glutamate levels approximately 250 % [$F(10,65) = 8.149$, $p < 0.001$]. Interestingly, a slower response in glutamate was observed when compared to the effects of exogenous glutamate and high [K^+].

Next, the effect of the reuptake inhibitor $_{DL}$-threo-β-benzyloxyaspartate ($_{DL}$-TBOA) was investigated (Fig. 6). It was observed that $_{DL}$-TBOA (1 mM, 500 nl) increased the glutamate levels to approximately 175 % of controls [$F(5,47) = 4.504$, $p = 0.003$]. The increase of glutamate was slower when compared to the effects of exogenous glutamate and high [K^+]. Furthermore, the elevation of $[Glu]_0$ was more persistent.

To investigate to which extent the basal $[Glu]_0$ was derived from exocytotic release, the influence of the sodium channel blocker tetrodotoxine (TTX) was investigated (Fig. 7). Blockade of the sodium channels terminates the depolarization processes and consequently the action-potential-dependent vesicular release of glutamate. It was observed that application of TTX (500 µM, 500 nl) decreased the glutamate levels to approximately 90 % of basal levels [$F(10,109) = 5.422$, $p < 0.001$].

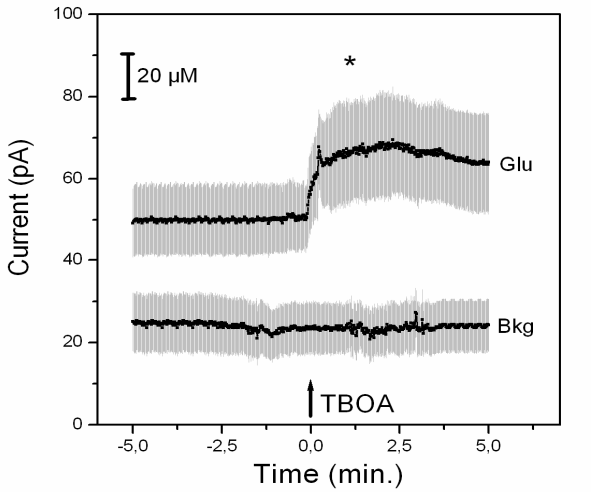

*Figure 6: Influence of $_{DL}$-TBOA on the extracellular glutamate levels. 500 nl of 1 mM $_{DL}$-TBOA (n = 7) was applied and the glutamate levels detected by the glutamate (Glu) and background (Bkg) microsensors were monitored. * Denotes a statistical significant difference (p < 0.05; One Way Anova followed by a Student-Newman-Keuls posthoc test).*

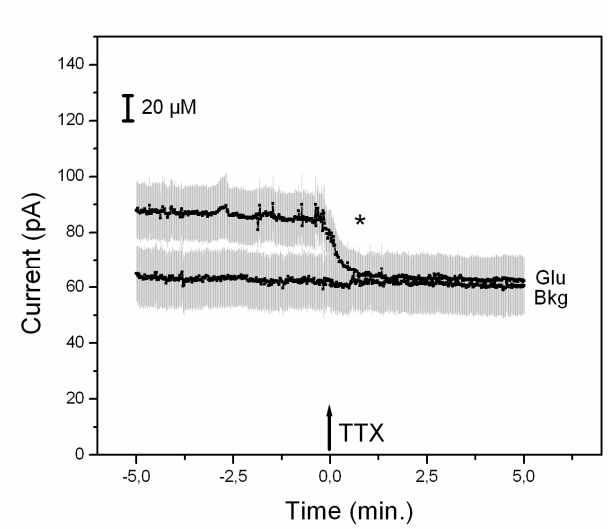

*Figure 7: Influence of TTX on the extracellular glutamate levels. 500 nl of 500 µM TTX (n = 10) was applied and the glutamate levels detected by the glutamate (Glu) and background (Bkg) microsensors were monitored. * Denotes a statistical significant difference (p < 0.001; One Way Anova followed by a Student-Newman-Keuls posthoc test).*

7.4 Discussion

Microsensor recordings in the striatum

In the present study the potential of the hydrogel-coated glutamate microsensor as an analytical tool *in vivo* was explored. Compared to other analytical techniques, such as microdialysis, the microsensor has several advantages. First, due to its small dimensions, it will induce less brain damage, which might result in the detection of a more physiological relevant extracellular glutamate pool (Khan and Michael, 2003; Drew et al., 2004). Secondly, the sensor is able to detect second to second changes of glutamate, as the total response time of the microsensor is approximately 8 seconds. Although this is far too slow to detect millisecond changes of fast synaptic glutamatergic neurotransmission (Greengard, 2001), it is much faster than most other recording techniques. Third, the microsensor displays a high specificity and selectivity for glutamate over a wide linear concentration range.

For detection of extracellular glutamate levels both a glutamate and background microsensor were used. The signal detected by the glutamate microsensor is derived from glutamate and from a non-specific electrochemical origin, whereas the background microsensor only detects the latter. Subtraction of both signals theoretically represents the extracellular glutamate concentration. However, these results need to be interpreted with some caution, as slight variations in the non-specific electrochemical signal can contribute considerably to the final current output of the sensor. In addition, the depth of anesthesia can also affect the output of the sensors.

Extracellular glutamate levels

As the extracellular glutamate levels are detected as current (pA), an important question is how this current is related to actual glutamate concentration in the brain. In chapter 5 we have observed that several crucial parameters significantly determined the sensitivity of the microsensor. Calibration of the microsensor for these parameters is necessary, which are: biofouling (Wisniewski et al, 2000), high concentrations of reducing agents, such as ascorbic acid and uric acid (O'Neill et al., 1998; Gilgun-Sherki et al., 2001; Rice, 2001) and restrictive oxygen levels of 2-7 %; (Hu and Wilson, 1997; Masomoto et al., 2003). Each microsensor was calibrated post *in vivo* under these conditions and the average sensitivity for glutamate was

approximately 0.55 pA/µM. This implies that the average difference between the current output of the glutamate and background sensor in this study represented a $[Glu]_0$ of approximately 18.2 µM ± 9.3 µM. This value is in good accordance with some microsensor observations (Kulagina et al., 1999; Oldenziel et al, 2006b, Rutherford et al., 2006; Stephens et al., 2006) and with observations in which an enzyme electrode was integrated into a microdialysis probe (Zhao et al., 1997; Guyot et al., 2001), but is much higher than detected in other studies. For comparison, most microdialysis studies report basal glutamate concentrations in the low micromolar range (1-5 µM). In addition, other microsensor studies (Hu et al., 1994; Rahman et al., 2005), and a study in which direct sampling by fused silica tubing is combined with capillary electrophoresis (Kennedy et al., 2002) also have reported these low basal glutamate values. Consequently, the true extracellular glutamate concentration in the brain is still a matter of debate. It is known that peak concentrations of several millimolar are reached in the synaptic cleft (Bergles et al., 1999) and that glutamate hardly diffuses out of the cleft, due to the presence of high densities of excitatory amino acid transporters (EAATs) (Danboldt, 2001; Del Arco et al., 2003). In addition, an interesting question is to what extent the different analytical methods monitor synaptically released glutamate. The spatial resolution of the analytical technique is of utmost importance in this debate. On one hand it has been claimed that an analytical technique with a poor spatial resolution underestimates the extracellular glutamate concentration, because it induces extensive brain damage, which interferes with the physiology of glutamatergic transmission (Khan and Michael, 2003). On the other hand it has been hypothesized that it overestimates the extracellular glutamate concentration, because glutamate can accumulate in the damaged area, which lacks the access of the highly active reuptake transporters (Nedergaard al., 2002; Cavelier et al., 2005). Compared to other analytical techniques, the hydrogel-coated glutamate microsensor has by far the best spatial resolution, and although the dimensions of the sensor (10 µm) are much larger than the width of the synaptic cleft (~ 50 nm), less brain damage and a closer approach of synaptic activity can account for the relatively high basal glutamate levels. The finding that TTX induced a significant decline in basal extracellular glutamate levels, as discussed below, supports this idea.

Influence of different pharmacological agents

To explore the potential of the microsensor, different pharmacological agents, which are known to influence the release of glutamate, were injected close to the sensor. First the influence of microinjection itself was investigated. It was observed that saline injections did not affect the microsensor recordings (Fig. 2). In contrast with other microsensor studies (Burmeister et al., 2001; Burmeister et al., 2002; Day et al., 2006), which have reported small dilution or pressure artifacts caused by microinjection. However, in those studies the distance between the micropipette and microsensors was smaller, i.e. 50-100 µm, instead of approximately 100 µm in the present study. In some of our experiments, when the micropipette was situated closer to the microsensors, we also observed those type of artifacts.

To investigate whether the microsensors were capable of detecting dynamic changes of glutamate in the brain, different volumes of exogenous glutamate were applied. Microinjection of exogenous glutamate was clearly and dose-dependently detectable by the sensors (Fig. 3). Note, it is possible that endogenous glutamate, which is triggered by exogenous glutamate via hetero-exchange and transporter-mediated release, also may contribute to the detection (Nedergaard et al., 2002; Volterra and Meldolesi, 2005). Remarkable are the fast dynamics of the glutamate peaks, indicating an effective uptake by the high-affinity membrane transporters (Danboldt, 2001). This is in agreement with the more sustained elevation in extracellular glutamate when the transporters were blocked by $_{DL}$-TBOA, as discussed below.

When different concentrations of elevated [K^+] were injected, dose-dependent increases of glutamate were observed (Fig. 4). Similar results were obtained with other microsensor studies (Hu et al., 1994; Burmeister et al., 2001; Burmeister et al., 2002). Elevated [K^+] causes massive depolarisation of neurons and, consequently, release of glutamate from both vesicular and cytosolic pools. In addition, astrocytes may also contribute to the release of glutamate, as high [K^+] causes release of glutamate as part of the internal cell volume regulation (Kimelberg et al., 1995; Leis et al., 2005). At present it is thought that the type 1 electroneutral Na-K-Cl cotransporter (NKCC1) plays an important role in this regulation (Chen and Sun, 2005).

The experiments with exogenous glutamate and elevated [K^+] indicate that the temporal resolution of the microsensor is of crucial importance for detecting dynamic changes of extracellular glutamate in the brain. To what extent the properties of the

microsensor itself affect the dynamics of the detected glutamate peaks is not known. On one hand it is expected that the relative slow response time of the microsensor (~ 8 sec) limits the rise time of the induced signal. On the other hand, it is expected that the balance between the (slow) diffusion of both exogenous glutamate and KCl through the brain and their fast uptake in brain cells also affects monitoring of glutamate (Westerink and de Vries, 2001; Syková, 2004). In this respect, the exact distance between the micropipette and the microsensors is critical and minor differences can induce large variations in the detected glutamate levels (Burmeister et al., 2002).

Next, the excitatory amino acid kainate was applied to investigate the applicability of the sensor in receptor mediated release (Fig. 5). Kainate induced a significant increase in extracellular glutamate, which was higher, with faster dynamics as observed in microdialysis studies (Butcher et al., 1987; Smolders et al., 1996; Bianchi et al., 1998). Likely the detected glutamate originates from vesicular origin, as activation of the kainate receptor elicits depolarization and firing of action potentials (Huettner, 2003; Lerma, 2003). However, the release of glutamate due to neuronal death cannot be ruled out completely, as kainate is also a potent neurotoxin. However, if this also occurs on such a short time-scale is currently not known. Remarkable are the slower dynamics of the kainate induced increase of glutamate in comparison to the KCl and exogenous glutamate data. This can be explained by the fact that kainate induces a receptor-mediated release, whereas high-K^+ causes an instantaneous depolarisation, and administration of exogenous glutamate is immediately detected by the sensor.

The non-transportable reuptake inhibitor $_{DL}$-TBOA, which is a blocker of the glutamate transporters EAAT1-3 (i.e. GLAST, GLT-1 and EAAC1), induced a significant elevation in extracellular glutamate (Fig. 6). An absolute increase of about 18 µM was observed, which is in good accordance with microdialysis experiments (Montiel et al., 2005). When compared to the previous data, a more sustained elevation was observed. This can be explained by the fact that $_{DL}$-TBOA and exogenous glutamate are interacting competitively with the membrane transporter, whereas the other compounds induce an immediate effect. Interestingly, the different experiments underline that the temporal resolution of the microsensor is of crucial importance for detecting dynamic changes of extracellular glutamate in the brain.

The $_{DL}$-TBOA data indicate that a constant tonic release of glutamate was present, although active release of glutamate from astrocytes by heteroexchange can not be ruled out completely, as the non-transportable $_{DL}$-TBOA partly behaves as a transportable inhibitor on astrocytes (Anderson et al., 2001). To investigate to what extent the basal extracellular glutamate levels were derived from neuronal origin, the potent sodium channel blocker TTX was applied (Fig. 7). Interestingly, TTX decreased the basal extracellular glutamate levels to a large extent (~ 90 % of basal levels), which implies that basal extracellular glutamate levels might be derived predominantly from exocytotic release. This observation is in contrast with microdialysis literature and confirms the results of two other studies using glutamate microsensors (Kulagina et al., 1999; Day et al., 2006). Apparently, the microsensor approaches the synaptic cleft more closely than the microdialysis probe does, which is most likely because its dimensions are more than a 1000 fold smaller than those of a microdialysis probe. At present, more refined experiments are in progress, i.e. lower volumes and concentrations of TTX in combination with other pharmacological agents, to further explore the potential of the microsensor.

7.5 Conclusion

In the present study the hydrogel-coated glutamate microsensor was applied on a routine base to the brain of anesthesized rats. It was observed that by injecting different pharmacological agents close to the microsensor (~ 100 µm), the sensor was capable of detecting characteristic changes in the release of glutamate. Moreover, the high temporal resolution allowed a second-to-second monitoring of extracellular glutamate. The observation that the recorded glutamate was largely TTX-dependent is of great significance and illustrates that the sensor is able to sample neuronally released glutamate. Therefore, the microsensor can contribute significantly to improve our understanding of the physiology of glutamatergic neurotransmission. When used in combination with other techniques, such as electrophysiological recordings, electrical stimulation or microdialysis, an even more powerful analytical tool may be generated. An attractive hypothesis is that microsensors monitor synaptically released glutamate, whereas microdialysis samples glutamate that is released from various other (astrocytic) sources. The latter hypothesis is currently under investigation.