Application of a glutamate microsensor to brain tissue
Oldenziel, Weite Hendrik

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2006

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.
Chapter 8

A step forward in the detection of extracellular glutamate?
General discussion and conclusion.
Abstract

The aim of the research presented in this thesis was to evaluate the hydrogel-coated glutamate microsensor technique and to apply it as an analytical tool on a routine base. During this process many methodological pitfalls were encountered. This chapter discusses the most crucial steps of these aspects. In addition, some recommendations for future research are made.
8.1 Introduction

The assessment of extracellular glutamate in the brain of living animals (in vivo) and in brain slices (in vitro) is of crucial importance to improve our understanding of glutamatergic neurotransmission. However, the analytical techniques that are currently applied for this purpose do not fulfi l the necessary requirements. It appears that most methods applied in vivo (e.g. microdialysis) do not detect neuronally derived glutamate, whereas a technique that detects extracellular glutamate within a slice is currently not even available.

Glutamate microsensors form a promising answer for both applications. However, despite the fact that research on the development of a glutamate microsensor has been performed for more than a decade now, its practical application is scarce. It appears that progress in the use of microsensors is hampered by technical difficulties in their construction and application. This is illustrated by the fact that reports on promising microsensor concepts are often not followed by routine applications (e.g. Hu et al., 1994; Ryan et al., 1997; Lowry et al., 1998; Kulagina et al., 1999; Mikeladze et al., 2002).

A promising microsensor concept was published by Kulagina et al. (1999). Until recently (but see Day et al., 2006), this microsensor was the only one that has reported the detection of TTX-dependent extracellular glutamate levels. This result formed the direct motive for the aim of this thesis: the introduction and evaluation of this specific analytical technique, and its application in neurochemical research. However, it appeared that many basal questions concerning the use of this microsensor were not answered. Therefore, we decided to investigate the encountered methodological problems step by step. As a consequence, this thesis is divided in three parts. First, the construction of the microsensor (chapters 2-4). Second, a critical evaluation of its analytical properties (chapter 5) and third, its practical application (chapters 6 and 7). In this chapter the results are summarized and discussed, and recommendations for future research are made.

8.2 Remarks on the construction of the microsensor

The reproducible construction of the microsensor appeared to be a critical step in its practical use. In particular, the process of polymerisation of the five component
hydrogel solution on the microsurface of the CFE appeared to be a difficult procedure to control. Many parameters can influence this process and therefore much attention was paid to this subject in the first three chapters (chapters 2-4). It appeared that the reproducible construction of the microsensor could be significantly improved by using an automated dipcoater (chapter 2).

In addition, a most critical property of the microsensor appeared to be the thickness of the hydrogel layer. Microscopic inspection revealed that a hydrogel layer with a certain constitution (smooth, uniform) and thickness (~3 μm) was required for an optimal performance of the microsensor. In chapter 2 a successful attempt was made to control the thickness of the hydrogel layer by monitoring its electrical resistance. This application was not carried out in later studies due to the fact that the resistance was also dependent on the composition of the hydrogel constitution, which was optimized and controlled first in chapters 3 and 4. However, as most construction steps are standardized now, it is worthwhile to include this method again in future research.

8.3 Evaluation of the analytical properties of the microsensor

During the characterization and evaluation of the sensor we observed that the sensor had specific analytical advantages and disadvantages (chapter 5). For example, of advantage were its selectivity, specificity, linear range and stability, whereas the interference by reducing agents (e.g. ascorbic acid and uric acid), biofouling and oxygen deprivation limited the performance of the sensor. Consideration of these parameters is of crucial importance for its practical application.

Reducing agents

A most critical issue appeared to be the interference by reducing agents, predominantly by ascorbic acid. This interference limited the sensitivity of the sensor to a large extent. Therefore, much attention has been paid to this subject throughout the different studies (chapters 2-5). Although the problem is not fully solved yet, currently it is at a certain level of control. Improving the construction of the sensor (chapter 2), optimizing the composition of the hydrogel (chapter 3) and purification of ascorbate oxidase (chapter 4) were of crucial importance for this process. Throughout the years we also have carried out many pilot studies to solve this problem (results
not shown), e.g. incorporation of discriminating layers outside and within the hydrogel (e.g. Nafion, PPD, PEA), incorporation of electrochemical active compounds into the hydrogel (e.g. FAD), enlargement of the CF surface by incorporation of carbon fragments, modifying the architecture of the hydrogel by incorporation of PEI, sucrose or glucose, etc. It appeared that none of these approaches was of additional advantage to prevent the interference by reducing agents.

**Nafion layer**

In our research we have applied Nafion commonly as an outer layer on the sensor. The application of Nafion, which is a commercially available perfluorsulfonic acid, serves two purposes. At first, it should form a barrier to diffusion of anionic components, which includes most reducing agents. Secondly, it should prevent biofouling on the sensor. However, it is concluded that the contribution of the Nafion layer to the performance of the microsensor is low: it does not discriminate between glutamate and reducing agents, while it reduces the sensitivity of the microsensor for glutamate significantly (chapter 3). In addition, its influence on the prevention of biofouling is limited (chapter 5). This is most likely explained by the way the Nafion layer is applied, i.e. dipcoating the sensor several times in a diluted Nafion solution. A thicker Nafion layer protects better against biofouling and excludes reducing agents to a larger extent, but it also restricts the access of glutamate to a higher degree. This results in a microsensor with a low sensitivity and slow response time (results not shown). For a thinner Nafion layer the opposite is valid. In this respect, an alternative for Nafion would be an option. Ideally, an outer layer is required which discriminates between glutamate and interfering compounds. However, most likely such a layer is not available, as glutamate and reducing agents such as ascorbic acid have rather similar physical chemical properties (e.g. molecular size, electrical charge).

This implies that primarily should be focussed on an outer layer that protects better against biofouling. Although biofouling did not critically limit the performance of the microsensor in the experiments we have performed so far, it is possible that it becomes a limiting step when the sensor is applied in more detailed pharmacological experiments. In the past we have performed some pilot studies with different outer layers (results not shown). At that time the construction of the sensor suffered from several other problems, which complicated the interpretation of the results. Therefore, it is worthwhile to have a closer look at this research again. As an example can be
mentioned the application of a hydrophobin layer on the hydrogel. Hydrophobins are a class of small proteins that self-assemble into an amphiphatic membrane at hydrophilic-hydrophobic interfaces (Scholtmeijer et al., 2001; Wang et al., 2005). Theoretically, this provides some distinct advantages for application in biosensor research.

**Selectivity**

A general aspect, valid for all type of amperometric biosensors, is that detected currents always should be interpreted with caution. Application of a biosensor in the brain is prone to the detection of non-specific signals. Therefore, we have paid much attention to this subject (chapter 5). When compared to several other sensors, the hydrogel-coated microsensor has two distinct advantages. First, the sensor is operated at a low voltage of −150 mV, which diminishes largely the detection of electrochemically active compounds. Secondly, the glutamate microsensor is applied in combination with a background sensor. This background sensor serves as a blank or reference sensor and indicates the magnitude of the non-specific current. This non-faradic current is derived from several parameters, such as the surface area of the electrode, surface double-layer capacitance, surface oxides, poisoning of the electrode surface by adherence of compounds, voltage drift, non-specific electrochemical reactions in the hydrogel, etc (Harris, 1995; Boulton et al., 1995; Gerhardt and Burmeister, 2000). To prevent such variation, it is necessary to construct a background sensor as similar as possible as the glutamate microsensor.

**Oxygen dependency**

As the sensor uses two oxygen demanding enzymes, its oxygen dependency is a critical issue. It appeared that the sensor could work properly at the dissolved oxygen levels present in the brain (i.e. 2-5 % pO₂) and that its sensitivity was hardly affected by physiological changes in oxygen levels that normally will occur.

To further investigate the oxygen dependency of the sensor, we also have performed some pilot experiments in which we administered oxygen and nitrogen to the snout of the rat (results not shown). With an oxygen microsensor we could clearly detect changes in dissolved oxygen levels during these experiments, whereas the output of the glutamate sensor was hardly affected (experiments in progress).
In order to investigate how the oxygen levels are affected by local drug application, we also have performed some pilot experiments in which drugs were locally applied via a micropipette (i.e. in a similar way as indicated in chapter 7) close to an oxygen microsensor (results not shown). Although small changes in oxygen levels were observed at some experiments, it is not likely that this has contributed largely to the in vivo results obtained with the sensor so far (as presented in chapter 7). It is concluded that although the oxygen dependency of the microsensor is an important issue, changes in oxygen levels in the brain do not seem to affect the glutamate recordings to a large extent.

In addition, a critical remark is made concerning the application of the sensor at conditions in which the oxygen levels are strongly affected. For example, it is well known that glutamate plays an important role in ischemic conditions (e.g. stroke or other brain damage processes), and much scientific research is performed to investigate the exact role of glutamate at such disorders. However, as the oxygen levels are often strongly decreased at these conditions, future studies have to answer if the microsensor can be applied for this type of research.

In this respect can be mentioned (as an example) the experiment in which the rat was euthanized, while extracellular glutamate levels were monitored with the microsensor (Fig. 10 of chapter 5). Post mortem rises of glutamate were observed previously in microdialysis studies (Geddes et al., 1999), but in our microsensor experiment we observed the opposite. Theoretically it is possible that the output of the sensor was limited by oxygen deprivation. Future experiments have to clarify to what extent oxygen deprivation contributed to these results. Combining microsensor recordings and simultaneous monitoring the dissolved oxygen levels with a microsensor would be an interesting option to study the exact oxygen dependency in vivo.

Response time
A critical parameter of the microsensor is its response time. Important to note is that the response time of the sensor has a close relationship with its interference by reducing agents, as discussed extensively in chapter 5. In brief, to prevent the interference by reducing agents, sensors with a relatively thick hydrogel layer are constructed. In a thicker layer more ascorbate oxidase is present, which prevents better against interference from ascorbic acid, and second, the diffusion of reducing
agents into the hydrogel layer is restricted to a larger extent. However, due to this thicker layer, the response time of the sensor to glutamate also increases dramatically. In addition, the sensitivity of the sensor is also affected strongly, i.e. the sensitivity at non-steady state conditions decreases, as the diffusion of glutamate into the hydrogel is restricted, while the sensitivity at steady state conditions increases, because there is more glutamate oxidase present in the hydrogel that can contribute to the response of the sensor. The equilibration time of this latter reaction is in the minute range.

Interestingly, in the presence of reducing agents these differences in response time and sensitivity disappeared, i.e. in the presence of high concentrations of reducing agents the response time decreased to approximately 8 seconds, whereas the sensitivity decreased to about 1 pA/μM. Note, this is an important aspect to realize if the sensor is applied at conditions with lower levels of reducing agents (e.g. in brain slices). For such conditions, sensors with a thinner hydrogel layer need to be constructed to maintain sufficient response time.

**Stability**

Another issue that needs further clarification is the stability of the sensor. At present it is not known how the behaviour of the sensor will be affected when its enzymes are continuously active. This is an important question to answer, in particular when the sensor is going to be applied to freely moving animals. In this regard it is necessary to investigate if it is possible to operate the animal at one day and to perform the microsensor experiment at another.

During the evaluation studies (chapter 5), we already have received some indication of the stability of the sensor, but additional experiments are required. An option to investigate this issue is to place the sensor in a beaker, which contains both glutamate and ascorbic acid, and to monitor glutamate levels over a period of several days. Another option would be to administer injections of glutamate and ascorbic acid continuously to a sensor placed in a flow injection analysis system.

**Storage conditions**

How storage conditions influence the performance of the microsensor is a subject that has not been paid much attention to until now. For example, we have observed differences in the storage stability of microsensors with and without a Nafion layer.
A step forward in the detection of extracellular glutamate? General discussion and conclusion

(results not shown). Microsensors without a Nafion layer could be stored at least two weeks prior to their use, whereas those coated with a Nafion layer could not be stored much longer than several days. The reason for this is not known. It is possible that a further drying of the Nafion layer increases the barrier to diffusion, which prevents the diffusion of glutamate into the hydrogel. Another option is that the Nafion layer degrades the enzymes, or the hydrogel layer, or the communication within the hydrogel layer. This aspect, as well as other influences on storage conditions, need further investigation.

Another aspect of concern is the storage of the microsensor after calibration. We have observed that the performance of the sensor gradually degraded over several days when stored in aCSF (results not shown). It is worthwhile to investigate different storage conditions in order to maintain a better sensor stability. For example, addition of antimicrobial agents or agents that preserve the activity and stability of the enzyme might be considered (e.g. addition of cofactors of enzymes or storage in certain buffer solutions). This would allow a greater flexibility in the use of the sensor after calibration.

8.4 Practical application of the microsensor

We have applied the microsensor both in vitro as well as in vivo. In this regard, the temporal and spatial resolution of the microsensor appeared to be important analytical aspects. The first concerns the response time of the sensor, whereas the latter concerns its physical dimensions. The high temporal resolution (a response time of approximately 8 seconds, depending on the conditions) allowed a second-to-second detection of extracellular glutamate, whereas its small dimensions (10 μm diameter) allowed precise implantation at specific brain areas of interest, for example the CA1 area of the hippocampus, without inducing much brain damage.

In Vitro

From our in vitro experiments in organotypic hippocampal slice cultures (chapter 5) and in acutely prepared slices (chapter 6) it was concluded that the microsensor formed an excellent analytical tool to study extracellular glutamate in brain slices. It appeared that the sensor was capable of detecting second-to-second changes of extracellular glutamate inside the slice at specific anatomical (sub)areas of interest,
whereas other methods only monitor derivatives of glutamate or detect glutamate that leaks out of the slice.

In comparison to in vivo studies, the conditions for specific detection of glutamate in vitro are more favourable: significant lower concentrations of reducing agents, higher dissolved oxygen levels and a high level of control of experimental conditions. Besides, in the slice experiments performed so far drugs are washed in for a certain period (e.g. 10 min). The sensitivity of the microsensor for glutamate at such steady state conditions is significantly higher when compared to non-steady state conditions (chapter 5). Due to these conditions, the sensitivity of the microsensor in vitro is approximately 10 times higher than in vivo. Despite, an aspect of concern is the temporal resolution of the sensor, i.e. the combination of lower levels of reducing agents and detection of glutamate at steady state conditions also increases the response time of the sensor significantly.

At present, experiments are in progress in which the microsensor is combined with electrical stimulation and local drug delivery (i.e. chemical stimulation of neuronal structures via micropipette injections), in order to monitor specific depolarization processes in the slice. At such conditions, the sensitivity of the microsensor is lower, whereas the response time of the microsensor becomes more critical. The potential use of the microsensor at such experiments is currently under investigation.

Do microsensors form a step forward in the detection of extracellular glutamate in brain slices?
In my opinion this question can be answered positive. At present, there is no other technique that can detect extracellular glutamate directly in a slice and therefore microsensor experiments may further improve our understanding of glutamatergic neurotransmission. Of particular interest would be to combine the microsensor with electrophysiological recordings. In such a way both depolarization processes and extracellular glutamate release may be monitored simultaneously.

In Vivo
From our in vivo experiments in anesthetized rats (chapters 5 and 7) it is concluded that the microsensor also forms a promising analytical tool for the detection of extracellular glutamate in vivo. Despite the fact that a combination of several limiting conditions (i.e. reducing agents, biofouling and oxygen deprivation), and more difficult
to control experimental conditions (i.e. implantation of the microsensors and micropipette at a close distance, physiological variations, etc) complicated the practical application of the microsensor, as discussed in chapter 5.

An unsolved question, that still needs a definitive answer, is the real extracellular concentration of glutamate in the brain. Previously (chapters 1, 5 and 7), this subject was discussed extensively. In brief, with our microsensor we have observed basal extracellular glutamate concentrations of approximately 20 μM. Although this value is in good accordance with other microsensor observations (e.g. Kulagina et al., 1999, and observations by the research group of Dr. Gerhardt: Rutherford et al., 2006; Stephens et al., 2006; Hasçup et al., 2006; Nickell et al., 2006), and also with observations in which a glutamate electrode is integrated into a microdialysis probe (Zhao et al., 1997; Guyot et al., 2001), it is much higher than observed in other studies, e.g. in microdialysis studies (Westerink, 1995; Miele et al., 1996; Danbolt, 2001; Hutchinson, 2002), other microsensor reports (Hu et al., 1994; Rahman et al., 2005), or studies in which direct sampling by fused silica tubing is combined with capillary electrophoresis (Bowser and Kennedy, 2001; Kennedy et al., 2002; Sauvinet et al., 2003). As a consequence, the true extracellular glutamate concentration in the brain is still a matter of debate. The spatial resolution of the analytical technique takes a centre place in this discussion (Drew et al., 2004; Cavaleri et al., 2005). It is hypothesized that a technique with smaller dimensions inflicts less brain trauma and, in turn, damages the physiology of glutamatergic neurotransmission to a lesser extent (Clapp-Lilly et al., 1999; Khan and Michael, 2003). Consequently, synaptic activity can be approached more closely and because the glutamate concentration in the synaptic cleft is in the millimolar range, it is possible that higher concentrations of extracellular glutamate are detected by such a technique, in contrast to a technique with larger dimensions. This would explain the relatively high glutamate values we, and others, have found. The fact that the glutamate detected by microsensors is TTX-dependent supports this idea, but more research is required to confirm this hypothesis.

Misinterpretation of the signal?
On the other hand, is it possible that the sensor overestimates the glutamate concentration? For example, is it possible that it detects non-specific electrochemical signals? Theoretically the background sensor should correct for this. However,
variation in the detection of such non-specific currents can cause mistakes in the interpretation of the signal. Although the absolute variation is not that high, it is noted that the sensitivity of the microsensor in vivo is low (due to limiting conditions such as the interference by reducing agents, biofouling and oxygen deprivation). This implies that only small changes of a few picoamperes can cause (large) mistakes in the interpretation of the signal.

Another complicating factor, that may cause a false interpretation of the signal, is the fact that detected currents have to be correlated to final glutamate concentrations. This correlation is based on post in vivo calibration at conditions that mimic the in vivo situation most closely, i.e. calibration of a biofouled sensor in the presence of high concentrations of reducing agents and in the presence of restrictive oxygen levels. It is possible that (small) mistakes are made by such a correlation, for example because the behaviour of the sensor in the calibration system is different in comparison to the in vivo situation, because the physiological conditions in the calibration system do not reflect the in vivo situation exactly, or because changes of physiological conditions occur throughout the experiment. Besides, the sensors have to be transferred from the animal to the calibration set-up, which also can cause some variation, e.g. damaging of the sensor, drying of proteins at the electrode surface, enzyme deterioration, cleaning of the electrode surface due to rinsing by the calibration procedure, etc.

In addition, there are several other factors that possibly can influence the basal current of the microsensor when it is implanted in the brain. Some examples are:

a) Sensor dependent variation. Variation in the sensitivity, selectivity and response time of the sensor, but also leakage currents, biofouling, damage of the sensor during implantation, etc, may change the current output of the sensor.

b) Variation in experimental procedures. The physical condition of the rat, the level of anaesthesia, changes in physiological parameters, variations in operation conditions, etc, may change the absolute current output of the sensor.

c) Variation due to electrochemical procedures. Variation in the performance of the reference electrode due to implantation or biofouling, influence of grounding, electrical noise, disturbances, etc, may change the experimental conditions.
Although there are many factors that can influence the performance of the sensor, most of these parameters also contribute to the current output of the background sensor. This diminishes the chance of detecting false positive signals and of overestimating the signal.

It is concluded that currently the question of how much the real extracellular glutamate level is in the brain can not be answered with absolute certainty and more research is needed. It is obvious that in this respect it has to be tried to eliminate as much as possible sources of variation that may contribute to the basal current output of the sensors. In addition, although the use of a background sensor is inevitable for monitoring non-specific currents, currently the exact glutamate concentration in an individual experiment can not be estimated by subtracting the current output of the background sensor from the output of the glutamate sensor. Future research on non-specific currents that are detected by both sensors have to create the possibility to use the background sensor in a self-referencing mode. In this respect, a comment is made concerning the use of the home-made amplifiers. Although they suited perfectly for the type of research performed until now, i.e. exploring the potential of the microsensor, an exact determination of the extracellular glutamate concentration in the brain and in brain slices requires detection with femtampere sensitivity, in combination with a high level of noise reduction. For this purpose better validated and commercially available, but expensive, amplifiers with higher specifications are probably required.

Do microsensors form a step forward in the detection of extracellular glutamate in the brain?

In my opinion this is indeed the case. It appeared that the temporal resolution of the microsensor was of crucial importance for detecting dynamic changes of extracellular glutamate. When the microsensor is compared with the microdialysis technique, it is observed that fast dynamic changes in glutamate as monitored with the microsensor (chapter 7) can not be detected with the microdialysis technique. Another very important finding was the fact that TTX dependent glutamate could be detected with the microsensor, which does not seem to be possible with microdialysis.

This implied that both the microsensor and the microdialysis technique monitor a different glutamatergic pool. Apparently, the microsensor seems to detect neuronally derived glutamate to a certain extent. It is still unknown which
glutamergic pool is sampled by the microdialysis technique. This question is currently under investigation.

8.5 Comparison with other microsensors

To evaluate the analytical properties of the sensor, it is also necessary to compare its performance with other glutamate microsensors. As mentioned in chapter 1, the only microsensor which is consistently used as an analytical technique on a routine base is the sensor of Dr. Gerhardt. When the performance of the hydrogel-coated microsensor is compared with this sensor a few striking differences can be observed (Burmeister et al., 2000-2002; Day et al., 2006; chapters 5 and 7). These differences are predominantly caused by the electron transfer mechanisms of the sensors, i.e. the Gerhardt sensor is a first generation type of sensor, whereas the hydrogel-coated sensor is classified as a derivative of the second generation type of biosensors. First, the response time of the Gerhardt sensor is faster in comparison to the hydrogel-coated sensor (~ 1 sec vs. ~ 8 sec). Second, due to its high operating potential, the Gerhardt sensor is prone to the detection of side oxidation reactions by easily oxidizable compounds, whereas the sensitivity of the hydrogel-coated sensor is often decreased by such compounds. Third, the dimensions of the Gerhardt sensor are much larger (a triangle shape from a 1 mm basis to a 2 μm tip at which several electrode sites of 50 x 150 μm² are localized), in comparison to the hydrogel-coated sensor (a 10 μm diameter electrode). An additional advantage of the Gerhardt sensor is that it can be used in self-referencing mode, i.e. it is possible to subtract the output of the background sensor from the signal of the glutamate sensor to verify the exact glutamate concentration.

Despite these differences between both sensors, they display a close similarity in their glutamate recordings. Similar type of dynamics were observed after application of high potassium, extracellular glutamate, reuptake blockade (DL-TBOA) and sodium channel blockade (TTX). Of particular interest is that both sensors monitor similar concentrations of basal extracellular glutamate, i.e. approximately 20 μM. It is very interesting that the only two microsensor techniques that are currently applied on a routine base detect such high levels of extracellular glutamate, whereas other techniques monitor low μM levels. Future research have to provide an answer to this challenging discussion.
With regard to the spatial resolution of both sensors, it is remarkable that they monitor similar type of glutamate recordings, as the Gerhardt sensor has much larger dimensions in comparison to the hydrogel-coated sensor. As mentioned above, it is hypothesized that the spatial resolution of an analytical technique plays a critical role in detecting physiologically derived glutamate. However, this hypothesis is questioned by these microsensor findings. Future research has to answer the question to what extent the spatial resolution of an analytical technique influences the glutamate recordings.

In this respect, a comparison with other microsensors would be interesting. Although not available at present, possible future results are expected from research groups using the recently commercially available Pinnacle sensor (Pinnacle, 2006), or from other groups applying their sensor on a routine base (e.g. Rahman et al., 2005).

8.6 Future research

It is obvious that the spatial and temporal resolution of the microsensor are beneficial for various types of studies. A promising future application of the microsensor would be a combination with other (analytical) techniques. By combining the microsensor technique with electrophysiological recordings or electrical stimulation, a better understanding of the physiology of glutamatergic neurotransmission may be achieved. This approach allows simultaneous monitoring of neuronal activity and detection of extracellular glutamate. A combination with microdialysis will also provide the needed complementary information on the interpretation of extracellular glutamate levels.

How the diameter of the sensor exactly influences the properties of the detected glutamate is another interesting question. A challenging approach to investigate this in more detail would be to perform experiments with microsensors with different carbon fiber diameters.

Another important aspect that needs to be developed is the application of the sensor in freely moving rats. Monitoring in conscious animals most likely allows a closer examination of physiologically derived glutamate and may provide more information than obtained in anesthetized animals. In addition, it can answer the question how anaesthetics affect extracellular glutamate levels.
An interesting future option is to modify microsensors in order to monitor other substrates of interest. Because most aspects of the construction of the microsensor are under control now, it is relatively easy to replace glutamate oxidase by another enzyme. For example, we already have constructed glucose microsensors, i.e. replacing glutamate oxidase by glucose oxidase. However, there are many more possibilities, as long as a selective oxidase enzyme is available for the analyte of interest.

8.7 Conclusion

Does the hydrogel-coated glutamate microsensor form a step forward in the detection of extracellular glutamate? I believe that his question can be answered positive. In both \textit{in vitro} and \textit{in vivo} experiments the glutamate microsensor appeared to be an analytical technique of great value and a significant improvement compared to most other techniques used at present. However, application of the microsensor technique has not reached maturity. Its practical use is attended with different technical problems and its application is not very user friendly, i.e. the small dimensions make the microsensor vulnerable, which requires cautious handling, and its construction is critical in order to produce a microsensor with sufficient analytical power.

The aim of the research presented in this thesis was to construct, evaluate and apply the microsensor in brain research. Looking back, I conclude that this aim is achieved, although more fundamental research was required as thought on forehand. It is hoped that the research presented in this thesis is of benefit for future studies in the field of microsensors, not only for our research group, but also for other groups who want to adopt this technique.

The next stage is that the microsensor needs to demonstrate its value in more detailed pharmacological studies, as well as in behavioural experiments. This is a challenging goal and will be the subject of my successors.