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Application of a glutamate microsensor to brain tissue

Oldenziel, Weite Hendrik

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10.2 Summary

Microsensors represent a promising new analytical method for studying neuronal processes in the brain of laboratory animals. Because they are able to monitor the second-to-second release of neurotransmitters in the vicinity of cellular activity, without inducing severe brain damage, they may improve our understanding of neuronal processes in the central nervous system (CNS) significantly. This may increase our knowledge of the physiology of the brain and, in turn, of the pathophysiology of several psychiatric, neuronal and neurodegenerative diseases.

For more than a decade now attention has been paid to the development of a microsensor that is able to detect extracellular concentrations of the amino acid l-glutamate in the brain. To that end, different types of microsensors have been developed. A promising concept is the hydrogel-coated glutamate microsensor. The research in this thesis describes the construction, evaluation and practical application of this specific microsensor.

Role of glutamate in the central nervous system

The amino acid l-glutamate is one of the most important neurotransmitters in the central nervous system (CNS). Approximately 50 % of all neurons use glutamate as a neurotransmitter, whereas almost all neurons display sensitivity to it. Consequently, glutamate is involved in many physiological processes, for example in the development and plasticity of the CNS, in cognitive processes, in the formation of memory, in the regulation of the cerebral blood flow, etc. For that reason, glutamate is also involved in many pathophysiological processes, such as epilepsy, schizophrenia, depression, Parkinson’s disease, Alzheimer’s disease, amyotrophic lateral sclerosis and stroke.

In addition, glutamate fulfills several other functions in the CNS. For example, it plays an important role in the energy metabolism of the brain, in the detoxification of several (hazardous) compounds and it is an important building block in the synthesis of proteins and peptides, including the anti-oxidant glutathione. Moreover, glutamate is a precursor for the inhibitory neurotransmitter γ-aminobutyric acid (GABA). Approximately 20 % of all neurons in the CNS use GABA as a neurotransmitter. Consequently, glutamate and GABA are regarded as the primary neurotransmitters in the CNS, in which glutamate generally has an excitatory function, whereas GABA predominantly fulfils an inhibitory role.

Monitoring of glutamate in the brain

Because glutamate plays an important role in many physiological and pathophysiological processes in the CNS, it is an important target for scientific research. In this respect, a

number of glutamatergic compounds are in development as potential new drugs for several of the previous mentioned disorders.

However, at present our knowledge concerning glutamatergic neurotransmission is limited. For example, recent discoveries have shown that glutamatergic neurotransmission is more complex than the neurotransmission of “classical” neurotransmitters, such as dopamine, noradrenaline, acetylcholine and serotonin. For example, it is only since a few years that a crucial role for astrocytes in glutamatergic neurotransmission is recognized. This lack of knowledge is most likely due to the fact that most analytical techniques, which currently are applied to monitor extracellular glutamate, are not able to discriminate between glutamate that originates from neurons or from another source. In this respect, the assessment of neuronally derived glutamate is of crucial importance to improve our understanding of glutamatergic neurotransmission.

Until now, microdialysis is the most frequently used analytical technique for the detection of extracellular glutamate directly in the brain. The mechanism of this technique is based on the principle of dialysis. The microdialysis probe consists of a semipermeable membrane that surrounds two tiny canullae, through which fluid flows in and out the probe. Extracellular compounds can diffuse into the perfusion fluid over the semipermeable membrane. It appears that microdialysis is an appropriate analytical technique to study neurotransmitters, such as the previous mentioned dopamine, noradrenaline, acetylcholine and serotonin. However, its application for monitoring glutamate, but also for compounds such as GABA, is questioned. It appears that glutamate detected by microdialysis does not fulfil the classical release criteria for exocytotic release, such as calcium dependency or the response to the sodium channel blocker tetrodotoxine (TTX).

At the end of the eighties and throughout the nineties our research group, but also an increasing number of other groups, have published several papers in which this issue was discussed. It was hypothesized that the spatial- and temporal resolution of the microdialysis technique was insufficient for the detection of neuronally derived glutamate. Note, the spatial resolution concerns the dimensions of the microdialysis probe, that has a diameter of 300-500 µm. These large dimensions cause substantial brain damage, which disturbs physiological processes and, consequently, the detection of glutamate from such processes. This implies that microdialysis most likely samples glutamate from damaged brain tissue. The temporal resolution concerns the response time of the detection. The microdialysis technique usually collects samples over a period of minutes. However, the neuronal processes in the brain are in the order of milliseconds to seconds. This implies that many processes are not detectable by the microdialysis technique.

Ideally, for a close monitoring of neuronal processes in the synaptic cleft an analytical technique is required with dimensions of a few nanometers and with a response time in the

order of microseconds. It is obvious that such a technique is currently not available. The best alternative at the moment is the use of microsensors.

Microsensors

Microsensors are in fact miniaturized biosensors. Biosensors exist since 1962 and are defined as sensing devices, which combine a biological recognition element (e.g. an enzyme, antibody or protein) with a transducer. The transducer converts the biological recognition reaction into a quantifiable signal. This may occur via several different principles, for example optically, calometrically, acoustically, or electrochemically. Although in theory many different type of biosensors can be constructed, by far the most frequently used type is the enzymatic amperometric biosensor. This type of sensor converts the activity of an enzyme in an electrical current.

When the dimensions of the transducer are minimized to a micrometer-scale, biosensors are referred to as microsensors. Consequently, a microsensor combines the selectivity of an enzyme, with the spatial and temporal resolution of a microelectrode. In this respect, a microsensor is regarded as a promising tool for detecting neuronally derived glutamate. However, although 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 development of a suitable microsensor is hampered by technical difficulties in the construction and application. This is illustrated by the fact that reports on promising microsensor concepts are often not followed by applications of these sensors on a routine base. One of the most critical problems is the transfer of electrons from the enzyme to the electrode surface. Different electrochemical active compounds, which are present in the brain, can interfere in this process. To solve this problem, different types ("generations") of biosensors have been developed. In addition, the practical application of the microsensor can also be limited by other factors, such as biofouling, stability problems, oxygen deprivation, etc.

Hydrogel-coated glutamate microsensor

At the end of the nineties a promising microsensor concept was developed by Kulagina et al. (1999). This sensor is referred to as a hydrogel-coated glutamate microsensor and consists of a carbon fiber electrode, that is coated with a hydrogel. The hydrogel contains three enzymes (glutamate oxidase, horseradish peroxidase and ascorbate oxidase), that are wired via the cross-linker poly (ethylene glycol) diglycidylether to an osmium redox polymer. The concentration of glutamate is recorded as current (picoamperes), which is generated via a complex electrochemical cascade. A promising finding was that this microsensor was able to detect TTX-dependent glutamate. This formed the direct motive for the aim of this thesis: the

introduction of this specific technique and its practical application in neurochemical research. However, it appeared that this introduction was more complex as thought on forehand. Many fundamental aspects of the sensor appeared to be unknown. Therefore, a step-wise approach was required from the introduction to the practical application of this sensor.

Contents of the thesis

The research presented in this thesis is divided into different chapters. The **first chapter** concerns a general introduction in which attention is paid to biosensors and the role of glutamate as a neurotransmitter. In the first paragraph of this chapter different aspects of biosensors are discussed, such as methodological considerations, commercialization, different generations of biosensors and their analytical advantages and disadvantages. The second paragraph discusses the hydrogel-coated glutamate microsensor: its design, the electrochemical cascade, analytical specifications, specific advantages and disadvantages, etc. In addition, the motive for the introduction of this specific microsensor is explained as well. The third paragraph discusses the physiology of glutamate in the CNS. Attention is paid to the different functions of glutamate in the brain, its specific role as a neurotransmitter and the regulation of glutamatergic neurotransmission. Particular attention is paid to recent insights in the functioning of astrocytes in glutamatergic neurotransmission. The last paragraph briefly summarizes the introduction and discusses the outline of the thesis.

The experimental section can be divided into three subjects, respectively the construction (chapters 2-4), evaluation (chapter 5) and practical application (chapters 6 and 7) of the sensor. During our research it appeared that the reproducible construction of the microsensor was a difficult task. Therefore, much attention was paid to this subject in the first few chapters (chapters 2–4). The different steps in the construction of the microsensor were investigated in **chapter 2**. It appeared that the application of the hydrogel determined the analytical properties of the microsensor most critically. It was also observed that the physical conditions during the process of hydrogel coating on the carbon fiber electrode were crucial. This has led to the development of an automatic dipcoater. The dipcoater allowed us to standardize the physical conditions during the coating procedure, which improved both the analytical properties, as the reproducible construction of the sensor significantly. The dipcoater also allowed the possibility of combining the coating procedure with other cross-link methods, in order to improve the polymerization and to follow this process in detail. The latter was performed by combining the coating procedure with amperometry, in which the growing thickness of the hydrogel-layer on the sensor surface in time was represented by an increase in electrical resistance. In chapter 2 we also observed that small changes in the composition of the hydrogel could influence the performance of the sensor dramatically.

This last observation was the reason to study the influence of the different hydrogel components on the analytical performance of the microsensor in more detail in **chapter 3**. It appeared that optimizing the composition of the hydrogel could improve the analytical properties of the microsensor significantly. It was observed that the balance between the osmium redox polymer and the enzymes, as well as the balance between the three different enzymes was of crucial importance. In addition, it was observed that different batches of ascorbate oxidase could change the performance of the sensor significantly. This was experienced as a serious handicap in the practical use of the microsensor, as the performance of the sensor changed significantly each time a new batch was applied.

For this reason the influence of ascorbate oxidase on the analytical performance of the sensor was investigated in more detail in **chapter 4**. It appeared that different batches of ascorbate oxidase displayed variation in their quantity of protein, salts and stabilisator. Apparently, this caused variation in the final amount of ascorbate oxidase that was incorporated into the hydrogel. It was observed that a simple enzyme purification procedure as buffer exchange could prevent this variation.

After these studies it was concluded that we were able to construct microsensors with, to a certain extent, reproducible analytical properties. However, before the microsensor could be applied as an analytical tool on a routine base, it was necessary to evaluate its performance critically. This was performed in **chapter 5**. Attention was paid to the selectivity, specificity, sensitivity, linearity, biofouling and oxygen dependency of the sensor. In addition, pilot experiments *in vitro* in brain slices (organotypic slice cultures), and *in vivo* in anesthetized rats were performed. Attention was also paid to the correlation of monitored currents to supposed concentrations of detected glutamate. From this chapter it was concluded that the microsensor could be applied as an analytical tool both *in vitro* as well as *in vivo*. This was further investigated in chapters 6 and 7.

In **chapter 6** the microsensor was applied *in vitro* on a routine base. The influence of several pharmacological agents, which are known to facilitate the release of glutamate from neurons and astrocytes, was investigated to explore the applicability of the microsensor. As far as we know this was the first study in which extracellular glutamate from brain slices was investigated with a microsensor. It was concluded that the microsensor offered many analytical advantages in comparison to currently applied methods. For example, the high spatial and temporal resolution of the sensor allowed implantation at specific subareas of the slice, in which the release of glutamate could be detected on a second-to-second timescale in the vicinity of cellular activity. Another advantage was that the circumstances in the slice for monitoring with a microsensor were relatively beneficial, i.e. experimental conditions and manipulations could be controlled easily, while low levels of reducing agents and high levels of oxygen were present.

In **chapter 7** the sensor was applied *in vivo* on a routine base. Different pharmacological agents were injected in the vicinity of the sensor to explore its potential as an analytical tool. Despite the fact that the circumstances *in vivo* were less favorable in comparison to the conditions *in vitro*, it appeared that the sensor *in vivo* was a promising analytical tool as well. The influence of the different pharmacological agents could be detected clearly and changes in extracellular glutamate could be monitored in the order of seconds. In particular, the observation that TTX induced a significant decrease in basal extracellular glutamate levels was of great significance and implies that the sensor is indeed able to detect glutamate derived from neurotransmission.

Finally, in **chapter 8** the microsensor was critically evaluated. Several critical aspects regarding the construction, evaluation and practical application of the sensor were discussed. Besides, the question if the microsensor technique forms a step forward in the detection of extracellular glutamate was answered. This final chapter had a more subjective and speculative character, in which the opinion of the author played an important role. In addition, recommendations for future research were made.