Chapter 2: (INTRODUCTION: Section B)

Microdialysis of glutamate: Analysis, interpretation and comparison with microsensors

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This chapter is based on the following paper:

Abstract
Glutamate sampled from the brain by microdialysis does not always fulfil the classic criteria for exocytotic release. In this regard the origin (neuronal vs. astroglial, synaptic vs. extrasynaptic) of glutamate collected by microdialysis as well as in the extracellular fluid (ECF) itself, is still a matter of debate. In this overview microdialysis of glutamate and the use of microsensors to detect extracellular glutamate are compared and discussed. During basal conditions glutamate in microdialysates is mainly derived from non-synaptic sources. Indeed recently several sources of astrocytic glutamate release have been described, including glutamate derived from gliotransmission. However during conditions of (chemical, electrical or behavioural) stimulation a significant part of glutamate might be derived from neurotransmission. Interestingly accumulating evidence suggests that glutamate determined by microsensors is more likely to reflect basal synaptic events. This would mean that microdialysis and microsensors are complementary methods to study extracellular glutamate.

Keywords: Microdialysis; Microsensor; Glutamate; Baclofen; TTX; TBOA; Extracellular
1. INTRODUCTION

1.1 Glutamate neurotransmission

Glutamate is the main excitatory neurotransmitters, in the central nervous system (CNS). Glutamate plays an important role in the physiology of the brain, but also in various pathophysiological concepts such as depression, schizophrenia and epilepsy. In addition, glutamate is implicated in the mechanism of action of a variety of centrally acting drugs.

Glutamate was first fully accepted as an amino acid neurotransmitter at the end of the 1970s. Except for being a neurotransmitter, glutamate is an intermediate in energy metabolism of the CNS, detoxifies ammonia via the formation of glutamine, is an important building block in the synthesis of proteins and peptides, including glutathione, is involved in fatty acid synthesis and is a precursor for the inhibitory neurotransmitter gamma-aminobutyric acid (GABA). For years, it has been difficult do differentiate between its role as neurotransmitter and its role in neuronal metabolism (Conti and Weinberg 1999; Watkins 2000). Glutamate is used by approximately 50-60% of all terminals in the CNS as a neurotransmitter (Coyle et al. 2003). Over half of the brain’s 100 billion neurons generate glutamate (in contrast to e.g. the brains 10 000 dopamine-generating neurons (McGeer et al. 1987). Furthermore 90% of all neurons display sensitivity to glutamate. Recently it was demonstrated that glutamate can co-release in addition to monoaminergic neurotransmission (Trudeau 2004). Glutamate is involved in most aspects of normal brain functioning, including cognitive processes and the formation of memory. It also plays a major role in neuron-development and synaptic plasticity of the CNS, including the migration, differentiation and death of cells, and in the induction and elimination of synapses. High concentrations in the extracellular fluid (ECF) caused by excessive glutamate release are associated with neuronal dysfunction resulting in a variety of neurodegenerative and psychiatric disorders including epilepsy, motor neuron disease, traumatic brain injury, Huntington's chorea, Parkinson's, Alzheimer's disease, amyotrophic lateral sclerosis and stroke (Nicholls 1993; Danbolt 2001). Abnormal changes in glutamate neurotransmission are also implicated in pathologies such as schizophrenia, depression, drug abuse and addiction (Palucha and Pilc 2005).

1.2. Microdialysis: pros and cons

Since two decades the microdialysis technique (Ungerstedt 1991; Westerink and Cremers 2007) is the most common method to sample and detect neurotransmitters in the living brain. The method has several important advantages. Concentration vs. time profiles can be obtained from freely moving individual animals and information on several compounds
can be obtained simultaneously. In addition the microdialysis probes can be used for local delivery of drugs to specific brain regions. Simultaneous sampling from multiple sites is possible by implanting more than one probe in the same animal. Microdialysis can be used to obtain direct measurements of free drug concentration in the brain following a systemic injection in vivo. The dialysis principle provides protein free samples, avoiding sample clean up procedures. With current analytical methods low detect limits are possible.

Several disadvantages of the microdialysis method are also evident. Microdialysis provides information on a time scale which is considerably slower than many dynamic processes in the CNS, having a low temporal response, with sample collection times typically varying from 1 to 30 min (although occasionally rapid sampling times down to a few seconds have been achieved: (Rossell et al. 2003; Rada et al. 2003). Another disadvantage of the method is the size of the probe (> 250 µm in diameter) that will induce extensive tissue damage. The dialysis probe creates a disrupted zone of tissue as inflammation, gliosis and swollen axons were observed within 1.4 mm away from the probe (Clapp-Lilly et al. 1999). (Borland et al. 2005) found that after acute implantation the release of dopamine was strongly inhibited in the vicinity of the probe, whereas the effect of reuptake inhibitors was artificially enhanced. As the dialysis probe is surrounded by scar tissue soon after implantation, the microdialysis method might represent a neuropathological rather than a neurophysiological condition. However, tissue inflammation and scar development can be reduced by making use of sterile perfusion fluids and careful swivel and tubing protocols (Zhou et al. 2002) and (Huff et al. 2003).

1.3. Criteria for determining neuronal release

As microdialysis probes induce extensive damage to brain tissue, the question arises whether the sampled neurotransmitter is derived from synaptic events. Therefore various criteria are used to critically evaluate the significance of neurotransmitters sampled by microdialysis. The criteria for determining neuronal release in the ECF is founded on the general functions of neurons, which are expected to be sensitive to potassium (K+) depolarization, sodium (Na+) channel blockage induced by e.g. tetrodotoxin (TTX), removal of calcium (Ca2+), and depletion of presynaptic vesicles by local administration of the selective neurotoxin α-latrotoxin. For the monoamines, dopamine, noradrenaline, serotonin and acetylcholine it was found that under a wide variety of experimental conditions, dialysate contents of these transmitters indeed arise predominately from exocytotic processes (Imperato and Di Chiara 1984; L'Heureux et al. 1986; Westerink et al. 1987; Consolo et al. 1987; Kalén et al. 1988). The dialysate levels of these transmitters
decrease rapidly when excitation–secretion coupled release mechanisms are inhibited (e.g. by calcium omission or blockade of voltage-gated sodium channels with TTX). However the neuronal origin of glutamate and GABA measured by microdialysis remains questioned, as most authors agree that glutamate does not and GABA only partly fulfill the classical release criteria for exocytotic release (Westerink et al. 1987; Kehr and Ungerstedt 1988; Westerink and de Vries 1989; Osborne et al. 1990; Bourdelais and Kalivas 1992; Smith and Sharp 1994; Timmerman and Westerink 1997).

1.4. Microsensors an alternative?
A possible alternative to microdialysis of neurotransmitters is the use of microsensors. Electrochemical sensors based on amperometry have been mainly used to measure intrinsically electroactive neurotransmitters (i.e. catecholamines and indolamines), since these compounds are readily oxidized at electrodes. Microsensors have been particularly useful in the measurement of dopamine, serotonin, purines and nitric oxide levels in both anesthetized and unanesthetized animals (Giros et al. 1996; Clément et al. 2004). Because the microelectrodes are 10 µm in diameter, they offer excellent spatial resolution since the tissue damage is much reduced. Moreover the response time of these sensors is often in the subsecond range.

For analytes that are not intrinsically electroactive (such as glutamate) specific enzymes need to be immobilized onto the surface of electrodes. A considerable number of enzymes, mainly oxidases and dehydrogenases, have been successfully incorporated into the design of electrochemical sensors. Most biosensors used in brain research use however oxidase enzymes with amperometric detection methods. Ensuing detection of the analyte can be accomplished by monitoring the consumption of an electroactive enzyme co-substrate, such as oxygen, or the formation of an electroactive product, such as hydrogen peroxide. A cross-linkable redox polymer can be used to form a hydrogel, which both immobilize enzymes onto the microelectrode and shuttle electrons between the entrapped enzymes and electrodes (Gregg and Heller 1991; Heller 1992; Mitala and Michael 2006). In recent years several enzyme-based electrochemical biosensors have been developed and described for monitoring hydrogen peroxide, choline, acetylcholine, glucose and glutamate in vivo in brain tissue of laboratory animals. Interestingly evidence is provided that microsensors have more easy access to the synaptic pools of neurotransmitters than microdialysis probes (Yang et al. 1998; Kulagina et al. 1999).
2. MICRODIALYSIS OF GLUTAMATE

2.1. Glutamate in the ECF of the brain

Glutamate is ubiquitously expressed throughout the mammalian CNS, with intracellular concentrations in the millimolar range, in which the cytosolic concentrations is about 5 to 10 mM and the vesicular concentration approximately 0.1 M. The high-affinity glutamate transporters (EAAT1-5) assure a high signal-to-noise ratio of neurotransmission and keep the extracellular concentrations of glutamate low in order to avoid the excitotoxic actions of glutamate (Bergles et al. 1999; Amara and Fontana 2002). Subsequently glutamate transporters are localized in the vicinity of the synapse, removing the released glutamate immediately (Diamond and Jahr 1997; Lehre and Danbolt 1998). Although glutamate concentration in the synaptic cleft may reach concentrations of several millimoles it has been calculated that glutamate transporters have the potency to bring concentrations in the ECF locally down to ± 20 nM (Zerangue and Kavanaugh 1996; Levy et al. 1998). Although these transporters are also present on glutamate neurons, the astroglial cells seem to play a dominant role in this respect. To assure a high signal-to-noise ratio for neurotransmission and to avoid excitotoxic actions of glutamate on neurons, the (extrasynaptic) ECF concentrations of glutamate are believed to be maintained at ± 1–3 µM (Barbour et al. 1994; Diamond and Jahr 1997; Turecek and Russel 2000).

To sample and determine glutamate in the ECF of conscious animals, the microdialysis method has been used in numerous studies. Glutamate in dialysates is first derivatized with fluorogenic reagents such as o-phtaldialdehyde, fluorescamine or naphthalene-2,3-dicarboxaldehyde and subsequently analyzed by HPLC in conjunction with fluorescence or electrochemical detection. Quantitative microdialysis studies have consistently reported ECF glutamate concentrations in the range of 1–5 µM (Miele et al. 1996; Niwa et al. 1996; Shiraishi et al. 1997; Lada et al. 1998; Zhang et al. 2004).

As the glutamate transporters are not evenly distributed over the glial membranes, it is to be expected that the glutamate concentration will also display a heterogenous concentration in the ECF. Moreover, there is accumulating evidence that astrocytes actively release glutamate which means that at certain sites in the ECF glutamate release will dominate over uptake. Consequently, different extracellular compartments of glutamate might exist in the ECF (see below).
2.2. The origin of glutamate in microdialysates during basal conditions

As mentioned in the introduction, most if not all authors agree that basal values of glutamate in microdialysis samples do not respond to TTX infusion or omission of calcium (Westerink et al. 1987; Timmerman and Westerink 1997). One explanation for the difficulty to detect glutamate of synaptic origin in dialysates is the fact that the “spill-over” of synaptically released glutamate to the ECF is limited even though the synaptic cleft is in continuity with the extrasynaptic space. The average peak concentration of glutamate attained in the cleft has been estimated to be about 1 mM, with a time constant of decay of 1 ms (Clements et al. 1992). The highly active glutamate transporters might explain this fast mechanism (Zerangue and Kavanaugh 1996; Levy et al. 1998). Accordingly glutamate will diffuse only very briefly into the sampling domain of the microdialysis probe. It is evident that the microdialysis probe simply due to its size has no access to the intracellular space of synapses. Another explanation for the unexpected behaviour of glutamate might be related to the traumatic brain injury caused by the large dimensions (200–500 µm diameter) of the microdialysis probe. Close examination of the tissue near the implantation site of the probe has produced evidence for disturbances in microcirculation, edema, changes in the rate of glucose utilization, loss of integrity of blood brain barrier, production of cytokines and eventual proliferation of glial cells (Benveniste and Diemer 1987; Clapp-Lilly et al. 1999; Khan and Michael 2003; Borland et al. 2005). Scar formation around the probe might act as a barrier to diffusion which further will separate the dialysis membrane and the synapse.

2.3. Is glutamate able to escape from the synaptic cleft during excessive neuronal stimulation?

Given the fact that glutamate apparently hardly escapes from the synaptic cleft during basal conditions, various authors have attempted to detect synaptic glutamate during excessive neuronal stimulation. The latter was achieved by means of chemical, electrical or behavioural stimulation of glutamate transmission. In addition some researchers succeeded to improve the temporal resolution of the microdialysis sampling to 1 min and even seconds by using capillary electrophoresis with laser-induced fluorescence detection (Lada et al. 1997; Rossell et al. 2003).

Chemical stimulation includes the infusion of excitatory agents via the microdialysis probe to increase the release of glutamate. The potassium-channel blocker 4-aminopyridine prolongs the opening of the potassium channels. Infusion of this compound resulted in an increase in glutamate levels in microdialysates, which was largely TTX dependent (Peña and Tapia 2000). Infusion of the glutamate high-affinity
transport blocker dl-threo-β-benzylhydroxy aspartate (TBOA), resulted in a pronounced increase of glutamate (to about 300% of controls). However, this increase in glutamate was not TTX dependent (Xi et al. 2003) which indicates that glutamate accumulation also under these conditions is primarily derived from astrocytes. A different but rather successful approach to detect synaptic glutamate was to use a dual-probe microdialysis method. During this approach the cell-body area of glutamatergic neurons are chemically stimulated by infusion of excitatory agents via the microdialysis probe, and the transmitter in the terminal area is then monitored simultaneously. In this regard, application of substance P in the substantia nigra increased extracellular levels of glutamate in the striatum (Reid et al. 1990). In comparable experiments, local administration of NMDA in the frontal cortex increased glutamate levels in the striatum (Palmer et al. 1989; Dijk et al. 1995).

In several studies, electrical stimulation of the cell-body areas of glutamate pathways has been successfully applied to increase extracellular glutamate levels in microdialysates of the corresponding nerve terminal areas. For example, stimulation of the medulla, sciatic nerve or raphe magnus increased glutamate in areas of the spinal cord (Kapoor et al. 1990; Paleckova et al. 1992; Sorkin and McAdoo 1993). Electrical stimulation of the nervus vagus increased glutamate levels in the nucleus tractus solitarius (Allchin et al. 1994), whereas stimulation of the nucleus paragigantocellularis increased glutamate in the ipsilateral locus coeruleus (Liu et al. 1999). Electrical stimulation of the prefrontal cortex induced an increase (in 5-s samples) of extracellular glutamate in the striatum of anesthetized rats (Lada et al. 1997). Application of a 10-s train of pulses induced a rapid increase in glutamate to 200–300% of controls that returned to basal values within 60 s. The increase in glutamate was calcium and TTX dependent. The stimulation of glutamate was suppressed by the metabotropic glutamate receptor agonist, 1-aminocyclopentane-trans-1,3-dicarboxylate (ACPD). (Tucci et al. 2000) demonstrated that electrical stimulation of the prefrontal cortex increased extracellular glutamate in the nucleus accumbens.

Certain types of behavioural stimulations are accompanied by characteristic changes (often an increase) in extracellular levels of glutamate in microdialysis experiments. In several studies, extracellular glutamate was recorded during restraint stress. Substantial increases in glutamate levels to 200–450% of basal values lasting for 10–20 min were reported for the prefrontal cortex, hippocampus, nucleus accumbens and striatum (see references in (Timmerman and Westerink 1997). Photic stimulation of the conscious rat increased extracellular glutamate in the visual cortex to 200% of controls for at least
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3 min (Reyes et al. 2002) and formalin injection into the hind paw resulted in an increase of extracellular glutamate that lasted for 2 min in certain subareas of the hypothalamus (preoptic area but not in lateral and ventromedial hypothalamus) (Silva et al. 2004). Results with a different time scale were presented by (Rossell et al. 2003), who stimulated a whisker of the rat and sampled glutamate in the motor cortex. By using capillary electrophoresis coupled to laser-induced fluorescence detection, they were able to determine glutamate in 1-s microdialysis samples. Upon whisker stimulation, glutamate was increased only in the first 1-s sample; in the 2-s sample glutamate again fell back to control values, although the whisker stimulation was continued. The results of the 1-s lasting increase in glutamate content contrast with the 2–20-min lasting increases in glutamate that were observed in the behavioural experiments discussed above. These data demonstrated the importance of the time scale in detecting physiological changes in extracellular glutamate. It is likely that glutamate displaying subsecond increases reflects different origin than glutamate that was enhanced in behavioural experiments lasting several minutes.

An intriguing question is whether the above-described behavioural-induced changes are calcium and TTX dependent. It was found that handling stress induced increases in glutamate that persisted in the presence of TTX (Timmerman and Westerink 1997). Even mild activation of rats induces changes in glutamate levels in the ECF. Grooming – induced by dropping water onto the rat's snout – increased extracellular glutamate in the striatum to ± 200% of controls (Miele et al. 1996). However, a similar increase was seen when the experiments were performed in the presence of TTX. In contrast, there is a report about feeding-induced increase in extracellular glutamate in the nucleus accumbens that was found to be TTX dependent (Saulskaya and Mikhailova 2002). The study that used formalin injection into the hind paw described increases in glutamate that were both TTX and calcium dependent (Silva et al. 2004). However, the specificity of this observation is questioned by the fact that other amino acids, such as arginine and aspartate also responded in a calcium- and TTX-dependent manner.

In conclusion, evidence is provided that glutamate from synaptic sources is detectable in microdialysates collected during excessive stimulation of glutamate pathways. The behavioural experiments have produced so far the most interesting but also complex results. A rapid increase in glutamate with a time scale of 1 s was detected apart from changes in glutamate that were lasting during several minutes. It is unclear whether these different processes relate to synaptic release at the present time, as in most of the experiments; the TTX and calcium dependency of the observed effects was not
consistently studied. It is tempting to speculate that changes of glutamate in subseconds time frame are related to synaptic activity, whereas changes in minutes might represent non-exocytotic release from yet unknown probably astroglial sources.

2.4. Non-exocytoxic release of glutamate from astroglial cells
The presence of a non-exocytotic extracellular pool of glutamate is well established (Herrera-Marschitz et al. 1996; Timmerman and Westerink 1997). Several authors have suggested or provided evidence that this glutamate pool might be derived from astroglial cells by non-vesicular release. Nowadays, it is recognized that in many aspects astrocytes are similar to neurons. Astrocytes communicate with each other by changing intracellular calcium, while gap junctions connect them with one another. Ca-signals could spread between astrocytes in the form of Ca$^{2+}$ waves. Spontaneous calcium oscillations in astrocytes can excite neighbouring neurons, but neurons can also activate astrocytes. Evidence is accumulating that astrocytes release gliotransmitters in response to changes in intracellular calcium. This process is called gliotransmission (Pascual et al. 2005). In Table 1 the various types of glutamate release are shortly summarized:

The fact that astrocytes can influence synaptic activity by release of glutamate points to a complex interaction between glutamate and astrocytes. Glutamate released between neurons and astrocytes activates Ca$^{2+}$ signalling in astrocytes, but astrocytes are responsible for the uptake of glutamate and thereby the control of the steady-state levels of extracellular glutamate. At present, the exact functional role of these processes has not been established.
Table 1: Mechanisms of glutamate release from astrocytes

<table>
<thead>
<tr>
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<th>Mechanism</th>
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<tbody>
<tr>
<td>1</td>
<td>Glutamate release in astrocytes can be mediated by a prostaglandin-dependent mechanism after a rise of Ca(^{2+}) (Bezzi et al. 1998).</td>
</tr>
<tr>
<td>2</td>
<td>Glutamate release is regulated by volume-sensitive organic anion channels (nonselective) (VSOACs), also referred as volume-regulated anion channels (VRACs). Hypo-osmotic solutions activate these channels as part of the volume regulation and allow the efflux of aspartate, taurine, glutamate, chloride, and other anions in a calcium-independent manner. Glutamate behaving as a volume transmitter can facilitate by this mechanism the communication between cells not connected by synapses (Rutledge and Kimelberg 1996; Del Arco et al. 2003; Takano et al. 2005).</td>
</tr>
<tr>
<td>3</td>
<td>Glutamate release by GAP-junction hemichannels (Contreras et al. 2002; Ye et al. 2003). These channels release both ATP and glutamate, and their opening probability is controlled by changes in intracellular Ca(^{2+}) concentrations (Hansson et al. 2000; Parpura et al. 2004; Volterra and Meldolesi 2005). Following the intracellular Ca(^{2+})-wave diffusion between gap-junction-linked glial cells, Ca(^{2+}) can facilitate the simultaneous release of glutamate from adjacent glial cells, which consequently modifies the extracellular non-synaptic glutamate concentration in a wide region (Araque et al. 1999; Bezzi and Volterra 2001; Haydon 2001).</td>
</tr>
<tr>
<td>4</td>
<td>Glutamate release controlled by the purinergic P2x(_7) receptor gated by ATP channels (Sperlagh et al. 2002; Wang et al. 2002; Duan et al. 2003; Fellin et al. 2006). This receptor is gated by ATP and has been proposed to be involved in the release of glutamate as well as d-aspartate. It shares some properties with the GAP-junction hemichannels, including the increase opening probability at low Ca(^{2+}) concentration, but their pharmacology is different (Sperlagh et al. 2002; Wang et al. 2005).</td>
</tr>
<tr>
<td>5</td>
<td>Sodium-dependent high-affinity heteroexchange mechanisms for glutamate with ascorbate, for glutamate and GABA, and for glutamate and glycine have been reported, but are poorly characterized (Bonanno and Raiteri 1994)</td>
</tr>
<tr>
<td>6</td>
<td>Exocytosis of vesicles using a protein docking system similar to synaptic vesicles (Araque et al. 2000; Bezzi et al. 2004; Montana et al. 2004; Chen and Sun 2005; Crippa et al. 2006; Jourdain et al. 2007) In hippocampal astrocytes, a synaptic-like microvesicle capable of uptake, storage and release of glutamate has been identified (Bezzi et al. 2004; Zhang...</td>
</tr>
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et al. 2004). The loading of these vesicles are similar to neurons, involving the activity of vesicular glutamate transporters; the fusion of these vesicles to the plasma membrane has been shown to be calcium –dependent. However unlike neurons the process is not dependent on the opening of voltage-gated calcium channels, but instead by IP$_3$-mediated calcium release from endoplasmic reticulum following activation of a variety of G-coupled receptors (Volterra and Meldolesi 2005). It is likely that the glutamate concentrations measured in vivo with microdialysis and microsensoring may originate, in part, from vesicular glutamate release from astrocytes. The exact contribution of vesicular glutamate released from astrocytes in the extrasynaptic space is unknown. Traditional attempts to inhibit vesicular release in neurons, including blockade of voltage-dependent sodium and calcium channels would not alter vesicular release from astrocytes.

| 7 | Reversal of sodium-dependent uptake by glutamate transporters. This mechanism does not seem to occur during normal brain functioning, but is believed to be responsible for the massive release of glutamate from neurons and astrocytes after traumatic or ischemic injury to brain tissue (Benveniste et al. 1984;Katayama et al. 1990). |
| 8 | Glutamate release coupled to the cystine–glutamate exchanger. This is a sodium-dependent glutamate transporter, which can operate in the reverse mode, i.e. cystine is exchanged for glutamate by transporting one molecule of cystine into the cell and one molecule of glutamate out of the cell. Accumulated cystine is reduced to cysteine and in turn converted to glutathione (Baker et al. 2002;Xi et al. 2003;Cavelier and Attwell 2005). |
| 9 | 3 m-Glu receptors controlled release (Rae et al. 2005) |
| 10 | pathological release of glutamate from neurons and astrocytes after traumatic or ischemic injury to brain tissue (Rae et al. 2005) |
| 11 | NAAG metabolism (Baker and Kalivas book) |
| 12 | glutamate may be synthesized from glutamine hydrolysis in the extrasynaptic space (Mena et al. 2005) |
2.5. Is glutamate released from astrocytes detectable in microdialysates?

The question arises whether the various mechanisms of glutamate release from astrocytes are detectable with microdialysis. Considering that non-exocytotic release of glutamate occurs via plasma membrane channels, glutamate will not likely respond to infusion of sodium-channel blockers (TTX). Similarly, most of the described release mechanism from astrocytes are calcium independent or make use of mobilization of intracellular calcium stores. Calcium omission during microdialysis experiments will probably not affect glutamate release from astrocytes. The use of specific channel agonists or blockers is needed for further characterization.

(Baker et al. 2002) provided evidence that by using specific agonist and antagonists, the cystine–glutamate exchanger or antiporter is detectable in microdialysates. Whether this exchanger contributes to extracellular glutamate release during normal brain function, or whether it occurs when energy metabolism of astrocytes is compromised, is currently a matter of debate (Nedergaard et al. 2002; Cavelier and Attwell 2005). Interestingly (Baker et al. 2002) also demonstrated that glutamate released by the cystine–glutamate antiporter was able to modify dopamine transmission.

Other types of glutamate release from astrocytes, for example, related to VSOACs, GAP-junction hemichannels, and the prostaglandin dependent as well as the purinergic P2X7 receptor mechanism have yet received little investigation in microdialysis studies.

Of particular interest is the role that mGlu receptors play in the regulation of glutamate. It is hypothesized that astrocytic mGlu receptors sense glutamate that is released during synaptic transmission to adjust its extracellular concentration by modulating uptake activity. Microdialysis studies showed that stimulation of group I or inhibition of group II mGluRs elevates extracellular glutamate levels in various brain regions (Swanson et al. 2001; Baker et al. 2002; Melendez et al. 2004). The fact that these effects were TTX independent supports the assumption that this type of release is derived from astrocytes.

3. MICROSENSORS AS ALTERNATIVE TO DETECTED GLUTAMATE

3.1. The principle of the microsensor

As microdialysis of glutamate provides only limited information about synaptic processes other methods with higher spatial and temporal resolution are needed. Glutamate microsensors (with a diameter of about 10 µm) might represent a promising alternative as the implantation requires at least 1000 times less volume in comparison with a
200 µm microdialysis probe. Moreover microsensors have often a much faster response time (subseconds to seconds) than microdialysis probes (seconds to minutes).

Electrochemical microsensors prepared from carbon fibres (diameter less than 10 µm) have been successfully used to study the release of catecholamines and serotonin in (sub)seconds from vesicles (Pothos et al. 2000), single cells (Kumar et al. 2001; Smith and Trimarchi 2001) and in the awake brain (Giros et al. 1996; Stuber et al. 2005). Unfortunately glutamate cannot be directly determined by these methods as it is not electrochemically active. However when specific enzymes (e.g. glutamate oxidase; GluOx) are applied to a fibre, wire or ceramic needle, glutamate is converted to H₂O₂, which can be detected amperometrically. A range of biosensor designs for glutamate, based on this principle, have been described for direct monitoring of glutamate in the ECF (Kulagina et al. 1999; Hu et al. 1999; Burmeister JJ et al. 2003; Oldenziel et al. 2004; Rahman et al. 2005).

The microsensor concept became of particular interest when (Kulagina et al. 1999) reported that the hydrogel-coated glutamate microsensor was able to detect TTX-sensitive glutamate in the brain. The glutamate microsensor we use in our laboratory is based on this concept. (Kulagina et al. 1999) optimized the glutamate microsensor by developing a sensor which utilizes an artificial redox mediator. Sensors based on this principle are described as “second generation” sensors. Importantly the use of a mediator decreases the potential needed for oxidation of the generated hydrogen peroxide resulting in a sensor with high selectivity and sensitivity.

Briefly, the hydrogel-based microsensors are constructed as follows (Kulagina et al. 1999; Oldenziel and Westerink 2005). Carbon fibres with a diameter of 10 µm are trimmed to a length of 300–500 µm. The microsensors are prepared by coating the fibre with a five-component redox-hydrogel, in which l-glutamate oxidase, horseradish peroxidase and ascorbate oxidase are wired via poly(ethylene glycol) diglycidyl ether to an osmium containing redox polymer (abbreviated as POs-EA). A thin Nafion coating completes the construction. The experiments performed in vivo are carried out amperometrically at a constant potential of −150 mV vs. Ag/AgCl. This rather low potential reduces the detection of various easily oxidizable substances endogenous to the extracellular space of brain tissue, i.e. the catecholamine and indolamine neurotransmitters, their acidic metabolites, urate and ascorbate. Furthermore, the potential at which these sensors operate is insufficiently negative to allow the microsensors to detect oxygen. As the enzymatic reaction occurs throughout an
extensive three-dimensional network, an optimal current density is achieved which increases the sensitivity of the sensor. A disadvantage of the concept is that the diffusion of glutamate throughout the hydrogel layer decreases the response time of the sensor to several seconds.

As the microsensors are directly implanted in the brain issue, the specificity of the method should be critically evaluated. Interfering molecules can hinder the performance of the glutamate microsensor at different levels: (1) by immediate oxidation or reduction of compounds at the carbon fibre electrode surface, (2) by interference within the redox cascade of the hydrogel and (3) by substrate aspecificity of glutamate oxidase.

These limitations caused by interfering compounds can to a certain extent be avoided by using a background microsensor. The background sensor is constructed in a similar fashion as the glutamate sensor except that glutamate oxidase is omitted from the hydrogel. The background sensor is placed in the tissue close to the glutamate sensor. When the tissue region is pharmacologically manipulated and a response at the microsensor is measured without affecting the amperometric current at the background sensor, the response can be attributed to glutamate. In this regard an interesting microsensor concept was developed by Dr G Gerhardt (Day et al. 2006). Their commercially available microsensor is based on a ceramic needle like device. As this type of sensor is a first generation type, detecting glutamate (hydrogen peroxide) only at high oxidation potential, it will display a low selectivity. To overcome this disadvantage the background sensor and the glutamate sensor were attached closed to each other on the same needle. This principle is called self-referencing.

3.2. Our experience with the hydrogel-coated microsensor
Despite the fact that research on the development of a glutamate microsensor has been performed for more than a decade now, the number of studies which have successfully applied glutamate microsensors in vivo or in vitro is rather limited. This is evident by the reports of promising microsensor concepts which are not followed by routine applications (e.g. (Hu et al. 1994;Ryan et al. 1997;Kulagina et al. 1999;Mikeladze et al. 2001). About 5 years ago we introduced the hydrogel-coated glutamate microsensor in our laboratory. The sensor based on the concept of (Kulagina et al. 1999) was critically evaluated and optimized (Oldenziel et al. 2004;Oldenziel and Westerink 2005). The next conclusions were finally drawn:
• The construction of the sensor is complex due to the fact that several individual compounds have to be cross-linked with each other to form the sensing layer. Owing to
the small size of the carbon fibre, the coating of the various components is difficult to control and standardize.

- The temporal response of these glutamate microsensors appears to be determined by the diffusion of glutamate into the enzyme-containing cross-linked polymer film. The response time depends on the thickness of the layer and is relatively slow: 5–10 s.
- The sensors are still sensitive to interfering compound such as ascorbate and uric acid, and the levels of these compounds in the brain are high and difficult to control.
- Theoretically the output of the sensor is limited by oxygen deprivation. When varying the oxygen content no evidence was found for oxygen dependency (Oldenziel et al. 2006b). However care should be taken when the sensor is applied at conditions in which the oxygen levels are strongly affected. For example, the well known post-mortem rise of glutamate that is observed during microdialysis (Geddes et al. 1999) was not detectable.

In contrast in our experiments with the glutamate microsensor we observed a decrease in the glutamate sensor current after euthanasia, which is explained by deprivation of oxygen in the tissue.

- A decrease in the sensitivity of the microsensor was observed in vivo. This is explained by biofouling as proteins settle onto the sensor and affect sensor signal stability and disrupt the enzyme activity.
- As it is questionable whether all non-specific electrochemical signals have been eliminated, it cannot be excluded that the microsensor overestimates the glutamate concentration. Theoretically the background sensor should correct for this error; however variation of the background sensor current can still cause misinterpretation of the signal.

Apart from these drawbacks we have successfully applied the glutamate microsensor in vitro as well as in vivo (Oldenziel et al. 2006c; Oldenziel et al. 2007). Basal values of the glutamate microsensor are higher than those of the background microsensor, which is indirect evidence that the microsensor is capable of detecting glutamate. Basal extracellular glutamate levels (mean ± SEM) were in vitro 1.7 ± 1.0 µM (CA1 area of the hippocampus; n = 19) and in vivo: 18.2 µM ± 9.3 µM (striatum; n = 85). This value is in good accordance with the microsensor observation reported by (Kulagina et al. 1999) (29 µM), but much higher than reported by (Rahman et al. 2005; Day et al. 2006), who found values between 1.4 and 2 µM. However recently (Rutherford et al. 2007), using the ceramic sensor, reported a significant difference in resting glutamate levels between the striatum (7 µM) and the prefrontal cortex (45 µM) of freely moving rats. The high values are somewhat unexpected as they are close to the excitotoxic concentrations of glutamate in the brain.
Finally the glutamate microsensor was evaluated in vivo in a series of pharmacological experiments carried out in anesthetized rats. In this approach compounds were administered with a micropipette connected to a pressure pulse controlled device for repeatable injections of picoliter and nanoliter volumes (PicoSpritzer®). The tip of the PicoSpritzer® was stereotactically implanted and positioned at a distance of approximately 100 µm from the glutamate microsensor.

The non-transportable reuptake inhibitor TBOA, which is a blocker of the glutamate transporters EAAT1-3 (i.e. GLAST, GLT-1, and EAAC1), induced a significant but rather slow increase in extracellular glutamate (Fig. 5). The gradual increase could be due to the fact that TBOA is interacting competitively with the membrane transporter. This data indicated that a constant tonic release of glutamate was present, although active release of glutamate from astrocytes by heteroexchange was not ruled out completely, as the non-transportable TBOA partly behaves as a transportable inhibitor on astrocytes (Anderson et al. 2001). The influence of TBOA on extracellular glutamate has not been investigated before with a microsensor. Interestingly the results were consistent with some recent microdialysis studies (Baker et al. 2002; Xi et al. 2003).

Importantly microinjection of TTX caused a rapid and dose-dependent decrease in the basal output of the glutamate microsensors and did not affect the background sensor (Fig. 6). This decrease suggests that – in contrast with the microdialysis method – the microsensor is indeed able to sample the synaptic pool of glutamate. These results confirm the observation by (Kulagina et al. 1999), who for the first time reported that glutamate detected by microsensors was TTX-dependent. Recently, similar data were reported by (Day et al. 2006) by using ceramic-based glutamate sensors. It is emphasized that for any glutamate microsensor it needs yet to be established which part of the recorded glutamate is TTX-dependent and which part is reflecting the non-synaptic pool.
Fig. 1. Influence of TBOA on the extracellular glutamate levels. 500 nl of 1 mM TBOA (n = 7) was locally applied and the currents produced by the glutamate (Glu) and background (Bkg) microsensors were monitored in the striatum. * denotes a statistical significant difference (p < 0.05; Oldenziel et al. 2006c).

Fig. 2. Influence of TTX on the extracellular glutamate levels. 500 nl of 500 µM TTX (n = 10) was locally applied and the currents produced by the glutamate (Glu) and background (Bkg) microsensors were monitored in the striatum. * denotes a statistical significant difference (p < 0.001; Oldenziel et al. 2006c).
4. CONCLUSIONS

4.1. Are glutamate microsensors an alternative to microdialysis?

Glutamate microsensors fulfil the desired criteria of improved spatial and temporal resolution. In contrast to the extensive damaged and functional disturbances that are produced by a microdialysis probe the damage caused by a microsensor is much more limited. Indeed, electron microscopy of the track created by a carbon fibre (10 µm) revealed that maximal tissue damage was confined to a radius of 2.5 µm followed by an annular region with a width of 4 µm that contained a mix of healthy and damage cellular elements (Peters et al. 2004). This might explain why microsensors – in contrast to microdialysis probes – are able to detect TTX-dependent glutamate release during basal conditions. This result might have a significant impact on future research of extracellular glutamate. It should be emphasized that the time resolution of the glutamate sensor is limited to 5–10 s which means that only relatively slow changes (e.g. certain behavioural and pharmacological effects) can be recorded.

The main disadvantage of the microsensor is the complexity of its construction. This is especially true for the carbon fibre based sensors. An alternative might be the commercial available ceramic-based sensor that has a more robust construction (Day et al. 2006). However as the latter sensor has larger dimensions it needs to be established to what extent this sensor is able to detect synaptic glutamate release.

4.2. Interpretation of extracellular glutamate: two recording methods, two different sources?

Results from microsensors and microdialysis of glutamate are substantially different. First microdialysis and microsensors technologies disagree on the absolute concentrations of neurotransmitters in the ECF. Microsensors detect considerable (10–50 times) higher values. Second, microsensors – in contrast to microdialysis – are able to sample TTX-sensitive glutamate under basal conditions. The absence of any effect of TTX infusion or calcium depletion on glutamate concentrations in microdialysates under basal conditions, suggests that the source of glutamate detected with this method may represent a different pool of origin, such as astrocytic glutamate released by gliotransmission. This would mean that both methods are complementary. However additional microsensor and microdialysis experiments are needed to substantiate this hypothesis and further define the various glutamate pools that contribute to the ECF.