Chapter 9

Summary and general discussion

In Chapter 1 a general introduction to glutamate as a neurotransmitter in the brain is given. Although synaptically released glutamate has been studied in great detail, the origin and function of basal extracellular glutamate measured in vivo outside the synaptic cleft, is questioned. It is generally agreed that extracellular glutamate in the brain is derived from both synaptic neuronal and glial release. The ability of glutamatergic synapses to undergo depolarization is strongly influenced by synaptic as well as nonsynaptic regulation of extracellular glutamate. The regulation of the latter two processes has been termed glutamate homeostasis (Kalivas 2009). Concurrently it is essential to understand and quantify these extracellular and synaptic glutamate concentrations.

The different pathophysibilities that are associated with glutamate and different glutamatergic therapeutic targets are mentioned. A greater understanding of the diversity of glutamate signalling mechanisms and the role of membrane and vesicular transporters may open up new therapeutic avenues, as will the clarification of the role played by glutamate and co-transmission. Although the glutamatergic system is highly complex, and many potential glutamate targets have failed clinical trails, there is the hope of positive results from forthcoming trails, which will open the way for improvements over existing therapies and provide drugs for currently untreatable disease.

In Chapter 2 an overview of glutamate microdialysis and the use of glutamate microsensors in detecting extracellular glutamate in the brain are given.

Discrepancies exist regarding estimation of the extracellular glutamate concentration, as measured by patch clamping recording, microdialysis or microsensors. These differences are likely due to the experimental setup and limitations of each procedure. Extracellular glutamate concentrations measured by patch recordings are close to the NMDA\(^1\) receptor-mediated current, and were estimated to be less than 100 nM (Herman and Jahr 2007), whereas direct measurements of glutamate from microdialysis or glutamate biosensors are estimated between 1- 5 \(\mu\)M (Cavelier and Attwell 2005). However discrepancies in absolute extracellular glutamate concentrations between various kinds'

\(^1\) N-Methyl-D-aspartic acid (NMDA)
of microsensors are also found. The hydrogel coated glutamate microsensors detected *in vitro* 1.3-1.9 µM and *in vivo* in the striatum 18.2-23.6 µM. The basal values reported by Gerhardt et al. (Hascup *et al.* 2008; Rutherford *et al.* 2007; Huettl *et al.* 2007) displayed a large variation: 2-40 µM. The current question of how much the real extracellular glutamate level is in the brain cannot be answered with absolute certainty. Kalivas *et al.* (2009) believes that the extracellular glutamate concentration is likely to be between the two estimates: in the 100-300 nM range.

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 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. Preliminary data showed that glutamate detected by microsensors is partly derived from synaptic sources. However later studies in our laboratory could not confirm these observations.

The temporal and spatial resolution of the microsensors is much better than for the microdialysis approach. Microdialysis probes usually detect neurotransmitter levels in the order of minutes. The response time of microsensors is in seconds and is mainly determined by the thickness of the applied protection and enzyme layers.

In contrast to microdialysis probes, which have diameters between 200 and 300 µm, microsensors are much more miniaturized. However it should be realized that even with small sensor dimensions (e.g. 10-25 µm) a certain degree of damage is to be expected. The resulting gliosis might have strong effects on the origin of the detected glutamate. This is especially the case for chronic implantations.

In contrast to the microdialysis approach a technical drawback of microsensors is the difficulty to administer properly pharmacologically active compounds close to the sensor surface. Especially the needle type sensors are difficult to approach by a single injection in the 100-300 nl-range. The development of a combined microsensor-injection needle design might compensate for this drawback.
Chapter 3 describes first and second generation glutamate sensors. Their structures, working mechanisms, interference prevention, \textit{in vitro} detection characteristics and \textit{in vivo} performance are described and compared. In brief, \textit{First generation sensors} (the platinum needle based and the multi-electrode array (MEA) sensors) have a simpler structure and are faster in glutamate detection. They show an acceptable sensitivity and noise level. However the selectivity of these sensors is still a matter of concern since high brain levels of ascorbic acid, uric acid, dopamine and presumably unknown compounds are interfering with the detection. Therefore all microsensors use protection layers to improve the specificity. Many types of protections layers are used, although some of them are more effective than others. In general microsensors use background sensors to correct for aspecific signals. In this regard the self-reference based sensor, in which the active and baseline sensors are localized on the same surface (Burmeister and Gerhardt 2001), is a promising approach.

\textit{Second generation sensors}, show a less precise detection and their fabrication is difficult to reproduce, even with a semi-automatic dip-coater. The complexity of the mixture of the various components that are used to construct the hydrogel layer might explain this observation. In general, second generation sensors detect higher basal levels of glutamate compared with the results obtained from first generation sensors.

For first as well as second generation sensors, the oxygen-dependency is a critical property (especially under anaesthesia). More research is needed to eliminate this drawback. Another drawback of the present sensors is the occurrence of biofouling limiting the sensitivity, selectivity and duration of experiments. Much more research is needed to optimize these properties.

Of great interest is to relate behaviour to glutamate signals. Various stimuli or conditions such as movements, auditory stimuli, sleep, pain, reward etc need to be studied in this respect. For these studies a robust amplifier that is not influenced by movements of the experimental animal is prerequisite. In this respect the availability of wireless sensors is a promising development.
In Chapter 4 we have investigated whether NMDA receptors localized on the somatodendritic sites of mesolimbic dopamine neurons can be used as a functional NMDA model. To that end a dual-probe microdialysis system was used. The NMDA receptors localized on dopamine neurons in the ventral tegmental area (VTA) were influenced by infusing glutamatergic compounds and the activity of these neurons was determined by recording dopamine in the ipsilateral nucleus accumbens (NAc). NMDA increased dopamine release in the ipsilateral NAc, concentration dependent when 100 up to 500 µM concentrations were applied in the VTA. Conversely the stimulation by NMDA was largely suppressed during co-infusion of various NMDA receptor antagonists, confirming that the NMDA-stimulated release of dopamine was mediated by NMDA receptors. The maximal decrease of dopamine caused by the NMDA receptor antagonists was in line with previous dual-probe microdialysis studies where a 10-20% decrease was found (Karreman et al. 1996; Westerink et al. 1996). The model therefore displays a limited sensitivity when a decrease in NMDA-receptor activity needs to be detected.

In contrast increased effect of glutamatergic excitation was clearly detectable as infusions of NMDA increased dopamine levels to about 150% of controls. A limitation of the present model is the observed behavioural activation that was observed during high dosages of NMDA infusion. The pharmacological and stress-induced activation of the
dopamine neurons are difficult to distinguished under these conditions. However in the concentration range until 100 µM NMDA the pharmacological effects of NMDA can be discriminated from the behavioural activation as stereotypic behaviour is virtually absent at this concentration. Increased behavioural activation was also observed after high doses of glutamate, TBOA\(^2\), HCA\(^3\), CPG\(^4\) and MSO\(^5\).

The NMDA receptors contain a primary agonist binding site for glutamate and a additional modulatory site for the endogenous monocarboxylic amino acids glycine and D-serine along with some additional sites sensitive to Mg\(^{2+}\), polyamines, glutathione, protons and other factors (Javitt 2004). Therefore NMDA receptors represent a target rich environment although relatively few of the targets have been exploited to date. In the present dual-probe in vivo NMDA model, we investigated whether the NMDA receptor ligands glycine and D-serine were able to stimulate the function of the receptor. Glycine or D-serine were infused into the VTA whereas dopamine was detected in the ipsilateral NAc. In this study the glycine modulatory site of the NMDA complex seemed to be more susceptible to glycine than D-serine as an increase in dopamine was observed after glycine infusion which was not observed with D-serine. This was rather unexpected as glycine was shown to have a lower affinity for the glycine-modulatory site of the NMDA receptor than D-serine (Schell et al. 1995).

In Chapter 5 the glutamatergic afferent input to the dopamine neurons in the VTA was manipulated by infusing glutamatergic pharmacological active compounds via a microdialysis probe (first probe), while concurrently the modulation on the output of the dopamine neurons as determined by the release of dopamine in the ipsilateral NAc by the second probe was investigated. In addition glutamate was determined in the first probe. Glutamate changes in the first probe and dopamine changes in the second probe were evaluated and compared, in order to relate changes in microdialysate glutamate concentrations to postsynaptic glutamatergic effects as expressed by dopamine levels.

Infusion of glutamate in the VTA did not alter dopamine levels in the ipsilateral NAc. This observation can be explained by rapid uptake of glutamate from the extracellular space by high affinity transporters. Blocking “high” affinity glutamate transporters by infusing TBOA in the VTA induced a significant concentration-dependent elevation in extracellular

\(^2\) DL-threo-ß-Benzyloxyaspartic acid (TBOA)  
\(^3\) L-Homocysteic acid (HCA)  
\(^4\) (S)-4-Carbozyphenylglycine (CPG)  
\(^5\) L-methionine sulfoximine (MSO)
glutamate in the VTA dialysates, however only a moderate increase was noticed in extracellular dopamine in the ipsilateral NAc. The combined treatment of TBOA and glutamate in the VTA was not different from the results obtained with the single glutamate administration. The results indicated that the exogenous glutamate could only partly reach the glutamate receptors that activate the dopamine neurons.

When extracellular glutamate levels were strongly reduced by infusion of the glutamate synthesis blocker MSO, no effect was seen on dopamine release in the NAc. From this observation we may conclude that an important part of the glutamate sampled by microdialysis is derived from glial cells and do not relate to synaptic events.

Of all possible glutamate release mechanism, the cystine-glutamate antiporter has gained special interest after an article published by Baker et al. (2002). In the latter report the reverse dialysis of cystine-glutamate exchange (system Xc\(^{-}\)) inhibitors reduced basal extracellular levels by ±60% in the NAc. In the dual-probe microdialysis study the hypothesis was first confirmed with cystine, which induced an increase in extracellular glutamate in the VTA and an increase in extracellular dopamine in the ipsilateral NAc. These increases confirm that the functional glutamate release is possibly coupled to a cystine-glutamate antiporter. In accordance with Baker et al. (2002) the cystine-glutamate exchanger inhibitors HCA and CPG induce a small but significant decrease (74% and 85% of controls, respectively) in glutamate levels in the VTA. However whether this decrease in extracellular glutamate is also present in the synaptic cleft could not be confirmed as dopamine levels in the ipsilateral NAc did not decrease after HCA infusion and unexpectedly increased after CPG infusion.

In the last decade, the members of group II mGluR (mGluR2/3) receptors are considered as potential therapeutic targets. Activation of the mGluR2/3 receptors provide a negative feedback mechanism to prevent excessive presynaptic glutamate release that have been implicated in the pathology of psychiatric disorders. In this chapter, the extracellular glutamate modulation by mGlu2/3 receptors was examined with the known agonist APDC\(^6\) and the antagonist APICA\(^7\). The agonist APDC and the antagonist APICA induced a decrease and increase in extracellular glutamate respectively, thereby confirming the modulation of glutamate neuronal activity by mGluR2/3 receptors in the mesolimbic pathway.

\(^6\) (2R,4R)-4-Aminopyrrolidine-2,4, dicarboxylate (APDC) \\
\(^7\) (RS)-1-Amino-5-phosphonoindan-1-carboxylic acid (APICA)
In this study several experiments displayed a clear mismatch between the extracellular levels of glutamate in the VTA and the activity of dopaminergic cells in the VTA (as measured by dopamine in the NAc).

Taken together, these observations suggest that care should be taken to relate glutamate in microdialysates to “neuronal glutamatergic activity” in the brain. During certain pharmacological conditions however, a minor part of glutamate sampled by microdialysis, might be directly related to synaptic events.

In chapter 6 we have investigated the effects of group II mGlu receptor (mGlu2/3) related compounds on extracellular levels of glutamate detected by dual-probe microdialysis (a similar model as used in Chapter 5) as well as microsensors in the prefrontal cortex. The Eli Lilly agonist compounds LY354740 and LY379268 did not induced the expected decrease in dialysate levels. This is not surprising as there are a lot of discrepancies between studies where these LY agonists are studied. In accordance with the hypothesized model the mGlu receptor antagonist LY341495 induced a significant increase in extracellular glutamate levels in the VTA, whereas dopamine dialysate levels in the NAc were even so increased.

The microsensor experiments, carried out in anesthetised rats, revealed a decrease in extracellular glutamate after systemic administration of the mGlu2/3 agonists LY354740 and LY379268. The latter two sets of data are in agreement with the presumed feed-back hypothesis

In Chapter 7 and 8 the hydrogel glutamate microsensor was used in hippocampal slices. In Chapter 7 the hydrogel glutamate microsensor was used in organotypic hippocampal slices. Organotypic slice cultures can be considered as an interesting means to have relative preserved neuronal circuitries and functional neuronal-glial interactions which represents more closely the in vivo situation. The influence of several pharmacological agents on the extracellular glutamate levels were investigated and compared to results obtained previously in acute hippocampal slices by Oldenziel et al. (2007). The sensors used in acute and organotypic slices had similar sensitivities in the flow injection calibrations. However, the sensors displayed different in situ glutamate sensitivities, indicating that both slice types are intrinsically different. Where most compounds modulated glutamate in acute hippocampal slices, little or no effect was seen in organotypic hippocampal slices. Elevated glutamate and potassium concentrations did
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induce an increase in extracellular glutamate concentrations, but other compounds such as veratridine, latrotoxin, kainate, TBOA, calcium free aCSF, exogenous L-cystine, and glutamine had no effect in organotypic slices. The typical nature of the organotypic slices seems to prevents the detection of the pharmacological-induced changes in glutamate. Apparently in organotypic slices, glutamate release and metabolism is more tightly regulated and controlled by a better developed network of astroglial cells.

In Chapter 8 the glutamate microsensor was further evaluated in acute slices. First rats of different age groups were used. A significant difference in extracellular glutamate concentration was seen with an increase in age. Second it was investigated whether the increase in extracellular glutamate during infusion of elevated potassium could be modified by co-infusion of various pharmacological compounds (TTX $^8$, 4AP $^9$, Cd$^{2+}$ $^{10}$, CPG HCA and MSO). The various compounds that were evaluated did not induce statistically significant differences between the two high potassium stimulations. Finally we investigated whether glutamate release can be evoked after electrical stimulation of the slices; both in the absence and presence of the transport inhibitor TBOA. The preliminary experiments performed at University of Amsterdam (head: Dr. Henk Karst) using electrical stimulation of acute slices could not detect changes in extracellular glutamate. Taken together the results of Chapter 7 and 8 showed that the glutamate pools in brain slices are difficult to approach with the hydrogel microsensors.

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8 Tetrodotoxin (TTX)
9 4-aminopyridine (4AP)
10 Cadmium (Cd$^{2+}$)