Cortical brain release of glutamate by ketamine and fluoxetine: An *in vivo* microdialysis study in the Flinders Sensitive Line rat

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Abstract

In vivo intracranial microdialysis is a valuable technique yielding novel and useful insight into normal or pathological neurochemical processes in the brain by means of sampling of interstitial fluid of cells in a living animal. It's most important advantage is that it can continuously monitor time-related changes in the concentration of neurotransmitters and their metabolites, other neuromodulators, energy substrates, as well as exogenous drugs in the extracellular fluid of specific brain areas of interest. While the development and standardization of the intracranial microdialysis technique in our laboratory was the main aim of the current study, a pilot application study was also performed during which the effect of several locally administered pharmacological agents on brain glutamate levels in a genetic rat model of depression was investigated. Abnormal neuronal glutamate levels have been implicated in various psychiatric conditions including major depressive disorder. The Flinders Sensitive Line (FSL) is a genetic line of Sprague-Dawley rat that displays various behavioral and neurochemical traits akin to that observed in depression. The Flinders Resistant Line (FRL) rat is used as the normal control.

The prefrontal cortex is an important brain area involved in the neuropathology of depression. Prefrontal cortical glutamate levels in a small number of FSL and FRL rats were therefore compared at baseline and following local administration of potassium chloride (100 mM), the latter in order to study changes in evoked glutamate release. Ketamine hydrochloride (9 mM) and fluoxetine (30 µM) respectively were also administered via reverse dialysis. Prior to initiating the microdialysis studies, an HPLC-fluorescence method was developed to analyze the levels of glutamate in the microdialysate.

As part of the development and standardization of the microdialysis technique, a number of validation studies were performed. This included refining the stereotaxic surgery procedure, determining the most appropriate anesthesia protocol, and standardizing the microdialysis procedure with regard to perfusion fluid, flow rate,

sample volume, duration of dialysis, and anatomical verification of probe location. The HPLC-fluorescence method for the analysis of glutamate was also developed and validated. This technique proved to be sensitive and specific for the determination of glutamate with a linearity of 0.991 in the concentration range of standards tested (0.1 – 10 μM) and an intra-assay repeatability (precision value) yielding relative standard deviations of less than 10.5%, Mean elution time was between 24 and 26 minutes for glutamate in the microdialysis sample and the limit of detection and quantification was both 0.1 μM.

Results from the application study indicated that baseline values of glutamate in the prefrontal cortex did not differ between FRL and FSL rats during the 1 hour period of dialysis. However, potassium chloride-evoked glutamate release was greater in FSL vs. FRL rats, although this difference was not statistically significant. Local perfusion by reverse dialysis of ketamine hydrochloride produced statistically significant increases in glutamate concentrations at certain time points in FSL rats. Although glutamate levels were also increased in FRL rats in response to ketamine, it was not statistically different compared to baseline levels. Fluoxetine perfusion did not affect glutamate release in either of the two rat groups.

In conclusion, we have successfully developed and established an intracranial *in vivo* microdialysis procedure in our laboratory, as well as standardized and validated a sensitive method to analyze glutamate in microdialysate samples. These techniques were then applied in a small number of FSL vs. FRL rats in order to confirm their application in a typical research scenario. Although the data were too limited to make any valid conclusions about glutamate concentrations in an animal model of depression or the effect of drugs on the release thereof, these novel techniques and analyses will be valuable in future studies.

Keywords: microdialysis, glutamate, Flinders sensitive line rats, depression, HPLC-fluorescence, prefrontal cortex

Opsomming

In vivo intrakraniale mikrodialise is ‘n waardevolle tegniek om inligting in te samel met betrekking tot normale en patologiese neurochemiese prosesse in die brein deur middel van monsterneming van die interstisiële vloeistof van selle in ‘n lewendige dier. Mikrodialise se belangrikste voordeel is dat daar op ‘n kontinue wyse monitering van tydsafhanklike veranderinge in die konsentrasies van neurotransmitters, metaboliete, ander neuromodulatore, energiesubstrate asook eksogene geneesmiddels in die ekstrasellulêre vloeistof van spesifieke breinareas kan plaasvind. Hoewel die ontwikkeling en validering van hierdie spesifieke mikrodialise tegniek die belangrikste doelwit van die huidige studie was, is daar ook ‘n loodsstudie gedoen om die effek van die lokale toediening van verskeie farmakologiese substanse op die brein glutamaatvlakke van ‘n genetiese rotmodel van depressie te bepaal. Abnormale neuronale glutamaatvlakke is in verskeie psigiatriese toestande, insluitend major depressie, waargeneem. Die Flinders sensitiewe lyn (FSL) is ‘n genetiese lyn Sprague-Dawley rot wat verskeie gedrags- en neurochemiese eienskappe ooreenstemmend met die wat in depressie waargeneem is, vertoon. Die Flinders weerstandige lyn (FRL) rot word as die normale kontrole gebruik.

Die prefrontale korteks is ‘n belangrike area in die brein geassosieer met die neuropatologie van depressie Prefrontale kortikale glutamaatvlakke is in twee klein groepe FSL en FRL rotte vergelyk om basislynwaardes te verkry asook glutamaatvrystelling as gevolg van die plaaslike toediening van kaliumchloried (100 mM). Ketamienhidrochloried (9 mM) en fluoksetien (30 µM) is ook via omgekeerde dialise toegedien. Die mikrodialise studie is voorafgegaan deur die ontwikkeling van ‘n hoëdrukvloeistofchromatografie met fluoresensie (HDVC-FC) as deteksiemetode om die glutamaatvlakke in die mikrodialisaat te analiseer.

Gepaardgaande met die ontwikkeling en standardisering van die mikrodialise tegniek, is ‘n aantal ander validasie studies ook uitgevoer ten einde die stereotaksiese sjirurgiese prosedure te standardiseer met betrekking tot die mees toepaslike anestesie protokol.

Hierdie validasie prosedures is ook aangewend om die mees geskikte perfusievloeistof, vloeitempo daarvan, monstergrootte, duur van dialise en die anatomiese verifikasie van die toetsstafie ("probe") se plasing te bepaal. Die HDVC-FD is ook ontwikkel en valideer. Laasgenoemde metode is bewys as sensitief en spesifiek vir die analise van glutamaat met 'n liniariteit van 0.991 in die konsentrasiereeks glutamaat getoets (0.1 – 10 µM) en 'n intra-analise herhaalbaarheid (presisie) met standaardafwykings van minder as 10.5%. Gemiddelde retensietye vir glutamaat in die mikrodialisaat was tussen 24 en 26 minute en die limiet vir deteksie en kwantifisering was in beide gevalle 0.1 µM.

Resultate verkry uit die toepassingstudie het aangetoon dat die basislynwaardes van glutamaat in die prefrontale korteks nie verskil het gedurende die 1 uur dialise periode nie. Hoewel statisties nie beduidend nie, was die kaliumchloried-ontlokte glutamaatvrystelling hoër in die FSL rotte as in die FRL rotte. Plaaslike toediening van ketamienhidrochloried by wyse van omgekeerde dialise het by sommige tydsintervalle statisties betekenisvolle verhoging ten opsigte van basislynglutamaatvrystelling in die FSL rotte veroorsaak. Alhoewel glutamaatvrystelling in respons op ketamien ook op sekere tydstippe verhoog was, was hierdie verhogings nie statisties betekenisvol in vergelyking met basislynwaardes nie.

Ten slotte, is daar dus geslaag in die ontwikkeling en validasie van die *in vivo* intrakraniale mikrodialise tegniek in ons laboratorium asook in die suksesvolle standardisering en validasie van 'n sensitiewe analise metode ten einde glutamaatvlakke in die mikrodialisaat te kan bepaal. Hierdie tegnieke is vervolgens in 'n klein groepie FSL en FRL rotte toegepas ten einde die geldigheid daarvan in 'n tipiese navorsingsopset te bepaal. Alhoewel die data van die toepassingstudie te beperk was om enige geldige gevolgtrekkings hieruit met betrekking tot glutamaatvlakke in 'n dieremodel van depressie of die effek van geneesmiddels op die vrystelling daarvan te kon maak, sal hierdie nuwe tegnieke en analitiese prosedures baie waardevol en bruikbaar wees in toekomstige studies.

Sleutelwoorde: mikrodialise, glutamaat, Flinders sensitiewe lyn rotte, depressie, HDVC-fluoresensie, prefrontale korteks.

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"For to me to live is Christ, and to die is gain."
Philippians 1:21

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Chapter 1: Introduction

1.1 Project motivation and project statement

Microdialysis is a technique for measuring extracellular concentrations of substances in tissues, usually *in vivo*, by means of a small probe equipped with a semipermeable membrane. Substances surrounding the semipermeable part of the probe diffuse with the concentration gradient in or out of the perfusate (Westerink, 1995). When substances are introduced into the extracellular space through the membrane, the procedure is referred to as retrodialysis or reverse dialysis (Plock and Kloft, 2005). Intracerebral microdialysis refers to the implantation of the probe into a selected brain region and is currently a very popular and valuable technique for the measurement of drug-induced changes in neurotransmitter concentrations and to correlate behavioral changes and neurotransmission. This technique, based on the principle of kinetic dialysis, involves stereotaxic placement of a dialysis probe with a porous membrane (pore size of 5 – 35 kDa) which enables sampling of the extracellular fluid in conscious, freely-moving subjects with subsequent analysis of the samples. The inlet of the dialysis probe is connected to a pump, providing a constant pulse-free flow rate (between 0.1 and 5 µl/min of the perfusion fluid (usually a physiological salt) solution. At the end of the probe outlet, low volume microdialysates (1 – 40 µl) are collected. Due to the absence of enzymes and proteins, the stability of compounds in the samples are improved and can be directly analyzed without further sample preparation (Plock and Kloft 2005).

Major depressive disorder is a wide spread global disorder causing morbidity and mortality on a global scale (Ustun *et al*., 2004), and while the monoamine hypothesis has long been accepted as the best theory explaining the mechanism of action of current employed antidepressants, accumulating evidence on the pathogenesis of mood disorders indicate the involvement of the excitatory amino acid, glutamate in these disorders (Machado-Vieira *et al*., 2009c, Sanacora *et al*., 2012). The glutamate

hypothesis of depression was introduced in the early 1990's with the findings that NMDA receptor antagonists have antidepressant properties (Trullas and Skolnick, 1990). More recently, with the integration of results from different fields of study, this hypothesis is referred to as the neuroplasticity hypothesis (Pittenger and Duman, 2008). Glutamate is released by approximately 40% of synapses in the brain and very important in the mediation of cognition and emotion (Coyle and Puttfarcken, 1993). Clinical studies have confirmed the abnormal regulation of glutamate in limbic and cortical areas in the brains of depressed patients (Ongur *et al*., 2008; Sanacora *et al*., 2012), while postmortem evidence of elevated frontal cortical glutamate levels in individuals with major depressive disorder and bipolar disorder (Hashimoto *et al*., 2007; Lan *et al*., 2009) confirmed these observations. Although the origins of plasma glutamate and the way it is linked to the pathophysiology of mood disorders are not yet clear, depressed individuals were shown to present with elevated glutamate concentrations and decreased plasma glutamine/glutamate ratios (Mauri *et al*., 1998; Mitani *et al*., 2006), while treatment with antidepressants decreased these elevated glutamate levels (Kucukibrahimoglu *et al*., 2009). In addition, animal models of stress have shown an increased release of glutamate and altered synaptic glutamate transmission in response to different environmental stressors (Cazakoff and Howland, 2010, De-Vasconcellis-Bittencourt *et al*., 2011), and although the source of the extracellular glutamate determined via microdialysis has been questioned (Van der Zeyden *et al*., 2008), results obtained in more recent studies by different methodologies, confirmed the stress-induced increased glutamate release found in microdialysis studies (Cazakoff and Howland, 2010; Musazzi *et al*., 2010).

There are quite a number of analysis methods available to quantitate glutamate concentrations in microdialysis samples, viz the standard technique of HPLC with fluorescence or electrochemical detection (Donzanti and Yamamoto, 1988; Kehr, 1998), LC-MS/MS (Buck *et al*., 2009), as well as capllary electrophoresis (Dawson *et al*., 1995). At the time of this study, we have been limited to the use of an HPLC-fluorometric method and this method was therefore developed and validated in our laboratoy prior to the determination of glutamate in the microdialysis samples.

In the current study, male Flinders Sensitive Line (FSL) rats, considered to be a valid animal model of depression (Overstreet *et al.*, 2005, Wegener, *et al.*, 2011) were employed. This genetic model presents with extensive predictive and face validity for depression (Overstreet 1993; Willner and Mitchell, 2002 and Neumann *et al.*, 2010) including, lower body weight, reduced physical activity, sleep disturbances and anxiety, anhedonia following stress (Overstreet *et al.*, 2005, Wegener *et al.*, 2011) and a heightened sensitivity to environmental stressors (Pucilowski *et al.*, 1993; Neumann *et al.*, 2010). Neurochemical alterations found, include a hyperresponsive cholinergic system (Overstreet, 2002), impaired serotonergic neurotransmission (Overstreet *et al.*, 1998; Overstreet *et al.*, 2003, Zangen *et al.*, 1997; Hasegawa *et al.*, 2006) and gamma amino butyrate (GABA) activity (Pepe *et al.*, 1988) as well as an increased response of NMDA-nitric oxide synthase (NOS) signaling following stress (Wegener *et al.*, 2010). This behavioral and neurochemical profile can usually be adjusted with antidepressants (Kokras *et al.*, 2008).

1.2 Project objectives

The current project aims to establish and validate the technique of *in vivo* microdialysis in our laboratory where after it will be applied to determine extracellular frontal cortical glutamate concentrations (basal, KCl-evoked and drug-induced) in a small group of the Flinders Sensitive Line rat (a genetic model for depression in rats) compared to the Flinders Resistant Line rat (healthy controls). The establishment of the microdialysis technique may aid our understanding of the pathophysiology of depression as well as other neuro-psychiatric disorders.

The specific aims of this study are the following:

- To set up and validate the technique of *in vivo* intracranial microdialysis in rats for application in amongst others, depressive disorders.
- To develop and validate an analytical method for the determination of glutamate in the microdialysate (30 µl per sample).
- To apply these techniques in a small group of FSL vs. FRL rats to determine glutamate release at baseline, following K^+ stimulation and perfusion with fluoxetine and ketamine.

1.3 Project design

The project comprises of three parts of which the first two components involves the establishment of the microdialysis technique and development and validation of the analytical method for determination of glutamate. The third part follows as application of these techniques and will involve the stereotaxic surgery and placement of probes into the prefrontal cortex of a small group of a genetic line of rats resembling depression (FSL rats) and their healthy controls (FRL). Following recovery after stereotaxic surgery, insertion of the dialysis probes will take place and determination of released glutamate, basal as well as K^+ - induced will be performed over a period of 5 hours. These samples will be analysed by means of an HPLC – fluorescence detection method.

A total amount of 20 rats (10 FSL and 10 FRL) will be used in the application study and they will be randomly divided in 4 groups, viz.: aCSF (n = 2) (this group is also referred to as the control/naive group). Experimental groups included aCSF with KCl (n = 2); aCSF with Fluoxetine (n = 3) and aCSF with Ketamine (n = 3) (see Fig 1.1).

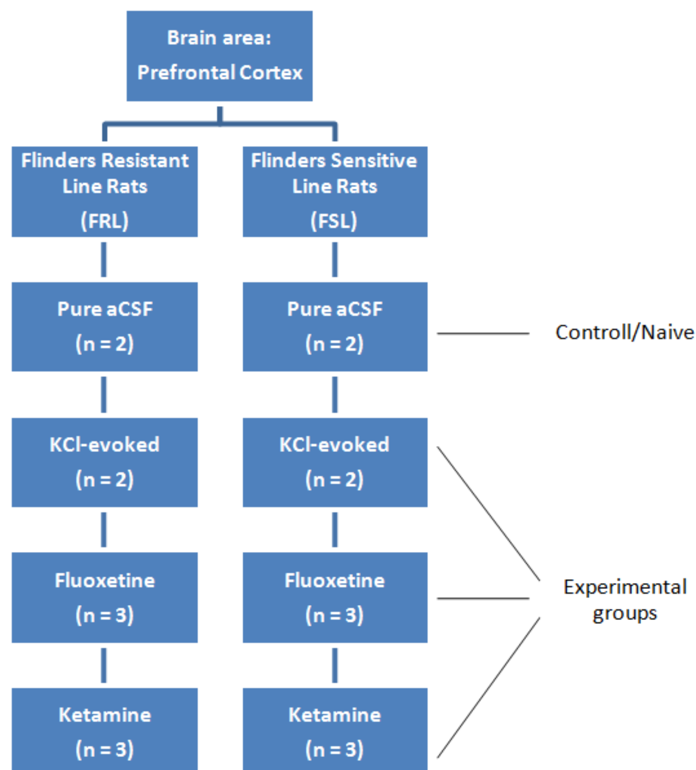

Figure 1.1 Project layout.

Chapter 2: Literature: Microdialysis and sample analysis

2.1 History

Microdialysis as a sampling method of the extracellular fluid (ECF) in live organisms can be traced back to the 1960's. One of the first experiments was described by Bito and co-workers in 1966. The pioneering microdialysis experiments were achieved by implanting a sterile dialysis sac into the cortex or subcutaneously in the neck of mongrel canines. After ten weeks the contents of the sacs were analysed for the presence of amino acids and ions. These results were compared to concentrations in the plasma and cerebrospinal fluid (CSF) of the same animals. Interestingly Bito *et al*. (1966) noted that the fluid in the brain sacs did not represent a dialysate from either the blood or CSF thus indicating a third compartment, the extracellular fluid (ECF). This method as described by Bito *et al*. (1966) had the disadvantage of providing only a single sample, multiple samples from one animal was not possible.

Six years later in 1972, Delgado *et al*. achieved and reported a technique for acquiring multiple samples over a timed period from a single animal. The authors devised the "dialytrode" to achieve this. The dialytrode consisted of a push-pull cannula with a small polysulfone membrane bag glued on its tip. Unfortunately Delgado failed to be the first to perfect the method of microdialysis.

It wasn't until the rapid advancement in highly sensitive HPLC analytical techniques in the beginning of the 1980's that the value of the microdialysis technique was realized. Microdialysis was first applied in animals in the fields of neurobiology, pharmacology and physiology by Ungerstedt and colleagues (see Ungerstedt *et al*. (1982a; 1982b; 1984). It soon followed as a sampling technique in humans as described by Meyerson *et al*. (1990), Hillered *et al*. (1990) and Ungerstedt *et al*. (1991).

Soon several books on *in vivo* monitoring including chapters on *in vivo* microdialysis were published and a monograph entitled “Microdialysis in the Neurosciences” was edited by Robinson and Justice in 1991.

A series of meetings concentrating on electrochemical detection and *in vivo* methods in neuropharmacology was initiated by professor C. Marsden in 1982, and succeeded in further developing microdialysis as a technique. These meetings eventually grew into a medium-sized international conference on “Monitoring Molecules in Neuroscience” held every second year.

Microdialysis as it is applied today has mainly been developed by Johnson *et al*. (1983) and Ungerstedt *et al*. (1983).

The introduction of a substance into the extracellular space via the microdialysis probe followed (Wang *et al*., 1993, Galvan *et al*., 2003) and this method of introducing a substance to the extracellular fluid is referred to as “reverse microdialysis” or “retro-dialysis”.

2.2 Principles of microdialysis

Microdialysis is a valuable method for the sampling of interstitial fluid of cells in a live animal or human. Microdialysis involves the surgical insertion of a “probe” into a discrete area of an organ or tissue. For the purpose of this dissertation the focus will be on the brain (intracerebral microdialysis), more precisely the prefrontal cortices of rats (Chefer *et al*., 2009).

An inserted probe is situated in the interstitial space being directly adjacent to the cells composing the tissue. The probe is connected to a precision perfusion pump feeding the probe (at constant velocity) with a physiological solution. The basic principle of microdialysis rests upon the first law of Fick. This law states that the flux in a concentration field goes from regions of high concentration to regions of low concentration, with a magnitude proportional to the concentration gradient.

For example: When the concentration of a substance in the interstitial fluid is higher than the concentration in the perfusate, this said substance will diffuse across the membrane which divides the two fluids. In a stationary system this diffusion will continue until the concentration in both the interstitial space and perfusate are equal. Exchange of molecules over the membrane is permitted in both directions thus making it possible to introduce exogenous compounds to the area where the probe is implanted. The “cut-off” point of the membrane prevents larger molecules from crossing the membrane. The dialysate is therefore devoid of proteins and enzymes having a molecule size larger than the cut-off point of the membrane used. The benefit of this is that the sample is devoid of any enzymes or proteins responsible for the metabolism of neurotransmitters and amino acids. Thus no metabolic degradation of neurotransmitters will occur once the neurotransmitters have crossed the dialysis membrane and collected in the tubes.

For the purpose of understanding the principle of the technique a brief description will be given of the probe. Basically the probe consists of a thin hollow piping with a semi permeable membrane tip. The membrane serves the purpose of separating the extracellular fluid and the dialysate, but allows small molecules to pass through the membrane (Figure 2.1).

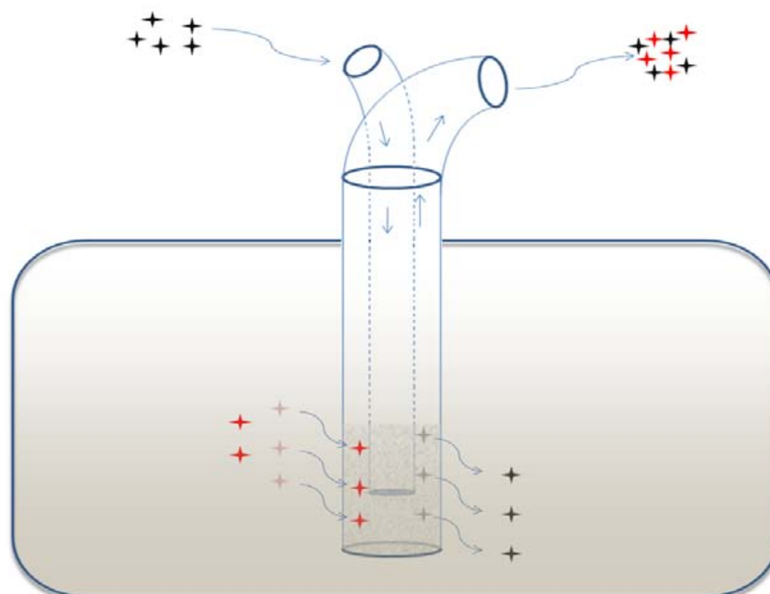

Figure 2.1 Graphical representation of *in vivo* microdialysis probe: The black stars represent an exogenous substance introduced to the probe via the inlet tubing. This

substance migrates across the membrane into the periprobe extracellular fluid. The red stars represents an endogenous substance (e.g. neurotransmitter) migrating across the membrane of the probe into the perfusion fluid and then subsequently collected as a sample.

The analyte concentration in the dialysate (C_{dial}) never represents the exact concentration of the analyte in the periprobe fluid (C_{tissue}). (The method of no-net-flux is an exception.) The probe is constantly perfused with the analyte-free solution (perfusate), thus preventing equilibrium to be established. The analyte concentration found in the dialysate will therefore only represent a fraction of the actual analyte concentration existing in the periprobe fluid. Thus $C_{tissue}>C_{dial}$, this ratio is called the relative recovery (RR). The relative recovery of a probe can be defined as the ratio between the concentration of the substance in the dialysate and the fluid surrounding the probe (Plock and Kloft, 2005). Typical samples obtained from microdialysis are in the microliter range, usually between 10 and 30 microliters. The low concentration of the substances in the dialysate necessitates the use of highly sensitive analytical methods, like LCMS/MS or HPLC (Brunner *et al.*, 2006).

2.3 Stereotaxic surgery

Insertion of the microdialysis probe via the cannula in the rodent brain involves the induction of surgical anesthesia followed by delicate stereotaxic surgery. The word "stereotaxic" originates from the Greek word "stereo" (solid) and "taxis" (arrangement, order). Stereotaxic surgery is therefore a surgical procedure performed in a solid spatial arrangement on a predetermined coordinated anatomical location and is a minimally invasive procedure.

The three main components that comprise stereotaxic surgery include:

- The planning (mapping) of the surgery by using an anatomical atlas e.g. Paxinos and Watson, 2005, and the calculation of subsequent coordinates which will be use to introduce the probe into the targeted area.
- Fixation of the rat (under anesthesia) in a suitable stereotaxic apparatus or frame.
- The actual stereotaxic localization and placement procedure.

The surgical procedure takes approximately 40 minutes from the induction of surgical anesthesia to the end of the procedure. The anesthetized animal's head is fixed in the stereotaxic frame. The head of the rat is prepared to expose a clean and sterile platform where the midline incision is to be made in the skin above the skull.

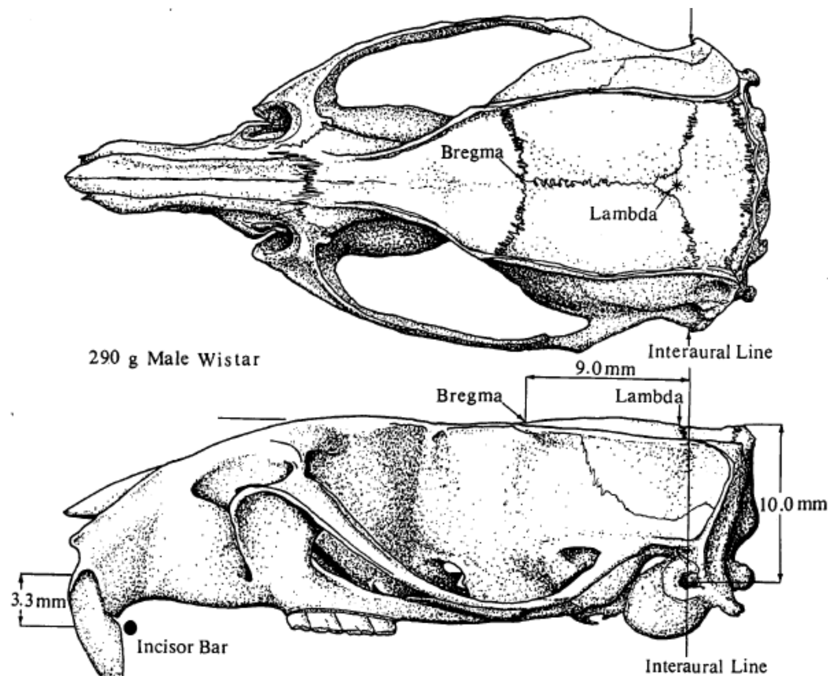

Figure 2.2 The location of bregma on the exposed skull surface of a rat. The bregma is used as a zero point and the 3-dimensional coordinates are calculated in accordance to the bregma suture. Illustration taken from Paxinos and Watson, 2005.

With the guide cannula fastened in the cannula holder of the stereotaxic frame, adjustments are made to position the tip of the cannula directly on top of the "bregma" suture of the skull (Fig. 2.2). The x (lateral), y (ventral) and z (anterior- posterior) coordinates are recorded and taken as the "zero" reference. Depending on the area to be investigated the cannula would be aimed at the specific coordinates for that area, obtained from Paxinos and Watson (2005), e.g. the prefrontal cortex is at 4.2 mm anterior-posterior (AP), 2.4 mm lateral (L) and 2.4 mm ventral (V) relative to the coordinates of the bregma suture.

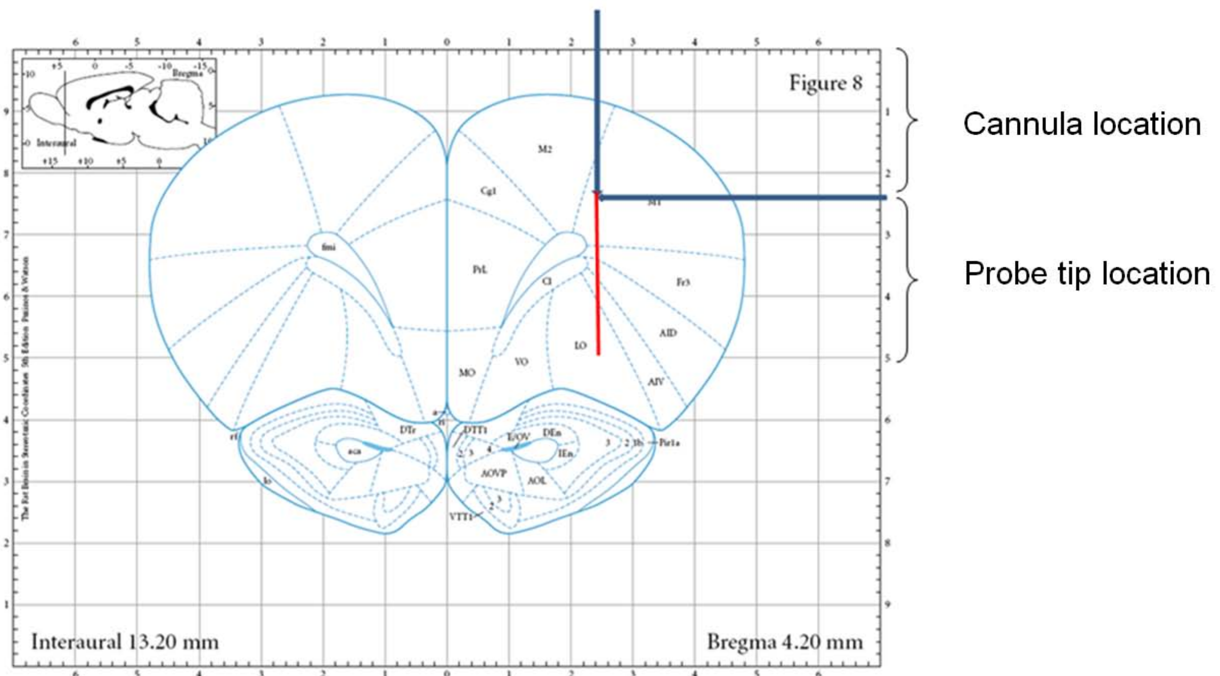

Figure 2.3 The coronal view of a rat brain showing the location of the cannula and probe. The coordinates: AP: 4.2 mm; L: 2.4 mm; V: 2.4 mm aimed at the frontal cortex. The blue arrows indicate the location of the cannula tip and the red line represents the probe tip protruding beyond the tip of the cannula (Modified figure taken from Paxinos and Watson, 2005).

The desired coordinates (Fig. 2.3) for the probe location are calculated and adjusted to the coordinates for the anterior-posterior and lateral axis and the spot on the skull is marked. This spot gives the location of where the opening should be made in the skull. A dental drill with a suitable burr is used to drill the hole, which exposes the dura mater. Two additional holes are drilled adjacent to this hole and anchor screws are fitted into these holes.

A sterile syringe needle is used to pierce the dura mater in the hole and the cannula is fitted flush in the hole and is lowered slowly to the desired y-coordinates (depth). Acrylic glue is applied around and under the anchor screws as well as around the cannula. The glue is “built up” to anchor the cannula firmly and in a steady position.
After the stereotaxic procedure the rat is granted enough time in its home cage to recover from the anesthesia and surgery.

2.4 Microdialysis procedure

The microdialysis procedure is usually performed on the third day after recovery from the initial surgery. A too lengthy recovery period (more than 72 hours) leads to cell death and hypercellularity in an extended area around the cannula (De Lange *et al.*, 1995). A too short recovery period does not allow for sufficient washout period of the anesthetic drug used during the surgery. Recovery periods of 3 days have been found to yield best results and are employed by many authors (Fukushima *et al.*, 2004; Volke *et al.*, 1999 and Wegener *et al.*, 2000). Following the time period allowed for recovery, the rat is lightly anesthetized and the probe is inserted into the guide cannula. Approximately 2 hours are granted for general habituation from the time that the rat is placed in the microdialysis cage while perfusion of the probe, usually with a physiological salt solution, is then initiated. Following this period, sample collection is initiated and approximately 1 hour is granted for the generation of a "baseline" measurement followed thereafter by approximately 4 hours of dialysis (either normal microdialysis or retrodialysis). Samples are collected in a refrigerated automatic sample collector set to pool each sample for a period ranging from 1- 20 minutes (Chefer *et al.*, 2009). Following collection of all the samples the rat is euthanized.

2.5 Microdialysis perfusion fluid

2.5.1 Composition

A physiological salt solution, e.g. Ringers solution, artificial cerebrospinal fluid (aCSF) etc. is usually used as perfusate (Chefer *et al.*, 2009). The ionic composition of the endogenous cerebrospinal fluid (CSF) is controlled by a very efficient homeostatic process (Rapoport, 1976). The ions prevalent in the highest concentrations are Na^+, K^+ and Cl^- followed by Mg^{2+}, Ca^{2+}, H^+, HCO_3^-, HPO_4^{2-} and SO_4^{2-} in lesser concentrations. These ions in the CSF play an important role in the polarizing and depolarizing of the neurons.

Microdialysis typically targets the ECF (extracellular fluid) of the organ in question, it is thus necessary for the perfusate to exhibit an ion composition directly related to the composition of the endogenous ECF.

In addition to the isotonic composition of the physiological salt solution, the composition of this solution can be altered on purpose to illicit a reaction that can be quantified (Chefer *et al.*, 2009), e.g. using K^+ evoked neuronal activation.

2.5.2 Temperature and flow rate

It is advisable to pre-heat the perfusate to the specimen's core body temperature. This is however not done by most investigators and the perfusion fluid is usually applied at room temperature. The effect of a temperature gradient between the CSF and the perfusion fluid is still a topic for debate, although de Lange *et al.* (1997) used a subcutaneous implanted cannula in order to equilibrate the aCSF to the body temperature of the specimen before entering the targeted tissue.

The flow rate of the perfusion medium also plays an important role in the resolution and the recovery of the samples. With higher flow rates the pressure inside the probe may exceed the pressure of the surrounding ECF and will result in a net fluid transport over the membrane counteracting the diffusion of molecules into the dialysate. Typically perfusion flow-rates used in microdialysis experiments range between 0.1 and 5 µl/min. Lower flow-rates may increase recovery but also yields smaller size samples (De Lange *et al.*, 1997). The smaller sample sizes necessitate special instruments capable of handling these small samples. Employing a flow rate of 1.5 µl per minute with a standard collection time of 20 minutes would typically yield a sample volume of 30 µl.

Perfusate collection is done in small HPLC-vial inserts. These collection tubes are loaded in a refrigerated micro-fraction collector, a precision instrument feeding an array of pre-loaded collection tubes at a precise pre-set time interval. Refrigeration counteracts the degradation of the contents of the perfusate and more importantly prevents vaporization of the samples.

2.6 Anesthesia for craniotomy procedure

Anesthetics and anesthesia techniques play an important role in the surgical outcome when a microdialysis probe/cannula is implanted intra-cranially. Being a craniotomy, special measures must be taken to ensure the best outcome of the surgery. All the

factors that may influence the anesthesia should be taken into account. A wide range of anesthetics and techniques are being used with varied degrees of success.

To obtain a good understanding of cerebral physiology, craniotomy and anesthesia used in neurosurgery one has to refer to the human medical literature.

The aim of the anesthesia (in the case of a craniotomy) is to provide optimal operating conditions while still maintaining adequate/balanced cerebral blood flow to provide the brain with sufficient levels of oxygen and glucose. The brain has a limited ability to store glucose and oxygen, thus to avoid neuronal damage, special care should be taken to keep the blood flow, glucose and oxygen within normal limits (Ravussin and Wilder-Smith, 1996).

To determine the most suitable anesthetic agent and technique to use, it is advisable to run an initial anesthesia trial to determine the anesthesia variability in the different specimens to be used. Differences in handling protocols, sex, strain, health and age may result in differences in induction, duration and recovery time (Ravussin and Wilder-Smith, 1996). It is important to determine these parameters for the specific drug and specimens in question.

The following is a list of the optimal operating conditions in the case of a craniotomy as defined by Ravussin and Wilder-Smith, (1996) and Young *et al.* (1998):

- **Prevent increase in intracranial pressure (ICP)**
  Increased intracranial pressure (ICP) results in brain tissue swelling which tends to cause pain and other complications. Haemorrhage may occur more easily with the insertion of the probe/cannula as a result of increased ICP. This may further lead to possible ischemia and brain damage. Basili *et al.* (2000) concluded that intra-operative and early post-operative hypertension may be associated with a higher incidence of post-operative intracranial haemorrhage. These data suggest that efforts to prevent hypertensive episodes may be justified.

- **Maintain brain blood perfusion within the normal limits.**
  The brain has a limited ability to store oxygen and glucose. When brain perfusion is inhibited too far below the normal limits brain damage may set in within a short period of time.

- **Decreased metabolic demands to protect the brain against ischemia.**
  Decreasing the brain's metabolic demand for glucose and oxygen allows more tolerance for a lower level of brain blood perfusion, allowing for the surgery to be done while maintaining a lower limit of intracranial pressure/blood pressure.

2.6.1 Route of administration:

The **intravenous** route is second only to the inhalation route regarding temporal and depth control of the anesthesia. The tail vein is used as intravenous route in rats. This administration route, especially when used in rodents, requires some advanced experience and skill.

The **intraperitoneal** route is especially useful in rodents due to the ease of performing intra-peritoneal injections. When using this route it is not feasible to perform multiple injections till the desired effect is achieved. The undesirable pharmacokinetic characteristics of this route of administration necessitate the use of a drug with a large safety margin to obtain satisfactory anesthesia as well as optimal recovery of the specimen.

The **intramuscular** route differs from the intraperitoneal route only by the ease of administration. Intramuscular administration tends to be more painful and stressful to the animal.

The **subcutaneous** route is not popular for use in anesthesia, mainly due to the undesirable pharmacokinetic qualities of this route.

Inhalation is probably the most popular route used in anesthesia. It exhibits a fair amount of advantages over the other routes of administration. Induction, depth of anesthesia and recovery can be controlled with superior temporal resolution when compared to the other routes. This route however requires specialized equipment which does not always prove to be cost-effective if it has to be newly acquired.

2.6.2 Assessment of anesthesia

The assessment of the level of anesthesia is important to ensure that the animal is completely anesthetized before proceeding with any surgical procedure.

The survival and recovery of the animal depends heavily on the assessment and correct application of the anesthesia and the stage of surgical anesthesia is not always easily recognized. The stages of anesthesia as described by Guedel, 1936 are not distinctly recognizable and more often than not "flow" into one-another making the transition difficult to recognize. The stages may also vary between animals. Unfortunately skilful assessment of anesthesia as a technique can only be acquired by sound experience.

In the book "Veterinary Anaesthesia" authored by Hall and co-workers (2001) it is stated that the experienced anesthetist most of the time relies mostly on the animal's response to noxious stimuli produced by the surgeon to indicate the adequate depth of unconsciousness. According to the authors the most effective depth is that taken to obliterate the animal's response to noxious stimuli, e.g. tail pinch and eye-ball contact without depressing the respiratory and/or circulatory systems.

Although it is difficult to distinguish between the different stages of anesthesia as first described by Guedel in 1936 using inhaled diethyl ether, these stages are still referred to as the so-called Guedel's signs and is used as an indication whether surgical anesthesia has been attained or not. The Guedel stages are as follows:

Stage 1 starts when the anesthetic drug is administered to the point when the animal loses consciousness. During this stage analgesia sets in.

Stage 2 starts when consciousness is lost and lasts until the regular breathing pattern is regained. This is the "excitement" stage which may attribute to struggling, holding breath, vomiting or coughing of the animal.

Stage 3 is the "surgical anesthesia" stage. This stage can be recognized by the breathing pattern settling back into a normal rhythm. To verify if the animal has reached this stage a number of tests can be done which will be discussed later in the chapter.
Stage 4 is the stage of "over dosage" and the animal will ultimately cease breathing and circulatory collapse will occur.

The stage of interest for the performance of surgery would be "Stage 3". This stage can be further subdivided into four "planes". (CCAC, Guide 1993)

Plane 1: The specimen exhibits regular respiration and is still able to display blink and swallowing reflexes. This is "light" anesthesia.

Plane 2: Respiration is still regular, but the specimen will no longer exhibit the blink reflex and the pupil will become fixed (unresponsive to light). This plane is known as "surgical" anesthesia.

Plane 3: The specimen now starts to display laboured breathing due to over-relaxation of the respiratory muscles. Assisted ventilation may become necessary. This is now "deep" anesthesia.

Plane 4: The specimen would typically stop breathing entirely and would subsequently perish if no assisted ventilation is applied.

Following a basic understanding of the stages of anesthesia one has to know how to recognize the specimen's state of anesthesia. A variety of reflex assessments may be applied to estimate the depth of anesthesia. Note that accurate assessment cannot be done by using only one reflex test. This is due to the inter-variability between animals. The specific anesthetic also plays an integral role in how the reflexes are altered. The

following reflex tests owe their popularity to the distinctness of the tests (Hall *et al.*, 2001).

- The **Pupillary reflex test** can be applied by shining a light in the eye of the specimen upon which the pupils will typically constrict. The reflex will be apparent at the start of Stage 3 and will gradually decrease onward and totally disappear when the approximate middle of Stage 3 has been reached.
- The **Palpebral reflex test** is applied by touching of the eyelid of the specimen. Usually this is done at the corner of the eye. When this reflex is present the specimen would typically blink. This reflex will disappear relatively early in Stage 3.
- The **Corneal reflex test** is considered to be inaccurate by Hall *et al.* (2001), partly due to the fact that this reflex can sometimes persist for a short time after cardiac arrest. The corneal reflex test is however still popular amongst researchers. The animal would typically blink when the cornea is lightly touched. This reflex would generally disappear early in Stage 3. Care should be taken not to damage the cornea when touching it.
- Amongst the most popular tests is the **Withdrawal reflex**. The gentle pulling of a limb of the specimen and pinching of the toe will cause the specimen to pull back. The presence of this reflex indicates that the specimen is still able to experience a painful stimulus. This reflex should be totally absent before any surgical procedure is commenced. Disappearance of this reflex is usually apparent early in Stage 3.

A number of factors directly involving the specimen and pharmacokinetics of the drug may have an impact on the anesthesia parameters. The effect of these factors on the anesthesia should be determined in a pilot study, prior to the execution of the final protocol. This aids the validation of a standardized anesthesia regime for the specific specimens used.

This is a brief list of the above mentioned factors:

- **Species:** Intra- and inter-species variability will for obvious reasons be present. A successful regime or protocol for one species would thus not necessarily be appropriate for use in other species.
- **Strain:** Different strains also play an important role. This would typically be the result of different genetic traits between the strains.
- **Age:** The metabolic processes and physiological systems will differ with age. Especially parenteral drugs will be influenced.
- **Weight:** The dose should be adjusted according to the subject's weight. It is noteworthy that very fat specimens may not be able to breathe as effectively as specimens with less fat. Moreover, fat tissue does not have the same blood perfusion or distribution properties as the rest of the body tissues. If body fat accounts for a considerable percentage of the total body weight, administering the same dose (as for a non-obese specimen calculated by mg/kg) may produce a relative overdose.
- **Sex:** Sex difference also plays a role in anesthesia.
- **Health of specimen:** Metabolic deficits, pre-existing disease or other pathological conditions may influence the outcome of the anesthesia.
- **Demeanour:** Handling the specimen will typically increase circulating adrenalin levels, heart rate and blood pressure. These will in turn have an effect on especially the induction of anesthesia.
- **Previous anesthesia:** For example, sodium pentobarbitone is not cleared as rapidly as some other anesthetic agents. Pentobarbitone may be present in the specimen's physiological system for several days despite fully recovered consciousness and normal behaviour. A second anesthetic procedure/attempt may thus be altered with regard to induction, duration and recovery.

2.6.3 Drugs used for anesthesia

Anesthetic agents fall into two categories, on the basis of its route of administration, viz. inhalational agents and parenteral agents.

2.6.3.1 Inhalational anesthetic agents:

Volatile anesthetic agents usually require a vaporizer and other sophisticated equipment for administration. This is one of the major shortcomings of inhalational anesthetics. Vaporizers and the accompanying instruments are very expensive. The concentration of the anesthetic agent can easily be adjusted which enables the user to accurately and quickly and easily alter the depth of anesthesia. Another advantage of inhalational anesthetic agents is the rapid recovery following the discontinuation of the anesthesia. The duration of anesthesia can be precisely controlled by stopping the inhalation of the agent. The recovery from an inhalational anesthetic agent is more rapid than from anesthetic drugs with other routes of administration (Flecknell, 1996).

- Isoflurane:

Isoflurane should only be used with its specified vaporizer. Isoflurane is often used in craniotomy surgical procedures (Walters, 1999), but is also fairly expensive. Vasodilatation and negative inotropic effects may decrease cardiac output and cause a dose-dependent hypotension. Mild respiratory depression may occur. Good muscle relaxation is achieved. Isoflurane exhibits little to no analgesic effects. A more rapid induction and recovery is produced by isoflurane in comparison to halothane (Rice *et al.*, 1986).

- Halothane:

Halothane is not used for neurosurgery for it has several unfavorable effects on the brain. Halothane can however be employed without the use of a vaporizer. It is important to not excessively expose the user to the vapors. Halothane is also fairly cheap and readily obtained (Flecknell, 1996).

2.6.3.2 Parenteral anesthetic agents:

Parenteral anesthetic agents are easily administered requiring merely a syringe and needle. One of the disadvantages of this group is that once the agent is injected it is almost impossible to control its effects. Recovery from parenteral agents depends on the drugs' metabolism or the redistribution from the blood to the tissues or a

combination of these processes. This leaves the user little to do in the event of an overdose or to improve the recovery outcome.

- Sodium pentobarbitone:

This agent is probably the most widely used agent for anesthetizing rats. It is very cheap, readily obtained and is administered intraperitoneally which is fairly easily done. However pentobarbitone has a threefold variation within a strain with respect to the duration of unconsciousness following a standard dose (Messier *et al.,*1999). This intra-strain variation together with a narrow safety margin and the accumulative effect of multiple doses poses a crucial challenge and limitation to the use of pentobarbitone in this setting.

Sodium pentobarbitone as mono-regime for neuro-anesthesia has some distinct disadvantages which makes it highly unfavorable for use in rats prepared for microdialysis as being discussed in the following paragraph.

Sodium pentobarbitone has an extended anesthesia induction period and additionally it has been found that the onset of surgical anesthesia in rats is invariably accompanied with severe respiratory depression (Flecknell, 1996). This leads to a high mortality rate when sodium pentobarbitone is used as sole anesthetic agent in rats. Sodium pentobarbitone also exhibits a narrow safety margin which increases the risk of overdosing. Analgesia is limited until the specimen is completely unconscious and excitation may occur during the recovery phase.

- Ketamine & xylazine

Ketamine should never be used as a sole anesthetic agent as it has poor analgesic effects. Concurrent use of a tranquilizing agent like diazepam, xylazine or acepromazine is recommended to provide smoother recovery and prevention of excessive muscle rigidity. Muscle tone is not attenuated during ketamine anesthesia and many of the reflexes used to assess the depth of anesthesia remain intact (Hall *et al.*, 2001). Usually the blink and swallowing reflexes remains responsive (Hall *et al.*, 2001). The animal is however unresponsive to pain. Ketamine increases heart rate by a positive inotropic effect on the myocardium, cardiac output, blood pressure and

central venous pressure, and may cause apneustic respiration. Ketamine also increases intracranial and intraocular pressures (Kolenda *et al*., 1996). The Ketamine and Xylazine combination as well as Hypnorm® (combination of fentanyl and fluanisone) are currently considered most effective by various authors (Messier *et al.,* 1999).

- Fentanyl & fluanisone & midazolam

The combination of fentanyl (a potent, short acting opioid analgesic) and fluanisone (an antipsychotic of the butyrophenone class) as in the above mentioned product Hypnorm® simultaneously administered with midazolam (a ultra-short acting benzodiazepine) provides good surgical anesthesia with muscle relaxation for about 20 – 40 minutes (NOAH Compendium of Data Sheets for Animal Medicines, 2012)

- Urethane

Urethane provides long periods of surgical anesthesia. The carcinogenic effect of urethane is one of the reasons why animals should not be allowed to recover from urethane anesthesia (Flecknell, 1996).

- Propofol

Propofol may pose some valuable advantages when used for neuro-surgery. Propofol is a relative new anesthetic drug which has gained in popularity since the late 80's when it was first introduced. Propofol is administered intravenously, either by bolus injection or continuous infusion.

The most distinct advantage of propofol is the favorable effects it has on the brain. This makes it a strong candidate for use during craniotomy surgery. Propofol anesthesia decreases mean arterial pressure (MAP), cerebral metabolic rate for oxygen ($CMRO_2$) and intracranial pressure (ICP), but the cerebral perfusion pressure (CPP) and cerebral autoregulation as well as CO_2 reactivity are maintained. Propofol has been proven to be neuroprotective in several *in vivo* and *in vitro* models of cerebral ischemia (Vasileiou *et al*., 2009). In addition propofol also exerts an inhibitory effect on platelet aggregation (Vasileiou *et al*., 2009). Propofol exhibits an improved outcome in animals anesthetized with propofol compared with nitrous oxide/fentanyl (Leslie *et al*., 2001).

Propofol has been found to have anxiolytic effects (Kurt *et al*., 2003, Vasileiou *et al*., 2009) and has a very fast onset of anesthesia as well as a rapid rate of recovery. The duration of anesthesia produced by propofol in rats is approximately 5 minutes (Flecknell, 1996). Furthermore propofol is void of any cumulative effects. These aforementioned pharmacokinetic and –dynamic properties of propofol make it possible to control the depth of anesthesia much easier than with other parenteral anesthetic agents. In rats propofol is typically administered as an IV bolus in the lateral tail vein. This dose can then be carefully titrated to the desired anesthesia depth. Generally a dose of 25% of the original calculated dose every 3 - 4 minutes would suffice as maintenance. Concurrent administration of IV diazepam can decrease propofol need by as much as 50%.

Cheng *et al*. (2008) found that propofol demonstrates less postoperative pain and less patient use of morphine compared to isoflurane. Cenic *et al*. (2000) suggested propofol sedation to be superior to sedation with morphine in intubated head-injured patients. Nausea and vomiting is strongly associated with intracranial surgery (Fabling *et al*., 1997). The anti-emetic effect of propofol largely attenuates the incidence of post-operative nausea and vomiting (Vasileiou *et al*., 2009).

Rapid administration can lead to hypotension, reduced myocardial contractility and respiratory depression. One of the disadvantages of propofol is that the hyperlipid emulsion promotes bacterial growth and once the bottle is opened it should be used within 6-8 hours.

2.7 Anatomical probe location verification

Microdialysis experiments would be futile without the precise verification of the probe location in the specimen's brain. This would defeat one of microdialysis's most important advantages, viz. its ability to sample discrete brain regions. Rapid probe location verification also aids the experimenter to screen the specimens before

analyzing the samples. This eliminates the unnecessary, expensive and time consuming analysis of samples which would be ultimately excluded from the study due to wrong probe location. Traditionally most researchers employed standard histological staining (i.e., cresyl violet, fast green perfusion and formalin fixation). These staining methods are time consuming and often expensive and complicated. Bert *et al.* (2004) described a novel, rapid and inexpensive method for the verification of probe track location in the rat as well as the mouse brain. The method employs a digital photomicrograph of a coronal section of the frozen brain of the specimen rodent. The appropriate coronal diagram obtained from a rodent brain atlas is now superimposed on the photomicrograph with the help of computer software. This allows the precise and rapid allocation of the dialysis probe track. In a study comparing the photomicrograph technique and the cresyl violet staining method, the photomicrograph technique yielded more rapid, accurate, reliable and less expensive results (Bert *et al.*, 2004).

2.8 Glutamate determination via microdialysis

While assessment of monoamine transmitters (eg. DA, NA, 5HT) and γ-amino butryic acid (GABA) can be accurately quantified with microdialysis, glutamate presents certain limitations. There are two pools of glutamate in the brain, the first being related to metabolic processes and the second being neuronal glutamate contained in the neuronal perikarya and dendrites (seen as a single unit) and the nerve terminals (seen as another unit) (Balazs *et al.*, 1972).

Glutamate found in the dialysate resulting from the microdialysis procedure can either be derived from the body plasma, the neuronal perikarya, the nerve terminals or a combination of these three sources. There is thus a slight posibility that the damage caused by the microdialysis probe to the brain tissue and the blood-brain barrier may be partly responsible that the glutamate obtained in the dialysate may be derived from any or a combination of the mentioned sources (Musazzi *et al.*, 2011).

It is however evident that glutamate does not diffuse from the synaptic cleft to reach the extracellular space in significant quantities due to glutamate transporters located on

astrocytic processes forming a sheath around synapses (Diamond & Jahr., 2000; Pfrieger & Barres., 1996). This is further supported by studies demonstrating that specific stimulation of presynaptic terminals (by inducing postsynaptic excitatory potentials) caused no increase in dialysate concentrations of glutamate (Segovia *et al.*, 1997b, Obrenovitch *et al.*, 2000). The question is therefore whether glutamate sampled by microdialysis represents synaptic release, carrier-mediated release, glial metabolism or a combination of the mentioned sources. The *in vivo* microdialysis criteria for determining synaptic release of neurotransmitters are based on the response to tetrodotoxin (TTX) (assessing involvement of nerve impules) and calcium (assessing calcium dependency) administration. On this basis most authors have concluded that glutamate does not fulfill this classical criteria for exocytotic release (Del Arco *et al.*, 2003).

However, other lines of evidence showed that extracellular glutamate concentrations may change in response to specific pharmacological and behavioural stimuli, this in turn could also be interpreted as a consequence of the activation of specific neurochemical circuits (Del Arco *et al.*, 2003). It is also widely believed that the glutamate concentrations reflected in the dialysate of microdialysis experiments are mainly of astrocytic origin. Del Arco and colleagues proposed that glutamate released into the extracellular compartment could diffuse and have long-lasting effects modulating glutamatergic neurone-astrocytic networks and their interactions with other neurotransmitter networks in the same brain area. To support Del Arco's hypothesis the assumptions have to be made that the activity of neurones is functionally linked to the activity of astrocytes, the existence of extrasynaptic glutamate receptors pertaining functional properties are different from glutamate receptors located at the synapses.

Another hypothesis suggests that the cystine/glutamate exchanger located in astrocytes is responsible for most of the basal glutamate measured with microdialysis (Jabaudon *et al.*, 1999; Baker *et al.*, 2001).

As already mentioned, some microdialysis studies showed increased extracellular concentrations of glutamate produced by specific drugs. Interestingly these increases were attenuated by TTX and dependent on the presence of calcium in the perfusion

medium although basal concentrations were not attenuated (Moghaddam, 1993; Grobin and Deutch, 1998). The above data have indeed been interpreted as neuronal release under the prerequisition of high stimulation. This glutamate could then diffuse from the synaptic cleft into the extracellular fluid (Del Arco *et al.*, 2003).

In a very recent study, Musazzi and coworkers (2011) concluded that "although extracellular glutamate measured by microdialysis may be only partly of neuronal origin, the results of separate experiments employing different methodologies substantially confirm the general results of microdialysis studies."

It is therefore clear that despite the questionable origin of glutamate measured by means of microdialysis and that it may complicate interpretation of results, the technique may still give useful information regarding glutamate release and possible changes in its activity induced by either specific conditions or treatments.

As stated previously, depressed patients were shown to present with elevated glutamate concentrations and decreased plasma glutamine/glutamate ratios (Mauri *et al.*, 1998; Mitani *et al.*, 2006), while treatment with antidepressants decreased these elevated glutamate levels (Kucukibrahimoglu *et al.*, 2009),

2.9 Quantification of glutamate

Various methods can be employed to quantitate amino acids in microdialysates obtained from microdialysis experiments and typically include LC-MS and HPLC coupled with various detectors (see section 1.1). Of these methods HPLC-coupled with fluorometric detection is the method utilized in this study and will be discussed specifically.

2.9.1 High performance liquid chromatography (HPLC) with fluorometric detection

Fluorescence spectroscopy is the most sensitive optical detection technique used with high-performance liquid chromatography (HPLC). Fluorescence of a molecule can occur naturally or can be achieved by labeling the molecule with a fluorescent label in a

derivatization reaction as typically used for amino acid analysis. The chemical modification can either be performed automatically prior to chromatographic separation (precolumn) or after the separation (postcolumn). In the flow cell of a fluorescence detector, the active molecule is exposed to a defined wavelength of light from a high energy light source, typically a xenon lamp. Several nanoseconds later, the excited analyte emits its energy at a less energetic, longer wavelength. A wavelength-selective fluorescence detector usually utilizes a photomultiplier which is positioned at an angle of 90° to the xenon lamp and detects the light that emitted from the fluorescing compounds (Holger and Verena. 2012).

Amino acids in their native form are usually very weak chromophores which mean that they do not absorb UV light. For fluorescence detection the amino acids thus have to be chemically modified (derivatized) in order to make sensitive detection possible.
In this study an o-phthalaldehyde (OPA) based reagent was used for the derivatization of glutamate as depicted in Figure 2.4.

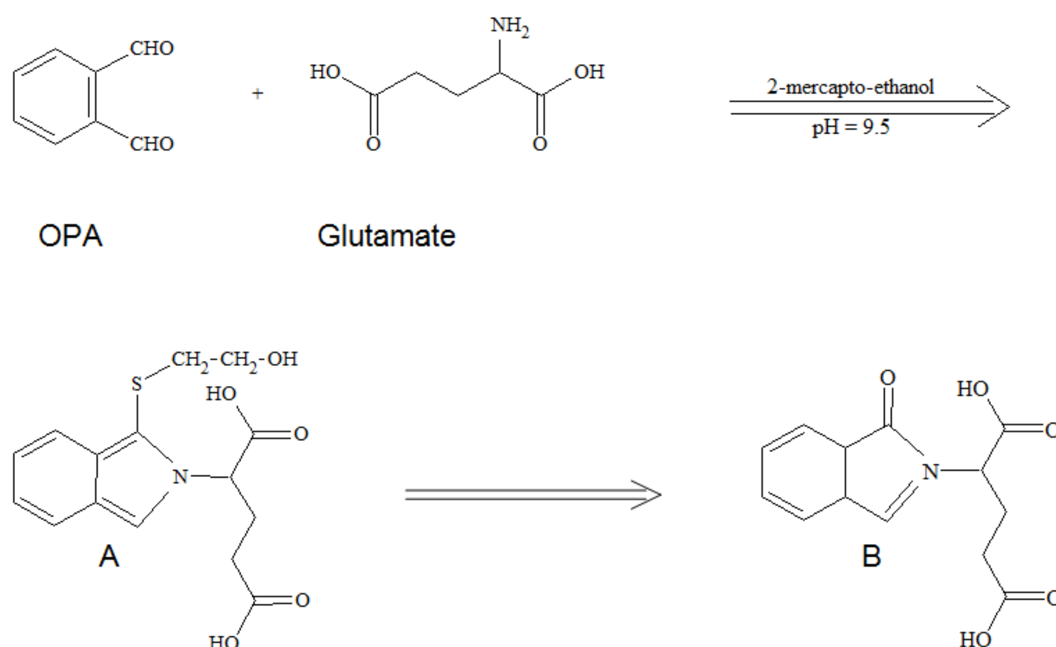

Figure 2.4 OPA as well as glutamate has no fluorescence activity in its native form. The OPA/Glutamate reaction rapidly yields fluorescent isoindoles (A and B).

These fluorescent isoindoles are quantitively detected and thus gives the researcher a means to detect and quantify the concentration of glutamate in a tissue extract or dialysate.

Chapter 3: Major depression and the role of glutamate

3.1 Introduction

The focus of the current study was to develop and validate the microdialysis technique in our laboratory. However, in order to confirm its successful setting up, it was necessary to perform a small pilot application study in rats. The technique was applied in a small number of Flinders Sensitive Line (FSL) rats, a genetic line of stress-sensitive rats presenting with depressive-like behaviors and compared to a group of healthy controls, the Flinders Resistant Line (FRL) rats. This would confirm the two main objectives of the study, viz. successful setting up of the *in vivo* cerebral microdialysis technique and the subsequent evaluation and quantification of glutamate in the dialysate from samples collected in the frontal cortex of the above two populations of animals. The following few paragraphs will therefore give a very concise review of the literature with regard to depression, the brain regions involved and the relevant neurobiology of depression, including the monoamines in short, but with special attention to the role of glutamate. Additionally the use of the FSL rat as a genetic model of depression will be discussed, followed by an overview of the current and possible new treatment strategies of depression.

3.2 Depression

Major depressive disorder is a wide spread global disorder causing significant morbidity and mortality (Ustun *et al.*, 2004), and despite many years of intense research little is known of the true cause of depression, or of the exact mode of action of antidepressant drugs. The monoamine hypothesis (see section 3.4.1) has long been accepted as the best theory explaining the mechanism of action of current employed antidepressants used in the treatment of depression. However accumulating pre-clinical and clinical evidence on the pathogenesis of mood disorders is strongly indicative of the involvement of the excitatory amino acid, glutamate in these disorders (Machado-Vieira

et al., 2009c, Sanacora *et al.*, 2012). Despite the availability today of many antidepressant compounds, these drugs have still several disadvantages. A great variation in intra-patient response to the treatment, treatment resistance and adverse effects are amongst the shortcomings of current treatment. Furthermore these drugs take up to 3 - 4 weeks to elicit an antidepressant effect and there is currently no "fast-acting" drug for the acute relief of depression. However the recent finding that ketamine (a non-competitive NMDA receptor antagonist) may produce acute relief of depression (aan het Rot *et al.*, 2010; Maeng *et al.*, 2007 and Machado-Vieira *et al.*, 2009c), is very promising. The exact mechanism of this rapid antidepressant response is however illusive but some of the possible mechanisms will be discussed later in this chapter.

3.3 Brain regions involved in depression

Distinct regions of the brain have been associated with the pathogenesis of depression, viz the frontal cortex, hippocampus, amygdaloid complex and nucleus accumbens (Frodl *et al.*, 2002). Various areas within the prefrontal cortex region have been shown to exhibit decreased blood-flow and metabolism in depressed patients, which were reversed by effective antidepressant therapy thus further strengthening the link between this brain area and depression (Drevets *et al.*, 1998). Lai and colleagues (2000) studied depressed geriatrics and demonstrated a reduction in the volume of the orbital frontal cortex in comparison to normal controls. The authors concluded that this phenomenon may play a critical role in the development of depression. These and other data provided significant evidence of the important role that the frontal cortex plays in the pathogenesis and treatment of depression.

3.4 Depression and the role of monoamines and glutamate

Although the role of noradrenalin and serotonin in the neurobiology and pharmacological management of depression is well accepted (Fava and Papakostas, 2008), especially considering the clinical efficacy of noradrenergic and serotonergic active antidepressants such as the tricyclic antidepressants (TCA's) and serotonin reuptake inhibitors (SRI's), these drugs remain at best 60-65% effective (Wells *et al.*, 2003), indicating that there is more to depression than simply a deficiency of NA and/or serotonin. Hashimoto *et al.* (2007) observed increased levels of glutamate specifically

in the frontal cortices of patients with bipolar disorder and major depression. Given the recent evidence that the glutamate NMDA receptor antagonists (e.g. ketamine) offer a rapid antidepressant response, a great deal of clinical and pre-clinical research has been undertaken to expand on the role of the glutamatergic system in mood regulation, in depression and in antidepressant response. This is directly applicable to the present study.

3.4.1 Monoaminergic neurotransmission

The monoamine theory of depression states that depression is due to deficiency of brain monoaminergic activity and that depression is treated by drugs that increase this activity (Schildkraut, 1965). This was a widely accepted theory, supported by neurobiological basic research as well as clinical studies. Different mechanisms may increase the availability of brain monoamines (noradrenaline, dopamine and serotonin), e.g. by blocking the reuptake of the monoamine from the synapse, inhibiting the intraneuronal metabolism of the monoamine or blocking the presynaptic inhibitory auto- or heteroreceptors. However, this theory suffered from a couple of inconsistencies and failed to explain several facts. First, there are drugs that can increase brain monoaminergic activity (e.g. cocaine and amphetamine) which are not effective clinically as antidepressants. Second, not all depressed patients respond equally to the same antidepressant. Third, and most importantly, these changes in the monoamine levels at the synapse take place within hours after the administration of the antidepressants, but the therapeutic response requires the continuous administration of these drugs for weeks (Posternak & Zimmerman 2005). These inconsistencies have lead to the modified monoamine theory of depression which suggests that the acute increase in the levels of the monoamines at the synapse may be only an early step in a potentially complex cascade of events that ultimately results in antidepressant activity (Pineyro & Blier, 1999).

The notion that perturbed monoaminergic transmission is causally implicated in depressive disorders has been emphasized in the literature for decades (Manji *et al.*, 2003). Clinically available antidepressants all act via monoaminergic mechanisms therefore research has concentrated on the role of G-protein-coupled receptors and

associated second messenger pathways. Accordingly, all those drugs elicit a broad-based enhancement in extracellular levels of monoamines while precursor depletion studies provide direct evidence for the importance of functionally intact monoaminergic pathways to the expression and maintenance of their antidepressant and other clinical actions (Richell *et al.*, 2005). It is however evident that although dysfunction within the monoaminergic neurotransmitter systems plays an important role in mediating some facets of the pathophysiology of mood disorders, they likely represent the downstream effects of other, more primary abnormalities (Manji and Lenox, 2000).

This notion also reflects current awareness that depressive states are often comorbid with other psychiatric disorders and that it would be a major simplification to attribute all the manifestations of depressive disorder to a disruption of corticolimbic monoaminergic systems which control mood, cognition and motor behavior. It would be beyond the scope of this section to give a complete report of what is currently known about the role monoamines play in depression, but the review of Millan (2006) may be referred to in this regard.

3.4.2 Glutamatergic neurotransmission

Glutamate (Fig 3.1.) is an acidic amino acid found abundantly in the central nervous system (CNS) and is known for its powerful excitatory effects on neurons in virtually every region of the CNS. As the major excitatory neurotransmitter, it plays a distinct role in an array of behavioral and physiological mechanisms, viz. learning and memory, as well as neuronal plasticity (Krystal, 2007) and emotion processes. Glutamatergic neurotransmssion is therefore very important in the pathophysiology of mood disorders (Zarate *et al.*, 2002, Sanacora *et al.*, 2008, Yuksel and Ongur, 2010) a finding which will be discussed in a little more detail in the next section.

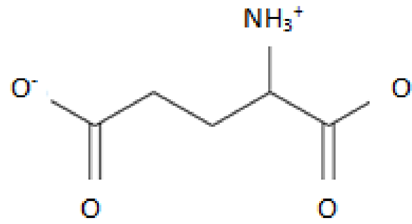

Figure 3.1 The chemical structure of glutamate.

Glutamate concentrations in the extracellular fluid of the CNS are in the order of 2.6 mM, while in plasma it may be much higher (160 mM) (de Vries & Alexandre, 2005). These two compartments are separated by the blood-brain barrier (BBB).

Apart from the role of glutamate as a neurotransmitter it also serves as an important fuel reserve (as discussed in the previous chapter). One molecule of glutamate yields 12 ATP molecules when oxidised to oxaloacetate. In the case of insufficient glucose concentrations the brain is able to mobilize glutamate as a source of fuel (Miller *et al.*, 1975). It is well established that ischemic episodes also cause marked mobilization of glutamate in the brain (Hawkins, 2009) which is neurotoxic to brain cells.

Excessive glutamate release resulting in high extracellular fluid concentrations are associated with severe neurological dysfunction culminating in conditions such as epilepsy, traumatic brain injury, motor neuron disease, Huntington's chorea, Parkinson's disease, Alzheimer's disease, stroke and amyotrophic lateral sclerosis (Maeng *et al.*, 2007). In addition schizophrenia, depression, anxiety, addiction and drug abuse have also been linked to abnormal changes in glutamate neurotransmission (Van Der Zeyden *et al.*, 2007).

In the present study the focus was on depression and glutamate. Serum glutamate levels in depressed patients have been found to be significantly higher than those in healthy controls (Kim *et al.*, 1982; Mauri *et al.*, 1998 and Hashimoto, 2009). Additionally Mitani *et al.* (2006) perceived a positive correlation between plasma glutamate levels and the severity of the depressive symptoms in depressed patients. Maes *et al.* (1998) demonstrated that a 5-week treatment with antidepressants significantly decreased serum glutamate levels in depressed patients compared to that in controls while Hashimoto *et al.* (2007) observed increased levels of glutamate specifically in the frontal

cortices from patients with bipolar disorder and major depression. These clinical findings confirm that glutamate plays an integral role in the pathogenesis of various neurological disorders including major depression.

Within the brain glutamate and glutamate neurotransmission can be targeted directly and indirectly in an attempt to evoke a desirable effect. In figure 3.2 the transmission, metabolism and sites of action of glutamate is depicted according to Tokita *et al.* 2012. In brief glutamate is released from the presynaptic glutamatergic neuron into the synaptic cleft where it may interact with four receptors (kainate, AMPA, NMDA and mGluR) contained on the presynaptic glutamatergic neurons, postsynaptic neurons and the glial cells. Glutamate can either interact with any the named receptors and/or could be taken up via excitatory amino acid transporters (EAATs) located on the glial cells. When glutamate is transported into the glial cell by the EAATs, glutamine synthetase combines glutamate and ammonia to yield glutamine. This glutamine is then transported back to the presynaptic glutamatergic neuron where the enzyme glutaminase transforms glutamine into glutamate and ammonia. The newly formed glutamate may now be released from the presynaptic glutamatergic neuron into the synaptic cleft.

This described pathway could be altered by blocking/modulating the receptors, altering the reuptake/release of the glutamate or inhibiting/accelerating the metabolism of glutamate within the cells.

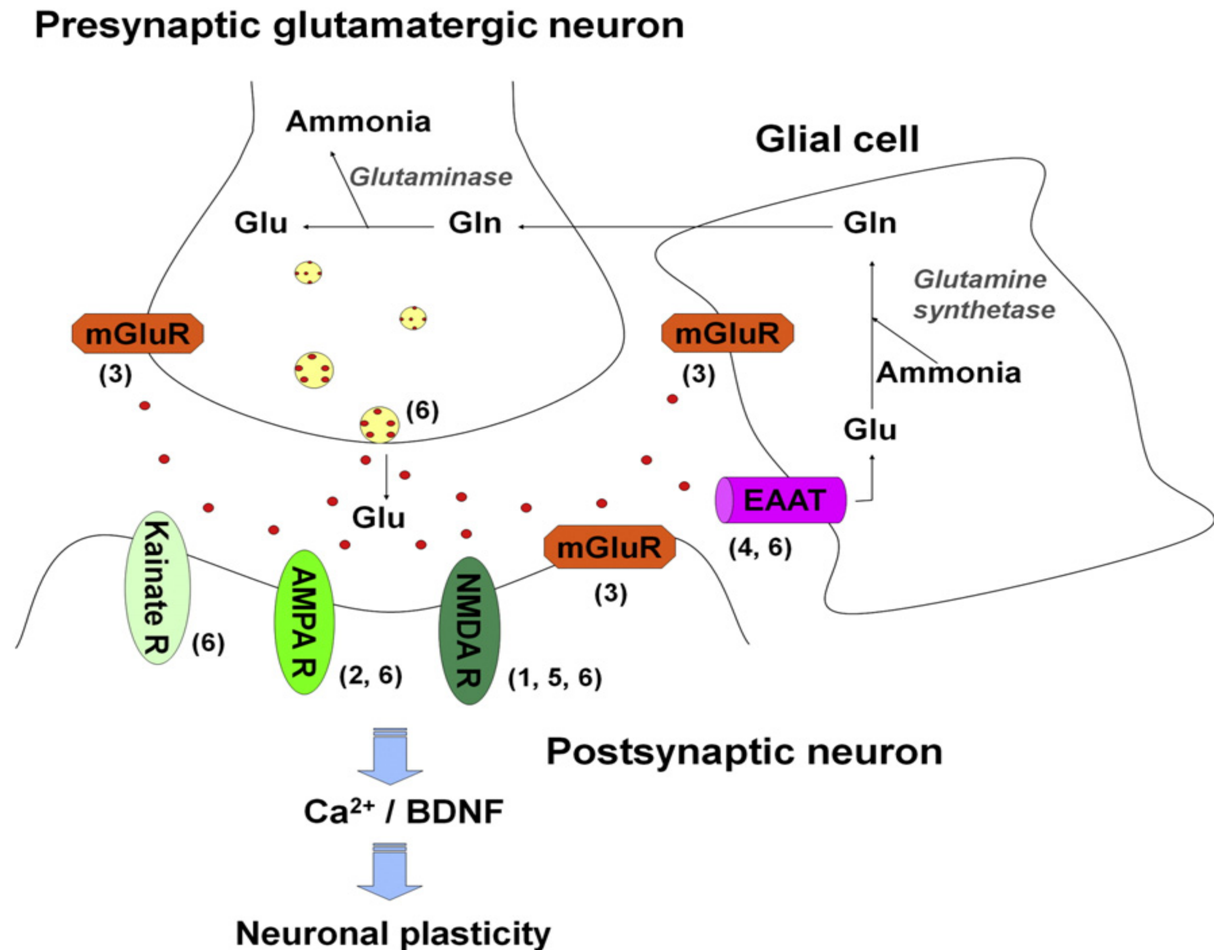

Figure 3.2 Major functional components for glutamatergic neurons and potential targets of glutamatergic agents exerting antidepressant-like actions. Glutaminase hydrolyzes glutamine to glutamate and ammonia in presynaptic neurons. Glutamate is released into the synaptic cleft and stimulates glutamate receptors (kainate receptors, NMDA receptors, AMPA receptors, and mGluRs) in postsynapses, presynapses, and glial cells. Glutamate is taken up by EAATs on glial cells. Glutamine synthetase converts glutamate and ammonia to glutamine, which is transported to presynaptic neurons. Glutamatergic agents are considered to act on the numbered targets in the Figure as follows targets: (1) NMDA receptor antagonists (ketamine, NR2B subunit antagonists, memantine, magnesium, and zinc); (2) positive modulators of AMPA; (3) group I mGluR ion. (Adopted from Tokita *et al.*, 2012)

There is some compelling evidence suggesting that glutamate receptor abnormalities may play a role in the pathophysiology of depressive disorder e.g. (Tokita *et al.*, 2012). Especially AMPA, mGluR and NMDA receptor modulators have been linked to

antidepressant action (Tokita *et al.*, 2012). However, for the purpose of this study, only ketamine which modulates glutamatergic neurotransmission via the NMDA receptor will be discussed.

3.4.3 Fluoxetine’s and ketamine's effects on glutamate release

Fluoxetine was one of the first selective serotonin reuptake inhibitors used in the treatment of depression and as such fits the monoaminergic hypothesis of depression, by increasing the amount of monoamine (serotonin) in the central synapses. While fluoxetine is an established and widely used antidepressant, its previously reported effects on glutamate's release was investigated for the purpose of this study.

The effect of fluoxetine on glutamate release within the prefrontal cortex is inconclusive, with no results from microdialysis studies. Bonanno and co-workers (2005) investigated the effect of fluoxetine on depolarization-evoked release of glutamate in freshly prepared hippocampus samples of drug-treated rats and found fluoxetine to markedly reduce the depolarization-evoked glutamate release. However, this finding was only the case with chronic fluoxetine treatment and not with acute treatment. Wang and colleagues (2003) found acute fluoxetine administration reduced 4-aminopyridine – evoked glutamate release in cerebrocortical synaptosomes.

In two different microdialysis studies, the effects of ketamine on glutamate release in rats after an acute intraperitoneal injection have been shown to be biphasic (Moghaddam *et al.*, 1997). At subanesthetic doses (10 – 30 mg/kg) ketamine increased glutamate outflow whereas with anesthetic doses (50 – 200 mg/kg) glutamate outflow was decreased (Moghaddam *et al.*, 1997). Lorrain *et al.*, (2003) investigated the effect of subcutaneous (SC) administered ketamine on prefrontal cortex glutamate levels and found that an acute 18 mg/kg injection evoked a significant release of glutamate. Lorrain and co-workers (2003) also administered a 1 mM ketamine solution directly to the medial prefrontal cortex (mPFC) but in contrast to the SC injection the local administration of ketamine had no effect on the glutamate release.

3.5 Drug treatment of depression

The current treatment of depression is based on the monoamine hypothesis, which links the pathogenesis of depression to a deficit of monoamines in the central nervous system (as discussed in section 3.4.1). The drugs currently used in the treatment of depression can be divided into four basic groups namely, monoamine oxidase inhibitors, tricyclic antidepressants, selective serotonin reuptake inhibitors and the serotonin norepinephrine reuptake inhibitors (Goodman & Gilman, 2011). The onset of action of these antidepressants is delayed (typically 2 weeks) and there is a multitude of cases where the depressive condition is resistant to these antidepressants (Machado-Vieira *et al*., 2009a). These drugs also exhibit some unfavorable side effect profiles, contributing to non-compliance and adversely affect outcome (Machado-Vieira *et al*., 2009a).

3.5.1 Current therapy

Monoamine oxidase inhibitors (MAOI's) include irreversible MAOI's e.g. tranylcypromine and reversible selective MAOI's e.g. moclobemide. The MAOI's act on the enzyme "monoamine oxidase" present in neuronal cells and are responsible for the degradation of the monoamines. Thus MAOI's increase the concentrations of all monoamines in the synapses by inhibiting monoamine oxidase (Goodman & Gilman, 2011).

Tricyclic antidepressants (TCAs), including amitriptyline, imipramine, clomipramine, dothiepin, lofepramine and trimipramine, owe their name to a common shared pharmacophore consisting of three rings of atoms. TCA's act by increasing the concentration of NA or serotonin in the synaptic cleft (Goodman & Gilman, 2011). TCA's can be subdivided into secondary or tertiary amines, which have different effects with respect to blocking the reuptake of noradrenalin (secondary amines, e.g. desipramine) or serotonin (tertiary amines, e.g. clomipramine) (Goodman & Gilman, 2011). Different metabolic pathways are responsible for the metabolism of the amines leading to a varied adverse effect profile for this group of drugs.

Currently the most widely utilized class of antidepressants is the "**selective serotonin reuptake inhibitors**" (SSRI's). Like the name of the group implies it acts by blocking/inhibiting the reuptake of serotonin into the pre-synaptic nerve terminals. This subsequently increases the concentration of serotonin in the synaptic clefts. This group

owes its popularity to its favorable adverse effect profile relative to the other antidepressant drugs. Typical SSRI's includes fluoxetine, sertraline, paroxetine, citalopram and escitalopram (Goodman & Gilman, 2011).

The "**atypical antidepressants**" group of antidepressants present with unrelated chemical structures to the above and exhibit different pharmacological actions. Maprotiline and mianserine are tetracyclic structures and selectively inhibit the reuptake of I-NE. Contrary to TCA's and SSRI's, tianeptine enhances serotonin uptake and is devoid of any significant activity on any receptors or other monoamine systems (Brink *et al.*, 2006). Trazodone is a serotonin antagonist and 5HT re-uptake inhibitor, while mirtazepine blocks alfa 2 adrenergic receptors. Reboxetine is a selective noradrenaline re-uptake inhibitor, while bupropion primarily inhibits dopamine reuptake. Duloxetine, venlafaxine and desvenlafaxine inhibit both serotonin and noradrenalin reuptake. A recently introduced antidepressant with a unique mode of action is agomelatine which has antagonistic and agonistic actions respectively on specific serotonin and melatonin receptors (Goodman & Gilman, 2011).

3.5.2 New strategies:

Ketamine is a non-competitive NMDA receptor antagonist and has long been used as an anesthetic agent in pediatric and adult patients (Goodman & Gilman, 2011). It is only in recent years that ketamine received an abundance of attention due to its apparent rapid anti-depressive effects. It has been found that a single low dose (sub-anesthetic) ketamine administration intravenously produces rapid relief of depression (aan het Rot *et al.*, 2010; Maeng *et al.*, 2007, Machado-Vieira *et al.*, 2009a). Maeng et al. (2008) demonstrated an acute as well as a sustained antidepressant effect in humans, a finding reproduced by several more recent studies (Murrough *et al.*, 2012). Ketamine has also been tested as an effective way to rapidly treat people with suicidal thoughts, a benefit usually not seen for weeks with traditional antidepressant treatments. However, clinical use of ketamine has been limited because it has to be administered intravenously under medical supervision and in some cases can cause short-term psychotic symptoms (Pittenger *et al.*, 2007; Zarate *et al.*, 2006).

In rats ketamine yielded a reduced immobility time during the forced swim test. Maeng *et al.* (2008) demonstrated acute as well as a sustained antidepressant effect in both

human subjects and mice. The mechanism of this fast onset of antidepressant activity has not been fully elucidated and studies to investigate the involvement of other signaling pathways to explain this rapid effect has in recent years increased. In addition to ketamine's affinity for NMDA receptors it also decreases Ser845-phosphorylated GluR1 (an AMPA receptor subunit), while the pretreatment with dihydroxy-6-nitro-7-sulfoamoylbenzo(f)-quinozaline (NBQX) (an AMPA receptor antagonist) attenuates ketamine-induced phosphorylation of GluR1 and antidepressant effects in the forced swim test (Maeng *et al*., 2008). Further studies also found that ketamine (in contrast to ECS, fluoxetine and imipramine) induces a rapid increase in the phosphorylated mTOR (mammalian target of rapamycin) levels in the prefrontal cortex of rats. It has been suggested that the mTOR pathway is possibly involved in the rapid antidepressant action of ketamine (see next section)

Mammalian target of rapamycin complex 1 (mTORC1) is involved in the regulation of cell growth and structurally and functionally preserved in all species. It controls protein synthesis and dysregulation of the mTOR signaling pathway has been implicated in several diseases including psychiatric disorders (Karolewicz *et al*., 2011). Four major inputs control mTOR activity: nutrients, such as amino acids; growth factors, e.g. insulin; cellular energy levels, and stress. mTOR function is influenced by the activity of neuronal surface receptors including NMDAR, mGluR5, and neurotrophic tyrosine kinase receptors (TrkB) which are vital for the induction of synaptic plasticity (Karolewicz *et al*., 2011). Animal models furthermore demonstrated that the antidepressant effects of ketamine, an NMDAR antagonist, are mediated by activation of the mTOR-dependent translation initiation pathway leading to increased synaptic signaling proteins and increased number and function of new spine synapses in the PFC in rats.

The pretreatment with NBQX also attenuated the phosphorylated mTOR increase (Li *et al*., 2010). The rapid antidepressant effect exerted by ketamine may therefore be partially explained by a rapid reversal of the deficits in the mTOR-dependent pathway (Karolewicz *et al*., 2011).

3.6 FSL rat model of depression

To be able to do research on depression without risking any possible unintended injury or harm to human beings, numerous animal models of this disorder have been developed. There are several genetic rat models of depression including the "congenital learned helplessness model", the "Fawn Hooded rat", the "high DPAT sensitivity animals", the "roman high-avoidance model" the "swim high-active animals" and finally the "Flinders Sensitive Line rat". The robustness of an animal model is determined by the degree to which it can emulate certain key characteristics of the human illness, including similarity between the behavior of the animal model and the symptoms of depression, termed face validity, the animal model's response to typical pharmacological treatment used to treat the disorder (i.e. antidepressant drugs) and referred to as predictive validity and finally how well the model reproduces the neurobiology of the illness, in this case depression, termed construct validity.

In the current study, male Flinders Sensitive Line (FSL) rats, considered to be a valid animal model of depression (Overstreet *et al.*, 2005, Wegener, *et al.*, 2011), were employed. The FSL rat (a genetic line of Spraque-Dawley rat) was initially bred to create an animal model with increased sensitivity to anticholinesterase agents, and has also been referred to as a hypercholinergic model (Overstreet, 2003). This genetic model presents with extensive predictive and face validity for depression (Overstreet 1993; Willner and Mitchell, 2002 and Neumann *et al.*, 2010) including, lower body weight, reduced physical activity, sleep disturbances and anxiety, anhedonia following stress (Overstreet *et al.*, 2005, Wegener *et al.*, 2011) and a heightened sensitivity to environmental stressors (Pucilowski *et al.*, 1993; Wegener *et al.*, 2010) (see Figure 3.3). Neurochemical alterations found (see Figure 3.4), include a hyperresponsive cholinergic system (Overstreet, 2002), impaired serotonergic neurotransmission (Overstreet *et al.*, 1998; Overstreet *et al.*, 2003, Zangen *et al.*, 1997; Hasegawa *et al.*, 2006) and gamma amino butyrate (GABA) activity (Pepe *et al.*, 1988) as well as an increased response of NMDA-nitric oxide synthase (NOS) signaling following stress (Wegener *et al.*, 2010). This latter evidence of a disturbance in glutamate-NMDA-nitric oxide signaling is further support for using FSL animals in this particular study. In most cases, the

aforementioned behavioral and neurochemical profile can be usually adjusted with antidepressants (Kokras *et al.*, 2008).

The exact glutamatergic profile of the FSL rat is still elusive. Recently Hascup and colleagues (2011) compared the resting levels of glutamate in the prefrontal cortex of both FSL and FRL rats (aged 3-6 months) and found that there was no significant difference in resting glutamate concentrations of these two strains (FSL: 5.0 ± 0.5 μM and FRL: 5.3 ± 1.0 μM). Suprisingly it was found that FSL rats aged 12 – 15 months had a significant increase in resting glutamate levels up to 17.0 μM (Hascup *et al.*, 2011). This age-related increase in resting glutamate levels were not shared by the FRL strain of which the resting glutamate levels stayed approximately the same.

Behavioral characteristics of FSL rats

Symptom/observation in depressed individuals	Behavioral measure in FSL rats
A. Core symptoms	
Suicidal ideation and/or attempt	
Psychomotor retardation	**Reduced bar pressing for rewards**
Anhedonia	*Normal ICSS threshold and Saccharin Intake*
Reduced appetite/loss of weight	**Lower body weight; reduced appetite**
Cognitive disturbance	*Normal accuracy in food-motivated task*
Elevated REM sleep	**Elevated REM sleep**
Reduced REM sleep latency	**Reduced REM sleep latency**
Reduced slow wave sleep	*Normal slow wave sleep*
B. Associated variables	
Reduced killer T cell activity	**Reduced killer T cell activity**
Other immune abnormalities	**Other immune abnormalities**
Higher incidence of IBS	**Greater gut sensitivity to antigen**
Higher incidence of asthma	**Greater airway sensitivity to antigen**
Anxiety of some types	**Anxiety in some tasks**

Bold in both columns indicates a match; *italics in one column and normal font in the other column indicates a mismatch.*

Figure 3.3 Behavioral characteristics of FSL rats. Adapted from Overstreet *et al.* 2005.

FSL rat and theoretical models of depression

Theoretical model	Key finding in FSL rat	Key observation in depression
Cholinergic model	**Increased cholinergic sensitivity**	**Increased cholinergic sensitivity**
	No antidepressant effect of cholinergic antagonists	**No change in swim test immobility with antagonist**
Serotonergic model	Reduced 5-HT_{1A} sensitivity	*Increased 5-HT_{1A} sensitivity*
		Reduced 5-HT sensitivity
Noradrenergic model	Altered sensitivity	*No change in sensitivity*
Dopaminergic model	**Reduced DA transporter**	**Reduced transporter**
GABAergic model	Decreased GABA levels	Not determined
Neuropeptide Y model	**Reduced NPY levels**	**Reduced NPY levels**
HPA axis model	Elevated cortisol	*Normal corticosterone*
Circadian rhythm model	**Phase advance in some**	**Phase advance**
	Reduced amplitude	*Normal amplitude*
Neurotrophin model	Reduced levels	*Normal levels*
	Increase with AD treatment	**Increase with AD treatment**

Figure 3.4 FSL rat and theoretical models of depression. Adapted from Overstreet *et al*. 2005.

Chapter 4: Method development

4.1 Introduction

The study was conducted to establish the microdialysis technique in our laboratory with the preliminary application of monitoring glutamate levels in the prefrontal cortices of rats. Ketamine and fluoxetine were administered via reverse-dialysis and their effects on glutamate levels were monitored.

All experiments and procedures were conducted under the approval and according to the guidelines set by the ethical committee of the North-West University (application number: 0028-08-S5)

.

Adult male FSL- and FRL-rats obtained from the Laboratory Animal Center of the Potchefstroom Campus were used in all the experiments.

Microdialysis was performed on each rat to obtain the samples (microdialysate) to be analyzed for glutamate using a validated method of high performance liquid chromatography (HPLC) coupled to a fluorescence detector.

4.2 Animals

Male Flinders sensitive line (FSL) rats, and a corresponding negative control line, the Flinders resistant line (FRL) rats, were originally obtained from Dr David H Overstreet, University of North Carolina, Chapel Hill, NC, USA. These rats were reared and housed in the Laboratory Animal Center of the Potchefstroom campus.

They were group-housed in cages with a height of 30 cm, a width of 32 cm and a depth of 32 cm, with a grid floor. Climate conditions were kept stable at 21 ± 0.5ºC and 50 ± 5% relative humidity. The light was provided over a 12 hour light – 12 hour dark cycle at a light intensity of 350 - 400 lux. Male Flinders Sensitive Line rats (FSL) and Flinders

Resistant Line rats (FRL) weighing 250 - 350 g were used in all experiments. All rats were between 2 – 3 months old.

From the day on which the stereotaxic surgery was performed onwards, the rats were housed singly in cages for the duration of the recovery period. These cages were of the same size as the previous cages they were housed in. The animals were always allowed ad-libitum access to food pellets and clean water.

4.3 Project design

The brain area targeted in this study is the prefrontal cortex. Glutamate concentrations were determined in the FSL-rat and its normal counterpart, the FRL-rat. Both lines of rats were randomly allocated into 4 groups which would ultimately differ by the substance administered via reverse dialysis. The 4 groups were those receiving: pure aCSF; aCSF with KCl; aCSF with fluoxetine and aCSF with ketamine.

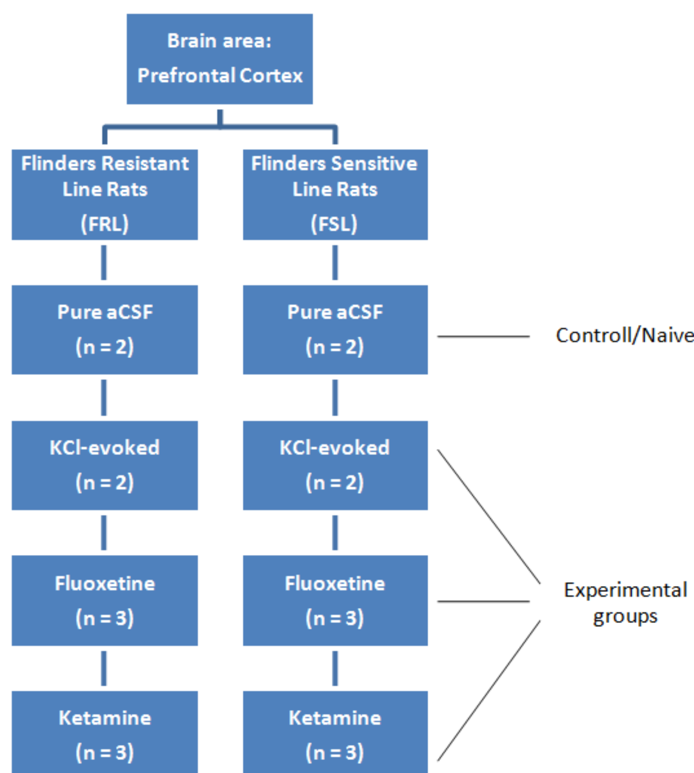

Figure 4.1 Project Layout

The following glutamate levels were analyzed in both FSL and FRL rats (see also Fig 3.1).

- Basal glutamate levels
- K^+-evoked glutamate levels
- In reaction to locally administered fluoxetine
- In reaction to locally administered ketamine

4.4 Anesthesia

The rats were anesthetized on two occasions. The first was to perform the stereotaxic surgery, and the second was to insert the microdialysis probe prior to the actual microdialysis procedure.

For the initial anesthesia, sodium pentobarbitone (6% solution, Kyron Laboratories Pty Ltd) was administered via intraperitoneal injection. A dose of 45 mg/kg body weight was administered. The rats were placed in a cage and left for the induction of the anesthesia to set in. Sodium pentobarbitone alone is however not sufficient for the induction of surgical anesthesia (as described in par 2.6.3.2), and therefore halothane (3 ml of a 100% Halothane solution) was administered via a syringe on a cotton bud placed in front of the nose of the rat, following the fixation of the rat's head in the Kopf stereotaxic frame. The corneal reflex test as well as the withdrawal reflex test was employed to verify the presence of the surgical anesthesia phase. The halothane dose was regulated by adjusting the distance of the halothane source from the nose of the rat. Surgery commenced once the satisfactory surgical anesthetic phase was achieved.

Following a 3 day recovery period of the rat from the initial surgery, the microdialysis procedure was performed. This involves the insertion of the probe into the previously implanted guide cannula. The safe and correct insertion of the probe requires the rat to be very docile and easy to handle. For this reason a second administration of a very light anesthesia is required. The rat was placed in a sealed container filled with halothane vapor (5 ml of 100% halothane solution). The lid of the container is made of plexi-glass in order to monitor the rat. As soon as the rat stopped moving around it was

immediately removed from the container and the probe was inserted quickly. It took about 10 seconds for the rat to recover full mobility.

4.4.1 Different regimes tested

Five separate anesthesia regimes were tested namely: pentobarbitone alone, halothane alone, ketamine and xylazine, propofol and pentobarbitone in combination with halothane.

- Pentobarbitone 6% as sole anesthesia (Kyron Laboratories Pty. Ltd.): This regime was found to be one of the most popular used for the purpose of rodent anesthesia as well as anesthesia for microdialysis surgery. It is also readily available and inexpensive. A dose range between 40 mg/kg and 60 mg/kg administered intraperitoneally were tested. All doses produced undesirable results. A large variation in induction time, duration of anesthesia and recovery of anesthesia was found, these results was in accordance to what Messier *et al.* (1998) found. The narrow safety window of pentobarbitone also proved cumbersome as severe respiratory depression and subsequent death was unpredictable and frequent. Thus pentobarbitone as sole anesthesia regime was discarded.

- Halothane alone (SafeLine Pharmaceuticals Pty. Ltd.): Halothane is an inhalation anesthetic agent and can be used without a vaporizer. The decision on testing the halothane arose from the fact that inhalation anesthesia produces a rapid induction as well as a rapid recovery. Halothane as sole anesthesia was also not feasible as the regulation of anesthesia was difficult due to rapid recovery during the microdialysis surgery.

- Ketamine (Kyron Laboratories Pty Ltd) and xylazine (Sigma-Aldrich®): This regime was tested and provided relative good results regarding the recovery after surgery and intra-operative parameters as described in paragraph 2.6. However, the muscle tone of the rat as well as the blink reflex (corneal reflex) amongst others used as indicator of anesthesia depth, was not attenuated during surgical anesthesia. Ketamine was also to be used during the experimentation as agent

administered via reverse dialysis, thus rendering ketamine as an anesthesia regime unfavorable.

- Propofol (Diprivan® 1% propofol, AstraZeneca): Being one of the safest anesthesia drugs and one used regularly in human craniotomy procedures it warranted investigation as anesthesia agent in rats. Propofol provides a host of advantages when used for craniotomy procedures. These advantages includes: rapid induction, rapid recovery, wide dosage safety margin, no increase in intracranial pressure and perfect surgical anesthesia. The half life of propofol in a rat is approximately 4 minutes thus necessitating a continuous infusion. Propofol is administered intravenously via the lateral tail vein of the rat. This proved to be difficult to achieve. The tail vein could not always be effectively catheterized. The exact and reliable catheterization could not be achieved in time and thus propofol was discarded as anesthesia protocol for the current study.

- Pentobarbitone and Halothane: Pentobarbitone and halothane as mono-therapies did not provide a sufficient and desirable anesthesia state for the stereotaxic surgery. A lower dose of pentobarbitone was employed (45 mg/kg) sufficient to produce a shallow anesthesia, this lower dose did not produce all the undesirable effects of pentobarbitone to its full extent. The pentobarbitone induced sleep to such an extent that the rat could be easily handled and placed in the stereotaxic frame. Following fixation of the rat in the stereotaxic frame, halothane could be administered via inhalation until the desired anesthesia plane has been achieved (see section 2.6.2). This surgical anesthesia was then maintained by adjusting the distance of the halothane source from the rat's nose, based on the observation of a combination of vital signs like respiratory depth and speed as well as cardiac function. The tongue was also monitored for signs of cyanosis.

This combination of pentobarbitone and halothane was the regime used in all the subsequent experiments.

4.5 Stereotaxic surgery

Implanting the microdialysis cannula involved complex stereotaxic surgery. The surgical procedure took approximately 40 minutes from the induction of surgical anesthesia to the end of the procedure. The anesthetized animal was fixed in the "Kopf-stereotaxic" frame (figure 4.2).

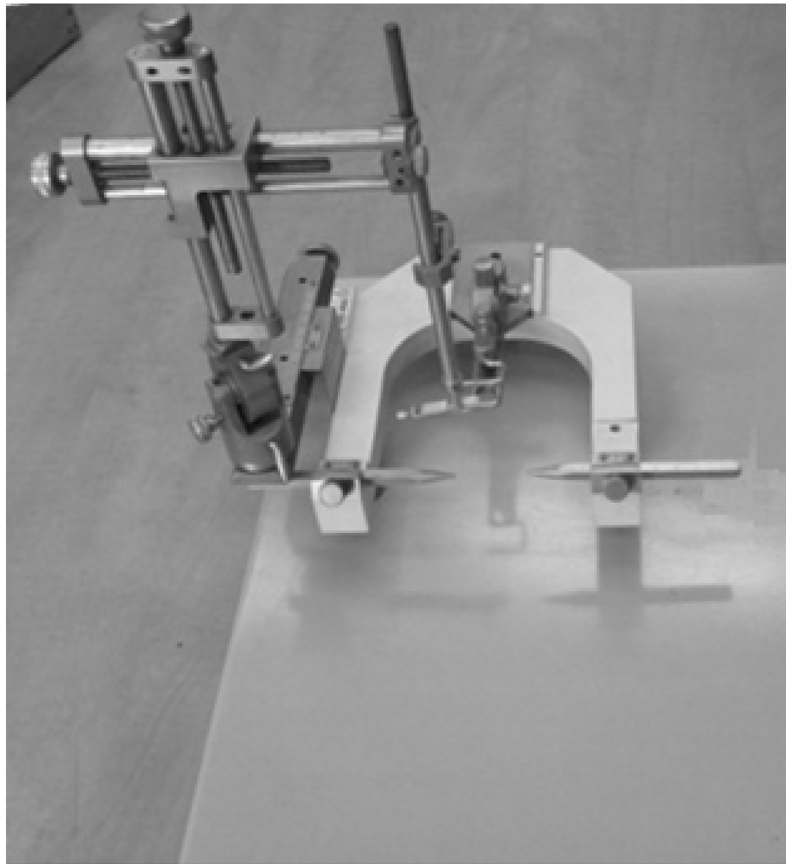

Figure 4.2 The Kopf-stereotaxic frame.

The rat was placed on a manually regulated heating pad to maintain its body temperature (37°C) during surgery. An electrical hair clipper was used to shear all the hair on top of the skull. The skin was cleaned and disinfected with an alcohol swab. A two centimeter longitudinal incision made along the skin with a 10 blade scalpel exposed the periosteum of the skull bone. The periosteum was then separated from the skull in a lateral direction to the incision. A drop of 20 volumes strength (6.08% w/v) hydrogen peroxide (African Medicines Pty. Ltd.) was applied to the exposed skull area;

this serves the purpose of disinfecting and slowing down the bleeding on the skull surface for easy identification of the skull sutures.

With the guide cannula (TSE systems) fastened in the cannula holder of the stereotaxic frame, adjustments were made to position the tip of the cannula directly on top of the "bregma" suture of the skull (see figure 2.2). The x (lateral), y (ventral) and z (anterior-posterior) coordinates were recorded and taken as the "zero" reference. In order to place the cannula in the frontal cortex of the brain, the cannula was then aimed at 4.2 mm anterior-posterior (AP), 2.4 mm lateral (L) and 2.4 mm ventral (V) relative to the coordinates of the bregma suture, according to the Paxinos and Watson (2005).

The desired coordinates for the probe location were calculated and adjusted to the anterior-posterior and lateral axis calculated coordinates and the spot on the skull was marked with a sharp needle. This spot gave the location of where the opening should be made in the skull. A dental drill (Meisinger™ 1.15 mm diameter) bit was used to drill the hole. Care was taken not to injure the dura of the brain, which may lead to excessive bleeding. Two smaller holes (0.69 mm diameter) were drilled in close vicinity of the cannula hole to house two stainless steel jeweler screws (0.9 mm x 1.92 mm) serving as extra anchoring to the cannula. The jeweler screws were fitted in the holes, but not screwed too deep as it would cause pressure and damage to the dura and brain.

A sterile syringe needle was used to puncture the dura in the hole to receive the cannula. The cannula was fitted flush in the hole and was lowered slowly to the desired y-coordinates (depth). Any blood or CSF fluids were removed with a cotton swab. Acrylic glue (Kemdent – Simplex Rapid™ liquid and powder) was applied around and under the anchor screws as well as around the cannula. The glue was "built up" to anchor the cannula firmly and in a steady position.

The dental acrylic set relatively fast. Care was taken not to move the cannula when attempting to unclamp it from the stereotaxic frame. One intraperitoneal sodium-

diclofenac (Adco-Diclofenac® 75 mg/3ml) injection (dose calculated by weight, 2 mg/kg) was given for pain directly after surgery. The rat was at this stage transferred to a heated recovery cage until full consciousness was regained. A recovery period of three days followed where after the microdialysis procedure commenced.

4.6 Microdialysis procedure

After the probe (TSE systems, 4 mm membrane length and 6 kD cut off) was inserted under light halothane anesthesia as described earlier (section 4.4.1), the rat was placed in the cage in which the microdialysis procedure was to be performed. This cage allowed the rat to move freely. Perfusion was initiated directly following the insertion of the probe. From this point in time to the time when the rat was euthanized was five hours. The first two hours allowed for general stabilization and habituation, and during this period no samples were collected. The following one hour, (3rd hour) was taken as the baseline measurement of glutamate concentration (time -60 - -20), this period yielded three samples at 20 minute intervals and these three measurements were used to calculate each group's own baseline value. The following 2 hours was used for the administration of the experimental substance via reverse dialysis (see figure 4.3). This period yielded seven samples taken at 20 minute intervals.

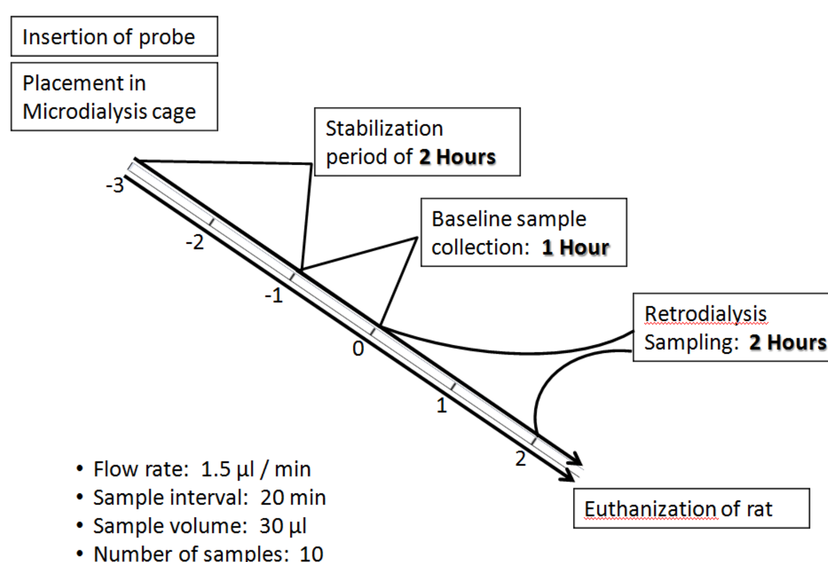

Figure 4.3 Dialysis procedure protocol.

4.7 Microdialysis perfusion fluid

4.7.1 Composition

For this study an isotonic aCSF was prepared with the following salts and concentrations thereof (Wegener *et al.*, 2000): 145 mM NaCl, 3 mM KCl, 1.2 mM $CaCl_2$, 1.0 mM $MgCl_2$.

Table 4.1 This table illustrates the aCSF composition used for each group of rats. All ingredients were dissolved in sterile, double distilled water. * Concentration calculated in accordance to Hervas *et al.* (1998) (see section 4.7.3.3 for explanation.) ** Concentration obtained from Hashimoto, (2009) (see section 4.7.3.2 for explanation.)

Substance for reverse dialysis	Naive (control)	KCl	Ketamine HCL	Fluoxetine HCl
NaCl (mM)	145.0	145.0	145.0	145.0
KCl (mM)	3.0	100.0	3.0	3.0
$CaCl_2$ (mM)	1.2	1.2	1.2	1.2
$MgCl_2$ (mM)	1.0	1.0	1.0	1.0
Ketamine HCl (mM)	--	--	9.0**	--
Fluoxetine (µM)	--	--	--	30.0*

4.7.2 Temperature and flow rate

For this study the aCSF was maintained at room temperature. A flow rate of 1.5 µl per minute was employed, with a standard collection time of 20 minutes, thus yielding a sample volume of 30 µl.

The relative recovery (RR) (as described in paragraph 2.2) was not calculated for the probes used in this study as the results were only aimed to be qualitative and for the purpose of comparing the FSL and FRL groups and subgroups in this study. Furthermore probes of the same batch were used to ensure uniformity.

4.7.3 Pharmacological agents used for the reverse dialysis

4.7.3.1 Potassium chloride

A widely used test for neuronal origin of chemicals sampled through microdialysis is to measure the changes in the levels of a neurotransmitter in reaction to exposure to depolarizing concentrations of potassium (Galvan *et al.*, 2003). A KCl concentration of 100 mM was administered to the designated rat group by reverse dialysis. This concentration roughly yielded ≈ 10% of the concentration diffusing through the membrane reaching a concentration fully comparable to that used *in vitro* (10 mM) to produce depolarization (Herrera-Marschitz *et al.*, 1997).

4.7.3.2 Ketamine hydrochloride

A 9 mM ketamine hydrochloride solution (Kyron Laboratories Pty. Ltd.) was prepared in aCSF and administered via reverse dialysis (Hashimoto, 2009). The 9.0 mM concentration was calculated to correspond to the sub-anesthetic doses cited in clinical studies where rapid relief of depression was documented after this dose of ketamine was administered intravenously (IV) (Machado-Vieira *et al.*, 2009a).

4.7.3.3 Fluoxetine hydrochloride

A 30 µM solution (Fluoxetine hydrochloride, Riedel-Vetranal®) was prepared in aCSF and administered via reverse dialysis. Hervas *et al.* (1998) found a 30 µM solution of

fluoxetine hydrochloride to yield a maximal increase in 5-HT concentrations following reverse dialysis

4.8 Sample collection and storage

The perfusate was collected in small HPLC-vial inserts. A typical sample yielded a perfusate volume of 30 µl. These collection tubes were loaded in a refrigerated micro-fraction collector. This is a precision instrument feeding an array of pre-loaded collection tubes at a precise pre-set time interval. Refrigeration counteracts the degradation of the contents of the perfusate and more importantly prevents vaporization of the samples.

4.9 Anatomical probe location verification

Following sample collection, the rats were euthanized by means of halothane overdose. The rats were placed in a sealed plexiglass box saturated with halothane vapor and left until consciousness was lost. As soon as the rat lost consciousness it was removed from the halothane box and decapitated and the brain was removed.

The photomicrograph technique described in par 2.7 (*Anatomical probe location verification*) was adopted in our laboratory and proved to be more than satisfactory as can be seen in figures 4.4 and 4.5. Bert *et al.* (2004) concluded (in a study comparing the photomicrograph technique and the cresyl violet staining method) the photomicrograph technique to yield more rapid, accurate, reliable and less expensive results.

During all the ensuing microdialysis experiments, the above procedure was followed and care was taken to ensure that the probe was located in the intended area. If this was not true, the microdialysate was not further analyzed and no data of the particular rat was used.

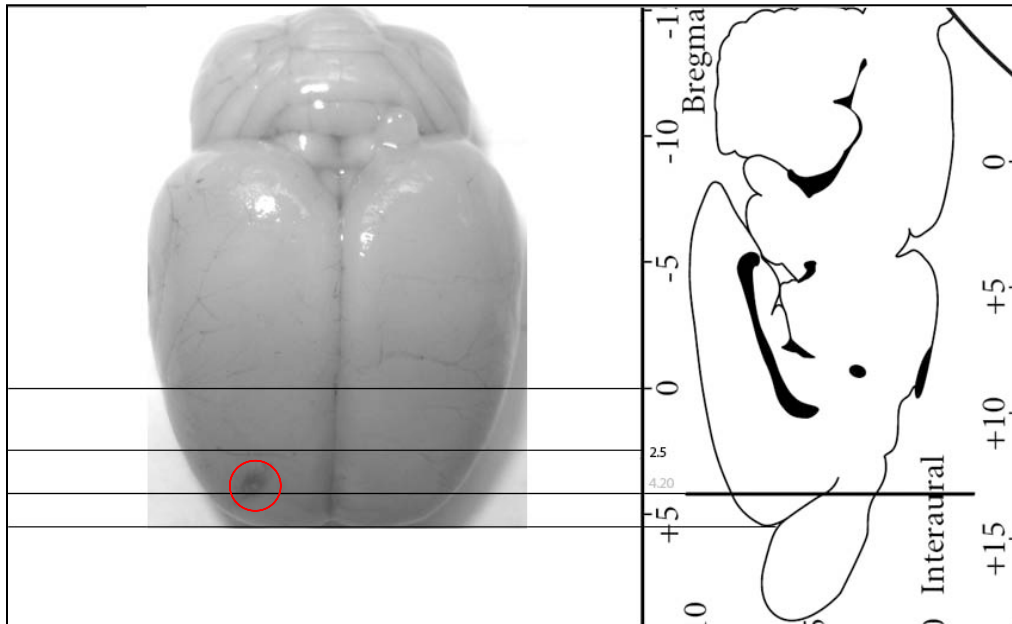

Figure 4.4 An example of anterior-posterior verification of the probe location. The scar where the probe was inserted is circled in red.

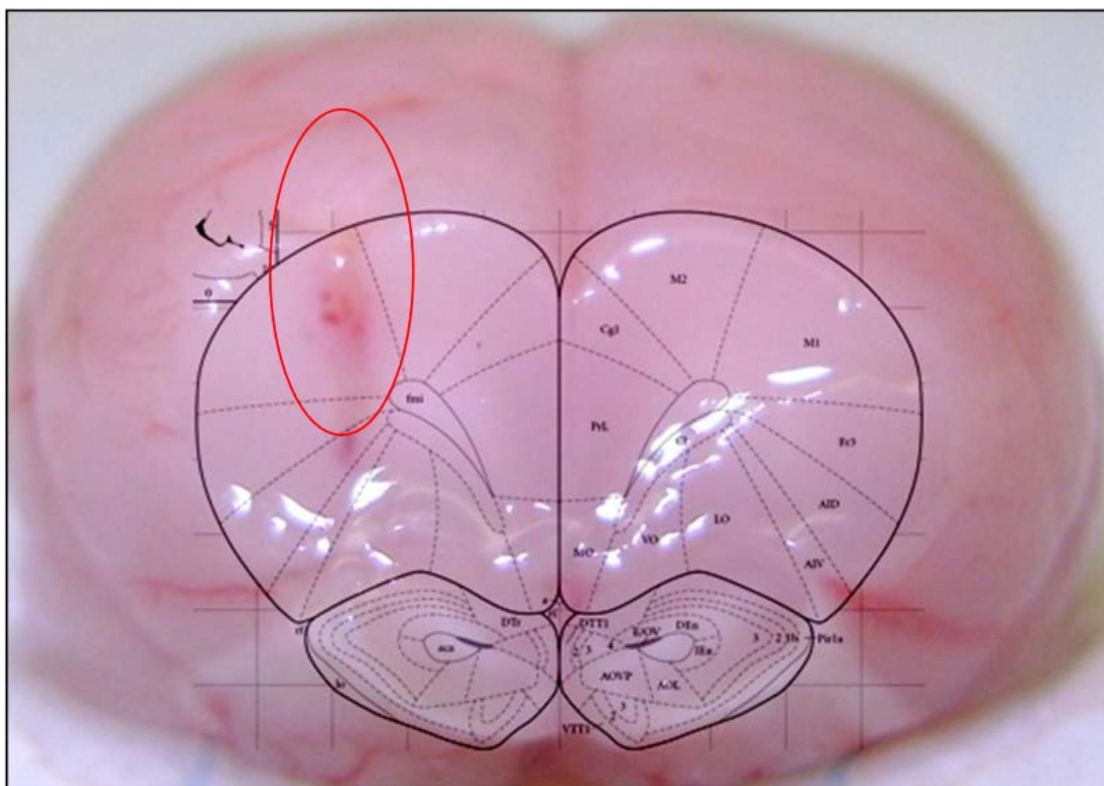

Figure 4.5 In this example the tract where the probe was located is visible circled in red. This superimposed image verifies the ventral and lateral probe location.

4.10 High performance liquid chromatography (HPLC) with fluorometric detection

The concentration of amino acids in the brain is in the micromolar to the submicromolar range and can be accurately determined by means of high-performance liquid chromatography. While glutamate and GABA are not naturally electroactive or have fluorescent or strong UV absorbance characteristics, they need to be derivatized in order to be detected (Shah *et al.*, 1999). In this study the derivatising agent, o-phthalaldehyde (OPA) which reacts with the primary amines in the presence of thiol, generating derivatives which are both electroactive and fluorescent (Zhang *et al.*, 2005) was used for the pre-column derivatization of the glutamate. (see Figure 2.4)

4.10.1 Apparatus

Table 4.2 HPLC apparatus and settings.

HPLC	**Agilent 1100 Series**
Column/Stationary phase	Supercosil LC-18-DB (150 x 4.6 mm, 3 µm)
Detector	Shimadzu RF-551 Spectrofluorometric detector: Ex wavelength: 350 nm Em wavelength: 450 nm
Pump	Agilent 1100 Isocratic Pump
Flow rate program	**Time (in minutes and seconds) Flow rate** 00:00 1.0 ml.min^{-1} 25:00 1.0 ml.min^{-1} 26:00 2.0 ml.min^{-1} 52:30 2.0 ml.min^{-1} 53:00 1.0 ml.min^{-1}
Autosampler	
Injection program	1. DRAW 5.0 µl from air 2. DRAW 45.0 µl from vial 100 (OPA) 3. EJECT 50.0 µl into sample 4. DRAW 70.0 µl from sample 5. EJECT 70.0 µl into sample 6. WAIT 2.00 minute 7. DRAW 50.0 µl from sample 8. INJECT (Inject volume: 50 µl)
Temperature	20ºC
Reporting integrator	Agilent Interface 35900E
Software	Chemstation Rev. A.06.02 data acquisition and analysis software

4.10.2 Mobile phase

The mobile phase used to separate the glutamate in the dialysate comprised of 70 mM Na_2HPO_4, 400 µM Na_2EDTA, 280 ml MeOH and 1800 ml distilled water. The pH was adjusted with H_3PO_4 to 5.26. The solution was filtered through a 0.45 µm hydrophilic polypropylene membrane filter and vacuum de-aerated prior to use.

Table 4.3 Mobile phase composition.

Compound	**Concentration**	**Amount / litre**
Na_2HPO_4	70 mM	9.9372 g
Na_2EDTA	400 µM	0.1169 g
MeOH	28% v/v	280 ml
H_2O	720 ml	720 ml
H_3PO4	Adjust pH to 5.26	

4.10.3 Sample preparation

The inserts containing the collected microdialysate were transferred from the refrigerated fraction collector to the -86ºC freezer where it resided until the HPLC-fluorometric analysis was performed.

On the day of analysis the samples were collected and inserted into the appropriate vials and loaded in the HPLC tray.

4.10.4 Sample injection

A sample injection program was created as follows:

1) DRAW 5.0 µl from air
2) DRAW 45.0 µl from vial 100 (OPA)
3) EJECT 50.0 µl into sample
4) DRAW 70.0 µl from sample
5) EJECT 70.0 µl into sample
6) WAIT 2.00 minute
7) DRAW 50.0 µl from sample
8) INJECT (Inject volume: 50 µl)

4.10.5 Analyte identification and quantification

Glutamate was identified by its characteristic retention time as determined by injections of a range of standard solutions. Sample peak areas were measured through the integrator system and compared with the standard calibration curve in order to quantify the glutamate's concentration (Chemstation software, Microsoft Excel).

See also addendum 1 for results of the validation of this analytical procedure.

4.10.6 Statistical analysis

Microdialysis results are expressed as mean percentage change from the average of three sequential baseline measurements (Time -60 - -20) ± standard error of the mean (SEM). The statistical analysis on extracellular glutamate levels was performed by comparing drug treated and control (FRL and FSL naive) animals as well as the 4 FRL groups against the 4 FSL groups for significant differences using analysis of variance with repeated measures over time (two-way ANOVA). The statistical analysis program Prism 5 for Windows (Graphpad Prism® version 5.0 Graphpad Software, San Diego, CA, USA) was used for all analyses and graph presentations. The Bonferroni post-test was employed and P-values of less than 0.05 were considered statistically significant.

Chapter 5: Results and Discussion

5.1 Introduction

Results of the application study are presented in the following section. Baseline values were calculated as the means of the first three samples (time -60 - -20) of each group of rats and the percentage of glutamate release was expressed with regard to these baseline values for each group. The "percentage of baseline" values obtained were then statistically analyzed where after each of the experimental groups (FRL-KCl-evoked, FRL-fluoxetine and FRL-ketamine) was compared with the control group (FRL-naïve). Likewise the FSL-experimental groups were compared to the FSL-naive group (Figures 5.9 and 5.10). The FRL and FSL counterparts of each experimental group were also compared (Figures 5.2, 5.4, 5.6 and 5.8). Time 0 minutes was defined as the exact time at which the substance, administered via retrodialysis, reached the brain. This time was calculated in advance by taking into account the volume of the tubing, syringe selector, swivel and probe, as well as the length of the tubing (from the syringe to the syringe selector to the swivel and from the swivel to the probe) and the flow rate. Time 0 minutes was regarded as a constant factor as long as the tubing lengths stayed the same.

5.2 Basal glutamate release in FRL compared to FSL

All the data in tables 5.1 to 5.4 were also graphically presented in figures 5.1, 5.3, 5.5 and 5.7 in order to get a clear picture of the changes in glutamate concentration over time in FRL vs. FSL rats.

Table 5.1 Basal prefrontal cortical glutamate concentrations of naive FRL and FSL rats, expressed as μM ± SEM.

Glutamate levels in the prefrontal cortex of FSL and FRL rats. Results are expressed as mean ± SEM			
Treatment	Time Minutes	Concentration (μM) FRL rats	Concentration (μM) FSL rats
aCSF (n = 2)	-60	0.140 ± 0.03	0.204 ± 0.11
	-40	0.102 ± 0.03	0.200 ± 0.07
	-20	0.130 ± 0.04	0.091 ± 0.01
	0	0.088 ± 0.03	0.073 ± 0.02
	20	0.096 ± 0.02	0.068 ± 0.02
	40	0.076 ± 0.02	0.096 ± 0.04
	60	0.075 ± 0.02	0.065 ± 0.00
	80	0.070 ± 0.01	0.066 ± 0.01
	100	0.067 ± 0.01	0.067 ± 0.00
	120	0.069 ± 0.01	0.058 ± 0.00

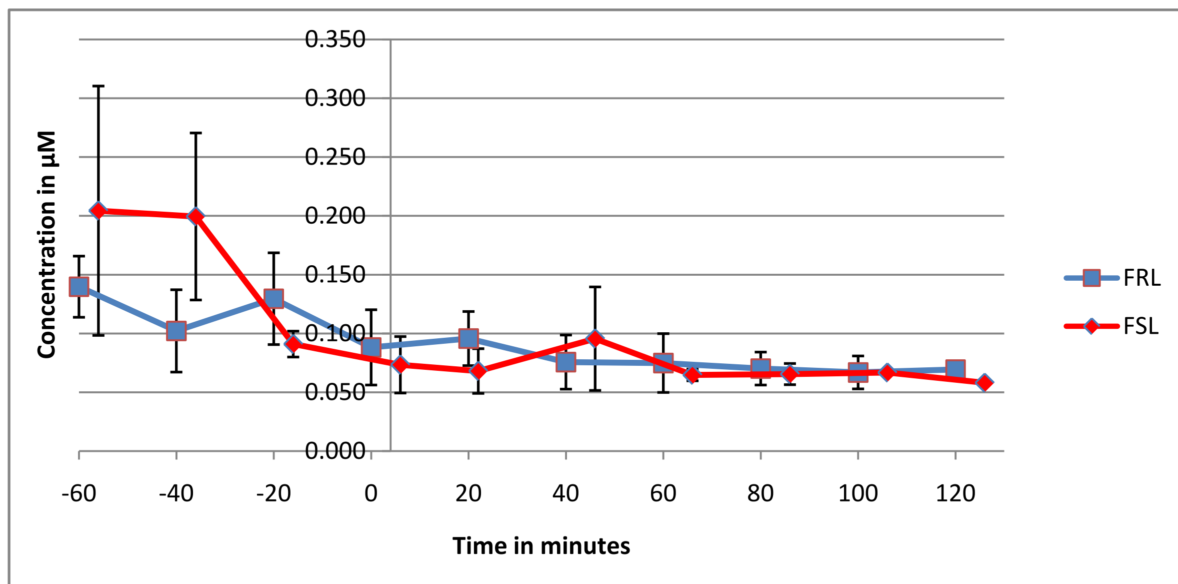

Figure 5.1 Basal prefrontal cortical glutamate concentrations of naive FRL and FSL rats, expressed as μM ± SEM.

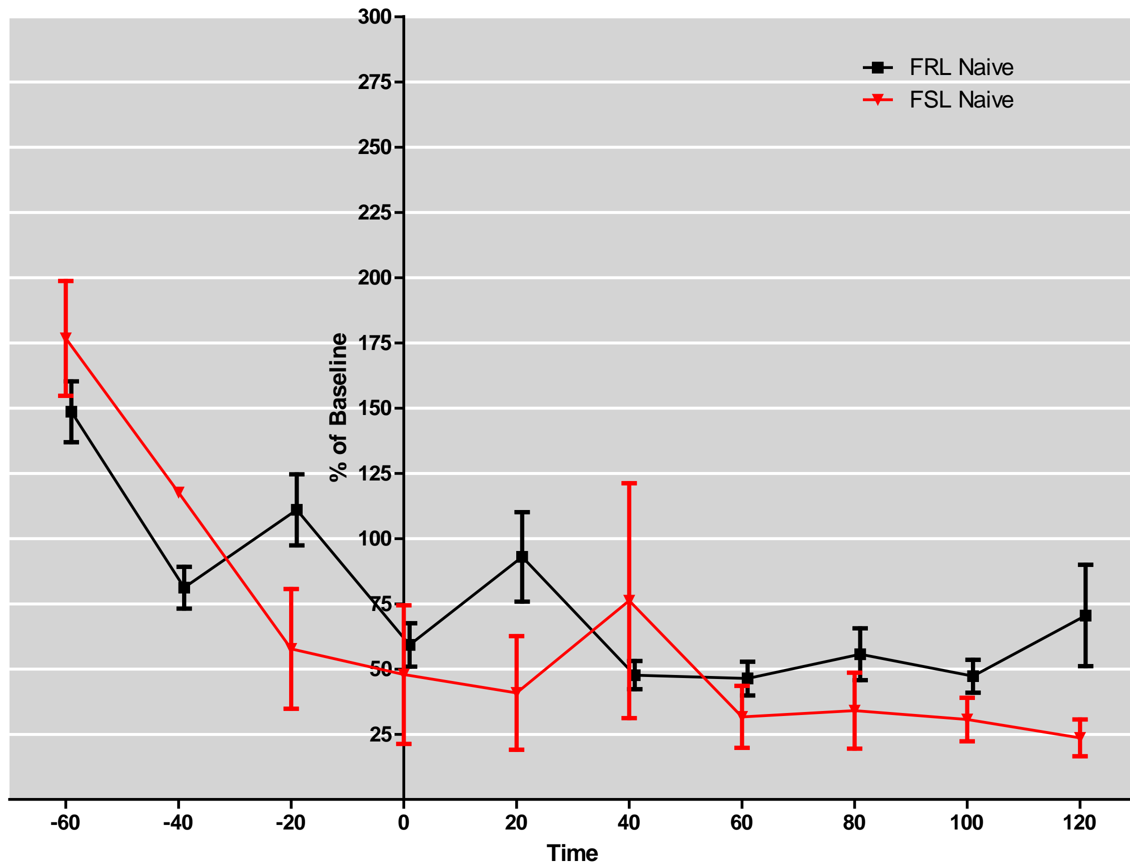

Figure 5.2 Basal prefrontal cortical glutamate levels of naive FRL and FSL rats expressed as % of baseline released.

Basal glutamate levels over a period of three hours were measured in naive FSL and FRL rats, with concentrations ranging from 0.06 – 0.20 μM found for FSL rats (n = 2) and 0.07 – 0.14 μM for FRL rats (n = 3). For the FSL rats, large variations were found between the first 2 baseline values at times -60 and -40 minutes (Fig. 5.2). Hereafter it stabilized at about 0.05 – 0.1 μM for the remaining two hours of perfusion with aCSF. The glutamate levels in the FRL rats were relatively stable with little variation during the first hour of aCSF perfusion (0.1 – 0.15 μM). Hereafter during the following two hours the levels decreased slightly and were stabilized at 0.05 – 0.1 μM. (see Table 5.1 and Figures 5.1 and 5.2).

Only one study, using micro-electrode array and comparing glutamate concentrations in FSL vs. FRL rats, could be found in the literature and this study described time-related differences in FSL vs. FRL rats (Hascup *et al.*, 2011). At ages between 3 and 6

months, this particular study found no significant differences in resting glutamate levels in the prefrontal cortices of the two groups of rats. However, a significant increase in the resting glutamate levels was noted in the FSL rats aged between 12 – 15 months (Hascup *et al*., 2011). Our results are therefore in accordance with this finding, as the rats used in the current study were also between 3 and 4 months of age.

Literature reports of glutamate baseline levels vary extensively, ranging from 1 – 3 µM (van der Zeyden *et al*., 2007). However Anneken & Gudelsky (2012) reported values of 1.15 µM while Moghaddam *et al*., (1997) reported basal glutamate levels of 0.8 µM. Both the latter studies analyzed basal glutamate by *in vivo* microdialysis in the prefrontal cortices of male Sprague Dawley rats. In a study where micro-electrode array was used, basal glutamate values ranged from 5.0 ± 0.5 µM in FSL and 5.3 ± 1.0 µM in FRL rats (Hascup *et al*., 2011). Therefore our values correspond very well with that found in the literature, although the small number of rats used in this study is a distinct limitation worth noting.

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5.3 KCl-evoked glutamate release in FRL compared to FSL

Table 5.2 Prefrontal cortical glutamate levels in response to local KCl-administration in FRL and FSL rats, expressed as μM ± SEM

Glutamate levels in the prefrontal cortex of FSL and FRL rats. Results are expressed as mean ± SEM			
Treatment	Time Minutes	Concentration (μM) **FRL rats**	Concentration (μM) **FSL rats**
	-60	0.106 ± 0.03	0.052 ± 0.00
	-40	0.086 ± 0.02	0.046 ± 0.00
	-20	0.119 ± 0.05	0.048 ± 0.00
KCl (n = 2)	0	0.133 ± 0.06	0.056 ± 0.00
	20	0.111 ± 0.04	0.068 ± 0.01
	40	0.096 ± 0.03	0.061 ± 0.01
	60	0.091 ± 0.03	0.059 ± 0.01
	80	0.079 ± 0.02	0.053 ± 0.01
	100	0.075 ± 0.00	0.049 ± 0.01
	120	0.056 ± 0.00	0.047 ± 0.01

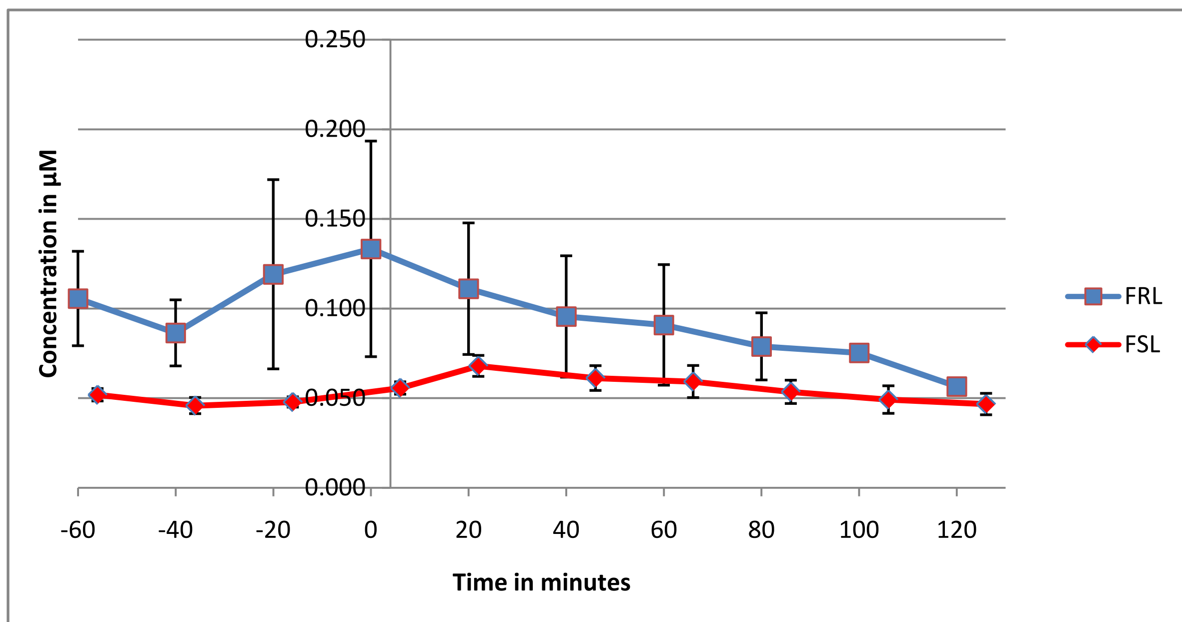

Figure 5.3 Prefrontal cortical glutamate concentrations in FRL and FSL rats, following the local administration of KCL, expressed as μM ± SEM.

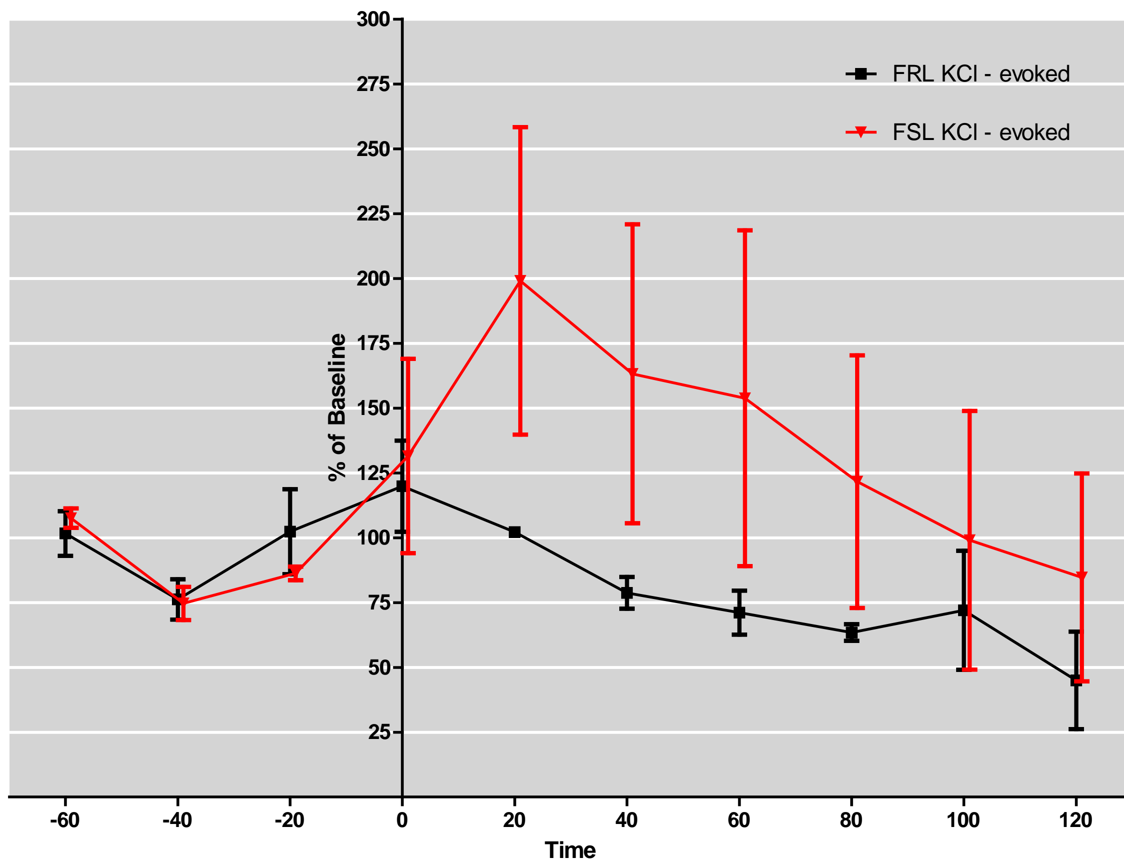

Figure 5.4 Effect of local perfusion of a 100 mM KCl solution on prefrontal cortical glutamate release in FRL (n = 2) and FSL (n = 2) rats respectively. The results are presented as % of baseline released.

An elevation of KCl concentration in the extracellular fluid depolarizes neuronal membranes and increases neurotransmitter release (Adams and Moghaddam, 2001). Therefore one would expect that the local perfusion of the PFC with 100mM KCl would have caused an increase in the basal glutamate release in both FRL and FSL rats. This was true for the FSL rats where an immediate increase in the release of glutamate was seen with a maximum increase of about 200% at 20 minutes, where after it decreased gradually returning to baseline levels at 100 minutes (see Figure 5.4). In FRL rats however, KCl perfusion only caused a transient release of glutamate, with glutamate levels returning slowly to baseline over the remaining two hours of dialysis (Fig. 5.4). Although there wasn't a statistically significant difference between the effects of KCl administration in FRL vs. FSL rats, this slight tendency of a higher response in the FSL (or pathological state) definitely require further investigation, as it may be an indication of the bolstering of KCl- evoked glutamate release in the pathological state.

5.4 The effect of local perfusion with fluoxetine on basal glutamate release in FRL compared to FSL.

Table 5.3 Prefrontal cortical basal glutamate levels following local perfusion with 30 μM fluoxetine in FRL and FSL rats, expressed as μM ± SEM.

Glutamate levels in the prefrontal cortex of FSL and FRL rats. Results are expressed as mean ± SEM			
Treatment	Time Minutes	Concentration (μM) FRL rats	Concentration (μM) FSL rats
	-60	0.280 ± 0.15	0.792 ± 0.53
	-40	0.319 ± 0.12	0.763 ± 0.41
	-20	0.313 ± 0.08	0.766 ± 0.42
Fluoxetine (n = 3)	0	0.365 ± 0.12	0.804 ± 0.43
	20	0.400 ± 0.08	0.765 ± 0.38
	40	0.366 ± 0.08	0.825 ± 0.43
	60	0.444 ± 0.02	0.796 ± 0.34
	80	0.354 ± 0.09	0.828 ± 0.32
	100	0.311 ± 0.11	0.852 ± 0.37
	120	0.324 ± 0.11	0.744 ± 0.31

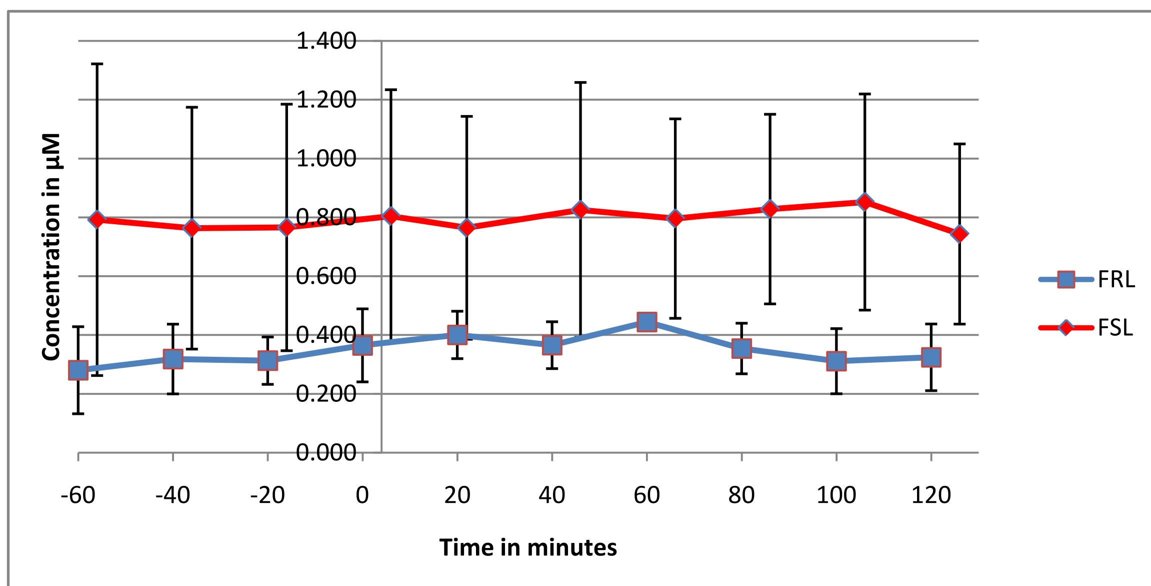

Figure 5.5 Prefrontal cortical basal glutamate levels following local perfusion with 30 μM fluoxetine in FRL and FSL rats, expressed as μM ± SEM.

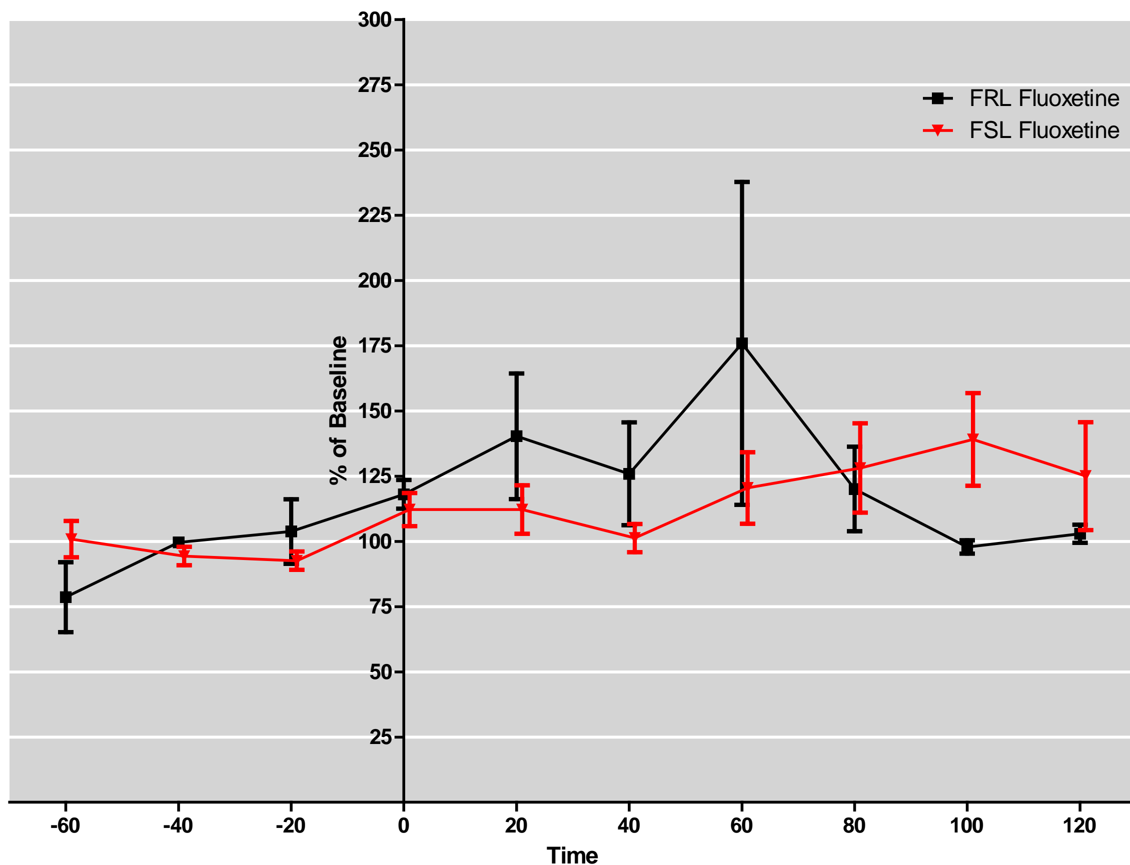

Figure 5.6 Effect of local perfusion of a 30 µM fluoxetine hydrochloride solution on basal glutamate release in the prefrontal cortex of FRL (n = 3) and FSL (n = 3) rats respectively. The results are presented as % of baseline released.

There were no significant differences in glutamate levels at any of the time points for either FRL or FSL rats following fluoxetine perfusion (Fig. 5.6). No difference was noted with respect to glutamate levels between FRL and FSL during the dialysation period (Fig. 5.6). Both FRL and FSL rats showed an immediate increase in glutamate levels as fluoxetine reached the brain (time=0), increasing to approximately 115% of baseline values (first three values) (Fig. 5.6).

The FRL rats showed a second glutamate peak at 20 - 40 minutes, increasing to more than 125% of baseline values (Fig. 5.6). Glutamate release peaked at 60 minutes in the FRL rat, reaching 175% of baseline value, where after levels returned to baseline for the remainder of the dialysis time (Fig. 5.6). The FSL rats stabilized at the initial increase (between 100-125% of baseline) for the first 60 minutes where after a slight increase (above 125%) was evident from 80 - 120 minutes.

In an *in vivo* microdialysis experiment done by Reznikov and co-workers (2007) it was found that following acute intraperitoneal (IP) fluoxetine (10mg/kg), basal glutamate levels were increased in the basolateral nucleus (BLA) and the central nucleus (CeA) of the brain. This acute fluoxetine injection did not have any effect on the restraint stress-induced increase in glutamate levels found in the amygdala. *In vitro* studies done on the effect of acute and/or chronic fluoxetine administration on glutamate release in the brain also showed contradicting results. Bonanno and co-workers (2005) found that chronic treatment (14 days) with fluoxetine reduced KCl- evoked release of glutamate, whereas acute treatment had no effect on the evoked glutamate release in the hippocampus. In the same study it was reported that chronic fluoxetine treatment had no effect on basal glutamate levels. In contrast to Bonanno *et al*. (2005), Wang and co-workers (2003) reported a reduction of 4-aminopyridine - evoked glutamate release after acute fluoxetine administration. It is worth mentioning that both Bonanno *et al*. (2005) and Wang *et al*. (2003) used *in vitro* superfused hippocampal synaptosomes to obtain their results.

As seen from the above discussion, there is no consensus on the effect of fluoxetine (administered either locally or systemically or chronic vs. acute) on the release of glutamate in either *in vivo* or *in vitro* studies. Our preliminary studies confirm a lack of effect of fluoxetine in this regard, although further studies are required.

5.5 The effect of local perfusion with ketamine on basal glutamate release in FRL compared to FSL.

Table 5.4 Basal glutamate release in prefrontal cortex in FRL and FSL rats following local perfusion with 9 mM ketamine, expressed as µM ± SEM.

Glutamate levels in the prefrontal cortex of FSL and FRL rats. Results are expressed as mean ± SEM			
Treatment	Time Minutes	Concentration (µM) **FRL rats**	Concentration (µM) **FSL rats**
	-60	0.268 ± 0.12	0.371 ± 0.27
	-40	0.268 ± 0.13	0.427 ± 0.35
	-20	0.461 ± 0.33	0.394 ± 0.29
Ketamine (n = 3)	0	0.144 ± 0.09	1.692 ± 1.61
	20	0.322 ± 0.14	1.483 ± 0.62
	40	0.300 ± 0.12	1.342 ± 0.49
	60	0.300 ± 0.14	0.774 ± 0.30
	80	0.209 ± 0.13	0.458 ± 0.22
	100	0.184 ± 0.08	0.287 ± 0.13
	120	0.139 ± 0.06	0.276 ± 0.13

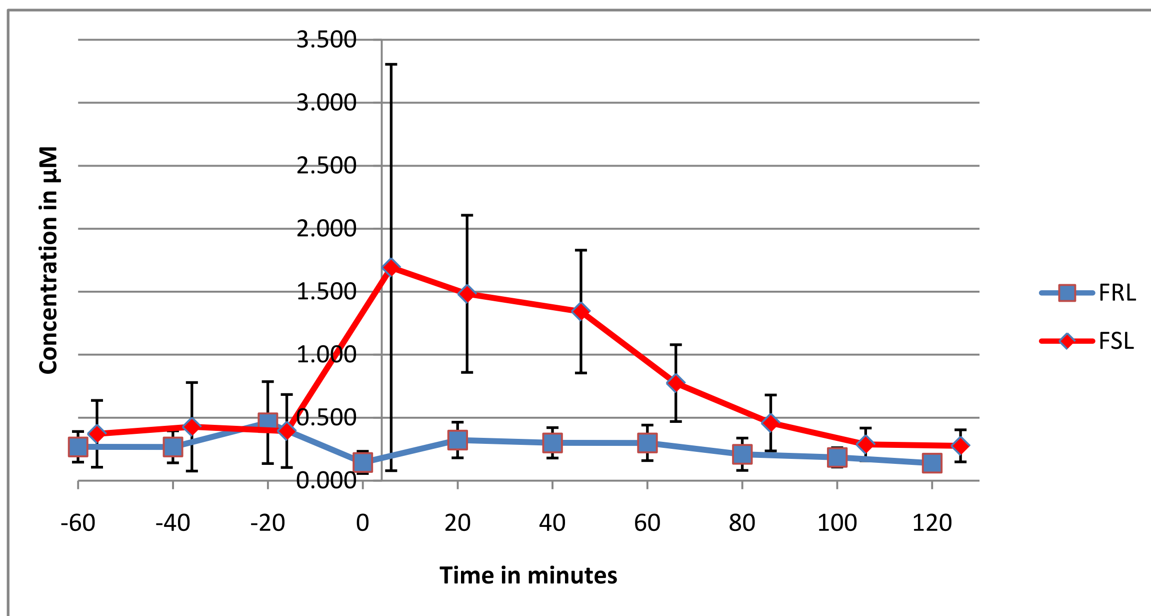

Figure 5.7 Basal glutamate release in prefrontal cortex in FSL and FRL rats following local perfusion with 9 mM ketamine, expressed as µM ± SEM.

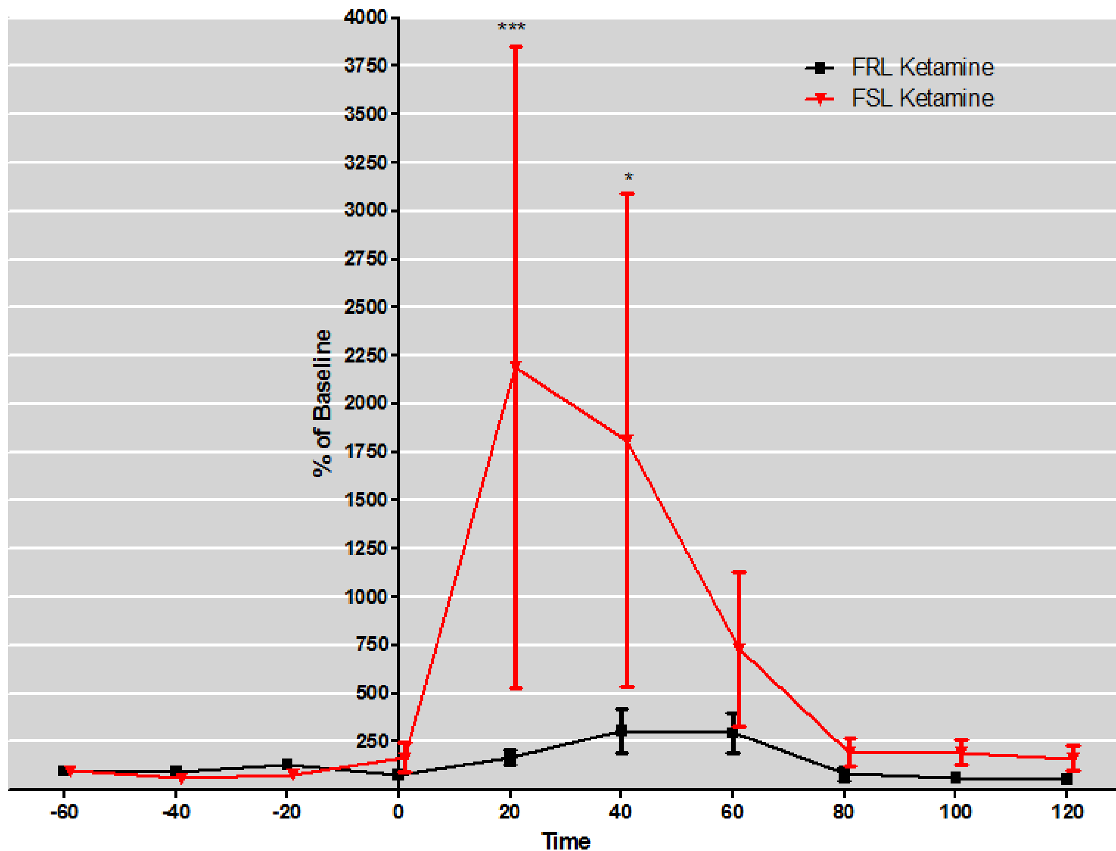

FIGURE 5.8 Effect of local perfusion of a 9 mM ketamine hydrochloride solution on prefrontal cortical glutamate release in FRL (n = 3) and FSL (n = 3) rats respectively. The results are presented as % of baseline released. $^{*}P < 0.05$ compared to FRL group (Bonferroni post-test). $^{***}P < 0.001$ compared to FRL group (Bonferroni post-test).

Local perfusion with a 9 mM ketamine solution (corresponding to the sub-anesthetic doses cited in clinical studies providing rapid relief of depression following IV administration, section 4.7.3.2) yielded some interesting results, with glutamate release increasing in both FRL and FSL rats. FRL rats displayed a gradual increase from 20 minutes onwards, culminating in two peaks of 312% and 303% of baseline value at 40 minutes and 60 minutes, respectively (Fig. 5.8). At 80, 100 and 120 minutes glutamate levels showed a gradual reduction and stabilized at approximately baseline concentrations (Fig. 5.8). This reduction ensued despite the continued administration of ketamine.

The FSL group responded with a rapid and marked increase following ketamine perfusion. At 20 and 40 minutes glutamate levels demonstrated a remarkable increase

to 3196% and 2590% of baseline, respectively (Fig. 5.8) that was statistically significant vs. ($^{*}P < 0.05$ and $^{***}P < 0.001$ respectively) compared to the FRL group at the same points (Fig. 5.8). At 60 minutes glutamate levels started to decrease to 988% despite the continuous administration of the ketamine hydrochloride. At 80 minutes the glutamate level decreased to 187% and reached a relative plateau at 120 minutes.

As stated previously (see section 3.4.2) the effect of ketamine on glutamate release after an IP injection have been shown to be biphasic (Moghaddam *et al.*, 1997). At subanesthetic doses (10 - 30 mg/kg) ketamine was found to increase glutamate outflow whereas anesthetic doses (50 - 200 mg/kg) decreased glutamate outflow (Moghaddam *et al.*, 1997). Lorrain *et al.* (2003) investigated the effect of subcutaneously (SC) administered ketamine on PFC glutamate levels and found that an 18 mg/kg injection evoked a significant release of glutamate. In the same study a 1 mM ketamine solution was locally administered in the medial prefrontal cortex (mPFC) but in contrast to the SC injection, the local administration of the ketamine had no effect on glutamate release.

In the current study, glutamate release was increased in both FRL and FSL groups of rats, although levels in FSL were significantly higher vs. FRL rats. This finding is in contrast with the results of the study of Lorrain *et al.* (2003) where a dose of 1 mM administered locally had no effect. In accordance with the results of the systemically administered ketamine, this may also be suggestive of a dose-dependent effect, because we have applied an almost 10-fold higher dose than what was used in the study of Lorrain *et al.* (2003). This dose in the current study corresponds to the subanesthetic dose clinically proven to rapidly relief depression (Machado-Vieira *et al.*, 2009a).

5.6 FRL prefrontal cortical glutamate release in response to local perfusion of KCl, fluoxetine and ketamine and pure aCSF.

In order to compare the effects of perfusion of the prefrontal cortex with KCl, fluoxetine and ketamine on glutamate release in naive FSL vs. FRL rats respectively (only aCSF-perfused), all four diagrams were plotted on the same graph for either the FRL or FSL groups (Fig. 5.9). Comparison of each group's experimental data with its control data obtained in naive FRL and FSL rats showed significant differences at specific time points when perfused with the pharmacological agents under study (Fig. 5.9).

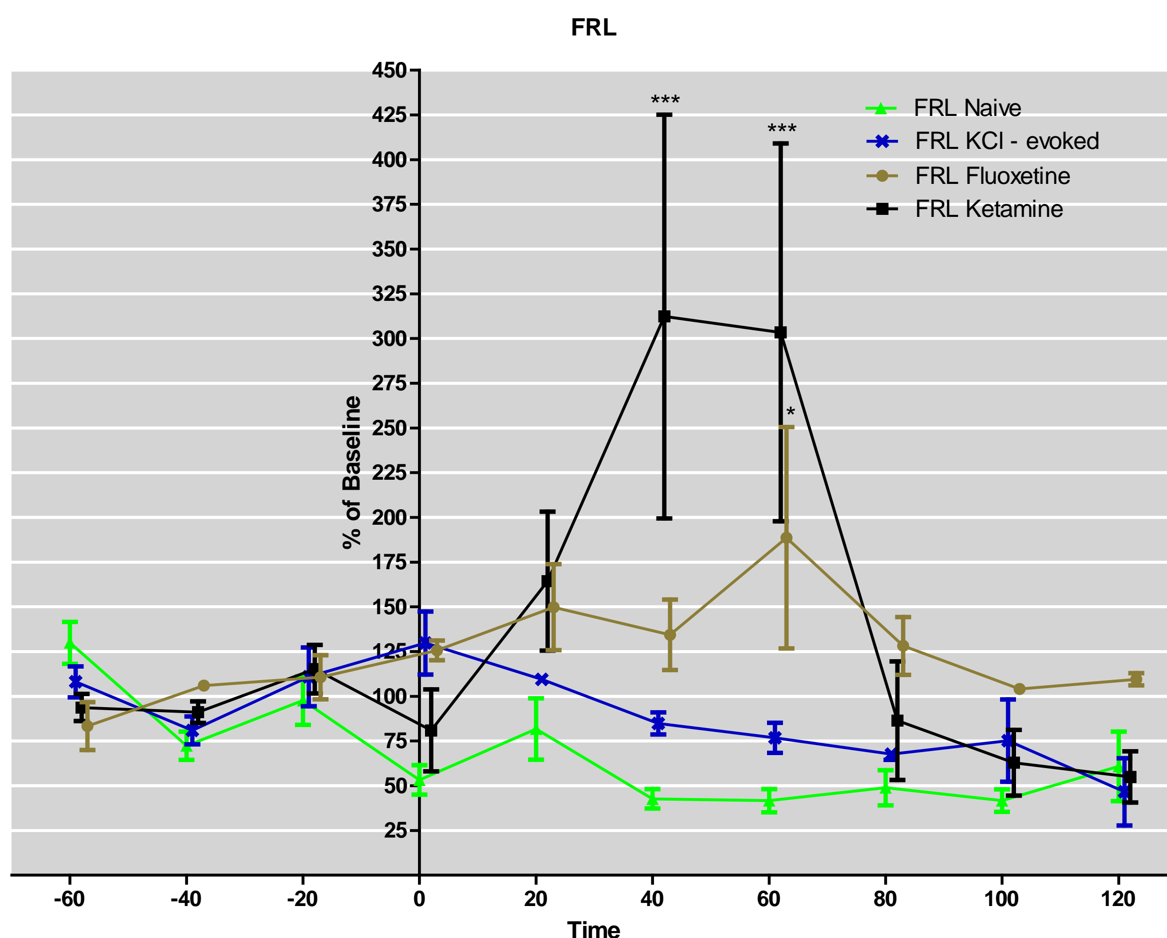

Figure 5.9 Effect of local perfusion of 100 mM KCl (n = 2), 30 µM fluoxetine hydrochloride (n = 3), 9 mM ketamine hydrochloride (n = 3) and pure aCSF (n = 2) (naive/control) respectively on prefrontal cortical glutamate levels in FRL rats. The results are presented as % of baseline released. $^{***}P < 0.001$ compared to FRL naive group (Bonferroni post-test).

Figure 5.9 represents the response of FRL rats to locally administered KCl, ketamine and fluoxetine to the prefrontal cortical brain area. The administration of potassium chloride (100 mM) caused no statistically significant changes in glutamate concentrations. This finding, although seemingly contradictory from what would be expected, is however in accordance with the finding of Herrera-Marschitz *et al.*, 1997), that glutamate release, different from that of other neurotransmitters, is only slightly increased by K^+ depolarization. Fluoxetine hydrochloride showed only a transient increase to 188% at t = 60. This transient increase was found statistically significant with a P-value of less than 0.05. Local administration of ketamine hydrochloride yielded a gradual increase over time to produce two statistically significant peaks at t = 40 min (312%) and t = 60 min (303%) compared to glutamate release in naive FRL rats (P‹0.001). At t = 80 min, t = 100 min and t = 120 min the glutamate levels were reduced again, stabilizing at baseline concentrations. This again was despite the continuous local administration of ketamine hydrochloride.

5.7 FSL prefrontal cortical glutamate release in response to local perfusion of KCl, fluoxetine and ketamine and pure aCSF.

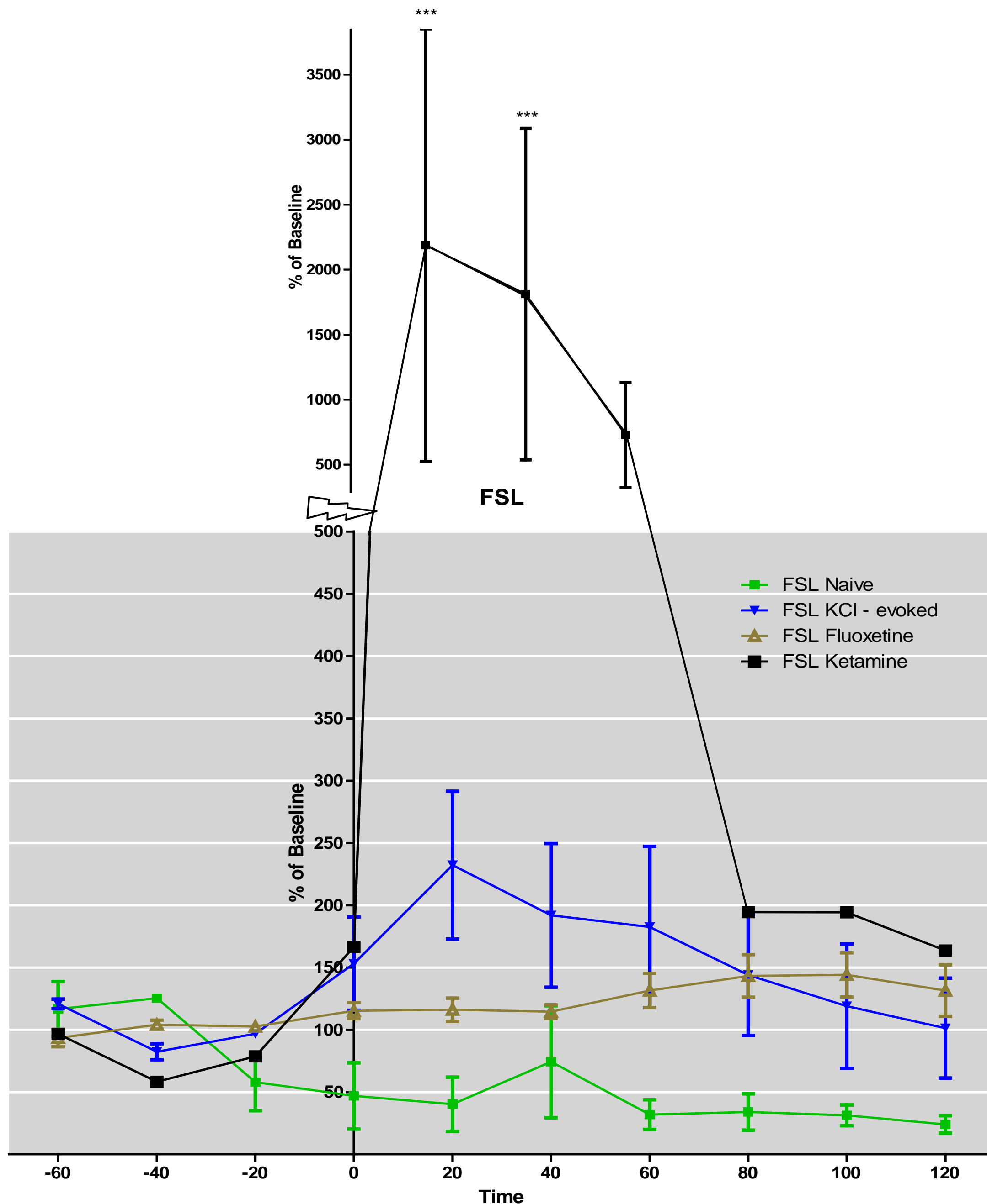

Figure 5.10 Effect of local perfusion of 100 mM KCl (n = 2), 30 μM fluoxetine hydrochloride (n = 3), 9 mM ketamine hydrochloride (n = 3) and pure aCSF (n = 2) (naive/ control) respectively on prefrontal cortical glutamate levels in FSL rats. The results are presented as % of baseline released. $^{***}P < 0.001$ compared to FSL Naive group (Bonferroni post-test).

In figure 5.10 prefrontal cortical response to different local pharmacological stimuli in FSL rats are plotted. The administration of potassium chloride (100 mM) caused an increase in the glutamate levels compared to those of naive FSL rats, although it was not statistically significant. This is in accordance with what has been described in the literature, i.e. that glutamate release is only slightly increased by K^+ depolarization (Herrera-Marschitz *et al.*, 1997). As stated previously (section 5.3), the slightly higher KCl- induced response in the FSL rats compared to the FRL rats may be indicative of a changed glutamate release during a pathological condition as mimicked by the FSL rat. Fluoxetine hydrochloride (30 µM) as well caused no statistical significant changes in glutamate concentrations compared to the naive FSL rats (Fig. 5.10). The local administration of ketamine hydrochloride yielded a striking contrast to the other groups. At t = 0 min when ketamine reached the prefrontal cortex, glutamate levels responded with a rapid and marked increase compared to the levels in the control (acSF) group (Fig. 5.10). At t = 20 min and t = 40 min the glutamate level rose to a significant ($p < 0.001$) 2188% and 1812% of basal values respectively compared to control (naive) FSL rats. At t = 60 min glutamate levels decreased to 729% despite the continuous administration of the ketamine hydrochloride. At t = 80 min the glutamate level decreased to 187% and reached a relative plateau for the remaining period (Fig. 5.10).

A very interesting phenomenon was the difference in the pattern of glutamate release induced by ketamine administration in the FRL and FSL rats (see Fig 5.8). In the FRL rats peak values were found only after 40 and 60 minutes and at these time points maximum values of 305% and 296% were reached. This observation was very different from what was seen in the FSL rats, where the first peak was reached at already 20 minutes post perfusion (2188%) and the second peak at 40 minutes (1812%). These values were also statistically significant higher than values obtained at the same time for FRL rats (Fig. 5.8). Although not statistically significant different from control values of glutamate, another peak was observed at 60 minutes (729%) in the FSL group. As previously mentioned (section 5.2) information regarding glutamate transmission in the FSL rat model of depression is deficient, but results of the current study indicate a completely different response to the local administration of ketamine on glutamate release in the FSL rat compared to the FRL rat. In the FSL rat a rapid, more than 20-fold increase in glutamate release was observed and maintained for almost an hour following ketamine administration. This finding, although in a very small sample,

supports the observation that the introduction of a stressor, or in this case ketamine, may unmask differences between FRL and FSL rats, not otherwise seen in stress-naive rats (Wegener *et al*., 2010). While it was not done in the current study, it would be interesting to see what the effects of fluoxetine and ketamine on KCl-evoked glutamate release would be, as this may provide valuable info regarding the pathological condition.

Both animal and clinical studies have confirmed the abnormal regulation of glutamate in limbic and cortical areas in animal models of stress (Cazakoff and Howland, 2010, De-Vasconcellis-Bittencourt *et al*., 2011) and in the brains of depressed patients (Ongur *et al*., 2008; Sanacora *et al*., 2012). Treatment with antidepressants was also shown to decrease the elevated glutamate levels in depressed individuals (Kucukibrahimoglu *et al*., 2009). One would therefore expect that antidepressants (fluoxetine and ketamine in the current study) would rather decrease glutamate release than the significant increase that was observed in the current study. However, while the literature is lacking results of basal glutamate levels in FSL vs. FRL rats, except for the age-related study that was done by Hascup *et al*. (2009), our finding is interesting, but definitely requires further investigation. As part of the validation of the microdialysis technique the current study investigated possible glutamatergic involvement in an animal model of depression and the effects of antidepressants on glutamate release. Some interesting results were observed in support of the notion that glutamate is involved in the pathology and action of antidepressants, although because of limited numbers of animals, no definite conclusions can be made at this stage and further studies are required to elucidate this involvement.

Chapter 6: Conclusion

6.1 Introduction

As stated in the project objectives (see section 1.2) we envisaged establishing and validating the technique of *in vivo* intracerebral microdialysis in our laboratory and to develop and validate a sensitive and reliable analytical method for the determination of glutamate in the microdialysate, which usually is a very small sample size. With regard to the technique of microdialysis we had to establish effective protocols for the anesthesia, surgery, recovery, the actual microdialysis procedure including retrodialysis as well as the probe location verification. The results of all these developmental work are described in chapter 4. The final objective was a small application study where these techniques could be applied in a small group of FSL vs. FRL rats in order to determine glutamate release at baseline (control/naive rats), following K^+ stimulation and perfusion with fluoxetine and ketamine.

In the following sections each of these objectives will be evaluated with regard to the successfull achievement thereof.

6.2 Microdialysis technique development and validation

The establishment of the microdialysis technique was successfully performed with reliable and repeatable protocols for all the different components of the technique and yielded accurate results.

Different anesthetic regimes were investigated, of which 5 of the most applicable regimes (see section 4.4.1) were implemented to obtain the most suitable regime for our conditions. The regime that proved to be the least harmful to the rats providing the most suitable results with regard to induction of surgical anesthesia, maintenance and recovery following surgery was the combination of pentobarbitone (45 mg/kg) and halothane regulated in response to vital signs.

The stereotaxic surgery was challenging with regard to the fixation of the rat in the Kopf-stereotaxic frame, as the steady and correct placement of the ear bars was essential for the successful surgery and probe placement. The identification of the bregma and the subsequent calculation of the correct coordinates for the specific brain area also necessitated more than a little practice and experience. If this was done incorrectly, probe placement would be off-target and data from this rat would be invalid. Regarding the microdialysis procedure, it was found that insertion of the microdialysis probe had to be performed swiftly and with great care to ensure the well-being of the rat and the reliable achievement of microdialysis.

6.3 Glutamate analysis by HPLC-fluorescence

The HPLC-fluorescence method for the analysis of glutamate was also successfully developed and validated. It is sensitive and specific for the quantification of the analyte (glutamate). Validation of this method gave a linearity of 0.991 in the concentration range of standards tested (0.1 – 10 µM) and an intra-assay repeatability (precision value) yielding relative standard deviations of less than 10.5%. Mean elution time was between 24 and 26 minutes for glutamate in the microdialysis sample and the limit of detection and quantification was both 0.1 µM.

6.4 Application study

The application study was done to verify whether the technique was successfully established in order to obtain quantifiable amounts of the excitatory amino acid neurotransmitter glutamate from the prefrontal cortices of FRL and FSL rats. In addition to the measurement of basal/resting glutamate concentrations (control/naive) the principle of retrodialysis (reverse dialysis) was also applied in order to verify the successful elicitation of an alteration in glutamate release in the prefrontal cortices of FSL and FRL rats following acute drug administration. This was achieved with the local administration of either KCl, or fluoxetine or ketamine via reverse dialysis.

There was no difference in basal glutamate levels of FRL vs. FSL rats, although my values were comparable to those reported in the literature. In accordance with results

from the literature of a relatively small increase of glutamate release following KCl administration (discussed in sections 5.6 and 5.7). KCl had practically no effect on glutamate release in the FRL rats, other than a small (less than 25%) transient increase at time 0. It however evoked release of glutamate in the FSL group of rats, though not statistically significant different from basal values. Fluoxetine had no effect on glutamate release in any of the FRL and FSL groups, a finding that was also difficult to verify from previous studies because of a lack of consensus in the few reported studies. Ketamine on the other hand increased glutamate release in both FRL and FSL rats compared to control values, but to a much higher extent in FSL than in FRL rats.

Despite the small group sizes, some interesting results were revealed while at the same time demonstrating that the technique of microdialysis and retrodialysis had been successfully implemented in our laboratory. Although the literature indicates that antidepressant effects involve an effect on the release of glutamate, no definite conclusions can be made with regard to the effects of fluoxetine and ketamine in this particular study. Further studies are required to substantiate the results regarding glutamate release in FSL v. FRL rats and the effects of antidepressants and other interventions there upon. Nevertheless, evidence of some interaction with glutamate was demonstrated warranting further studies. This is also true for the comparison of FRL vs. FSL rats, where the only statistically significant difference between glutamate release in FRL and FSL rats was found with the perfusion with ketamine into the PFC.

Finally, the development and implementation of the microdialysis technique as well as the standardization and validation of a sensitive method of glutamate analysis was successfully established, while data from the application study indicate that reliable results may be achieved by using these techniques. While the application study definitely suffers from a few shortcomings, of which the limited number of animals is probably the most important, and additional development and refinement is required, this method may make a positive contribution to the elucidation of the pathological mechanisms in depressive disorder and the search for new treatments.

Addendum 1

1.1 Validation of fluorescence HPLC method for the quantification of glutamate

1.1.1 Specificity and selectivity

Selectivity of an analytical method is defined as the ability of the method to differentiate and quantify an analyte in the presence of other compounds. The specificity is defined as a method's ability to produce a response for only one analyte (United States Department of Health and Human Services, 2001). In this study (using HPLC) specificity is achieved by chromatographic separation resulting in relatively clean chromatogram spectra.

Method: Four solutions were prepared and injected into the HPLC, with the first comprising of a standard concentration of glutamate in the same aCSF solution employed in the experiment, and the second solution a blank only solution of aCSF containing no glutamate. The peak and elution time of the glutamate was recorded via the HPLC method. After the glutamate standard was analysed, a microdialysis sample (unknown concentration glutamate) was analysed and lastly the same microdialysis sample was spiked with a known concentration of glutamate and analysed again.

Result: The four resulting HPLC-fluorescence chromatograms are presented below.

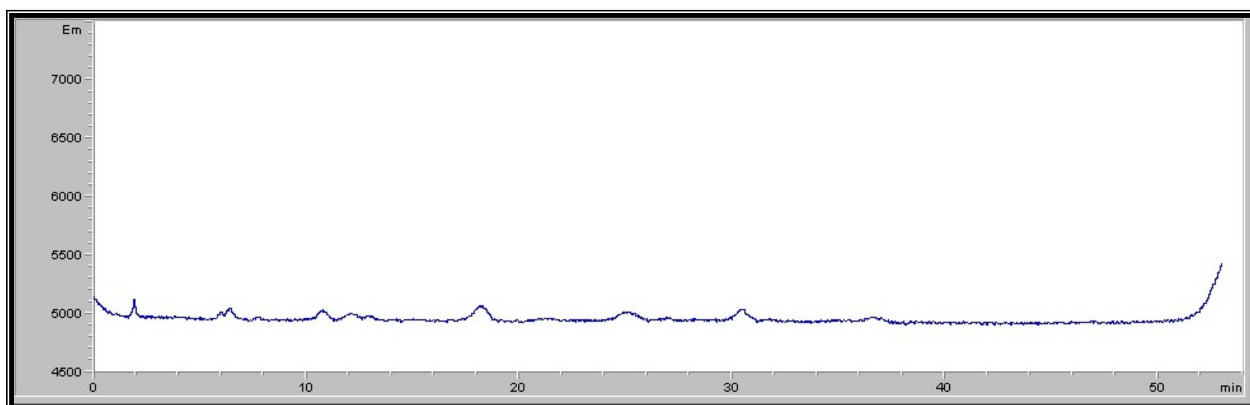

Figure A1.1 Chromatogram of a blank aCSF solution.

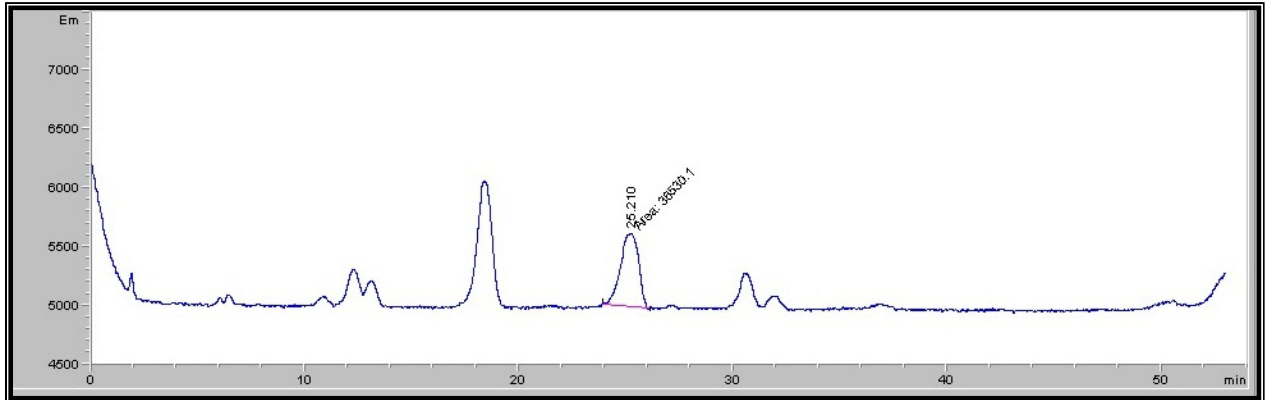

Figure A1.2 Chromatogram of a 1 μM Glutamate solution, at time 25.21 minutes the glutamate peak eluted.

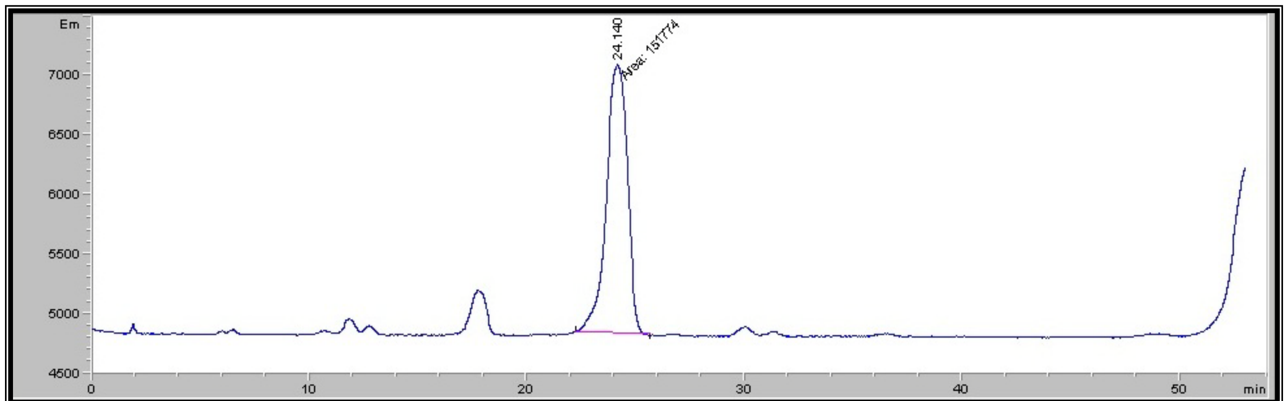

Figure A1.3 Chromatogram of a 10 μM Glutamate solution, at time 24.14 minutes the glutamate peak eluted.

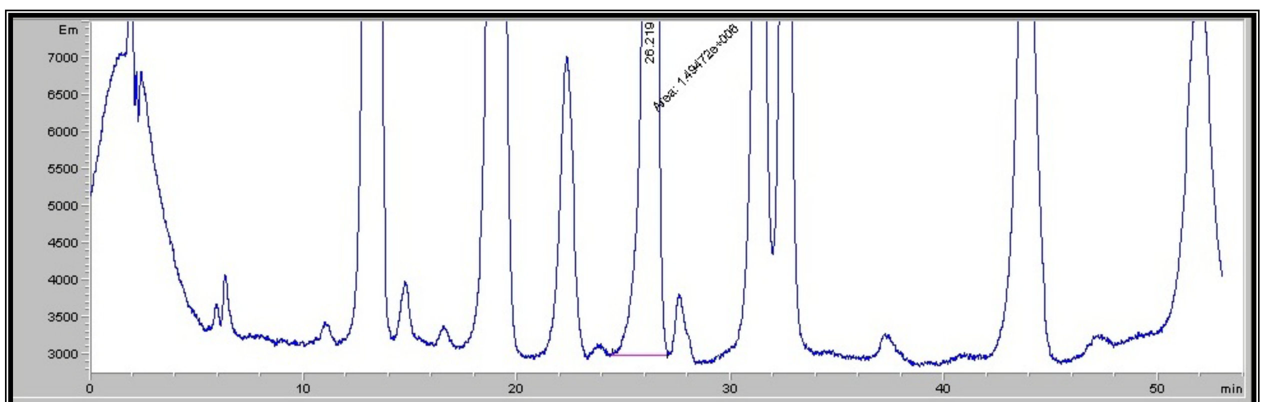

Figure A1.4 An example of a typical analyzed microdialysis sample, at time 26.219 minutes the glutamate peak eluted.

Conclusion: Very small and negligible contamination/system peaks are visible in the blank aCSF solution. The glutamate eluted between 24 and 26 minutes.

1.1.2 Linearity

The USP defines linearity as the ability of an analytical method to obtain a response that is directly proportional to known concentrations of analytes in a sample within a given range (USP, 2008). To establish proof of linearity the USP recommends a minimum of five concentrations and a concentration range of 80-120% of the test concentration to be analysed. A correlation coefficient (r^2) of not less than 0.95 is recommended for good linearity (USP, 2008).

Method: A concentration range (between 0.1 and 10 μM) consisting of 8 distinct concentrations typical of the concentrations usually found in the brain (including an endogenous concentration) of glutamate standard was prepared for analysis. The peak areas were recorded and plotted on a graph. The linearity was calculated using Equation 1.

$$y = mx + c \qquad \textbf{(Equation 1)}$$

where

y = peak area (PA)

m = gradient

x = concentration

c = y-intercept

Results:

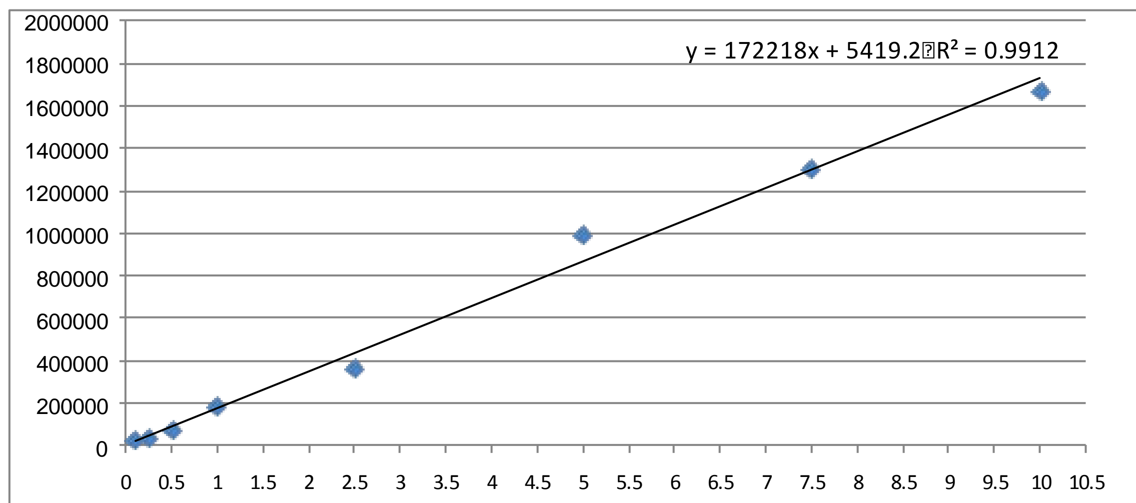

Figure A1.5 Glutamate (glutamate peak area) vs. Concentrations in µM

Conclusion: A correlation coefficient (r^2) of not less than 0.95 is recommended for good linearity (USP, 2008) and the correlation coefficient of 0.991 obtained in the current study was well above the recommended value in the range of standards (0.1 – 10 µM) used.

1.1.3 Repeatability/Precision

Precision is the closeness among different test results after repeated measurements (USP, 2008). The USP recommends a minimum of three concentrations with three replicates of each concentration for the assessment of precision or repeatability. The repeatability is subdivided into intra-assay repeatability and the intermediate repeatability.

Intra-assay repeatability: This is precision expressed under constant conditions over a short period of time.

Method: Precision is expressed as the percentage relative standard deviation (% RSD) of a series of measurements for the target substance.

$$\% \text{ RSD} = \frac{\text{SD of mean concentration}}{\text{Mean concentration}} \times 100 \qquad \textbf{(Equation 2)}$$

Results:

Table A1.1 Percentage standard deviation for concentrations 0.25, 0.5 and 1.0 µM.

Concentration (µM)	% STD
0.25	10.3 %
0.5	10.1 %
1.0	3.4 %

Conclusion: Lindholm (2004) describes a precision value with **% STD** lower than 15% to be good precision. The above table indicates a precision value of 10.3%, 10.1% and 3.4% for the respective concentration which can be accepted as good precision.

Intermediate repeatability: Intermediate precision is expressed as variation in different day's measurements.

Method: The method is the same as with intra-assay precision, the only difference is that three concentrations were analysed on three separate days.

Results: This test was not performed because each rat was measured against its own baseline value obtained from calculating the average reading of the first 3 samples taken, thus making the intermediate repeatability test unnecessary.

1.1.4 Lower limit of detection (LOD) and quantification (LOQ)

The lower limit of detection and quantification was found to be 0.1 µM

1.1.5 Recovery

The % recovery of the HPLC method for glutamate in the concentration range analysed varied between 70 – 96% which is acceptable for biological samples and was both consistent and reproducible.

Addendum 2

	A
aCSF	Artificial Cerebrospinal fluid
AIDS	Acquired immune deficiency syndrome
AMPA	Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ATP	Adenosine Triphosphate
	B
BBB	Blood-Brain barrier
BLA	Basolateral nucleus
	C
CCP	Cerebral Perfusion pressure
CeA	Central nucleus
$CMRO_2$	Cerebral Metabolic Rate (CMR) for oxygen
CNS	Central nervous system
	D
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders, 4th Edition
	E
ECF	Extracellular fluid
EAAT	Excitatory amino acid transporter
	G
GABA	Gamma-aminobutyric acid
	H
HIV	Human immunodeficiency virus
HPA-axis	Hypothalamic Pituitary Adrenocortical axis
HPLC	High performance liquid chromatography
	I
ICP	Intracranial pressure

IP	Intraperitoneal
IV	Intravenous
	K
KA	Kainic acid
KCl	Potassium chloride
	L
LC-MS-MS	Tandem liquid chromatography mass spectrometry
LOD	Limit of detection
LOQ	Limit of quantification
LTD	Long-term depression
LTP	Long-term potentiation
	M
MAP	Mean Arterial Pressure
MD	Microdialysis
MDD	Major Depressive disorder
mGluR	Metabotropic glutamate receptor
mTOR	Mammalian target of rapamycin
	N
NMDA	N-methyl-D-aspartate
NPY	Neuropeptide - Y
	R
r^2	Linearity correlation coefficient
RSD	Relative standard deviation
	S
SC	Subcutaneous
	T
TTX	Tetrodotoxin

References

AAN HET ROT, M., COLLINS, K.A., MURROUGH, J.W., PEREZ, A.M., REICH, D.L., CHHARNEY, D.S., & MATHEW, S.J. 2010. Safety and Efficacy of Repeated-Dose Intravenous Ketamine for Treatment-Resistant Depression. *Biological Psychiatry*, 67:139-145.

ADAMS, B.W. & MOGHADDAM, B. 2001. Effect of Clozapine, Haloperidol, or M100907 on Phencyclidine-Activated Glutamate Efflux in the Prefrontal Cortex. *Biological Psychiatry*, 50:750-757.

ALBIN, R.L., GREENAMYRE, J.T. 1992. Alternative excitotoxic hypotheses. *Neurology* 42:733–738.

ANNEKEN, J.H. & GUDELSKY, G.A. 2012. NMDA produces a delayed and sustained increase in the extracellular concentration of glutamate in the rat hippocampus. *Neuropharmacology*,(63):1022-1027.

BAKER, D.A., SHEN, H. & KALIVAS, P.W. 2002. Cystine/glutamate exchange serves as the source for extracellular glutamate: Modifications by repeated cocaine administration. *Amino Acids*, 23:161-162.

BALAZS, R., MACHIYAMA, Y. & PATEL, A.J. 1972. "Compartmentation and the metabolism of g-aminobutyrate.," in *Metabolic compartmentation in the brain.*, R. Balazs & J. E. Cremer, eds., John Wiley & Sons, New York, pp. 57-70.

BASILI, A., MASCHA, E., KALFAS, I. & SCHUBERT, A. 2000. Relation between perioperative hypertension and intracranial hemorrhage after craniotomy. *Anesthesiology*, 93:48–54.

BENVENISTE, H., DREJER, J., SCHOUSBOE, A. & DIEMER, N.H. 1984. Elevations of the extracellular concentrations of glutamate and aspartate in rat hippocampus during transient cerebral ischemia monitored by intracerebral microdialysis. *Journal of Neurochemistry*, 43:1369-1374.

BERMAN, R.M., CAPPIELLO, A., ANAND, A., OREN, D.A., HENINGER, G.R., CHARNEY, D.S. & KRYSTAL, J.H. 2000. Antidepressant Effects of Ketamine in Depressed Patients. *Biological Psychiatry,* 47:351-354.

BERT, L., FAVALE, D., JEGO, G., GREVE, P., GUILLOUX, J.P., GUIARD, B.P., GARDIER, A.M., SUAUD-CHAGNY, M.F. & LESTAGE, P. 2004. Rapid and precise method to locate microdialysis probe implantation in the rodent brain. *Journal of Neuroscience Methods*, 140:53-57.

BIANCHI, L., LA CORTE, L. & TIPTON, K.F. 1999. Simultaneous determination of basal and evoked output levels of aspartate, glutamate, taurine and 4-aminobutyric acid during microdialysis and from superfused brain slices. *Journal of Chromatography B: Biomedical Sciences and Applications*, 723:47-59.

BITO, L., DAVSON, H., LEVIN, E., MURRAY, M. & SNIDER, N. 1966. The concentrations of free amino acids and other electrolytes in cerebrospinal fluid, in vivo dialysate of brain, and blood plasma of the dog. *Journal of Neurochemistry*, 13:1057-1067.

BONANNO, G., GIAMBELLI, R., RAITERI, L., TIRABOSCHI, E., ZAPPETTINI, S., MUSAZZI, L., RAITERI, M., RACAGNI, G. & POPOLI, M. 2005. Chronic Antidepressants Reduce Depolarization-Evoked Glutamate Release and Protein Interactions Favoring Formation of SNARE Complex in Hippocampus. *The Journal of Neuroscience*, 25(13):3270-3279.

BRINK, C.B., HARVEY, B.H. & BRAND, L. 2005. Tianeptine: A novel atypical antidepressant that may provide new insights into the biomolecular basis of depression. *Recent Patents on CNS Drug Discovery*,(1):29-41.

BRUNNER, M. & DERENDORF, H. 2006. Clinical microdialysis: Current applications and potential use in drug development. *TrAC Trends in Analytical Chemistry*, 25:674-680.

CANADIAN COUNCIL ON ANIMAL CARE (CCAC). Standards for Experimental Animal Surgery. 1993.

CAZAKOFF, B.N. & HOWLAND, J.G. 2010. Acute stress disrupts paired pulse facilitation and long-term potentiation in rat dorsal hippocampus through activation of glucocorticoid receptors. *Hippocampus*, 20:1327-1331.

CHEFER, V.I., THOMPSON, A.C., ZAPATA, A. & SHIPPENBERG, T.S. 2009. Overview of Brain Microdialysis. *Current Protocol in Neuroscience*, 7:7.1.

COOPER, J., BLOOM, F. & ROTH, R. 2003. The Biochemical Basis of Neuropharmacology, Oxford University Press, New York.

COYLE, J.T. & PUTTFARCKEN, P. 1993. Oxidative stress, glutamate, and neurodegenerative disorders. *Science*, 262(5134):689-695.

DAWSON, L.A., STOW, J.M., DOURISH, C.T. & ROUTLEDGE, C. 1995. Analysis of glutamate in striatal microdialysates using capillary electrophoresis and laser-induced fluorescence detection. *Journal of Chromatography A*, 700:81-87

DE LANGE, E.C.M., DANHOF, M., DE BOER, A.G. & BREIMER, D.D. 1997. Methodological considerations of intracerebral microdialysis in pharmacokinetic studies on drug transport across the blood-brain barrier. *Brain Research Reviews*, 25:27-49.

DE LANGE, E.C.M., DANHOF, M., ZURCHER, C., DE BOER, A.G., & BREIMER, D.D. 1995. Repeated microdialysis perfusions: periprobe tissue reactions and BBB permeability. *Brain Research*, 702(1-2):261-265.

DE VASCONCELLOS-BITTENCOURT, A.P., VENDITE, D.A., NASSIF, M., CREMA, L.M., FROZZA, R., THOMAZI, A.P., NIETO, F.B., WOFCHUK, S., SALB EGO, C., DA ROCHA, E.R. & DALMAZ, C. 2011. Chronic stress and lithium treatments alter hippocampal glutamate uptake and release in the rat and potentiate necrotic cellular death after oxygen and glucose deprivation. *Neurochemical Research*, 36:793-800.

DE VRIES, E. & ALEXANDRE, P. 2005. The Blood-Brain Barrier and Its Microenvironment: Basic Physiology in Neurological Disease. New York; London: Taylor & Francis Group. 519p.

DEL ARCO, A., SEGOVIA, G., FUXE, K. & MORA, F. 2003. Changes in dialysate concentrations of glutamate and GABA in the brain: an index of volume transmission mediated actions? *Journal of Neurochemistry*, 85:23-33.

DELGADO, J.M., DEFEUDIS, F.V., ROTH, R.H., RYUGO, D.K. & MITRUKA, B.M. 1972. Dialytrode for long term intracerebral perfusion in awake monkeys. *Archive of International Pharmacodynamics*, 198:9-21.

DIAMOND, J.S. & JAHR, C.E. 2000. Synaptically Released Glutamate Does Not Overwhelm Transporters on Hippocampal Astrocytes During High-Frequency Stimulation. *Journal of Neurophysiology*, 83:2835-2843.

DONZANTI, B.A. & YAMAMOTO, B.K. 1988. An improved and rapid HPLC-EC method for isocratic separation of amino acid neurotransmitters from brain tissue and microdialysis perfusates. *Life Sciences*, 43:913-922.

DREVETS, W.C. 1998. Functional Neuroimaging Studies of Depression: The Anatomy of Melancholia. *Annual Medical Reviews*, 49:341-361.

FABLING, J.M., GAN, T.J., GUY, J., BOREL, C.O., EL-MOALEM, H.E. & WARNER, D.S. 1997. Postoperative nausea and vomiting. A retrospective analysis in patients undergoing elective craniotomy. *Journal of Neurosurgery and Anesthesiology*, 9(4):308-312.

FAVA, M. & KENDLER, K.S. 2000. Major Depressive Disorder. *Neuron*, 28(2):335-341

FAVA, M. & PAPAKOSTAS, G.I. 2008. Monoaminergic-based pharmacotherapy for depression. *Biology of Depression,* 187-209.

FLECKNELL, P. 1996. Laboratory Animal anaesthesia. 2nd ed. Academic Press Limited: London. 274 p.

FRODL, T., MEISENZAHL, E., ZETZSCHE, T., BOTTLENDER, R., BORN, C., GROLL, C., JÄGER, M., LEINSINGER, G., HAHN, K., MÖLLER, H.J., 2002. Enlargement of the amygdala in patients with a first episode of major depression*. Biological Psychiatry,* 51: 708–714.

FUKUSHIMA, T., NAKA-AKI, E., GUO, X., LI, F., VANKEIRSBILCK, T., BAEYENS, W.R.G., IMAI, K., & TOYO'OKA, T. 2004. Determination of fluoxetine and norfluoxetine in rat brain microdialysis samples following intraperitoneal fluoxetine administration. *Analytica Chimica Acta*, 522(1):99-104.

FURGESON, J. 2001. SSRI antidepressant medications adverse effects and tolerability. *The Primary Care Companion,* 3:22-27.

GALVAN, A., SMITH, Y., & WICHMANN, T. 2003. Continuous monitoring of intracerebral glutamate levels in awake monkeys using microdialysis and enzyme fluorometric detection. *Journal of Neuroscience Methods*, 126:175−185.

GARCIA, L.S.B., COMIM, C.M., VALVASSORI, S.S., REUS, G.Z., BARBOSA, L.M., ANDREAZZA, A.C., STERTZ, L., FRIES, G.R., GAVIOLI, E.C., KAPCZINSKI, F. & QUEVEDO, J. 2008. Acute administration of ketamine induces antidepressant-like effects in the forced swimming test and increases BDNF levels in the rat hippocampus. *Progress in Neuro-Psychopharmacology & Biological Psychiatry,* 32:140-144.

GARCIA, L.S.B., COMIM, C.M., VALVASSORI, S.S., REUS, G.Z., STERTZ, L., KAPCZINSKI, F., GAVIOLI, E.C. & QUEVEDO, J. 2009. Ketamine treatment reverses behavioral and physiological alterations induced by chronic mild stress in rats. *Neuro-Psychopharmacology & Biological Psychiatry*, 33:450-455.

GOODMAN & GILMAN 2011. GOODMAN & GILMAN'S THE PHARMACOLOGIC BASIS OF THERAPEUTICS. 12th ed. p.2084.

GROBIN, C.A. & DEUTCH, A.Y. 1998. Dopaminergic Regulation of Extracellular y-Aminobutyric Acid Levels in the Prefrontal Cortex of the Rat. *The Journal of Pharmacology and Experimental Therapeutics*, 285:350-357.

GUEDEL, A.E. 1936. Anesthesia: A teaching outline. Stage of Anesthesia. *Anesthesia & Analgesia*. 15:1-4.

HALL, L. W., CLARKE, K. W., & TRIM, M. 2001. Veterinary Anaesthesia. 10th ed. W.B. Saunders: England. 561 p.

HAMBERGER, A., JACOBSON, I., MOLIN, S.O., NYSTRÖM, B., SANDBERG, M. & UNGERSTEDT, U. 1982. Metabolic and transmitter compartments for glutamate. In: (Ed H Bradford) Neurotransmitter interaction and compartmentation. Plenum, New York, pp 359-378.

HASCUP, K.N., HASCUP, E.R., STEPHENS, M.L., GLASER, P.E.A., YOSHITAKE, T., MATHE, A.M., GERHARDT, G.A. & KEHR, J. 2011. Resting Glutamate Levels and

Rapid Glutamate Transients in the Prefrontal Cortex of the Flinders Sensitive Line Rat: A Genetic Rodent Model of Depression. *Neuropsychopharmacology*, 36:1769-1777.

HASEGAWA, S., NISHI, K., WATANABE, A., OVERSTREET, D.H. & DIKSIC, M. 2006. Brain 5-HT synthesis in Flinders Sensitive Line rat model of depression: and autoradiographic study. *Neurochemistry International* , 48:358-366.

HASHIMOTO, K. 2009. Emerging role of glutamate in the pathophysiology of major depressive disorder. *Brain Research Reviews*, 61(2):105-123.

HASHIMOTO, K., SAWA, A., & LYO, M. 2007. Increased Levels of Glutamate in Brains from Patients with Mood Disorders. *Biological Psychiatry*, 62:1310-1316.

HAWKINS, R.A. 2009. The blood-brain barrier and glutamate. *The American Journal of Clinical Nutrition*, 90:867S-874S.

HEALY, D., 1999. The three faces of the antidepressants: a critical commentary on the clinical-economic context of diagnosis. *The Journal of Nervous and Mental Disease*, 187(3):174-80.

HERRERA-MARSCHITZ, M., GOINY, Z., YOU, B., MEANA, J.J., PETTERSSON, E., RODRIQUEZ-PUERTAS, R., XU, Z.Q., TERENIUS, L., HOKFELT, T. & UNGERSTEDT, U. 1997. On the release of glutamate and aspartate in the basal ganglia of the rat: Interactions with monoamines and neuropeptides. *Neuroscience & Biobehavioral Reviews*, 21(4):489-495.

HERRLING, P. 1997. "Chlinical Results with Antagonists.," in *Excitatory amino acids.*, Academic Press, San Diego.

HERVAS, I. & ARTIGAS, F. 1998. Effect of fluoxetine on extracellular 5-hydroxytryptamine in rat brain. Role of 5-HT autoreceptors. *European Journal of Pharmacology*, 358:9-18.

HILLERED, L., PERSSON, L., PONTEN, U. & UNGERSTEDT, U. 1990. Neurometabolic monitoring of the ischemic human brain using microdialysis. *Acta Neurochir.* 102: 91-97.

HOLGER, F. & VERENA, J. 2012. Fluorescence Method Development Handbook. 16p.

JABAUDON, D., SHIMAMOTO, K., YASUDA-KAMATANI, Y., SCANZIANI, M., GAHWILER, B.H. & GERBER, U. 1999. Inhibition of uptake unmasks rapid extracellular turnover of glutamate of nonvesicular origin. *Neurobiology*, 96:8733-8738.

JOHNSON, R.D. & JUSTICE, J.B. 1983. Model studies for brain dialysis. *Brain Research Bulletin*, 10:567.

KANAI, Y. & HEDIGER, M.A. 2004. The glutamate/neutral amino acid transporter family SLC1: molecular, physiological and pharmacological aspects. *Pflugers Archive European Journal of Physiology*, 447:469-479.

KAROLEWICZ, B., CETIN, M. & ARICIOGLU, F. 2011. Beyond The Glutamate N-methyl D-aspartate Receptor in Major Depressive Disorder: The mTOR Signaling Pathway. *Bulletin of Clinical Psychopharmacology*, 21:1-6.

KEHR, J. 1998. Determination of glutamate and aspartate in microdialysis samples by reversed-phase column liquid chromatography with fluorescence and electrochemical detection. *Journal of Chromatography B: Biomedical Sciences and Applications*, 708:27-38-

KEHR, J. 1993, *Fluorecence detection of Amino Acids derivatized with o-phthaladehyde (OPA) based reagents.*

KEHR, J., YOSHITAKE, T. 2006. Monitoring brain chemical signals by microdialysis. In: Encyclopedia of Sensors (Eds CA Grimes, EC Dickey and MV Pishko) American Scientific Publishers, USA, pp 287-312.

KESSLER, R.C.,WANG, P.S., 2008. The descriptive epidemiology of commonly occurring mental disorders in the United States. *Annual Review of Public Health* 29, 115-129.

KIM, J.S., SCHMID-BURGK, W., CLAUS, D., & KORNHUBER, H.H. 1982. Increased serum glutamate in depressed patients. *European Archives of Psychiatry and Clinical Neuroscience*, 232:299-304.

KOKRAS, N., ANTONIOU, K., DALLA, C., BEKRIS, S., XAGORARIS, M., OVERSTREET, D.H. & PAPADOPOULOU-DIFOTI, Z. 2008. Sex-related differential response to clomipramine treatment in a rat model of depression. *Journal of Psychopharmacology*, 101-177

KOLENDA, H., GREMMELT, A. & RADING, S. 1996 Ketamine for analgosedative therapy in intensive care treatment of head injured patients. *Acta Neurochir*, 138:1193-1199.

KRYSTAL, J.H. 2007. Neuroplasticity as a target for the pharmacotherapy of psychiatric disorders: new opportunities for synergy with psychotherapy. *Biological Psychiatry*, 62(8):833-834.

KUCUKIBRAHIMOGLU, E., SAYGIN, M.Z., CALISKAN, M., KAPLAN, O.K., UNSAL, C. & GOREN, M.Z. 2009. The change in plasma GABA, glutamine and glutamate levels in fluoxetine or S-citalopram-treated female patients with major depression. *European Journal of Clinical Pharmacology*,(65):571-577.

LAI, T.J., PAYNE, M.E., BYRUM, C.E., STEFFENS, D.C., & KRISHNAN, K.R. 2000. Reduction of Orbital Frontal Cortex Volume in Geriatric Depression. *Biological Psychiatry*, 48:971-975.

LAN, M.J., MCLOUGHLIN, G.A., GRIFFIN, J.L., TSANG, T.M., HUANG, J.T., YUAN, P., MANJI, H., HOLMES, E. & BAHN, S. 2009. Metabonomic analysis identifies molecular

changes associated with the pathophysiology and drug treatment of bipolar disorder. *Mol Psychiatry*, 14:269-279.

LI, N., LEE, B., LIU, R.J., BANASR, M., DWYER, J.M., IWATA, M. & LI, X.Y. 2010. mTOR-dependent synapse formation underlies the rapid antidepressant effects of NMDA antagonists. *Science*, 329:959-964.

LILES, J.H. & FLECKNELL, P.A. 1992. The use of non-steroidal anti-inflammatory drugs for the relief of pain in laboratory rodents and rabbits. *Laboratory Animals*, 26(4):241-255.

LINDHOLM, J. 2004. Development and validation of HPLC Methods for Analytical and Preparative Purposes. Uppsala University.

LORRAIN, D.S., BACCEI, C.S., BRISTOW, L.J., ANDERSON, J.J. & VARNEY, M.A. 2003. Effects of ketamine and N-methyl-D-aspartate on glutamate and dopamine release in the rat prefrontal cortex: Modulation by a group II selective metabotropic glutamate receptor agonist LY379268. *Neuroscience*, 117:697-706.

MACHADO-VIEIRA, R., SALVADORE, G., DIAZGRANADOS, N., & ZARATE, C.A. 2009. Ketamine and the next generation of antidepressants with rapid onset of action. *Pharmacology & Therapeutics*, 123(2):143-150.

MACHADO-VIEIRA, R., YUAN, P., BRUTSCHE, N., DIAZGRANADOS, N., LUCKENBAUGH, D., MANJI, H.K. & ZARATE, C.A. 2009. Brain-Derived Neurotrophic Factor and Initial Antidepressant Response to an N-Methyl-D-Aspartate Antagonist. *Journal of Clinical Psychiatry*, 70:1662.

MACHADO-VIEIRA, R., ZARATE, C.A., SALVADORE, G., IBRAHIM, L.A. & DIAZGRANADOS, N. 2009. Targeting glutamatergic signaling for the development of novel therapeutics for mood disorders. *Current Psychiatry Reports*,(15):1595-1611.

MAENG, S. & ZARATE, C.A. 2007. The Role of Glutamate in Mood Disorders: Results from the Ketamine in Major Depression Study and the Presumed Cellular Mechanism Underlying its Antidepressant Effects. *Current Psychiatry Reports*, 94:467-474.

MAENG, S., ZARATE JR., C.A., DU, J., SCHLOESSER, R.J., MCCAMMON, J., CHEN, G., MANJI, H.K., 2008. Cellular mechanisms underlying the antidepressant effects of ketamine: role of alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptors. *Biological Psychiatry* 63, 349–352.

MAES, M., VERKERK, R., VANDOOLAEGHE, E., LIN, A., & SCHARPE, S. 1998. Serum levels of excitatory amino acids, serine, glycine, histidine, threonine, taurine, alanine and arginine in treatment-resistant depression: modulation by treatment with antidepressants and prediction of clinical responsivity. *Acta Psychiatrica Scandinavica*, 97:302-308.

MANJI H.K & LENOX R.H. 2000. Signaling: cellular insights into the pathophysiology of bipolar disorder**.** *Biol. Psychiatry,* 48:518-530.

MANJI, H.K., QUIROZ, J.A., SPORN, J., PAYNE, J.L., DENICOFF, K., GRAY, N.A., ZARATE C.A. & CHARNEY, D.S. 2003. Enhancing neuronal plasticity and cellular resilience to develop novel, improved therapeutics for difficult-to-treat depression, *Biol. Psychiatry,* 53:707– 42.

MAURI, M.C., FERRARA, A., BOSCATI, L., BRACIN, S., ZAMBERLAN, F., ALECCI, M., & INVERNIZZI, G. 1998. Plasma and Platelet Amino Acid Concentrations in Patients Affected by Major Depression and under Fluvoxamine Treatment. *Neuropsychobiology*, 37:124-129.

MESSIER, C., EMOND, S. & ETHIER, K. 1999. New Techniques in Stereotaxic Surgery and Anesthesia in the Mouse. *Pharmacology Biochemistry and Behavior*, 63(2):313-318.

MEYERSON, B.A., LINDEROTH, B., KARLSSON, H. & UNGERSTEDT, U. 1990. Microdialysis in the human brain: extracellular measurements in the thalamus of parkinsonian patients. *Life Sciences*. 46: 301-308.

MILLAN, M.J. 2006. Multi-target strategies for the improved treatment of depressive states: Conceptual foundations and neuronal substrates, drug discovery and therapeutic application. *Pharmacology & Therapeutics,* 110:135 – 370.

MILLER, A. L., HAWKINS, R. A. & VEECH, R. L. 1975. Decreased rate of glucose utilization by rat brain in vivo after exposure to atmospheres containing high concentrations of CO2. *Journal of Neurochemistry* 25:553-558.

MITANI, H., SHIRAYAMA, Y., YAMADA, T., MAEDA, K., ASHBY, R.C. & KAWAHARA, R. 2006. Correlation between plasma levels of glutamate, alanine and serine with severity of depression. *Progress in Neuro-Psychopharmacology & Biological Psychiatry*, 30:1155-1158.

MOGHADDAM, B. 1993. Stress Preferentially Increases Extraneuronal Levels of Exitatory Amino Acids in the Prefrontal Cortex: Comparison to Hippocampus and Basal Ganglia. *Journal of Neurochemistry,* 60:1650-1657.

MOGHADDAM, B., ADAMS, B., VERMA, A. & DALY, D. 1997. Activation of Glutamatergic Neurotransmission by Ketamine: A Novel Step in the Pathway from NMDA Receptor Blockade to Dopaminergic and Cognitive Disruptions Associated with the Prefrontal Cortex. *The Journal of Neuroscience*, 17(8):2921-2927.

MURROUGH, J.W., PEREZ, A.M., PILLEMER, S., STERN, J., PARIDES, M.K., AAN HET ROT, M., COLLINS, K.A., MATHEW, S.J., CHARNEY, D.S. & IOSIFESCU, D.V.

2012. Rapid and Longer-Term Antidepressant Effects of Repeated Ketamine Infusions in Treatment-Resistant Major Depression. *Biological Psychiatry*, *Available online 27 July 2012.*

MUSAZZI, L., RACAGNI, G. & POPOLI, M. 2011. Stress, glucocorticoids and glutamate release: Effects of antidepressant drugs. *Neurochemistry International*, 59(2):138-149.

NEUMANN, I.D., WEGENER, G., HOMBERG, J.R., COHEN, H., SLATTERY, D.A. & ZOHAR, J. 2010. Animal models of depression and anxiety: what do they tell us about human condition? *Progress in Neuro-Psychopharmacology & Biological Psychiatry*,

NOAH COMPENDIUM OF DATA SHEETS FOR ANIMAL MEDICINES. 2012. Hypnorm. http://www.noahcompendium.co.uk/VetaPharma_Ltd/Hypnorm/-55964.html.

OBRENOVITCH, T.P., URENJAK, J., ZILKHA, E. & JAY, T.M. 2000. Excitotoxicity in neurological disorders - the glutamate paradox. *International Journal of Developmental Neuroscience,* 18:281-287.

ONGUR, D., JENSEN, J.E., PRESCOT, A.P., STORK, C., LUNDY, M., COHEN, B.M. & RENSHAW, P.F. 2008. Abnormal glutamatergic neurotransmission and neuronal-glial interactions in acute mania. *Biological Psychiatry*, 64:718-726.

OVERSTREET, D.H. 1993. The Flinders Sensitive Line rats: A genetic animal model of depression. *Neuroscience and Biobehavioral Reviews,* 17(1):51-68.

OVERSTREET, D.H., COMMISSARIS, R.C., DE LA GARZA, I.R., FILE, S.E., KNAPP, D.J. & SEIDEN, L.S. 2003. Involvement of 5-HT1A receptors in animal tests of anxiety and depression: Evidence from genetic models. *Stress*, 6(2):101-110.

OVERSTREET, D.H., FRIEDMAN, E., MATHE, A.A. & YADID, G. 2005. The Flinders Sensitive Line rat: a selectively bred putative animal model of depression. *Neuroscience & Biobehavioral Reviews*, 29:739-759.

OVERSTREET, D.H., FRIEDMAN, E., MATHE, A.A., & YADID, G. 2005. The Flinders Sensitive Line rat: A selectively bred putative animal model of depression. *Neuroscience & Biobehavioral Reviews*, 29(4-5):739-759.

PAXINOS, G. & WATSON, C. 2005. The Rat Brain: In Stereotaxic Coordinates. 5th ed. p.367

PEPE, S., OVERSTREET, D.H. & CROCKER, A.D. 1988. Enhanced benzodiazepine responsiveness in rats with increased cholinergic function. *Pharmacology Biochemistry and Behavior*, 31:15-19.

PFRIEGER, F.W. & BARRES, B.A. 1996. New views on synapse-glia interactions. *Current Opinion in Neurobiology,* 6:615-621.

PIEPPONEN, T.P. & SKUJINS, A. 2001. Rapid and sensitive step gradient assays of glutamate, glycine, taurine and [gamma]-aminobutyric acid by high-performance liquid chromatography-fluorescence detection with o-phthalaldehyde-mercaptoethanol derivatization with an emphasis on microdialysis samples. *Journal of Chromatography B: Biomedical Sciences and Applications*, 757(2):277-283.

PINEYRO G. & BLIER, P. 1996. Regulation of 5-hydroxytryptamine release from rat midbrain raphe nuclei by 5-hydroxytryptamine1D receptors: effect of tetrodotoxin, G protein inactivation and long-term antidepressant administration. *J. Pharmacol. Exp. Ther.* 276(2):697–707.

PITTENGER, C., SANACORA, G. & KRYSTAL, J.M. 2007. The NMDA receptor as a therapeutic target in major depressive disorder. *CNS Neuroloical Disorder Drug Targets*, 6:101-115.

PITTENGER, G. & DUMAN, R.S. 2008. Stress, depression and neuroplasticity: a convergence of mechanisms. *Neuropsychopharmacology*,(33):88-109.

PLOCK, N. & KLOFT, C. 2005. Microdialysis - theoretical background and recent implementation in applied life-sciences. *European Journal of Pharmaceutical Sciences*, 25(1):1-24.

POSTERNAK, M.A. & ZIMMERMAN, M. 2005. Is there a delay in the antidepressant effect? A meta-analysis. *J Clin Psychiatry* 66:148– 158.

PRICE, M.T., OLNEY, J.W., LOWRY, O.H., & BUCHSBAUM, S. 1981. Uptake of exogenous glutamate and aspartate by circumventricular organs but not other regions of brain. *Journal of Neurochemistry*, 36:1774–1780.

PUCILOWSKI, O., OVERSTREET, D.H., REZVANI, A.H. & JANOWSKY, D.S. 1993. Chronic mild stress-induced anhedonia: greater effect in a genetic rat model of depression. *Physiology & Behavior*, 54:1215-1220.

RAPOPORT, S.I. 1976. Blood-brain Barrier in Physiology and Medicine, Raven Press, New York.

RAVUSSIN, P. & WILDER-SMITH, O.H.G. 1996. Intravenous anaesthetics in neurosurgery. *Current Opinion in Anesthesiology*, 9:381-384.

REID, K.H., PASKITTI, M., GUO, S.Z., SCHMELZER, T., & IYER, V. 2003. Experience with ketamine and sodium pentobarbital as anesthetics in a rat model of cardiac arrest and resuscitation. *Resuscitation*, 57:201-210.

REZNIKOV, L.R., GRILLO, C.A., PIROLI, G.G., PASUMARTHI, R.K., REAGAN, L.P. & FADEL, J. 2007. Acute stress-mediated increases in extracellular glutamate levels in the rat amygdala: differential effects of antidepressant treatment. *European Journal of Neuroscience*, 25:3109-3114.

RICHELL, R.A., DEAKIN, J.F. W. & ANDERSON, I.M. 2005. Effect of acute tryptophan depletion on the response to controllable noise stress. *Biol Psychiatry,* 57:295– 300.

SANACORA, G., TRECCANI, G. & POPOLI, M. 2012. Towards a glutamate hypothesis of depression an emerging frontier of neuropsychopharmacology for mood disorders. *Neuropharmacology*, 62:63-77.

SANACORA, G., ZARATE, C.A., KRYSTAL, J.H. & MANJI, H.K. 2008. Targeting the glutamatergic system to develop novel, improved therapeutics for mood disorders. *Nature Reviews Drug Discovery*, 7(5):426-437.

SCHILDKRAUT, J.J. 1965. The catecholamine hypothesis of affective disorders: a review of supporting evidence. *Am. J. Psychiatry* 122: 509–522.

SEGOVIA, G., PORRAS, A. & MORA, F. 1997. Effects of 4-Aminopyridine on Extracellular Concentrations of Glutamate in Striatum of the Freely Moving Rat. *Neurochemical Research*, 22:1491-1497.

SHAH, A.J., DE BIASI, V., TAYLOR, S.G., ROBERTS, C., HEMMATI, P. & MUNTON, R. 1999. Development of a protocol for the automated analysis of amino acids in brain tissue samples and microdialysates. *Journal of Chromatography B: Biomedical Sciences and Applications*, 735(2):133-140.

STEGINK, D.L., FILER, L.J., & BAKER, G.L. 1985. Plasma glutamate concentrations in adult subjects ingesting monosodium L-glutamate in consomme. *The American Journal of Clinical Nutrition*, 42:220-225.

STEGINK, L.D., FILER, L.J., & BAKER, G.L. 1982. Plasma and Erythrocyte Amino Acid Levels in Normal Adult Subjects Fed a High Protein Meal with and without Added Monosodium Glutamate. *The Journal of Nutrition*, 112:1953-1960.

STEGINK, L.D., FILER, L.J., & BAKER, G.L. 1983. Plasma Amino Acid Concentrations in Normal Adults Fed Meals with Added Monosodium L-Glutamate and Aspartame. *The Journal of Nutrition*, 113:1851-1860.

THE BLOOD-BRAIN BARRIER AND ITS MICROENVIRONMENT; BASIC PHYSIOLOGY IN NEUROLOGICAL DISEASE, 2005. Taylor & Francis Group: New York; London p.519.

THOMSON, A.M., WEST, D.C., LODGE, D. 1985. An *N*-methyl-D-asparte receptor-mediated synapse in rat cerebral cortex: a site of action of ketamine? *Nature* 313:479–481.

TOKITA K, YAMAJI T, HASHIMOTO K., 2012. Roles of glutamate signaling in preclinical and/or mechanistic models of depression. *Pharmacology Biochemistry & Behavior,* 100(4):688-704.

TRULLAS, R. & SKOLNICK, P. 1990. Functional antagonists at the NMDA receptor complex exhibit antidepressant actions. *European Journal of Pharmacology*, 185(1):1-10.

TSAI, P. & HUANG, P. 2000. Circadian Variations in Plasma and Erythrocyte Glutamate Concentrations in Adult Men Consuming a Diet with and without Added Monosodium Glutamate. *The Journal of Nutrition*, 130:1002S-1004S.

UNGERSTEDT, U. 1984. Measurement of neurotransmitter release by intracranial dialysis. In: (Ed CA Marsden) Measurement of neurotransmitter release in vivo. *Methods in neurosciences*, 6: 81-105.

UNGERSTEDT, U. 1991. Microdialysis-principles and applications for studies in animals and man. *Journal of International Medicine*, 230:365-373.

UNGERSTEDT, U., HERRERA-MARSCHITZ, M., JUNGNELIUS, U., STÅHLE, L., TOSSMAN, U., ZETTERSTRÖM, T. 1982. Dopamine synaptic mechanisms reflected in studies combining behavioural recordings and brain dialysis. In: (Eds KOTISAKA, M., SHOMORI, T., TSUKADA, T., WOODRUFF, G.M. *Advances in dopamine research*. Pergamon Press, New York, pp 219-231.

UNITED STATES DEPARTMENT OF HEALTH AND HUMAN SERVICES, 2001. Guidance for Industry: Bioanalytical Method Validation. http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM070107.pdf.

USTUN, T.B., AYUSO-MATEOS, J.L., CHATTERJI, S., MATHERS, C., & MURRAY, C.J.L. 2004. Global burden of depressive disorder in the year 2000. *British Journal of Psychiatry*, 184:386-392.

VAN DER ZEYDEN, M., OLDENZIEL, W.H., REA, K., CREMERS, T.I. & WESTERINK, B.H. 2007. Microdialysis of GABA and glutamate: Analysis, interpretation and comparison with microsensors. *Pharmacology Biochemistry and Behavior*, 90(2):135-147.

VOLKE, V., WEGENER, G., VASAR, E., & ROSENBERG, R. 1999. Methylene blue inhibits hippocampal nitric oxide synthase activity in vivo. *Brain Research*, 826(2):303-305.

WANG, Y., WONG, S.L. & SAWCHUK, R.J. 1993. Microdialysis calibration using retrodialysis and zero-net flux: application to a study of the distribution of zidovudine to rabbit cerebrospinal fluid and thalamus. *Pharm. Res*. 10: 1411–1419.

WANG, S.J., SU, C.F., KUO, Y.H. 2003. Fluoxetine depressed glutamate exocytosis in the rat cerebrocortical nerve terminals (synaptosomes) via inhibition of P/Q-type Ca^{2}-channels. *Synapse,* 48:170-177.

WEGENER, G. & MATHE, A.A. 2011. Selectively bred rodents as models of depression and anxiety. *Current topics in behavioral neurosciences*,1866-3370.

WEGENER, G., LINNET, K., ROSENBERG, R., & MORK, A. 2000. The effect of acute citalopram on extracellular 5-HT levels is not augmented by lithium: an in vivo microdialysis study. *Brain Research*, 871(2):338-342.

WEGENER, G., VOLKE, V. & ROSENBERG, R. 2000. Endogenous nitric oxide decreases hippocampal levels of serotonin and dopamine in vivo. *British Journal of Pharmacology*, 130:575-580.

WELLS, B.G., DIPIRO, J.T., SCHWINGHAMMER, T.L. & HAMILTON, C.W. 2003. Pharmacotherapy Handbook. 5 ed. 958p.

WESTERGREN, I., NYSTROM, B., HAMBERGER, A. & JOHANSSON B.B. 1995. Intracerebral Dialysis and the Blood-Brain Barrier. *Journal of Neurochemistry,* 64:229-234.

WESTERINK, B.H.C. 1995. Brain microdialysis and its application for the study of animal behaviour. *Behavioural Brain Research*, 70(2):103-124.

WILLNER, P. & MITCHELL, P.J. 2002. The validity of animal models of predisposition to depression. *Behavioural Pharmacology,* 13(3):169-188.

YOUNG, W.L. 1998. Inhalational-vs-intravenous neuroanaesthesia. *European Journal of Anesthesiology,* 15 (supplement 17):18-34.

YUKSEL, C. & ONGUR, D. 2010. Magnetic resonance spectroscopy studies of glutamate-related abnormalities in mood disorders. *Biological Psychiatry*, 68(9):785-794.

ZANGEN, A., OVERSTREET, D.H. & YADID, G. 1997. High serotonin and 5-hydroxyindoleacetic acid levels in limbic brain regions in a rat model of depression: Normalization by chronic antidepressant treatment. *Journal of Neurochemistry*, 69(6):2477-2483.

ZARATE, C.A., QUIROZ, J., PAYNE, J. & MANJI, H.K. 2002. Modulators of the glutamatergic system: implications for the development of improved therapeutics in mood disorders. *Psychopharmacology Bulletin*, 36(4):35-83.

ZARATE, C.A., SINGH, J.B., CARLSON, P.J., BRUTSCHE, N., AMELI, R., LUCKENBAUGH, D., CHARNEY, D.S. & MANJI, H.K. 2006. A Randomized Trial of an N-methyl-D-aspartate Antagonist in Treatment-Resistant Major Depression. *Archive of General Psychiatry*, 63:856-864.

ZHANG, M.Y. & BEYER, C.E. 2006. Measurement of neurotransmitters from extracellular fluid in brain by in vivo microdialysis and chromatography-mass spectrometry. *Journal of Pharmaceutical and Biomedical Analysis*, 40(3):492-499.