Resting Glutamate Levels and Rapid Bursts of Glutamate Release in the Prefrontal Cortex of the Flinders Sensitive Line Rat-A Genetic Rodent Model of Depression

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Running Title: **Glutamate Dynamics in Flinders Sensitive Line Rat**

Abstract

Despite the numerous drugs targeting biogenic amines for major depressive disorder (depression) the search for novel therapeutics continues due to their poor response rates (~30%) and slow onset of action (2-4 weeks). To better understand glutamate's role in depression, we utilized an enzyme-based microelectrode array (MEA) that was selective for glutamate measures with fast temporal (2 Hz) and high spatial (15 x 333 μm) resolution. These MEAs were chronically implanted into the prefrontal cortex of 3-6 month and 12-15 month Flinders Sensitive Line (FSL) and control Flinders Resistant Line (FRL) rats, a validated genetic rodent model of depression. While no changes in glutamate dynamics were observed between 3-6 month FRL and FSL rats, a significant increase in resting glutamate levels was observed in the 12-15 month FSL rats compared to the 3-6 month FSL and age-matched FRL rats on days 3-5 post-implantation. Our MEA also recorded, for the first time, a unique phenomenon in all four rat groups of fluctuations in resting glutamate that we have termed glutamate transients. While these events lasted only seconds, they did occur throughout the testing paradigm. The average concentration of these glutamate burst events was significantly increased in the 12-15 month FSL rats compared to 3-6 month FSL and age-matched FRL rats. These studies lay the foundation for future studies of both tonic and phasic glutamate signaling in rat models of depression to better understand the potential role of glutamate signaling in depression.

Keywords: electrode, biosensor, freely moving, antidepressant, basal glutamate, major depressive disorder

Introduction

Major depressive disorder (depression) is a chronic, recurring mental illness affecting ~340 million people worldwide and is considered to be an underlying cause in 35-40% of suicides (Kulkarni and Dhir, 2009). Depression is characterized by lowered mood and loss of interest in daily activities leading to social, occupational, and educational impairments. These impairments cause decreased quality of life, decreased productivity and increased health care costs (Skolnick *et al*., 2009). Children, adolescents and young adults, who have significant risk of recurrence, are increasingly diagnosed with the disorder (McCauley *et al.*, 1993).

Depression treatments include pharmacotherapy, psychotherapy, magnetic therapy and electroconvulsive methods. Pharmacological treatments often target biogenic amines, but despite the plethora of commercially available antidepressants, the search for novel therapeutic targets and specific drugs is an ongoing endeavor for several reasons: 1) Most depressed patients are not adequately treated with monotherapy. Placebo controlled trials show efficacy rates of approximately 30% and few people achieve total remission of depressive symptoms despite using multiple therapies (Rakofsky *et al*., 2009). Recent data from the STAR*D trial, revealed remission rates of ~30% for citalopram monotherapy, and this only increased to ~50-70% with the most intensive combination of therapies (Insel and Wang, 2009). 2) Current antidepressants, even when effective, often are discontinued due to side-effects, including sexual dysfunction, nausea, weight-gain, and precipitation of a manic phase. 3) Current antidepressants have a slow onset of clinical response (2-4 weeks) that prolongs the suffering and can result in dire consequences for patients with suicidal ideation (Skolnick *et al*., 2009; Nierenberg, 2001). 4) All antidepressants currently carry a warning for their ability to increase suicidal thoughts in a small percentage of adolescents and young adults. Although this effect appears to lessen after age 25, it discourages some physicians from using antidepressants in adolescents (Stone *et al*., 2009).

Glutamate, the major excitatory neurotransmitter in the mammalian central nervous system, is implicated in several neurodegenerative and neuropsychiatric disorders (Danbolt, 2001). Recent clinical and post-mortem studies of depressed patients have found pathophysiological evidence of altered glutamatergic neurotransmission (Witkin *et al*., 2007). Depressed patients have increased serum and plasma levels of glutamate compared to controls (Mauri *et al*., 1998; Altamura *et al*., 1993; Kim *et al*., 1982), and the severity of the depression is correlated with increases in plasma glutamate (Mitani *et al*., 2006). Post-mortem brain tissue shows increased glutamate levels in depressed patients compared to controls (Hashimoto *et al*., 2007). This clinical data has lead to the development of several therapeutic strategies targeting glutamatergic receptors for the treatment of depression (Kugaya and Sanacora, 2005; Paul and Skolnick, 2003). Sub-anesthetic doses of ketamine, an N-methyl-D-aspartate receptor antagonist, have anti-depressant effects with immediate onset of action (Maeng and Zarate, 2007; Berman *et al*., 2000) probably due to its ability to increase synaptogenesis (Li *et al*., 2010). Riluzole inhibits presynaptic glutamate release and open label trials have shown clinical efficacy in the treatment of depression (Brennan *et al*., 2010). Based on this clinical evidence, a current hypothesis proposes that increased extracellular glutamate causes excessive glutamatergic neurotransmission that is deleterious to neuronal function and contributes to depression (McNally *et al*., 2008; Pittenger *et al*., 2007).

In preclinical settings, several animal models for depression have been developed (Pollak *et al*., 2010). Of those, the Flinders Sensitive Line (FSL) Rat is one of several extensively validated genetic rodent models of depression with many traits similar to those observed in depressed individuals. Studies of mature (3 month) FSL rats support decreased appetite and weight when compared with control, Flinders Resistant Line (FRL) rats (Overstreet *et al*., 1993). FSL rats show high degrees of psychomotor retardation compared to FRL control littermates. They are less active in novel, open field environments and bar-presses at a lower

rate for water or food reward (Overstreet and Russell, 1982). However, there was no evidence for anhedonia or cognitive disturbances (Overstreet *et al*., 2005) in FSL rats. Furthermore, FSL rats display severe immobility on the forced swim task (Kulkarni and Dhir, 2009; Overstreet *et al*., 1994) that is reversed with chronic treatment of antidepressants (Yadid *et al*., 2000). For a complete review of the studies conducted in this rat model, the reader is referred to Overstreet *et al*., 2005.

Since alterations in glutamatergic neurotransmission may be an underlying cause in the pathology of depression, we decided to study resting glutamate levels in the FSL rats compared to the control, Flinders Resistant Line (FRL), rats. We utilized an enzyme-based microelectrode array (MEA) selective for measuring glutamate with low limits of detection (<0.2 μM), fast temporal resolution (500 milliseconds) (Burmeister *et al*., 2004, 2002, 2000; Burmeister and Gerhardt, 2001) and minimal damage to surrounding brain tissue (50-100 μm) (Hascup *et al*., 2009). Using these MEAs in combination with pharmacological treatments, we have shown that resting glutamate levels in the medial prefrontal cortex (PFC) are at least 50% neuronally derived. Furthermore, pre-application of tetrodotoxin (TTX) completely abolishes the physiological glutamate response to a tail-pinch stressor (Hascup *et al*., 2010). These MEAs were chronically implanted into the PFC of young (3-6 month) and old (12-15 month) FSL and FRL rats allowing us to measure extracellular levels of resting glutamate over several days (Hascup *et al*., 2008; Rutherford *et al*., 2007) in a clinically relevant brain structure implicated in depression (Canbeyli, 2010).

Materials and Methods

Animals

A colony of FSL and FRL rats were maintained at the Department of Physiology and Pharmacology, Karolinska Institutet, Stockholm, Sweden. Male rats were separated into 3-6 month (young, FRL n=7; FSL n=6) and 12-15 month (old, FRL n=4; FSL n=4) age groups for all experiments. Male Fischer 344 rats were separated into 3-6 month (n=7) and 18 month (n=6) age groups. All animals were group housed in a 12 hour light/dark cycle at a constant room temperature of 23 ºC with *ad libitum* access to food and water. Animals were treated in accordance with protocols approved by the animal Ethical Committee of Stockholm, the Federation of European Laboratory Animal Science Associations and all procedures were conducted in conformity with the Karolinska Institutet's Guidelines for animal welfare. Efforts were made in order to minimize the number of animals used and reduce their sufferings. Animals were allowed at least one week to acclimate to the testing environment and researchers prior to any experiments. All appropriate animal care (food, water, bedding, cage cleaning, etc.) was performed by the animal resource center staff. Following surgery, rats were individually housed under the same conditions. Upon completion of the studies, rats were euthanized with an overdose of anesthetic. The brains were removed and sliced for visual inspection of MEA placement.

Microelectrode array design

MEAs were assembled and selected for *in vivo* recordings as previously described (Burmeister *et al*., 2002, 2000.). Preparation of the MEA for recording has been extensively described in Hascup *et al*., 2006 and Burmeister *et al*., 2002. Briefly, the platinum recording sites were dip coated with Nafion® (Sigma-Aldrich Corp., St. Louis, MO) to repel anions (Burmeister and Gerhardt, 2001). Two of the MEA recording sites were coated with an L-

glutamate oxidase (EC 1.4.3.11) (Seikagaku America, Inc., East Falmouth, MA) coating solution (Nickell *et al*., 2005). In order to induce cross-linking of L-glutamate oxidase and to increase the adhesion to the microelectrode, glutaraldehyde and BSA (Sigma-Aldrich Corp., St. Louis, MO) were added to the L-glutamate oxidase solution. The L-glutamate oxidase layer was required by our technique to measure glutamate, as it causes the enzymatic break-down of glutamate to α-ketoglutarate and the electroactive reporter molecule, H_2O_2. When a potential of +0.7 V vs. a Ag/AgCl reference electrode was applied, the reporter molecule, H_2O_2, was oxidized yielding two electrons at the MEA recording surface. The resulting current was then amplified and recorded by a Mark II FAST16 recording system (Quanteon, LLC, Nicholasville, KY). The remaining two MEA recording sites (self-referencing or sentinel sites) were coated similar to the glutamate recording sites, with the exception that the coating solution did not contain L-glutamate oxidase. This meant the sentinel sites could not record any converted glutamate but could record any electroactive interferents (at +0.7 volts versus a Ag/AgCl reference electrode) not repelled by Nafion®. When the current recorded on the sentinel sites was subtracted from the current on the glutamate recording sites, the resulting signal represents the resting glutamate levels in brain tissue (Hascup *et al*, 2010; Burmeister *et al*, 2002; Burmeister and Gerhardt, 2001).

Reference electrodes

A miniature Ag/AgCl reference electrode was prepared by first stripping the Teflon off the silver wire (200 µm bare, 275 µm coated; A-M Systems, Inc., Carlsberg, WA) ¼ inch on each end. One of the stripped ends was soldered to a gold-plated socket (Ginder Scientific, Ottawa, ON) and the other end was coated with AgCl by placing the tip of the stripped sliver wire (cathode) into a 1 M HCl plating bath saturated with NaCl containing a Pt wire (anode) and applying +9 V DC using a power supply to the cathode vs. the anode for 5-10 minutes.

Ceramic MEA preparation

The MEA paddle was modified for use in freely moving animals as extensively described in previous literature (Hascup *et al*., 2008; Rutherford *et al*., 2007, Hascup *et al*., 2006). Briefly, the pedestal was the portion of the system that was chronically implanted on the rat's head and contained the MEA. During recording sessions, the pedestal was connected to the miniaturized Rat Hat, containing the 4-channel amplifier that was in turn connected to a low torque commutator.

MEA calibration

MEAs were calibrated to determine their sensitivity and selectivity against ascorbic acid as previously described (Hascup *et al*., 2010, 2009, 2008, 2006; Nickell *et al*., 2005). Selectivity ratios for glutamate over ascorbic acid were calculated in addition to the slope (sensitivity), limit of detection (LOD), and linearity (R^2) for glutamate for all MEAs. For this study, we used 21 MEAs consisting of 37 active glutamate recording sites. The MEAs used in this study had an average glutamate sensitivity of 7.2 pA/μM, selectivity ratio of 80 to 1, and signal-to-noise ratio of 0.2 μM.

MEA implantation

MEA implantation has been extensively described in previous literature (Hascup *et al*., 2010, 2009; Rutherford *et al*., 2007). The MEA pedal assembly was implanted in the right PFC (MEA tip coordinates: AP: +3.2 mm; ML: 0.8 mm, DV: -5.0 mm vs. bregma) with the incisor bar set so that the skull was level (-2.3 mm), which was based on the atlas of Paxinos and Watson (1998). Rats were allowed to recover for a minimum of two days prior to initial recordings.

Recording protocol

Typical recording sessions involved allowing the rat to freely roam around the recording chamber for 30 minutes to acclimate to the surroundings before connecting the pedestal to the

Rat Hat potentiostat and the start of data acquisition. Once the recordings were started, the rat underwent a minimum 90 minute acclimation period, or until a stable baseline was established. Once a stable baseline was obtained, the recording continued for one additional hour that provided us with the necessary data for analysis of resting glutamate and fluctuations in glutamate spillover events or bursts.

Data Analysis

The Mark II FAST16 recording system saved amperometric data at 2 Hz for all four recording channels. Unless otherwise noted, calibration data, in conjunction with a MATLAB graphic user interface program developed by Jason Burmeister, L.L.C., was used to calculate resting glutamate levels and fluctuations in glutamate spillover. Resting glutamate was calculated by taking a 300 second baseline average so fluctuations from spontaneous increases in glutamate spillover did not arbitrarily increase resting glutamate levels (Figure 1A). To analyze rapid glutamate transients, the MATLAB graphic user interface was set to detect peaks above a signal to noise ratio of 2 with a moving baseline of 10 points (or 5 seconds). A new baseline was measured prior to the start of each glutamate transient. Measures derived from the MATLAB graphic user interface file included: 1) resting glutamate (μM), 2) transient interval (seconds), 3) transient duration (seconds) and 4) transient maximum amplitude (μM) from baseline. A two-way analysis of variance (ANOVA) followed by a Bonferonni post-hoc was used to analyze resting glutamate, transient interval, transient duration and transient maximum amplitude over days. A one-way ANOVA followed by a Tukey's post-hoc was used to analyze the averages of transient interval, transient duration and transient maximum amplitude on all days tested. A two-tailed t-test was used to compare the same parameters 3-6 and 18 month Fischer 344 rats and to age-matched FSL rats. Data are shown as mean ± standard error of the mean (SEM) and significance was defined as $p < 0.05$.

Results

Resting Glutamate

Resting glutamate levels were determined on days 3-5 post-implantation in the PFC of 3-6 month (young) and 12-15 month (old) FRL and FSL rats as shown in Figure 1B. As previously described, the self-referencing electrode current was subtracted from the glutamate recording electrode current (and divided by the glutamate recording site slope obtained from *in vitro* calibration). As shown in Figure 1B, consistent resting glutamate levels on days 3-5 post-implantation were seen for the 3-6 month FRL (6.3 ± 1.2 μM, 5.3 ± 1.0 μM, 5.4 ± 0.6 μM; n=7) and FSL (5.3 ± 1.0 μM, 5.5 ± 0.5 μM, 5.0 ± 0.6 μM; n=6) and the 12-15 month FRL (3.8 ± 1.4 μM, 4.4 ± 1.8 μM, 5.2 ± 2.7 μM; n=4) and FSL (19.3 ± 3.4 μM, 17.3 ± 2.9 μM, 17.4 ± 3.7 μM; n=4) rats. A two-way ANOVA revealed that the day post-implantation had no effect on resting glutamate levels ($F=0.11$; $DFn=2$, $DFd=49$; $P=0.90$), however, a significant effect of rat group was observed ($F=42.92$; $DFn=3$, $DFd=49$; $P<0.0001$) and no effect of interaction ($F=0.2$; $DFn=6$, $DFd=49$; $P=0.98$). Using a Bonferonni, post-hoc, we compared resting glutamate levels between animal groups. There was no difference in resting glutamate levels between 3-6 month FRL and FSL rats as well as the 3-6 month FRL and 12-15 month FRL rats. However, we did observe significantly increased ($p<0.001$) resting glutamate levels in the 12-15 month FSL rats compared to the 12-15 month FRL rats on all testing days. Also, 12-15 month FSL rats had significantly ($p<0.001$) higher resting glutamate levels compared to 3-6 month FSL rats on all testing days.

Rapid Glutamate Transients

Rapid glutamate transients were observed throughout the duration of the tests in all rat groups over all days tested. Figure 2A shows an amperometric trace highlighting these rapid glutamate spillover events compared to the sentinel recording site. This release was only

observed on the L-glutamate oxidase coated sites and not the self-referencing recording sites confirming that it was from spillover of glutamate and not noise or artifact. As shown in the trace, the spontaneous increases in glutamate spillover were robust compared to baseline and occur intermittently throughout the duration of the experiment. The self-referenced subtracted glutamate signal from this trace is shown in Figure 2E. Figure 2B-E shows a series of representative traces of rapid glutamate transients in the 3-6 month FRL (2B) and FSL (2C) and the 12-15 month FRL (2D) and FSL (2E) rats. Arrows beneath each trace indicate a glutamate transient. Each trace shows 4 to 5 glutamate transients during an approximate 100 second interval. As evidenced by the traces, the young FRL and FSL and the old FRL rats have similar glutamate transient amplitudes. However, the old FSL rats show dramatically increased concentrations of glutamate during these transient events. No change in animal behavior was observed during these transient events.

A large number of rapid glutamate transients were observed over the three testing days in the 3-6 month FRL (2241; n=7) and FSL (2041; n=6) and the 12-15 month FRL (1235; n=4) and FSL (1296; n=4) rats. Table 1 shows the average number of rapid glutamate transients per minute, transient interval, transient duration and maximum amplitude of on each testing day for each rat group. A two-way ANOVA revealed that the day post-implantation had no effect on either the number of rapid glutamate transients per minute ($F=0.12$; $DFn=2$, $DFd=49$; $P=0.89$), transient interval ($F=0.07$; $DFn=2$, $DFd=49$; $P=0.93$), transient duration ($F=1.58$; $DFn=2$, $DFd=49$; $P=0.21$), or transient amplitude ($F=0.30$; $DFn=2$, $DFd=49$; $P=0.74$). Among rat groups, no differences in rapid glutamate transients per minute ($F=0.02$; $DFn=2$, $DFd=49$; $P=0.99$) or transient interval ($F=0.80$; $DFn=3$, $DFd=9$; $P=0.57$) were observed. But transient duration ($F=15.22$; $DFn=3$, $DFd=49$; $P<0.0001$) and amplitude ($F=23.90$; $DFn=3$, $DFd=49$; $P<0.0001$) were significantly different among rat groups. Since no interaction was observed for transient duration ($F=1.30$; $DFn=6$, $DFd=49$; $P=0.27$) or amplitude ($F=0.55$; $DFn=6$, $DFd=49$; $P=0.77$), a

Bonferonni, post-hoc was used to determined that transient duration was increased in the 12-15 month FSL rats compared to age matched controls ($p<0.001$, day 3; $p<0.05$, day 4 post-implantation) and the 3-6 month FSL rats ($p<0.001$, day 3; $p<0.01$, day 4 post-implantation). Also, the transient maximum amplitude in the 12-15 month FSL rats was significantly increased compared to age-matched controls ($p<0.05$ day 3, $p<0.001$ day 4, $p<0.01$ day 5 post-implantation) and the 3-6 months FSL rats ($p<0.01$ day 3, $p<0.001$, day 4, $p<0.01$, day 5 post-implantation).

Since no differences in rapid glutamate transients were observed within rat groups over days, we averaged the results of days 3, 4 and 5 post-implantation into a single data point for each rat within a testing group as shown in Figure 3. Using a one-way ANOVA, no difference in the transient interval ($F=1.49$, $df=3$, $P=0.25$) was observed in the 3-6 month FRL (61 ± 9 sec) and FSL (56 ± 2 sec) and the 12-15 month FRL (72 ± 13 sec) and FSL (82 ± 15 sec) rats as shown in Figure 3A. The average transient duration was shown in Figure 3B. A one-way ANOVA indicated a significant effect of transient duration ($F=6.77$; $df=3$; $P=0.003$). A tukey's post-hoc analysis revealed no differences in the transient duration of young FRL (15 ± 2 sec) and FSL (15 ± 3 sec) rats, but the duration was significantly longer ($p<0.05$) in the 12-15 month FSL (34 ± 7 sec) compared to age-matched FRL (15 ± 2 sec) rats and 3-6 month FSL rats ($p<0.01$). Finally, the maximum amplitude of the rapid glutamate transients is shown in Figure 3C. A one-way ANOVA indicated a significant difference ($F=12.43$, $df=3$, $P=0.0002$) in the average maximum amplitude of glutamate transients. A tukey's post-hoc analysis revealed no difference between 3-6 months FRL (0.4 ± 0.1 μM) and FSL (0.4 ± 0.1 μM) rats. However, the average maximum amplitude was significantly ($p<0.001$) increased in the 12-15 months FSL (1.9 ± 0.5 μM) rats compared to age-matched FRL (0.4 ± 0.1 μM) rats. Also, the average maximum amplitude of the rapid glutamate transients was significantly increased ($p<0.001$) in the 12-15 month FSL rats compared to the 3-6 month FSL rats.

Similar glutamate transients were observed in the dentate gyrus of 3-6 and 18 month Fischer 344 rats, a strain commonly used for aging studies and not a model of depression. Table 2 presents rapid glutamate transients in these rats compared with age-matched FSL rats on day 3 post-implantation. No differences in resting glutamate or rapid glutamate transients were observed between the 3-6 and 18 month Fischer 344 rats. However, transient duration was increased in the 3-6 month ($t=4.8$, $df=11$; $p<0.001$) and 12-15 month ($t=3.7$, $df=8$; $p<0.001$) FSL rats compared to age-matched Fischer 344 rats. Also, the amplitude of glutamate transients bursts was increased in the 12-15 month ($t=3.5$, $df=8$; $p<0.01$) compared to 18 month Fischer 344 rats. Thus, our data support the effects are likely unique to the FSL rats and not just to age-related changes in glutamate signaling.

Discussion

An increasing body of evidence exists for the role of glutamate in depression. Thus, abnormal expression of genes involved in regulation of glutamate synthesis, uptake and metabolism, increased glutamate levels and dysregulated function of glutamatergic receptors were suggested to be involved in pathogenesis and pathophysiology of depression. (Terracciano *et al*., 2010; McNally *et al*., 2008). To experimentally test the hypothesis of abnormal glutamate signaling in depression we examined resting extracellular glutamate levels and the neurochemical activity of glutamate in the mPFC of awake FSL rat, a validated genetic model of depression. FSL rats were selectively bred from a Sprague-Dawley background and have phenotypic traits similar to depressed individuals including decreased motivation, body weight (also observed in this study; data not shown) and psychomotor activity. These rats show severe immobility on the forced swim task that was reversible after chronic treatment with antidepressants (Overstreet *et al*., 2005).

The concept of glutamatergic dysregulation is an emerging field in depression research. Because of this, few studies of rodent models of depression exist for direct comparison. Takahashi and colleagues (2011) demonstrated that a single dose of riluzole (10 mg/kg, po) significantly decreased resting glutamate levels in the PFC of olfactory bulbectomized (OBX) rats by *in vivo* microdialysis. Sartorius and colleagues (2007) used a model of learned-helplessness combined with magnetic resonance spectroscopy and found an increased glutamate/γ-aminobutyric acid (GABA) ratio that was returned to control levels with treatment of either desipramine (10 mg/kg, ip) or electroconvulsive therapy. Finally, Garcia and colleagues (2009) have observed similar findings in a vesicular glutamate transporter 1 (vGluT1) knockout mouse model of depression. Using ^{13}C magnetic resonance spectroscopy vGluT1 heterozygous mice also had an increased glutamate/GABA ratio. For a more thorough review

of the pathophysiology of glutamatergic dysregulation in rodent models of depression the reader is referred to Mitchell and Baker (2010).

While no differences in glutamatergic regulation were observed within each group, an intriguing finding from this study was that only the 12-15 month FSL rat group showed differences in resting glutamate and rapid glutamate transients. This may indicate that FSL rats have a predisposition to depression as they age (Overstreet *et al*., 2005). In fact, Braw and colleagues (2006) found that pre-pubertal (40 day old) FSL rats have lowered anxiety-like behaviors in the open-field and elevated plus mazes compared to age-matched FRL controls. By three months of age, FSL and FRL rats spend the same amount of time in the open areas of these behavioral tests. But in other behavioral studies, such as the social interaction and active avoidance tasks, 3 month old FSL rats exhibit increased anxiogenic behavior compared to age-matched FRL rats (Overstreet *et al*., 2004; Overstreet *et al*., 1990). These studies raise the possibility of more drastic changes in the anxiety/depression profile of FSL rats as they continue to age (Braw *et al*., 2006); which may explain the why only 12-15 month FSL rats exhibited differences in resting and rapid glutamate transients. This time point (or earlier between 7-11 months) could mark a transition period from predisposition to depression, to depression, or even an elevated severity of anxiety/depression. In fact, Mitani and colleagues (2006) showed that plasma levels of glutamate were positively correlated to the severity of depression in depressed individuals. To test this hypothesis, one would need to examine glutamate regulation in these rats at a more intermediate time span (7-11 months) and later in development (18-20 months).

This increase in extrasynaptic glutamate and rapid glutamate transients observed in the 12-15 month FSL rats could derive from any of several sources. Excessive synaptic stimulation may overwhelm the high-affinity glutamate uptake transporters located primarily on glia thereby leading to the overflow of glutamate into the extracellular space (Pittenger *et al*., 2007). An alternate hypothesis has been proposed by Sanacora and colleagues (2005) based on imaging

studies supporting a reduction of glia in the cortex of depressed patients. This could lead to fewer high-affinity glutamate transporters thereby reducing their ability to efficiently clear synaptic spillover of glutamate. Alternately, decreases in metabotropic autoreceptors could further increase spillover of glutamate. Several recent studies in the FSL rats support this line of reasoning. For example, a decrease in hippocampal volume was observed in FSL rats compared to control FRL rats (Chen *et al*., 2009). Matrisciano and colleagues (2008) have also shown reduced expression and receptor signaling of the presynaptic, inhibitory $mGluR_{2/3}$ autoreceptors in the hippocampus of FSL rats. While these studies were performed in the FSL hippocampus, it has been shown that the rat prefrontal cortex receives glutamatergic afferents from the hippocampus (Carr and Sesack, 1996). Loss of the $mGluR_{2/3}$ receptors in the hippocampus could cause disinhibition of the glutamatergic efferents projecting to the prefrontal cortex, leading to increased glutamate release, which we observe in resting glutamate as well as increased glutamate spillover in the 12-15 month FSL rats.

This is the first reported observations of rapid glutamate transients, and is a result of the high temporal, low spatial and minimal damage of the MEAs. Our laboratory has also recorded these rapid glutamate transients in the hippocampus of awake Fischer 344 rats, a strain typically used in aging studies. We also have data to support that these signals are greatly affected by TTX (Stephens *et al*., manuscript in preparation). Furthermore, we previously reported that local application of either TTX or ω-conotoxin (MVIIC; calcium channel blocker) reduces resting glutamate levels by 40 to 50% in the prefrontal cortex of freely behaving rats as well as abolishes the glutamatergic response from a tail-pinch stressor (Hascup *et al*., 2010). Since recent studies support that gliotransmission was not calcium dependent (Agulhon *et al*., 2010) this data suggests a possible neuronal origin for the rapid glutamate transients reported in this manuscript.

The main finding from this study regarding increased resting glutamate levels in the 12-15 month FSL rats was similar to previous reports of glutamatergic alterations in depressed patients as previously discussed. An important consideration of all animal models is the translation to the human condition, which is dependent upon the animal model's validity. In fact, an animal model that perfectly matches the human condition is highly unlikely, and therefore, emphasis on animal models of disease should focus on specific components (Malkesman and Weller, 2009). For example, studies support that the FSL rat is not relevant to the norepinephrine model of depression and the data consistent with the serotonin and dopamine models are mixed (Overstreet *et al*., 2005). Our data supports that only 12-15 month FSL rats might be relevant to the glutamate model of depression. Thus, the results of the present studies should be investigated in additional animal models of depression, but the present studies point to phasic changes in the glutamate system in this model that may be highly relevant to treatment of the human condition and the effects of chronic, untreated or undiagnosed depression.

Disclosure/Conflicts of Interests

Greg A. Gerhardt is the sole proprietor of Quanteon, LLC.

Jan Kehr is the sole proprietor of Pronexus Analytical AB.

Kevin N. Hascup, Erin R. Hascup, Michelle L. Stephens, Paul E.A. Glaser, Takashi Yoshitake and Aleksander A. Mathé have no conflicts of interest to declare.

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References

Agulhon C, Fiacco TA, McCarthy KD (2010). Hippocampal short- and long-term plasticity are not modulated by astrocyte Ca2+ signaling. *Science* **327**: 1250-1254.

Altamura CA, Mauri MC, Ferrara A, Moro AR, D'Andrea G, Zamberlan F (1993). Plasma and platelet excitatory amino acids in psychiatric disorders. *Am J Psychiatry* **150**: 1731-1733.

Berman RM, Cappiello A, Anand A, Oren DA, Heninger GR, Charney DS*, et al.* (2000). Antidepressant effects of ketamine in depressed patients. *Biol Psychiatry* **47**: 351-354.

Braw Y, Malkesman O, Dagan M, Bercovich A, Lavi-Avnon Y, Schroeder M*, et al.* (2006). Anxiety-like behaviors in pre-pubertal rats of the Flinders Sensitive Line (FSL) and Wistar-Kyoto (WKY) animal models of depression. *Behav Brain Res* **167**: 261-269.

Brennan BP, Hudson JI, Jensen JE, McCarthy J, Roberts JL, Prescot AP*, et al.* (2010). Rapid enhancement of glutamatergic neurotransmission in bipolar depression following treatment with riluzole. *Neuropsychopharmacology* **35**: 834-846.

Burmeister JJ, Moxon K, Gerhardt GA (2000). Ceramic-based multisite microelectrodes for electrochemical recordings. *Anal Chem* **72**: 187-192.

Burmeister JJ, Gerhardt GA (2001). Self-referencing ceramic-based multisite microelectrodes for the detection and elimination of interferences from the measurement of L-glutamate and other analytes. *Anal Chem* **73**: 1037-1042.

Burmeister JJ, Pomerleau F, Palmer M, Day BK, Huettl P, Gerhardt GA (2002). Improved ceramic-based multisite microelectrode for rapid measurements of L-glutamate in the CNS. *J Neurosci Methods* **119**: 163-171.

Burmeister JJ, Coates TD, Gerhardt GA (2004). Multisite microelectrode arrays for measurements of multiple neurochemicals. *Conf Proc IEEE Eng Med Biol Soc* **7**: 5348-5351.

Canbeyli R (2010). Sensorimotor modulation of mood and depression: an integrative review. *Behav Brain Res* **207**: 249-264.

Carr DB, Sesack SR (1996). Hippocampal afferents to the rat prefrontal cortex: synaptic targets and relation to dopamine terminals. *J Comp Neurol* **369**: 1-15.

Chen F, Madsen TM, Wegener G, Nyengaard JR (2009). Imipramine treatment increases the number of hippocampal synapses and neurons in a genetic animal model of depression. *Hippocampus*.

Danbolt NC (2001). Glutamate uptake. *Prog Neurobiol* **65**: 1-105.

Garcia-Garcia AL, Elizalde N, Matrov D, Harro J, Wojcik SM, Venzala E*, et al.* (2009). Increased vulnerability to depressive-like behavior of mice with decreased expression of VGLUT1. *Biol Psychiatry* **66**: 275-282.

Hascup ER, af BS, Hascup KN, Pomerleau F, Huettl P, Stromberg I*, et al.* (2009). Histological studies of the effects of chronic implantation of ceramic-based microelectrode arrays and microdialysis probes in rat prefrontal cortex. *Brain Res* **1291**: 12-20.

Hascup ER, Hascup KN, Stephens M, Pomerleau F, Huettl P, Gratton A*, et al.* (2010). Rapid Microelectrode Measurements and the Origin and Regulation of Extracellular Glutamate in Rat Prefrontal Cortex. *J Neurochem* **115(6)**: 1608-1620.

Hascup KN, Rutherford EC, Quintero JE, Day BK, Nickell JR, Pomerleau F, Huettl P, Burmeister JJ, and Gerhardt GA (2006) Second-by-Second Measures of L-glutamate

and other neurotransmitters using enzyme-based microelectrode arrays, In: (Michael AC and Borland LM (eds). *Electrochemical Methods for Neuroscience* CRC Press, Boca Raton, FL, pp 407-450.

Hascup KN, Hascup ER, Pomerleau F, Huettl P, Gerhardt GA (2008). Second-by-second measures of L-glutamate in the prefrontal cortex and striatum of freely moving mice. *J Pharmacol Exp Ther* **324**: 725-731.

Hashimoto K, Sawa A, Iyo M (2007). Increased levels of glutamate in brains from patients with mood disorders. *Biol Psychiatry* **62**: 1310-1316.

Insel TR, Wang PS (2009). The STAR*D trial: revealing the need for better treatments. *Psychiatr Serv* **60**: 1466-1467.

Kim JS, Schmid-Burgk W, Claus D, Kornhuber HH (1982). Increased serum glutamate in depressed patients. *Arch Psychiatr Nervenkr* **232**: 299-304.

Kugaya A, Sanacora G (2005). Beyond monoamines: glutamatergic function in mood disorders. *CNS Spectr* **10**: 808-819.

Kulkarni SK, Dhir A (2009). Current investigational drugs for major depression. *Expert Opin Investig Drugs* **18**: 767-788.

Li N, Lee B, Liu RJ, Banasr M, Dwyer JM, Iwata M*, et al.* (2010). mTOR-dependent synapse formation underlies the rapid antidepressant effects of NMDA antagonists. *Science* **329**: 959-964.

Maeng S, Zarate CA, Jr. (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. *Curr Psychiatry Rep* **9**: 467-474.

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 Psychiatr Scand* **97**: 302-308.

Malkesman O, Weller A (2009). Two different putative genetic animal models of childhood depression--a review. *Prog Neurobiol* **88**: 153-169.

Matrisciano F, Caruso A, Orlando R, Marchiafava M, Bruno V, Battaglia G*, et al.* (2008). Defective group-II metaboropic glutamate receptors in the hippocampus of spontaneously depressed rats. *Neuropharmacology* **55**: 525-531.

Mauri MC, Ferrara A, Boscati L, Bravin S, Zamberlan F, Alecci M*, et al.* (1998). Plasma and platelet amino acid concentrations in patients affected by major depression and under fluvoxamine treatment. *Neuropsychobiology* **37**: 124-129.

McCauley E, Myers K, Mitchell J, Calderon R, Schloredt K, Treder R (1993). Depression in young people: initial presentation and clinical course. *J Am Acad Child Adolesc Psychiatry* **32**: 714-722.

McNally L, Bhagwagar Z, Hannestad J (2008). Inflammation, glutamate, and glia in depression: a literature review. *CNS Spectr* **13**: 501-510.

Mitani H, Shirayama Y, Yamada T, Maeda K, Ashby CR, Jr., Kawahara R (2006). Correlation between plasma levels of glutamate, alanine and serine with severity of depression. *Prog Neuropsychopharmacol Biol Psychiatry* **30**: 1155-1158.

Mitchell ND and Baker, GB (2010). An update on the role of glutamate in the pathophysiology of depression. *Acta Psychiatr Scand* **122**: 192-210.

Nickell J, Pomerleau F, Allen J, Gerhardt GA (2005). Age-related changes in the dynamics of potassium-evoked L-glutamate release in the striatum of Fischer 344 rats. *J Neural Transm* **112**: 87-96.

Nierenberg AA (2001). Do some antidepressants work faster than others? *J Clin Psychiatry* **62 Suppl 15**: 22-25.

Overstreet DH (1993). The Flinders sensitive line rats: a genetic animal model of depression. *Neurosci Biobehav Rev* **17**: 51-68.

Overstreet DH, Friedman E, Mathe AA, Yadid G (2005). The Flinders Sensitive Line rat: a selectively bred putative animal model of depression. *Neurosci Biobehav Rev* **29**: 739-759.

Overstreet DH, Janowsky DS, Pucilowski O, Rezvani AH (1994). Swim test immobility co-segregates with serotonergic but not cholinergic sensitivity in cross-breeds of Flinders Line rats. *Psychiatr Genet* **4**: 101-107.

Overstreet DH, Keeney A, Hogg S (2004). Antidepressant effects of citalopram and CRF receptor antagonist CP-154,526 in a rat model of depression. *Eur J Pharmacol* **492**: 195-201.

Overstreet DH, Rezvani AH, Janowsky DS (1990). Impaired active avoidance responding in rats selectively bred for increased cholinergic function. *Physiol Behav* **47**: 787-788.

Overstreet DH, Russell RW (1982). Selective breeding for diisopropyl fluorophosphate-sensitivity: behavioural effects of cholinergic agonists and antagonists. *Psychopharmacology (Berl)* **78**: 150-155.

Paul IA, Skolnick P (2003). Glutamate and depression: clinical and preclinical studies. *Ann N Y Acad Sci* **1003**: 250-272.

Paxinos G, and Watson C. (1998) The rat brain: stereotaxic coordinates. Academic Press: New York.

Pittenger C, Sanacora G, Krystal JH (2007). The NMDA receptor as a therapeutic target in major depressive disorder. *CNS Neurol Disord Drug Targets* **6**: 101-115.

Rakofsky JJ, Holtzheimer PE, Nemeroff CB (2009). Emerging targets for antidepressant therapies. *Curr Opin Chem Biol* **13**: 291-302.

Rutherford EC, Pomerleau F, Huettl P, Stromberg I, Gerhardt GA (2007). Chronic second-by-second measures of L-glutamate in the central nervous system of freely moving rats. *J Neurochem* **102**: 712-722.

Sartorius A, Mahlstedt MM, Vollmayr B, Henn FA, Ende G (2007). Elevated spectroscopic glutamate/gamma-amino butyric acid in rats bred for learned helplessness. *Neuroreport* **18**: 1469-1473.

Skolnick P, Popik P, Trullas R (2009). Glutamate-based antidepressants: 20 years on. *Trends Pharmacol Sci* **30**: 563-569.

Stone M, Laughren T, Jones ML, Levenson M, Holland PC, Hughes A*, et al.* (2009). Risk of suicidality in clinical trials of antidepressants in adults: analysis of proprietary data submitted to US Food and Drug Administration. *BMJ* **339**: b2880.

Takahashi K, Murasawa H, Yamaguchi K, Yamada M, Nakatani A, Yoshida M*, et al.* (2011). Riluzole rapidly attenuates hyperemotional responses in olfactory bulbectomized rats, an animal model of depression. *Behav Brain Res* **216**: 46-52.

Terracciano A, Tanaka T, Sutin AR, Sanna S, Deiana B, Lai S*, et al.* (2010). Genome-Wide Association Scan of Trait Depression. *Biol Psychiatry*.

Witkin JM, Marek GJ, Johnson BG, Schoepp DD (2007). Metabotropic glutamate receptors in the control of mood disorders. *CNS Neurol Disord Drug Targets* **6**: 87-100.

Yadid G, Nakash R, Deri I, Tamar G, Kinor N, Gispan I*, et al.* (2000). Elucidation of the neurobiology of depression: insights from a novel genetic animal model. *Prog Neurobiol* **62**: 353-378.

Table 1: Rapid Glutamate Transients in FRL and FSL rats.

	Age		3-6 Months		12-15 Months	
	Rat		FRL	FSL	FRL	FSL
	N		7	6	4	4
Glutamate Transients / Minute	Days Post-Implantation	3	1.8 ± 0.3	1.9 ± 0.3	2.1 ± 0.5	1.7 ± 0.5
		4	1.8 ± 0.2	1.8 ± 0.3	1.8 ± 0.7	1.7 ± 0.3
		5	2.0 ± 0.2	1.9 ± 0.4	1.7 ± 0.5	2.0 ± 0.2
Glutamate Transient Interval (sec ± SEM)		3	59.6 ± 7.6	56.6 ± 3.1	63.2 ± 13.1	94.8 ± 23.5
		4	59.2 ± 6.2	52.0 ± 1.1	77.3 ± 21.3	88.2 ± 17.0
		5	69.3 ± 15.0	58.1 ± 7.0	73.1 ± 12.9	65.2 ± 4.5
Glutamate Transient Duration (sec ± SEM)		3	18.2 ± 2.0	16.0 ± 2.9	13.4 ± 2.8	43.9 ± 12.7***,###
		4	18.0 ± 2.3	13.2 ± 2.9	15.2 ± 2.4	33.6 ± 7.8*,##
		5	18.2 ± 2.4	14.3 ± 3.2	15.4 ± 2.9	24.5 ± 2.4
Glutamate Transient Amplitude (µM ± SEM)		3	0.3± 0.1	0.3 ± 0.1	0.5 ± 0.1	1.6 ± 0.3*,##
		4	0.4 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	2.3 ± 0.8***,###
		5	0.3 ± 0.1	0.5 ± 0.2	0.4 ± 0.1	1.9 ± 0.7**,##

*p<0.05, **p<0.01, ***p<0.001 vs 12-15 month FRL rats; #p<0.05, ##p<0.01, ###p<0.001 vs 3-6 month FSL rats.

Table 2: Rapid Glutamate Transients in the Dentate Gyrus of Fischer 344 rats.

Rat	Fischer 344 (n=7)	FSL (n=6)	Fischer 344 (n=6)	FSL (n=4)
Age (months)	3-6	3-6	18	12-15
Resting Glutamate (μM)	7.0 ± 1.6	---	12.2 ± 4.2	---
Glutamate Transient Interval (sec ± SEM)	69.4 ± 7.2	---	100.0 ± 20.0	---
Glutamate Transient Duration (sec ± SEM)	2.9 ± 0.5	↑↑↑	6.3 ± 1.8	↑↑↑
Glutamate Transient Amplitude (μM ± SEM)	1.0 ± 0.3	---	0.4 ± 0.2	↑↑

Values for FSL Day 3 post-implantation are located in Table 1. Symbols indicate: --- no difference, ↑↑ $p<0.01$, ↑↑↑ $p<0.001$

Titles and Figures to Legends

Figure 1: Resting Glutamate Levels

A) An amperometric trace of resting glutamate determination from a 12-15 month FSL rat. Resting glutamate levels were calculated from a 300 second baseline average, prior to burst analysis studies, using the equation shown in the figure. This baseline was calculated while little to no burst events were observed so fluctuations from spontaneous increases in glutamate spillover did not arbitrarily inflate resting glutamate values. B) Resting levels of glutamate in young and old FRL and FSL rats. A two-way ANOVA was used to compare resting glutamate levels in 3-6 month FRL (n=7) and FSL (n=6) rats, 12-15 month FRL (n=4) and FSL (n=4) rats and 3-6 and 12-15 month FSL rats. A two-way ANOVA indicated no effect of recording day ($F=0.11$; $DFn=2$, $DFd=49$; $P=0.90$), however, a significant effect of rat group was observed ($F=42.92$; $DFn=3$, $DFd=49$; $P<0.0001$). A Bonferonni post-hoc revealed 12-15 month FSL rats had significantly elevated levels of resting glutamate compared to age-matched FRL rats (***$p<0.001$) as well as 3-6 month FSL rats ($^{###}p<0.001$).

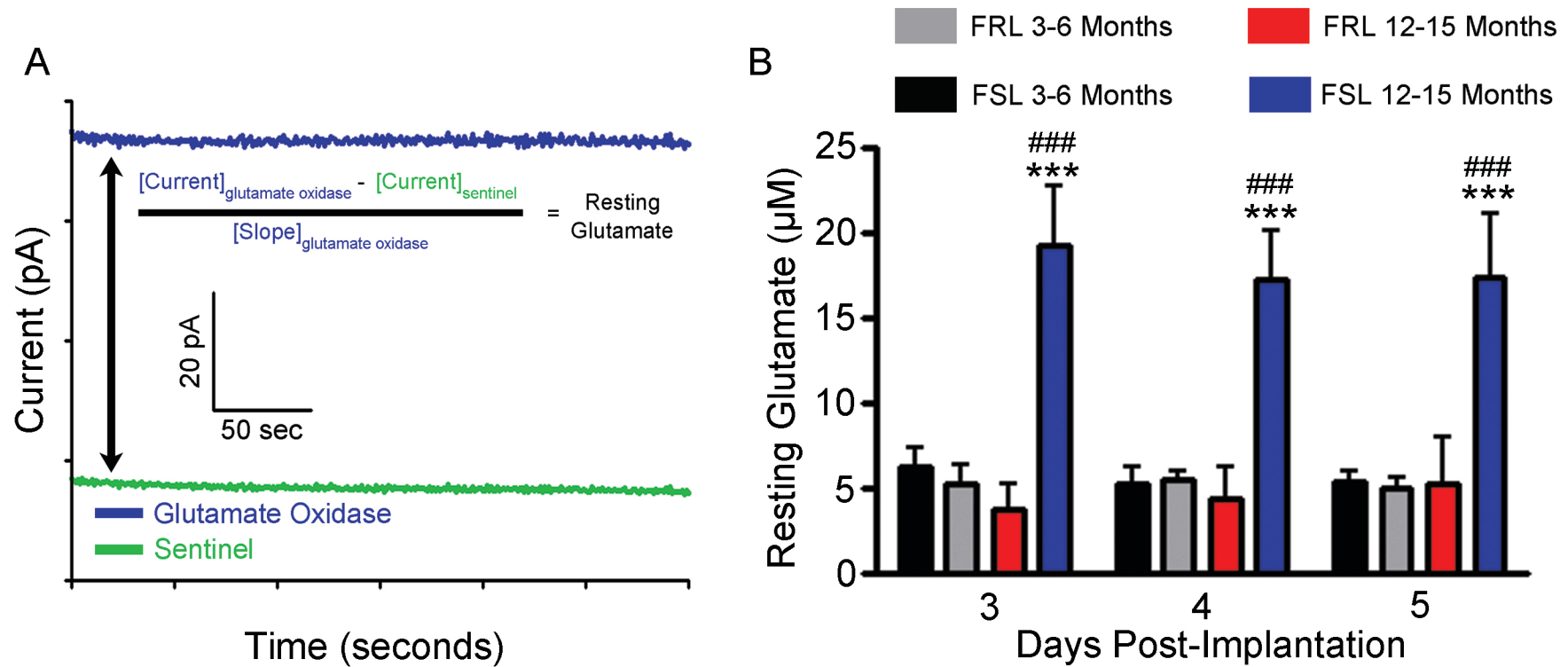
Figure 2: Rapid Glutamate Transients

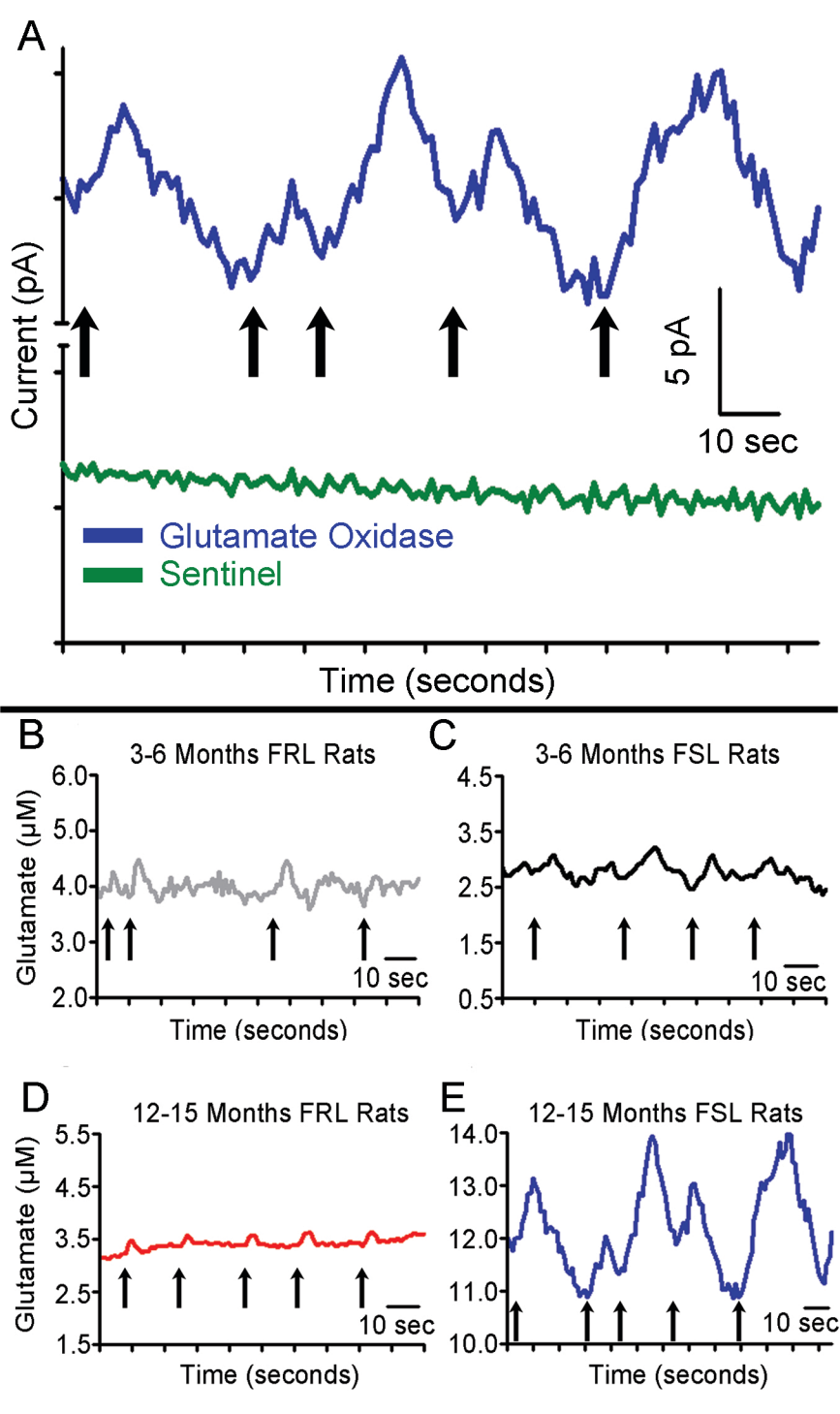
A) An amperometric trace of spontaneous glutamate bursts from the same 12-15 month FSL rat shown in Figure 1A. Rapid fluctuations in current were observed on the L-glutamate oxidase coated site (blue) and not on the sentinel recording site (green). Arrows indicate the start of a spontaneous burst spillover event which was detected using the MATLAB graphic user interface. Note, the y-axis was segmented to emphasize these spontaneous release events only occurred on the glutamate oxidase coated sites and not the self-referencing sites. Self-referenced representative traces of increased glutamate bursts in 3-6 month FRL (B) and FSL (C) and the 12-15 month FRL (D) and FSL (E) rats. All rat groups displayed intermittent glutamate bursts with multiple events occurring in rapid succession. The increased glutamate

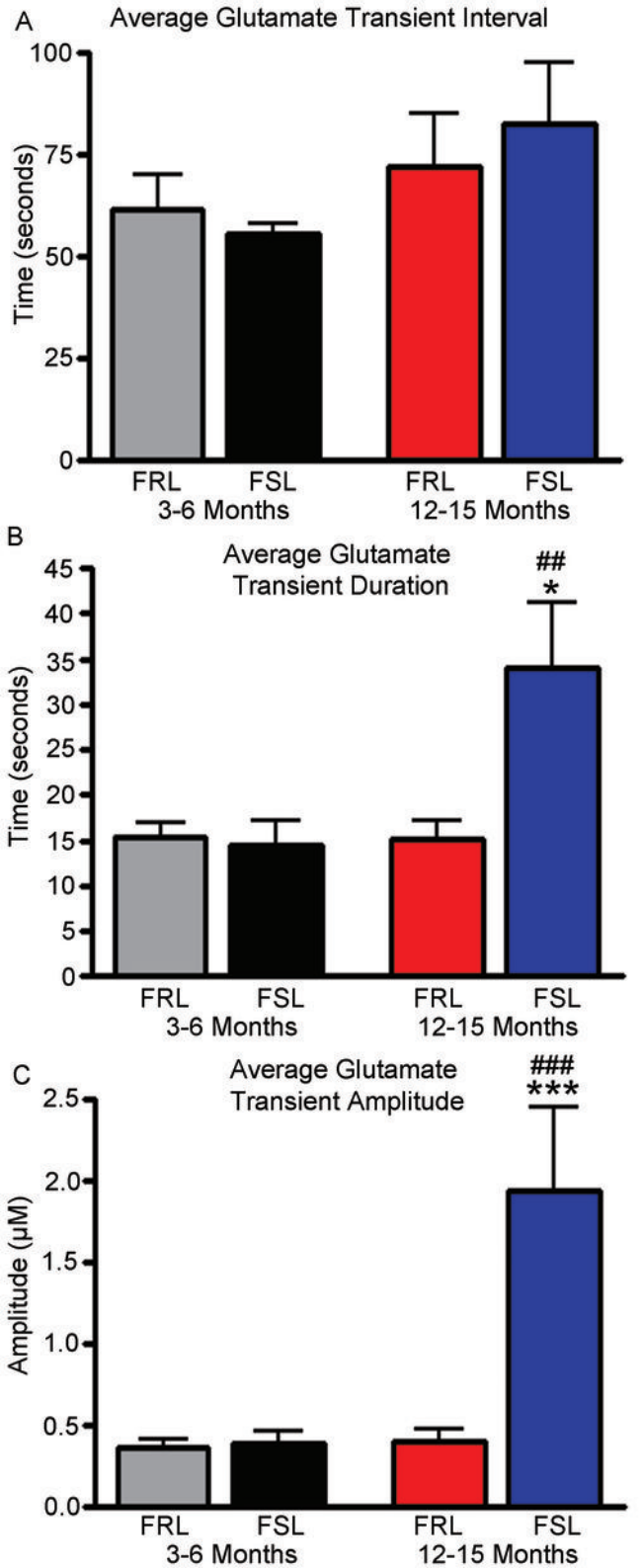
spillover was similar in the 3-6 month FRL and FSL and the 12-15 month FRL rats, however, the 12-15 month FSL rats showed larger average concentrations of glutamate spillover. Arrows indicate the start of an increased glutamate spillover event.

Figure 3: Analysis of Rapid Glutamate Transients

The glutamate transient interval (A), duration (B) and maximum amplitude (C) were averaged across days post-implantation. On average, the inter-burst interval occurred twice per minute in all four rat groups. The average burst duration was similar in the young FRL (n=7) and FSL (n=6) and the old FRL (n=4) rats. But, a one-way ANOVA with a Tukey's post-hoc indicates that the 12-15 month FSL rats (n=4) had significantly longer burst duration compared to age-matched FRL rats (*$p<0.05$) and young FSL rats ($^{\#\#}p<0.01$). Similar to burst duration, the average maximum amplitude of glutamate release was similar in the 3-6 month FRL and FSL and the 12-15 month FRL rats. Using the same calculations as (B), the 12-15 month FSL rats had significantly higher average concentrations of glutamate during spillover events compared to age-matched FRL rats (***$p<0.001$) and 3-6 month FSL ($^{\#\#\#}p<0.001$) rats.


A
Current (pA)
[Current]glutamate oxidase - [Current]sentinel
[Slope]glutamate oxidase
= Resting Glutamate
20 pA
50 sec
Glutamate Oxidase
Sentinel
Time (seconds)
B
FRL 3-6 Months
FRL 12-15 Months
FSL 3-6 Months
FSL 12-15 Months
Resting Glutamate (μM)
25
20
15
10
5
0
###
***
3
4
5
Days Post-Implantation


A
Current (pA)
5 pA
10 sec
Glutamate Oxidase
Sentinel
Time (seconds)
B
3-6 Months FRL Rats
Glutamate (μM)
6.0
5.0
4.0
3.0
2.0
10 sec
Time (seconds)
C
3-6 Months FSL Rats
4.5
3.5
2.5
1.5
0.5
10 sec
Time (seconds)
D
12-15 Months FRL Rats
Glutamate (μM)
5.5
4.5
3.5
2.5
1.5
10 sec
Time (seconds)
E
12-15 Months FSL Rats
14.0
13.0
12.0
11.0
10.0
10 sec
Time (seconds)


A
Average Glutamate Transient Interval
Time (seconds)
100
75
50
25
0
FRL
FSL
FRL
FSL
3-6 Months
12-15 Months
B
Average Glutamate
Transient Duration
##
*
Time (seconds)
45
40
35
30
25
20
15
10
5
0
FRL
FSL
FRL
FSL
3-6 Months
12-15 Months
C
Average Glutamate
Transient Amplitude
###
***
Amplitude (μM)
2.5
2.0
1.5
1.0
0.5
0.0
FRL
FSL
FRL
FSL
3-6 Months
12-15 Months