

# Brain Rewarding Stimulation Reduces Extracellular Glutamate Through Glial Modulation in Medial Prefrontal Cortex of Rats

Gen Murakami<sup>1</sup>, Masato Nakamura<sup>1</sup>, Masatoshi Takita<sup>2</sup>, Yasushi Ishida<sup>3</sup>, Takatoshi Ueki<sup>4,5</sup> and Daiichiro Nakahara<sup>\*,1,5</sup>

<sup>1</sup>Division of Psychology and Behavioral Neuroscience, Department of Integrated Human Sciences, Hamamatsu University School of Medicine, Hamamatsu, Japan; <sup>2</sup>Cognition and Action Research Group, National Institute of Advanced Industrial Science and Technology, Tsukuba, Japan; <sup>3</sup>Department of Psychiatry, Faculty of Medicine, University of Miyazaki, Miyazaki, Japan; <sup>4</sup>Department of Anatomy and Cell Biology, Graduate School of Medical Sciences, Nagoya City University, Nagoya, Japan; <sup>5</sup>Department of Biofunctional Imaging, Medical Photonics Center, Hamamatsu University School of Medicine, Hamamatsu, Japan

Growing evidence implicates a critical involvement of prefrontal glial modulation of extracellular glutamate (GLU) in aversive behaviors. However, nothing is known about whether prefrontal glial cells modulate GLU levels in rewarding behaviors. To address this question, we measured GLU efflux in the medial prefrontal cortex (PFC) of rats associated with rewarding behaviors. We used intracranial self-stimulation (ICSS) of the medial forebrain bundle (MFB) as the rewarding behavior. GLU was indirectly measured using microdialysis combined with on-line fluorometric detection of NADH resulting from the reaction of GLU and NAD<sup>+</sup> catalyzed by GLU dehydrogenase with a time resolution of 1 min. ICSS caused a minute-by-minute change of extracellular GLU in the medial PFC, with a slight decrease during the stimulation, followed by an increase afterward. This bidirectional change was tetrodotoxin insensitive and abolished by the gliotoxin fluorocitrate. To confirm and extend the previous studies of aversion-induced increase of extracellular GLU in the medial PFC, we also measured prefrontal GLU efflux associated with an aversive stimulation, immobilization stress. The temporal change in extracellular GLU caused by this stress was markedly different from that observed during ICSS. A rapid increase in GLU was detected during the aversive stimulation, followed by a large increase afterward. This bimodal change was tetrodotoxin insensitive, similar to that detected for ICSS. These findings indicate a bidirectional regulation of extracellular GLU by prefrontal glial cells associated with rat ICSS behavior, and reveal that glial modulation of GLU neurochemistry in the medial PFC contributes to rewarding as well as aversive behaviors in rats. *Neuropsychopharmacology* (2015) **40**, 2686–2695; doi:10.1038/npp.2015.115; published online 27 May 2015

## INTRODUCTION

Clinical and preclinical studies support a role of glial cells, particularly in the prefrontal cortex (PFC), in the pathophysiology of major depressive illness. Post-mortem analyses of the brains of patients with depression reveal reductions in the number or density of glial cells in the PFC (Cotter *et al*, 2002; Rajkowska, 2000). The results of a microarray study indicated that expression of the glial glutamate (GLU) transporters excitatory amino-acid transporter 1 (EAAT1; human analog of GLU transporter 2) and 2 (EAAT2; human analog of GLU transporter 1) are decreased in the anterior cingulate cortex and dorsolateral PFC in patients with depressive illness (Choudary *et al*, 2005; Oh *et al*, 2014). By contrast, elevated GLU levels have been found in

prefrontal cortical areas of postmortem tissue of depressed subjects (Hashimoto *et al*, 2007; McEwen *et al*, 2012). Thus, because GLU clearance from the extracellular space is thought to be a major function of EAATs located on glial cells (Haydon and Carmignoto, 2006), the morphological atrophy of glial cells observed in the PFC of patients with depression may result in decreased transporter expression, thereby reducing the capacity to regulate synaptic concentrations of GLU (Reznikov *et al*, 2007) and leading to increased GLU levels. Parallel findings have been reported in rodent models of depression. For example, rats with selective pharmacological ablation of glial cells in the PFC display depressive-like symptoms (Banasr and Duman, 2008). Fewer astrocytes (Banasr *et al*, 2010) and EAATs (Zink *et al*, 2010) and increased GLU efflux in the PFC (Bagley and Moghaddam, 1997; Mogensen and Divac, 1993; Steciuk *et al*, 2000) have been observed in rat models of stress-induced depression. Furthermore, behavioral deficits in rat models of chronic stress-induced depression are reversed by chronic treatment with the GLU transport enhancers, such as ceftriaxone (Mineur *et al*, 2007) or riluzole (Banasr *et al*, 2010).

\*Correspondence: Professor D Nakahara, Department of Biofunctional Imaging, Medical Photonics Center, Hamamatsu University School of Medicine, 1-20-1 Handayama, Hamamatsu 431-3192, Japan, Tel: +81 53 435 2466, Fax: +81 53 435 2466, E-mail: nakahara@hama-med.ac.jp Received 15 September 2014; revised 16 March 2015; accepted 24 March 2015; accepted article preview online 29 April 2015

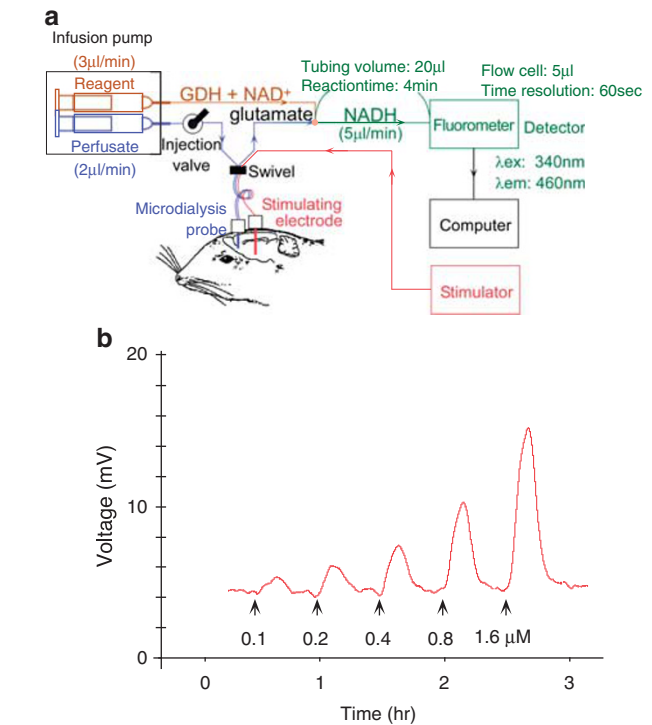
Although lowered mood, such as sadness, hopeless, and pessimism, is the fundamental hallmark of depression, anhedonia is equally integral to the disorder (Eshel and Roiser, 2010). Anhedonia refers to the decreased ability to experience pleasure in rewarding activity, and has been proposed to result from deficits in the brain reward system (Nestler and Carlezon, 2006). The intracranial self-stimulation (ICSS) paradigm is a widely used method for assessing function of the brain reward system (D'Suza and Markou, 2010). Using this ICSS paradigm, researchers reported that blockade of glial cell activity by a selective inhibitor of the glial GLU transporter produces a reduced rate of response to ICSS, reflecting a state of anhedonia (Bechtholt-Gompf *et al*, 2010). This effect was reproduced when the drug infusion was restricted to the PFC (John *et al*, 2012), indicating a critical involvement of prefrontal glial dysfunction in anhedonia-like outcomes. As mentioned above, one of most important features of glial function is to control extracellular levels of GLU by release (Baker *et al*, 2002; Kalivas, 2009) and uptake (Danbolt, 2001) mechanisms to maintain optimal synaptic transmission under physiological conditions. However, it is currently unknown whether prefrontal glial cells modulate extracellular GLU concentrations *in vivo* associated with a variety of behavioral states. We hypothesize that, in direct opposition to anhedonia, the expression of a hedonic phenotype may be associated with an increase in the capacity of glial cells to regulate synaptic concentrations of GLU, thereby decreasing extracellular GLU levels. This hypothesis has not yet been explored.

Microdialysis can be used for *in vivo* extracellular sampling from awake, freely moving rats to monitor chemical changes in the neuronal environment. In contrast to monoamines, amino acids such as GLU and GABA in microdialysis samples are usually insensitive to tetrodotoxin (TTX) or to the removal of calcium ions from the perfusate buffer (Fillenz, 1995; Timmerman and Westerink, 1997), demonstrating that the primary source of GLU is not neuronally derived. Thus, GLU levels in microdialysates largely reflect glial activity. Therefore, in the present study, we used a relatively high time-resolution method of measuring on-line microdialysis samples every 1 min (Figure 1) to explore the glial modulation of extracellular GLU *in vivo* in the PFC of animals during ICSS. We also investigated a minute-by-minute change of GLU following the induction of aversion, a state opposite of reward, that was produced by immobilization (IMB) to confirm and extend previous studies showing an aversion-induced increase in extracellular GLU levels of the PFC (Moghaddam and Jackson, 2004).

## MATERIALS AND METHODS

### Animals and Surgery

All procedures for animal treatment and surgery were performed in accordance with the guidelines established by the Institute for Experimental Animals of Hamamatsu University School of Medicine and were approved by the university's committee for animal experiments. Male Wistar rats (Japan SLC, Shizuoka, Japan), weighing 230–250 g at the time of surgery were individually housed under a 12 h light/dark cycle (lights on at 0700 hours) in a temperature-



**Figure 1** (a) Schematic diagram of a microdialysis system for on-line measurement of extracellular GLU concentrations combined with electrical self-stimulation. The inlet and outlet of the microdialysis probe were connected, through a liquid swivel (Instech, model 375/D/22QE, Plymouth Meeting, PA) with Teflon tubing, to a microinfusion pump and a fluorescence detector. The electrode was connected, through an electric swivel (Air Precision, model T13EEG-6, France), to a stimulator. (b) Response characteristics of the analysis-detection system to GLU concentration standards. Forty microliters each of 0.1, 0.2, 0.4, 0.8, and 1.6 μM GLU was microinjected into the system at the points denoted by arrows. GLU, glutamate.

controlled environment (23 °C), with food and water available *ad libitum*. The animals were anesthetized with sodium pentobarbital (50 mg/kg, *i.p.*) and placed in a Narishige stereotaxic apparatus with the skull level between bregma and lambda. Holes were made through the skull with a dental drill, and a guide cannula was implanted in the medial PFC using coordinates (3.5 mm anterior to bregma, 0.7 mm lateral to the midline, and 0.6 mm ventral to the dura) derived from the atlas by Paxinos and Watson (1986). A monopolar stimulating electrode was implanted in the MFB at the level of the lateral hypothalamus (3.8 mm posterior, 1.6 mm lateral, and 8.5 mm ventral). The stimulating electrode consisted of a stainless-steel wire (0.2 mm in diameter) coated with polyurethane, except at the tip. A reference electrode, a watch screw (1.2 mm in diameter), was embedded into the frontal bone. The electrode/cannula assembly was fixed to the skull with dental cement and anchor screws, and a dummy probe was placed inside the cannula.

### Microdialysis and On-Line Enzyme-Fluorometric Analysis

The microdialysis probe was the same as that described previously (Nakahara *et al*, 1993). The length of the dialysis

tubing (Cuprophane; 0.23 mm in diameter, molecular cutoff of 35 000 Da, Nikkiso, Japan) was 4 mm. Unless otherwise stated, the dialysis probe was perfused with Ringer's solution (138 mM NaCl, 2.4 mM KCl, 1.2 mM CaCl<sub>2</sub>, pH 7.0) using an infusion pump with a 2.5-ml syringe at a flow rate of 2 µl/min. The *in vitro* recovery of GLU of these probes was  $19.4 \pm 0.1\%$  (mean  $\pm$  SEM,  $n = 9$ ) at 37 °C. GLU concentrations in the dialysate were determined by the following on-line enzyme-fluorometric detection system (Figure 1a), which was similar to that described by others (Obrenovitch *et al*, 1990; Takita *et al*, 2002) but with higher time resolution. The dialysate was transferred into the mixing cross through Teflon tubing with an inside diameter of 100 µm (TT-100, Eicom, Kyoto, Japan). The reagent solution consisted of 85 U/ml GLU dehydrogenase, 0.22 mmol/l NAD<sup>+</sup>, 0.29% v/v hydrazine, and 1.0 mmol/l ADP in TEA buffer (64.5 mM, pH 8.0). The reagent solution was pumped using an infusion pump with a separate 2.5-ml syringe at 3 µl/min and was transferred into the mixing cross to react with the dialysate. The mixing of the reagent and dialysate in the tubing caused GLU and NAD<sup>+</sup> to react, catalyzed by GLU dehydrogenase, and the resulting NADH flowed to a highly sensitive fluorometer equipped with a 5-µl flow cell (FP-920, Jasco, Tokyo, Japan). The flow rate of 5 µl/min for the dialysate-reagent mixture and the 5-µl flow cell provided a 1 min resolution, since this is the time required for complete replacement of the content in the flow cell. The dead volume of this tubing permitted a reaction time of 4 min. The fluorescence of NADH was automatically measured at excitation–emission wavelengths of 340–460 nm with a 60-s interval using commercially available software (MacLab System, AD Instruments, NSW, Australia).

### Intracranial Self-stimulation

Two or three days prior to dialysis experiments, the rats were placed into a 25 × 30 × 28.5 cm transparent acrylic box and trained to press a lever for rewarding stimulation of the MFB using conventional shaping procedures. Depression of the lever delivered a 0.3 s train of biphasic rectangular pulses with a pulse duration of 0.1 ms and a frequency of 100 pulses/s on a continuous reinforcement schedule. The minimal current intensity required for sustained and stable lever pressing was determined for each rat. A stable performance was operationally defined as a minimum of 1000 lever presses in a 20-min session. Animals producing stable self-stimulation with little or no motoric side effects were selected for further testing. These rats were trained to press the lever for another 40 min before being returned to their home cages.

### Experimental Protocol

Experiments began after 7–10 days of postoperative recovery. The dummy probe was replaced with the dialysis probe, which was continuously perfused with normal Ringer's solution. Four experiments were conducted. In all of them, animals were perfused with normal Ringer's solution 2–3 h prior to the start of the experiment to allow for neurochemical stabilization. In the first experiment, animals were exposed to ICSS ( $n = 12$ ) for 20 min. In addition, two rats were deprived of food for 24 h and then given food for

15 min. Prefrontal GLU levels were measured 3 h before, during, and 2 h after exposure to ICSS or eating. In the second experiment, animals underwent ICSS for 20 min (the first ICSS) under the same conditions as those used in the first experiment. After 2 h of recovery, these animals again experienced the same ICSS conditions (the second ICSS) but in the presence ( $n = 5$ ) or the absence ( $n = 5$ ) of the voltage-dependent sodium channel blocker TTX (10 µM). For TTX administration, 60 min before the second ICSS, the normal Ringer's solution was switched to a Ringer's solution containing 10 µM TTX. Rats were kept in the operant box between the first and second stimulations. For the third experiment, on the evening of the day before the experiment, the animals were administered a Ringer's solution containing 500 µM fluorocitrate, a selective inhibitor of glial metabolism, through the dialysis probe for 3 h. Fluorocitrate-treated animals ( $n = 4$ ) continued to display active sniffing and locomotion during and several hours after the administration. The following morning, 20 min ICSS was delivered to the animals. Pilot experiments showed that this drug treatment was sufficient for its effect on GLU concentrations to reach a plateau. In the fourth experiment, animals underwent IMB during 20 min (the first IMB). IMB involved fastening the limbs with sticky tape to a wooden board placed in the operant box. Rats were returned to their home cages after the first IMB. After 2 h of recovery, the animals again experienced the same IMB conditions (the second IMB), but this stimulation was conducted in the absence ( $n = 6$ ) or the presence ( $n = 5$ ) of 10 µM TTX. Throughout experiments, GLU levels in the dialysate were continuously monitored with a temporal resolution of 1 min. GLU concentrations were calibrated at the end of the experiment using 40 µl of 1 µM L-GLU as a standard.

### Drugs

The drugs used included TTX (Sigma Chemical, St Louis, MO), D,L-fluorocitric acid barium salt (Tocris, Neuramin, Bistol, U.K.), a selective inhibitor of glial metabolism, GLU dehydrogenase (Boehringer Mannheim, Germany), NAD<sup>+</sup> (Boehringer), ADP disodium salt (Oriental Yeast, Tokyo, Japan), and hydrazine monohydrate (Wako Pure Chemical, Osaka, Japan). Amino acids for standards were obtained from Kanto Chemical (Tokyo, Japan). Fluorocitrate was prepared from its barium salt after precipitation with sodium sulfate as described by the study by Paulsen and Fonnum (1989).

### Statistical Analysis

GLU concentrations in the dialysate were estimated after calibration with the standards and are expressed as mean  $\pm$  SEM. The change in GLU concentrations during rewarding stimulation was evaluated by comparing the concentration during the stimulation with the immediately preceding baseline value, after applying a smoothing algorithm to remove the oscillations of GLU signals caused by pulsatile flow through the measuring system. The total amount of GLU was calculated as the area under the concentration–time curve (AUC) minus the pre-stimulation baseline value for a 20 min stimulation period and for a 20 min post-stimulation period. Because GLU efflux returned to baseline

levels during intertrial periods, basal (resting) GLU efflux was calculated as the total amount recovered during a 20 min period, which started 60 min after each stimulation. Statistical analysis of the AUC data was performed using one- or two-way analysis of variance (ANOVA), followed by the least significant difference test, as appropriate. Statistical evaluation of TTX or fluorocitrate effects on basal GLU efflux was conducted using Student's paired *t*-tests. *P*-values < 0.05 were regarded as significant.

### Histology

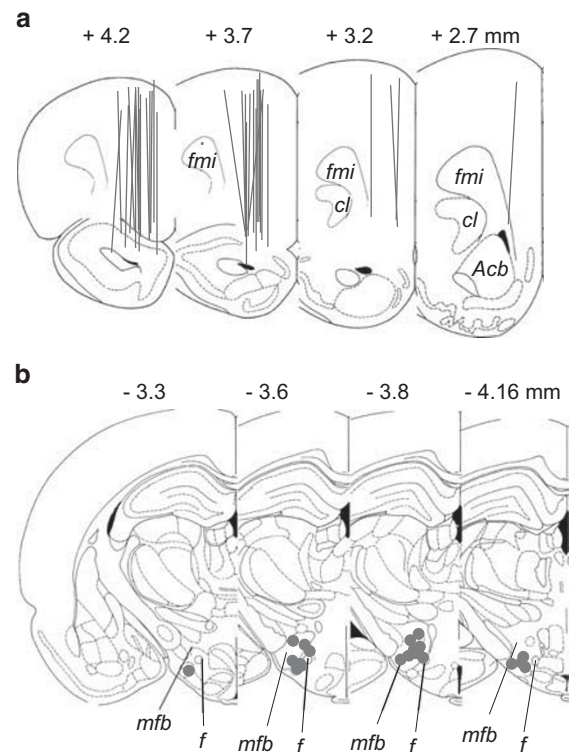
Following the experiments, rats were deeply anesthetized with an overdose (100 mg/kg, i.p.) of sodium pentobarbital and perfused intracardially with 10% formalin in saline. Brains were removed and placed in 10% formalin for at least 1 week. Frozen brain blocks were coronally cut into 30- $\mu$ m slices and then stained with thionine for verification of cannula and stimulating electrode placements.

### RESULTS

Standard solutions of 0.1–1.6  $\mu$ M L-GLU in Ringer's solution were infused into the injection valve attached to the measuring system (Figure 1a), and the output voltage was recorded continuously (Figure 1b). This GLU range corresponds to those levels likely found with microdialysis techniques under physiological and experimental conditions such as ICSS (You *et al*, 2001) or IMB (Lowy *et al*, 1995; Steciuk *et al*, 2000). We also examined the selectivity of the measuring system for GLU by measuring the signal produced by a number of compounds expected to be present in extracellular fluid. The detector did not respond to any these substances (data not shown). Histological examination of the brain sections identified most dialysis membrane tracks as positioned within the medial PFC (Figure 2a), and all the electrode tips implanted for ICSS located in ventral aspects of the MFB at the level of the lateral hypothalamus (Figure 2b).

### GLU Efflux During ICSS

Figure 3a and b shows the results of the first experiment, examining the temporal pattern of GLU efflux in the dialysate from the medial PFC during ICSS. The mean resting GLU concentration in the dialysate before stimulation was  $2.0 \pm 0.3 \mu\text{M}$  ( $n = 12$ ). ICSS for 20 min evoked an initial slight, but not statistically significant, decrease in GLU efflux during the first 10–15 min, followed by a significant increase, which peaked after stimulation cessation, and a gradual return with some fluctuation to baseline. Thus, ICSS was accompanied by a bidirectional change in GLU efflux, consisting of an initial slight GLU decrease followed by a large positive efflux (Figure 3a and b and Supplementary Movie). We also investigated the effect of feeding as a natural reward on GLU efflux in the medial PFC and detected a temporal pattern in GLU efflux similar to that observed with ICSS (Figure 3c). This observation is consistent with those of Rada *et al* (1997) and Saulskaya and Mikhailova (2002), who found feeding-induced decreases of extracellular GLU levels in the nucleus accumbens. Thus, a transient reduction in extracellular GLU followed by a prolonged enhancement

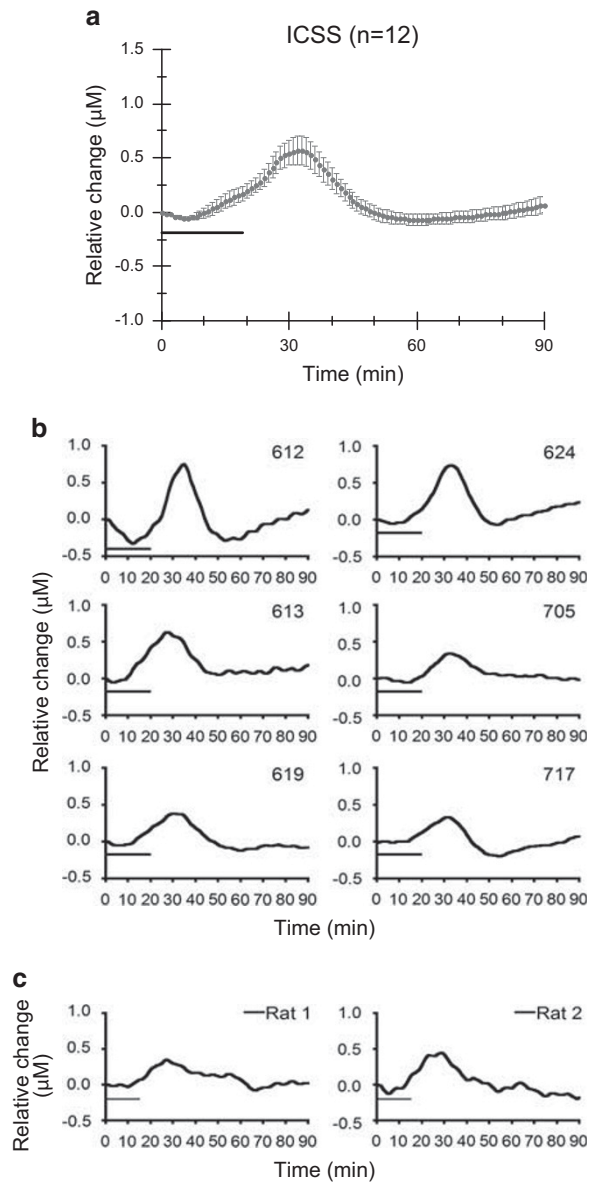


**Figure 2** (a) Histological localization of the 4-mm microdialysis membranes in the medial PFC. Most of the dialysis membrane tracks were positioned within the medial PFC, but the tips of some of the probes invaded the anterior olfactory tubercle ventral to the medial PFC. (b) Histological localization of the stimulation electrode tips in the MFB. All the electrode tips were located in the ventral aspect of the MFB at the level of the lateral hypothalamus. Fewer lines (a) and black circles (b) are depicted than the number of rats used in these experiments because of overlapping placements among several rats. The numbers above each section indicate the distance (mm) posterior (–) to bregma. The drawings are adopted from Paxinos and Watson (1986). Acb, nucleus accumbens; cl, claustrum; f, fornix; fmi, forceps minor of the corpus callosum; MFB, medial forebrain bundle; PFC, prefrontal cortex.

reflects a common feature of prefrontal GLU levels associated with artificial (ICSS) and natural (food) rewarding stimuli.

### Sensitivity of Stimulated GLU Efflux to TTX

Figure 4 shows the results of the second experiment, investigating whether the change in GLU efflux in response to ICSS was dependent on neuronal activity. In the absence of TTX, ICSS elicited changes in GLU efflux similar to those observed during the second stimulation delivered after a 2 h interval (Figure 4a and b). TTX (10  $\mu\text{M}$ ) was added to the perfusion medium 60 min prior to the second ICSS. The mean resting level of GLU in the presence of TTX was not different from that observed immediately before addition of TTX (with TTX,  $1.9 \pm 0.3 \mu\text{M}$ ; without TTX,  $1.9 \pm 0.4 \mu\text{M}$ ;  $n = 5$ ), indicating that non-neurotransmitter pools of GLU largely contribute to resting levels of GLU in the dialysate (for reviews, see Fillenz, 1995; Lupinsky *et al*, 2010; Timmerman and Westerink, 1997). Addition of TTX to the perfusion medium significantly decreased, but did not



**Figure 3** Time courses of GLU efflux in the medial PFC in response to intracranial self-stimulation (ICSS) and eating. (a) The average ( $\pm$  SEM) of the GLU responses to ICSS in 12 animals. (b) Representative individual GLU responses from six animals (identification numbers in upper right of each graph). Thick horizontal bars indicate the stimulation duration (20 min). (c) Individual representative GLU responses from two animals when they ate food intermittently. Horizontal bars indicate the duration of eating (15 min). GLU, glutamate; PFC, prefrontal cortex.

abolish, the GLU response to ICSS (two-way ANOVA:  $F_{(1,4)} = 10.43$ ,  $p < 0.05$ ; Figure 4c and d). Furthermore, the bidirectional response of extracellular GLU was more apparent than that seen in the absence of TTX, with a significant decrease in amplitude observed during ICSS ( $p < 0.05$ ; Figure 4d). Finally, TTX-sensitive effluxes were estimated by subtracting values obtained in the presence of TTX from those in the absence of TTX (Figure 4e). The TTX-sensitive efflux estimated for ICSS increased monophasically, with a slow rate of increase and a long duration. Taken together, these results suggest that the change in GLU

efflux during ICSS is the sum of the TTX-sensitive increase and TTX-insensitive decrease of GLU, which reflect the regulation of GLU by neurons and glial cells, respectively. In the absence of TTX, this increase and decrease in GLU negate one another, resulting in an overall slight decrease in measured GLU.

### Effect of Fluorocitrate

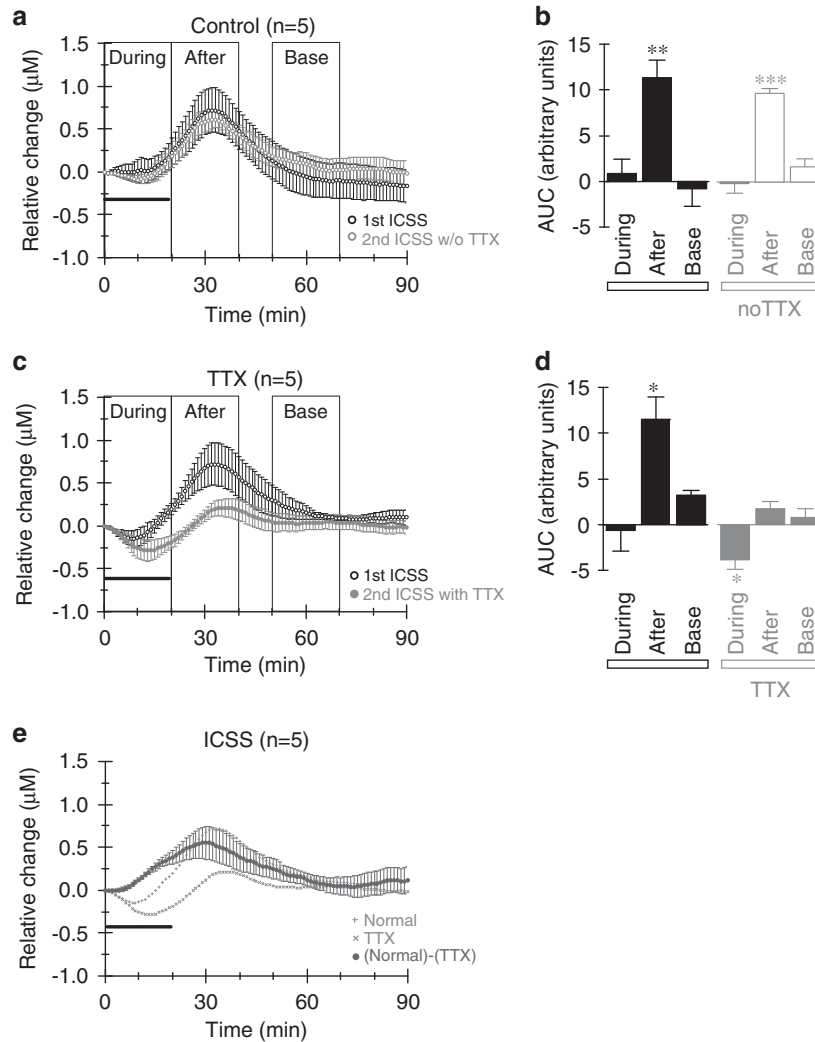
Figure 5 shows the results of the third experiment. To clarify whether glial cells have a role in the GLU efflux during ICSS, the gliotoxin fluorocitrate (500  $\mu$ M), a selective inhibitor of glial metabolism that has no effect on neuronal activity (Paulsen and Fonnum, 1989; Prosser *et al*, 1994), was administered through the dialysis probe. Pretreatment with fluorocitrate significantly blunted the post-stimulation changes in GLU efflux of the medial PFC evoked by ICSS (Figure 5a and b) (two-way ANOVA (drug treatment  $\times$  time interaction):  $F_{(2,48)} = 5.15$ ,  $p < 0.01$ ). Neither the TTX-sensitive component nor the TTX-insensitive efflux was observed. In the presence of fluorocitrate, the mean resting level of GLU in dialysate was  $12.3 \pm 0.9 \mu$ M ( $n = 4$ ), which was markedly higher than those observed ( $2.02 \pm 0.2 \mu$ M,  $n = 22$ ) in the first and second experiments ( $t = 19.0$ ,  $df = 24$ ,  $p < 0.001$ ). This result strongly suggests that a low TTX-sensitive GLU efflux level in response to ICSS would be masked by the effect of fluorocitrate.

### GLU Efflux During IMB and its Sensitivity to TTX

Figure 6 shows the results of the fourth experiment, investigating whether the change in GLU efflux in response to IMB, a state of aversion, was dependent on neuronal activity. When animals were subjected to IMB for 20 min in the absence of TTX, there was a rapid and significant increase in GLU efflux, which peaked during the stress period, followed by another sharp increase, which peaked shortly after the rats were freed from IMB, and then a gradual return to baseline (Figure 6a and b; one-way ANOVA:  $F_{(2,22)} = 17.31$ ,  $p < 0.01$ ). Thus, IMB was also associated with a bimodal pattern of GLU efflux, but the temporal pattern was clearly different from that seen during ICSS. TTX (10  $\mu$ M) added to the perfusion medium 60 min prior to the second IMB significantly decreased the GLU response to the IMB stress but maintained the bimodal temporal pattern of the response (Figure 6c and d; two-way ANOVA:  $F_{(1,4)} = 8.43$ ,  $p < 0.05$ ). The TTX-sensitive efflux estimated for the IMB stress increased monophasically, with a time course similar to that observed for ICSS (Figure 6e). Therefore, the stress-induced bimodal increase in GLU efflux consists of a TTX-sensitive monophasic increase derived from neurons and a TTX-insensitive bimodal increase derived from glial cells.

### DISCUSSION

In the present study, using a continuous GLU monitoring system, we measured the minute-by-minute change of GLU efflux in the dialysate from the medial PFC before, during, and after ICSS in the MFB. We found a bidirectional change in GLU efflux in response to ICSS characterized by an initial small decrease followed by a large increase. To the best of our



**Figure 4** Time course for the effect of tetrodotoxin (TTX) on GLU efflux in the medial PFC induced by intracranial self-stimulation (ICSS). (a) The average ( $\pm$  SEM) response from five TTX-untreated (control) animals. (c) The average ( $\pm$  SEM) response from five TTX-treated animals. (b and d) The area under the concentration–time curve (AUC) calculated for the period delimited by the thin rectangles. Statistical analysis was performed using one-way, repeated measures ANOVA followed by the least significant difference test for multiple comparisons. The F-values for the main effects were as follows. Control group:  $F_{(2,8)} = 8.83$ ,  $p < 0.01$  for the 1st ICSS;  $F_{(2,8)} = 22.33$ ,  $p < 0.001$  for 2nd ICSS. TTX group:  $F_{(2,8)} = 6.30$ ,  $p < 0.05$  for 1st ICSS;  $F_{(2,8)} = 4.91$ ,  $p < 0.05$  for 2nd ICSS. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  compared with respective basal values. (e) The time course for the estimated TTX-sensitive efflux of GLU in the medial PFC for ICSS. Thick horizontal bars indicate the duration of the stimulation (20 min). ● (green), estimated TTX-sensitive efflux of GLU; + (red), GLU response during perfusion with normal Ringer's solution; × (red), GLU response during perfusion with Ringer's solution containing TTX. ANOVA, analysis of variance; GLU, glutamate; PFC, prefrontal cortex. A full colour version of this figure is available at the *Neuropsychopharmacology* journal online.

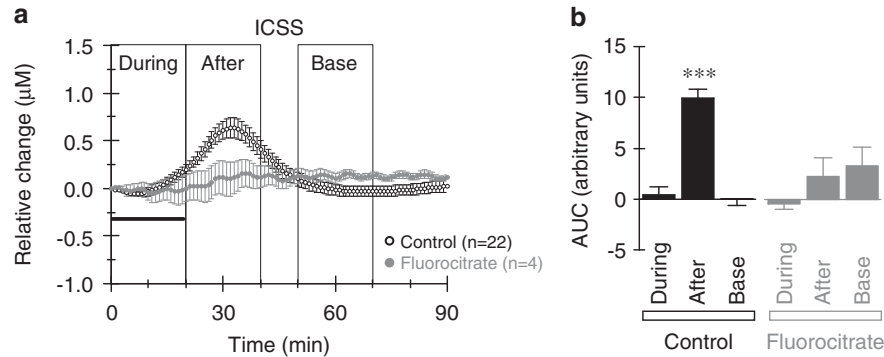
knowledge, this is the first demonstration of relatively rapid changes in GLU efflux from the medial PFC during ICSS. This bidirectional change was TTX insensitive and abolished by the gliotoxin fluorocitrate. These results clearly demonstrate that the ICSS-evoked bidirectional pattern of extracellular GLU requires the presence of functioning glia.

Because ICSS reward was associated with a decrease in extracellular GLU levels during the stimulation, which became more apparent in the presence of TTX, we predicted that aversion, a state opposite of reward, was linked to an increase in GLU levels, as described previously (Bagley and Moghaddam, 1997; Jackson and Moghaddam, 2001; Lupinsky *et al*, 2010; Lowy *et al*, 1995; Mogensen and Divac, 1993). Therefore, we investigated the minute-by-minute change in extracellular GLU in the medial PFC following

IMB stress, reflecting an aversive state. Our analysis identified a bimodal change in cortical GLU efflux in response to the stress. This change consisted of an initial rapid increase followed by a markedly larger increase, which was different from that observed during ICSS. Thus, the aversion experienced during the IMB period was associated with a TTX-independent increase in extracellular concentrations of GLU, indicating that this GLU was likely derived from glial cells, similar to the decrease of GLU detected during ICSS.

#### TTX-Insensitive GLU Efflux Induced by Reward and Aversion

The temporal patterns for the TTX-insensitive GLU efflux observed during ICSS and IMB are thought to reflect a net



**Figure 5** Time course for the effect of fluorocitrate on GLU efflux in the medial PFC induced by intracranial self-stimulation (ICSS). (a) Averaged ( $\pm$  SEM) responses from control ( $n=22$ ) and fluorocitrate-treated ( $n=4$ ) groups during ICSS. Control data were obtained from the first and second experiments. The bar indicates the stimulation duration (20 min). (b) The area under the concentration–time curve (AUC) calculated for the period delimited by the thin rectangles. The time course data were analyzed with a one-way repeated measures ANOVA followed by the least significant difference test for multiple comparisons. The F-values for the main effect were as follows. ICSS:  $F_{(2,42)}=32.07$ ,  $p<0.001$  for control group;  $F_{(2,6)}=0.94$ , NS for fluorocitrate-treated group. \*\*\* $p<0.001$  compared with the basal value. ANOVA, analysis of variance; GLU, glutamate; PFC, prefrontal cortex; NS, not significant.

sum of two opposite processes: extracellular release and uptake of GLU by high-affinity transporters in glial cells. The cystine-GLU exchanger in glial cells supplies mostly nonvesicular GLU in the extracellular space, which stimulates GLU receptors located on presynaptic and postsynaptic neurons (Baker *et al*, 2002; Kalivas, 2009). However, glial GLU transporters, primarily EAAT-2 which is almost exclusively expressed by astrocytes (Tanaka *et al*, 1997), limit GLU spilled from the synapse into the extrasynaptic compartment and restrict entry of nonvesicular GLU into the synapse (Danbolt, 2001). These release and uptake mechanisms in glial cells play active roles in the maintenance and modulation of neuronal activity and synaptic transmission (Anderson and Swanson, 2000; Persson and Ronnback, 2012). The astrocytic GLU transporter blocker dihydrokainic acid infused into the lateral ventricle reduces sensitivity to reward (anhedonia) and induces the expression of the early immediate gene *c-Fos* in various reward-related regions, including the medial PFC (Bechtholt-Gompf *et al*, 2010). In addition, blockade of astrocytic GLU uptake in the medial PFC, followed by an increase in extracellular GLU levels and neuronal activity, is sufficient to produce anhedonia (John *et al*, 2012). Taken together with these findings, our data suggest that uptake mechanisms in glial cells may differentially regulate the activity of prefrontal neurons by altering the extracellular GLU levels depending on behavioral states, such that levels decrease during ICSS and increase during IMB.

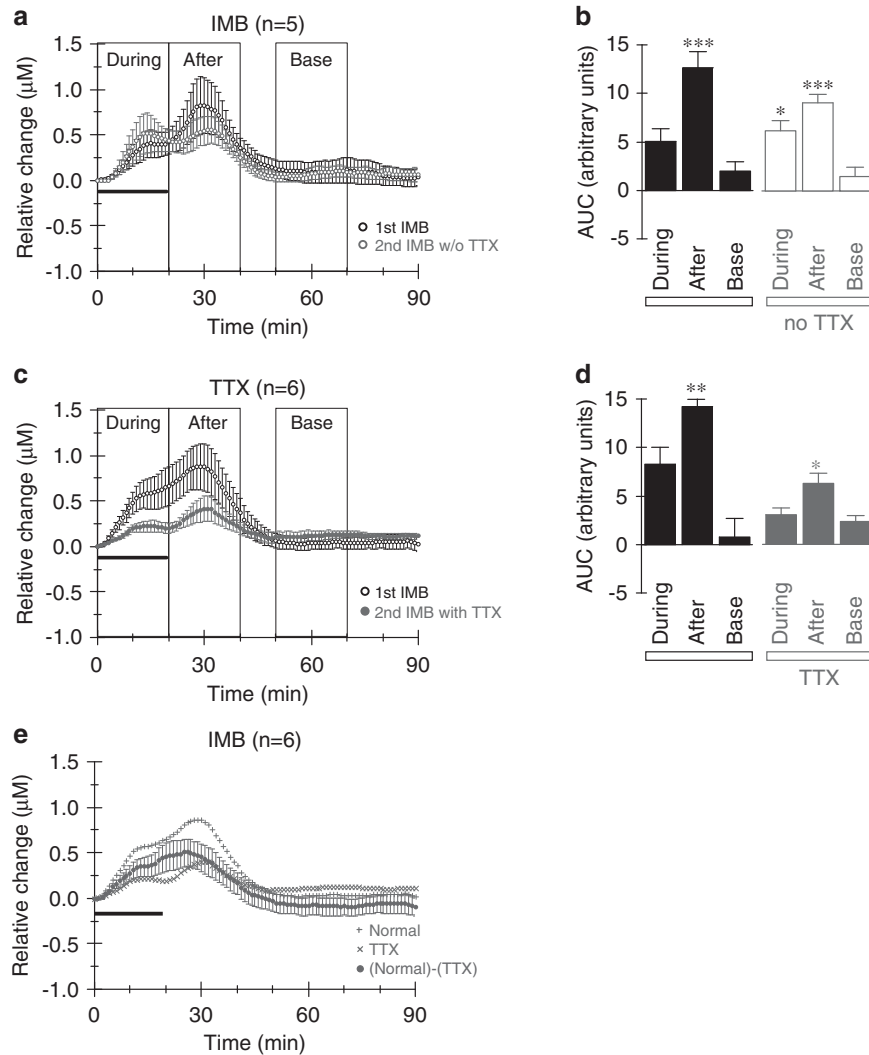
### TTX-Sensitive GLU Efflux Induced by Reward and Aversion

In contrast with the glia-derived TTX-insensitive components, neuron-derived TTX-sensitive GLU changes following ICSS and IMB were both estimated to be monophasic and similar with respect to a slow and prolonged increase. Various arousing stimuli, such as stressful stimuli and appetitive behavior, activate neural metabolism, which is reflected by increased brain temperature (Abrams and Hammel, 1964; Kiyatkin *et al*, 2002; Kiyatkin and Wise, 2001). Because the temporal dynamics are also slower and

more prolonged than changes in neuronal activity and greatly exceed the duration of stimuli, this activated metabolism may be associated with TTX-sensitive GLU changes in response to rewarding and aversive stimuli. The physiological roles of the long-lasting TTX-sensitive GLU changes are obscure and should be clarified in future studies.

### Clinical Implications

Major depression is one of the most common and debilitating mental disorders (Kulkarni and Dhir, 2009). Although most patients with depressive illness respond to combinations of drug therapy, cognitive behavioral psychotherapy, and electroconvulsive therapy, some severely depressed patients do not respond. Those individuals may be helped with deep brain stimulation (DBS) (Holtzheimer and Mayberg, 2011; Mayberg *et al*, 2005; Schlaepfer *et al*, 2008). As mentioned above, anhedonia, a core symptom of major depression, appears to be associated with dysfunction in the brain reward circuit (Nestler and Carlezon, 2006), which is predominantly comprised of the ventral tegmental area, nucleus accumbens, striatum, and PFC connected by the MFB. Intriguingly, a recent pilot study reported beneficial (anti-anhedonic) effects of DBS at the superolateral branch of the MFB, a central part of the brain reward circuit, for the treatment of therapy-resistant depression (Schlaepfer *et al*, 2013). Stimulating this region via DBS likely activated myelinated axons descending from the frontal lobe into the ventral tegmental area, which in turn exerted its activating effects on forebrain structures by way of ascending dopaminergic projections through the MFB (Schlaepfer *et al*, 2013), to ameliorate the dysfunctional reward circuit by modulating pathological neural activity. High frequency stimulation, a frequency applied for clinical use of DBS, can affect glial cells, in addition to neurons in the mouse brain, which propagate calcium waves over considerable distances (Bekar *et al*, 2008). Owing to this propagation of calcium waves in glial networks, the glial effects may spread at a distance from the stimulation site (Vedam-Mai *et al*, 2012). In the present study, we found that ICSS of the MFB induced a decrease in extracellular GLU levels via glial cells in the



**Figure 6** Time course for the effect of tetrodotoxin (TTX) on GLU efflux in the medial PFC induced by immobilization (IMB). (a) The average ( $\pm$  SEM) response from five TTX-untreated animals. (c) The average ( $\pm$  SEM) response from six TTX-treated animals. (b and d) The area under the concentration–time curve (AUC) calculated for the period delimited by the thin rectangles. The time course data were analyzed with a one-way repeated measures ANOVA followed by the least significant difference test for multiple comparisons. The F-values for the main effect were as follows. Control group:  $F_{(2,8)} = 11.3$ ,  $p < 0.01$  for 1st IMB;  $F_{(2,8)} = 10.43$ ,  $p < 0.01$  for 2nd IMB. TTX group:  $F_{(2,10)} = 7.60$ ,  $p < 0.01$  for 1st IMB;  $F_{(2,10)} = 4.47$ ,  $p < 0.05$  for 2nd IMB. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  compared with respective basal values. (e) The time course for the estimated TTX-sensitive efflux of GLU in the medial PFC for IMB animals. The thick horizontal bars indicate the duration of stimulation (20 min). ● (green), estimated TTX-sensitive GLU efflux; + (red), GLU response during perfusion with normal Ringer's solution; × (red), GLU response during perfusion with normal Ringer's solution containing TTX. ANOVA, analysis of variance; GLU, glutamate; IMB, immobilization; PFC, prefrontal cortex. A full colour version of this figure is available at the *Neuropsychopharmacology* journal online.

medial PFC, a region distant from the stimulation site. Given these findings, a beneficial (anti-anhedonic) effect on application of DBS to the MFB in patients with depressive illness might be partly explained by a normalization of increased extracellular levels of GLU caused by dysfunction of glial GLU transporters in the PFC, with the wave propagation playing a pivotal role.

### Technical Limitations

Microdialysis combined with on-line GLU analysis has a time resolution of minutes. Owing to this low temporal resolution, the method is best suited for the quantitative analysis of basal and slowly changing tonic GLU concentrations. By contrast, recently developed enzyme-based

microelectrode arrays (MEA) measure GLU concentrations on a subsecond time-scale and are best suited for examining basal and rapidly changing phasic GLU levels (Hascup *et al*, 2008; Rutherford *et al*, 2007). Therefore, electrochemical monitoring techniques such as MEA, which are alternatives to microdialysis, will enable us to identify and explore more rapid changes in GLU signals that may occur during ICSS.

In conclusion, in the present study, which used a microdialysis-based on-line GLU monitoring system, we demonstrated, for the first time, a bidirectional regulation of extracellular GLU by glial cells in the medial PFC as a potential mechanism that influences the rewarding effects of ICSS. We also provided a basis to further the understanding of how prefrontal glial mechanisms can regulate rewarding and aversive states of an animal.



## FUNDING AND DISCLOSURE

This study was supported by Grants-in-Aid for Scientific Research, the Ministry of Education, Culture, Sports, Science and Technology of Japan (Nos. 08610078, 09410024). The authors declare no conflict of interest.

## ACKNOWLEDGMENTS

We thank Dr Kazue Semba for valuable comments on the manuscript, Dr Ryuko Ohashi for assistance during the experiment, and Ms Shino Sugiyama for secretarial assistance.

## REFERENCES

- Abrams R, Hammel HT (1964). Hypothalamic Temperature in Unanesthetized Albino Rats during Feeding and Sleeping. *Am J Physiol* **206**: 641–646.
- Anderson CM, Swanson RA (2000). Astrocyte glutamate transport: review of properties, regulation, and physiological functions. *Glia* **32**: 1–14.
- Bagley J, Moghaddam B (1997). Temporal dynamics of glutamate efflux in the prefrontal cortex and in the hippocampus following repeated stress: effects of pretreatment with saline or diazepam. *Neuroscience* **77**: 65–73.
- Baker DA, Xi ZX, Shen H, Swanson CJ, Kalivas PW (2002). The origin and neuronal function of in vivo nonsynaptic glutamate. *J Neurosci* **22**: 9134–9141.
- Banasr M, Chowdhury GM, Terwilliger R, Newton SS, Duman RS, Behar KL et al (2010). Glial pathology in an animal model of depression: reversal of stress-induced cellular, metabolic and behavioral deficits by the glutamate-modulating drug riluzole. *Mol Psychiatry* **15**: 501–511.
- Banasr M, Duman RS (2008). Glial loss in the prefrontal cortex is sufficient to induce depressive-like behaviors. *Biol Psychiatry* **64**: 863–870.
- Bechtolt-Gompf AJ, Walther HV, Adams MA, Carlezon WA Jr, Ongur D, Cohen BM (2010). Blockade of astrocytic glutamate uptake in rats induces signs of anhedonia and impaired spatial memory. *Neuropsychopharmacology* **35**: 2049–2059.
- Bekar LK, He W, Nedergaard M (2008). Locus coeruleus alpha-adrenergic-mediated activation of cortical astrocytes in vivo. *Cereb Cortex* **18**: 2789–2795.
- Choudary PV, Molnar M, Evans SJ, Tomita H, Li JZ, Vawter MP et al (2005). Altered cortical glutamatergic and GABAergic signal transmission with glial involvement in depression. *Proc Natl Acad Sci USA* **102**: 15653–15658.
- Cotter D, Mackay D, Chana G, Beasley C, Landau S, Everall IP (2002). Reduced neuronal size and glial cell density in area 9 of the dorsolateral prefrontal cortex in subjects with major depressive disorder. *Cereb Cortex* **12**: 386–394.
- Danbolt NC (2001). Glutamate uptake. *Prog Neurobiol* **65**: 1–105.
- D'Suza MS, Markou A (2010). Neural substrates of psychostimulant withdrawal-induced anhedonia. In: Self DW, Staley JK (eds) *Behavioral Neuroscience of Drug Addiction: Current Topics in Behavioral Neuroscience* 3. Springer-Verlag: Berlin Heidelberg-Berlin Heidelbergpp 119–178.
- Eshel N, Roiser JP (2010). Reward and punishment processing in depression. *Biol Psychiatry* **68**: 118–124.
- Fillenz M (1995). Physiological release of excitatory amino acids. *Behav Brain Res* **71**: 51–67.
- Hashimoto K, Sawa A, Iyo M (2007). Increased levels of glutamate in brains from patients with mood disorders. *Biol Psychiatry* **62**: 1310–1316.
- 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.
- Haydon PG, Carmignoto G (2006). Astrocyte control of synaptic transmission and neurovascular coupling. *Physiol Rev* **86**: 1009–1031.
- Holtzheimer PE, Mayberg HS (2011). Deep brain stimulation for psychiatric disorders. *Annu Rev Neurosci* **34**: 289–307.
- Jackson ME, Moghaddam B (2001). Amygdala regulation of nucleus accumbens dopamine output is governed by the prefrontal cortex. *J Neurosci* **21**: 676–681.
- John CS, Smith KL, Van't Veer A, Gompf HS, Carlezon WA Jr, Cohen BM et al (2012). Blockade of astrocytic glutamate uptake in the prefrontal cortex induces anhedonia. *Neuropsychopharmacology* **37**: 2467–2475.
- Kalivas PW (2009). The glutamate homeostasis hypothesis of addiction. *Nat Rev Neurosci* **10**: 561–572.
- Kiyatkin EA, Brown PL, Wise RA (2002). Brain temperature fluctuation: a reflection of functional neural activation. *Eur J Neurosci* **16**: 164–168.
- Kiyatkin EA, Wise RA (2001). Striatal hyperthermia associated with arousal: intracranial thermorecordings in behaving rats. *Brain Res* **918**: 141–152.
- Kulkarni SK, Dhir A (2009). Current investigational drugs for major depression. *Expert Opin Investig Drugs* **18**: 767–788.
- Lowy MT, Wittenberg L, Yamamoto BK (1995). Effect of acute stress on hippocampal glutamate levels and spectrin proteolysis in young and aged rats. *J Neurochem* **65**: 268–274.
- Lupinsky D, Moquin L, Gratton A (2010). Interhemispheric regulation of the medial prefrontal cortical glutamate stress response in rats. *J Neurosci* **30**: 7624–7633.
- Mayberg HS, Lozano AM, Voon V, McNeely HE, Seminowicz D, Hamani C et al (2005). Deep brain stimulation for treatment-resistant depression. *Neuron* **45**: 651–660.
- McEwen AM, Burgess DT, Hanstock CC, Seres P, Khalili P, Newman SC et al (2012). Increased glutamate levels in the medial prefrontal cortex in patients with postpartum depression. *Neuropsychopharmacology* **37**: 2428–2435.
- Mineur YS, Picciotto MR, Sanacora G (2007). Antidepressant-like effects of ceftriaxone in male C57BL/6J mice. *Biol Psychiatry* **61**: 250–252.
- Mogensen J, Divac I (1993). Behavioural changes after ablation of subdivisions of the rat prefrontal cortex. *Acta Neurobiol Exp (Wars)* **53**: 439–449.
- Moghaddam B, Jackson M (2004). Effect of stress on prefrontal cortex function. *Neurotox Res* **6**: 73–78.
- Nakahara D, Ozaki N, Nagatsu T (1993). In vivo microdialysis of neurotransmitters and their metabolites. In: SH P, Naoi M, T N, S P (eds) *Methods in Neurotransmitters and Neuropeptide Research*. Elsevier: AmsterdamAmsterdamp 219–248.
- Nestler EJ, Carlezon WA Jr (2006). The mesolimbic dopamine reward circuit in depression. *Biol Psychiatry* **59**: 1151–1159.
- Obrenovitch TP, Sarna GS, Millan MH, Lok SY, Kawachi M, Ueda Y et al (1990). Intracerebral dialysis with on-line enzyme fluorometric detection: a novel method to investigate the changes in the extracellular concentration of glutamic acid. In: Kriegstein J, Oberpichler H (eds) *Pharmacology of Cerebral Ischemia 1990*. Wiss-Verlag: Stuttgart, Germanypp 23–31.
- Oh DH, Oh D, Son H, Webster MJ, Weickert CS, Kim SH (2014). An association between the reduced levels of SLC1A2 and GAD1 in the dorsolateral prefrontal cortex in major depressive disorder: possible involvement of an attenuated RAF/MEK/ERK signaling pathway. *J Neural Transm* **121**: 783–792.
- Paulsen RE, Fonnum F (1989). Role of glial cells for the basal and Ca<sup>2+</sup>-dependent K<sup>+</sup>-evoked release of transmitter amino acids investigated by microdialysis. *J Neurochem* **52**: 1823–1829.

- Paxinos G, Watson C (1986). *The Rat Brain in Stereotaxic Coordinates* Academic: New York, NY, USA.
- Persson M, Ronnback L (2012). Microglial self-defence mediated through GLT-1 and glutathione. *Amino Acids* **42**: 207–219.
- Prosser RA, Edgar DM, Heller HC, Miller JD (1994). A possible glial role in the mammalian circadian clock. *Brain Res* **643**: 296–301.
- Rada P, Tucci S, Murzi E, Hernández L (1997). Extracellular glutamate increases in the lateral hypothalamus and decreases in the nucleus accumbens during feeding. *Brain Res* **768**: 338–340.
- Rajkowska G (2000). Postmortem studies in mood disorders indicate altered numbers of neurons and glial cells. *Biol Psychiatry* **48**: 766–777.
- Reznikov LR, Grillo CA, Piroli GG, Pasumarthi RK, Reagan LP, Fadel J (2007). Acute stress-mediated increases in extracellular glutamate levels in the rat amygdala: differential effects of antidepressant treatment. *Eur J Neurosci* **25**: 3109–3114.
- Rutherford EC, Pomerleau F, Huettl P, Strömberg 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.
- Saulskaya NB, Mikhailova MO (2002). Feeding-induced decrease in extracellular glutamate level in the rat nucleus accumbens: dependence on glutamate uptake. *Neuroscience* **112**: 791–801.
- Schlaepfer TE, Bewernick BH, Kayser S, Mädler B, Coenen VA (2013). Rapid effects of deep brain stimulation for treatment-resistant major depression. *Biol Psychiatry* **73**: 1204–1212.
- Schlaepfer TE, Cohen MX, Frick C, Kosel M, Brodesser D, Axmacher N et al (2008). Deep brain stimulation to reward circuitry alleviates anhedonia in refractory major depression. *Neuropsychopharmacology* **33**: 368–377.
- Steciuk M, Kram M, Kramer GL, Petty F (2000). Immobilization-induced glutamate efflux in medial prefrontal cortex: blockade by (+)-Mk-801, a selective NMDA receptor antagonist. *Stress* **3**: 195–199.
- Takita M, Kawashima T, Kaneko H, Suzuki SS, Yokoi H (2002). Sensitization of glutamate release and N-methyl-D-aspartate receptor response by transient dopamine pretreatment in prefrontal cortex of rats. *Neurosci Lett* **317**: 97–100.
- Tanaka K, Watase K, Manabe T, Yamada K, Watanabe M, Takahashi K et al (1997). Epilepsy and exacerbation of brain injury in mice lacking the glutamate transporter GLT-1. *Science* **276**: 1699–1702.
- Timmerman W, Westerink BH (1997). Brain microdialysis of GABA and glutamate: what does it signify? *Synapse* **27**: 242–261.
- Vedam-Mai V, van Battum EY, Kamphuis W, Feenstra MG, Denys D, Reynolds BA et al (2012). Deep brain stimulation and the role of astrocytes. *Mol Psychiatry* **17**: 124–131.
- You ZB, Chen YQ, Wise RA (2001). Dopamine and glutamate release in the nucleus accumbens and ventral tegmental area of rat following lateral hypothalamic self-stimulation. *Neuroscience* **107**: 629–639.
- Zink M, Vollmayr B, Gebicke-Haerter PJ, Henn FA (2010). Reduced expression of glutamate transporters vGluT1, EAAT2 and EAAT4 in learned helpless rats, an animal model of depression. *Neuropharmacology* **58**: 465–473.

Supplementary Information accompanies the paper on the Neuropsychopharmacology website (<http://www.nature.com/npp>)