Transcranial photoacoustic imaging
of NMDA-evoked focal circuit dynamics in rat hippocampus

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Abstract

Transcranial functional photoacoustic (fPA) voltage-sensitive dye (VSD) imaging promises to overcome current temporal and spatial limitations of current neuroimaging modalities. The technique previously distinguished global seizure activity from control neural activity in groups of rats. To validate the focal specificity of transcranial fPA neuroimaging *in vivo*, we now present proofs-of-concept that the results differentiate between low- and high-dose N-methyl-D-aspartate (NMDA) evoked neural activity in rat hippocampus. Concurrent quantitative EEG (qEEG) and microdialysis recorded real-time circuit dynamics and glutamate concentration change, respectively. We hypothesized that location-specific fPA VSD contrast would identify the neural dynamics in hippocampus with the correlation to NMDA evoked focal glutamate release and time-specific EEG signals. To test the hypothesis, we infused 0.3 to 3.0 mM NMDA at 2 μl/min over 60 min via an implanted microdialysis probe. The dialysate samples collected every 20 min during the infusion were analyzed for focal changes in extracellular glutamate release. Transcranial fPA VSD imaging provided NMDA-evoked VSD responses with positive correlation to extracellular glutamate concentration change at the contralateral side of the microdialysis probe. The graded response represents the all-or-none gating system of the dentate gyrus (DG) in hippocampus. Quantitative EEG (qEEG) successfully confirmed induction of focal seizure activity during NMDA infusion. We conclude that transcranial fPA VSD imaging distinguished graded DG gatekeeping functions, based on the VSD redistribution mechanism sensitive to electrophysiologic membrane potential. The results suggest the potential future use of this emerging technology in clinics and science as an innovative and significant functional neuroimaging modality.

Introduction

59 Whereas electrophysiological and invasive neurochemical techniques have been valuable in assessing
60 activity within specific neural circuits in brain, there is a distinct need for a non-invasive means to
61 image real-time regional functional activity in deep brain *in vivo*. The hippocampal circuit has rows
62 of excitatory pyramidal neurons all aligned in close proximity with similar orientation. The dentate
63 gyrus (DG) is classically considered as a gate keeper for the propagation of excitatory activity into
64 the hippocampus as the excitatory neurons of the DG are less excitable than other types of
65 hippocampal neurons. Therefore, low amplitude excitatory inputs do not generate action potentials in
66 DG neurons and fail to open the DG gate [1]. In contrast to sub-threshold excitatory stimulus, strong
67 repetitive excitatory inputs can break the DG gate and induce seizure activity [1]. This all-or-none
68 gating function of the DG is consistent with the hippocampus being the lowest seizure threshold
69 region of the mammalian brain.

70   Previous optical imaging approaches to local cortical circuits [2-4] applied invasive procedures
71 to overcome superficial imaging depth, and further extension to deeper brain regions such as the
72 hippocampus was prohibitive. The imaging of electrophysiological or neurochemical dynamics
73 within the hippocampus primarily has been accomplished by optical imaging of freshly-sliced brain
74 tissue [5] or magnetic resonance imaging spectroscopy (MRS) for non-invasive quantification of
75 glutamate at high spatial resolution [6,7]. However, optical neuroimaging and MRS suffer from shallow
76 imaging depth and from slow imaging speed, respectively. The application of two-photon
77 microscopy to measure calcium ion dynamics in specific deep brain structures, including
78 hippocampus, has poor temporal resolution [8]. Therefore, non-invasive, real-time neuroimaging
79 modality having a high spatiotemporal resolution would be a significant advance, but such an
80 approach would require neurochemical and electrophysiological characterization to validate the
81 neural activity.

82   Photoacoustic (PA) imaging is a hybrid approach combining optics and acoustics where the
83 signal corresponding to the neural activity is detected in the form of acoustic transcranial imaging
84 with optical absorbance as an image contrast [9,10]. PA imaging is based on thermo-elastic perturbation
85 of a target evoked by light absorbance from a pulsed laser illumination, which generates radio-
86 frequency (RF) acoustic pressure waves detected by piezoelectric ultrasound transduction or optical
87 interferometry. With this unique imaging mechanism, several attractive applications have been
88 proposed for preclinical and clinical research with tomographic and microscopic imaging modes,
89 including detection of endogenous contrast of cancer indicators, e.g., melanoma [11], breast
90 microcalcifications [12-14], monitoring of cancer angiogenesis [15], oxygen metabolism [16], and
91 quantification of lipid content [17,18], among others. Recently, we presented the functional PA (fPA)
92 neuroimaging of near-infrared VSD redistribution mechanism differentiating the graded membrane
93 potential in lipid vesicle model and chemo-convulsant seizure in rodent brain *in vivo* [19].

94   Here, we validated the transcranial fPA neuroimaging of hippocampal electrophysiology
95 activated by stimulation of N-methyl-D-aspartate (NMDA) receptors in rat brain *in vivo* through
96 intact skull and scalp. The tri-modal neural sensing approach utilized microdialysis, quantitative
97 electroencephalography (qEEG), and transcranial fPA neuroimaging to simultaneously monitor
98 electrophysiological changes associated with glutamatergic neurotransmission (Figure 1). To
99 stimulate the hippocampus, we infused NMDA by reverse microdialysis that triggered localized
100 changes of extracellular glutamate concentration. We quantified the change of extracellular glutamate
101 concentration evoked by reverse microdialysis by direct microdialysis and high-performance liquid
102 chromatography (HPLC). We combined fPA neuroimaging and qEEG with microdialysis to evaluate

103   the effects associated with graded DG gatekeeping at hippocampus, in the presence of glutamatergic
104   excitation. Concurrently, the electrophysiological signatures were captured with transcranial fPA
105   neuroimaging. Each of the three modalities thus provided information with different degrees of
106   specificity, including spatiotemporal specificity from fPA imaging, temporal specificity from qEEG,
107   and neurochemical specificity from microdialysis in the target neural circuit.

108

Results

110   **Transcranial fPA neuroimaging of rat hippocampus.** Transcranial fPA neuroimaging of
111   hippocampal circuit dynamics was performed during NMDA infusion at rat hippocampus (Figure 2).
112   The representative fPA sagittal planes obtained at 790 nm presented a sufficient sensitivity at the
113   depths of interest, i.e., 3.6 mm, at the contralateral position to the microdialysis probe (Figure 3a).
114   The sagittal PA imaging plane of hippocampus revealed the cross-sections of transverse sinus and
115   inferior cerebral vein in the superficial depth range (Figure 3b). From the temporal dimension, time-
116   averaged VSD responses were reconstructed pixel-by-pixel for pre-injection phase (-10 – 0 min)
117   followed by the results for time bins in 10 min interval: 0 – 10 min, 10 – 20 min, and 20 – 30 min.
118   Note that the total recording duration (40 min) was limited by internal memory of the fPA
119   neuroimaging system. Figure 3c shows the representative VSD responses in hippocampal cross-
120   sections collected during 0.3 mM and 3.0 mM NMDA infusion. As a result, the hippocampus with
121   0.3 mM NMDA infusion did not present such a significant change ($n = 3$), which implies a failure to
122   overcome the high activation threshold of DG gatekeeping with 0.3 mM NMDA infusion. Otherwise,
123   the substantial amount of circuit dynamics was detected at hippocampus with 3.0 mM NMDA
124   infusion ($n = 6$). In the 3.0 mM NMDA infusion group, the peak VSD responses were presented
125   during the first or second 10 min durations, and their peak intensities corresponds to the maximal
126   glutamate concentration change measured in the microdialysis, e.g., 734.48 % and 493.91 % (center
127   and right columns in Figure 3c). Further quantitative multi-modal correlation will be given in the
128   following subsections.

129   **NMDA-evoked glutamatergic neurotransmission.** Intrahippocampal infusion of NMDA diluted
130   into artificial cerebrospinal fluid at the required concentrations was performed through the
131   microdialysis probe. Concentrations of glutamate release in the dialysate were calculated from
132   values of three baseline samples collected at 20 min intervals before NMDA infusion. During
133   infusion into hippocampus (bregma -3.8 mm, lateral 3 mm, depth 3.6 mm; Figure 4a), samples
134   continued to be collected every 20 min for the remainder of the study. Glutamate release was
135   calculated as a % of basal values. Figure 4b shows the effect of NMDA infusion on
136   glutamate release in the hippocampus of anesthetized rats. The results demonstrate the dose-related
137   response of NMDA infusion at 0.3 ($n = 3$), 1.0 ($n = 1$), and 3.0 mM ($n = 6$) into the hippocampus,
138   where 0.3 mM NMDA caused a 17.93±19.05 % increase in glutamate levels that remained elevated
139   for the duration of the infusion; the 1.0-mM NMDA infusion appears to cause doubling of the
140   glutamate levels in the hippocampus compared to baseline (i.e., 112.42 %). This increase observed at
141   the 1.0-mM remained elevated through to the end of the NMDA infusion. However, at 3.0-mM, the
142   NMDA infusion raised the glutamate level up to 392.99±250.54 %, above baseline level, which
143   peaked during the second 20-min sampling period.

144   **EEG of electrophysiological activity in hippocampus.** The DG is a hippocampal region
145   specifically subjected to a barrage of excitatory inputs. However, the majority of excitatory activity
146   does not propagate through the DG and into the hippocampus as the DG performs a gating function [1].

147   Excessive activation of the DG disrupts its gating function and induces acute seizures in naïve
148   animals [1]. Hippocampal infusion of 3.0 mM NMDA induced a maximal VSD and significant rise in
149   extracellular glutamate concentration, suggesting a potential break in the DG gate that may lead to
150   acute seizure activity. We therefore utilized qEEG during the same hippocampal 3.0 mM NMDA
151   infusion protocol to identify if the maximal VSD response and extracellular glutamate concentrations
152   were associated with significant electrophysiological activity, a tri-modal approach that validates the
153   temporal and spatial resolution of fPA imaging during focal NMDA infusion.

154   In patients with focal epilepsy, gamma and theta activity from scalp EEG are an indicator of
155   the seizure onset zone and ictal onset [20,21]. Here, the temporal specificity of qEEG recorded circuit
156   responses in real-time, before and after focal NMDA infusion. The 3.0 mM NMDA infusion into the
157   hippocampal circuit induced focal seizure activity recorded on qEEG (Figure 4c black asterisk and
158   S1). When NMDA was delivered to the hippocampal circuit by the microdialysis probe (Figure 4a)
159   [22], a significant change in qEEG spectral power was presented, especially in the gamma range (Figure
160   4c). In the theta range, spectral power increased during the seizure (Figure 4d) further supporting the
161   identification of the focal seizure activity. The seizure trace on EEG demonstrated both a gamma and
162   theta component (Figure 4f). Importantly, the ictal event occurred during the same temporal window
163   as the maximum VSD response and the greatest increase in extracellular glutamate concentration.
164   The dose-related VSD and extracellular glutamate concentration response with qEEG suggest that the
165   3.0-mM NMDA infusion resulted in a break in the DG gate associated with focal seizure activity.

166   However, qEEG lacks spatial resolution, and recordings from deep brain structures cannot be
167   isolated without implantation of invasive depth electrodes. Therefore, in order to maintain the tri-
168   modal experimental paradigm we opted not to place depth electrodes. We made the stimulation
169   focal, according to the previous proof-of-concept results by fPA detection onset of generalized
170   seizures through an intact skull and scalp [19].

171   **Quantitative multi-modal correlation.** The fPA VSD responses at the contralateral side to the
172   microdialysis probe (blue dots in Figure 3c) were measured and plotted as a function of the
173   corresponding changes in extracellular glutamate concentration (Figure 5). Note that the regions-of-
174   interest ROIs to quantify the VSD responses were in 1 x 1 mm^2 size in the sagittal cross-section of rat
175   hippocampus. The reference phase for VSD response quantification was obtained from the baseline
176   phase: 5 – 10 min. In fPA imaging, 3.0 mM NMDA infusion at hippocampus yielded significant
177   elevation of VSD response: 0.08±0.35, 2.24±1.06, and 1.17±1.56 for baseline (-10 – 0 min), NMDA1
178   (0 – 20 min), and NMDA2 (20 – 30 min) phases, respectively (Figure S2a). The increase of
179   glutamate concentration change was correspondingly presented from -4.67±4.48 %, 369.75±254.91
180   %, to 392.99±250.54 %. In addition, when re-analyzing the data for -50 – 100 %, 100 – 500 %, and
181   500 – 1,000 % bins of fractional glutamate concentration change, high positive correlation was
182   obtained with VSD responses: 0.38±0.55 (n_{sample} = 9), 0.97±1.40 (n_{sample} = 5), and 3.17±0.32 (n_{sample}
183   = 4) for 11.04±29.60 %, 279.85±141.64 %, to 666.49±73.63 % of glutamate concentration changes
184   (red lines in Figure 5). The goodness of fit (R^2) among the mean values was 0.95 with slope and y-
185   intercept at 0.12 and 0.00, respectively. The qEEG found the increased bursts of gamma power
186   during 3.0 mM NMDA infusion, reaching up to ~261 % increase with a focal seizure (Figure 4c and
187   S1).   Otherwise, focal 0.3 mM NMDA infusion at hippocampus did not presented any circuit
188   activity increase statistically significant. The VSD responses were -0.11±0.02, -0.15±1.71, and -
189   0.07±0.64 in baseline, NMDA1, and NMDA2 phases, respectively, which are correlated to the low
190   glutamate concentration changes in hippocampus: -1.65±6.90 %, 3.90±10.41 %, and 17.93±19.05 %
191   (Figure S2b). Also, the VSD responses presented strong positive correlation when re-analyzed in -10
192   – 0 %, 0 – 10 %, and 10 – 50 % bins of fractional glutamate concentration changes: -1.00±1.00,

193   0.10 ± 0.35, and 0.58 ± 0.48 ($n_{sample} = 3$ for each) with -6.84 ± 3.38 %, 4.43 ± 4.05 %, and 22.59 ± 11.64 %
194   of fractional glutamate concentration changes, respectively (blue lines in Figure 5). The R^2 was 0.88
195   with slope and y-intercept at 0.05 and -0.46, respectively.

196   **Brain histology.** Brain tissue was extracted after all *in vivo* experimental protocols. The brain was
197   frozen-sectioned into 300-μm thickness slices and evaluated to confirm probe placement using the
198   hemorrhage caused by the microdialysis probe insertion for the NMDA infusion and collections as a
199   marker. Figure 6a shows the bright-field images of the coronal plane of the hippocampus at -3.8 mm
200   from bregma, respectively. White arrows indicate the hemorrhage caused by the microdialysis probe
201   positioned at the following coordinates in hippocampus: bregma -3.8 mm, lateral 3 mm, and depth
202   3.6 mm. The VSD staining of brain tissue was confirmed with near-infrared fluorescence
203   microscopy. Uniform VSD fluorescence was found in the VSD perfusion animal, while negative
204   control (VSD-) presented negligible fluorescence emission (Figure 6b), which again confirms the
205   results in our previous publication [23].

206

Discussion

208   The application of fPA neuroimaging was expanded to neuroscience [24-26] as label-free transcranial
209   fPA imaging of neurovascular coupling proposed as a means to quantify hemodynamic changes [27,28].
210   However, this approach does not yield quantitative neural activities that directly correspond to
211   electrical activity. Hemoglobin provides an effective contrast signal in fPA neuroimaging, but the
212   neurovascular coupling in brain is comparatively slow compared to electrophysiological neural
213   activities.   Instead, there has been extensive investigations into more effective exogenous contrast
214   agents [29-33]. This approach has enabled several neuroimaging approaches with functional voltage
215   sensors. Deán-Ben et al. showed real-time PA tomography of a genetically encoded calcium
216   indicator, GCaMP5G, using zebrafish *in vivo* [34]. Sheryl Roberts, et al. also proposed a new
217   metallochromic calcium sensor for PA imaging (CaSPA) which has a high extinction coefficient, a
218   low quantum yield, and high photo-bleaching resistance for brain and heart imaging [35]. Ruo et al.
219   reported PA imaging of neural activity evoked by electrical stimulation and 4-aminopyridine-induced
220   epileptic seizures using hydrophobic anions such as dipicrylamine (DPA) in mouse brain [36].
221   However, these voltage sensors requires PA imaging at the visible spectral range (488 nm and 530
222   nm for GCaMP5G; 550 nm for CaSPA; 500 nm and 570 nm for DPA), which are suboptimal when
223   imaging deep brain such as hippocampus positioned at 5 mm – 8 mm depth including intact scalp and
224   cortex in rat [22,37].

225   Recently, we proposed transcranial fPA recordings of brain activity *in vivo* with a near-
226   infrared VSD, delivered through the blood-brain barrier (BBB) via pharmacological modulation, as a
227   promising tool to transcend optical neuroimaging limitations, particularly as it relates to sensing
228   depth [19,23,38]. The studies demonstrated that transcranial fPA neuroimaging distinguishes *in vivo*
229   seizure activity in stimulated rat brains from that of control groups in real time. However, the results
230   were limited by the use of a global chemo-convulsant, causing perturbation across the entire brain
231   caused by the intraperitoneal administration of penetylenetetrazole (PTZ). In this paper, we presented
232   follow-on advances in fPA VSD neuroimaging by focal neural stimulation of heterogeneous neural
233   circuits, with concomitant validations from qEEG and glutamate quantification using microdialysis,
234   respectively.   The set of experiments described here yield key findings as follows: (1) The
235   microdialysis-dependent low- and high-dose NMDA infusion into the central nervous system (CNS)
236   lead to a wide range of focal extracellular glutamate concentration increase in hippocampus up to

237 ~800 %. (2) The neurochemical response (microdialysis) was well-correlated to the phenotypes in the
238 electrophysiological sensing (qEEG). The NMDA activation in the hippocampus triggers an all-or-
239 none type of circuit dynamics that lead to the initiation of a focal seizure in the hippocampal circuit.
240 (3) Transcranial fPA neuroimaging data successfully identified hot spots of focal NMDA receptor
241 activation, as presented in the qEEG recordings. The hippocampal circuitry provided the proportional
242 excitation of glutamatergic neurotransmission with concomitant NMDA infusion. The DG of the
243 hippocampus is positioned as a gatekeeper to regulate the vast excitatory cortical inputs from
244 propagating into the hippocampus [1]. Characteristically, the DG displays a high activation threshold; a
245 trait that is mediated by its profuse innervation by inhibitory GABAergic neurons and relatively
246 hyperpolarized resting membrane potential of its pyramidal neurons [39]. In epilepsy the DG fails to
247 gate the propagation of excitatory inputs into the hippocampus, resulting in overexcitation and
248 seizures. In naïve animals, *in vivo* optogenetic activation of the DG disrupts its gating function and
249 induces seizures that increase in severity depending on the duration of the stimulus [1]. In this study,
250 the disruption of DG gating by strong stimuli has been clearly demonstrated by utilizing the fPA
251 VSD neuroimaging techniques during focal NMDA infusion.

252 Further investigations are required to advance our current perspectives available with tri-
253 modal sensing, including fPA, qEEG, and microdialysis. Glutamate produces fast-rising brief
254 depolarizations in pyramidal neurons. Therefore, the use of fPA neuroimaging will enable us to more
255 precisely assess glutamate and GABA dynamics in order to formulate a more complete profile of
256 circuit activation. Once homeostasis is disrupted, neuronal activity is sensitive to changes both of
257 excitatory and inhibitory mechanisms. Faster neurochemical recording is another approach that may
258 prove useful in assessing the impact of these measures. Although microdialysis successfully
259 provided quantitative, focal neurochemical concentrations, the sampling rate of 1 sample per 20 min
260 was slow. Techniques offering faster temporal resolution may allow more meaningful comparison of
261 the neurochemical changes yielded by microdialysis and the electrophysiological events monitored
262 by qEEG and fPA neuroimaging [40]. One such technique, using custom built hardware and the
263 Amplex Red method, achieved fluorescence-based quantification of glutamate from samples taken
264 every 5 seconds, though reliability appeared to be limited when higher glutamate concentrations were
265 measured [41]. Implantable glutamate biosensors allow sub-second readouts of neurochemical
266 concentrations. However, current limitations include sensitivity, selectivity, and high cost; recent
267 developments in materials, effective modeling, and sensor design may soon alleviate some of these
268 limitations [42,43].

269 From the results, transcranial fPA neuroimaging was able to differentiate the circuit activity
270 defined with qEEG and microdialysis. However, future developments should serve to further advance
271 the efficacy of the fPA neuroimaging in neuroscience. (1) We expect that improved signal processing
272 for extracting neural activity from the ubiquitous blood context will enable better characterization of
273 brain function. The present *in vivo* experiments confirmed the possibility of background suppression,
274 as also presented in our previous study [19]. Enhanced signal processing and/or use of multi-spectral
275 wavelengths may allow significantly improved spectral differentiation of electrophysiological
276 activities in the brain at higher temporal resolution, leading to development of novel quantitative
277 metrics for real-time brain activity measures. Having isotropic resolution with 2-D PA probes would
278 be also an interesting direction to pursue as a follow up to the present work. The use of 2-D PA probe
279 would not only allow real-time volumetric information, but also enable the suppression of off-axis
280 interference. Even though we presented that neural activity can be successfully discerned with our
281 current 1-D PA probe, its sensitivity might be affected by off-axis interferences especially from the
282 elevation direction because of the limited acoustic lens focusing at a fixed depth. The neuroimaging
283 using 2-D PA probe would reject those interferences by the advanced electrical beamforming

284   capability in axial, lateral, and elevation directions. Having an improved PA imaging system would
285   provide significant breakthrough in terms of spatiotemporal resolution in fPA neuroimaging. Even
286   though our current laser system yields both 4 fps (frame-per-second) of temporal resolution and PA
287   signal sensitivity at rat hippocampus, further optimization of temporal resolution would provide finer
288   spatiotemporal specificity. On the other hand, we consider employing larger animal model for this
289   fPA VSD neuroimaging research. Pig models have been an ideal subject to pave the way to human
290   translation of neuroengineering technologies, thanks to their analogous brain structure and
291   physiology and scalp and skull thicknesses to those in humans, with alleviated ethical issues [44]. We
292   already validated the transcranial fPA neuroimaging in the pig model, and will continue to pursue
293   this research direction [45].

294   In all, the transcranial fPA neuroimaging at hippocampus in *in vivo* rat brain was successfully
295   correlated with electrophysiologic and neurochemical measurements using qEEG and microdialysis:
296   focal NMDA infusion triggers glutamate release that excites the neural circuit, and at threshold doses
297   it causes runaway excitation in the hippocampus by overcoming DG gating. This is reflected in the
298   lower seizure threshold of the hippocampus. Therefore, the transcranial fPA neuroimaging is a
299   promising technology for the visualization of focal neural events in real time.

300

Material and Methods

302   **Animal preparation.** For the proposed *in vivo* experiments, 8-9-week-old male Sprague Dawley rats
303   (Charles Rivers Laboratory, Inc., MA, United States) weighing 275-390g were used. The use of
304   animals for the proposed experimental protocol was approved by the Institutional Animal Care and
305   Use Committee of Johns Hopkins Medical Institute (RA16M225). Rats were housed in groups of 3
306   per cage with free access to food and water and maintained on a 12hr light / 12hr dark cycle.

307   On the day of the study the rats were weighed and anesthetized with urethane (1.2mg/kg,).
308   Urethane was given incrementally with alternating intra-peritoneal (ip) and subcutaneous (sc) dosing.
309   Three (3) ml of isotonic saline was given sc on each side of the body to keep the animal hydrated
310   during the experimental procedure. Body temperature was maintained until animal was fully
311   anesthetized and ready for surgery. For fPA and qEEG studies, an iv catheter was inserted into a tail
312   vein prior to surgery for dye administration during the studies. Once a stable plane of anesthesia was
313   established, hair was shaved from the scalp of each rat to have acoustic coupling for transcranial fPA
314   recording. The rat was placed into a stereotaxic device (Stoeling Co. Wood Dale, IL). This fixation
315   procedure was required to prevent any unpredictable movement during fPA or EEG recording of
316   neural activities. A CMA12 microdialysis probe (Harvard Apparatus, Holliston, MA, USA) was
317   implanted into the CA_3 region of the right hippocampus (stereotaxic coordinates: 3 mm lateral and
318   3.8 mm posterior to bregma, and 3.6 mm below the surface of the dura, Figure 3a) [22]. The probe
319   active exchange surface was 2×0.5 mm. The probe was secured to the skull using dental acrylic
320   cement. The fPA and qEEG probes were placed on the contralateral side of the microdialysis probe.

321   **Fluorescence quenching-based near-infrared voltage-sensitive dye.** In the present *in vivo* study,
322   we used the fluorescence quenching-based near-infrared cyanine VSD, IR780 perchlorate (576409,
323   Sigma-Aldrich Co. LLC, MO, United States) as used in our previous *in vivo* study differentiating a
324   chemo-convulsant seizure activity [19], and it has the analogous chemical structure of PAVSD800-2,
325   our new VSD validated in our previous *in vitro* study [38]. This VSD yields fluorescence quenching
326   and de-quenching depending on membrane polarization and subsequent change in the local VSD

327　molecule density, leading to a reciprocal change of PA contrast with non-radiative relaxation of
328　absorbed energy.

329　**Functional fPA neuroimaging.** We used real-time PA data acquisition to record
330　electrophysiological neural activities *in vivo* as in our previous study [19]: an ultrasound research
331　system consisted of an ultrasound linear array transducer connected to a real-time data acquisition
332　system (SonixDAQ, Ultrasonix Medical Corp., Canada). To induce the PA signals, pulsed laser light
333　generated by a second-harmonic (532 nm) Nd:YAG laser pumping an optical parametric oscillator
334　(OPO) system (Phocus Inline, Opotek Inc., USA) provided 690-900 nm of tunable wavelength range
335　and 20 Hz of the maximum pulse repetition frequency. A bifurcated fiber optic bundle, each 40 mm
336　long and 0.88 mm wide, was used for laser pulse delivery. The PA probe was situated between the
337　outlets of the bifurcated fiber optic bundles using a customized, 3-D printed shell for evenly
338　distributing laser energy density in the imaging field-of-view. The alignment of outlets was focused
339　specifically at 20 mm depth. The PA probe was positioned in the contralateral sagittal plane of
340　microdialysis probe (3 mm) to cover the hippocampal cross-section. The distance between the PA
341　probe and the rat skin surface was 20 mm filled with acoustic gel, and the resultant energy density
342　was at ~3.5 mJ/cm^2 , which is far below the maximum permissible exposure (MPE) of skin to laser
343　radiation by the ANSI safety standards [46]. A wavelength of 790 nm was used, at which sufficient
344　absorbance can be obtained by the near-infrared VSD, i.e., IR780 perchlorate. Also, excitation at that
345　wavelength prevented the undesired time-variant change of blood oxygen saturation, since the
346　wavelength corresponds to the isosbestic point of Hb and HbO$_2$ absorption spectra. Detailed
347　information of neural activity reconstruction using normalized time-frequency analysis can be found
348　in our previous publication [19].

349　***In vivo* microdialysis**. *In vivo* microdialysis sampling was carried out as previously described [47,48].
350　For infusion experiments, NMDA (Sigma-Aldrich Chemicals, St. Louis, Mo) was weighed,
351　solubilized, and diluted to the desired concentration in artificial cerebrospinal fluid (NaCl, 147
352　mmol/L; KCl, 2.7 mmol/L; CaCl$_2$, 1.2 mmol/L; MgCl$_2$, 0.85 mmol/L) (Harvard Apparatus, Holliston,
353　MA, USA) on the study day. Once the probe was inserted and secured, it was perfused with artificial
354　cerebrospinal fluid pumped at a flow rate of 2 μl/min. Samples were collected at 20 min intervals,
355　and immediately transferred to a -80°C freezer until assayed. To allow sufficient time for the
356　glutamate levels to equilibrate, three baseline samples were collected an hour following initiation of
357　infusion. Following these samples, NMDA was infused into the brain directly through the dialysis
358　probe with the same pump parameters as used for the baseline samples. Dialysate samples were
359　assayed for glutamate by a two-step process using HPLC-ECD on an Eicom HTEC-500 system
360　(EICOM, San Diego, CA, USA). After passing the samples through a separation column, they were
361　processed via a column containing immobilized L-glutamate oxidase enzyme, resulting in the release
362　of hydrogen peroxide. The hydrogen peroxide concentration was then determined using a platinum
363　working electrode. Chromatographic data were acquired online and exported to an Envision software
364　system (EICOM, San Diego, CA, USA) for peak amplification, integration, and analysis.

365　**Quantitative EEG.** All EEG recordings utilized a three-electrode paradigm: 1 recording, 1 reference
366　(aligned to the site of activation) and 1 ground over the rostrum. The electrodes (IVES EEG; Model #
367　SWE-L25 – IVES EEG solutions, MA, USA) were fixed with minimal cyanoacrylate adhesive
368　(KrazyGlue), similar to previous protocols [49]. Data acquisition was performed using Sirenia software
369　(Pinnacle Technologies Inc., Kansas, USA) with synchronous video capture. Data acquisition had a
370　14-bit resolution, 400 Hz sampling rate, and a band pass filter between 0.5 Hz and 50 Hz. The
371　acquisition files were stored in an .EDF format and scored manually, using real-time annotations

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372   from the experiments. EEG power for 2-second epochs was done using an automated fast Fourier
373   transformation module in Sirenia software [50].

374   ***In vivo* experimental protocol.** The *in vivo* protocols were designed for simultaneous multi-modal
375   sensing of the neural activity at hippocampus: microdialysis-qEEG and microdialysis-fPA
376   neuroimaging. Figure 2 shows a detailed schematic protocol for each group representing the response
377   to the administration of NMDA, Lexiscan and VSD (i.e., IR780 perchlorate). fPA and qEEG data
378   acquisition were performed for 40 min to correlate with three microdialysis samples collected at 20-
379   min intervals. Graded NMDA infusion concentrations were applied to identify the dose-dependent
380   glutamatergic excitation of hippocampal circuit: 0.3 mM ($n = 3$) and 3.0 mM ($n = 6$). VSD and
381   Lexiscan followed the data acquisition sequence with 3-min delay, thereby 5 min of baseline phase
382   was guaranteed for the VSD response reconstruction in fPA neuroimaging before starting NMDA
383   infusion. The dosing protocol for Lexiscan and VSD administration was as follows: through an iv tail
384   vein catheter, 150 µl of Lexiscan (0.4mg/5ml) was injected, followed by 200 µl of VSD at 2 mg/ml
385   concentration, flushed immediately with 150 µl of 0.9% isotonic saline. The EEG signal was
386   recorded an identical preparation procedure as the fPA neuroimaging, including animal preparation
387   and administration of IR780, Lexiscan and experimental duration time for all recordings.

388   **Brain histology.** Rats used for the above protocol were sacrificed, and whole brains immediately
389   harvested and placed in 10 % formalin. All brains were allowed to fix in fresh 10 % formalin for at
390   least 48 hours with gentle agitation on a conical rotator. Subsequently, the brains were processed
391   through a series of sucrose gradients (15 %, 20 %, 30 % for 12-24 hours each) for cryoprotection.
392   Brains were sectioned frozen at 300 µm thickness. Tissue sections were mounted on slides in
393   ProLong Diamond Anti-face mountant. Slides with sections were imaged using an Olympus OM-D
394   E-M5 Mark II for bright field image and using LI-COR Odyssey for fluorescence visualization.

395

Author Contributions

DFW, AAG and MB originally conceived of the NMDA administration and of microdialysis with PA idea and helped to design the overall research plan with initial funding for the *in vivo* experiments. DFW critically revised the focus of the results and final version and interpretation. EMB, LML, and SDK helped to design the overall research plan, helped plan specific experiments and all contributed to the review and writing of the manuscript. Jeeun K, SDK, JSE, BJS, HV planned and carried out *in vivo* experiments, analyzed the research outcomes, and wrote key elements of the first draft of the manuscript. Jeeun K analyzed and interpreted the PA measurements and completed the first manuscript, HV and JE the NMDA dosing and microdialysis, and SDK and BS the EEG experiments. MB provided resources and personnel and vital collaboration for the microdialysis part of the experiment. HV and LML devised the VSD vehicle preparation. APM performed histopathological analysis. MMH planned and supervised confirmation of VSD penetration into brain tissue also contributed to the final version of the manuscript. AAG critically revised experimental design, draft, and final versions of manuscript, and interpretation of results. Jin K developed, funded, and participated in the current PA system design. AR participated in early planning and critically read and edited the manuscript. He has contributed both technically and materially to support this research. EMB led the development, system specification, design specification, and funding of the current PA imaging system. Secured the funding of the needed imaging experiments throughout the lifetime of the project, including taking responsibility of 2 full-time research members specifically for this work, Jeeun K and APM. Further, he has contributed intellectually by mentoring these members and providing input on manuscripts, and participating on PI meetings.

Conflict of Interest Statement

The subject matter described in this article is included in patent applications filed by the University of Connecticut and Johns Hopkins University. LML is a founder and owner of Potentiometric Probes LLC, which sells voltage sensitive dyes.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.
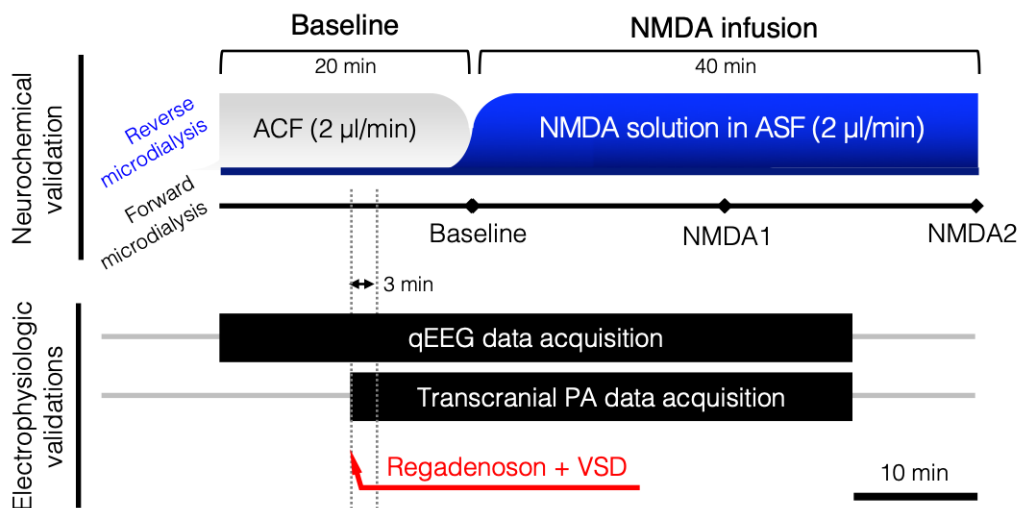
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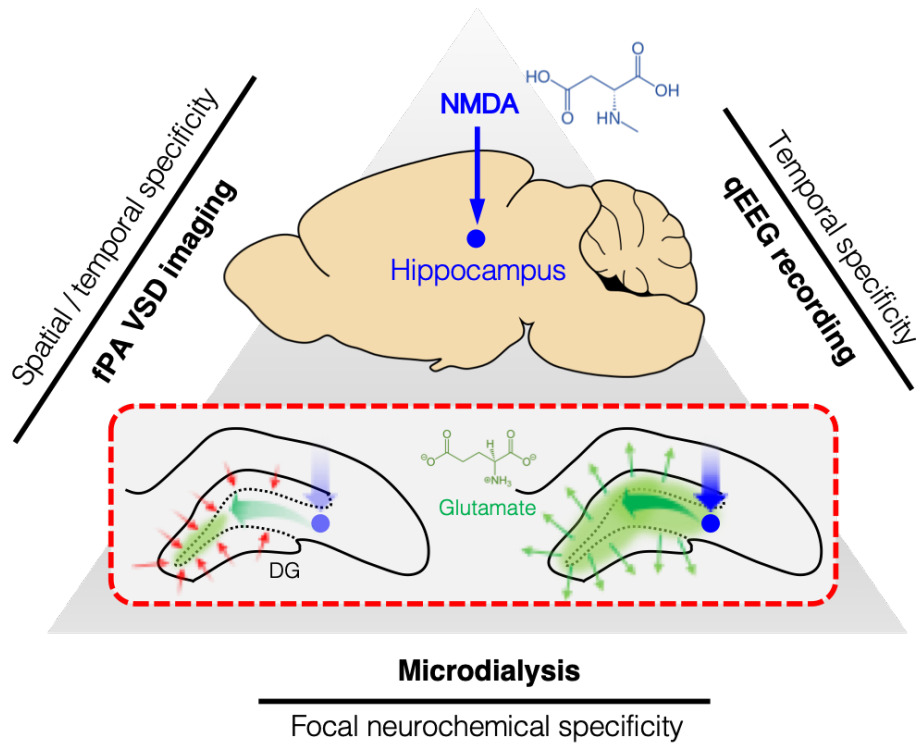
Figures

Figure 1 Tri-modal sensing of rat hippocampus. Red dotted rectangular describes dentate gyrus (DG) gating breakdown at hippocampus to a focal NMDA infusion. NMDA, N-methyl-d-aspartate; VSD, voltage-sensitive dye.
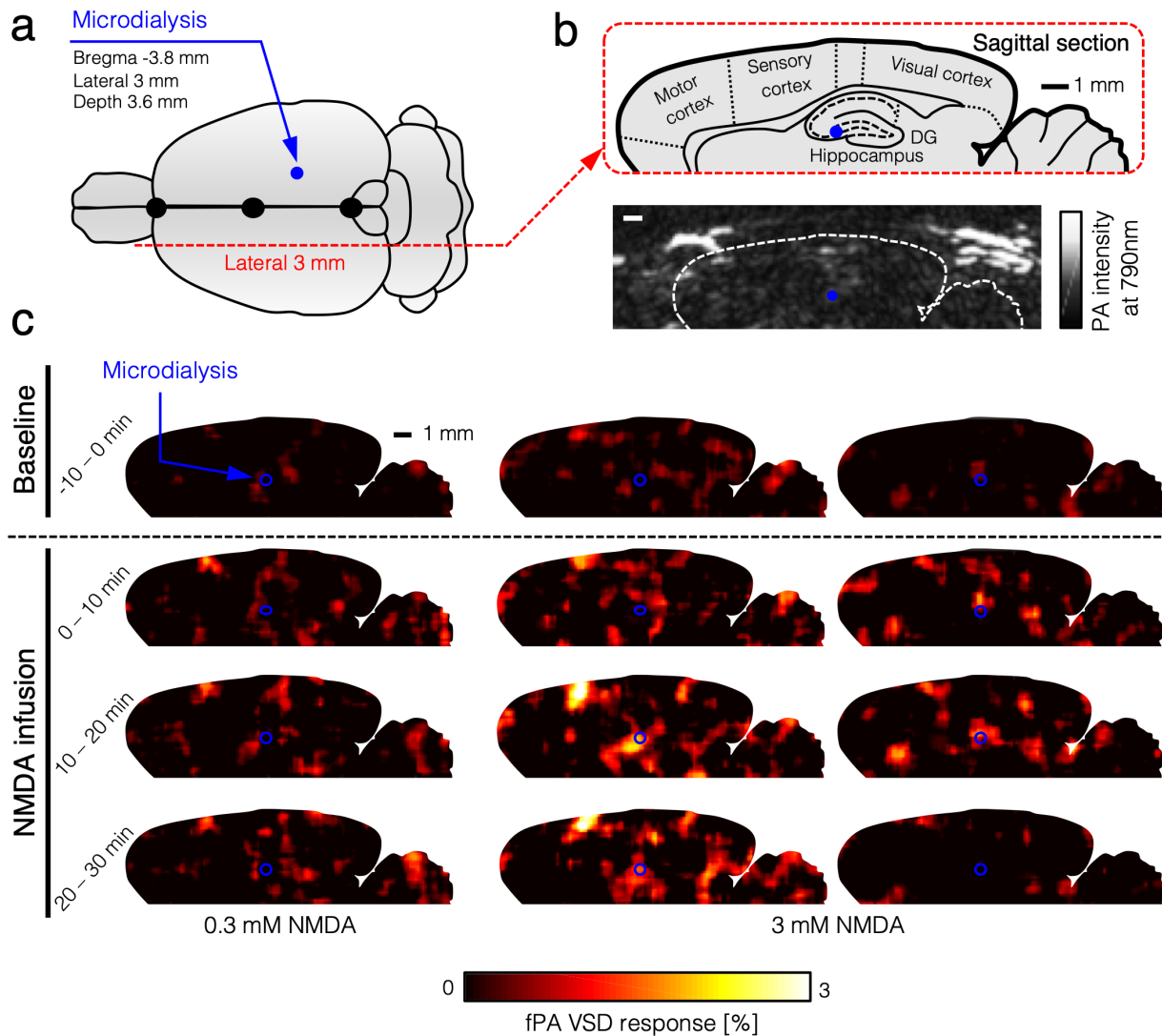

Figure 2 *In vivo* experimental protocol. Tri-modal monitoring of rat hippocampus using reverse/forward microdialysis, transcranial PA imaging, and qEEG. ACF, artificial cerebrospinal fluid.

Figure 3. Transcranial PA neuroimaging of the hippocampal circuit dynamics following focal NMDA infusion. (**a**) Illustration of stereotaxic coordinates for the microdialysis probe and sagittal cross-sections for real-time PA recording. (**b**) The sagittal PA imaging plane was selected in the contralateral side of the microdialysis probe infusing NMDA into the brain (3 mm lateral). (**c**) Time-averaged VSD response maps during -10 – 0 min (baseline phase); 0 – 10 min, 10 – 20 min, and 20-30 min (NMDA infusion phases). Note that the blue points indicate microdialysis probe in contra-lateral positions. Maximal glutamate concentration increases for representative fPA images were 34.23 % (left), 734.48 % (center) and 493.91 % (right).

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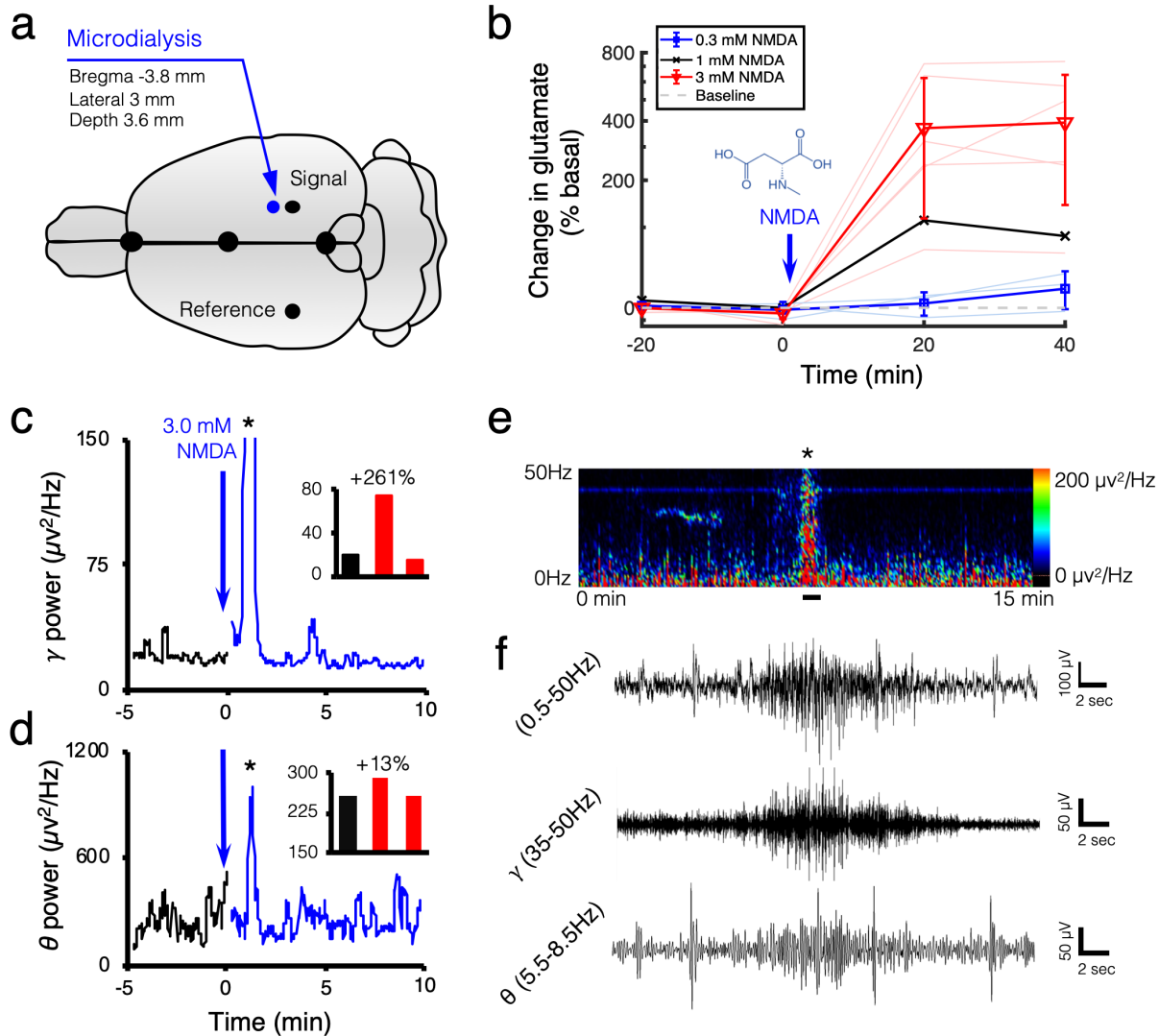
Figure 4. Extracellular glutamate concentration and concomitant electrophysiology during NMDA-evoked activity in the hippocampus. (**a**) Illustrates stereotaxic coordinates for the microdialysis probe and recording leads. Ground electrode was implanted over the rostrum. (**b**) 0.3 (n = 3), 1.0 (n = 1), and 3.0 mM (n = 6) NMDA infusion into the hippocampus caused maximal glutamate increases of 17.93±19.05%, 112.42%, and 392.99±250.54% respectively as compared to % baseline. Gray dotted line indicates the baseline. (**c**) Gamma (35-50Hz) power shows a 5-min baseline recording (black trace), followed by two consecutive 5 min traces (blue trace) following focal NMDA infusion in the hippocampus. EEG demonstrated a 261% increase in gamma power, as compared to % baseline, and an onset of epileptiform discharges after focal NMDA infusion (see inset bar graph; asterisk denotes epileptiform activity). (**d**) Respective theta power after NMDA infusion represents the theta component of the focal seizure. (**e**) 15-min spectral power heat map demonstrates the spectral power changes associated with NMDA infusion and subsequent epileptiform activity (denoted by black asterisk in e). (**f**) The representative raw EEG trace during the occurrence of the epileptiform event (solid black line in e) for full (05.-50Hz), gamma (0.5-50Hz), and theta (5.5-8.5Hz) power; respectively. For the expanded time scale of the focal hippocampal seizure see Supplemental 1.

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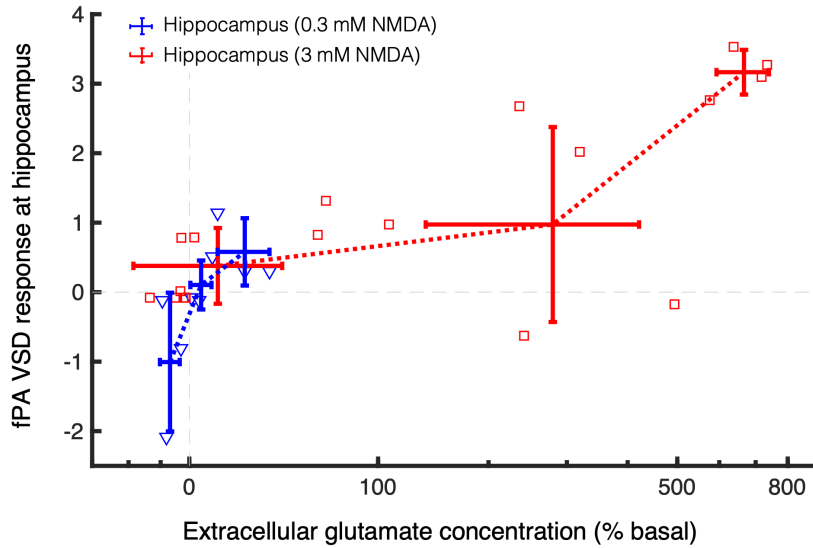
Figure 5. fPA VSD response at hippocampus as a function of extracellular glutamate concentration change. Grey dotted lines indicate the basal level in fPA VSD response and extracellular glutamate concentration.

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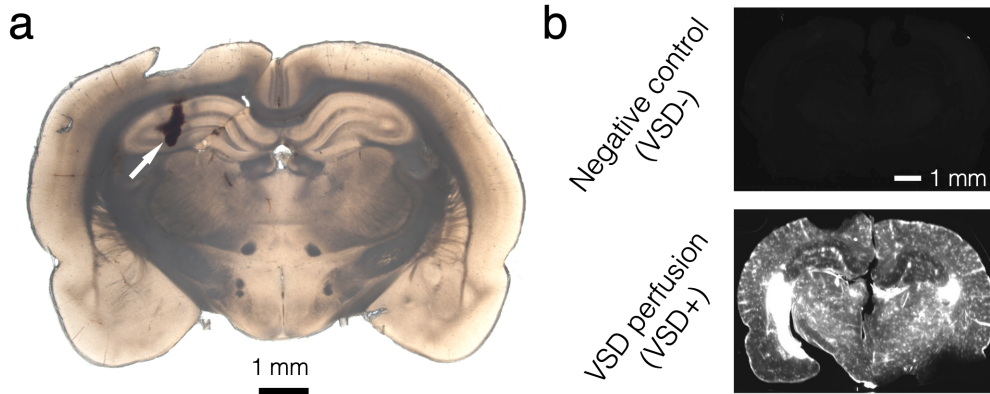
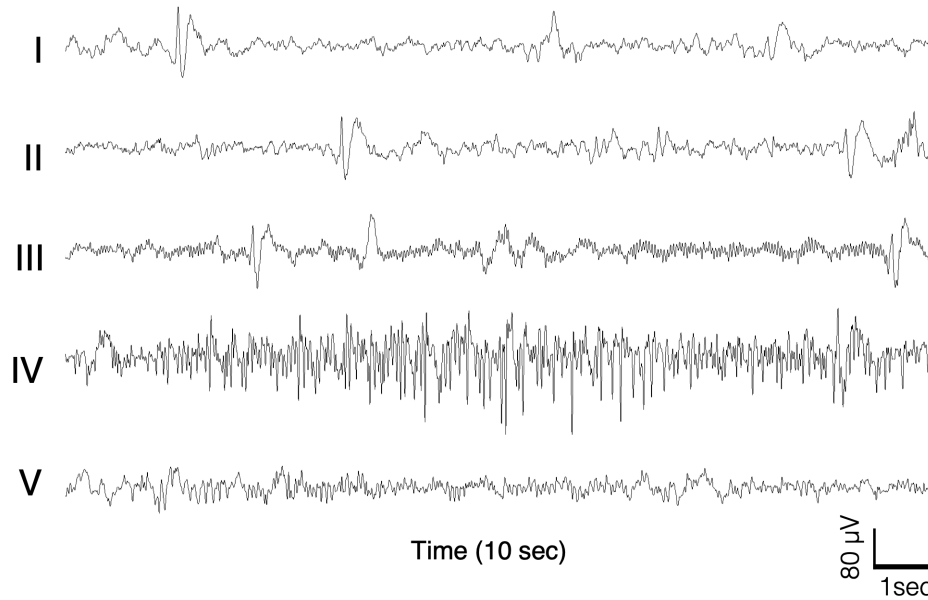**Figure 6. Histopathological confirmation** (**a**) Microdialysis probe at hippocampus. White arrow indicates the wound caused by microdialysis probe installation. (**b**) Frozen-sectioning histopathological confirmation of systematic VSD delivery throughout brain tissue region.

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480   **Supplementary Material**

481

482   **Figure S1. Expanded time scale of EEG recording during NMDA infusion into the hippocampus. (a)** 10
483   sec expanded time scale of raw EEG traces during hippocampal NMDA infusion. (I) Baseline (II) immediately
484   after onset of NMDA infusion (III) immediately before ictal event (IV) during focal seizure event (V) and
485   sustained short duration high frequency hippocampal discharges after the focal seizure event.

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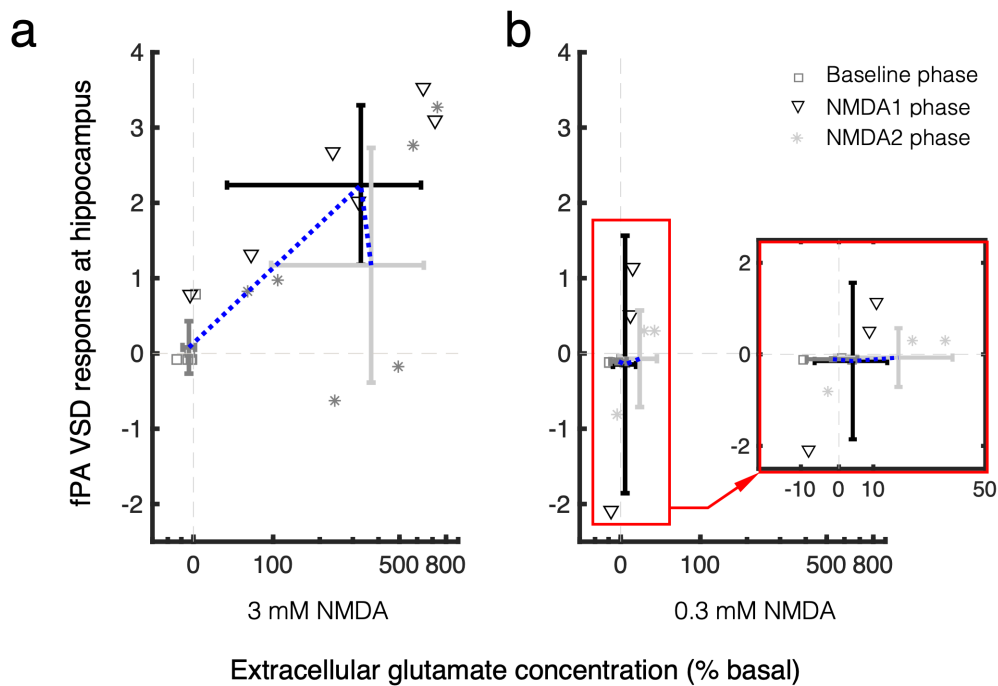
488 **Figure S2. fPA VSD responses in baseline, NMDA1, and NMDA2 phases as a function of extracellular**
489 **glutamate concentration change at hippocampus.** (**a**) 3.0 mM NMDA infusion. (**b**) 0.3 mM NMDA
490 infusion. 0.3 mM NMDA data in red rectangular is magnified and presented together (see inset graph). Grey
491 dotted lines indicate the basal level in fPA VSD response and extracellular glutamate concentration.

17

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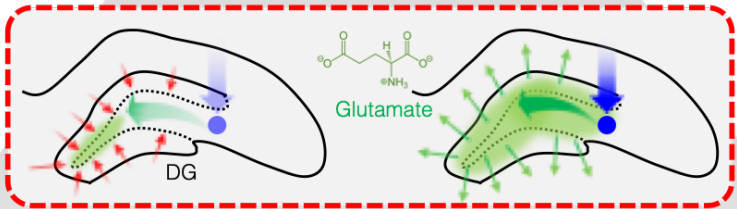563 27 Hu S, Wang LV. Neurovascular photoacoustic tomography. *Front Neuroenergetics* 2010; **2**: 10.

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623

a Microdialysis

Bregma -3.8 mm
Lateral 3 mm
Depth 3.6 mm

Lateral 3 mm

b Sagittal section

Motor cortex | Sensory cortex | Visual cortex

Hippocampus | DG

1 mm

PA intensity at 790nm

c

Microdialysis

Baseline
-10 – 0 min

1 mm

NMDA infusion

0 – 10 min

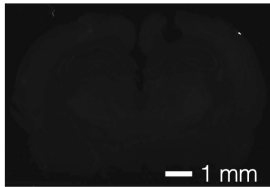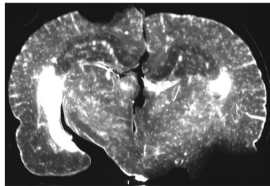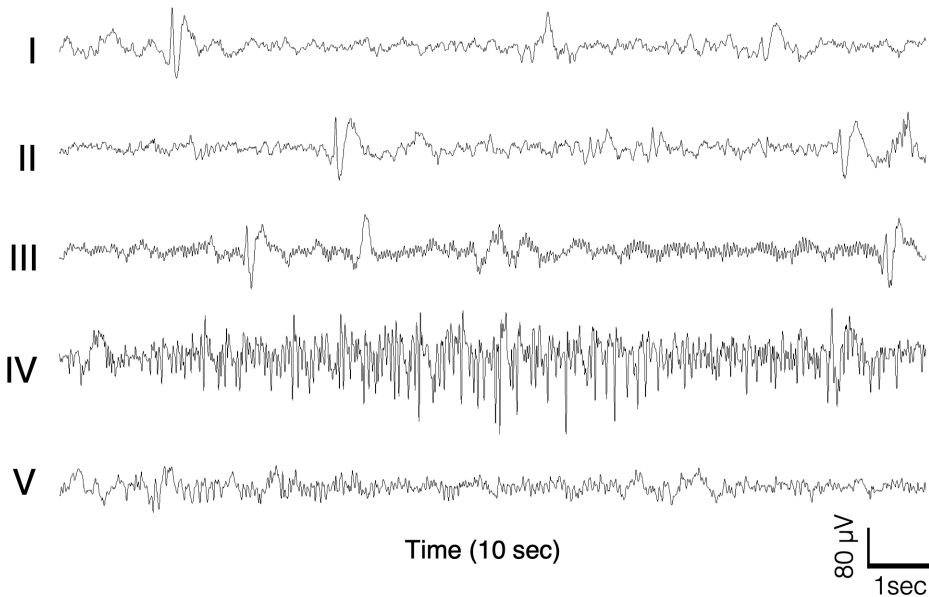10 – 20 min

20 – 30 min

0.3 mM NMDA

3 mM NMDA

0 ———— 3

fPA VSD response [%]

a

b

Negative control (VSD-)

1 mm

VSD perfusion (VSD+)

I

II

III

IV

V

Time (10 sec)

80 μV

1sec