Assessing the Functional Coupling between Extracellular Glutamate and Nitric Oxide \textit{in vivo} in the Brain using Microelectrodes

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Introduction

Nitric Oxide (NO) is a messenger involved in various physiologic processes in the brain, such as modulation of neurotransmitter release, long term potentiation and neurovascular coupling [1]. Unlike most signaling molecules, NO diffusion is not restricted by cell membranes, which means that its concentration dynamics cannot be controlled by vesicle storage/exocytose or intracellular uptake processes [2]. Instead of being released from vesicles like classical neurotransmitters, NO is enzymatically synthesized, mainly in synaptic terminals containing NMDA receptors physically coupled to the neuronal isoform of NO synthase (\textit{nNOS}). At this location NO production is triggered by activation of \textit{nNOS}, via calcium influx into the post-synaptic terminal through N-Methyl-D-Aspartate (NMDA) receptors activated by glutamate [1].

Since the activation of NMDA receptors is upstream from NO synthesis, the relationship between the extracellular glutamate and NO levels is important to understand the dynamics of NO in the brain. It is predictable that the spatio-temporal shape of a NO signal arising from a population of NO synthesizing synapses is strongly affected by the local dynamics of extracellular glutamate. However, experimental data regarding the relationship between extracellular glutamate changes and the resulting NO signals \textit{in vivo} is scarce. Additionally, the pathway of NO synthesis upon exogenous application of glutamate might not exclusively involve a localized calcium entrance into the post-synaptic terminals. It has been shown that potent glutamatergic stimulation can induce depolarizing responses such as spreading depression [3].

In this work we have studied the relationship between extracellular glutamate and NO signals in the cortex and in the CA1 sub-region of the rat hippocampus \textit{in vivo} by measuring both molecules with a sub-second temporal resolution using carbon fiber microelectrodes (CFMs) and ceramic-based microelectrodes arrays (MEAs). DC potential measurements were also performed, in order to evaluate the magnitude of tissue depolarization upon stimulation.

Methods

Home-made CFMs for NO measurement (150-250 \textmu \text{m tip length}) were coated twice with Nafion® by dipping the microelectrode tip into a Nafion® solution (5wt.%) for 2 seconds and drying for 4 min at 170 °C. CFM tips were electropolimerized with 5 mM solution of ortho-phenylenediamine (o-PD) by applying a potential of +0.7 V vs. Ag/AgCl for 30 min.[4]. Measurements of
glutamate were performed using MEAs biosensors containing four recording sites (S2 configuration), as previously described [5]. Briefly, a small drop of a solution containing 1% BSA, 0.125% glutaraldehyde and 0.1 U/µL glutamate oxidase (GlutOx) was cast on the surface of sites 1 and 2 and a solution of 1% BSA and 0.125% glutaraldehyde was cast in sites 3 and 4, (sentinels). Before experiments, MEAs sites were electropolimerized with 5 mM solution of meta-phenylenediamine (m-PD) by applying a triangular wave (+0.25 to +0.75 V, 0.05 Hz) during 20 min. The electrochemical system used for all recordings was the Fast Analytical Sensing Technology (FAST-16 mk-III, Quanteon L.L.C., KY). The hold potential of the CFM working microelectrode for NO measurement was +0.9 V vs. Ag/AgCl. Glutamate measurement using MEAs was done at +0.7 V. For simultaneous glutamate and NO measurement with MEAs, sites 1 and 2 were coated with GlutOx, for glutamate measurement, whereas sites 3 and 4 were coated with BSA for NO measurement. Extracellular DC potential recordings were performed using a glass micropipette connected to a high impedance voltmeter (Criso). Data were acquired using a digital recorder (PowerChrom, eDAQ Pty Ltd). In vivo experiments were carried out in the cerebral cortex and in the CA1 region of rat hippocampus. The microelectrodes were coupled to micropipettes filled with stimulus solutions and inserted in the brain regions of interest.

Results and Discussion

NO signals recorded using CFMs following local glutamate application (2 mM solution) in the CA1 sub-region of the hippocampus showed a peak profile having a rise time ranging from 2-15 s, a half-width of 2-22 s and a half-life of decay of 1-6 s depending on the duration and quantity of locally applied L-glutamate (<0.3 nmol). These signals were nearly abolished following administration of the nNOS inhibitor 7-nitroindazole (50 mg/kg i. p.). Furthermore, the a dose-response curve of the amplitude of NO peaks as a function of the amount of locally applied glutamate shows an EC50 of 39 pmol and maximal amplitude of 377 nM. The results suggest a close association between extracellular glutamate levels and NO production which was further supported by simultaneous glutamate and NO measurements using MEAs (Figure 1). The temporal profile of glutamate and NO changes following local application of glutamate is similar, the NO signal showing an almost 1000-fold lower amplitude than glutamate (Figure 1A and B). A linear relationship was observed between glutamate and NO signal amplitudes (Figure 1C). Additionally, DC potential measurements performed simultaneously with CFM NO measurements showed a small decrease in the DC potential (2 mV) which accompanied the NO signal, thereby excluding the occurrence of massive depolarization events such as spreading depression. However, local application of glutamate quantities above 0.3 nmol (using a 20 mM solution) frequently induced large DC potential decreases (30 mV), indicating a massive tissue depolarization that could be reproduced by local application of 1 M KCl, a stimulus known to evoke spreading depression. In this experimental condition, the temporal correlation between glutamate and NO signals was apparently lost. Although glutamate had a single peak shape, the NO signals recorded with CFMs showed a biphasic temporal profile which could not be fully attributable to NO, as suggested by the lack of effect of 7-nitroindazole.
The results reveal (a) a linear quantitative relationship between extracellular glutamate and downstream NO peak intensity; (b) a temporal coupling between glutamate and NO profiles, supporting the proposed, functional coupling between extracellular glutamate and NO production, and, ultimately, its role as a diffusible neuromodulator associated with glutamatergic neurotransmission. However, this coupling might be lost upon strong stimulation able to induce spreading depression.

![Graph showing glutamate and NO concentrations](image)

Figure 1. Simultaneous glutamate and NO measurements in vivo in the rat brain. (A) Simultaneous glutamate and NO recordings following successive local applications of 2 mM glutamate at 150 µm from the MEA. Subtraction of the currents measured at site 3 (+0.7V) by site 4 (+0.2V) was done to improve the signal-to-noise ratio of the NO recording. Panel B shows, in an expanded time scale, typical glutamate and NO signals recorded simultaneously upon local application of 125 nL of 2 mM glutamate. Panel C shows the linear relationship between glutamate and NO concentrations.

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References