

## ORIGINAL ARTICLE

## GLUTAMINE AUGMENTS NEURONAL NETWORK ACTIVITY IN RAT HIPPOCAMPAL SLICES

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**Background:** In recent past, a huge number of in vitro electrophysiological techniques have been developed to explore underlying mechanisms of most complicated functions of brain. Neurophysiologist and neuroscientist use different compositions of artificial cerebrospinal fluid (aCSF) usually based on ionic and energy demands of neurons but these compositions lack amino acids such as aspartic acid, taurine and glutamine. **Methods:** We used in vitro electrophysiological recording technique to estimate the effects of glutamine, an amino acid and precursor of neurotransmitters glutamate and GABA, on hippocampal sharp wave ripple activity (SPW-R) in rats. We evoked SPW-Rs in hippocampal slices applying high frequency stimulation. **Results:** We found that glutamine significantly enhanced the incidence and amplitude of sharp wave ripples. However, duration of sharp wave and ripples' frequency did not change significantly. It is interesting that glutamine neither prolonged sharp wave ripple activity nor transformed these into pathological events such as recurrent epileptiform discharges. **Conclusion:** Our data indicate that addition of glutamine in aCSF may optimize the experimental conditions for in vitro electrophysiology without disturbing excitatory – inhibitory balance. This study may provide a better experimental paradigm for exploring the underlying mechanisms of neurological disorders and for searching new therapeutic options to cure these neurological conditions.

**Keywords:** Hippocampus; SPW-Rs; Glutamine; CA3; CA1

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## INTRODUCTION

Hippocampus plays a pivotal role in cognitive functions such as in formation of declarative memories and spatial orientation in humans and animals. It transfers and stores temporary memory traces into stable and long-lasting memories in mental cortex for long term use through a process term as memory consolidation.<sup>1–3</sup> These cognitive functions of the hippocampus strongly depend on neuronal network oscillations. In healthy individuals, synchronous neuronal activity such as theta ( $\theta$ ; 4–12 Hz)-oscillations and gamma ( $\gamma$ ; 30–100 Hz)-oscillations have been observed during exploratory behaviour which represent the acquisition of new information. On the other hand, during slow wave sleep, immobility and consummatory behaviour sharp wave ripple activity (SPW-Rs; 140–200 Hz) has been observed in the hippocampus. SPW-Rs are thought to consolidate memory traces in the cortex during memory consolidation.<sup>4–6</sup> In pathophysiological conditions, neurons of hippocampus display hyper-synchronous network discharges having frequency ranges from 250–500 Hz which is an electrophysiological representation of epilepsy with impaired memory function. These network oscillations are linked to the different behavioural states of animals and are important for acquisition, formation and consolidation of new memories as well as retrieval of past information.<sup>7–10</sup> SPW-Rs are also observed spontaneously in ventral in hippocampal slices of mice and rats and can be induced by stimulus protocols similar to the those

which are used to induce long lasting synaptic changes in rat hippocampus such as long term potentiation (LTP).<sup>11,12</sup> Several scientific reports demonstrated that different neurotransmitters and neuromodulators modulated these network oscillations and process of memory consolidation.<sup>10,13–16</sup>

An excitatory neurotransmitter glutamate is widely spread in most parts of the CNS and CSF.<sup>14,17</sup> Moving through glutamate transporters it accumulates in astrocytes after release from glutamatergic neurons. Inside astrocyte glial glutamate-synthetase converts it to glutamine. Glutamine then releases and enter neuron where it again transformed to different neurotransmitters such as glutamate and GABA.<sup>18,19</sup> In vivo infusion of glutamine shows prolonged increase in the release of glutamate followed by induction of LTP in performant path.<sup>20</sup> Previously published results suggests that spatial memory and LTP in dentate gyrus also depend on activation of metabotropic glutamate receptors.<sup>21</sup> It has been observed that glutamate dependent activation NMDA receptors in CA1 region of hippocampus cause post synaptic induction of LTP.<sup>22–24</sup> All these synaptic modulation of glutamate release in hippocampal pathways and implicated changes in LTP proposes for its association in consolidation of memory.<sup>25</sup> In addition to glutamate, GABA<sub>A</sub> receptors agonists suppress both stimulus induced and spontaneous SPW-Rs.<sup>26,27</sup>

In recent decade, an overwhelming development has been made in the field of neurophysiology. Electrophysiological techniques

have provided valuable tools for illustration several complicated brain functions. In addition, progress made in *in vitro* electrophysiology unveiled the secrets underlying the brain physiology and/or pathophysiology. Different types of recording chambers, microelectrodes, amplifiers for intensifying neuronal signals, numerous dyes and different compositions of artificial cerebrospinal fluid (aCSF) have been introduced to optimize the neurophysiological research. Most of the neuroscientists around the globe, while performing *in vitro* electrophysiology, focus to maintain ionic concentrations of different ions present in cerebrospinal fluid, pH, temperature, oxygen concentration and glucose in aCSF. Although *in vivo* CSF also contains several amino acids such as glutamine, taurine and aspartic acid in addition to the above-mentioned entities. So here we were interested to know whether addition of glutamine, a precursor for very important neurotransmitter glutamate and GABA, in the aCSF would affect the neuronal activity in the rat hippocampal slices or not. For this, we evaluated the effects of glutamine on stimulus evoked SPW-R activity in rat hippocampus *in vitro*.

## MATERIAL AND METHODS

Electrophysiological field potential recordings were carried out on horizontal hippocampal slices of adult Wistar rats (female, having weight <200 g and age of 6–8 weeks). After deep ether anaesthesia animals were decapitated and their brains were immediately removed and transferred into ice cooled artificial cerebrospinal fluid (aCSF). Horizontal hippocampal slices of 400  $\mu$ m thickness were prepared as previously published procedures<sup>15</sup> and transferred to an interface chamber having continuous perfusion of aCSF composed of (in mM): NaCl 129, KCl 3, MgSO<sub>4</sub> 1.8, CaCl<sub>2</sub> 1.6, NaH<sub>2</sub>PO<sub>4</sub> 1.25, NaHCO<sub>3</sub> 21, glucose 10, and continuous supply of 95% O<sub>2</sub> and 5% CO<sub>2</sub> at 35 $\pm$ 1  $^{\circ}$ C (osmolarity; 295–300 mosmol/kg, pH 7.4, flow rate;  $\sim$  2ml/min). Electrophysiological recordings were done after 2–3 hours recovery time. Animal procedures performed were in accordance to guidelines approved by the Animal Ethics Committee of the institution. Glutamine was purchased from a local representative of Sigma Aldrich and was dissolved in aCSF in concentration of 1 mM.

Extracellular field potentials (FPs) were recorded from stratum pyramidale of CA1 and CA3 using microelectrodes<sup>15,16</sup> under interface conditions. Electrophysiological signals were obtained in Spike2 software (Cambridge Electronic Design, UK). These signals were filtered using low pass filter at 3 kHz and stored for offline analysis in computer disk using an interface CED 1,401 (Cambridge Electronic Design, UK). SPW-Rs were

electrically evoked in rat hippocampal slices by high frequency stimulation (HFS).<sup>15,16</sup>

Different components of SPW-Rs such as incidence, duration and amplitude of sharp wave and ripples frequency were analysed, using the different digital filters in Spike2 software. Ripples were detected by band pass filter of 90–400 Hz. Frequency of ripple oscillations was calculated using custom-made software. For sharp wave detection, raw data were filtered at 20 Hz using low pass filter in Spike2 software. All data were shown as mean $\pm$ standard error to the mean (SEM). Statistical significance was determined by applying one way ANOVA using Microcal Origin 6.0 (Microcal Origin, Northampton, MA).  $p < 0.05$  (\*) was considered an indicative of significant difference.

## RESULTS

Sharp wave ripple activity was induced in the area CA3 of rat hippocampus by applying HFS to the stratum radiatum (SR) of the area CA1 (Figure-1A). After application of 2–4 trains of HFSs, SPW-R activity appeared in the area CA3 and subsequently propagated to the area CA1 of hippocampus (Fig-1B). The incidence of stimulus SPW-Rs varying from slice to slice presenting an average value of 11.6 $\pm$ 0.3 events per min (n=11 slices from 8 animals). Unlike spontaneously occurring SPW-Rs, stimulus induces SPW-Rs appeared in identical incidence on both areas CA3 and CA1 (Figure-1C) as each individual SPW-R event after its induction in the area CA3 propagated into the area CA1. The amplitude of sharp waves in the area CA3, at their sites of origination, was significantly higher than at their sites of propagation in the CA1 region (Figure-1C;  $p < 0.05$ ). This is in line with other similar studies on stimulus induces SPW-Rs.<sup>15</sup> Similarly sharp wave presented longer duration in the area CA3 than in the Area CA1. However, their ripples frequencies were not significantly different in both regions (Figure-1C).

After stable induction of SPW-Rs by HFS protocol, glutamine (1 mM) was mixed in aCSF and was continuously perfused to the slices for one hour (Fig-2A). We observed that glutamine had stabilized the neuronal network activity. It augmented the incidence of sharp wave ripple activity significantly from 11.6 $\pm$ 0.3 to 16.5 $\pm$ 0.43 events per min (Figure-2 & Figure-3A; n=11,  $p < 0.05$ ). Interestingly, we noticed that glutamine mediated augmentation of neuronal network activity was not fully reversed during washing out of the drug. As our data showed, incidence of the sharp wave ripples is significantly higher than control even after one hour of wash out period (Figure-2B & Figure-3; n=11,  $p < 0.05$ ). Glutamine also significantly increased the amplitude of sharp waves in both areas CA1 and CA3 (Figure-2B &

Figure-3B;  $n=11$ ,  $p<0.05$ ). In the area CA3, amplitude of SPW-sharp waves was  $2.8\pm 0.18$  mV in control condition. The amplitude was significantly enhanced to  $3.2\pm 0.22$  mV after one hour application of glutamine (Figure-2B & Figure-3B;  $n=11$ ,  $p<0.05$ ). Unlike incidence, amplitude was reversible during washing out of the drug (Figure-3B). Similarly, in the area CA1, glutamine showed a significant augmentation in the amplitude of sharp waves (Figure-2B & Figure-3B;  $n=11$ ,  $p<0.05$ ). After one hour application of 1 mM glutamine, amplitude was increased significantly from  $1.66\pm 0.17$  mV to  $2.7\pm 0.21$  mV in the CA1 region ( $p<0.05$ ). Glutamine didn't alter duration of sharp waves in both area CA1 and CA3 significantly (Figure-3C;  $p>0.05$ ). Duration of sharp waves in the area CA3 altered from  $52.3\pm 2.6$  ms to  $51.2\pm 2.4$  ms in an insignificant manner (Figure-3C;  $n=11$ ,  $p>0.05$ ) while in the area CA1 it changed from  $40.4\pm 2.4$  ms to  $40.1\pm 1.6$  ms (Figure-3C). This change in duration of SPW-Rs in the area CA1 was also insignificant ( $n=11$ ,  $p>0.05$ ). Like duration of sharp wave, glutamine didn't alter ripples' frequency significantly in both CA1 and CA3 (Figure-3D;  $n=11$ ,  $p>0.05$ ).

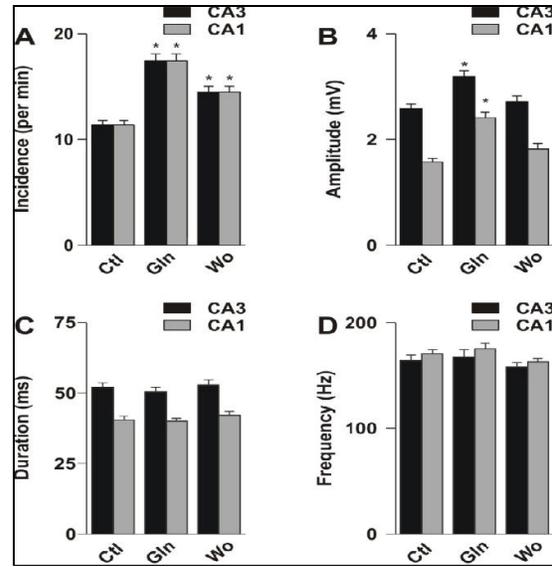


Figure-3: Graphical representation of effects of glutamine on sharp wave ripples

DISCUSSION

This study shows that glutamine significantly increases the incidence and amplitude of SPW-Rs in both area CA1 and CA3. Interestingly increase in incidence is not fully reversible during one hour of washing out of the drug. This shows that glutamine increases the viability of slices and augment the neuronal activity when it is mixed in the aCSF. On the other hand, glutamine does not change the duration of sharp waves or ripples' frequencies. We used stimulus induced SPW-Rs model to investigate the effect of addition of glutamine in aCSF. This is a quite reliable model to conduct mechanistic studies of synchronized network activities in vitro. This model provides a very useful tool to investigate the effects of imbalance between neuronal excitation and inhibition. As a very small increase in GABAergic inhibition via GABA<sub>A</sub> or GABA<sub>B</sub> activation tends to stop this activity<sup>16,28</sup> while a partial disinhibition of GABAergic tone or an increase in excitatory tone transforms this activity into the hyper-synchronized neuronal network activities like recurrent epileptiform discharges (REDs) and seizure like events (SLEs).<sup>27,28</sup> Glutamine doesn't change duration and frequencies of these synchronized oscillatory activity. In electrophysiological recordings, duration and frequencies of neuronal network activity are key features to distinguish between normal physiological and pathophysiological representations. In EEG, highly synchronized neuronal network activities in the brain presenting duration  $\geq 100$  ms usually indicate seizures or recurrent epileptiform discharges (REDs). Similarly, hyper-synchronized neuronal network oscillations having frequencies  $>200$  Hz often reflect pathological conditions such as epilepsies. It

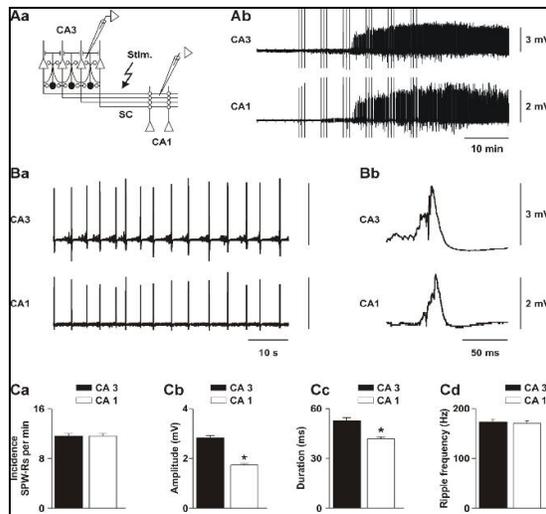


Figure-1: Induction of sharp wave ripples by high frequency stimulus.

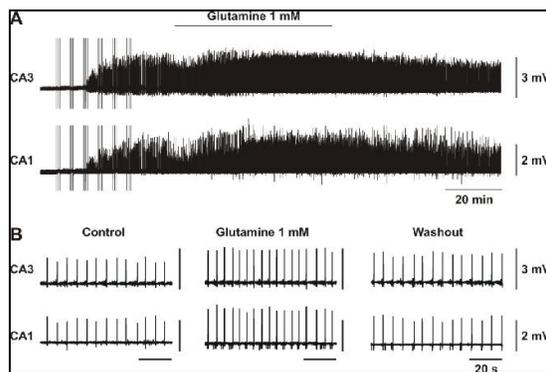


Figure-2: Effect of glutamine on sharp wave ripples

is reported that a partial increase in excitatory tone of hippocampal neurons via augmenting glutamatergic transmission or via disinhibiting the GABAergic inhibition increases both during of SPW-Rs and ripples' frequency. Glutamine is a precursor of the most important excitatory neurotransmitter glutamate and inhibitory neurotransmitter GABA. In this study, application of glutamine does not increase neither duration of sharp waves nor ripples' frequencies. On the other hand, it does not suppress the SPW-Rs indicating that addition of glutamine in aCSF does not disturb the excitatory-inhibitory balance. Interestingly, it increases the incidence of SPW-Rs activity thus leads us to draw a conclusion that addition of glutamine in aCSF during in vitro studies may improve and optimize the experimental paradigm.

Aa; A schematic representation of experimental paradigm showing neuronal connections between area CA1 and CA3 of hippocampus. Note that the positioning of recording electrodes in the area CA1 and CA3 and stimulation electrode (Stim) placed on Schaffer collateral (SC) in the area CA1. Ab; Simultaneous extracellular recordings of the area CA3 (top trace) and CA1 (bottom trace) exhibiting appearance of sharp wave ripples by HFS. SPW-R activity appeared after 3<sup>rd</sup> HFS train. Moreover, stimulation was stopped after 6<sup>th</sup> HFS train. Ba; Raw traces of field potentials recorded from the area CA3 (top) and CA1 (bottom) in extended time frame showing SPW-Rs. Bb; Analog to Ba showing a single SPW-R event. Ca-d; Graphical representation of incidence, amplitude, duration and ripples' frequency of SPW-Rs in the area CA3 and CA1. Note that the amplitude and duration of SPW-Rs are significantly higher in the area CA3.

A; Field potential recordings of CA3 and CA1 region showing the effect of glutamine on SPW-R activity. B; Analog to A, field potentials recorded from CA3 (top) and CA1 (bottom). Left traces show control condition while middle traces represent application of 1 mM glutamine Right traces show the effect of one hour of washing out of glutamine in extended time frame. Note that glutamine increased the incidence and amplitude of sharp waves in both areas CA1 and CA3.

A; Bar graphs representing effect of glutamine (Gln) on incidence of sharp wave ripples in area CA1 and CA3. Note that glutamine significantly enhanced the incidence of sharp wave ripples in both regions and this effect was not fully reversible after one hour of wash out of drug ( $n=11$ ;  $p<0.05$ ). B; Graphs showing that amplitude of sharp waves was significantly and reversibly enhanced by application of glutamine (Gln) in both areas ( $n=11$ ;  $p<0.05$ ). C & D; Bar graphs indicating effects of glutamine on duration of sharp wave and ripples' frequency. Note that both were

significantly unaltered during glutamine application ( $n=11$ ;  $p>0.05$ ).

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## AUTHORS' CONTRIBUTION

MUR and MM performed the experiments. MAS designed the research project, supervised the work and wrote the manuscript.

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