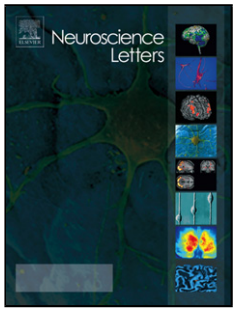


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# Excitatory-inhibitory imbalance in the brain of the wobbler mouse model of amyotrophic lateral sclerosis substantiated by riluzole and diazepam

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## Highlights:

- Drugs have distinct effects on the brain of the wobbler mouse model of ALS
- Riluzole demonstrated more pronounced effects on wobbler mice compared to controls
- Diazepam displayed reduced effects on wobbler individuals compared to controls
- Drug effects on paired-pulses and initial facilitation during trains were comparable

**Abstract**

Amyotrophic lateral sclerosis (ALS) is a devastating neurodegenerative disease. So far, no cure exists, prompting studies in disease mechanisms to facilitate development of new treatment strategies. In this study, we employed the wobbler mouse model of ALS focusing on a symptomatic group of animals. We studied the neurophysiological changes conferred by riluzole or diazepam application, two drugs employed in ALS. Riluzole is an antiglutamatergic agent and the only drug to offer some effect on the life expectancy of ALS patients. To target the inhibitory system, we utilized diazepam as a GABAergic modulator. Acute brain slices were prepared from the wobbler mouse model and

analyzed using extracellular field recordings in the hippocampus. During Schaffer collateral stimulation, riluzole caused a marked reduction in the paired-pulse ratio ($p < 0.0001$). Importantly, this reduction was more pronounced in wobbler slices (e.g. 184.2 ± 8.9% at 20 ms interval without riluzole , and 124.3 ± 9.8% in the presence of riluzole) compared to control slices (at 20 ms: from 198.7 ± 5.8% to 160.5 ± 6.7%). Diazepam caused less pronounced effects at wobbler slices and reduced the paired-pulse ratio more in control animals compared to wobbler individuals ($p < 0.0001$). Comparable results were obtained during trains of stimulations (10 pulses at 20 Hz). Importantly, paired-pulse ratios as well as synaptic facilitation were overall similar in control and wobbler slices, without the drugs present, indicating that the differences were only revealed pharmacologically. In summary, the present data support excitatory-inhibitory imbalances in the brain of the wobbler mouse and further consolidate this mouse as an animal model of ALS.

**Keywords**

ALS; hippocampus; electrophysiology; short-term plasticity

**Introduction**

Amyotrophic lateral sclerosis (ALS) is a devastating neurodegenerative disease affecting upper and lower motor neurons. The disorder is accompanied by a noteworthy incidence of cognitive dysfunction and overlap between frontotemporal dementia and ALS is increasingly acknowledged [13, 16]. So far, riluzole has been the only drug to offer some extension of life expectancy of ALS patients [16]. However, the effect is limited, only increasing life expectancy with approximately three months [3, 24].

Excitatory-inhibitory imbalances are likely to contribute to the disease manifestations. Using proton magnetic resonance spectroscopy, ALS patients have been shown to display elevations in glutamate-

glutamine (Glx) levels which were reduced in riluzole-treated patients [11]. Additionally, the levels of GABA were reduced in the motor cortex of ALS patients as compared to healthy controls [11]. Hyperexcitability is often seen in ALS [1]. It has been characterized by degeneration of inhibitory cortical circuits together with increased excitation of excitatory systems [38]. Interestingly, cortical hyperexcitability was demonstrated to develop prior to clinical symptoms, suggesting that it may contribute to the neurodegeneration in familial ALS [39]. Similarly, in sporadic ALS, a cortical origin of the disorder has been suggested, based on cortical hyperexcitability preceding the development of lower motor neuron dysfunction [21].

Animal models offer important possibilities to study selected disease mechanisms. Such studies can be combined with pharmacological tools to dissect pathophysiological alterations and facilitate improved treatment strategies. The wobbler mouse model of ALS displays several of the cellular and phenotypic signs of ALS found in human patients [4, 25, 34]. Interestingly, cortical hyperexcitability in the wobbler model has been explained by reduced GABAergic inhibition [27]. In line with these observations, our laboratory has previously documented increased excitation in CA1 together with a reduced number of inhibitory interneurons in the hippocampal formation [37]. Riluzole has successfully been shown to reduce motor impairments in wobbler mice [12]. Other ALS models are based on mutations in the gene coding for superoxide dismutase-1, since several mutations in this gene have been identified in ALS patients [17]. In the SOD1(G93A) mouse model of ALS, a loss of excitatory input has been reported together with almost unchanged inhibitory input. Together these regulatory changes resulted in an excitatory-inhibitory imbalance, preceding motor neuron degeneration [35].

Pathological changes have been identified in the hippocampal formation of ALS patients [26, 29, 36, 40]. Studies in animal models of ALS also support the hippocampal formation as a key area [22, 23, 32, 37]. In the SOD1(G93A) model, the hippocampus was specifically pin-pointed as being the brain area most sensitive to glutamatergic modulation in ALS-type degeneration [5]. In line with our

observations on GABAergic interneurons in the wobbler model [37], others have described loss of hippocampal interneurons, which were concomitant to learning impairments in the SOD1(G93A) mouse [32]. Obviously, other areas of the brain also contribute. For instance, it has been suggested that hyperexcitability in the motor cortex is a common disease mechanism in ALS and frontotemporal dementia (FTD) spectrum disease [1]. For this study, we selected the hippocampus, a key area in learning and memory, and well-suited for studies of neuronal function.

It has been demonstrated that cognitively impaired ALS patients display shorter survival time and that 28% of ALS patients show cognitive impairments [14]. Others report cognitive impairments in as many as 50% of patients with ALS [33]. In a recent review it was emphasized that the high incidence of cognitive dysfunction, together with the impact on prognosis, should prompt further research in strategies to manage cognitive deficits in ALS [16].

To increase the understanding of brain-related changes in ALS, we here employed the wobbler mouse model of ALS to analyze effects of the anti-glutamatergic drug riluzole and the GABAergic drug diazepam on neuronal mechanisms in a symptomatic group of animals. Electrophysiological recordings revealed stronger effects of riluzole, together with reduced effect of diazepam, in wobbler compared to control hippocampus, substantiating excitatory-inhibitory imbalances in the brain of the wobbler mouse.

## Materials and methods

### Animals

Wobbler mice were bred on a (C57BL6/J) background, and kept with a cycle of 12 : 12 hours of light : darkness, with unrestricted access to food and water. Additionally, to ensure the best possible conditions for the wobbler mice, the wobbler mice (*Vps54*$^{wr/wr}$) and their control littermates (*Vps54*$^{+/+}$ and *Vps54*$^{+/wr}$) had their diet enriched with peanut butter. The wobbler phenotype only

develops in homozygous mutant mice, with no symptoms of disease in heterozygous [4, 31]. We used animals of the symptomatic age (P45-60) of both sexes. Housing and handling were performed in accordance with institutional, national, and EU guidelines for the care and use of laboratory animals. Procedures were approved and monitored by the veterinarian at the Department of Biomedicine, Aarhus University, Denmark. Genotyping was performed as previously described [37].

**Electrophysiology**

Electrophysiological recordings were performed as previously described [7, 37]. Briefly, 400 µm thick coronal slices were acutely prepared from deeply anaesthetized and decapitated animals and analyzed using extracellular field recordings. We employed a MultiClamp 700B together with Digidata 1440A with concomitant Clampex software (Molecular Devices). We controlled timing with a Master-8 (A.M.P.I., Jerusalem, Israel). Slices were prepared in and continuously perfused during experiments with standard artificial cerebrospinal fluid (ACSF) composed of (in mM) 126 NaCl, 2.5 KCl, 1.25 $NaH_2PO_4$, 2.5 $CaCl_2$, 1.3 $MgCl_2$, 10 D-glucose, 26 $NaHCO_3$, bubbled with carbogen (5% $CO_2$/95% $O_2$).

**Data analysis**

We analyzed the data in Clampfit (Molecular Devices) using previously described methods [37]. The initial slopes of the responses were calculated at about 30-70% of the response in Clampfit and further analyzed in Microsoft Excel. Figures were prepared using Microsoft Excel and Adobe Illustrator. For all groups of experiments, we used slices from three or more animals. “n” indicates the number of slices and is used for statistics. Comparisons were performed by two-way ANOVA. Level of significance was $p = 0.05$. Calculated p-values for main effects are listed in the respective figures together with the F and p-values from the corresponding tests for interactions. No significant interactions (level set at 0.05) were detected for the train analysis; however, a few significant interactions were detected for the paired-pulse analysis (Figure 2B+C and Figure 3A). In comparisons with significant interactions, simple main effects analyses for the individual paired-pulse intervals

were performed by unpaired t-tests. * indicates $p < 0.05$ for simple main effect. Statistical analysis was performed using STATA 12, (StataCorp, Tx, USA) and Microsoft Excel.

**Compounds**

Riluzole and diazepam were obtained from SigmaAldrich and dissolved in DMSO to stock solutions at 100 mM. Drugs were added to the recording solutions to obtain a final concentration at 30 µM. Final DMSO concentration was 0.03%. Other chemicals and salts were obtained from regular commercial sources.

## Results

**Paired-pulse facilitation and trains of afferent stimulation**

We employed the established wobbler mouse model of ALS [4, 9, 34]. We have previously characterized paired-pulses and trains of repetitive stimulations in hippocampal wobbler synapses using this model and found no major impairments [37]. However, a few of the last pulses in the 10 pulse trains (20 Hz and 50 Hz) reached statistical significance in pre-symptomatic juvenile animals (P17-21) indicating subtle changes in the short-term plasticity. It should be noted, that since we record from native tissue, with no pharmacological inhibitors, both excitatory and inhibitory networks contribute to our recorded data. Additionally, in our previous study, we documented increased hippocampal excitation, which was more pronounced in the symptomatic group of wobbler animals [37].

In this study we focused on a symptomatic group of mice and expanded our analysis of the short-term plasticity using pharmacological agents. As control experiments, we first analyzed both paired-pulses and repetitive trains of stimulation in standard buffer without any drugs and found similar responses in slices from the two types of animals ($p > 0.05$). This first line of analysis confirmed our

previous studies [37] and revealed that in the absence of pharmacological agents, the short-term plasticity appears overall unaffected in the symptomatic wobbler mouse (Figure 1).

**The disease-modulating drug riluzole displays stronger effects on wobbler synapses compared to controls**

A well-balanced excitatory-inhibitory balance is crucial for normal brain function. Riluzole has been reported to display a range of effects at excitatory mechanisms, and to inhibit the persistent sodium current, however the drug also displays other effects, depending on the experimental paradigm [2, 8]. In CA1 of rat brain slices the effect was shown to be selectively mediated by use-dependent inhibition of glutamatergic fibers, with no effect on GABA-mediated inhibition [19]. To characterize the effect of the drug in wobbler slices and investigate possible changes in excitatory mechanisms, we studied short-term plasticity in the presence of riluzole. We selected 30 µM as a saturating concentration [19].

Riluzole caused a significant reduction in the paired-pulse ratio in control slices (Figure 2A). At 20 ms the normalized ratios were 198.7 ± 5.8% (n = 6) in plain ACSF, and only 160.5 ± 6.7% (n = 7) in the presence of riluzole. In wobbler slices the drug markedly reduced the ratio (Figure 2B). At 20 ms the levels were 184.2 ± 8.9% (n = 6) without riluzole, and only 124.3 ± 9.8% (n = 6) in the presence of riluzole. When comparing the ratio in the presence of riluzole on control and wobbler slices, we revealed that the drug displayed stronger effects on wobbler slices, especially at shorter interpulse intervals (Figure 2C).

During 20 Hz afferent stimulation riluzole significantly reduced the slopes of the fEPSPs when normalized to the first response in the train. At control slices we revealed significant reductions, e.g.at pulse four, the values were 219.6 ± 15.0% (n = 5) in normal ACSF, but only 163.7 ± 10.2% (n = 7) in the presence of riluzole (Figure 2D). In wobbler tissue, the effect was more pronounced e.g. at pulse four, the levels were 216.0 ± 21.4% (n = 5) in standard ACSF and 101.7 ± 11.0% (n = 6) in 30 µM riluzole (Figure 2E).

Intriguingly, when comparing the relative slopes in the presence of riluzole, the levels were significantly lower in wobbler slices (Figure 2F). In summary, this line of data revealed that riluzole is a strong modulator of hippocampal transmission with significantly larger reductions of the normalized fEPSPs recorded in wobbler slices.

**Diazepam results in more pronounced effects in control slices compared to wobbler**

Diazepam is a member of the benzodiazepines targeting $GABA_A$ receptors, acting as positive allosteric modulators [20, 30]. It is tempting to suggest benzodiazepines to ameliorate excitatory-inhibitory imbalances in ALS. In fact, benzodiazepines are used to treat spasticity associated with ALS [13, 15]. Additionally, diazepam has been shown to pharmacologically reverse reduced intracortical inhibition in patients with ALS [6]. Here we employed a saturating concentration of diazepam (30 µM) for a broad targeting of the GABAergic system in the wobbler model, using similar experimental protocols as for the previous analyses of riluzole.

When analyzing control slices, paired-pulses of stimulations revealed significant reductions of fEPSPs as result of diazepam application (Figure 3A). At 20 ms the ratio was reduced from 198.7 ± 5.8 % (n = 6) in plain ACSF to 148.9 ± 6.6% (n = 6) in the presence of 30 µM diazepam. In wobbler slices we observed similar facilitation in the presence of the drug (Figure 3B), with 20 ms values reaching 184.2 ± 8.9% (n = 6) in plain ACSF and 182.5 ± 10.2% in diazepam. We compared the responses in the two groups of animals and revealed that diazepam displayed the most pronounced effects at control slices as compared to wobbler tissue (Figure 3C).

We challenged the synapses with 10 pulses of 20 Hz stimulation and analyzed the effect of diazepam under these conditions. In control slices, diazepam caused a marked reduction in the initial facilitation during the trains of stimulation (Figure 3D). At pulse four, the relative slope was 219.6 ± 15.0% (n = 5) in ACSF and only 153.6 ± 12.1% (n = 6) in the presence of the drug. However, when performing similar analysis in wobbler slices, we observed comparable facilitation in plain ACSF and in the presence of diazepam (Figure 3E). At pulse four, the relative slope was 216.0 ± 21.4% (n = 5) in

ACSF and 206.9 ± 18.5% (n = 5) in diazepam. A comparison of the normalized fEPSP slopes revealed that diazepam displayed stronger effects on control slices compared to wobbler slices (Figure 3F). Based on this line of studies, we conclude that diazepam displays more pronounced effects in control compared to wobbler hippocampus, as revealed by paired-pulse analysis and trains of stimulations.

**Discussion**

In this study we revealed distinct effects of riluzole and diazepam on short-term plasticity in the wobbler brain. We suggest that wobbler mice harbor a hyperactive or upregulated glutamatergic system, which makes them more susceptible to inhibition by riluzole compared to control brain. On the other hand, diazepam displays more pronounced effects on control slices compared to wobbler, which is likely a result of impaired inhibitory systems in wobbler brain. It is clear, that the brain requires a well-balanced excitatory and inhibitory drive, to sustain normal functions.

ALS may be a disease with a long preclinical period [10]. Animal models represent an important possibility to analyze early and selected neurophysiological changes which are difficult to study in humans. By employing two pharmacological tools and the wobbler mouse model, this study reveals important mechanistic details on ALS-like mechanisms in the brain.

The brain receives increasing focus in ALS. Cognitive dysfunction has a high incidence in ALS and there is an increased recognition of its impact on prognosis [16]. Importantly, cognitively impaired ALS patients have been shown to display shorter survival time [14]. The fact that cortical hyperexcitability has been shown to develop before lower motor neuron dysfunctions [21] and prior to clinical symptoms [39] suggests that imbalances in the brain may represent some of the first pathological mechanisms in ALS.

Riluzole displays a range of effects with several affecting the glutamatergic system [2, 8]. Importantly, detailed studies in CA1 from rodent slices have previously documented that riluzole

acts by a use-dependent mechanism on glutamatergic fibers, with no effect on GABA-mediated inhibition [19]. In line with our studies, riluzole was also reported to decrease paired-pulse facilitation of the EPSP [19]. In a previous report, we have described increased excitation in the wobbler hippocampus and this may explain why riluzole has a more prominent effect on wobbler tissue (Fig. 2). It is likely that the beneficial effects of riluzole in ALS involve its antiglutamatergic effects [8]. ALS patients often display hyperexcitability [1] and this has been shown to involve increased excitation of excitatory systems [38]. The stronger effect of riluzole in wobbler tissue therefore nicely aligns with the wobbler mouse as a model of ALS-like mechanisms.

The reduced effect of diazepam in wobbler slices (Fig. 3) may be explained by reduced numbers of inhibitory interneurons, as described previously [27, 37]. Additionally, wobbler mice display decreased VGAT immunoreactivity [27] indicating decreased density of GABAergic synapses. Feedforward inhibition of the second pulse during paired stimulations, mediated by GABAergic interneurons, has previously been demonstrated in CA1. By enhancing $GABA_A$ receptor-mediated inhibition by pentobarbital, a reduced paired-pulse facilitation was observed [18]. Similar feedforward inhibition would also affect the synaptic facilitation during trains of stimulation and likely also involve larger neuronal networks as well as feedback inhibition. Our findings are most likely explained by an impaired GABAergic system in the wobbler brain and consequently a reduced impact of diazepam. Impairments of inhibitory systems have previously been reported for ALS patients [28, 38], thereby supporting the wobbler mouse as an ALS animal model.

The fact that the neurophysiological changes are mainly revealed pharmacologically indicates that there are underlying changes which are somehow concealed when using this experimental paradigm. However, when analyzed using pharmacological agents targeted at the excitatory or inhibitory system, the brain network responds differently in the two groups of animals.

In conclusion, the present data substantiate excitatory-inhibitory imbalances in the brain of the wobbler mouse and further consolidate this mouse as an animal model of ALS-like mechanisms.

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**Figure legends**

**Fig. 1. Paired-pulses and trains of stimulations of Schaffer collaterals in wobbler hippocampus.**

(A) Analysis of paired-pulse facilitation in CA1 of slices prepared from control and wobbler brain and analyzed in standard ACSF with no drug present. The paired-pulse ratio was calculated by normalizing the slope of the second response to the slope of the first. Error bars represent SEM. Insert illustrates the placement of the stimulation and recording electrodes used for all electrophysiological recordings in the present study. Neuronal layers are also labelled. DG indicates the dentate gyrus. Examples of traces with 20 ms interpulse interval are shown to the right. (B) Trains with 10 pulses at 20 Hz, without drug application, reveal similar responses in control and wobbler slices. Examples of traces from pulse one and four are shown to the right. Scale bars represent 1 mV/5 ms. “I” denotes “interactions” with calculated values afterwards.

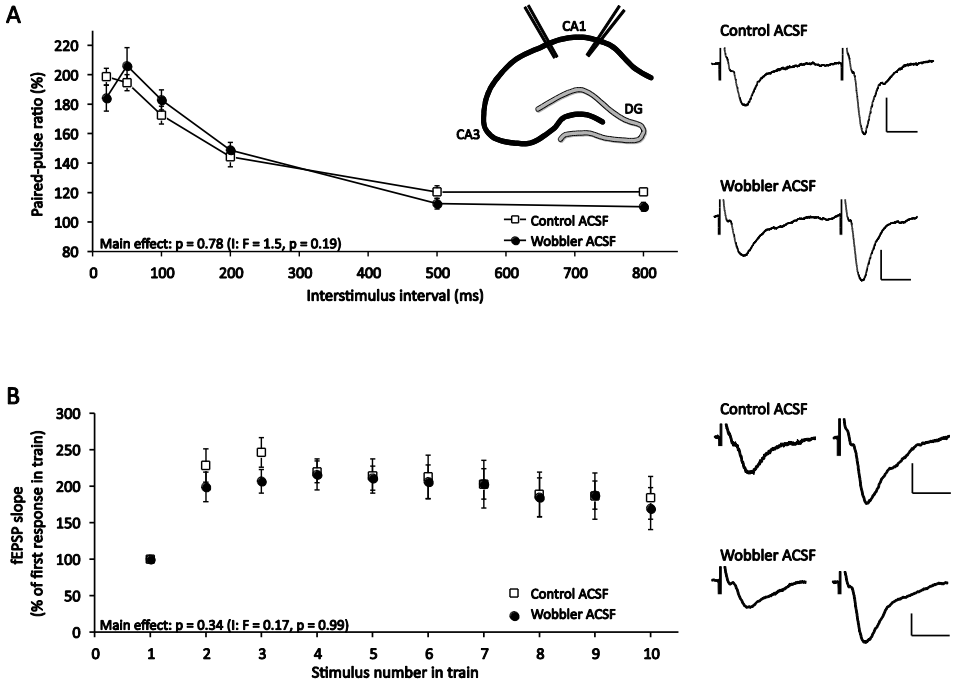


**Fig. 2. Riluzole displays stronger effects in wobbler slices compared to control slices.** (A) Paired-pulse facilitation was reduced in the presence of riluzole when analyzed in control slices. The paired-pulse ratio was calculated by normalizing the slope of the second response to the slope of the first. (B) The ratio was markedly reduced in wobbler slices. (C) Riluzole exerts a more pronounced decrease in facilitation in wobbler slices, compared to control. Traces for 20 ms interpulse interval are displayed in the top of Panel A-C. (D) 10 pulses of stimulation applied in the presence of riluzole revealed a reduced facilitation in control slices. (E) 20 Hz trains in wobbler slices revealed a prominently reduced facilitation in the presence of riluzole. (F) A stronger effect on wobbler slices is apparent when comparing the facilitation in the two groups. Traces for pulse 1 and 4 are displayed in the top of Panel D-F. Scale bars represent 1 mV/5 ms. "I" denotes "interactions" with calculated values afterwards.

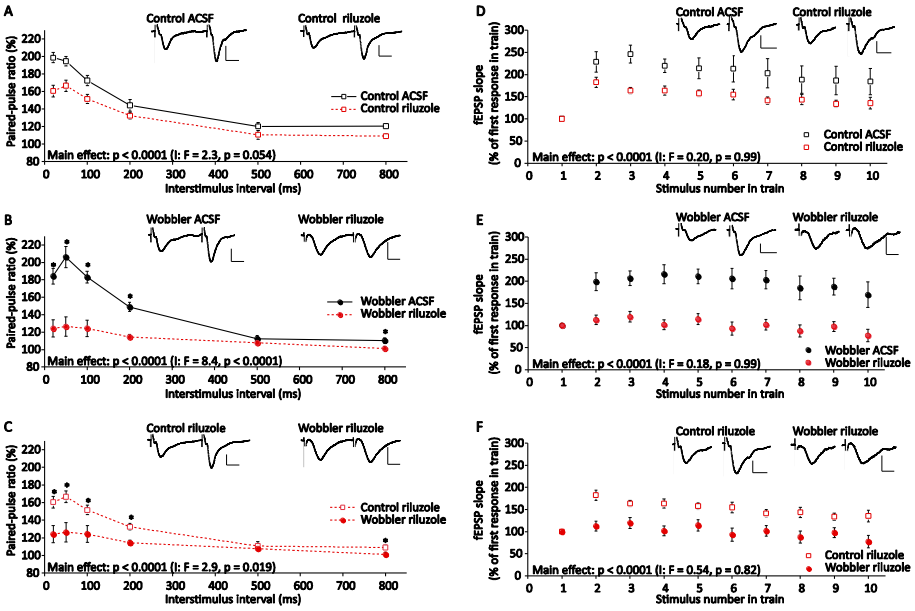


**Fig. 3. Reduced effects of diazepam in wobbler slices compared to control slices.**

(A) Paired-pulses were applied in the presence of diazepam and responses were recorded at CA1 synapses of control slices. The paired-pulse ratio was calculated by normalizing the slope of the second response to the slope of the first. Diazepam markedly reduced the ratio. (B) In wobbler slices, the ratios were apparently unaffected by diazepam. (C) When comparing the facilitation in the presence of diazepam, the drug displayed more pronounced effects on controls compared to wobblers. Traces for 20 ms interpulse interval are displayed in the top of Panel A-C. (D) 10 pulses of stimulation applied in the presence of diazepam revealed a markedly reduced facilitation in control slices. (E) In wobbler slices, the facilitation was apparently unaffected by diazepam. (F) When comparing the normalized fEPSP slopes in the two groups of animals diazepam displays a larger effect in control. Traces for pulse 1 and 4 are displayed in the top of Panel D-F. Scale bars represent 1 mV/5 ms. “I” denotes “interactions” with calculated values afterwards.