

Acetyl-L-carnitine restores synaptic transmission and enhances the inducibility of stable LTP
after oxygen-glucose deprivation

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Abstract

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2 Hypoxic circumstances result in functional and structural impairments of the brain. Oxygen-
3 glucose deprivation (OGD) on hippocampal slices is a technique widely used to investigate
4 the consequences of ischemic stroke and the potential neuroprotective effects of different
5 drugs. Acetyl-L-carnitine (ALC) is a naturally occurring substance in the body, and it can
6 therefore be administered safely even in relatively high doses. In previous experiments, ALC
7 pretreatment proved to be effective against global hypoperfusion. In the present study, we
8 investigated whether ALC can be protective in an OGD model. We are not aware of any
9 earlier study in which the long-term potentiation (LTP) function on hippocampal slices was
10 measured after OGD. Therefore, we set out to determine whether an effective ALC
11 concentration has an effect on synaptic plasticity after OGD in the hippocampal CA1 subfield.
12 A further aim was to investigate the mechanism underlying the protective effect of this
13 compound. The experiments revealed that ALC is neuroprotective against OGD in a dose-
14 dependent manner, which is manifested not only in the regeneration of the impaired synaptic
15 transmission after the OGD, but also in the inducibility and stability of the LTP. In the case of
16 the most effective concentration of ALC (500 μ M), use of a phosphoinositide 3-kinase (PI3K)
17 inhibitor (LY294002) revealed that the PI3K/Akt signaling pathway has a key role in the
18 restoration of the synaptic transmission and plasticity reached by ALC treatment.
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34 Keywords: oxygen-glucose deprivation, acetyl-L-carnitine, long-term potentiation, PI3K/Akt,
35 neuroprotection, ischemia
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1. Introduction

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3 Cerebral ischemia results in failure of the bioenergetic processes. The key element of this
4 phenomenon is the breakdown of the mitochondria, which leads to the failure of ATP
5 production and the excessive release of Ca^{2+} . It causes not only excitotoxicity, but also the
6 generation of reactive oxygen species, the release of proapoptotic signal proteins and the
7 subsequent neuronal death (Ten and Starkov 2012). It has been well established that the
8 hippocampus, and especially the CA1 subfield, is extremely sensitive to hypoxic-
9 hypoglycemic conditions (Kirino 1982, Pulsinelli et al. 1982), which can occur, for example,
10 during ischemic stroke or cardiac arrest. Hippocampal slices are widely used to investigate the
11 injury induced by ischemic events and to measure the effects of different pharmacological
12 interventions against the neuronal damage (Picconi et al. 2006, Molz et al. 2015). Oxygen-
13 glucose deprivation (OGD) mimicking brain ischemia can result in the reversible or
14 irreversible depression of neurotransmission, depending on the duration of this insult.
15 Electrophysiological recordings from slice preparations allow continuous monitoring of the
16 changes in the evoked electrical responses which can occur as a result of the ischemic event
17 or potential neuroprotective agents (Picconi et al. 2006, Nistico et al. 2008). Furthermore, the
18 hippocampus is a suitable and generally used model system for the study of synaptic
19 plasticity, and especially long-term potentiation (LTP), since it is involved in learning and
20 memory (Ho et al. 2011). As a result of the vulnerability to hypoxic-hypoglycemic
21 circumstances of this structure, the effects of ischemia are manifested in functional and
22 morphological damages, e.g. LTP impairment and the loss of dendritic spines (Kocsis et al.
23 2014), or in irreversible harmful processes ending in cell death.

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42 Acetyl-L-carnitine (ALC) is an endogenous compound, which is a short-chain acetyl ester of
43 L-carnitine (Bremer 1983, Bieber 1988) synthesized in the human brain, liver and kidney by
44 the enzyme ALC transferase. This molecule affects the overall energy metabolism and cell
45 functions, including the regulation of the lipid, carbohydrate and protein metabolism
46 (Rapoport 1999), it boosts mitochondrial ATP production and it protects the mitochondria
47 against oxidative stress (Zanelli et al. 2005). ALC is actively transported across the blood-
48 brain barrier to the brain (Burlina et al. 1989), where it has a relative high concentration (Shug
49 et al. 1982). Several studies have demonstrated the neuroprotective effect of ALC against
50 different neurodegenerative diseases, such as Alzheimer's disease, ischemia or neuropathic
51 pain (Di Cesare Mannelli et al. 2009, Zhou et al. 2011, Xu et al. 2015). We previously
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1 investigated the neuroprotective effect of ALC against global hypoperfusion in a 2-vessel
2 occlusion (2VO) model (Kocsis et al. 2014, Kocsis et al. 2015). The effects of ALC were also
3 tested on striatal slices in an *in vitro* ischemia model, where it prevented the loss of the
4 recorded field excitatory postsynaptic potentials (fEPSPs) through the activation of M2
5 muscarinic receptors and the choline uptake system (Picconi et al. 2006). Moreover, the
6 mechanisms underlying the neuroprotective effect of ALC exhibit a great deal of variety,
7 since it provides a substrate reservoir for cellular energy production, facilitates the uptake of
8 acetyl-CoA into the mitochondria during fatty acid oxidation, enhances different synthesis
9 processes (Fiskum 2004, Di Cesare Mannelli et al. 2010), has antioxidant and anti-apoptotic
10 properties (Zanelli et al., 2005), and induces nerve regeneration by increasing the production
11 and binding of the nerve growth factor (Foreman et al. 1995).

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21 In the present study, our aim was to examine the potential neuroprotective effect of ALC
22 against *in vitro* global ischemia delivered to hippocampal slices. To the best of our
23 knowledge, there has not been any study so far in which the LTP function was measured on a
24 hippocampal slice after OGD. We therefore set out to determine whether an effective ALC
25 concentration has an effect on synaptic plasticity after OGD in the hippocampal CA1 subfield.
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2. Experimental procedures

2.1 Animals and housing conditions

Male Wistar rats weighing 200-250 g (N = 21) supplied by Charles River Laboratories, were kept under constant environmental conditions (23 °C; humidity 55 ± 5%; a 12-h/12-h light/dark cycle) and were housed individually in standard plastic cages, where they had free access to food and water. Every effort was made to minimize the number of animals used and their suffering. The principles of animal care (NIH publication No. 85-23), and the protocol for animal care approved both by the Hungarian Health Committee (1998) and by the European Communities Council Directive (2010/63/EU) were followed. Before the experimental procedures, all the rats were in normal health and had no neurological deficits.

2.2. *In vitro* slice preparation

The preparation and maintenance of rat hippocampal slices were described previously (Kocsis et al., 2014; Kocsis et al., 2015). Briefly, the animals were decapitated and the middle parts of the hippocampi were placed in ice-cold artificial cerebrospinal fluid (aCSF) composed of (in mM): 130 NaCl, 3.5 KCl, 1 NaH₂PO₄, 24 NaHCO₃, 1 CaCl₂, 3 MgSO₄ and 10 D-glucose (all from Sigma, Germany), saturated with 95% O₂ + 5% CO₂. Coronal hippocampal slices (350 µm) were prepared with a vibratome (Leica VT1200S, Germany). Slices were transferred to a holding chamber and allowed to recover in the solution used for recording (differing only in that it contained 3 mM CaCl₂ and 1.5 mM MgSO₄) for at least 1 h.

2.3. Electrophysiological recordings

For the electrophysiological experiments, slices were transferred to a Haas recording chamber, where the flow rate of the recording solution (34 °C) was 1.5–2 ml×min⁻¹. A bipolar concentric stainless steel electrode (Neuronelektrod Ltd, Hungary) was placed in the stratum radiatum between the CA1 and CA2 regions of the hippocampal slices. The stimulus intensity was adjusted to between 30 and 60 µA (constant current, 0.2-ms pulses delivered at 0.05 Hz) to evoke the half-maximum response. fEPSPs were recorded with a 1.5-2.5 MΩ resistance glass micropipette filled with aCSF. The recordings were amplified with a neutralized, high input-impedance preamplifier and filtered (1 Hz-3 kHz). The fEPSPs were digitized (AIF-03, Experimetria Ltd. Hungary), acquired at a sampling rate of 10 kHz, saved to a PC and analyzed off-line with Origin Pro 8 software (OriginLab Corporation,

1 Northampton, MA, USA). The fEPSPs were monitored until the amplitudes were generally
2 stable, and a 10-min-long baseline was then recorded, which was followed by a 15-min OGD.
3 The fEPSPs were allowed to recover for 40 min after the OGD, and at the end of this period
4 theta burst stimulation (TBS) was applied (bursts of 4 impulses at 100 Hz with a burst interval
5 of 350 ms) for LTP measurements. After the TBS, changes in fEPSP amplitudes were
6 recorded for a further 35 min (Fig. 1).
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10 11 12 **2.4. *In vitro* ischemia**

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15 *In vitro* ischemia was delivered by switching for 15 min to an OGD aCSF solution in which
16 sucrose replaced glucose, and gassed with 95% N₂ + 5% CO₂. After the OGD, the slices were
17 perfused with normal aCSF again until the end of the measurements. In the preliminary
18 experiments, OGD was delivered for different periods (5, 8, 12, 15, 16, and 17 min) in order
19 to determine the appropriate length of the ischemia for our study. After shorter terms of OGD,
20 the fEPSPs returned, but the aim was to find the limit when the fEPSPs no longer displayed
21 recovery. Electrical noise could not be excluded completely during the electrophysiological
22 measurements, and it also was present after the elimination of the fEPSPs. This is the reason
23 why the recorded amplitudes never reached the value of zero. Both fEPSP amplitudes and
24 initial slopes were recorded and quantified in all of the measurements; however as no
25 appreciable differences between these two parameters were observed, only the amplitudes are
26 expressed in the figures.
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37 38 **2.5. Application of ALC**

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41 ALC (Sigma, Germany) was applied for 25 min at the desired final concentration (125, 250 or
42 500 μM) in aCSF or OGD aCSF for the control (10 min) and the OGD period (15 min),
43 respectively. In the preliminary studies, ALC was tested in all of the concentrations utilized in
44 the further experiments (25-min wash-in), and none of them influenced the amplitudes (Fig.
45 3). Nevertheless, all the recordings also were self-controlled, since after the stabilization of
46 the fEPSPs, the recordings started with a 10-min period when the slices were still perfused
47 with normal aCSF solution. This was followed by an additional 10 min, when the aCSF
48 contained ALC. Since no differences were still observed, this ALC wash-in period was
49 regarded as the control baseline during the data analysis. The wash-in of the ALC was
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1 continued during the 15-min OGD, and it was then followed by the recovery period when
2 normal aCSF solution was perfused onto the slices (Fig. 1).
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4 **2.6. Application of LY294002**

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7 In order to investigate the mechanisms underlying the protective effect of ALC, a
8 phosphoinositide 3-kinase (PI3K) inhibitor, LY294002 (Tocris, United Kingdom) was
9 utilized, in a final concentration of 50 μ M. It was dissolved in aCSF or OGD aCSF containing
10 500 μ M ALC, and the solution was washed onto the slices during the control and the OGD
11 period, respectively.
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16 **2.7. Statistical analysis**

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19 In OGD measurements, the fEPSP amplitudes were expressed as a percentage of the 10-min
20 baseline value before OGD, while to express the potentiation after LTP induction the last 10
21 min of the recovery period following the OGD was regarded as a baseline.
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25 For data presented as means \pm S.E.M., statistical analysis was performed with the use of the
26 non-parametric Wilcoxon test and the Mann-Whitney U test. The significance level was
27 established at $p < 0.05$.
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31 **3. Results**

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35 In the first part of the experiments, our aim was to determine the accurate duration of OGD
36 sufficient for the elimination of the fEPSPs. After shorter OGD periods (5 min or 8 min), the
37 amplitudes displayed a complete recovery. In the case of the 5-min OGD, the fEPSPs
38 demonstrated not only regeneration after the ischemia, but also facilitation (presumably post-
39 ischemic LTP). The increased amplitudes were stable until the end of the recording period. A
40 12-min period of ischemia was likewise not enough to abolish the fEPSPs, but 16 or 17-min
41 periods of OGD resulted in complete elimination of the amplitudes (Fig. 2). A 15-min period
42 of OGD ($N = 9$) was the shortest which abolished the fEPSPs, and the following experiments
43 were carried out with this model. A slight increase of the fEPSPs was detected immediately
44 after the OGD in almost all measurements of the different experimental groups, and the
45 amplitudes started to decrease only thereafter. This phenomenon is probably due to an
46 increased sensitivity of the postsynaptic NMDA receptors due to the removal of Mg^{2+} ions
47 from the ionophore part during an ischemic event, an excitotoxic circumstance that may have
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resulted first in an abnormal facilitation of the synaptic transmission in the CA1 region, and then by the progression of OGD lead to the complete elimination of the fEPSPs.

The potential neuroprotective effect of ALC against 15-min OGD was tested in different concentrations. Based on our preliminary studies, we chose 125, 250, and 500 μM ALC for the OGD measurements. Before these experiments, the potential neuromodulatory effect of ALC was tested on control slices. None of the applied concentrations influenced the fEPSPs (Fig. 3).

125 μM ALC was ineffective in half of the group ($N = 6$), while in the other half of the experiments ($N = 6$) it resulted in a slight recovery. Increase of the fEPSPs after OGD was slow and reached its maximum early. The average of the amplitudes in the last 10 min of the recordings was significantly lower ($58.91 \pm 3.70\%$) as compared with the control level. As a result of 250 μM ALC ($N = 12$), the fEPSPs increased continuously and reached the control level within 25 min. In the last 10 min of the recording period, the amplitudes stabilized at a significantly higher level ($107.59 \pm 2.37\%$) relative to the control. 500 μM ALC ($N = 12$) resulted in an even faster recovery of the fEPSPs. There was no significant difference between the values for the last 10 min of the 250 and 500 μM ALC groups, but both were significantly higher than that for the 125 μM ALC group (Fig. 4).

After the 40-min recovery period, LTP was induced by TBS and the fEPSP amplitudes were measured for an additional 35 min. The level of the LTPs was normalized to the last 10 min of the recovery period. The LTP inducibility was tested in the 15-min OGD group, but because of the irreversible elimination of the fEPSPs, no potentiation was observed. The illustration of these results in Fig. 5 was therefore not adequate. From the 6 samples showing partial restoration in the 125 μM ALC group, we observed LTP in only 2 cases. The fEPSPs showed a weak potentiation in the first 10 min after TBS ($116.87 \pm 1.62\%$), then started to decrease and at the end of the recordings they had fallen back to the control level ($102.79 \pm 0.96\%$). There was no significant difference between the control period and the last 10 min of the recording. 250 μM ALC ($N = 12$) resulted in a higher potentiation, although the amplitudes showed a slight decrease for 15 min after TBS and stabilized only thereafter ($123.96 \pm 1.97\%$). This potentiation was significantly higher as compared with the values for the 125 μM ALC group. 500 μM ($N = 12$) was the most effective ALC concentration since it resulted not only in complete restoration of the fEPSPs after OGD, but also in a stable LTP. As a

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result of the TBS, the amplitudes increased, stabilized immediately and remained stable until the end of the recording period ($141.12 \pm 0.90\%$). This LTP is similar to that of tested in control conditions ($140.75 \pm 0.40\%$) on hippocampal slices ($N = 8$). In the 500 μM ALC-treated group, the average of the fEPSP amplitudes in the last 10 minutes of the recordings was significantly higher relative to the control level and to the values of the last 10 min of the 125 and 250 μM ALC groups (Fig. 5).

LY294002, a PI3K inhibitor, was used to reveal the mechanisms underlying the protective effect of ALC, which was manifested in the recovery of the fEPSPs and a stable LTP after OGD. As a result of 500 μM ALC, the amplitudes started to decrease later during the ischemia as compared with the OGD group, but this effect was abolished in the presence of the inhibitor ($N = 8$). After the OGD period, the fEPSPs did not recover when ALC was applied with LY294002 (Fig. 6). LTP inducibility was also tested in the group that received 500 μM ALC + LY294002, but the complete and irreversible elimination of the fEPSPs resulted in the same results as recorded in the 15-min OGD group. Fig. 7 shows representative traces from the different experimental groups. There were no pathological responses to the stimulation none of the cases, even after the TBS.

4. Discussion

Hypoxic conditions, reperfusion and reoxygenization are central elements of many disorders in the nervous system, e.g. ischemic stroke, traumatic brain injury and heart attack (Ronaldson and Davis 2013). During an ischemic event, the limited blood flow in different parts or in the whole brain results in a complex cascade of molecular processes, which leads to cell death (Thompson and Ronaldson 2014). There is still no effective solution with which to prevent the structural and functional impairments of stroke patients, and it is therefore very important to search for alternative treatments which could rescue the nerve tissue. ALC is a naturally occurring substance which has been demonstrated to be neuroprotective in different diseases (Malaguarnera 2012). ALC is widely distributed in the tissues; its synthesis occurs in the brain, intestine, liver and kidney (Pettegrew et al. 2000). The therapeutic application of this compound is promising since it readily crosses the blood-brain barrier, affects many bioenergetic processes, exhibits antioxidant, antiapoptotic and neuromodulatory effects and enhances nerve regeneration (Jones et al. 2010). Among the numerous mechanisms

1 underlying the neuroprotective action of ALC, it is likely that this nutrient can serve as an
2 alternative source of acetyl-CoA during or after cerebral ischemia. ALC enhances the aerobic
3 energy metabolism impaired by the breakdown of the pyruvate dehydrogenase complex under
4 hypoxic conditions (Zanelli et al. 2005).
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8 In previous experiments, we investigated the potential neuroprotective action of ALC against
9 global hypoperfusion in a 2VO model of rats. We found that ALC was effective only when it
10 was administered before the ischemic insult, but not as post-treatment (Kocsis et al., 2014).
11 These findings are in accordance with the results of other research groups. In the present
12 study, we examined the effect of this compound against *in vitro* global ischemia on
13 hippocampal brain slices with electrophysiological techniques. Our aim was not only to find
14 the effective concentration of ALC which restores the synaptic transmission after OGD, but
15 also to measure the LTP function after the recovery period.
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23 After determination of the accurate period of OGD sufficient for the elimination of the
24 fEPSPs, we tested ALC in different concentrations as pretreatment. In preliminary studies,
25 ALC applied after OGD did not have protective effect, which was in accordance with a
26 previous report (Picconi et al., 2006). Hence, the compound was washed into the slices before
27 and during the OGD period.
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33 Though 15-min OGD resulted in the complete elimination of fEPSPs, which was not followed
34 by recovery, there might be differences between each slice as regards the extent of the
35 damage or the number of the potentially salvageable neurons, which may underlie the results
36 revealed in the 125 μ M ALC-treated group. Indeed, this concentration was high enough in
37 some cases to achieve a detectable slight neuroprotective effect, probably corresponding to
38 slices with a higher number of salvageable neurons remained following the 15-min OGD. The
39 ineffectiveness of 125 μ M ALC in the other half of the experiments suggests that this
40 concentration may be in the edge of the least effective concentration of ALC in this model.
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48 The damages caused by OGD to the neuronal network of the hippocampal CA1 subfield could
49 be partially restored with 125 μ M ALC only at the level of fEPSPs, but not at that of a
50 complex process such as LTP. Nevertheless, in 2 cases of the 125 μ M ALC group, we
51 detected a slight and decaying potentiation. These results, similarly to the partial restoration of
52 the fEPSPs, may also be accounted for the potential differences in the status of the particular
53 slices exposed to ischemia.
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250 and 500 μM ALC displayed similar protective effects in the recovery period, but we found differences between the LTP results of these groups. Despite the complete recovery in the 250 μM ALC group, the potentiation of the fEPSP amplitudes was not stable after TBS. 500 μM ALC exerted its neuroprotective effect via the restoration of the fEPSPs and also preservation of the synaptic integrity and plasticity.

The PI3K/protein kinase B (Akt) pathway is important during the development of the central nervous system and plays a critical role in mediating survival signals in a wide range of circumstances (Brunet et al. 2001). Furthermore, it has been well established that this signaling pathway mediates synaptic plasticity and memory (Horwood et al. 2006), and is also involved in the cognitive impairment caused by chronic cerebral hypoperfusion (Shu et al. 2013).

Among several mechanisms through which ALC exerts its protective action, the importance of the PI3K/Akt pathway has already been demonstrated. In cortical neuronal cell cultures exposed to oxidative stress-induced neurodegeneration, ALC and α -lipoic acid pretreatment resulted in activation of the PI3K, PKG and ERK1/2 pathways, which have essential roles in cell survival (Abdul and Butterfield 2007). In the present study, we explored the hypothesis that ALC exerts its neuroprotective effect via the PI3K/Akt pathway, which is manifested not only in the recovery of the fEPSPs after OGD, but also in the potentiation of the amplitudes after TBS. Despite the complete recovery and stable LTP reached following 500 μM ALC treatment, LY294002 administration abolished this protective effect.

These results suggest that the PI3K/Akt pathway is necessary for the neuroprotection induced by ALC in different ways. This ALC-activated signaling pathway promotes cell survival mechanisms through which the synaptic transmission recovers after OGD. Additionally, activation of the PI3K/Akt pathway contributes to the enhancement of the synaptic plasticity revealed by the LTP measurements. Nevertheless, there are many other mechanisms connected with the neuroprotective effect of ALC, which can simultaneously take part in the results revealed in our experiments.

Among the various beneficial effects of ALC, its natural presence in the body underlies the feasibility of this compound in patients with high safety. Experiments measuring the potential protective effect of ALC in different animal models of diseases are promising and provide a broad range of information for clinical research. Clinical trials with ALC in patients have so

1 far rather focused on neurodegenerative diseases (e.g. Alzheimer's disease); however, the
2 numerous studies revealing its neuroprotective effects in ischemic models emphasize the
3 importance of the assessment of the neuroprotective efficacy of ALC also in clinical trials of
4 stroke as well in the future.
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10 **5. Conclusions**

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12 In this study, we demonstrated that ALC can exert its protective effect on a complex process
13 such as LTP, a phenomenon underlying the basis of learning and memory, which provides
14 further relevance of its therapeutic application. Overall, these results also contribute to the
15 better understanding of the relationship between ALC and the neuroprotection following
16 ischemia.
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41 **7. Conflict of interest**

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43 The authors declare that they have no conflict of interest.
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8. Legends to Figures

Fig. 1

Schematic timeline of the experimental protocol. ALC was washed onto the slices during the 10-min control and the 15-min OGD period. At the end of the recovery period, LTP was induced by TBS. The last 10 min of the recovery period (symbolized by the gray part of the black line) was regarded as the control baseline of the LTP period.

Fig. 2

Results of the preliminary experiments conducted to determine the sufficient period of OGD. 5-min OGD resulted in complete recovery of the fEPSPs ($N = 2$). In some cases, a noteworthy facilitation was observed after the 5-min OGD ($N = 2$), which was presumably a post-ischemic LTP. The 8- and 12-min OGD were still not enough to eliminate the fEPSPs; these periods resulted in complete or partial regeneration, respectively ($N = 2$ and 4, respectively). Longer OGD periods (16 min ($N = 2$) and 17 min ($N = 2$)) completely abolished the fEPSPs. Data points are means \pm S.E.M. of the normalized amplitudes of the fEPSPs.

Fig. 3

The potential neuromodulatory effect of 125 ($N = 6$), 250 ($N = 6$), and 500 μ M ALC ($N = 6$) was tested on control hippocampal slices (25-min-long ALC application). None of the concentrations influenced the fEPSPs. Data points are means \pm S.E.M. of the normalized amplitudes of the fEPSPs.

Fig. 4

ALC was neuroprotective against 15-min OGD in a dose-dependent manner. The 15-min OGD ($N = 9$) was the shortest period which was not followed by any recovery, and ALC was therefore tested in this model. 125 μ M ALC resulted in a slight recovery in only half of the group ($N = 6$), but in the other half ($N = 6$) it was ineffective. 250 and 500 μ M ALC ($N = 12$ each) were protective: the fEPSPs recovered completely after the OGD. Data points are

1 means \pm S.E.M. of the normalized amplitudes of the fEPSPs. Asterisks denote significant
2 differences between the control and the last 10 min of the recovery period (**p < 0.01, ***p <
3 0.001, Wilcoxon test), # denotes significant differences between the last 10 min of the
4 recovery period of each experimental group (### p < 0.001, n.s.: not significant; Mann-
5 Whitney U test).
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10 11 12 **Fig. 5**

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15 The effect of ALC on LTP inducibility. Despite the slight regeneration of the fEPSPs in the
16 125 μ M ALC group, LTP could be induced in only 2 cases. The potentiation was weak, and
17 the amplitudes started to decrease immediately, and reached the control baseline at the end of
18 the recordings. 250 μ M ALC ($N = 12$) resulted in a significantly higher LTP, but showed a
19 slight decrease for 15 min after TBS and stabilized only thereafter. 500 μ M ALC ($N = 12$)
20 was the most effective concentration. It was manifested in significant potentiation, which was
21 stable over time. This LTP is similar to that of tested on hippocampal slices ($N = 8$) in control
22 conditions (inset in the upper right quadrant). Data points are means \pm S.E.M. of the
23 normalized amplitudes of the fEPSPs. Asterisks denote significant differences between the
24 control and the last 10 min of the LTP period (***p < 0.001, Wilcoxon test); # denotes
25 significant differences between the last 10 min of the LTP period of each experimental group
26 ($### p < 0.001$, n.s.: not significant; Mann-Whitney U test).
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41 **Fig. 6**

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43 The PI3K/Akt pathway has a key role in the neuroprotective effect of ALC. The 15-min OGD
44 ($N = 9$) completely eliminated the fEPSPs, which was blocked by 500 μ M ALC ($N = 12$)
45 treatment, but in the presence of LY294002 ($N = 8$) this protective effect of ALC was
46 abolished. Data points are means \pm S.E.M. of the normalized amplitudes of the fEPSPs.
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54 **Fig. 7**

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57 Representative traces of fEPSPs from the different experimental groups recorded in the
58 control, the last 10 min of the recovery, and the last 10 min of the post-TBS period. 125 μ M
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1 ALC was effective against the ischemic insult in half of the experiments ($N = 6$), but only 2 of
2 them showed a slight potentiation after TBS. This LTP was unstable and the amplitudes
3 decreased to the pre-TBS level at the end of the recordings (3rd row). Both 250 and 500 μM of
4 ALC resulted in a complete recovery of the fEPSPs; however, stable LTP could only be
5 recorded in the 500 μM ALC-treated group. This effect of ALC was abolished in the presence
6 of LY294002.
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9. References

- 1
2 Abdul HM, Butterfield DA (2007) Involvement of PI3K/PKG/ERK1/2 signaling pathways in
3 cortical neurons to trigger protection by cotreatment of acetyl-L-carnitine and alpha-
4 lipoic acid against HNE-mediated oxidative stress and neurotoxicity: implications for
5 Alzheimer's disease. *Free radical biology & medicine* 42:371-384.
6
7 Bieber LL (1988) Carnitine. *Annual review of biochemistry* 57:261-283.
8
9 Bremer J (1983) Carnitine--metabolism and functions. *Physiological reviews* 63:1420-1480.
10
11 Brunet A, Datta SR, Greenberg ME (2001) Transcription-dependent and -independent control
12 of neuronal survival by the PI3K-Akt signaling pathway. *Current opinion in*
13 *neurobiology* 11:297-305.
14
15 Burlina AP, Sershen H, Debler EA, Lajtha A (1989) Uptake of acetyl-L-carnitine in the brain.
16 *Neurochemical research* 14:489-493.
17
18 Di Cesare Mannelli L, Ghelardini C, Calvani M, Nicolai R, Mosconi L, Toscano A, Pacini A,
19 Bartolini A (2009) Neuroprotective effects of acetyl-L-carnitine on neuropathic pain
20 and apoptosis: a role for the nicotinic receptor. *Journal of neuroscience research*
21 87:200-207.
22
23 Di Cesare Mannelli L, Ghelardini C, Toscano A, Pacini A, Bartolini A (2010) The
24 neuropathy-protective agent acetyl-L-carnitine activates protein kinase C-gamma and
25 MAPKs in a rat model of neuropathic pain. *Neuroscience* 165:1345-1352.
26
27 Fiskum G (2004) Mechanisms of neuronal death and neuroprotection. *Journal of*
28 *neurosurgical anesthesiology* 16:108-110.
29
30 Foreman PJ, Perez-Polo JR, Angelucci L, Ramacci MT, Tagliatela G (1995) Effects of
31 acetyl-L-carnitine treatment and stress exposure on the nerve growth factor receptor
32 (p75NGFR) mRNA level in the central nervous system of aged rats. *Progress in*
33 *neuro-psychopharmacology & biological psychiatry* 19:117-133.
34
35 Ho VM, Lee JA, Martin KC (2011) The cell biology of synaptic plasticity. *Science* 334:623-
36 628.
37
38 Horwood JM, Dufour F, Laroche S, Davis S (2006) Signalling mechanisms mediated by the
39 phosphoinositide 3-kinase/Akt cascade in synaptic plasticity and memory in the rat.
40 *The European journal of neuroscience* 23:3375-3384.
41
42 Jones LL, McDonald DA, Borum PR (2010) Acylcarnitines: role in brain. *Progress in lipid*
43 *research* 49:61-75.
44
45 Kirino T (1982) Delayed neuronal death in the gerbil hippocampus following ischemia. *Brain*
46 *research* 239:57-69.
47
48 Kocsis K, Knapp L, Gellert L, Olah G, Kis Z, Takakuwa H, Iwamori N, Ono E, Toldi J,
49 Farkas T (2014) Acetyl-L-carnitine normalizes the impaired long-term potentiation
50 and spine density in a rat model of global ischemia. *Neuroscience* 269:265-272.
51
52 Kocsis K, Knapp L, Meszaros J, Kis Z, Farkas T, Vecsei L, Toldi J (2015) Acetyl-L-carnitine
53 and oxaloacetate in post-treatment against LTP impairment in a rat ischemia model.
54 An in vitro electrophysiological study. *Journal of neural transmission* 122:867-872.
55
56 Malaguarnera M (2012) Carnitine derivatives: clinical usefulness. *Current opinion in*
57 *gastroenterology* 28:166-176.
58
59 Molz S, Olescowicz G, Kraus JR, Ludka FK, Tasca CI (2015) Purine receptors are required
60 for DHA-mediated neuroprotection against oxygen and glucose deprivation in
61 hippocampal slices. *Purinergic signalling* 11:117-126.
62
63 Nistico R, Piccirilli S, Cucchiaroni ML, Armogida M, Guatteo E, Giampa C, Fusco FR,
64 Bernardi G, Nistico G, Mercuri NB (2008) Neuroprotective effect of hydrogen
65

- peroxide on an in vitro model of brain ischaemia. *British journal of pharmacology* 153:1022-1029.
- Pettegrew JW, Levine J, McClure RJ (2000) Acetyl-L-carnitine physical-chemical, metabolic, and therapeutic properties: relevance for its mode of action in Alzheimer's disease and geriatric depression. *Molecular psychiatry* 5:616-632.
- Picconi B, Barone I, Pisani A, Nicolai R, Benatti P, Bernardi G, Calvani M, Calabresi P (2006) Acetyl-L-carnitine protects striatal neurons against in vitro ischemia: the role of endogenous acetylcholine. *Neuropharmacology* 50:917-923.
- Pulsinelli WA, Brierley JB, Plum F (1982) Temporal profile of neuronal damage in a model of transient forebrain ischemia. *Annals of neurology* 11:491-498.
- Rapoport SI (1999) In vivo fatty acid incorporation into brain phospholipids in relation to signal transduction and membrane remodeling. *Neurochemical research* 24:1403-1415.
- Ronaldson PT, Davis TP (2013) Targeted drug delivery to treat pain and cerebral hypoxia. *Pharmacological reviews* 65:291-314.
- Shu Y, Zhang H, Kang T, Zhang JJ, Yang Y, Liu H, Zhang L (2013) PI3K/Akt signal pathway involved in the cognitive impairment caused by chronic cerebral hypoperfusion in rats. *PloS one* 8:e81901.
- Shug AL, Schmidt MJ, Golden GT, Fariello RG (1982) The distribution and role of carnitine in the mammalian brain. *Life sciences* 31:2869-2874.
- Ten VS, Starkov A (2012) Hypoxic-ischemic injury in the developing brain: the role of reactive oxygen species originating in mitochondria. *Neurology research international* 2012:542976.
- Thompson BJ, Ronaldson PT (2014) Drug delivery to the ischemic brain. *Advances in pharmacology* 71:165-202.
- Xu S, Waddell J, Zhu W, Shi D, Marshall AD, McKenna MC, Gullapalli RP (2015) In vivo longitudinal proton magnetic resonance spectroscopy on neonatal hypoxic-ischemic rat brain injury: Neuroprotective effects of acetyl-L-carnitine. *Magnetic resonance in medicine* 74:1530-1542.
- Zanelli SA, Solenski NJ, Rosenthal RE, Fiskum G (2005) Mechanisms of ischemic neuroprotection by acetyl-L-carnitine. *Annals of the New York Academy of Sciences* 1053:153-161.
- Zhou P, Chen Z, Zhao N, Liu D, Guo ZY, Tan L, Hu J, Wang Q, Wang JZ, Zhu LQ (2011) Acetyl-L-carnitine attenuates homocysteine-induced Alzheimer-like histopathological and behavioral abnormalities. *Rejuvenation research* 14:669-679.

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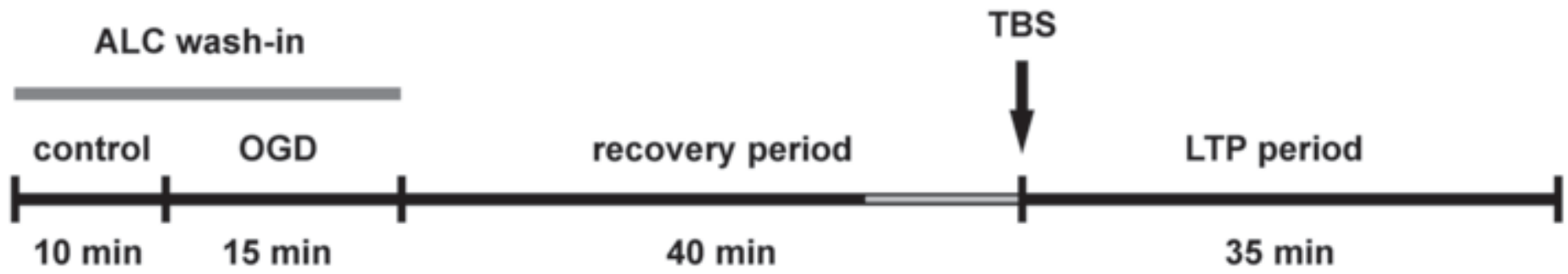


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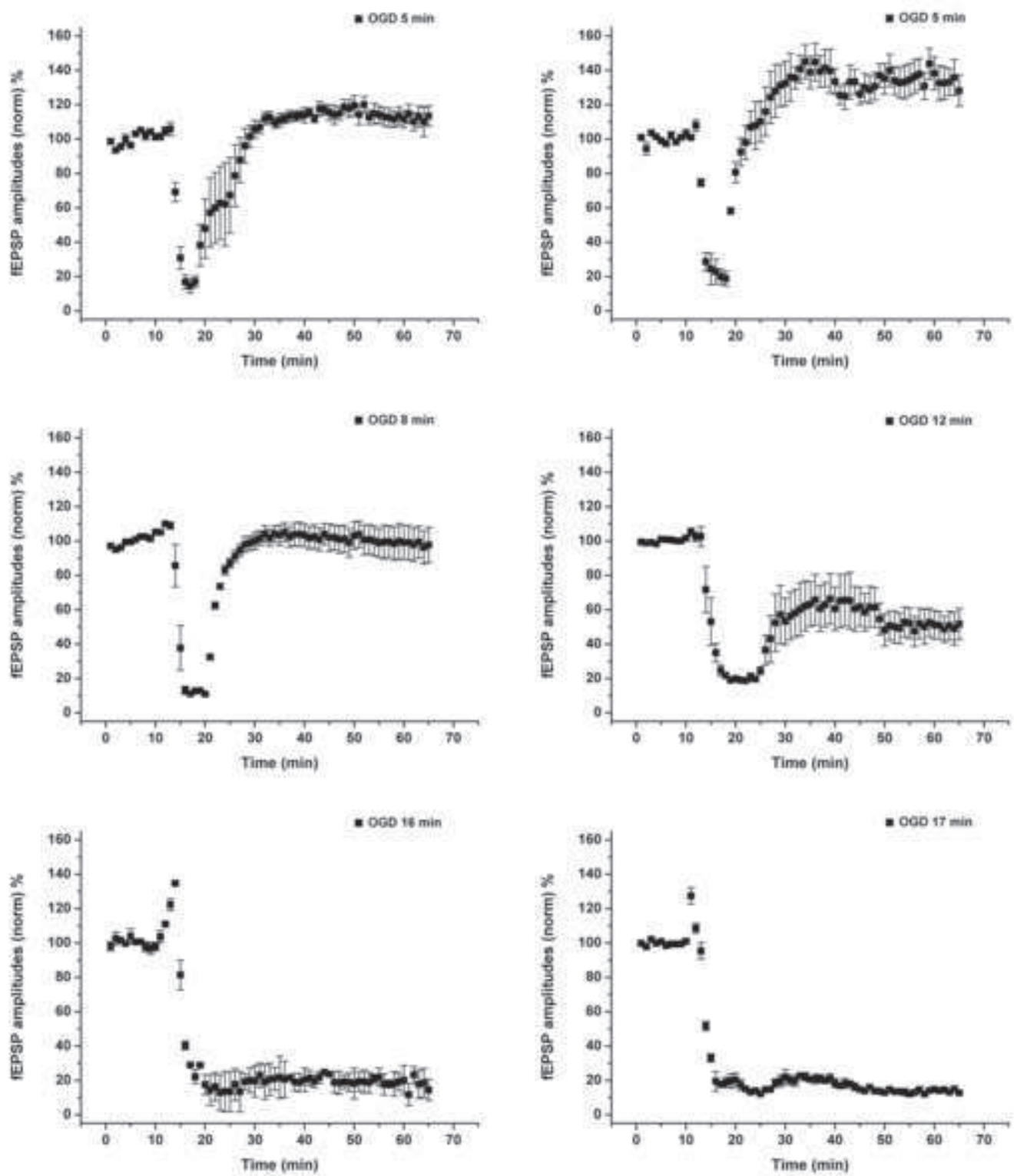


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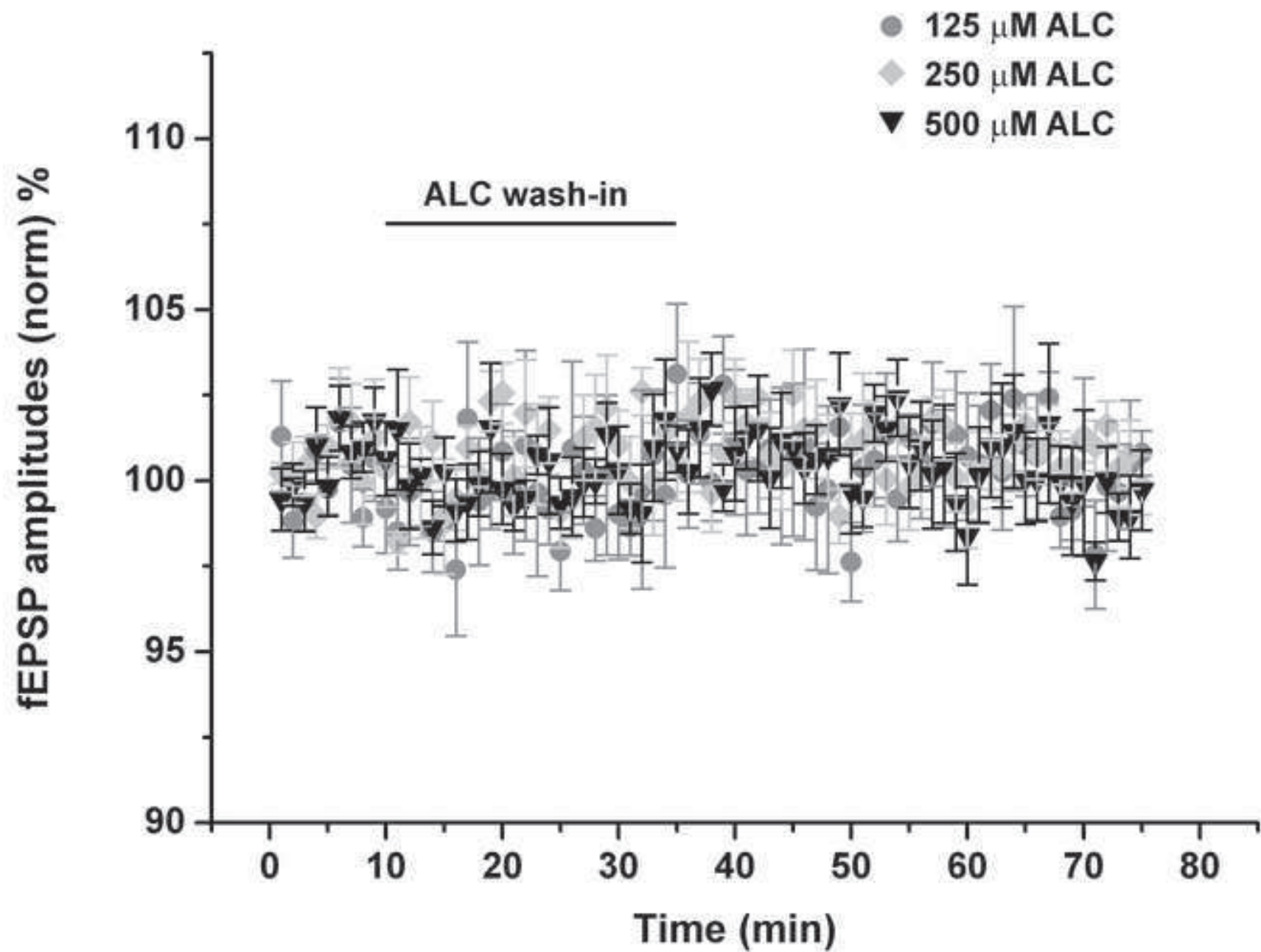


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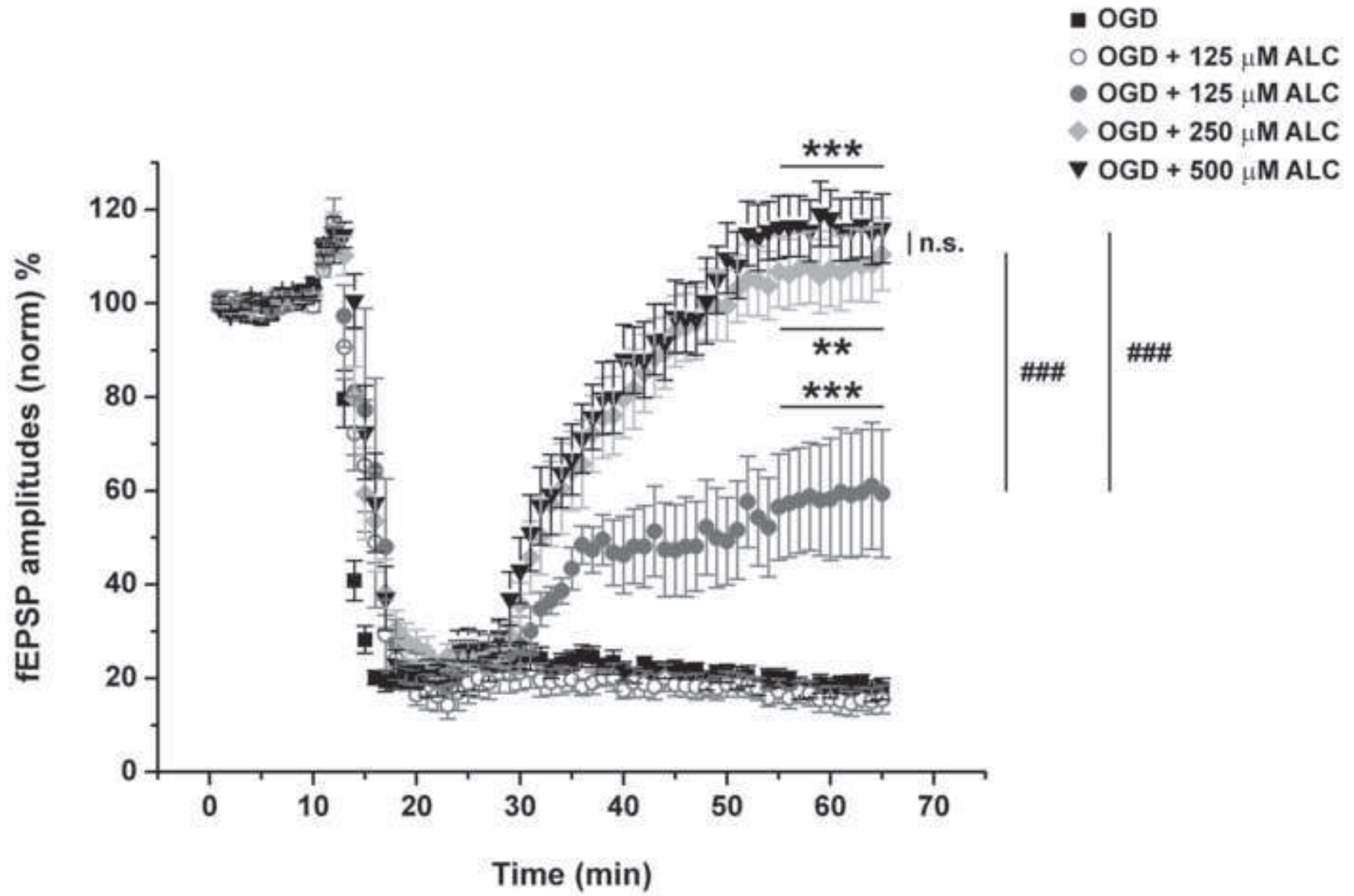


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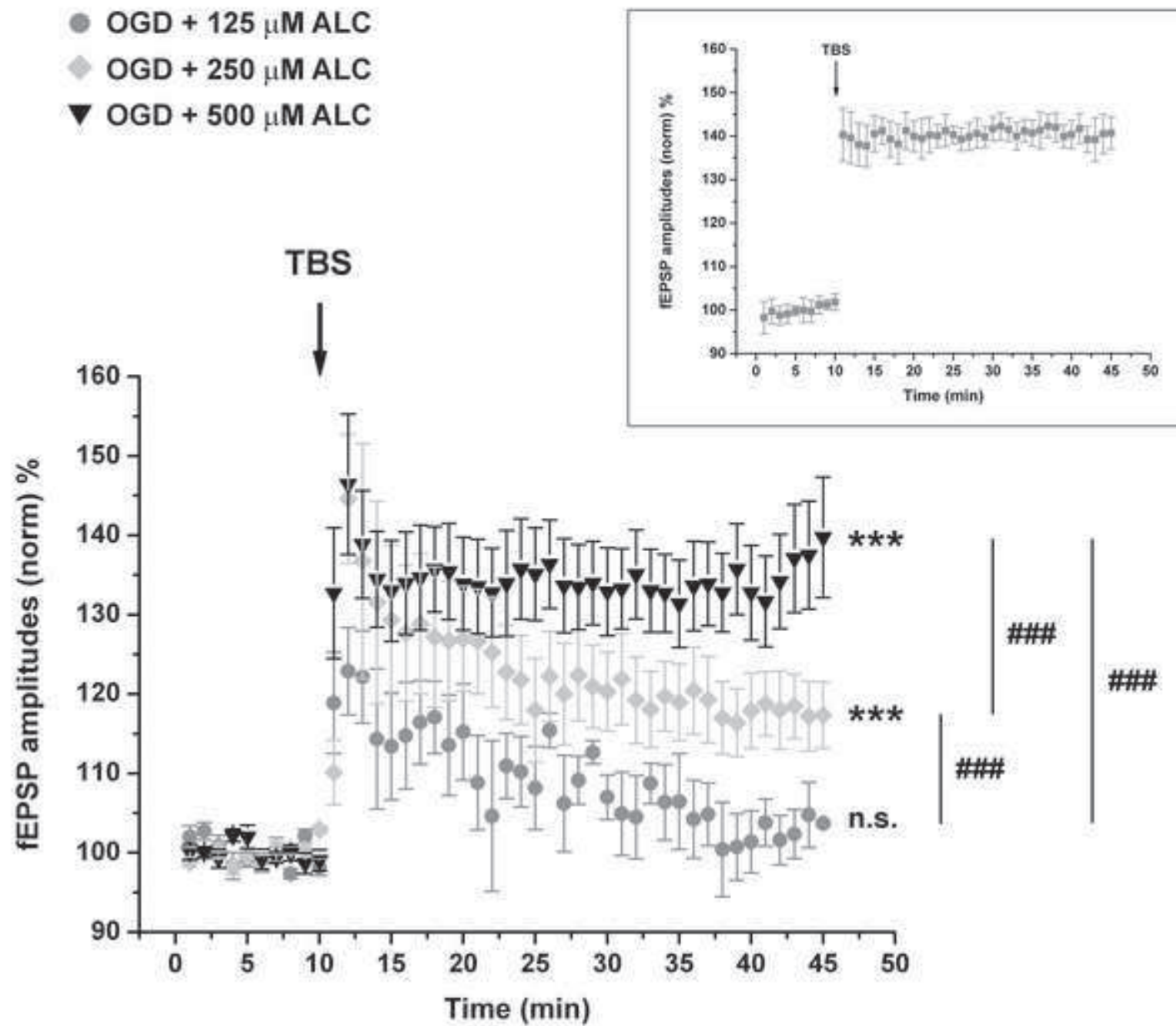


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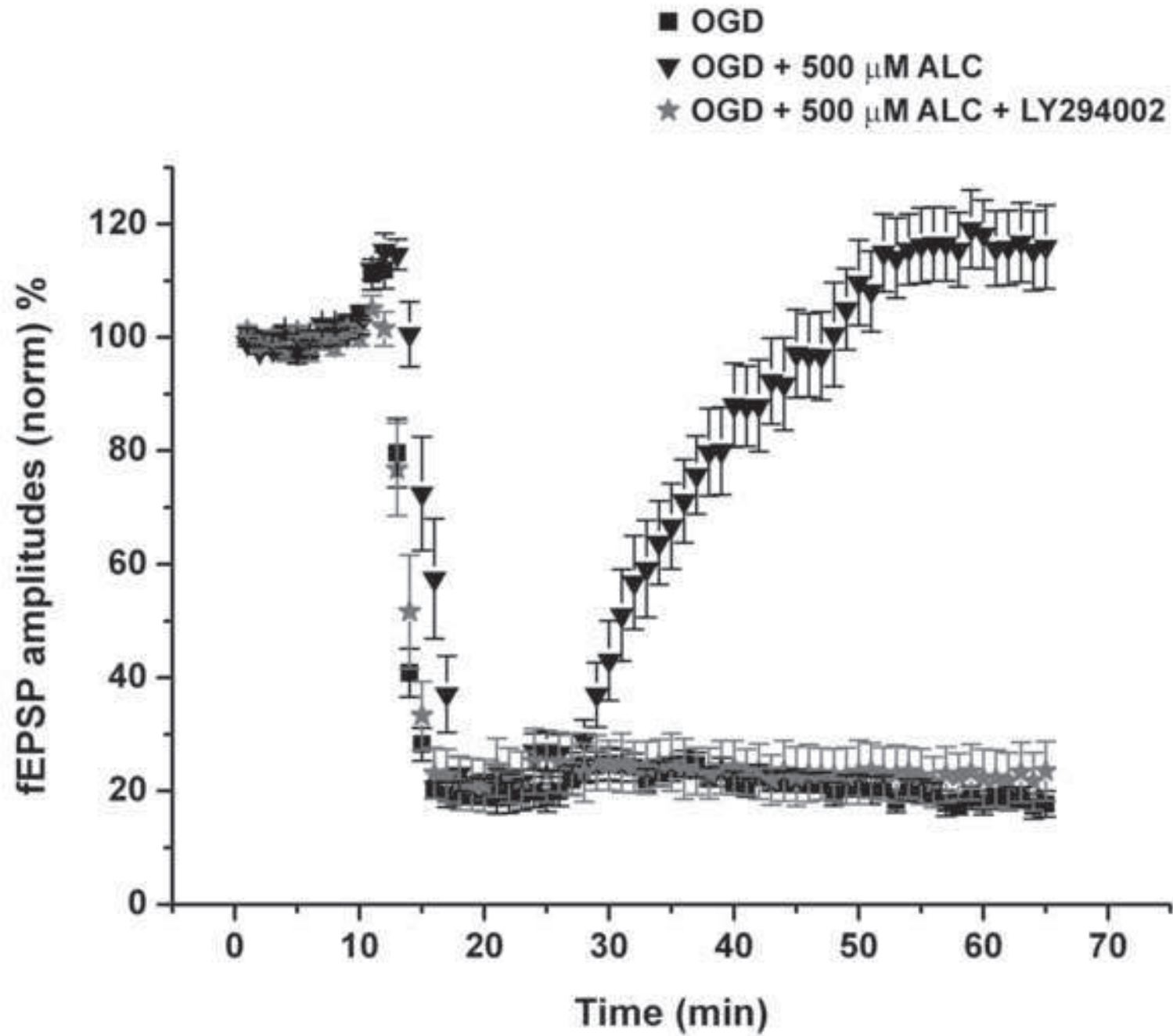


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