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Acute hypoxic exposure and prolyl-hydroxylase inhibition improves synaptic transmission recovery time from a subsequent hypoxic insult in rat hippocampus

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Abstract

In the CNS short episodes of acute hypoxia can result in a decrease in synaptic transmission which may be fully reversible upon re-oxygenation. Stabilization of hypoxia-inducible factor (HIF) by inhibition of prolyl hydroxylase domain (PHD) enzymes has been shown to regulate the cellular response to hypoxia and confer neuroprotection both in vivo and in vitro. Hypoxic preconditioning has become a novel therapeutic target to induce neuroprotection during hypoxic insults. However, there is little understanding of the effects of repeated hypoxic insults or pharmacological PHD inhibition on synaptic signalling. In this study we have assessed the effects of hypoxic exposure and PHD inhibition on synaptic transmission in the rat CA1 hippocampus. Field excitatory postsynaptic potentials (fEPSPs) were elicited by stimulation of the Schaeffer collateral pathway. 30 min hypoxia (gas mixture 95% N,5% CO) resulted in a significant and fully reversible decrease in fEPSP slope associated with decreases in partial pressures of tissue oxygen. 15-30 min of hypoxia was sufficient to induce stabilization of HIF in hippocampal slices. Exposure to a second hypoxic insult after 60 min resulted in a similar depression of fEPSP slope but with a significantly greater rate of recovery of the fEPSP. Prior single treatment of slices with the PHD inhibitor, dimethylallylglycine (DMOG) also resulted in a significantly greater rate of recovery of fEPSP post hypoxia. These results suggest that hypoxia and ‘pseudohypoxia’ preconditioning may improve the rate of recovery of hippocampal neurons to a subsequent acute hypoxia.

Key words

Prolyl hydroxylase inhibition, hypoxia, Hippocampus, CA1 region, synaptic transmission, pre-conditioning

Abbreviations

EPO, erythropoietin; fEPSP, field excitatory post synaptic potential; DMOG; dimethylallylglycine; HIF, hypoxia inducible factor; PHD; prolyl hydroxylase domain; VEGF, vascular endothelial growth factor.

1. Introduction

In the central nervous system approximately 40% of cerebral oxygen is utilised for synaptic transmission (Astrup et al., 1982). Given the high demand for O, the relationship between hypoxia and synaptic signalling is very important whereby neurons can alter synaptic transmission in response to hypoxic conditions within minutes. Depending on many factors including the duration of hypoxia, neurons can fully recover upon reoxygenation (Fowler et al., 2003; Lipton & Whittington, 1978). The depression of synaptic transmission during hypoxia is primarily mediated by adenosine, the concentration of which is greatly increased during cerebral ischemia (Loghi Pasini et al., 2000). The release of adenosine from cells in response to reduced regional blood flow, which is not significant to induce glutamate excitotoxicity, suggests adenosine may play some role in alleviating the potential for excitotoxicity (Matsumoto et al., 1992; Duarte et al., 2016).

The recovery of synaptic transmission after a period of hypoxia and repeated hypoxia has been previously investigated by a number of laboratories. We have previously demonstrated that rats treated with intermittent hypoxia for 7 days showed improved recovery times for synaptic transmission in both the CA1 and dentate gyrus regions of the hippocampus (Wall et al., 2014). Sebastião et al., (2001) demonstrated an inhibitory role for adenosine in the recovery from 90 min hypoxia, an effect that was reversed by tetrodotoxin. Frenguelli et al., (2003) also demonstrated a role for adenosine A1 receptors (desensitisation) in the recovery from hypoxia, although Vikolinsky et al., (1999) showed some of these effects to be irreversible.

Other previous studies have demonstrated that application of hypoxic or sub-lethal ischemic conditions prior to stroke significantly reduces infarct severity in neonates and adult rats (Vannucci et al., 1998; Gidday et al., 1999; Miller et al., 2001; Bernaudin, et al., 2002a). It has been proposed that tolerance to ischemic insults by hypoxia preconditioning is due to activation of hypoxia inducible factors (HIF) and HIF target genes including VEGF, EPO, GLUT-1 and adrenomedullin (Bergeron et al., 1999; Bernaudin, et al., 2002b). Under normoxic conditions the activation of HIF-1α is blocked by prolyl hydroxylase domain proteins (PHDs). Whether these factors would have a role to play in acute hypoxia (30 to 60 min) remains to be seen.

Since their discovery, PHDs have become a novel therapeutic target for hypoxic injuries. Pharmacological inhibition of PHDs via 2-OG competitive antagonism or iron chelation has become an attractive strategy to precondition neurons for a subsequent hypoxic stress. In vitro, various types of PHD inhibitors have been shown to stabilize HIF-1α, either by 2-OG antagonism (N-oxalylglycine, DMOG, 3,4-dihydroxybenzoate, DHB), iron chelation (deferoxamine, DFO) or heavy metal substitution of iron (CoCl2) (Epstein et al., 2001; Huang et al., 2003; Siddiq et al., 2005). We have recently demonstrated fast acting (within minutes) mechanisms of action of PHD inhibition on synaptic signalling and plasticity (Corcoran et al., 2013; Corcoran & O’Connor, 2013; Lanigan & O’Connor, 2018). In the following studies we have set out to investigate the effects of an initial hypoxic (95% N,5% CO) exposure or pre-treatment with the PHD inhibitor, DMOG, on the rate of recovery of synaptic transmission from a subsequent hypoxic exposure (see methods).

2. Results

2.1. Acute hypoxia decreases synaptic signalling and increases HIF stabilization

We recorded fEPSPs from the stratum radiatum pyramidal neurons of the CA1 region of the rat hippocampus every 30 s. During hypoxia (95% N,5% CO) superfusion, the fEPSP slope was significantly decreased to 8.0±3.5% control (n=7, P<0.001) within 15 min. 20 min after re-oxygenation the fEPSP slope recovered to 92.4±9.5%
control (Figure 1A). Using fluorescence quenching oxymetry, we monitored the oxygen tension at the surface and within the slice at a depth similar to our recording electrode (~100 µm). Partial pressures of oxygen (PO) were measured during control O, hypoxia and subsequent reoxygenation. 100 µm below the surface we detected a rapid reduction of PO which reached 7.8±5.9 mmHg 5 min after hypoxia superfusion (Figure 1Bii). At the surface of the slice PO fell to 45±6.7 mmHg (Figure 1Bii). We then measured the levels of HIF-1α to determine if our hypoxia paradigm resulted in phenotypic changes associated with hypoxia superfusion. Immunoblotting of hippocampal slices exposed to 30 min hypoxia showed a 6.1±0.8 fold increase in HIF-1α expression compared to control slices (n=4, P<0.01). 30 min reoxygenation of slices resulted in a significant degradation of HIF-1α compared to hypoxia, although still significantly higher than control tissue. Application of DMOG (1 mM) for 30 min also significantly increased HIF-1α expression (Figure 1C).

Figure 1
Hypoxia (95% N:5% CO) exposure for 30 min causes a reversible decrease in synaptic signalling in the CA1 and stabilizes HIF-1α expression (A) Stable fEPSPs were recorded before hypoxia superfusion (represented by the black bar) for 30 min. Time matched control were shown in closed circles, 30 min hypoxia in open circles. Hypoxia induced near full inhibition of fEPSP in the CA1 after 5 min. Re-oxygenation resulted in a full recovery of fEPSP slope. Insets show representative fEPSPs (1) pre-hypoxia, (2) hypoxia and (3) recovery at the points indicated. (B, i) Measurements of PO on the hippocampal slice surface indicate a significant decrease in oxygen tension within 5 min of exposure to hypoxia (black line). Oxygen tension exceeded 200 mmHg 5 min after re-oxygenation. (B, ii) Initial decline of PO for recordings on the surface of the slice (open circles) and 100 µm below the surface (black triangles). Note the gradient difference from surface to 100 µm (C). Homogenates from slices taken before hypoxia (N), 30 min after hypoxia (H), 30 min following re-oxygenation (R) and 30 min in the presence of DMOG (1mM) showing stabilization of HIF-1α during hypoxia, which is rapidly degraded upon re-oxygenation. Denitrosometric analysis was performed by normalizing the HIF-1α band to the α-tubulin band. Values are expressed as fold increases of control O. slices and expressed as the mean±SEM. **P<0.01, n=7 (A & B), n=4 (C).

2.2. Hypoxic pre-conditioning improves the recovery rate from a subsequent hypoxic insult
In another set of experiments fEPSPs were evoked every 30 s in the CA1 region of the hippocampus for 90 min before superfusion of media equilibrated with 95% N:5% CO (hypoxia). fEPSP slope decreased significantly to 9.1±0.9% control (n=6, P<0.001) 15 min after hypoxia. fEPSP slope returned to baseline (95.5±4.4%) 15 min after reoxygenation (Figure 2A). In a parallel set of experiments an initial 30 min superfusion with 95% N:5% CO resulted in a significant decrease in fEPSP slope to 9.6±1.7% control (not significantly different from the first set of experiments). Again fEPSP slope returned to baseline upon reoxygenation (95.5±3.8%), 30 min after reoxygenation, a second 30 min hypoxia was initiated. The second insult resulted in a similar decrease in fEPSP slope (8.1±2.8% 15 min after 30 min hypoxia). The fEPSP slope returned to 100.1±6.3% control with a higher recovery rate upon reoxygenation. To determine changes in recovery rate we analysed the time taken to return to pre hypoxia baseline synaptic transmission (t) and the time taken to reach 50% of the maximum recovery (t). The t was significantly reduced to 4.7±0.6 min (versus 10.4±1.5 min in single hypoxia perfused slices) following reoxygenation of preconditioned slices (Figure 2B, C). Additionally, t was significantly reduced to 6.8±0.7 min (versus 13.9±1.8 min in single hypoxia perfused slices; n=6, P<0.01) after reoxygenation (Figure 2B, C).

Figure 2
Prior hypoxia (95% N:5% CO) exposure improves recovery time from a subsequent hypoxia insult in the CA1. (A) Time course for normalised fEPSP slope recorded from the CA1 region over 150 min. In the control experiments stable baseline fEPSPs were maintained for 90 min before induction of a single 30 min hypoxia exposure (●). Full recovery was observed upon re-oxygenation in the CA1. In a second set of time matched experiments slices were exposed to two 30 min hypoxia exposures separated by 30 min control O (○). (B) fEPSP recordings from A were normalized to time 0 at reoxygenation (110 min) to determine the rate of recovery in single (○) and double (●) hypoxia exposed slices. (C) Measurement of time to 50% recovery (t) and time to maximum recovery (t) following reoxygenation in the CA1 region. Preconditioned slices in the CA1 exhibited significantly improved rates of recovery compared to slices exposed to one hypoxia event. All values are expressed as the mean±SEM. **P<0.01, n=6.

2.3. DMOG preconditioning improves the recovery rate from acute hypoxia
Application of DMOG resulted in a 10% depression of fEPSP similar to that observed by Batti et al., (2013). Therefore in these experiments the baseline was normalised to 100% before the hypoxia exposure. In the presence of DMOG, 30 min hypoxia resulted in a significant decrease in fEPSP slope to 16.2±2.4% which was not significantly different to hypoxia alone treated slices (n=6, P>0.05). Reoxygenation resulted in an increase of fEPSP slope to 90.6±3.8% control which was not significantly different to hypoxia alone treated slices 30 min after reoxygenation (Figure 3A). To determine changes in recovery rate we analysed the time taken to return to pre- hypoxia baseline (t) and the time taken to reach 50% of the maximum recovery (t). In DMOG treated slices t was significantly decreased to 6.5±0.4 min (n=6, P<0.01) following reoxygenation. t was also significantly decreased to 8.7±0.5 min (n=6, P<0.01, compared to single hypoxia perfused slices) after reoxygenation (Figure 3B,C).
3. Discussion

In these experiments we have observed a significant increase in the recovery rate of synaptic transmission in the hippocampal CA1 region following a prior acute superfusion of 95% N/5% CO (hypoxia) or DMOG application. These responses were all associated with an up-regulation of HIF-1α and a reduction in PO levels both on the surface and 100 µm below the surface of the slices.

Figure 3
Acute DMOG treatment improves recovery time from a subsequent hypoxia insult in the CA1.
(A) Time course for normalised iEPSP slope recorded from the CA1 region over 80 min. Stable baseline iEPSPs were maintained for 80 min before induction of a 30 min hypoxia exposure (●). Application of DMOG (1 mM) resulted in a 10% depression of synaptic signalling (see Batti et al., 2013). The baseline was normalised to 100% before the hypoxia exposure (○). (B) iEPSP recordings were normalized to time 0 at reoxygenation (50 min) to determine the rate of recovery in hypoxia (●) and hypoxia + DMOG treated slices (○). (C) Measurement of time to 50% recovery (t1) and time to maximum recovery (t2) following re-oxygenation. Slices treated with DMOG + hypoxia had significantly improved rates of recovery compared to slices exposed to hypoxia alone. All values are expressed as the mean±SEM. *P<0.01 n=6.

In vitro electrophysiological recordings require the perfusate to be constantly bubbled with 95%/O/5% CO gas. Whilst the high O content of this gas mixture can produce a hyperoxic environment, this methodology is routinely used for electrophysiological recording in acute hippocampal slices and has been shown to be best practice to preserve function within the slice (Aitken et al., 1995). PO, in the intact CNS ranges from below 10 to 35 mmHg in normobaric air but there is significant variance and in particular for the hippocampus (Garcia et al., 1985). Therefore brain tissue exceeding 40 mmHg in the CNS might be considered hyperoxic (D’Agostino et al., 2007). However the partial pressure of O decreases from the surface of the slice to the middle layers (see Figure 1). Experiments carried out in our laboratories show that within 5 min of acute hypoxia (95% N/5% CO) exposure, oxygen tension levels reduced to 8 mmHg, 100 µm below the hippocampal slice surface near where the recording electrode is placed and 45 mmHg at the surface (Batti et al., 2010; Batti & O’Connor, 2010). Therefore it is important to know the PO levels at the depth of the recording electrode in the brain tissue.

As an early consequence of short-term hypoxia, synaptic transmission is reduced. This suppression of synaptic activity may be due to a compensatory mechanism that restores the balance between oxygen supply and consumption in favour of maintaining resting potentials and thus preserves the neuron’s structural integrity (Hochachka et al., 1996). On the other hand, more long-term synaptic failure has been associated with irreversible neuronal damage although the molecular mechanisms are still very unclear. Also there is mounting evidence to suggest that during acute hypoxia it is not the hypoxia per se which damages neurons but the reoxygenation of the cells (Duarte et al., 2016). However for more prolonged durations of hypoxia neurons depolarise and may swell, although synaptic transmission can be irreversibly disturbed without accompanying cytotoxic edema (Bolay et al., 2002; Hofmeijer & Van Putten, 2012; Feber et al., 2016). As well as the more well-known adenosine release changes during hypoxia, reoxygenation following hypoxia can lead to an increase in superoxide flash generation which will increase oxidative stress (Wang et al., 2008). These superoxide flashes which occur in individual mitochondria under normoxic conditions can become harmful to neurons under hypoxia-reoxygenation conditions generating reactive oxygen species. Therefore much research has investigated the actions of anti-oxidants on improving neuronal viability associated with hypoxia- reoxygenation injury and OGD (Yao et al., 2011).

Pearson et al., (2001) have previously shown that with repeated exposure to hypoxia and thus re-oxygenation, there is a reduction in the protective effect of the depression in synaptic transmission. This effect however did not seem to involve desensitisation of adenosine A1 receptors. In fact adenosine release was decreased with repeated episodes of hypoxia and this may be a factor in increased neuronal vulnerability. In contrast however where rapid hypoxia-induced adenosine release has previously been measured in real time in the CA1 with concomitant recordings of iEPSPs, recovery of the iEPSP occurred despite a significant surge in extracellular adenosine upon reoxygenation (Feng et al., 2003). This they indicated may be a desensitisation of A1 receptors during metabolic stress. They also suggested that the rise in adenosine was not as a result of excitotoxic glutamate release. Methods to block this desensitisation during hypoxia could be neuroprotective. Furthermore it has previously been reported that acute hypoxia can lead to changes in the density and efficiency of A1 receptors. This effect was blocked by the A1 receptor antagonist DPCPX (Coelho et al., 2006). They suggest that this acute hypoxia and desensitisation of A1 receptor mediated changes in synaptic transmission may be due to internalisation of the receptors in the nerve terminals. Whilst adenosine can inhibit excitatory synaptic transmission through A1 receptors, it has also been shown to protect other tissues from hypoxia usually by means of controlling their metabolism (Duarte et al., 2016). This study may also indicate that activation of A1 receptors during transient hypoxia can play an important role in the recovery from hypoxia (re-oxxygenation) and subsequent metabolic changes.

30 min hypoxia or DMOG superfusion increased HIF-1α in our tissues. To date the only reported function for HIF-1α is as a transcription factor and we hypothesise that it is playing this role in the brain also. While HIF-1α levels are inducible, its binding partner HIF-1beta/ARNT is constitutively expressed and it is only when the two isoforms dimerize that they form a functional transcription factor. While beyond the scope of this current study, we intend to measure the dimerization of HIF-1α and HIF-1β in response to hypoxia and DMOG in future studies. Several studies to date have implicated the HIF-β-β2 system in the mechanism of remote ischemic preconditioning (βPC), protection conferred by transient brief episodes of ischemia at a remote site before a subsequent prolonged ischemia/reperfusion injury of the target organ). For example studies have shown mice with genetically reduced levels of PHD2 showed greater resistance to cardiac ischaemia due to enhanced HIF-1α levels (Hynes et al., 2010), as did animals with activation of HIF by pharmacological PHD inhibition (GSK360A) or VHL deficiency (Ong et al., 2014). Others have shown enhancement of cardiac protection by pharmacological (DMOG) and genetic enhancement of HIF-1α (Eckle et al., 2008). To date the majority of research has been carried out in the periphery, however the fundamental reasoning behind this peripheral work can be
translated to the CNS. For example, evidence suggests that transient ischemic attacks (TIAs) may precondition patients against later strokes (Moncayo et al., 2000).

Previously Furling et al., (2000) using glutathione peroxidase transgenic mice also showed improved recovery in synaptic transmission. These mice exhibiting a small increase in glutathione peroxidase-1 (GP1; a cellular antioxidant enzyme) showed improved tolerance compared to non-Tg slices even after multiple hypoxia episodes. Also preconditioning cells with acute hypoxia-reoxygenation insults (15 min hypoxia/ 15 min normoxia) may improve cell viability to subsequent hypoxic stress (Yao et al., 2011). In a paper by Sebastiao et al., (2001) they investigated the recovery of synaptic transmission in the CA1 after 90 min hypoxia. In the presence of DPCPX recovery was attenuated, an effect which could be reversed in the presence of tetrodotoxin or stopping stimulation of fibres but not by postsynaptic blockade with CNQX.

NMDA receptor block also improved recovery from hypoxia in the presence of DPCPX. They conclude that adenosine release during hypoxia can prevent glutamate activating NMDA receptors. We cannot say if this is the case in our experiments.

We were also interested in the effect of PHD inhibition on the rate of synaptic transmission recovery during superfusion of 95%/5%/5%CO2 and reoxygenation. We have previously demonstrated acute effects of PHD inhibitors on synaptic signalling and synaptic plasticity (LTP) in the rat hippocampus (Corcoran et al., 2013; Lanigan et al., 2018), whereby DMOG, DFO and EDHB can have depressive effects on synaptic signalling in the hippocampus possibly acting through NMDA receptors. In the current experiments DMOG also resulted in a 10% decrease in synaptic transmission. We have also previously shown that chronic (7 days) treatment with intermittent hypoxia and DMOG application can alter the recovery time from hypoxia (Wali et al., 2014). PHD inhibition has become a novel target for preconditioning systems for ischemic as well as therapeutic intervention following ischemic attacks (Miyata et al., 2011; Ogle et al., 2012). The benefits of such preconditioning have been overwhelming positive both in vitro (Lomb et al., 2007, 2009; Batti et al., 2010; Watters et al., 2011) and in vivo (Kasiganesan et al., 2007; Chen et al., 2008; Nagel et al., 2011). In addition PHD inhibition has been shown to decrease oxidative stress induced cell death of cortical neurons (Niatsetskaya et al., 2010). It is possible that the neuroprotective properties of hypoxic pre-treatment and PHD inhibition with agents such as DMOG, may also play a role in the improved recovery rates we observed in these experiments. Preconditioning experiments such as these may reduce the oxidative stress associated with reoxygenation from acute hypoxia and allow the neurons to return to maximum function more rapidly. The protective effects of PHD inhibitors may occur without a role for HIF-1α as Park et al., (2012) have demonstrated that hypoxia can regulate glutamate receptors through a HIF-independent mechanisms albeit in a C. Elegans animal model. In unpublished experiments using PHD2 KO mice we observed an increased synaptic depression during acute hypoxia suggesting an important role for prolyl hydroxylases in acute hypoxia. More work will be required to demonstrate conclusively whether it is increased HIF-1α signaling or O2 metabolic consumption that is behind our preconditioning in both the hypoxia and pseudohypoxia experiments. Indeed, DMOG can inhibit cellular O2 consumption before HIF signaling (e.g., Zhdanov et al., 2015).

In conclusion we have shown that hypoxia and pseudohypoxia (DMOG treatment) preconditioning improves the rate of recovery of synaptic transmission in rat hippocampal slices to a subsequent acute hypoxia. Understanding the mechanisms involved in this effect may be important in elucidating the complex changes in neurons during hypoxia.

4. Experimental Procedures

4.1. Wistar rats
Male Wistar rats were obtained from Charles Rivers Laboratories International and housed in the University College Dublin (UCD) Biomedical facility. All experimental procedures were approved by the Animal Research Ethics Committee of the Biomedical Facility at UCD.

4.2. Extracellular recording techniques
Field excitatory postsynaptic potentials (fEPSPs) were evoked by stimulation of the Schaffer-collateral pathway of the CA1 region of the hippocampus using aCSF-filled monopolar glass electrodes. fEPSPs were elicited at a frequency of 0.033 Hz and recorded from the dendritic field of the CA1 pyramidal neurons using aCSF-filled monopolar glass electrodes (0.5 to 2MΩ). The stimulating electrode was connected to a S48 stimulator (Grass Instrument; Massachusetts, USA) via a Grass SIU5 stimulus isolation unit. The recording electrode was connected to an Axopatch 1D via a CV-4 head stage, which amplified evoked responses 1000-fold. fEPSPs were acquired at 20 kHz and filtered at 5 kHz. Stimulus strength was adjusted to give 50% maximal response as determined by an input/output curve. Recordings were acquired and analysed using the software package WinWCP (J. Dempster, Stratfucyde).

4.3. Superfusion of N2/O2 and PO2 recording
During fEPSP recordings, generation of an in vitro hypoxic environment was induced by switching the gas aerating the aCSF from 95% O2/5% CO2 (control O2) to 95% N2/5% CO2 (pseudohypoxia). Re-oxygenation was induced by switching the gas from 95% N2/5% CO2 to 95% O2/5% CO2. Partial pressures of oxygen (PO2) were recorded on and from within the hippocampal slices using a PO monitor (OXYLITE) during hypoxia superfusion and re-oxygenation. The oxygen probe was positioned on the surface and inside the hippocampal slice at a depth similar to the recording electrode (100 μm). Changes in PO2 were recorded every 5 s. Measurement of PO2 above 200 mmHg could not be detected as this was outside the range of the probe. N2/CO2 was bubbled in the ACSF for 30 min at a flow rate of 3 ml/min and required 210 s to equilibrate with the recording chamber of 1 mL volume. The PO2 returned to levels greater than 200 mmHg after 200 s.

4.4. Immunoblotting
Hippocampal slices were taken after electrophysiology experiments and homogenised in RIPA buffer supplemented with protease cocktail inhibitor, phosphatase inhibitor cocktail II and phosphatase inhibitor cocktail III. Samples were then centrifuged for 10 min at 10,000 rpm at 4°C. The pellet was discarded and supernatant used for protein concentration assay (BCA Protein Assay Reagent, Pierce) and loading sample preparation. Proteins (20 mg) were separated by 10% SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was incubated with primary antibodies against HIF-1α (1:500, Novus Biologicals), anti-a tubulin (1:1,000, Sigma-Aldrich), and secondary anti mouse-HRP linked antibody (Cell Signalling).

4.5. Data Analysis
All fEPSP slope measurements are presented as a percentage of the baseline recordings. Baseline recordings were determined by the average of fEPSP slope over 20 min prior to drug application or superfusion of 95%/5%/5%CO2. Statistical significance was determined using either the non-parametric paired Wilcoxon matched t-test or paired Student t-tests. In Figures 3 and 4 One-way ANOVA with a post-Bonferroni test was carried out. Data are presented as mean±SEM. P<0.05 was considered significant.
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