

RESEARCH REPORT 2002/2003

FAN GMBH AND IFN PROJECT GROUP NEUROPHARMACOLOGY

HEAD: PROF. DR. KLAUS G. REYMANN

Summary

The main topics of the FANgGmbH in close collaboration with the IfN-Project Group Neuropharmacology are "cerebral ischemia" and "phenomons of brain plasticity" investigated both under physiological and under pathophysiological conditions. For this aim we use different approaches. The method of extracellular field potential recordings in both acutely isolated and cultured hippocampal slices allow us to test potential neuroprotective drugs after oxygen/glucose-deprivation (OGD) *in vitro*. With different Ca²⁺ sensitive dyes we try to elucidate mechanisms of hypoxic/hypoglycemic cell death in cultured hippocampal slices. This preparation also enables us to study mechanisms of regeneration after ischemic and traumatic injury, e.g. by proliferation of endogenous stem cells. With different approaches *in vivo* we investigate the underlying mechanisms of neuronal death after ischemia, we test new neuroprotective strategies targeting at ion transporters and proteases, and we elucidate the possibilities of CNS regeneration after an ischemic insult. In order to study mechanisms of learning and memory we use the model of long-term potentiation (LTP) in acutely isolated hippocampal slices. These experiments are partially performed in combination with measuring intracellular, dendritic Ca²⁺ concentration changes during LTP-induction with different fluorescent dyes by confocal laser scan microscopy, thereby characterizing which Ca²⁺ sources might be involved in LTP.

Strategies for treatment of cerebral ischemia

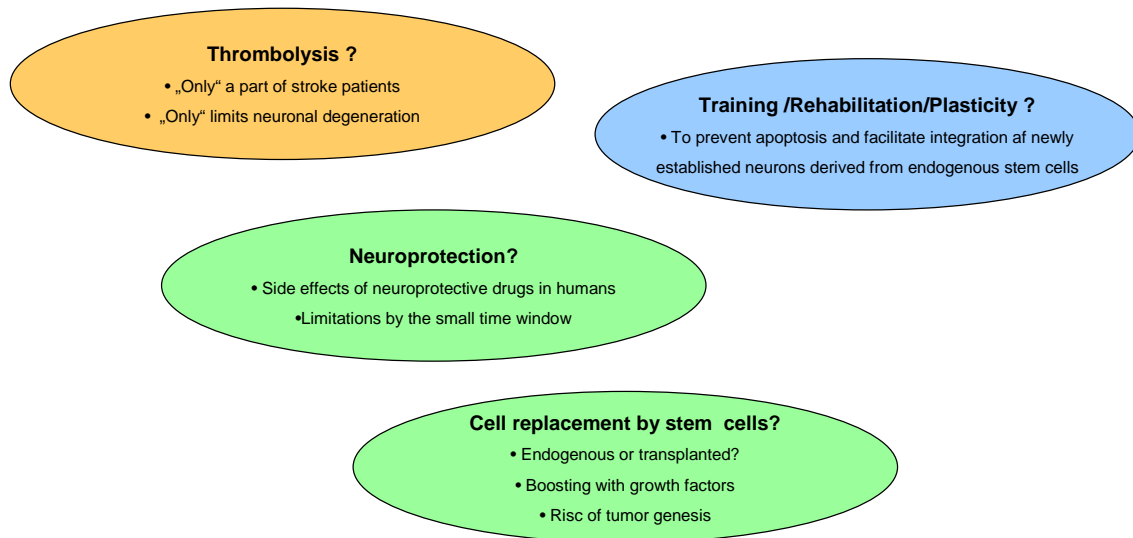


Fig. 1: Strategies for treatment of cerebral ischemia

1. Ion exchangers and ion channels as novel targets for neuroprotective drugs in cerebral ischemia

U.H. Schröder, M. Martínez-Sánchez, F. Striggow S. Busse,

Cerebral ischemia and, under in vitro conditions, oxygen/glucose deprivation (OGD) lead to a massive influx of Ca^{2+} and Na^+ into neurons, resulting in damage and subsequent death. The role of particular ion channels involved is still a matter of debate and there are only few studies regarding the relevance of ion exchangers. By means of ion-sensitive dyes (Fura-2, Mag-fura-2 and Fura-FF for Ca^{2+} , SBFI for Na^+) we studied calcium and sodium dynamics in pyramidal neurons in organotypic hippocampal slice cultures (OSC) and monitored short-term survival in OSCs and freshly isolated slices from adult rats as well as long-term survival in OSCs by propidium iodide (PI) staining 24 h after the insult. The mechanisms of interest are depicted in figure 1. Blockade of the NMDA-receptor is not only neuroprotective (Breder et al. 2000) but also strongly reduces Ca^{2+} -influx (Martinez et al., 2002). Inhibition of all voltage gated Ca^{2+} -channel subtypes by mibefradil, in contrast, neither affected neuronal survival nor Ca^{2+} -influx. Diminution of the Na^+ -influx by tetrodotoxin and lidocain was also protective (Breder et al. 2000, Martinez et al., 2002). Na^+ -and Ca^{2+} -gradients are coupled by the

Na⁺/Ca²⁺ exchanger, which pumps Ca²⁺ out of the cells under physiological conditions (Figure1). The selective Na⁺/Ca²⁺ exchange inhibitor KB-R7943 does not only improve recovery of population spike amplitudes in OSCs and adult slices and reduce PI staining after OGD, but also strongly limits Ca²⁺-influx (Schröder et al. 1999, Breder et al. 2000, Martinez et al., 2002). This suggests that the Na⁺/Ca²⁺ exchanger, operating in reverse mode, contributes to hypoxia/hypoglycemia-induced injury in pyramidal neurons. The competitive AMPA/Kainate receptor blocker NBQX neither affected somatic Na⁺- or Ca²⁺-gradients but did confer neuroprotection. Similar results were obtained with the mitochondrial Na⁺/Ca²⁺ exchange inhibitor CGP-37157 (Martinez et al., 2002). Thus our data suggest that the protective effects of MK-801, KB-R7943 and lidocaine are due to inhibition of somatic Ca²⁺ load, while NBQX and CGP-37157 might block detrimental ionic mechanisms in the dendrites. Intracellular Ca²⁺ stores do not significantly contribute to OGD-induced intracellular Ca²⁺-rise or neurodegeneration (Martinez et al., 2002).

Initial studies indicate that the activation ATP-regulated-K⁺-channels can also reduce OGD-induced injury after an insult. Low nanomolar concentrations of the novel ATP-regulated-K⁺-channel opener Y-26763 improve neuronal recovery in OSCs and adult slices without affecting mitochondrial function, although isoforms of ATP-regulated-K⁺-channel are present in the plasma membrane as well as in mitochondria. High nanomolar concentrations in contrast elicit a concentration-dependent decrease of mitochondrial oxygen consumption, but only in the presence of high Ca²⁺ concentrations (Schröder et al. 2003). As the activation of these channels stabilizes the membrane potential it is likely that the channels confer protection by this effect. Stabilization of the membrane potential will reduce NMDA-receptor activation by stabilizing the magnesium block, reduce Na⁺ channel activity and counteracts the reversal of the Na⁺/Ca²⁺ exchanger. Thus ATP-regulated-K⁺-channels may be a promising target to reduce injury after ischemic insults.

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2. Expression of Protease Activated Receptors in the brain and their role in ischemia

P. Henrich-Noack , M. Riek

On the basis of our former work concerning the role of thrombin/protease activated receptors (PARs) in neuronal degeneration after transient global ischemia or after hypoxic challenge of cultured slices, we started investigating the involvement of this protease and its receptors in

the pathology of focal ischemia. Our former research focussed on the hippocampal structure. During the course of that project we found that thrombin can enhance or induce damage (high concentration) as well as reduce cell death (low concentration) after oxygen-glucose deprivation in cultured hippocampal slices. In vivo, application of the antagonist hirudin shortly before transient global ischemia resulted in increased neuronal survival but at the same time hirudin reduced the protective tolerance effect of short ischemic challenges when applied before the preconditioning stimuli. Semi-quantitative RT-PCR showed that pro-thrombin mRNA is selectively increased after transient global ischemia but PAR 1-3 mRNA are not significantly changed early after clamping both common carotid arteries.

In our current work we checked how focal ischemia changes mRNA expression of PARs in cortical/striatal regions. The model of focal ischemia represents the pathology of stroke, which is the most prominent form of ischemia that appears in clinic. Blood flow was interrupted by transient occlusion of the middle cerebral artery by intracerebral injection of endothelin-1 (eMCAO) in freely moving animals. Subsequently, analysis of mRNA expression of PAR 1-4 was performed 12 hrs, 48 hrs and 7 d post ischemia. The results reveal a quite different pattern than seen in experiments with brain slices or with global ischemia, which underlines the idea that different pathologies underly global and focal ischemia. In the damaged area PAR1-3 were downregulated, with different time-schedules (PAR1 at 12 + 48 hrs; PAR3 at 48 hrs + 7 d) and with different extents, PAR2 being only slightly influenced (Rohatgi et al., 2003). PAR4 was highly up-regulated in the ipsilateral hemisphere but showed on the contralateral side rather the opposite change, i.e. downregulation on day 7 post ischemia. PAR1-3 were also slightly downregulated in the contralateral hemisphere. These results underline the notion that the PAR/thrombin system might have multiple, even opposing functions depending crucially on the kind of insult (global vs focal), the post-ischemic time-point and the location (ipsilateral/contralateral).

Pharmacological studies also revealed some differences regarding the role of thrombin in focal ischemia compared to former results from global ischemia. Like in experiments with global ischemia, our current data suggest that thrombin is a mediator of the tolerance phenomenon. We could reduce infarct size by thrombin-application as a preconditioning stimulus 7 days before eMCAO. This time schedule (and thrombin concentration) was a quite different from tolerance induction in global ischemia. Interestingly, hirudin, the thrombin-specific antagonist, given shortly before focal ischemia did not induce protection against eMCAO-induced damage but led to a tendency towards deterioration (Noack et al, 2003).

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3. Endogenous stem cell proliferation, differentiation and migration in the hippocampal formation of gerbils after global cerebral ischemia.

W. Schmidt, K. Reymann

Both ischemic and traumatic brain injury leads to a massive loss of neurones in the CNS. Since the first reports of stem cells in the adult brain, these progenitor have been considered to play a central role in replacing neurones after brain injury. Nevertheless little is known about these repair mechanisms after ischemic and neurotraumatologic injuries yet. We investigated whether transient global cerebral ischemia in adult gerbils, which causes neurodegeneration selectively in CA1 pyramidal neurons, affects endogenous stem cells in the dentate area as well as CA1 region. Immunfluorescence together with confocal laser microscopy of anti-bromo-deoxyuridine (BrdU) antibodies after incorporation of BrdU into DNA during cell replication was used to demonstrate the proliferation and migration of stem cells. The two key findings of this study are that (1) global ischemia markedly increases neurogenesis in hippocampal subgranular zone (SGZ) from immature neuronal progenitor cells (β III-tubulin) to mature neurons (NeuN) and that (2) there is also an appearance of newly born neurons in the degenerated CA1 pyramidal cell layer, as demonstrated by immunfluorescence. These results provide evidence for an increased neurogenesis in the gerbil CA1 pyramidal cell layer four weeks following global cerebral ischemia, which could promote morphological and functional recovery after cerebral ischemia (Schmidt and Reymann, 2002).

Funding: FANgGmbH

4. Immunohistochemical characterization of repair mechanisms in organotypic hippocampal slice cultures after hypoxia and traumatic injury.

K.Dinkel, O. Chechneva, A. Laskowski, M. Martínez-Sánchez

We are also using organotypic hippocampal slice cultures as an *in vitro* model, for examining the temporal profile of degenerative and regenerative processes induced by mechanical lesion of Schaffer collaterals or oxygen-glucose deprivation (OGD). Our investigations are mainly focused on stem cell proliferation and differentiation (Neurogenesis) in the different hippocampal areas. Studies were carried out on a model for focal traumatic brain injury (transection of Schaffer collaterals) and a model for ischemia (oxygen-glucose deprivation). In both models Propidium iodide incorporation was used as a marker for neuronal

degeneration, whereas incorporation of bromodeoxyuridine (BrdU) was used to identify proliferating cells. Differentiation of newly generated cells was determined using immunohistochemical double labelling confocal (BrdU/specific neuronal or glial marker) followed by confocal laser scan microscopy.

The reorganisation process after *mechanical lesion of Schaffer collaterals* in organotypic hippocampal slice cultures was followed over the course of time. 1d –4d after lesioning the Schaffer collaterals, degenerative neurons could be detected only at the lesion site (CA3 area). No secondary damage was evident in other areas (e.g. CA1, the projection area of Schaffer collaterals) in 1-14d after injury. After 1 day focal trauma induced cell proliferation all over the slice with a cluster at the lesion site. A large portion of these proliferating cells were identified as progenitor cells (Nestin). Newly generated microglia (IB4) appeared first at 5 days after lesion (dal), new glial precursor cells (S-100 β) at 3 dal. Proliferation of immature neurons (early neurogenesis) was detected 2 dal (Doublecortin), newly generated neurons were identified 3 dal β -III Tubulin) (Fig. 2). Our results demonstrate an increased proliferation and neurogenesis in OHCs after mechanical lesion of Schaffer collaterals (Laskowski et al., 2003). However only a small portion of proliferating cells differentiated into neurons. This indicated that the endogenous repair/replacement mechanism is working rather inefficiently. CNS growth factors may be able to enhance endogenous neurogenesis. We therefore currently investigate the effect of bFGF on proliferation. Primary results showed stimulation of the cell proliferation after adding the factor to the medium for 4 days.

We also investigated whether *oxygen and glucose deprivation* (OGD), a process that mimics ischemic conditions *in vitro*, could also influence the proliferation of progenitor cells in the postnatal rat hippocampus. One day after the insult, cultures displayed a significant increase in both neuronal necrotic cell death and cell proliferation. We found that OGD induced a significant increase of progenitor cells and immature neurons. Furthermore it was demonstrated that precursor cells of newly generated neurons were not exclusively localized in the dentate area We could also show that the neuroprotective compound MK-801, a N-methyl-D-aspartate (NMDA) receptor antagonist, modulates OGD-induced cell death and cell proliferation: MK801 induced proliferation under normoxic conditions, but seemed to decrease proliferation under OGD conditions. In summary, OGD in the hippocampus is followed by a widely distributed increase of cell proliferation and development of certain populations into the early immature neuronal phenotype. (M. Martínez-Sánchez, 2003). As seen in focal trauma, OGD-induced neurogenesis seems rather small compared to the neurons

lost by the insult Therefore studies are currently on the way to investigate the capacity of exogenous growth factors to enhance neurogenesis after ischemia *in vitro*

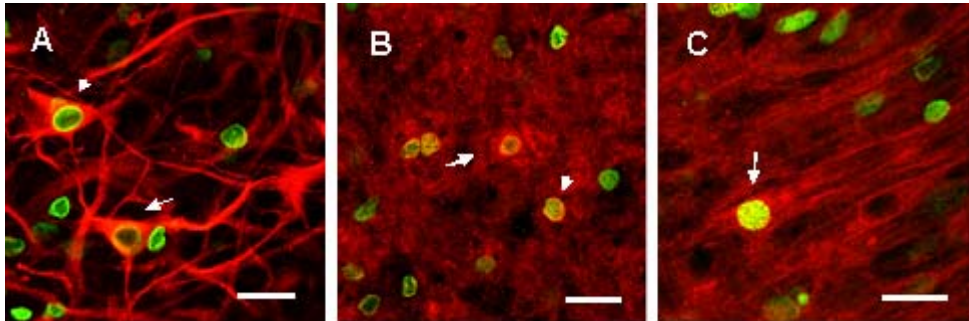


Fig. 2: Progenitor cells (BrdU(green)/Nestin+ (red) cells) in control cultures (A) Immature newly generated neurons (BrdU (green)/DCX+ (red) cells) detected 2-3 days after BrdU administration (B). Newly generated neurons (β -III Tubulin (red)/BrdU (green) detected 5-6d after BrdU administration. Scale bar 20 μ m.

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5. Characterization of tetanus-induced Ca^{2+} responses in hippocampal CA1 neurons

T. Jäger, D. Balschun, T. Behnisch

LTP-like stimulation induces a biphasic Ca^{2+} signal in dendrites, which propagates at short distances. Our results point to the coincidental involvement of NMDA-receptor- and mGluR-mediated Ca^{2+} increase for the generation of the synaptically-driven Ca^{2+} waves (Jäger et al., 2002). The Ca^{2+} signal propagation might represent a special form of intracellular signalling in neurons during LTP induction, necessary for synaptic tagging (Frey et al.) or for induction of local, dendritic protein synthesis and/or potentiating neighbouring synapses.

As a continuation of the collaboration with K.-P. Huang (NIH, Bethesda, USA)(Pak et al. 2000, PNAS 97, 11232-11237) we investigated whether deletion of the postsynaptic PKC substrate neurogranin (Ng) in mice has any functional consequences on the tetanus-induced intracellular calcium rise. Ng $-/-$ mice displayed a significantly lower $[Ca^{2+}]_i$ amplitude and area in response to a 100 Hz 'tetanization. Our results are in agreement with a shifted frequency response curve and impaired spatial learning of Ng $-/-$ mice as compared with wild-type littermates (Huang et al. 2003).

Funding: SFB 426, FANgGmbH

6. Electrophysiological phenotyping of mutant mice

D. Balschun

In collaboration with the Departments of Neurochemistry and Neurophysiology and several other German research groups [see (Altrock et al., 2003)] Bassoon mutant mice (*Bsn*^{-/-}), lacking the central region of Bassoon, were checked for morphological, electrophysiological and behavioral phenotypic changes. We investigated different types of short-term plasticity in the CA1 region of the hippocampus and found a lower excitability and a decreased synaptic depression in response to trains of stimulation between 2 Hz and 100 Hz (Experiments together with M. Sokolov in the Department of Neurophysiology). The data obtained by all groups together led to the hypothesis of a functional inactivation of a significant fraction of glutamatergic synapses in *Bsn*^{-/-} mice (Altrock et al., 2003). In contrast to the CA1 region and the dentate gyrus, the CA3 region is predominated by presynaptic, NMDA-independent types of plasticity. Therefore, we checked *Bsn*^{-/-} mice for changes of short- and long-term synaptic plasticity in that region. In agreement with our findings in the CA1 region we found functional changes in some types of short-term plasticity, such as a markedly reduced PTP amplitude while others such as paired-pulse facilitation were unchanged. In addition, *Bsn*^{-/-} did not express LTP, but only short-term potentiation (STP).

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7. Proteomics of late LTP

D. Balschun

In collaboration with the Departments of Neurochemistry/Neurophysiology and the Free University of Amsterdam (group of Ka Wan Li) we analyzed the proteome of a synaptic fraction obtained from rat hippocampi that were collected six hours after induction of a robust LTP *in vivo*. Samples from animals receiving baseline control stimulation according to the same time schedule served as controls. After 2-D gel electrophoresis, computer-based image analysis and MALDI TOF/TOFR mass spectroscopy we identified 32 differentially regulated protein spots in the molecular weight range from 10-100 kDa, 26 spots were upregulated and 6 were downregulated as compared to controls. The identity of these proteins is currently being determined (Smalla et al. 2003).

8. Modulation of the consolidation of spatial memory and LTP *in vivo*

D. Balschun, K. Reymann

As a cooperation with H. Besedovsky (University Marburg), W. Wetzel (Special Lab Behavioral Pharmacology) and the Department of Neurophysiology we examined the role of interleukin-6 in hippocampal long-term potentiation and spatial learning. We found a specific upregulation of IL-6 gene expression during dentate LTP *in vivo*. Application of an IL-6 neutralizing antibody (IL-6 Ab) 90 min after tetanization and learning, respectively, resulted in a clear-cut improvement of the consolidation of LTP and memory. Our results imply IL-6 in the physiological regulation of those processes.

In collaboration with W. Wetzel (see there for details) and the Department of Neurophysiology the role of group I mGluRs in LTP and spatial learning was further elaborated. Application of the mGlu5-specific antagonist MPEP 30 min before tetanization and training, respectively, resulted in an impairment of dentate LTP and spatial learning (Balschun & Wetzel, 2002) (Fig. 3). The importance of mGlu5 activation was further corroborated by the pronounced improvement of memory retention seen after post-training infusion of DFB, an allosteric potentiator of mGluR5. In collaboration with Drs. Kulla, Manahan-Vaughan and Nicoletti we found an NMDA receptor dependent up-regulation of mGluR5 in the dentate gyrus and CA1 both 24 and 48 h after tetanization of the perforant path. This data suggest that mGluR5 is not only involved in the induction of LTP, but is also a component of the plastic changes that follow the induction of LTP.

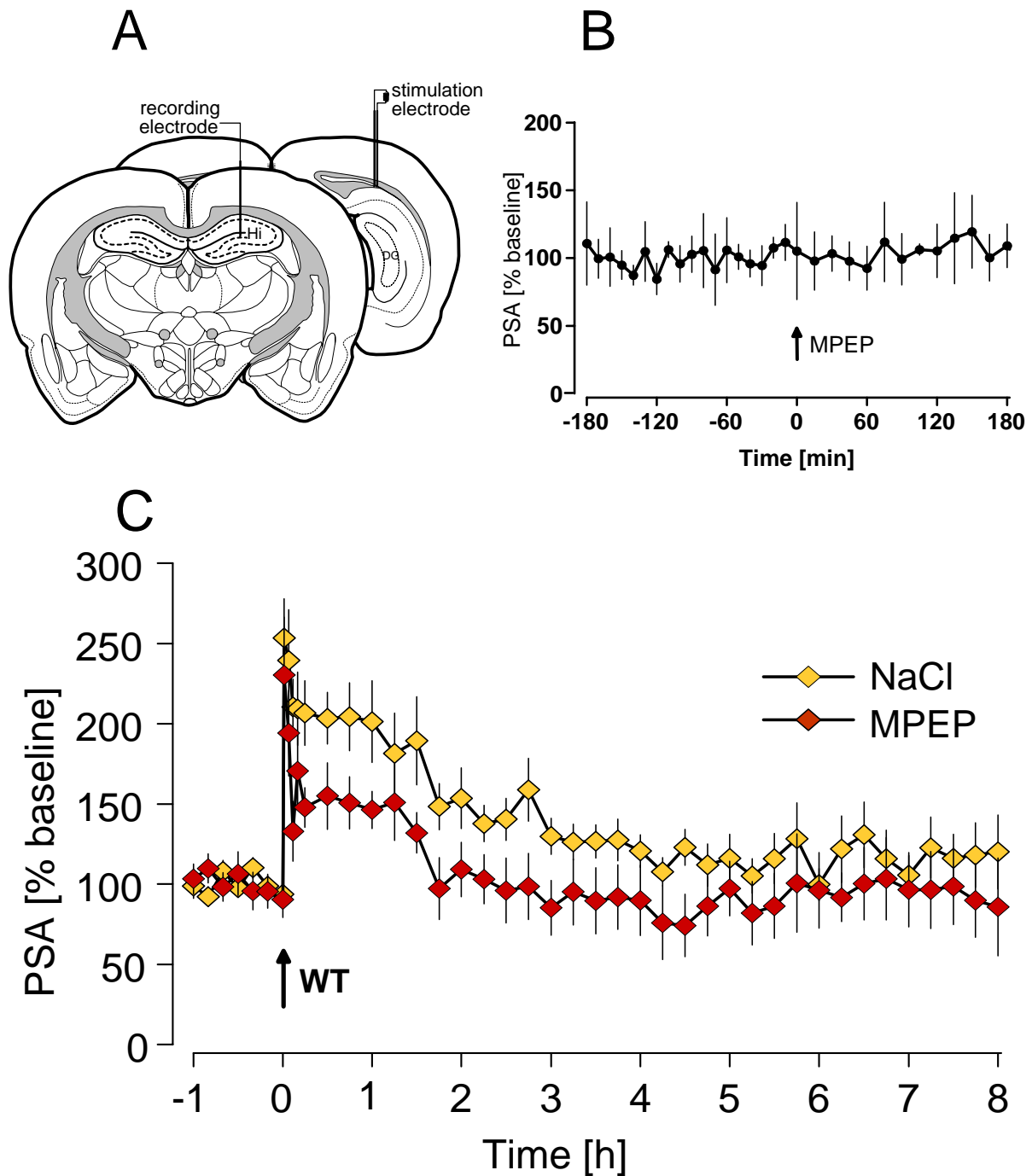


Fig. 3: The subtype-specific mGluR5 antagonist MPEP applied icv to freely moving animals, impaired an LTP induced by a weak tetanization paradigm (3 bursts of 15 pulses, 200 Hz) of the perforant path. (A) Schematic diagram of electrode placement for the recording from the hilus (HI) of the dentate gyrus of the right hemisphere. (B) Application of MPEP 30 min before tetanization resulted in a significantly earlier decay of potentiation as compared with controls. The values of the MPEP and control group were statistically different for 6 hours starting at 15 min after tetanization, Mean \pm SEM is given.

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