Effect of an Inhibitor of Neuronal Nitric Oxide Synthase 7-Nitroindazole on Cerebral Hemodynamic Response and Brain Excitability in Urethane-Anesthetized Rats

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Summary

The role of neuronal nitric oxide synthase (nNOS) in the pathophysiology of epilepsy and seizures remains disputable. One of the reasons why results from the acute *in vivo* studies display controversies might be the effect on the regulation of cerebral blood flow (CBF) during pharmacologically induced alterations of NO system. We examined neurovascular coupling in the rat sensorimotor cortex in response to transcallosal stimulation under nNOS inhibition by 7-nitroindazole (7-NI). Adult Wistar rats were anesthetized with urethane and epidural silver EEG electrodes were implanted over sensorimotor cortices. Regional CBF was measured by Laser Doppler Flowmetry (LDF). We catheterized a common carotid artery to measure arterial blood pressure (BP). 7-NI did not significantly affect blood pressure and heart rate. Electrophysiological recordings of evoked potentials (EPs) revealed no effect on their amplitude, rhythmic potentiation or depression of EPs. Transcallosal stimulation of the contralateral cortex induced a frequency dependent rise in CBF. Although 7-NI did not significantly affect basal CBF and cortical excitability, hemodynamic responses to the transcallosal stimulation were diminished implicating a role of nNOS in neurovascular coupling. Urethane anesthesia is suitable for future epileptological experiments. Our findings demonstrate that NO contributes to the hemodynamic response during brain activation.

Key words

Cerebral hemodynamic response • Brain excitability • Neuronal nitric oxide synthase • 7-nitroindazole • Rat

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Introduction

Nitric oxide (NO) is synthesized by a family of enzymes nitric oxide synthases (NOS) present in most of the cells of the body. There are four members of the NOS family: neuronal NOS (nNOS), endothelial NOS (eNOS), inducible NOS (iNOS) and mitochondrial NOS (mtNOS). The last one mentioned is an isoform of nNOS present in the inner mitochondrial membrane. All the isoforms share between 50 and 60 % sequence homology. The nNOS and eNOS are Ca^{2+}-calmodulin-dependent enzymes constitutively expressed in mammalian cells that generate an increase of NO lasting a few minutes. In contrast, iNOS is Ca^{2+}-calmodulin-independent and its regulation depends on *de novo* synthesis. iNOS is expressed following immunological or inflammatory stimulation in macrophages, astrocytes, microglia and other cells producing high amounts of NO lasting hours or days (Buchholzer and Klein 2002, Guix *et al.* 2005). L-Arginine is used as a substrate by NOS to produce NO and citrulline in a process requiring NADPH and O_2.

The ubiquitous localization of NO indicates its implication in a wide range of physiological processes, but it can also produce harmful effects due to its reactivity, mainly with proteins, under various pathophysiological conditions. A multitude of regulatory processes have been attributed to the nitric oxide since its first recognition as a signaling molecule in the central nervous system. NO is involved in the regulation of

synaptic transmission and plasticity (Bon and Garthwaite 2003, Garthwaite *et al.* 1988), blood flow (Lindauer *et al.* 1999a), mitochondrial respiration (Brown 2001), as well as in inflammatory processes (Bal-Price and Brown 2001). NO as neuromodulator interferes with glutamate, GABA, monoamine, adenosine and cholinergic neurotransmission (Buchholzer and Klein 2002, Prast *et al.* 1998, Saransaari and Oja 2004, Trabace *et al.* 2004, Wall 2003). The importance of NO in the brain is determined by its broad effects on neuronal, glial and vascular compartments and its involvement in neurodegenerative diseases (Guix *et al.* 2005).

The main NO cellular signaling pathway is through the activation of guanylate cyclase (GC) with the subsequent production of cyclic guanosine-3', 5'-monophosphate (cGMP) and protein phosphorylation (Guix *et al.* 2005). The mechanisms linking the rise in cyclic GMP content to the various effects of NO in the CNS are not fully understood, although it seems that the final step is a reduction of cytosolic calcium. Another action of NO independent of soluble guanylate cyclase (sGC) is the modulation of oxidative phosphorylation in the mitochondria by competitive and reversible inhibition of cytochrome c oxidase, the terminal heme-containing enzyme in the mitochondrial respiratory chain. This suggests a crucial role of NO in regulation of energy generation. The action of NO is limited by its biologic half-life which is *in vivo* up to one second and by the relative short distance that this molecule can traverse.

The function of the nervous tissue is strictly dependent on aerobic metabolism. That is the reason why the regional CBF increases to balance oxygen demands during functional activation (so called neurovascular coupling or hemodynamic response) (Hoffmeyer *et al.* 2007, Lindauer *et al.* 1999b). The mechanism of the neurovascular coupling is region-specific and includes both astrocytic and neuronal compartments. Astrocytes are sensing the neuronal activity *via* receptors for many neurotransmitters which are located near the synapses. Their activation leads to the changes of astrocytic Ca^{2+} levels and to the production of arachidonic acid metabolites – prostaglandin E_2 and epoxyeicosatrienoic acids which induce arteriole dilation (Haydon and Carmignoto 2006). In parallel to astrocytic regulatory system neurons expressing nNOS during the activation produce NO which directly diffuses to vascular smooth muscle cells (VSMCs) and dilates the arterioles. The vasorelaxant action of NO on the VSMCs involves the generation of cGMP and cGMP-dependent protein kinase

(PKG)-dependent modulation of ion channels, inducing hyperpolarization of the membrane. Moreover, NO activates sarcoplasmic-endoplasmic reticulum Ca^{2+} ATPases, depleting the Ca^{2+} levels of the cytosol (Guix *et al.* 2005).

NO is considered to play a role in the pathophysiology of epilepsy, although the results of experiments carried out by several authors are often discrepant. Available *in vivo* data show a lack of effect, a decrease or increase in seizure susceptibility following the administration of NOS inhibitors. The effects of NOS inhibitors are dependent on the type of inhibitor, the route of administration, the model of seizures and the species of animals used in experiments (Wojtal *et al.* 2003). Nevertheless, our recent findings suggest a crucial role of NO produced by nNOS in the initiation of epileptic seizures *in vitro*. The NOS inhibitor 7-nitroindazol (7-NI, putative nNOS inhibitor) in combination with potent NO scavenger (4-(carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl 3-oxide (c-PTIO)) was able to prevent the initiation of seizure-like events in low-magnesium model of seizures in acute hippocampal slice and organotypic hippocampal slice culture preparations (Kovacs *et al.* 2009).

One of the reasons why results from the acute *in vivo* studies display controversies might be the changes in the regulation of CBF during pharmacologically induced alterations of the NO system. However, the knowledge from this field is limited because of technical aspects. Anesthetics significantly influence blood pressure as well as cerebral hemodynamics. Furthermore, the recordings in freely moving animals are technically demanding and for that reason the results should be interpreted with caution due to frequent movement artefacts. In neurovascular coupling research α-chloralose anesthesia is frequently used. However, due to its pharmacological properties and common occurrence of negative side effects (e.g. spreading depression) it is not suitable for epileptological studies. Therefore the current study was conducted to elucidate whether urethane anesthesia, which is frequently used to study epileptic events in anesthetized rats, is also convenient to study cerebrovascular dynamics.

Methods

Animals

Adult male Wistar rats (250-350 g, n=37) were used in our study. Rats were randomly divided into

experimental groups (11 to monitor arterial blood pressure under urethane anesthesia, 10 to monitor blood pressure in conscious animals and 16 to monitor spontaneous brain activity, brain excitability and changes of CBF). Rats were housed in standard plastic cages in temperature-controlled environment (22±1 °C), humidity 50-60 % with a 12-h light/dark cycle (lights on at 6 am) with free access to food and water. All experiments were performed in agreement with the Animal Protection Law of the Czech Republic, and the project was approved by the Animal Care and Use Committee of the Institute of Physiology of the Academy of Sciences of the Czech Republic. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Drug treatment

7-NI obtained from Sigma-Aldrich (Czech Republic), was dissolved in dimethyl sulfoxide (DMSO). All rats were given either 7-NI or its vehicle intraperitoneally in a volume of 1 ml/kg body weight. The dose of 7-NI (25 mg/kg) was selected on the basis of previous studies and because it is the most widely used dose in neuroprotection studies. Solutions were freshly prepared at the beginning of each experiment. Urethane was obtained from Sigma-Aldrich (Czech Republic) and dissolved to 20 % in distilled water. Heparine (Zentiva, Czech Republic) was diluted to its final concentration 0.1 % by sterile 0.01 M phosphate buffered saline.

Experimental procedures

Continuous recording of blood pressure and heart rate

Under urethane anesthesia (1.2 g/kg i.p.) a chronic catheter was implanted into the common carotid artery in order to measure systemic arterial blood pressure. The carotid artery was carefully prepared from a small ventral midline neck incision. A vascular microclip was placed over both internal and common carotid artery. A ligation was placed on the common carotid artery as caudally as possible and the second ligation was prepared for the future fixation of the catheter near the bifurcation. After arteriotomy a plastic catheter (PE50) was inserted into the central portion of common carotid both ligations were tightened. To prevent thrombosis the catheter was filled up with 1 % heparin solution. The catheter was then passed under the skin and the other ending was pulled out from a small nuchal incision and temporarily closed with a plug until connected to a recording tube (Zicha *et al.* 2008). This allowed us to measure BP in freely moving

animals. Animals were placed into the stereotactic frame and their body temperature was maintained at 37.1±0.1 °C by heating pad. The catheter was connected to the pressure sensor (BLPR2, WPI – Germany) and BP was continuously recorded with Spike2 software (1 kHz). Heart rate was analyzed offline using power spectral algorithm in Spike2 software.

In order to assess the effect of urethane on cardiovascular dynamics same animals (n=10) were implanted carotid catheter under isoflurane anesthesia (1.5-2 %) and then allowed to recover for 4 h. After recovery animals were placed into the plastic box for the acute recordings of blood pressure and heart rate.

Measurements of brain activity, brain excitability and cerebral blood flow

Epidural recording electrodes were stereotactically implanted over both cerebral hemispheres (L 3.0 mm, R-C 1.0 mm; 2x L 3.0 mm, R-C 3.0 mm; L 4.0 mm, R-C 5.5 mm) under urethane anesthesia. Bipolar stimulation electrodes were placed over the right somatosensory cortex (L 2.5 mm, R-C 1.0 mm anterior and 1.0 mm posterior in relation to bregma, L=2.5 mm). A reference and a ground electrode were placed over the cerebellum. For the CBF measurements skull was thinned to improve laser light transmission. Therefore skull was carefully drilled contralaterally to the stimulating electrodes until internal cortical lamina was reached. The Laser Doppler probe was then placed directly on internal cortical lamina of the remaining bone and connected to the LDF (LDPM – PF5010, Perimed, Sweden) and digitalized at 256 Hz. EEG data were acquired at 2 kHz and bandpass filtered at 2 to 500 Hz (RA16PA preamplifier and Pentusa Base Station; Tucker-Davis Technologies, Gainesville, FL, U.S.A.).

To assess cerebrovascular reactivity electric stimulation of right sensorimotor cortex was performed by gradually increasing stimulations (3, 5, 10 and 15 Hz, length 4 s, intensity 3 mA). Every stimulation was followed by a 3 min recovery period. The stimulation session was then repeated after 10 min and results were averaged for each frequency from both measurements. To assess the effect of 7-NI on spontaneous brain activity and brain excitability, the whole set of two stimulation sessions (see above) was repeated 30 min after treatment.

Data were analyzed offline using MATLAB software (Mathworks, Inc., Natick, MA, U.S.A.). For analysis of single evoked responses, the amplitude from the first negative wave (N1) to the following positive

wave (P2) was measured. To evaluate the effect of treatment on the brain plasticity the sum of all evoked responses during one stimulation train was calculated ($\sum$ of EP). The baselines of CBF were measured in 10 s window preceding the stimulation. The CBF responses to the stimulations were assessed by measuring peak amplitude.

Statistical analysis

The data were tested for normality and statistically evaluated using ANOVA for repeated measures (in the case of more than two groups) and t-test where appropriate (two groups). All data are expressed as mean ± standard error of mean (S.E.M.).
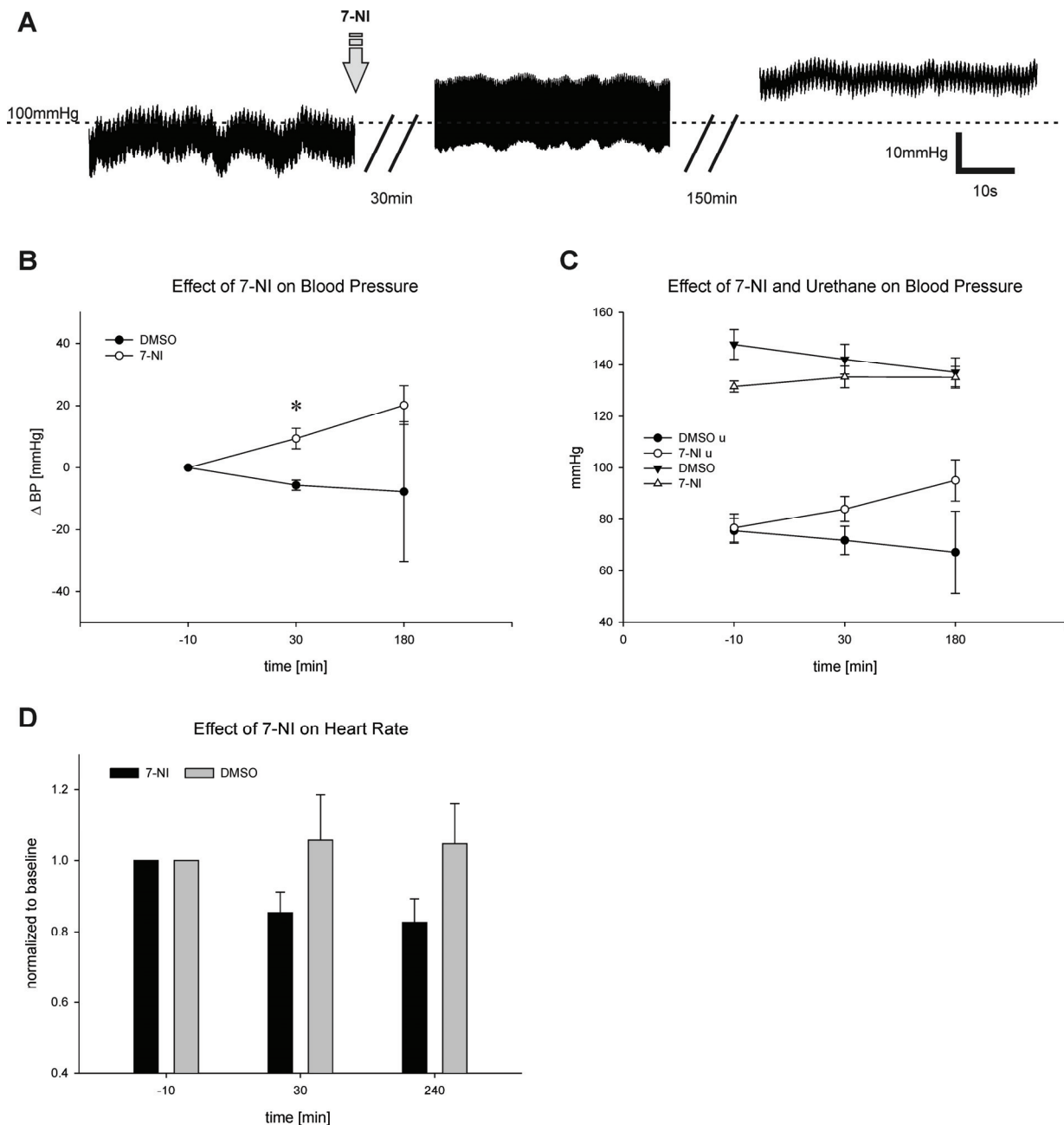
Fig. 1. Effect of 7-NI on physiological parameters – blood pressure and heart rate. (**A**) Blood pressure recording in a typical subject before and after 7-NI administration. (**B**, **C**) The systemic blood pressure in 7-NI treated rats was significantly higher 30 min after drug application in comparison to control animals: 83.95±4.84 mmHg versus 71.79±5.50 mmHg and at 30 and 180 minutes (95.06±8.05 mmHg) after the 7-NI treatment when compared to initial values (76.59±5.46 mmHg). Urethane significantly lowered baseline arterial blood pressure values in comparison with unanesthetized animals (131.555±2.197 mmHg). (**D**) 7-NI did not induce significant changes in HR. Data shown in D have been normalized to the mean of their pretreatment magnitude. * shows statistically significant differences between 7-NI treated animals and control (DMSO) animals.
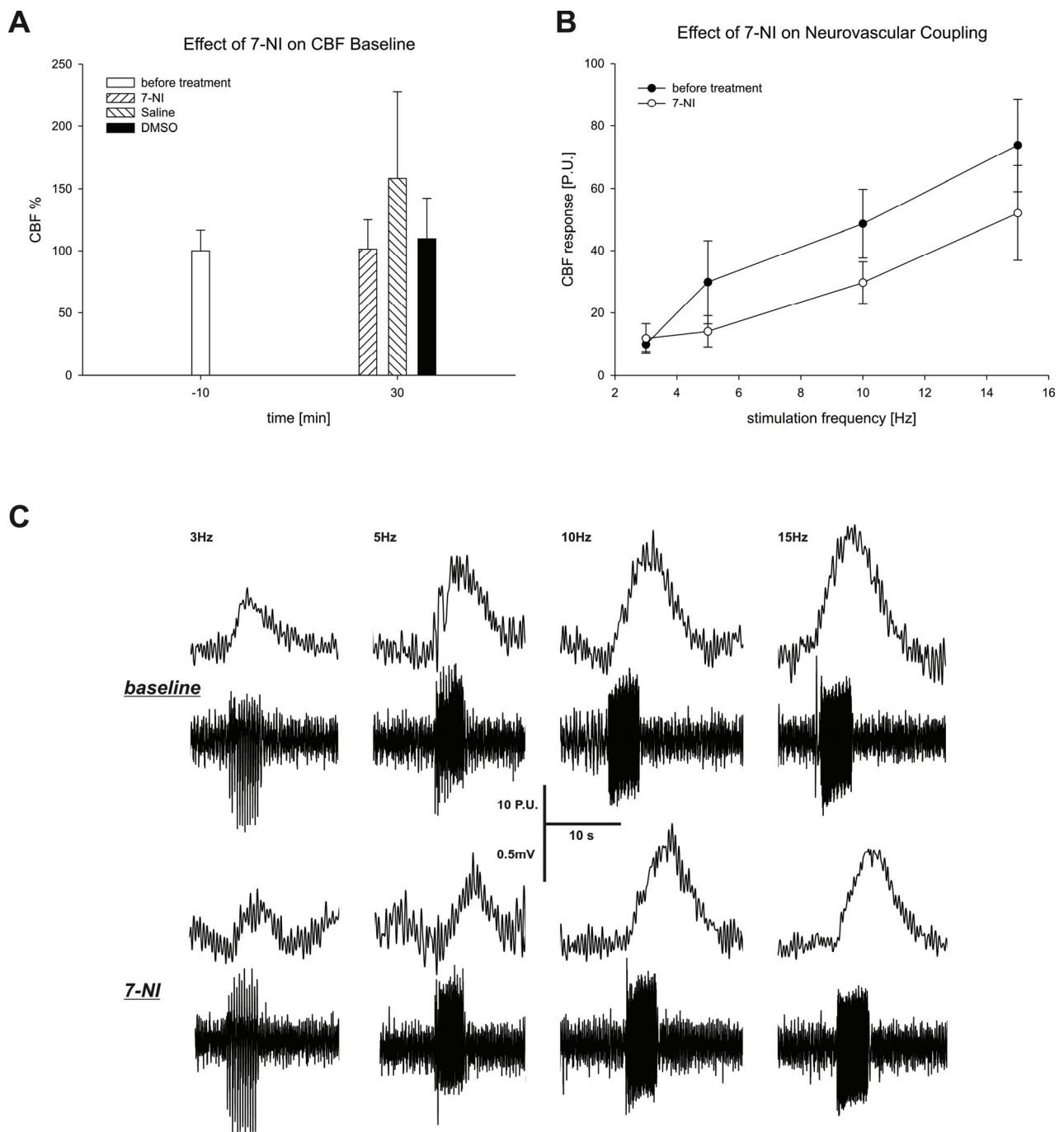
Fig. 2. Effect of 7-NI on cerebral hemodynamics. (**A**) Baseline regional cerebral blood flow did not change significantly during nNOS inhibition by 7-NI, neither did it change in control animals. (**B**) Average across subject hemodynamic responses plotted against the stimulation frequency. Transcallosal stimulation of the contralateral cortex induced a frequency dependent rise in rCBF which was nonsignificantly reduced during nNOS inhibition. (**C**) CBF measurings in a typical subject before and after the 7-NI treatment.

Results

Effect of 7-NI on physiological parameters

During the experimental period, mean values of arterial blood pressure significantly increased after 7-NI treatment under urethane anesthesia (Fig. 1B). The systemic blood pressure in 7-NI-treated rats was significantly higher 30 min after the drug injection in comparison to control animals (DMSO-treated): 83.95 ± 4.84 mmHg vs. 71.79 ± 5.50 mmHg (Fig. 1A).

Since our experiments were done under urethane anesthesia we measured the effect of urethane anesthesia on 7-NI-induced alterations of arterial blood pressure. Urethane significantly lowered arterial blood pressure

(76.58±5.46 mmHg) in comparison with conscious animals (131.56±2.2 mmHg). However, 7-NI induced a nonsignificant increase in blood pressure 30 min after the treatment in conscious animals (Fig. 1C).

Heart rate was not changed either by the application of DMSO or by 7-NI. However, a slight decrease in HR was observed following 7-NI injection (Fig. 2).

Effect of 7-NI on cerebral blood flow

We tested the effect of the inhibition of nNOS by 7-NI on resting CBF. Regional CBF did not significantly change during nNOS inhibition by 7-NI, neither did it change in control animals (DMSO-treated, Fig. 3).
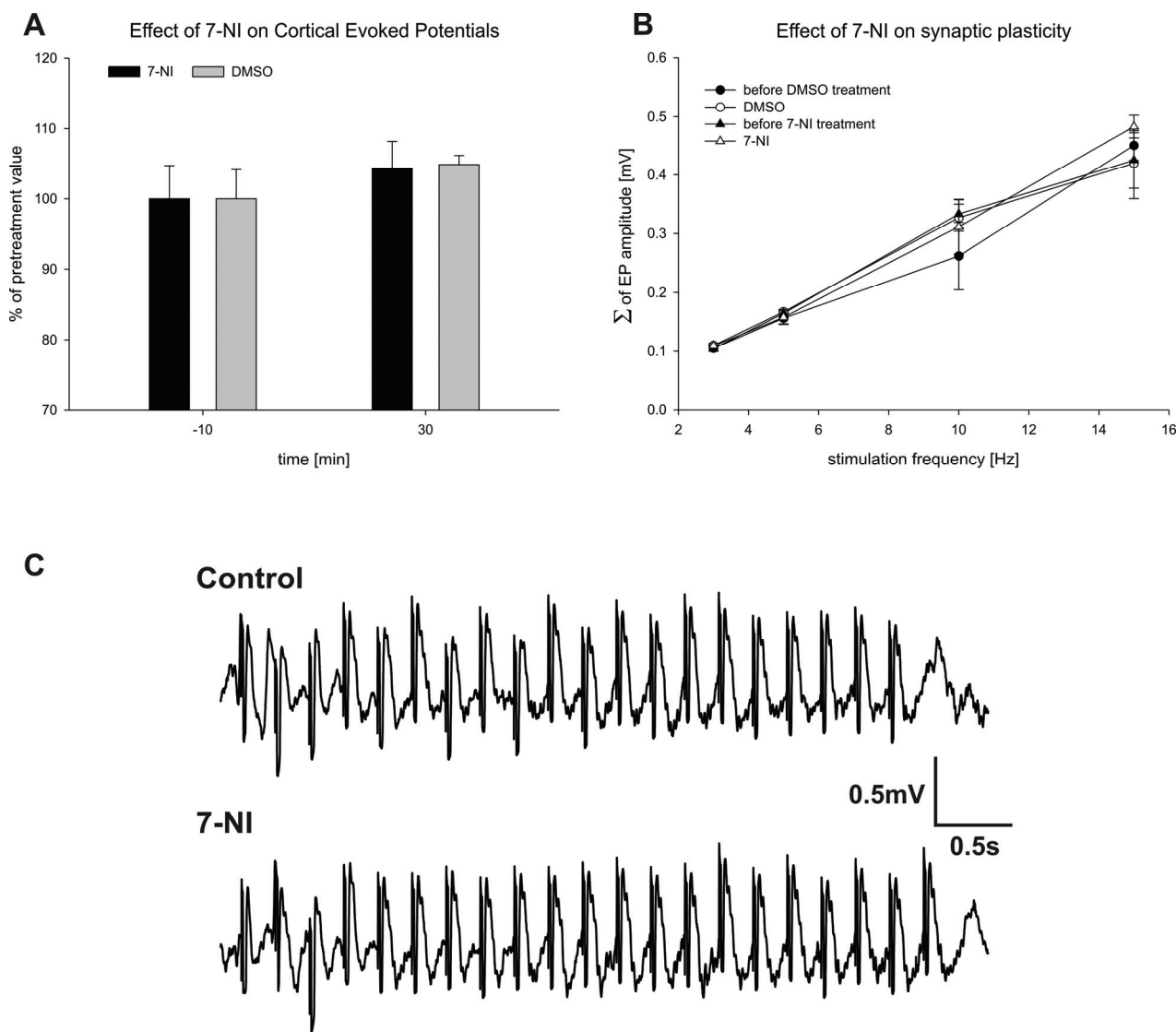
Fig. 3. Effect of 7-NI on cortical excitability. (**A**) The amplitude of cortical EPs in response to transcallosal stimulation did not change in both treatment groups 30 minutes after the application. (**B**) 7-NI did not induce changes of cortical EPs amplitude at any stimulation frequency. (**C**) A typical recording of cortical EPs at 5 Hz showing no changes in EP amplitude during stimulation trains.

Effect of 7-NI on neuronal excitability

To make sure that 7-NI does not cause changes in cortical excitability, cortical EPs were measured. Figure 3 shows the effect of 7-NI on EPs. There were no significant changes in the EPs evoked by transcallosal stimulation at any pharmacological situation, suggesting no effect of 7-NI on cortical neuronal excitability (Fig. 3A). Analysis of rhythmic stimulation did not reveal any effect of 7-NI on synaptic plasticity. The sum of the EPs during stimulation trains (3, 5, 10 and 15 Hz) linearly increased with frequency (Fig. 3B).

Effect of 7-NI on neurovascular coupling

Transcallosal stimulation of the contralateral cortex induces a frequency (dose) dependent rise in CBF. The application of 7-NI did not abolish the rise in CBF in response to the transcallosal stimulation. The amplitude of the hemodynamic responses increased with higher stimulation frequency in both groups. The dose response (the relation of CBF amplitude on stimulation frequency) demonstrates a linear relationship. However, we have observed a little decline of the dose-response curve after 7-NI treatment (Fig. 3).

Discussion

The results of the present study suggest that 7-NI has a significant effect on systemic blood pressure which was significantly increased both 30 and 180 min after 7-NI treatment under urethane anesthesia. In conscious animals 7-NI induced an increase of blood pressure, however, this increase did not reach the level of statistical significance. Changes of blood pressure were accompanied by non-significant changes of heart rate. The wide range of effects of nNOS inhibition on systemic parameters reported in previous studies may result from differences in the measuring techniques, rat strain employed, the anesthesia, the inhibitor used, its relative degree of neuronal versus endothelial NOS inhibition achieved and solvent used (Cholet *et al.* 1997, Hoffmeyer *et al.* 2007, Lindauer *et al.* 1999b, Stefanovic *et al.* 2007, Toda *et al.* 2009, Togashi *et al.* 1992, Zagvazdin *et al.* 1996). Non-specific inhibitors of NOS have been shown to increase blood pressure (Rees *et al.* 1989). In contrast, 7-NI was proposed to be a specific nNOS inhibitor, because it had no effect on systemic blood pressure in anesthetized mice (Moore *et al.* 1993). However, this inhibitor induced a significant increase in blood pressure in our experiments. The role of nNOS in BP regulation is unclear.

The 7-NI injection slightly but non-significantly decreased basal CBF in our study. Other studies in conscious rats have reported that 7-NI decreased baseline CBF (Gotoh *et al.* 2001, Montecot *et al.* 1997) probably due to vasoconstriction of cerebral arterioles mediated by reduced production of NO by nNOS. However, in anesthetized rats 7-NI did not induce changes in resting CBF measured by autoradiography in the somatosensory cortex (Cholet *et al.* 1996). Furthermore, using functional magnetic resonance imaging Stefanovic *et al.* (2007) found that 7-NI had no effect on CBF in α-chloralose anesthetized animals. Although 7-NI caused a slight decrease of the basal CBF values in our study, transcallosal stimulation of the sensorimotor cerebral cortex of one hemisphere caused a response in the homologous brain region on the opposite side which was observed as a corresponding rise in CBF. Rhythmic transcallosal stimulation is used to elicit a release of neurotransmitters especially glutamate which mimics enhanced brain activity. The regional CBF is likely controlled by a feed forward mechanism involving neuronal signaling *via* neurotransmitters mainly glutamate (Attwell and Iadecola 2002, Hoffmeyer *et al.* 2007). As mentioned earlier the mechanism of neurovascular coupling is region-specific and includes both astrocytic and neuronal compartments. In parallel to the astrocytic regulatory system, which is based on the release of arachidonic acid metabolites, the neurons expressing nNOS produce NO (activated by Ca^{2+}) during the activation which directly diffuses to vascular smooth muscle cells and dilates the arterioles (Hoffmeyer *et al.* 2007).

In our experiment 7-NI had no influence on amplitudes of cortical evoked potentials. We have found no further effect of 7-NI on rhythmic potentiation or depression of the EPs. This finding is in accordance with previous experiments mainly done on somatosensory evoked potentials (Hoffmeyer *et al.* 2007, Lindauer *et al.* 1993, Lindauer *et al.* 1999). Furthermore, no concomitant changes in cerebral glucose metabolism during neuronal activity under the inhibition of nNOS were reported by Cholet *et al.* (1997). However, others observed a decrease in somatosensory-evoked potential amplitudes after nNOS inhibition (Ngai *et al.* 1995, Stefanovic *et al.* 2007) in α-chloralose anesthetized rats. Variability of these findings might be explained by different placement of cortical electrodes for EP recording (Ngai *et al.* 1998) and the involvement of polysynaptic path in somatosensory EPs in contrast to monosynaptic transcallosal path.

Although 7-NI did not significantly affect basal CBF and cortical excitability, hemodynamic responses to the transcallosal stimulation were diminished implicating a role of nNOS in neurovascular coupling. In the present study, we have observed a significant increase of CBF during gradual frequency-increasing stimulation even after the injected dose of 7-NI (25 mg/kg). However, the dose-response (dependency of amplitude of the CBF response on stimulation frequency) indicates a little decline from control measurements confirming a role of

nNOS in hemodynamic response to transcallosal stimulation. Similar results have been shown also by others using different anesthesia and stimulation protocols (Hoffmeyer *et al.* 2007, Lindauer *et al.* 1999).

The research of neurovascular coupling *in vivo* is commonly performed on α-chloralose anesthetized animals because it appears to have little effect on cardiovascular reflexes, peripheral resistance and cerebral vasoreactivity (Lindauer *et al.* 1993, Maggi and Meli 1986, Smith and Hutchins 1980). Early studies have shown that α-chloralose preserves robust and stable hemodynamic and metabolic coupling to sensory stimulation (Lindauer *et al.* 1993). However, α-chloralose has a broad effect on systemic parameters; it induces respiratory depression, metabolic acidosis, and bronchial hyperreactivity (Arfors *et al.* 1971). The action of α-chloralose has been shown to involve the potentiation of GABA$_A$-induced currents by increasing the affinity for GABA (Garrett and Gan 1998), whereas at low concentration α-chloralose does not affect glutamatergic, glycinergic or cholinergic synaptic transmission (Wang *et al.* 2008). Animals with α-chloralose anesthesia show increased brain excitability and the rhythmic stimulation of the cortex frequently induces spreading depression. For that reason a topically administrated cocktail of ionotropic glutamate receptor blockers is commonly used in neurovascular coupling research (Hoffmeyer *et al.* 2007, Lindauer *et al.* 1999). Austin *et al.* (2005) showed that in rats under α-chloralose (10 to 30 mg/kg per hour) sensory stimulation induced varying electroencephalographic and BOLD responses over a longer period of measurements. Altogether, the above mentioned findings demonstrate that α-chloralose is not a suitable anesthesia for electrophysiological animal experiments in epileptology. Contrarily, urethane has been often used in neurophysiologic studies e.g. to describe firing properties of hippocampal neurons, electrically induced hippocampal gamma oscillations and epileptic activity (Garcia-Hernandez *et al.* 2010, Mikkonen *et al.* 2002, Stringer and Sowell 1994). Urethane has only modest effects on multiple neurotransmitter-gated ion channels at concentrations close to the anesthetic half maximal effective concentration (EC$_{50}$) and thus urethane is suitable for maintaining anesthesia during electrophysiologic recordings (Hara and Harris 2002). Our results indicate that urethane besides its hypotensive effect has minimal if any influence on hemodynamic responses.

In conclusion, our data suggest that urethane is a suitable anesthetic for further research of neurovascular coupling during epileptic events. Inhibition of neuronal nitric oxide synthase by 7-NI influenced hemodynamic responses but did not affect blood pressure, heart rate or brain excitability in urethane anesthetized rats.

Conflict of Interest
There is no conflict of interest.

Acknowledgements

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