

Constitutive Nitric Oxide Synthase Activity in the Prefrontal Cortex of Rats as an Index of Emotional State before Death

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INTRODUCTION

The prefrontal cortex (PFC), as a part of the 'limbic circuit', plays a fundamental role in emotional and cognitive processes (Lane et al., 1997; Maguire et al., 1998). This has been shown both in experimental animal models and in humans, using modern imaging techniques (Lane et al., 1997; Maguire et al., 1998; Middleton & Strick, 1997). Numerous excitatory amino acids (EAA) neurons are present in PFC, and glutamate plays a key role in excitatory neurotransmission in the central nervous system (McDonald, 1996). Nitric oxide (NO) plays a significant, indirect role in the stimulation of glutamate receptors, particularly the ionotropic (iGluR) and metabotropic (mGluR) glutamate receptor types. The stimulation of both types of glutamate receptors increases after the generation of NO (Gage et al., 1997; Kendrick et al., 1997), indicating that NO may act as a modulator of glutamatergic neurotransmission.

Both glutamate and the activity of the enzyme NO-synthase (NOS) respond to noxious, stressful environmental influences. An increased level of

glutamate was demonstrated post mortem in the brain tissue of rats that had been subjected to strong sensory stimulation (Hauser et al., 1999). An increased level of glutamate was also detected in the intracellular space of PFC and in the hippocampus of rats that were irritated by pain stimuli (Bagley & Moghaddam, 1997). Immunohistochemical investigation of the rat hippocampus demonstrated the increased expression of NOS in response to heat stress (LeGreves et al., 1997). NO-synthase was increased in the periaqueductal gray of rats that were exposed for 15 min to a predator (cat) (Chiavegatto et al., 1998).

In the present work, post-mortem constitutive NO synthase (cNOS) activity, as well as glutamate concentration, was assessed in the PFC of rats that had experienced simultaneous different short-term aversive sensory (acoustic, visual, and mechanical) stimulation. The extreme character and simultaneous application of stimuli, applied for a time much shorter than that reported previously (Baltrons & Garcia, 1997; Le Greves et al., 1997), were chosen because they correspond to the reality of forensic cases. In such cases, the victims very often are exposed to complex stimuli of extreme intensity just before death.

Investigations dealing with the problem of post-mortem assessment of a very short, intense fear reaction caused by extreme stimulation immediately before death are lacking (Gos &

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Hauser, 1996). Biochemical 'frozen frames' of neurotransmission could help in the reconstruction of events just before death—namely, was a victim of a crime aware of a life-threatening situation shortly before dying. By using the present experimental paradigm, a possible application of the biochemical assessment of human brain tissue could be developed for future forensic investigations.

EXPERIMENTAL

Animals

Tests were carried out on 84 male Wistar rats (weighing 270 g to 380 g each), whose brain mass ranged from 1.6 to 2.1 g. During the experiments, the animals were given free access to water and a standard laboratory diet.

Stimuli

Paired rats were stimulated simultaneously with three types of stimuli in a single stimulation session lasting 15 s. The character of the stimuli applied might approach that reported in forensic cases, in which the victim was very often submitted to strong, aversive stimuli of different modalities (mainly mechanical, visual, and acoustic) acting concomitantly before sudden and violent death (for example, traffic accidents, explosions, murders). By using the present experimental paradigm, the authors aimed to copy real-life situations within the limits accepted by an Ethics Committee.

The acoustic stimulus was produced by using two acoustic whistlers and by scraping the metal grid with a metal rod. The characteristics of the acoustic stimulus were measured using a Swan 910 acoustic analyzer.

The first whistler generated the main harmonic

A at 2624 Hz at the acoustic level 100 dB. The second harmonic B revealed the value of the acoustic level 80 dB, and the third harmonic C the acoustic level of 64 dB. The sharp break in the spectrum D was located near 11 kHz. The maximum level of the high frequency band E was about 60 dB. This value of the acoustic level was valid for frequencies between 14 and 16 kHz.

The second whistler generated the main harmonic at 3840 Hz at the acoustic level 107 dB. This whistler also generated ultrasound frequencies at the high acoustic level. For example, a frequency of 22700 Hz means that the acoustic level was 50 dB.

The scraping device generated a stable spectrum of a noise-type sound having a main frequency of 200 Hz at the acoustic level 54 dB and a continuous spectrum, which was nearly flat close to the upper frequency of the acoustic analyzer.

The mechanical stimulus was produced by exerting pressure upon the animals with a plastic rod having one flat surface and the other rectangular. This rod was a model of a typical blunt weapon. The mean areas were 30 cm² for the flat surface and 0.5 cm² for the rectangular surface. The force was measured using a dynamometric method, with calibration of the force-measuring device using standard masses. The mean value of the force was 3 N. The force was applied to the backs and sides of the animal's bodies. The stimuli were repeated using a frequency of 1 Hz.

The visual stimulus was produced using a stroboscope lamp S 100 W with a regulated frequency. The characteristics of this stimulus were measured using a standard photoelectric photometer (Carl Zeiss Jena), equipped with a detector used for visual photometry. The visual stimulus was generated using 6 Hz as the frequency of flashes. The mean value of light intensity at the point located 30 cm from the lamp was 10000 lx. This value was stable, with a deviation of less than 5%.

The stimulation of rats took place in a transparent Plexiglas cage with a movable lid.

After the stimulation period, the cage was closed and pressurized carbon dioxide gas was infused into the cage through plastic tubing. Death occurred within 1 min.

For each stimulation session, a pool of stressed animals was accompanied by a control pool of unstressed animals. Paired control rats remained for 12 h overnight in the same cage as that described for the stimulated rats. On the morning of the next day, the lid of the cage was moved down without disturbing the animals, and the same termination procedure was performed. During the entire termination procedure, the behavior of the animals was observed from the neighboring room. Animals that slept during the termination were selected as unstressed controls.

Sample Preparation

In an effort to obtain the brain structures for the cNOS activity assay as soon as possible, two separate groups were used (group I: PFC + Hippocampi; group II: Amygdala + Cerebellum, see Table 1). Each group consisted of stressed and control animals. In each of the two stressed animal pools, the stress procedure was identical. The brains of the animals from the stressed pool of group I (n=16) were dissected for PFC and hippocampi (one sample of PFC and one of hippocampi were lost during the biochemical assay procedure). The same dissection was performed on animals from the control pool of group I (n=16). From this pool, only the cNOS activity values obtained from the eight animals that slept during the termination procedure were included in the results for this pool (Table 1).

The mass of brain tissue needed for applying biochemical methods excluded the simultaneous assay of cNOS activity and glutamate concentration in the same PFC. Therefore, the glutamate concentration assay was performed in PFC that

were obtained from a third group of animals (group III, see Table 2), consisting of both stressed and control animal pools. The stress-evoking paradigm in the third group was the same as that in the two groups of animals that were disposed for the cNOS activity assay. The brains of animals from stressed (n=14) and control (n=14) pools from group III were dissected for PFC. From this pool, only the glutamate concentrations obtained from the six animals that slept during the termination procedure were included in the results (Table 2).

Brains were removed from all animals and dissected on ice. For PFC collection, the frontal parts of the hemispheres were dissected at the level bregma 3.2, according to the coordinates of Paxinos and Watson (1998), and supplied for the biochemical assay after the removal of their basal parts at the level of rhinal fissures. The brain structures were weighed and placed in liquid nitrogen, using a container of the Dewar CP65 type (Taylor-Wharton). The time span from the moment of the death of the animals until freezing the brain structures did not exceed 5 min.

NOS Activity Assay

The NOS activity in the homogenates of brain tissue was assayed by monitoring the conversion of [³H]L-arginine to [³H]L-citrulline, with modification of published techniques (Hudetz et al., 1998; Northington et al., 1997). The frozen tissue samples were homogenized in ice-cold Tris/HCl buffer (0.5 M, pH 7.5) and then centrifuged for 3 min at 10000 × g at 4°C. The supernatant obtained was used for the NOS activity assay. Briefly, 50 µL of the supernatant was preincubated for 5 min with or without L-methyl-arginine (1 mM) before the addition of [³H]L-arginine (ca. 260000 cpm). Incubations were performed for 30 min at 37°C in Tris/HCl buffer (0.5 M, pH 5.5) containing 0.03

mM unlabeled L-arginine, 1 mM NADPH, 0.03 mM tetrahydrobiopterin, 100 units calmodulin, and 2 mM CaCl₂. The reaction was terminated with Tris/HCl buffer (0.5 M, pH 5.5) at 4°C. The [³H]L-citrulline was separated from [³H]L-arginine using a Dowex 50 chromatography column. The radioactivity in the column eluate was counted by liquid scintillation spectrometry (LKB TACK BETA) using Ultima Gold XR scintillation fluid (Canberra Electronic E.U.). The NOS activity was defined as the difference between the controls and the L-methyl-arginine blanks, indicating the NOS-dependent formation of [³H]L-citrulline. The results were expressed as the amount of radiolabeled L-citrulline formed during 30 min of incubation per milligram protein (cpm/30/min/mg protein).

The glutamate concentration in the PFC was evaluated using glutamate dehydrogenase and NAD, according to the Bernt and Bermeyer (1974) method used hitherto (Hauser et al., 1999).

The Student's t test was used to compare the mean values from the tests after the evaluation of

the normal distribution and the equality of variances (with p=0.05) matched in pooled results.

All efforts were made to minimize animal suffering and to reduce the number of animals used. All procedures were approved by the Medical University of Gdansk Animal Use and Care Committee in accordance with European Community standards and guidelines.

RESULTS

The respective cNOS activities and glutamate concentrations are presented in Table 1 and Table 2. After 15 s of simultaneous mechanical+acoustic+visual stimulation, both the PFC and the amygdala of stimulated rats showed statistically significant increases of cNOS activity versus control animals. Such an increase was not obtained for the hippocampi and cerebella. The concentration of glutamate in PFC of stimulated versus control animals was also significantly increased.

TABLE 1

Activity of cNOS (measured as the amount of [³H]L-citrulline) in tissues from animals subjected to sensory stimuli (S) for 15 s and in tissues from unstimulated control animals (N)

	GROUP I				GROUP II			
	Prefrontal cortex		Hippocampi		Amygdala		Cerebellum	
	S	N	S	N	S	N	S	N
n	15	8	15	8	12	7	12	7
[³ H]L-citrulline (cpm/30 min/mg protein)								
Mean	124980	89650	148910	142375	227300	178700	324920	300710
SD	23620	23940	51880	30789	43500	29000	83800	83710
p	<0.01		>0.05		<0.05		>0.05	

n – number of individuals; SD – standard deviation; p – significance level

TABLE 2

Glutamate concentrations in the prefrontal cortex from animals subjected to sensory stimuli (S) for 15 s and from unstimulated control animals (N)

	GROUP III	
	S	N
n	14	6
Glutamate concentration (nmol/mg protein)		
Mean	185.5	164
SD	17.1	10.8
p	<0.02	

n – number of individuals; SD – standard deviation; p – significance level

DISCUSSION

The results of the experiments carried out here demonstrated a very rapid increase in cNOS activity in the PFC and the amygdala of rats after exposure to stressful sensory stimulation compared with control animals. The absence of this stress-induced cNOS upregulation in the hippocampi and cerebellum argues against a generalized metabolic effect. As the responsive brain regions, the PFC and the amygdala, process cognitive and emotional aspects of environmental influences, the observed changes may be linked to the characteristics of the presented stimuli.

Because the method applied here does not permit an explanation of the mechanism involved, whether the increase in activity is related to endothelial nitric oxide synthase (eNOS) or to neuronal nitric oxide synthase (nNOS) is not clear. Neuronal NOS is located exclusively in the soluble fraction of cell homogenates, but only a very small

part of eNOS is located there (Giulivi et al., 1998; Northington et al., 1997). Although the present study did not use high-speed or differential centrifugation to separate the supernatant and particulate fractions, the percentage of endothelium versus brain tissue in the sample would argue in favor of nNOS activity. Presumably, the sample used here also includes the nNOS activity of astrocytes (Baltrons & Garcia, 1997; Yamada et al., 1997).

The sensory stimuli had an aversive character and caused fear (McNish et al., 1997). Fear is accompanied by an increase in the blood flow of the PFC (Lane et al., 1997). Up-regulated nNOS activity in neurons and perivascular nerves may play a key role in the regulation of blood flow distribution (Hudetz et al., 1998; Jun-Ge et al., 1997).

In a preliminary study, (Hauser et al., 1999), increased post-mortem levels of glutamate were seen in brain hemispheres after 1 and 10 min

duration of the same aversive stimulation as that presented here. In the present study, such increases were detected in the PFC of rats after 15 s of stimulation (Table 2). An increase in extracellular glutamate occurs in PFC during pain stimulation (Bagley & Moghaddam, 1997). Coupling of the NMDA receptor-mediated calcium influx and nNOS activation is postulated to be due to a physical coupling of the receptor and the enzyme by an intermediary adaptor (PSD 95), in addition to the well-known Ca^{2+} /calmodulin mechanism. Presumably, NMDA receptor-mediated NO release into the synaptic space must be preceded by the translocation of nNOS to synaptic structures by binding to PSD 95 (Jaffrey et al., 1998).

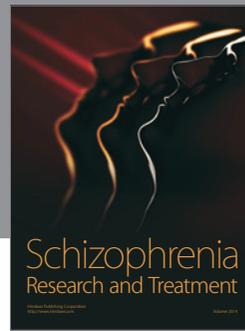
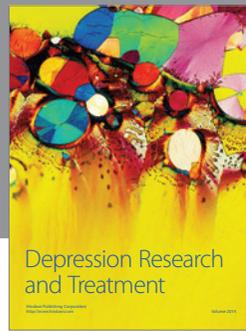
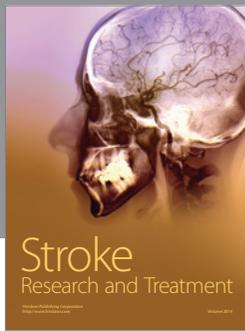
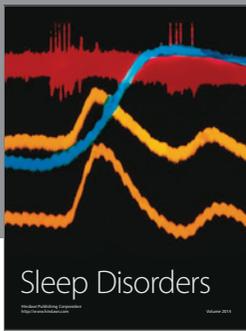
The catalytic activity of NOS measured in the homogenates, in the presence of excess calcium and NADPH, represents near-maximum activity and may not correlate with that in vivo. Nevertheless, in the experimental model applied here, the measured NOS activity—as well as the glutamate concentration—in the brain tissue could be taken as indices of the emotional state before death. The increase of cNOS activity in the amygdala—a key structure of the central fear response (LeDoux, 1998)—argues in favor of such a hypothesis (Table 1).

In this study, the very short emotional arousal existing in experimental conditions immediately before death has been assessed for the first time. Whether similar increases are found in man remains unknown and requires further post-mortem biochemical investigation of human brain structures. Insight into the emotional state that is created in the victim's brain immediately before death could be very helpful for analyzing the circumstances leading to death. Additionally, further experiments should be carried out to clarify the dependence between cNOS activity, the morphological origin of cNOS, and the level of glutamate in the PFC during the response to different types of aversive stimuli.

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