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ORIGINAL ARTICLE

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The concentration of glutamate in cerebral tissue as a factor for the assessment of the emotional state before death. A preliminary report

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Abstract In this paper the concentration of glutamate in the hemispheres of the brain, the cerebellum and the brain stems of rats exposed to sensory stimulation before death and of control rats are presented. Statistically significant differences in the concentration of this neurotransmitter were found in the hemispheres of the brain in the cases of stress lasting 10 min and 1 min and in the cerebellum after a stress of 10 min. This suggests that it may be possible to diagnose the post-mortem state of emotional tension related to sensoric aversive input which takes place directly before death.

Key words Rats · Central nervous system · Emotional response · Glutamate

Introduction

The components of the limbic system (mainly the amygdala and the hippocampal complex), the thalamus, the striatum and the cerebral cortex [1,2] take part in the activation of the functions connected with emotional stimulation. In the stress response to emotional stimuli the paraventricular hypothalamic nucleus and the locus ceruleus function together with the aforementioned structures [1, 3]. There are also reports of the part played by the cerebellum in the cognitive processes which presumably plays a part in the concentration of the attention, in the undertaking of activities and in the forming of the short-term memory as well as the emotional response [4].

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One of the most important ligands involved in the emotional arousal is glutamate [5] and two kinds of glutamate receptor have been identified. The first group consists of the ionotropic receptors iGluR (non-NMDA and NMDA) situated in the neurons of the sensory and associative areas of the cerebral cortex, in the neurons of the entorhinal cortex and in the postsynaptic membranes of the cells of the hippocampal complex [6–8]. They also exist in the amygdala [5], the striatum [9, 10], the cerebellum [11] and the nucleus accumbens [12]. In the modulation of glutamate release, a fundamental but indirect role is played by nitric oxide. Produced by the postsynaptic neurons it operates as a retrograde messenger potentiating [13, 14] or reducing [15] glutamate release from the presynaptic neurons into the synaptic space. In the second group are the metabotropic receptors mGluR which are located in the cellular membranes of the Purkinje cells of the cerebellum and in the pyramidal neurons of the CA3 area of the hippocampus [6, 16, 17], in the striatum [10] and the nucleus accumbens [12]. Both types of GluR were also identified in astrocytes [18].

The receptors iGluR and mGluR are responsible for the long-term potentiation (LTP) and depression (LTD) of synaptic activity, which are the exponents of the process of learning (LTP) and memory formation (LTP, LTD). The exact molecular mechanism of these phenomena was described in a review article [19]. LTP has been demonstrated in the cerebral cortex, the entorhinal cortex, the hippocampal complex, in the connections between the thalamus and the sensory areas of the cortex and the amygdala [5, 6], in the striatum [9] and in the cerebellar parallel fiber synapses [11]. It is presumed that LTP also takes place in the synaptic connections between the mossy fibres and the granule cells of the cerebellar cortex [7]. It is assumed that LTP also takes place in the neuronal network between the amygdala, the hippocampal complex and the entorhinal cortex. On the other hand a different process of modification of synaptic activity (long-term depression – LTD) consists of a long-term weakening of synaptic conductivity [6]. LTD has been demonstrated in the cerebellum and in the striatum where plays a role in

learning and perfecting the coordination and accuracy of movements [6, 7, 10]. There have been reports that the LTD process also takes place in the hippocampus [15].

In the plasticity of synaptic transmission astrocytes probably also take part. They are not only involved in the glutamate turnover [20] but also release it when stimulated by specific AMPA/kainate and mGluR receptors agonists [21]. Recent investigations suggest the continuous bidirectional communication between neurons and astrocytes based on reciprocal glutamatergic signalling [21].

Because of the role played by glutamate in emotional arousal, it was decided to measure the concentration of this neurotransmitter in various parts of the brains of rats after the action of stress-inducing factors and in conditions of absolute calm. The investigation was carried out to determine whether a post-mortem assessment of the emotional state immediately before death could be made.

Materials and methods

The tests were carried out on 64 male Wistar rats with a body weight ranging from 270 g to 580 g and a brain weight of 1.6–2.1 g. During the experiment the animals were given free access to water and to standard laboratory diet. The rats were terminated after exposure to sensory stimuli before death ($n = 32$) and in the control group after no exposure ($n = 32$). The rats from the control group, 2 in each experiment, were housed in a plexiglass cage of our own construction, where they remained throughout the night for a period of over 12 h. The following day in the morning the cage was closed in such a way as not to disturb the animals (they were usually asleep) and carbon dioxide was passed from pressurised cylinders into the cage through permanently installed plastic pipes. At this time the animals were observed through a peephole from a neighbouring room. In order to increase the rate of flow of the carbon dioxide and to speed up the death of the animals, a water pump was used to suck out the air from the cage for the first 20 s. Death followed in the time of 1 min. The animals were immediately decapitated, the cranial cavities were opened and the brains were removed, weighed and placed in defined parts in liquid nitrogen, using a container of the Dewar CP65 type (Taylor-Wharton). The duration of the whole period from the moment of death to the moment of placing the tissues in the low temperature did not exceed 5 min.

Rats were stimulated using three types of stimuli: acoustic, optical and mechanical. The first type of stimulus was produced using two methods i.e. an acoustical whistler and a scraping device. Optical stimulus was produced by stroboscope lamp S 100 W with regulated frequency. Mechanical stimulus was produced by a plastic rod with one surface flat and another rectangular. The mean area of flat surface was about 30 cm^2 and about 0.5 cm^2 for the rectangular area. The acoustic stimuli were measured by an acoustic analyzer Swan 910. The source and the microphone were located inside the sound-isolated chamber with negligible levels of external acoustic disturbances. The optical stimulus was measured using a standard photoelectric photometer (Carl Zeiss, Jena) with the detector used for visual photometry.

The force in the case of mechanical stimuli was measured using a dynamometric method with calibration of a force measuring device using standard masses.

The whistler with two balls inside the resonating compartment was measured in the same manner as described. The main harmonic A at 2624 Hz has an acoustic level of 100 dB. The second harmonic B has an acoustic level 80 dB and the third harmonic C of 64 dB. The sharp break in the spectrum D is located near 11 kHz. The maximum level of high frequency for band E is about 60 dB. This value for the acoustic level is valid for frequencies between 14 and 16 kHz.

Optical stimulus was generated using a frequency of flashes at 6 Hz. The mean value of light intensity at the point located 30 cm from the lamp was 10 000 lx. This value was stable with a deviation less than 5%.

Mechanical stimuli were produced exerting pressure upon the animals with the rod. The mean value of the force was about 3 N with significant deviations coming from manual generation of this kind of stimuli: stimulation was mainly on the backs and sides of the animal. Stimuli were repeated with a frequency of about 1 Hz. The mean dispersion of force was about 1 N, depending on the operator.

After the period of stimulation the cage was closed and filled with carbon dioxide. After termination the same procedure was followed as for the control group.

The brains of all the animals were divided into three parts: i.e. both hemispheres, the cerebellum and the brain stem (severed at the level of the inferior colliculus of the quadrigeminal lamina – nuclei of the pons).

Determination of the glutamate concentration

Brain tissue (1 g) was homogenised manually in 5 ml of ice-cold isolation medium containing 20 mM of Tris-Cl buffer, pH 7.4 and 0.2% Triton X-100. After homogenisation 5 ml ice-cold 1 M $HClO_4$ was added, the preparation was intensively mixed and centrifuged for 10 min at $3000 \times g$. The pellet was saved for protein determination. To 5 ml of the supernatant 0.5 ml of 1.9 M K_3PO_4 was added, mixed and centrifuged for 10 min at $3000 \times g$. The supernatant obtained (50 µl) was used for the glutamate assay using glutamate dehydrogenase and NAD following the method described by Bernt and Bergmeyer [22]. Briefly to 0.6 ml of glycine-hydrazine buffer (0.5 M glycine + 0.4 M hydrazine, pH 9.0) the following reagents were added: 50 µl of sample (or 50 µl of H_2O for blank) 30 µl of 33.5 mM ADP, 60 µl of 27 mM NAD and 0.21 ml H_2O. Samples were mixed and absorbance was read. Then 50 µl (4.5 U/ml) of glutamate dehydrogenase was added and after 45 min the absorbance was read again. For calculation the glutamate concentration the following formula was used: Δ A glutamate = Δ A sample – Δ A blank.

In order to determine the concentration of protein, the pellet was dissolved in 12 ml 1 M NaOH, diluted 10 times with distilled water and 50 µl was used to determine the concentration of protein using the Peterson method [23] and using bovine serum albumine as a standard.

Results

The concentration of glutamate found in the brain tissues of the rats after stimulation for 10 min (Table 1) and 1 min (Table 2) were compared with those obtained in the control rats.

The ‘*t*’-test was used to compare the mean values from the tests after evaluation of the normal distribution and the equality of variances (with $P = 0.05$) matched in pooled results.

Statistically significant differences were obtained between the concentrations of glutamate in the hemispheres of rats subjected to sensory stimuli for 10 min and 1 min and in the hemispheres of the control animals. The differences were very significant ($P = 0.001$) in the case of 10 min. Significant differences also occurred between the concentrations of glutamate in the cerebella after a 10-min stress period. However, no differences were apparent in the cerebella after 1 min of stress and in the brain stems after both the 10 min and 1 min stress periods.

Table 1 Concentration of glutamate (nmol/mg protein) in the hemispheres, cerebella and brain stems of animals subjected to sensory stimuli (S) for 10 minutes and of those from the control group (N)

	Hemispheres		Cerebellum		Brain stem	
	S	N	S	N	S	N
1.	168	123	127	104	117	101
2.	186	140	134	132	111	10
3.	182	115	140	98	101	103
4.	197	157	121	111	105	161
5.	179	124	131	116	137	96
6.	223	170	115	106	106	109
7.	249	193	132	127	107	92
8.	185	182	120	121	106	87
9.	249	186	132	107	93	89
10.	227	165	126	136	116	108
11.	197	181	131	137	105	100
12.	191	165	133	100	103	77
mean	202.75	158.42	128.50	116.25	108.92	101.92
SD	27.43	26.81	7.00	14.05	10.92	20.74
P	0.001		0.02		> 0.05	

Table 2 Concentration of glutamate (nmol/mg protein) in the hemispheres, cerebella and brain stems of animals subjected to sensory stimuli for 1 minute (S) and of those from the control group (N)

	Hemispheres		Cerebellum		Brain stem	
	S	N	S	N	S	N
1.	267	193	142	112	72	66
2.	191	157	115	104	78	60
3.	153	167	83	94	66	65
4.	151	134	103	109	65	74
5.	241	134	76	84	40	55
6.	131	128	66	59	49	47
7.	149	127	69	57	49	44
8.	–	112	67	60	54	35
9.	137	112	76	91	69	60
10.	149	126	107	97	61	64
11.	153	166	108	97	75	52
12.	136	114	84	94	67	63
13.	227	185	91	96	76	73
14.	177	161	102	96	58	71
15.	180	194	85	85	70	75
16.	165	165	97	84	–	75
17.	161	166	72	103	69	72
18.	191	167	122	115	103	90
19.	199	137	133	117	101	90
20.	198	165	136	100	119	77
mean	176.58	150.50	96.70	92.70	70.58	65.40
SD	37.47	26.33	23.81	17.42	19.56	14.16
P	0.02		> 0.05		> 0.05	

Discussion

The investigations carried out showed an increased glutamate level in the hemispheres of the brain and in the cerebella of stimulated rats versus control animals. The increase in the hemispheres of the brain occurred in rats stimulated for 1 min and 10 min, but in the cerebella only of those stimulated for 10 min. The results indicate a direct connection between the glutamate level and the state of emotional arousal.

The mechanism of the increase in the glutamate level and its possible role can only be considered hypothetically. The procedure used does not allow for differentiation between intracellular and extracellular glutamate. It has been reported that thesis an increased glutamate efflux in the extracellular space of the prefrontal cortex and the hippocampi of rats exposed to pain stimuli [24]. In the present experiments changes were shown after sensoric stimulation had been applied. Glutamate plays a key role in LTP and LTD [19] and the molecular mechanisms of these complex processess share similarities [15]. One of these is the activation of the receptors iGluR (NMDA and AMPA) and the receptors mGluR [9–11,15]. The effect is the influx of Ca^{2+} to the postsynaptic neurons, or the release of Ca^{2+} from the intraneuronal pool [9, 10, 15]. The experimental model applied varied from the conditioning of fear. Thus the synaptic exponents of learning LTP and LTD could not have taken place. The intensity of applied sensoric stimuli guaranted they were aversive [25].

The increase in the glutamate level could be connected with the increase in cerebral blood flow at time of stress [26]. The glutamate efflux has also been reported from oedematous astrocytes [21]. It is most probable that the elevated glutamate reflects a 'pure' response to the stimuli applied, just as in microdialysis studies with living animals [24]. The increase would occur in both intra- and extracellular space. A comparison of the p coefficients from both tables could indicate that this increase in the active structures is dependent on the time of stimulation. Glutamate plays a large role in excitatory neurotransmission in the central nervous system [1, 5]. In situations of emotional arousal a massive release glutamate takes place from the presynaptic endings [1, 5]. This might lead to the uncontrolled cytotoxic influx of Ca^{2+} to the postsynaptic neurons [18, 24]. This is prevented by the uptake of glutamate from the synaptic space by astrocytes [18].

In the final phase of the circuit, (presynaptic neurons-intracellular space- astrocytes) glutamate becomes transformed into glutamine [20]. This is returned to the neurons which metabolize it into glutamate (glutamate-glutamine cycle) [20, 21]. Additionally the astrocytes can also release glutamate, a process which is either dependent on or independent of Ca^{2+}[21]. Until now it has not been established which of the pools, extracellular (neurons, astrocytes) or intracellular, prevailed in the glutamate levels evaluated.

The levels of glutamate in the hemispheres of the brain of control rats and those stimulated for 1 min and for 10

min were significantly higher than those in the cerebella and the brain stems ($P = 0.001$). The differences shown in the levels of glutamate in cerebella occurred only during the 10-min stress time. In the brain stems these differences were not revealed. This may be the result of the different distribution and activity of glutamatergic neurons in the parts of the brain tested. Glutamate can also constitute the metabolic and not the transmitter pool [27]. A more accurate interpretation of the results obtained will become possible after the immunohistochemical studies.

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