

# **Effects of different perinatal treatments on the neurobehavioral development of newborn rats**

**PhD. thesis**

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# 1. Introduction

Perinatal period is essential in the development of the central nervous system. Injuries in this period can cause severe damage with permanent disabilities. However, very little is known about the short-term effects of neuronal lesions, despite the prognostic significance of the early changes. The nervous system – especially in young age – has a huge potential of plasticity and regeneration, which can prevent the negative effects of neuronal damage. Examination of the environmental factors affecting this regeneration and remodeling process is very important. In rats, the first two weeks of age is the critical period of neuronal maturation. Pups reach adult level in development at the third postnatal week. In our present studies we used four different types of treatments in newborn rats. Toxic lesion was induced by monosodium-glutamate and hypoxic/ischemic lesion was induced by carotid occlusion and hypoxic exposure. As positive influence we treated neonatal rat with the neurotrophic pituitary adenylate cyclase activating polypeptide (PACAP), and finally, we used enriched environment. Neurobehavioral development was examined with complex functional tests.

## 1.1. Monosodium-glutamate (MSG)

Glutamate, the major excitatory neurotransmitter in the brain, is also categorized as an excitotoxin. Monosodium glutamate (MSG) is used as a food additive, which causes only minor alterations in adults. However, administration of MSG to neonatal rodents brings about several profound changes. It leads to degeneration of the neurons in the arcuate nucleus, inner retinal layers and various other brain areas.

## 1.2. Hypoxic/ischaemic lesion

Perinatal hypoxic-ischemic injury is a serious problem in human neonates, with a high risk of future behavioral and neurological deficits, in spite of improvements in obstetric and neonatal intensive care. The most commonly used rodent model of neonatal hypoxia-ischemia is the unilateral ligation of the carotid artery followed by a period of hypoxic exposure.

## 1.3. Pituitary adenylate cyclase activating polypeptide (PACAP)

PACAP was isolated in 1989. It exists in forms of 38 and 27 amino acid residues, with PACAP38 being predominant. Since its discovery, several effects have been attributed to

PACAP, and numerous studies show its neurotrophic and neuroprotective actions. It is suggested that the peptide acts in the neural tube during patterning to control cell proliferation and gene expression. Several other studies show that PACAP participates in regulating mitosis, differentiation and apoptosis during neurogenesis.

#### **1.4. Enriched environment**

The discovery that rats raised as pets show better performance in various problem-solving tasks than animals raised in laboratory conditions pointed out the importance of environmental effects in the development of the nervous system. Expanded field, compared to standard laboratory cages, with continuous new stimuli has positive environmental effects, thus improves development.

#### **1.5. Aims of the present study**

1. Examination of neurobehavioral development of newborn rats using standard neurological tests in:
  - Toxic (monosodium-glutamate induced) lesion
  - Hypoxic/ischemic injury
  - Neurotrophic factor (PACAP) treatment
  - Enriched environment
2. Examination of possible neuroprotective effects of PACAP treatment or environmental enrichment in monosodium-glutamate induced toxic lesion.

## 2. Materials and methods

### 2.1. Animals

TREATMENT	NUMBER OF ANIMALS
Monosodium-glutamate	30 treated and 15 control in reflex and motor coordination tests 14 treated and 12 control in the open-field test 12 treated and 12 control in novelty-seeking tests
Hypoxia/ischaemia	12 treated and 12 sham operated in reflex, motor coordination and open-field tests
PACAP	18 PACAP 38, 20 PACAP 6-38-treated and 14 control in reflex and open-field tests 15 MSG and PACAP 38 treated animal in reflex and motor coordination tests
Enriched environment	8 control and 4 MSG treated impoverished animal (standard cage), 12 control and 16 MSG treated enriched animal is reflex, motor coordination and open-field tests

Animal housing, care and application of experimental procedures were in accordance with institutional guidelines under approved protocols (No: BA02/2000-20/2006, University of Pecs).

### 2.2. Treatments

2.2.1. MSG treatment: Rat pups received a high dose of 4 mg/g, or low dose of 2 mg/g MSG subcutaneously, dissolved in physiological saline on postnatal days 1, 3, 5, 7 and 9 or on postnatal days 1, 5 and 9. Control animals received only physiological saline on the same days.

2.2.2. Hypoxic-ischemic injury: Neonatal unilateral carotid artery ligation was induced in 7 day-old Wistar rat pups. The left common carotid artery was ligated through a midline cervical incision. Following surgery pups were placed in an airtight chamber containing a gas mixture of 8% oxygen and 92% nitrogen for 2 hours.

2.2.3. PACAP treatment: From the day of birth to two weeks of age, all animals in the litters received a daily subcutaneous injection of 1 µg PACAP 38, or 10 µg PACAP antagonist (PACAP6-38) dissolved in sterile physiological saline.

2.2.4. Enriched environment: First group of animals was kept in standard housing conditions (dimensions: 43x30x20 cm). Second group was kept in an expanded cage (88x50x44 cm). In this large cage, several objects of different colour, size and shape were placed, and half of them were changed each day.

### **2.3. Examination of neurobehavioral development**

Examinations of neurobehavioral development were carried out daily. Inspections were made for maturation of physical characteristics such as eye opening, incisor eruption and ear unfolding. Weight was also recorded. Pups were tested for the following neurological signs and reflexes:

#### 2.3.1. Righting reflex:

(a) Rats were placed on supine position and the time in seconds to turn over to prone position and place all four paws in contact with the surface was recorded.

(b) animals were dropped head down onto a soft surface and the first day of landing on four paws was recorded.

2.3.2. Negative geotaxis: animals were placed head down on an inclined board. The day they began to turn around and climb up the board with their forelimbs reaching the upper rim was observed. Time in seconds to reach the upper end of the board was recorded daily.

2.3.3. Sensory reflexes: the ear and the eyelid were gently touched with a cotton swab and the first day of the ear twitch reflex and the contraction of the eyelid was recorded.

2.3.4. Limb placing: the back of the forepaw and hindpaw was touched with the edge of the bench while the animal suspended, and the first day of lifting and placing the paws on the table was noted.

2.3.5. Limb grasp: the fore- and hindlimbs were touched with a thin rod, and the first day of grasping onto the rod was recorded.

2.3.6. Gait: the animals were placed in the center of a white paper circle of 13 cm in diameter, and the day they began to move off the circle with both forelimbs was recorded. From the day of the appearance, the time in seconds to move off the circle was recorded daily.

2.3.7. Auditory startle: the first day of the startle response to a clapping sound was observed.

## **2.4. Motor coordination tests**

Rat pups were tested for motor coordination twice a week between 2-5 weeks of age.

2.4.1. Grid-walking and foot-fault test: Rats were placed on a stainless steel grid floor (20x40 cm with a mesh size of 4 cm<sup>2</sup>) elevated 1 meter above the floor. For a 1-minute observation period, the total number of steps was counted. The number of foot-fault errors, when the animals misplaced a forelimb or hindlimb that it fell through the grid, was also recorded.

2.4.2. Rota-rod test: Animals were tested on a commercially available treadmill for small animals with diameter of 14 cm, attached to a rotating motor. The test was performed at a speed of 13 rpm. The pups were placed on the rotating drum and the time the animal could stay on the rota-rod was measured (max. 2 minutes).

2.4.3. Walking initiation: Rats were placed on a horizontal surface in the center of two concentric circles with diameters of 10 and 45 cm. The time taken to move off the circles was recorded.

2.4.4. Rope suspension test: Animals were suspended by both their forepaws on a horizontal nylon rope, stretched horizontally 40 cm over a foam pad. The time the animals fell off the rope was recorded.

2.4.4. Inclined board test: Animals were placed on a wooden board, and the board was gradually elevated by 5 degrees. The maximum angle at which the animals could maintain position on the inclined board for 5 seconds was recorded.

2.4.6. Vertical screen test: Rats were placed on a screen (50 x 22 cm, grid openings 15 mm) in the horizontal position. The screen was turned to vertical position, and the rat was observed for 5 seconds. A score of 0 was given if the rat gripped to the screen for the full 5 seconds; a score of 1 was given if the rat fell during the 5 second period and a 2 was assigned if the rat fell immediately.

2.4.7. Elevated body swing test: Animals were held by the tail 3 cm from the bottom of the surface. During the 30-second trial, the direction and number of times the animals moved more than 10 degrees to either the left or right side of the vertical axis was recorded.

2.4.8. Traversing a square bridge: Rats were placed on a square bridge (1x1x40 cm, elevated 40 cm above the floor). The duration the rat stayed on the bridge was measured.

## **2.5. Open-field activity**

Animals were observed for locomotor behavior in an open-field at 2, 3, 4, 6 and 8 weeks of age. Pups were placed in an open-field consisting of a 42x42 cm box with 21 cm high walls around. The floor was divided into 8x8 areas. Subjects were placed individually in the center, always facing the same direction, and were video-recorded for 5 minutes. Recordings were evaluated in a blinded fashion. The following parameters were measured: distance travelled, ambulation time, head lifting, rearing and grooming behavior. Speed was calculated from the ambulatory time and the total travelled distance. The time spent in the first zone next to the wall or in one of the four corners was also recorded.

## **2.6. Novelty-seeking behavior**

Testing consisted of four 5-min trials at 3 weeks of age in the open-field. There were no objects in the field during trial 1, which served as a familiarization period to the novel environment. In trial 2, two identical pink glasses filled with sand with white covers were placed in the open-field. The same two objects remained in the open-field during trial 3. In trial 4, one of the objects (familiar object) was replaced with a novel object, which was a quadrangular white plastic container with a blue top. The behavior of each animal was video-recorded for 5 minutes, and behaviors were evaluated later in a blinded fashion. Total activity time, number of fields crossed, time spent in the center and in the periphery were recorded during each trial. Central area was regarded as the fields contacting the object and their neighboring ones. During trial 4, the time spent in the proximity of the familiar and the novel objects were recorded separately. Habituation index was calculated as a percentage of areas crossed during the first 90 seconds in relation to the total crossed fields.

## **2.7. Histological analysis**

2.7.1. MSG: Neonatal MSG treatment causes morphological and biochemical alterations in various brain areas, including destruction of the neurons in the arcuate nucleus and in the retina. After completing the behavioral testing, animals were anesthetized and perfused with 4% paraformaldehyde at six weeks of age. Brains were removed and 50 µm-thick serial frontal vibratome sections were made from the hypothalamus. Sections



were stained with toluidine-blue, and parallel sections were processed for tyrosine-hydroxylase (TH) immunohistochemistry.

2.7.2. Hypoxic/ischemic injury: After completing the behavioral testing, animals were anesthetized and perfused with 4% paraformaldehyde. Brains were removed and 50  $\mu$ m-thick serial frontal vibratome sections were made. Sections were stained with cresyl-violet. For morphometric analysis, a total of 20 sections, with a 0.5 mm distance, were used between 3.8 mm anterior and 5.2 mm posterior to bregma point. Total brain area as well as the area of the cortex, hippocampus, striatum and the lateral ventricle of both contralateral and ipsilateral sides were measured.

## **2.8. Statistical analysis**

Results in appearance of physical and neurological signs were compared with ANOVA followed by Newman-Keul's post hoc analysis. Daily values were compared with Student's t-test. Improvements in the daily performance in righting reflex, negative geotaxis and gait were evaluated by ANOVA repeated measures, while the different groups were compared with Newman-Keul's. Correlation between body weight and appearance of neurological signs was evaluated with Spearman's correlation test. Nonparametric data of the open-field were evaluated by Kruskal-Wallis test followed by Dunn post hoc analysis. Parametric data were analysed with Newman-Keul's test following variance analysis. Mann-Whitney test was also used in evaluating result of the open-field of hypoxic animals.

# **3. Results**

## **3.1. MSG treatment**

We used the dose of 4 mg/g given on postnatal days 1,5 and 9. During the treatments, 2 control and 12 MSG-treated rats died. There was no significant difference in any test between female and male pups. Histological investigation of the arcuate nucleus revealed severe loss of neurons in the arcuate nucleus of the MSG-treated rats compared to control animals.

3.1.1. Physical characteristics: There were no significant differences in the tested signs of somatic development such as the days of eye-opening, incisor eruption and ear unfolding. However, the average body weight of the MSG-treated animals was significantly lower

starting from day 6 throughout the observed 6 weeks. The retarded somatic development was also reflected in the shorter body length of the MSG-treated pups: body length of MSG-treated rats was significantly shorter from the end of the first week.

3.1.2. Neurological reflexes: Among the examined neurological reflexes, forelimb placing, forelimb grasp and air righting reflexes appeared significantly later in MSG-treated pups compared to control rats. Righting reflex was performed in a significantly longer time by MSG-treated pups throughout the observation period. Control animals had better daily performance in negative geotaxis and gait.

3.1.3. Motor coordination, strength: In the grid-walking test, control and MSG-treated pups made the same number of steps during the 1-minute observation period. However, MSG-treated pups had a higher number of mistakes. This higher number of foot-faults was the result of more mistakes with both the fore- and hindlimbs. Worse performance was also observed in the rota-rod test.

3.1.4. Open-field activity: MSG-treated rats were more active during the first 3 postnatal weeks. A markedly increased activity was observed on week 2, when control animals moved very little. There was no difference in the speed of movement between the two groups. At 3 weeks of age, the locomotor activity of all animals increased markedly. The speed of the MSG-treated pups increased significantly when compared to control animals and this led to significantly larger explored area. At 4 weeks of age, most differences disappeared. At 8 weeks of age, MSG-treated pups moved significantly less than their control littermates. The number of headlifts and rearings were not significantly different during most of the observation period, the number of headlifts was lower in MSG-treated rats only at 3 weeks of age, which was compensated with a slightly higher number of rearings. The time spent at walls (thigmotaxis) was lower in MSG-treated pups at 2 weeks of age, although this difference was not statistically significant due to the high number of control animals that did not move away from the central starting position. At following weeks, MSG-treated pups spent significantly less time at the walls.

3.1.5. Novelty-seeking behavior: During trials 2 and 3, when the same objects were present in the arena, MSG-treated rats showed significant hyperactivity compared to the control group. The activity of control rats significantly increased during trial 4, when the novel object was introduced. MSG-treated rats showed an opposite behavior: activity was

reduced during trial 4. Control rats showed no change in the habituation index during trials 2 and 3, and rats were more active during the first 90 seconds of the trials. Habituation significantly decreased during trial 4 meaning that rats were almost equally active during the first and second halves of the observation time when exposed to the novel object. MSG-treated rats showed an opposite pattern of habituation: it decreased during trial 3, and increased during trial 4. Grooming behavior also showed opposite patterns in control and MSG-treated animals. Comparison of the times spent in the center (in proximity with the objects) and in the periphery shows that during trial 2, all animals spent significantly more time in the periphery than in the center. The ratio between the times spent in the center and periphery did not change during trial 3 in control rats, but a significant increase in the time spent in the center was observed during trial 4. During trial 4, there was no longer a significant difference between central and peripheral times, so animals spent significantly more time in the proximity of the objects when the novel object was introduced, compared to trial 3. In MSG-treated rats, the difference between the central and peripheral times disappeared already during trial 3, when the same two objects were present as during trial 2. Calculating the times spent in the proximity of the novel and familiar objects during trial 4 shows that control rats spent significantly more time exploring the novel object, while MSG-treated rats spent less time in the proximity of the novel object.

### **3.2. Hypoxic/ischemic injury**

Only 2 hypoxic-ischemic pups died during the first 24 hours after the insult, and 1 further pup died during the 3<sup>rd</sup> postoperative week. Retardation of somatic development was reflected in significantly less weight gain in hypoxic animals. Although pups with the same average birth weight were used, body weights were significantly lower than in control animals starting from 1 day after the hypoxic insult.

3.2.1. Neurological reflexes: Only eye opening was delayed among the physical characteristics in hypoxic-ischemic animals. In addition, several neurological reflexes, such as negative geotaxis, ear twitch reflex, forelimb and hindlimb grasp and gait reflex appeared significantly later compared to normal pups. Hypoxic-ischemic injury caused not only delay in the appearance of some reflexes but animals performed certain tasks, such as righting reflex, negative geotaxis and gait in significantly longer times.

3.2.2. Motor coordination, strength: In the rope test, hypoxic animals had significantly shorter fall off latencies at 3 and 4 weeks of age when they were allowed to use only the right forelimb, and differences were significant only at 4 weeks of age when they were grasping to the rope with both forelimbs. Interestingly, the rotarod test did not reveal significant differences. In the grid-walking test, hypoxic animals had significantly less total number of steps at 2 and 4 weeks of age. The number of foot faults was not different between control and hypoxic groups with fore- and hindlimbs on the ipsilateral side. Hypoxic animals had a higher number of mistakes with the contralateral forelimb than with the ipsilateral forelimb, and the number of faults with the contralateral forelimb was significantly higher than in control animals throughout the observation period. In the inclined board test, significant difference was found only at 2 weeks of age. The time animals could stay on a narrow bridge was longer in control rats only at 2 weeks of age. At later stages, all animals could stay on the bridge for the entire observed 2 minutes or could reach the platform.

3.2.3. Open-field activity: In the open-field, general activity and movement pattern did not show gross differences between the two groups. Pups moved very little at 2 weeks of age. The number of head-lifts and rearings significantly increased by 3 weeks of age, but no significant differences could be observed between control and hypoxic animals. By 6 weeks of age, further increase was only observed in the number of rearings, and hypoxic animals reared significantly more than control rats. Similarly, animals traveled very short distances at 2 weeks of age, with hypoxic animals traveling slightly, but significantly longer distances than control pups. Both groups showed a significant improvement by 3 weeks, with no differences between the 2 groups. At 6 weeks of age, hypoxic pups covered more distance than at 3 weeks and this was significantly different from the control rats as well. Similar pattern was observed in the speed of the animals: hypoxic rats moved significantly faster at 2 and 6 weeks of age, while no difference was observed at 3 weeks of age. The time spent at walls and in corners was significantly more in hypoxic animals only at 2 weeks of age. Control animals spent more time at the walls and in the corners at later stages, while the time spent along the walls did not show further changes in hypoxic animals and they spent less time in the corners as they grew older.

3.2.4. Histological evaluation: Brains of hypoxic pups showed a severe atrophy in most

cases. The total ipsilateral hemisphere was approximately 58% of the uninjured, contralateral hemisphere. Most severe reduction was observed in the hippocampus, which was only 11% on the ipsilateral side compared to the contralateral side. Approximately 50% reduction was observed in the cortex and the striatum. A compensatory enlargement of the lateral ventricle could be also observed.

### **3.3. PACAP and antagonist (PACAP 6-38) treatment**

3.3.1. Physical features and neurological reflexes: PACAP treatment accelerated maturation of facial characteristics with an average of 1-1.5 days, while PACAP6-38 retarded eye opening and ear unfolding. Among the neurological reflexes, the disappearance of the crossed extensor reflex and the appearance of air righting reflex and hindlimb placing were not influenced by PACAP treatment when compared to control groups. All other reflexes showed significant, 1.5-2.5 days advance in the PACAP-treated animals. Anti-PACAP retarded only the appearance of hindlimb placing. PACAP treatment caused significant advance not only in the day of appearance of most reflexes, but animals achieved certain tasks in shorter times. PACAP-treated animals had significantly better daily performance throughout the whole observation period than control animals also in negative geotaxis, i.e.: to reach the upper rim of the inclined board, while there was no difference between animals treated with PACAP6-38 or vehicle. The time taken to move off a circle 13 cm in diameter (gait) was also significantly shortened by PACAP between days 12-20, while significant difference could be observed between animals treated with anti-PACAP or vehicle only at 18 days of age. Measuring the righting reflex revealed PACAP-antagonist caused a significant retardation in this sign: there was a big difference between control animals and antagonist-treated animals between days 2-6, while PACAP had no effect.

3.3.2. Open-field activity: In the open-field, the typical movement pattern observed at 2 weeks was similar in all 3 groups: animals moved around for a few seconds in the center and then moved to the walls, where they spent most of their times in one area. All animals showed improvement in ambulation at 3 weeks when compared to the scores obtained at 2 weeks. The number of areas entered by rats treated with PACAP was almost the double of the number entered by control and anti-PACAP-treated animals at 3 weeks. The time spent along the walls in the open-field did not show difference between the

groups at 2 weeks: animals spent approximately 90% of their time along the walls. However, this time was significantly reduced in animals treated with PACAP at 3 weeks: they spent only two-thirds of their time along the walls. No improvement was observed in vehicle- or anti-PACAP-treated animals when compared to the 2-week values. Animals treated with vehicle or anti-PACAP spent more than 50% of their time in corners at 3 weeks, while PACAP-treated animals only 25%. At 3 weeks: PACAP-treated animals spent only 14% of their time in one area, while control and anti-PACAP-treated animals spent more than 35% of their time in one area.

### **3.4. Effect of PACAP in MSG-induced lesion**

In this experiment rats were administered 4 mg/g MSG at 1, 5 and 9 days of age combined with 1 µg PACAP 38 subcutaneously from 1-14 days.

3.4.1. Physical signs and neurological reflexes: MSG treatment led to retardation of forelimb placing, forelimb grasp and air righting reflexes, while in PACAP-treated animals, these reflexes appeared significantly earlier, similarly to normal rats. By the end of the observation period, MSG-treated rats weighed by almost one third less than normal rats. The average body weight of PACAP- treated animals was about 1 gr more than MSG-treated controls every observed day starting from day 6, but differences were not significant. MSG-treated pups performed worse in righting and gait reflexes which were ameliorated by PACAP treatment at 2 weeks of age.

3.4.2. Motor coordination tests: Foot-fault test has been shown to be a good indicator of MSG-induced retardation of motor coordination development and the number of foot faults was less in the PACAP-treated group than in the MSG-treated pups, but only at 2 weeks of age.

3.4.3. Histological analysis: Severe loss of tyrosine-hydroxylase immunopositive neurons was found in the arcuate nucleus after MSG treatment. In the PACAP-treated group, the number of neurons was higher, but it was not significantly different from the MSG-treated control pups.

### **3.5. Enriched environment**

3.5.1. Physical signs and neurological reflexes: Body weight of the animals kept in enriched environment was significantly less from 10 days of age. Other physical parameters didn't show any difference. Appearance of neurological reflexes was

enhanced by enrichment, and the animals also showed a slightly better performance than standard housed animals.

3.5.2. Motor coordination: Enriched animals moved significantly more in the grid walk test and made less mistakes than standard housed rats. Performance in the walk initiation at 3 weeks of age was also better in enriched animals. At 4 and 5 weeks of age, enriched animals managed to perform better in the rota-rod test than impoverished animals.

3.5.3. Open-field: No significant difference could be observed in the ambulation between the groups. However, at 3 weeks of age, enriched animals moved slightly more. Movement speed showed no difference, the observed alteration was caused by the significantly increased ambulation of the enriched animals. At 3 weeks of age, enriched animals reared also more than standard housed animals, with no significant differences at later ages. At 4 and 5 weeks of age, standard housed animals showed more head-lifting than the enriched rats.

### **3.6. Effect of enriched environment in MSG-induced lesion**

In both animal groups, half of the animals were treated with low-dose MSG (2 mg/g) in postnatal day 1, 5 and 9.

3.6.1. Physical signs and neurological reflexes: MSG treatment significantly retarded somatic development, animals weighed less than saline treated controls. This difference disappeared in enriched environment, MSG treated and control animals weighed the same. Appearance of the physical parameters was not altered by this dose of MSG. Appearance and performance of the neurological reflexes were better in MSG treated enriched animals than MSG treated standard housed rats, but this difference was not significant.

3.6.2. Motor coordination: In the foot-fault test MSG treated animals performed significantly worse than saline treated animals in the standard housed group. This negative effect was prevented by the enriched environment, MSG treated enriched animals made significantly less foot-faults than impoverished MSG treated rats, and performed similarly to enriched saline treated control rats.

3.6.3. Open-field: MSG treatment caused hyperactivity at young age, which appears as significantly higher ambulation and greater movement speed. These alterations disappeared in enriched environment.

## 4. Discussion

### 4.1. MSG treatment

Severe growth retardation was observed in the MSG-treated pups: both body weight and length were significantly lower than those of the control animals. Also, MSG treatment led to higher premature mortality. In our study, we found that several measures of neurobehavioral development were delayed while others were not altered by MSG-treatment. Although there are relatively few data available on the effect of MSG on these reflex performances, there are some other activity measures that have been reported to remain unchanged in MSG-treated rats. As far as motor activity is concerned, results obtained by different investigators vary. Most pronounced differences in our study were observed in the rota-rod test and in the foot-fault test. Worse performance in the rota-rod test has already been shown. The foot-fault test is extensively used after lesions to show differences in motor coordination. MSG-treated pups made significantly more mistakes in spite of making the same number of steps.

In the present study we showed that neonatal MSG treatment caused a temporary hyperactivity during early postnatal period followed by a slight hypoactivity at 8 weeks of age. MSG treatment also reduced the time of thigmotaxis after week 3. Novelty-seeking behavior was significantly altered after MSG treatment: treated animals responded to a novel object in the opposite pattern compared to control rats. Control animals were clearly interested in the novel object introduced in the last trial: they spent more time with ambulation with a decreased habituation, covered a larger area, spent less time with grooming and spent more time in the proximity of the novel object. In contrast to control rats, MSG-treated pups were less active, spent more time with grooming and spent significantly less time near the novel object during trial 4.

The efficacy of the MSG-treatment was confirmed by the histological examination of the arcuate nucleus, which, similarly to the findings of others, showed neuronal degeneration and lack of TH-immunostaining.

Locomotor activity is known to increase when animals are subjected to a novel environment and this behavior is closely related to the hippocampus. The markedly changed behavior of MSG-treated rats in the novel environment may be related to the



structural and neurochemical changes in the hippocampus. Also, the close relationship of the mesolimbic dopaminergic system with the novelty-seeking behavior and with the arcuate nucleus is well-established. Neonatal MSG treatment interferes with the dopaminergic system at various brain levels. It can be assumed that the destruction of the arcuate nucleus and other changes in the dopaminergic pathways upon MSG administration may also be responsible for the observed behavioral changes. Various consequences of MSG treatment have been reported gender-dependent, while others do not show sexual dimorphism. In our study, no differences were found in any measured parameter between males and females, similarly to other studies on the development of neural reflexes or on motor activity.

## **4.2. Hypoxic-ischemic injury**

We showed that an approximately 50% atrophy of the hypoxic hemisphere results in retarded neurobehavioral development as shown by delayed appearance and worse performance of some neurological reflexes, and retarded development of motor coordination. However, in spite of the permanent cerebral atrophy, most animal reached control levels by 6 weeks of age in most tests, except for the footfault test. In the open-field, hypoxic animals were more active at 2 and 6 weeks of age. The rat model of neonatal hypoxia-ischemia is known to result in extensive cerebral atrophy, but in contrast to human neonates, rats that underwent neonatal hypoxic-ischemic injury do not show gross functional deficits. This can be due to a higher degree of plasticity of the neonatal rat brain compared human brain. However, there are several functional tests that show short-and long-term deficits after exposure to neonatal hypoxia-ischemia. Animals with severe cortical damages can show recovery in some deficits. In accordance with other reports, we observed that animals subjected to hypoxia have retarded somatic development as shown by the significantly lower daily weights. Our results show that hypoxic animals perform worse in negative geotaxis, righting and gait reflexes as measured by the reflex times. Righting reflex was slower throughout the whole observation period, gait reflex was slower at days 10 and 12 and geotaxis at days 14 and 16. At later stages, differences between hypoxic and normal rats disappeared, which indicates that hypoxic pups showed a considerable degree of recovery.

According to our observations, one of the most reliable tests was the contralateral forelimb footfault test, since hypoxic animals made more mistakes throughout the whole observation period. The finding that the forelimbs are more affected than the hindlimbs might be explained by the size of the injury in the area of cortical representation for the hindlimbs. This is in accordance with results reported for adult rats after unilateral middle cerebral artery occlusion, where the behavioral signs were less damaged in the hindlimbs. However, differences between hypoxic and control rats could not be shown throughout the 5 weeks of observation period, and most hypoxic animals recovered to normal levels by 5 weeks of age or earlier. While adult rats with unilateral cerebral ischemia also show a certain degree of recovery in sensorimotor deficits, neonatal rats recover significantly better. This may be explained by the higher level of plasticity of the neonatal brain.

Regarding open-field activity, both hyper- and hypoactivity have been described following neonatal hypoxia-ischemia. Our observations show that rats subjected to hypoxia are hyperactive at 2 weeks of age, when normal pups hardly move at all. This may also explain why hypoxic animals spent more time at the walls at 2 weeks of age: a natural reflex of the pups is to quickly find the wall where they feel more safe. Since normal pups hardly move at all, several of them stayed in the center where they were originally placed and did not move to the wall at all, while hypoxic animals moved more and found the wall more quickly. Since the difference in this thigmotactic behavior disappeared at later stages, it was probably due to higher activity level in hypoxic pups rather than increased anxiety at 2 weeks, although this latter possibility cannot be excluded either. Consistent with our previous observations, there is a significant increase in locomotion at 3 weeks of age also in normal pups, which may explain why no significant differences were found between control and hypoxic animals at this age. However, hypoxic animals were more active at 6 weeks of age as shown by the number of rearings, the distance traveled, ambulation time and their speed. Interestingly, in spite of increased activity in the open-field, hypoxic animals made less steps on a horizontal wire. This indicates that when locomotion requires a higher level of coordination, like on a wire, hypoxic animals do not show hyperactivity or can be even hypoactive. Although the immature nervous system is capable of considerable compensatory reorganization following injury, most studies have described severe cerebral atrophy after hypoxic-

ischemic insult. It has been reported that the contralateral hemisphere occasionally becomes hypertrophic. Our results show that most severe atrophy occurred in the hippocampus, where the hypoxic hippocampus was only approximately 10% of the normal side. Other brain areas, as well as the total brain area showed approximately 50% reduction. These results correlate with those of others.

### **4.3. Effects of PACAP and PACAP antagonist**

In the present study we showed that neonatal rats treated with PACAP from the day of birth showed significant improvement when compared to vehicle-treated pups in the day of appearance of physical features, and most neurobehavioral signs.

PACAP treated animals showed accelerated facial development, while pups treated with PACAP6-38 had delayed ear unfolding and eye opening. Most neurological signs and reflexes were accelerated in PACAP treated animals, while anti-PACAP treated animals showed retardation only in hindlimb placing. PACAP treated pups performed righting significantly better between days 6-10, while anti-PACAP treated animals were slower between days 2-6 than control pups. PACAP-treated animals performed negative geotaxis and moving off a circle much faster than control animals, while anti-PACAP did not have an effect on them. These observations show that PACAP treatment causes acceleration of the development of complex motor skills. PACAP treatment caused significant increase also in exploratory behavior at 3 weeks of age. PACAP treated animals moved more, spent less time resting in one area, returned to and spent more time in the center. In contrast, control and PACAP6-38-treated animals spent more time at the walls. To stay in proximity with the perimeters of the environment (thigmotaxis) is part of the animal's defensive repertoire and can indicate anxiety. The higher frequency of headlifts may already indicate an increased interest in the environment by PACAP-treated rats. Increased activity in the open-field clearly demonstrates increased locomotor and exploratory behavior of PACAP-treated animals, but it may partly be due to less anxiety.

### **4.4. Effects of PACAP in MSG-induced lesion**

In summary, our results show that the dose of PACAP that effectively enhances neurobehavioral development in normal rats was able to counteract the retarding effect of MSG on righting, forelimb placing and grasp reflexes and caused a significant amelioration of the righting and gait reflex performance and motor coordination at 2

weeks of age. However, the assigned dose was not able to counteract the effects of MSG on the body weight and the neuronal loss in the arcuate nucleus. This might be explained by findings of others that have shown in vivo neurotrophic effects of PACAP only by local treatments. However, the slight protective effect of PACAP found in the present study calls for other investigations to find a possible optimal treatment paradigm with PACAP for neonatal nervous injuries.

#### **4.5. Enriched environment**

Environmental effects play an essential role in the development of the central nervous system. Enriched environment causes neurochemical and morphological changes in the brain. Thickness of the cerebral cortex, density of dendritic spines, expression of different neurotrophic factors and their receptors increase, and many brain areas show a higher level of neurogenesis.

However, not only morphologic changes occur, also functional differences can be found. Enriched environment causes improved performance in learning tests, social behavior and exploration, and show decreased anxiety. There is evidence that enriched environment can counteract the negative effects of different neuronal injuries. Enrichment in the adolescence can ameliorate the effects of prenatal stress. However, most studies examining the effects of enriched environment were done on adult rats, where enrichment was induced only in postweaning period or in adulthood. Very little is known about the early, postnatal effects of enriched environment.

In our study we found that altogether enriched environment has no significant effect on the neurobehavioral development, in spite its proven enhancement of different growth factors. Decrease in body weight may be due to rat pups moving more in the expanded field while playing with each other or the toys. In the motor coordination tests, which require more advanced skills, enriched environmental animals showed a better performance than standard housed rats. In the open-field test at 3 weeks of age, enriched animals were more active, which was significant in habituation.

#### **4.6. Effects of enriched environment in MSG-induced toxic lesion**

Only low-dose MSG treatment was used in this experiment. This treatment was able to cause severe neurodegeneration in the arcuate nucleus and in the retina. This dose, however, causes only minor alterations in the neurological, motor coordination and open-

field tests. These changes were greatly ameliorated by the enriched housing. According to our data, it is suggested that enriched environment does not change the normal neurobehavioral development, but causes some kind of reserve capacity in the brain, which results in an increased resistance against different neuronal lesions, and better recovery following injury.

## **Summary of new findings**

We managed to set up a standard testing method from different tests described in the literature in order to investigate neurobehavioral development, motor coordination and exploratory behavior in newborn rats.

We described the early negative effects of monosodium-glutamate treatment, which causes significant delay on the neurobehavioral development. These effects could be prevented in most cases with environmental enrichment and (neurotrophic factor) PACAP treatment.

This short-term developmental delay was also found in the perinatal hypoxic/ischemic injury, and the alterations might have prognostic significance.

We have shown that environmental enrichment on its own does not influence general neurobehavioral development, but results in improved motor coordination skills in more complex tests.

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