NEUROPROTECTIVE EFFECTS OF ENVIRONMENTAL ENRICHMENT

PH.D. THESIS

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INTRODUCTION

ENVIRONMENTAL ENRICHMENT

Early life events are critical in the development of the central nervous system. Positive influences can support the inner potential of the developing brain, which can have protective effects even after long periods. Early postnatal period is one of the critical age windows, in addition to prenatal life and adolescence, that is open to plastic changes and to positive or negative environmental influences. The first description of enriched environment is dated back to 1947, when Hebb described that rats raised as pets performed better in problem-solving tasks. Rats kept in enriched conditions are less vulnerable to various toxic, ischemic and traumatic injuries. Various morphological and biochemical changes account for the improved functional performance observed in these animals.

MUSICAL ENVIRONMENTAL ENRICHMENT

Musical environmental enrichment is one of the sensory subtypes of enriched environment. The auditory environmental factors take effect through the auditory pathway and stimulate the central nervous system. The effect is not limited to the auditory cortex, but it results in a more complex activation of the brain.

SOCIAL ISOLATION

In contrast to the protective effects of enriched environment, impoverished environment has been demonstrated to have deleterious effects on brain structure and function and is known to worsen the outcome in several injuries. Impoverished environment can be mimicked by housing rats in social isolation.

We not only examined the environmental factors alone, but also as combination of different treatments. We examined the effects of both protective (pituitary adenylate cyclase activating polypeptide) and negative (monosodium-glutamate) treatments.

PITUITARY ADENYLATE CYCLASE ACTIVATING POLYPEPTIDE (PACAP)

Pituitary adenylate cyclase activating polypeptide (PACAP) is a pleiotropic and multifunctional neuropeptide widely distributed throughout the body. It is the member of VIP-secretin-glucagon peptide family. It has two biologically active isoforms: PACAP27 and PACAP38. PACAP is involved in the regulation of various physiological and pathophysiological processes. Numerous studies have shown that PACAP is involved in the development of the central nervous system and has neuroprotective effects.

Another aim of our study was to investigate whether environmental enrichment influences PACAP levels of different brain areas in rats.

MONOSODIUM-GLUTAMATE (MSG)

This agent is also known as umami or E621 food additive flavoring agent. MSG has toxic effects only if it reaches the central nervous system in high concentration, which happens only in the newborn rodent brain, when MSG can pass through the immature blood-brain barrier. Glutamate toxicity occurs through overexcitation of the N-methyl-D-aspartate (NMDA) receptors. This then leads to increased calcium ion influx and finally cell death. MSG toxicity in rodents is known to cause degeneration of the retina, optic nerve, arcuate nucleus and various parts of the cortex.

High dose subcutaneous MSG treatment leads to severe injuries in newborn rats.

ASPHYXIA

Hypoxic injuries in the perinatal period are still among the most feared perinatal events, for their long-term consequences. In spite of intensive neonatal care, perinatal asphyxia and hypoxia during delivery represent a major clinical problem in neonatology. Long-term consequences of perinatal hypoxic injuries (hypoxic-ischemic encephalopathy) cause a huge burden on the individual, the family and the society.

The development of newborn rats during the first three postnatal weeks follows a general pattern of reflex appearance and maturation of motor skills. Three main factors act together in this important period: environmental-, genetic factors and pharmacological effects.

Based on this in the early postnatal development not only the toxic and ischemic effects, but the social isolation or the environmental enrichment are also important.

AIMS

Our aims were

1. to investigate the effects of environmental enrichment on the neurobehavioral development and motor coordination of newborn rats
2. to examine the effects of environmental enrichment on a disturbed developmental animal model:
    a. neonatal toxic (monosodium-glutamate) model
    b. neonatal asphyxia model
3. to describe the effects of musical environmental enrichment on the development of newborn rats:
    a. normal developmental model
    b. delayed (monosodium-glutamate treated) developmental model
4. to examine the levels of PACAP27 and -38 in different parts of the brain under environmental enrichment
    a. in young rats
    b. in adult rats
5. to examine the levels of PACAP38 in the milk of guinea pigs under environmental enrichment
6. to investigate of retinal morphology and gender differences in:
    a. environmental enrichment model
    b. social isolation model

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

RAT MODEL

A local Wistar rat colony was used for our experiments. Animal housing, care and application of experimental procedures were in accordance with institutional guidelines under approved protocols (No: BA02/2000-15024/2011, University of Pécs following the European Community Council Directive). Animals of both sexes were cross-fostered immediately after birth, to minimize litter differences. Litter size was 8 ± 1 pups in all groups. Pups stayed with their mothers during the whole examination period and were weaned after 5 weeks of age. All experimental animals were kept in the same room, under the same illumination and other outside environmental conditions (12 h light-dark cycle, food and water ad libitum). All of the litters contained control and treated animals.

GUINEA PIG MODEL

The aim of this study was to measure the PACAP38 levels of the milk of guinea pigs. Each group contained 3 female and 1 male guinea pigs. In average females had 2±1 pups after the delivery, but we only examined the milk of the mothers.

TREATMENTS

ENVIRONMENTAL ENRICHMENT

Rat litters were placed in one of the following cages immediately after birth. Regular (control, impoverished environment) cage with 43 cm × 30 cm × 20 cm dimensions (n = 30). Control rats were only shortly handled, for the duration of the neurobehavioral testing. A second group of animals (n = 37) was placed in a large cage, the floor of which was 88 cm × 50 cm with 44 cm high walls (88 cm × 50 cm × 44 cm). For complex environmental enrichment we exposed the rats to intensive multisensory stimulation. The cage contained different toys, objects, running tunnels and rotating rods with various shapes, materials (wood, plastic, metal), colors and shades. Half of the objects were changed daily, while the other half were left unchanged to avoid a stressful change of the environment.

In our first experiment when we examined the PACAP27 and -38 levels in the brain, we did two series. In the first, we put the rat litters after birth to (1) a regular cage for 3 weeks (n=4), or to (2) complex environmental enrichment for 3 weeks (n=5). The second part of this experiment was 6 months long. We examined three groups: (1) animals in standard environment (n=5), (2) complex environmental enrichment for 3 weeks, but then in the rest of the time they lived under regular circumstances (n=4), (3) rats grew up under standard circumstances, but at the age of 6 months we put them into environmental enrichment for 3 weeks (n=5).

In our guinea pig experiments we used two types of environments. The environmentally enriched animals (n=25 milk sample from this group) lived in a two times bigger cage then the control guinea pigs (n=22 milk sample from this group). The enriched animals had toys, objects, running tunnels and rotating rods with various shapes, materials (wood, plastic, metal), colors and shades. Half of the objects were changed daily (similarly to the rat model), while the other half were left unchanged to avoid a stressful change of the environment.

MUSICAL ENVIRONMENTAL ENRICHMENT

In the musical enrichment investigation we had to separate the three animal groups to three different rooms. In the first room, the rats lived in standard size cages in a silent environment. In the second room the animals lived in similar cages, but they listened to Mozart sonatas (Mozart: 17. piano, symph 24; Concertos; The great composers I-II; The horn concertos). The cages were positioned to the same distance from the music source. Rats are active at night, so we played the music to them from 6:00 PM till 6:00 AM. The average loudness of the music was set to 60 dB. In the third room we put the rats under similar circumstances like in room 2, but the type of music was different. There we played heavy metal music from different bands with 60 dB loudness in average.

We divided the animals to subgroups. Some of them received physiologic saline treatment, some others were treated by 4 mg/g MSG. In our experiment we had n=17 physiological saline treated rat in the silent room, n=16 MSG treated one in the classical music room and n=9 animal in the heavy metal room.

MONOSODIUM-GLUTAMATE

MSG was dissolved in 100 µL physiological saline and it was given by subcutaneous injection on postnatal days 1, 5 and 9, according to previous descriptions. For our retinal studies we used 2 mg/g bodyweight MSG, for neurobehavioral developmental investigations we applied 4 mg/g bodyweight MSG. This higher dose would injure the retina too severe that would not be able

to be counteracted by environmental factors. For the investigation of neurobehavioral development we had to use the higher 4 mg/g bodyweight MSG, because the lower dose had no significant effects on the development. Control animals received the same volume of physiological saline on the same postnatal days.

In this study we had n=30 rats under standard circumstances. We examined n=37 animals from complex environmental enrichment supplemented with toys in a bigger cage. Half of the pups were treated by physiological saline (control cage: n=16, enriched environmental cage: n=17 rats), the other half received subcutaneous MSG treatment (control cage: n=14, enriched environmental cage: n=20 animals).

PERMANENT BILATERAL COMMON CAROTID ARTERY OCCLUSION

Retinal ischemia was induced by permanent bilateral common carotid artery occlusion (BCCAO), a model of chronic cerebral hypoperfusion. Under isoflurane anesthesia, both common carotid arteries were exposed through a midline cervical incision. Arteries were gently separated from the surrounding connective tissue and vagus nerve, then, they were ligated with a 4.0 filament. The ligations were permanent, reperfusion did not occur. Wounds were closed using surgical stitches.

In this experiment we examined a total number of 49 adult Wistar rats (250-300 grams). (1) A group of animals underwent anesthesia and all steps of the surgical procedure, except ligation of the carotid artery (sham-operated controls) (n=7). The operated animals were kept under different circumstances for 2 weeks: (2) standard size cage (n=7 female, n=7 male), (3) complex enriched environment (n=7 female, n=7 male), and (4) standard size cage with socially isolated rats (1 rat/cage) (n=7 female, n=7 male).

ASPHYXIA

Female Wistar rats were inspected for vaginal sperm plug and then the pregnant animals were observed on gestational day 22. As soon as delivery started, mothers were sacrificed by neck dislocation under anesthesia. Pups were delivered by caesarian section from the uterine horns and stimulated to breathe. Control cesarean-delivered pups were delivered immediately, while other pups were delivered following a 15-min asphyctic period, kept at constant temperature (37 °C). Asphyxia was achieved by leaving the pups in the uterus for 15 min without breathing. Surviving rats were given to surrogate mothers after a survival period of 40 min at 37 °C. Neurobehavioral maturation and development of motor coordination were tested based on earlier descriptions. Only the surviving animals are included in the final evaluation. During the acute post-asphyctic phase, more than 50% of pups died in the asphyxia group, compared to the 10% of

the control group. Also, during the observation period, 4 pups died in the asphyxia group, while none in the control group. Gender was evenly distributed in the groups (55% males, 45% females).

We processed only those animals data who survived the whole experimental period (n=25 non-asphyctic animals, n=21 asphyctic rats). In our experiment we divided both groups to two subgroups: standard conditions (n=10 non-asphyctic rats, n=9 asphyctic animals), and environmental enrichment (n=15 non-asphyctic rats, n=12 asphyctic animals).

EXAMINATION OF NEUROBEHAVIORAL DEVELOPMENT

Examinations of neurobehavioral development were started on the first postnatal day (PND) and were carried out daily between 12 and 15 p.m. until PND 21. Neurobehavioral testing was performed in a blinded fashion, the investigator was not aware of the nature of handling. Neurobehavioral maturation and development of motor coordination were tested based on earlier descriptions.

The development of the pups was followed by many different examinations to get a wide view about the neurobehavioral development of the rats. Bodyweight was measured daily until 3 weeks of age, then twice a week until 5 weeks of age. Physical development was followed by inspections of maturation of physical characteristics such as eye opening, incisor eruption and ear unfolding. Pups were also tested for the following neurological signs and reflexes.

RIGHTING REFLEXES

(a) Surface righting reflex: rats were placed in supine position and the time was recorded in seconds to turn over to prone position and place all four paws in contact with the surface.

(b) Air righting: subjects were dropped head down onto a bed of shavings from a height of 50 cm, and the day of first landing on four feet was recorded.

NEGATIVE GEOTAXIS

Animals were placed head down on an inclined grid (45°) of 30 cm. The hind limbs of the pups were placed in the middle of the grid. The day they began to turn around and climb up the board with their forelimbs reaching the upper rim was observed. In cases where the animal did not turn around and climb up the board within the observed 30 s, the test was considered negative. From the day of the appearance of the negative geotaxis, the time in seconds to reach the upper end of the board was recorded daily.

CROSSED EXTENSOR REFLEX

The left rear paw was pinched and the animal was observed for the extension of the right leg. The day of disappearance of the crossed extensor reflex in its pure form, when it was replaced by a more complex behavioral response, was noted.

EAR TWITCH REFLEX

The ear was gently touched with a cotton swab and the first day of the ear twitch reflex was recorded.

EYELID REFLEX

The eyelid was gently touched with a cotton swab and the first day of the contraction of the eyelid was recorded.

LIMB PLACING REFLEX

The back of the forepaw and hind paw 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.

LIMB GRASP REFLEX

The fore- and hind limbs were touched with a thin rod, the first day of grasping onto the rod was recorded.

GAIT

Animals were placed in the center of a white circle of 13 cm. The day the pup was able to leave the circle with both forelimbs was recorded. In cases the animal did not leave the circle for 30 s, the test was considered to be negative. From the day of the appearance, the time in seconds to move off the circle was recorded daily.

AUDITORY STARTLE REFLEX

The first day of the startle response to a clapping sound was observed.

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

GRID-WALKING AND FOOTFAULT TEST

Rats were placed on a stainless steel grid floor (20 × 40 cm2 with a mesh size of 4 cm2) elevated 1 m above the floor. For a 1-min observation period, the total number of steps was counted (calculated by total right and left forelimb steps). The number of foot-fault, when the animals misplaced a forelimb or hind limb that it fell through the grid, was also recorded during a 1-min period (mistakes of all four limbs were counted separately during the examination).

ROPE SUSPENSION TEST

Animals were suspended by both their forepaws on a horizontal, 4-mm-diameter nylon rope, stretched horizontally 40 cm over a foam pad. The time the animals could hang on the rope was recorded (maximum 30 s).

ROTA-ROD TEST

Animals were tested on a commercially available treadmill with diameter of 14 cm for small animals, 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 (maximum 2 min).

WALKING INITIATION TEST

Rats were placed on a horizontal surface in the center of two concentric circles with diameters of 10 and 45 cm (inner and outer circles). The time taken to move off the circles was recorded.

INCLINED BOARD TEST

Rats 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 s was recorded [graded from 1 (lowest angle) to 5 (highest angle)].

MILK SAMPLE COLLECTION (MILKING)

We made this experiment in the guinea pig model. We took the first sample 2 days after the delivery, to avoid colostrum sampling. From the third day every morning between 8:00-8:30 AM we removed the mother from its pups for 30 minutes. We applied mechanical pressure for the milking, we took samples from both mammary glands. The daily milk samples were 1 ml/animal in average that we collected to Eppendorf tubes. We could get milk until the 13th day after the delivery in average.

HISTOLOGICAL EXAMINATION

Two weeks after the BCCAO, rats were sacrificed under isoflurane anesthesia. The eyes were immediately dissected in ice-cold phosphate buffered saline and fixed in 4% paraformaldehyde dissolved in 0.1 M phosphate buffer (Sigma, Budapest, Hungary). Tissues were embedded in Durcupan ACM resin (Sigma, Budapest, Hungary), cut at 2 μm and stained with toluidine blue (Sigma, Budapest, Hungary). Sections were mounted in DPX Mountant for histology (Sigma, Budapest, Hungary) and examined in a Nikon Eclipse 80i microscope. Photographs were taken with a digital CCD type camera using the Spot program, from central retinal areas of nearly the same eccentricities (1–2 mm from the optic disc). Files were then further processed with the Adobe Photoshop 7.0 program. Samples for measurements were derived from at least six tissue blocks per animal (n = 4–5 measurements from one tissue block). The following parameters were measured: (i) cross-section of the retina from the outer limiting membrane to the inner limiting membrane (OLM-ILM); (ii) the width of the outer and inner nuclear and outer and inner plexiform layers (ONL, INL, OPL, IPL, respectively); (iii) the number of cells/100 μm section length in the ganglion cell layer (GCL) and the number of cells/1,000 μm2 in the outer nuclear layer (ONL). Due to the lack of statistical or morphological difference between genders in the sham and BCCAO-standard groups, results are not shown separately for male and female animals in these groups.

RADIOIMMUNOASSAY (RIA)

We decapitated the rats under isoflurane anesthesia, removed the brains and processed the different areas (hypothalamus, brain stem, diencephalon, cerebellum, temporal-, occipital-, frontal- and parietal lobes) separately. Samples were homogenized and centrifuged (12000 rpm, 4 ^{o}C, 30 minutes). We used the supernatant for the PACAP27 és PACAP38 RIA examinations. PACAP38 and PACAP27 RIA procedures: "88111-3" antiserum was raised against a conjugate of Cys23-PACAP24-38 and bovine thyroglobulin coupled by carbodiimide in rabbit. For PACAP27 measurement "88123-3" antiserum was raised against a conjugate of PACAP27 and bovine thyroglobulin coupled by carbodiimide in rabbit. Ovine PACAP24-38 C-terminal fragment and PACAP27 were iodinated (125I, Institute of Isotopes, Budapest, Hungary) by iodogen and the reaction mixtures were separated on a reverse-phase HPLC column. The mono-iodinated peptides (125I-PACAP 24-38 and 125I–PACAP27) served later on as RIA tracers. Ovine PACAP38 and PACAP27 (Sigma) were used as RIA standards in a range of 0-1000fmol/ml. Assays were prepared in 1ml phosphate buffer (0.05mol/l, pH: 7.4) containing 0.1mol/l NaCl, 0.05% NaN3, 0.25% bovine serum albumin (Sigma). 100µl antiserums ("88111-3": working dilution: 1:10000 and "88123-3": working dilution: 1:45000), 100µl RIA tracers (5000cpm/tube) and 100µl standards or unknown samples were measured into polypropylene tubes with assay buffer. After 48-72h incubation at 4ºC, the antibody-bound peptides were separated from the free ones by addition of 100µl separation solution (10g charcoal, 1g dextran, 0.2g commercial fat-free milk powder in 100ml distilled water). Following centrifugation (3000rpm, 20min, 4ºC) the tubes were gently decanted and the radioactivity of the precipitates were measured in a gamma counter. PACAP38 and PACAP27 concentrations of the samples were read from calibration curves. PACAP levels were measured as fmol/mg PACAP in the tissue samples. In order to better demonstrate the differences, these values are expressed as relative changes (percentage PACAP fmol/mg), taking the control group results as 100%.

STATISTICAL ANALYSIS

Data are expressed as mean ± standard error of the mean (SEM). The statistical analysis of the appearance of physical signs was performed by two-way analysis of variance (ANOVA) followed by Neuman-Keul`s posthoc analysis. To compare the daily data we used Student's t-test after homogeneity check. The daily changes of negative geotaxis, righting reflexes

and gait time were evaluated by analysis of variance, the reflex time of the groups were also examined by Newman-Keul's test after the analysis of variance.

For the evaluation of the retina slides data from morphometric analysis results were presented as mean ± SEM. Statistical comparisons were made using the ANOVA test followed by Tukey-B's post hoc analysis. Results were considered significant when $p<0.05$. Levels of significance are indicated as follows: * $0.01<p<0.05$; ** $0.001<p<0.01$ and *** $p<0.001$.

RESULTS

EFFECTS OF ENRICHED ENVIRONMENT ON THE NEONATAL MONOSODIUM-GLUTAMATE TREATMENT

SOMATIC DEVELOPMENT

BODY WEIGHT GAIN

There was no difference in the body weight between saline and MSG-treated pups kept in enriched environment. Enriched environment alone did not lead to increased weight gain in saline-treated animals. These data clearly show that enriched environment can prevent the reduced weight gain in a neonatal excitotoxic lesion.

PHYSICAL PARAMETERS AND REFLEX DEVELOPMENT

There was no significant difference between control saline and control MSG-treated groups in the appearance of physical parameters such as eye opening, incisor eruption and ear unfolding. Among the other neurological reflexes, slight, but not significant delays were observed in the day of the appearance in almost all reflexes in the MSG-treated control animals. In contrast, most signs appeared earlier in animals kept in enriched environment, but differences were not significant.

MOTOR COORDINATION

Among the motor coordination tests, one of the most reliable indicators in our previous studies has been the grid walking/foot fault test. On counting the number of steps, we found that the enriched rats moved more than the control group on the 3rd week, while there was no

difference on the 4th week. These data show that the development of motor coordination required for walking on a grid is delayed in MSG-treated animals, but this delay can be prevented by environmental enrichment. In rope suspension and inclined board tests we found that pups in enriched environment performed slightly better than control animals, but differences were not significant. Walk initiation test from inner circle showed that control MSG-treated rats took markedly longer times to move out of the inner circle on both weeks 3 and 5, this also refers to developmental impairment.

EFFECTS OF ENVIRONMENTAL ENRICHMENT ON ASPHYXIA-INDUCED NEUROBEHAVIORAL DEVELOPMENT

Both acute mortality and death during the observation period were high among the asphyctic pups in contrast to the control group.

SOMATIC DEVELOPMENT

BODY WEIGHT GAIN

Asphyctic pups had significantly less weight gain than control groups, especially during the second week of the observation period. However, there was no difference in the body weight of asphyctic pups growing up in enriched environment after the first week. Enriched environment alone did not lead to increased weight gain in control animals. These data indicate that enriched environment can prevent the reduced weight gain in a neonatal asphyctic lesion.

PHYSICAL PARAMETERS AND REFLEX DEVELOPMENT

There was no significant difference in the day of the appearance of eye opening, ear unfolding and incisor eruption between control pups kept in small cages or in environmental enrichment. However, there was significant delay in rats undergoing perinatal asphyxia. Delays of 1–2.5 days in asphyctic pups compared to the respective control groups could be observed.

Environmental enrichment could significantly diminish the developmental delay in the crossed extensor reflex. Limb placing testing showed that asphyxia caused a significant delay in the appearance of both forelimb and hind limb placing. Environmental enrichment led to an earlier appearance of forelimb and hind limb placing reflexes and could counteract the negative effects of the asphyctic lesion. Similar results were obtained in the grasp reflexes. Both forelimb and hind

limb grasp reflexes appeared significantly later in pups undergoing asphyxia, while environmental enrichment could decrease this delay. This ameliorating effect by enrichment was significant in case of forelimb grasp.

MOTOR COORDINATION

In the foot-fault test, as expected, asphyctic pups made significantly more mistakes on the elevated grid. Enriched control rats made fewer mistakes than small cage control pups, and a similar tendency could be observed between small cage asphyctic and enriched asphyctic groups.

EFFECTS OF MUSICAL ENRICHMENT ON THE NEONATAL MONOSODIUM-GLUTAMATE TREATMENT

SOMATIC DEVELOPMENT

Musical environmental enrichment had no effect on the physiologic saline treated animals compared to the control group lived under silent circumstances.

BODY WEIGHT GAIN

During the first two postnatal weeks the 4mg/g bodyweight MSG treatment had significant effects on the weight gain of both the classic and the heavy-metal music group. MSG treated animals had significant lower weight than the control physiologic saline treated animals. We did not find any difference between the groups that listened to different types of music. Based on this finding two different types of music had no different effects.

PHYSICAL PARAMETERS AND REFLEX DEVELOPMENT

The eye opening appeared significant later among the MSG treated rats placed to the classical music room than the saline treated control rats and MSG treated heavy-metal animals. The appearance of ear unfolding showed similar results: it appeared significantly later in the MSG treated classical group than the saline treated animals in the silent room. In this sign we could not find significant difference between the heavy-metal and classical MSG treated rats.

The forelimb grasp reflex showed similar tendency as we found in the eye opening and ear unfolding signs. When we examined the air righting reflex of the animals we found that the heavy-metal MSG treated rats showed it earlier than the other animal groups in our experiment. The auditory startle reflex also showed significant difference between the animals. The classical music MSG treated group showed auditory response significantly later than the physiologic saline treated ones that lived under silent circumstances or the MSG treated heavy-metal rats.

ENVIRONMENTAL ENRICHMENT DIFFERENTIALLY ALTERS PACAP LEVELS IN THE CNS OF YOUNG AND ADULT RATS

In our experiments we could reliably detect PACAP38 and PACAP27 by the sensitive and specific RIA method that was used in our earlier studies. The absolute level of PACAP38 was about 10 times higher than those of PACAP27. In order to compare all age groups under different conditions, we give our results in percentage of control levels.

PACAP LEVELS IN THE CNS – 3-WEEK OLD RATS

Our results from 3-week-old rats show that both PACAP27 and PACAP38 levels were significantly lower in the brainstem, cerebellum, hypothalamus and telencephalon of the animals that were kept under environmental enrichment conditions.

The different parts of the telencephalon were also measured separately and we found that both PACAP27 and -38 levels showed significantly lower levels in all areas of the telencephalon, such as the frontal, temporal, occipital and parietal lobes.

PACAP LEVELS IN THE CNS – ADULT RATS

In the second part of our investigation we measured PACAP-LI in 6-month-old animals after exposure to early postnatal or late (adulthood) environmental enrichment. In rats exposed to a 3-week long postnatal enrichment and sacrificed later in adulthood we found that in most brain areas the PACAP levels did not markedly differ between controls and enriched groups. As an exception, PACAP27-LI showed higher level in the brainstem and lower level in the diencephalon compared to the control group. PACAP38-LI showed lower levels in the telencephalon. In contrast, a 3-week long environmental enrichment in adulthood preceding sacrifice markedly increased the levels of both peptides in most brain areas. This was significant in the brainstem, cerebellum, diencephalon and telencephalon in case of PACAP27, and in telencephalon in case of PACAP38.

EFFECTS OF ENVIRONMENTAL ENRICHMENT ON THE PACAP LEVELS OF THE MILK IN GUINEA PIGS

It was not known earlier if environmental enrichment can influence the PACAP concentration in the milk. We examined this in a guinea pig model.

In the guinea pig milk samples we found significant lower level of PACAP 38 than in the cow milk. The PACAP concentration altered with the environment: under complex environmental enrichment we found significant lower PACAP38 levels in the guinea pig milk samples.

GENDER-DEPENDENT EFFECTS OF ENRICHED ENVIRONMENT AND SOCIAL ISOLATION IN ISCHEMIC RETINAL LESION IN ADULT RATS

EFFECTS OF BILATERAL COMMON CAROTID ARTERY OCCLUSION ON THE REGENERATION UNDER REGULAR CIRCUMSTANCES

Retinas of sham-operated animals showed normal appearance. All retinal layers were visible, such as pigment epithelial layer, photoreceptor layer (PL), outer and inner plexiform layers (OPL and IPL), outer and inner nuclear layers (ONL and INL) and ganglion cell layer (GCL). There was no difference in retinal structure between male and female animals in this group. Ligation of the carotid arteries for 2 weeks led to a severe degeneration in the retinal structure. Individual retinal layers were significantly reduced in thickness, most marked reduction was observed in the INL and IPL. As a consequence the distance between outer and inner limiting membranes (OLM and ILM), corresponding to the entire retinal thickness, was significantly decreased. Several structural abnormalities were also observed. In the nuclear layers (ONL and INL), numerous empty spaces were found between the intact neuronal perikarya. Numerous cells in the GCL also displayed severe degeneration. This was well reflected in the reduced number of cells in the ONL and GCL. No differences could be observed between the standard housed male and female BCCAO-operated groups.

EFFECTS OF BILATERAL COMMON CAROTID ARTERY OCCLUSION ON THE REGENERATION UNDER ENVIRONMENTAL ENRICHMENT

Retinas from rats kept under enriched conditions for 2 weeks following BCCAO showed a markedly better preserved retinal structure than retinas from the standard ischemic group. This ameliorative effect of enriched environment could be observed in both males and females. Inner and outer nuclear retinal layers were less degenerated in both genders than in the standard ischemic group, resulting in a significantly greater OLM-ILM distance. Enriched environment did not have a significant effect on the thickness of the plexiform layers. In contrast to the standard ischemic groups, the effect of enriched environment resulted to be gender-dependent. The structure of the nuclear layers was not so well preserved in female animals. There

was a tendency toward a smaller thickness in female retinas, reaching statistical significance in the OPL and INL. The empty areas could still be observed in the INL of female retinas, while they almost entirely disappeared in males. The number of cells in the GCL in 100μm retinal length was higher in the enriched environment BCCAO male group compared to the standard cage BCCAO retinas.

EFFECTS OF BILATERAL COMMON CAROTID ARTERY OCCLUSION ON THE REGENERATION UNDER SOCIAL ISOLATION

No marked differences in the retinal structure could be observed between the socially isolated male and standard housed groups after BCCAO. Retinal tissue from female rats with BCCAO housed in social isolation showed more severe degeneration compared to the male rats kept under the same conditions. The morphometric analysis showed significant differences between the two groups in the distance of OLM-ILM and ONL. The OPL thickness was significantly decreased in both of social isolated male and female groups compared to the standard housed BCCAO group. The ONL and INL seemed intermingled with empty cell body-shaped holes in both layers. The most severe degeneration was observed in the ONL, INL and IPL. Quantitative analysis demonstrated significant differences in the GCL and ONL between the standard ischemic and social isolated groups. However, no statistical difference was observed between males and females in this parameter.

- Complex environmental enrichment has a protective effect against retarded body weight gain induced by MSG.
- Enriched environment has no effect on most of the reflexes in normal development, but MSG induced gait reflex performance and placing reflex appearance was in enriched environment.
- In motor coordination tasks the MSG treated enriched animals have significant better performance than the MSG treated control rats.
- In asphyctic lesion the enriched environmental group has significantly higher body weight gain than the asphyctic control animals.
- Asphyctic animals growing up in enriched environment show significantly better performance in crossed extensor reflex, grasp reflexes and placing reflexes than the control asphyctic group, and the eye opening happened earlier as well.
- Musical environmental enrichment affects on the neurobehavioral development of the animals even if it is just in a mild level. Different types of music have slightly different effects.
- In the central nervous system of 3-week old animals PACAP27 and -38 levels are significant decreased after environmental enrichment, but in 6-month old rats following late environmental enrichment the PACAP levels are increased.
- PACAP38 level is significantly lower in milk samples of guinea pigs in environmental enrichment.
- Enriched environment is protective in case of ischemic retinal lesion.
- Social isolation has negative effects on ischemic retinal injury.
- Environment has gender dependent effects. Enriched environment has less positive effects in females and social isolation makes them more vulnerable in the ischemic retina model.

Environmental factors have major effects on the central nervous system, so conscious application of it in the field of human medicine would be beneficial. Our prospects that results revealed in the basic science can be helpful to make enriched environment an additive way of treatment and enhance the rehabilitation processes of neurological diseases.

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