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Investigation of anxiolytic-like effects of distinct stressors and their co-morbidity with drug abuse in experimental animal models

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Ph. D. thesis

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INTRODUCTION

The physiological response to aversive stimuli – the stress response – becomes a pathological risk factor when it is long-lasting, frequently repeated or excessively strong. Continuous stress exposure or excessively strong stressors are relatively scarce, and occur only in specific situations. In contrast, repeated exposures to stressors are part of everyday life, the effects of which can accumulate and lead to various psychopathologies including anxiety. Given the strong association between stress responses and anxiety, one can hypothesize that the complexities of the former are reflected by a similarly complex stress-anxiety relationship; i.e. the similarity between the stressful situation and the testing environment should have an impact on stress-induced anxiety. In the present dissertation, one of our aims was to investigate the content-dependence of different stressors [a chronic non-social (immobilisation), a chronic social (aggressive encounter) and an acute traumatic stressor (electrical footshock)] via their anxiolytic-like effects in animal models.

Post-traumatic stress disorders (PTSD) is a severe anxiety disorder that can develop after exposure to any event that results in psychological trauma. This event may involve the threat of death to oneself or to someone else, or to one's own or someone else's physical, sexual, or psychological integrity, overwhelming the individual's ability to cope. If going along the path of the former paragraph that is the strong correlation between the formation of anxiety and the homologous stressful and challenging situation, PTSD would rarely be developed. Even if one experiences a trauma once, it is very unlikely to be again in a similar situation. Even though, incidental effects of trauma can be developed in heterogeneous conditions, which is indicated by i.e. its comorbidity with drug abuse. This comorbidity may arise from self-healing behaviour of traumatized patient. Furthermore, it should also be noted that the neuronal systems underlying the development of addiction and PTSD are highly overlapped, so when one is appeared, the other one might be easier evolved. Thus, our second aim was to investigate the effect of traumatic stress on two important parameters of addictive behaviour, the carving and tolerance.

AIMS

Various aspects of repeated stress exposure are intensively studied, including the relationship between the effects of subsequent stressors. However, manifestation of anxiety due to a specific stressor, and the similarity between the stress and the challenge situation has not been examined. Hence, the following questions were applied in the course of the study.

1. *Can the anxiolytic reaction be context-specific?*

   Animals were exposed to social and non-social stressors and tested in different challenge situations. Changes in HPA axis were also monitored.

2. *Is the footshock stress suited for modelling PTSD in our laboratory?*

   Subjects received foot-shock stress one month before the conditioned fear test.

3. *What are the effects of stress caused by electric shock on the HPA axis?*

   Baseline and novelty stress-ensuing corticosterone level was measured in different time points after footshock.

According to human data, the co-morbidity of PTSD with drug use disorders is higher than with other anxiety disorder. However, it is not clarified, where and how the PTSD influences the symptoms of addiction.

4. *How can one of the most determinative symptoms of addiction, the craving be influenced by PTSD?*

   In this experiment, drug-seeking behaviour of shocked and non-shocked rats was monitored on morphine-conditioned place preference test.

5. *How can the other relevant symptom of addiction, the tolerance be modulated by PTSD?*

   The physiological reaction to the regular, fixed dose of morphine was investigated with biotelemetry. The cease of the effect was taken as a sign of the tolerance.

METHODS

Animals

In all experiments adult male Wistar rats (350-400g, Toxicoop, Budapest, Hungary) were used. In resident-intruder tests, intruder rats weighed 250-300g. In psychosocial stress, stimulus rats weighed 450-500g. Standard laboratory food and tap water were available *ad libitum*. Experiments were always carried out in the first 3 hours of the dark (active) phase of rats. Behaviour of rats was video recorded using a light sensitive camera and dim red illumination. Experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and were reviewed and approved by the Animal Welfare Committee of the Institute of Experimental Medicine.

Types of stressors

Psychosocial stress

Psychosocial stress was administered in the home-cage of residents. The opponents were separated from residents by a transparent, perforated Plexiglas partition. This allowed sensory but not physical contacts between opponents. On each of the subsequent days, subjects were moved to the home-cage of a different resident, and the partition was removed.

Restraint stress

The animals were placed into transparent plastic tubes (5–6 cm inner diameter) having a 4 cm long conical head part ending with a large breathing-hole (2 cm inner diameter). This procedure minimized the space around the animal, prevented turning, and provided strong stress without being harmful.

Footshocks

Rats were transferred to a separate quiet room, where they received footshocks *via* gridded floor of a Plexiglas cage. Light intensity in cage was approximately 400 lx. Two shocks trains were administered per minute for 5 min, i.e. each rat received 10

shocks. Each shock train was 1s-long and consisted of 0.01 s shocks separated by 0.02s-long breaks. Current potential and intensity were 100 V and 3 mA, respectively. Control rats were placed into a similar box for 5 min, but shocks were not delivered.

Novelty stress

Animals were placed into an unfamiliar context for 30 min. The apparatus was a squared, gray-coloured wooden area.

Behavioural testing

Elevated plus-maze test

The elevated plus-maze is the most widely used and accepted test for monitoring anxiety in medical research, assessing the animal's behaviour during novelty-related stress. The apparatus was made of wood and consisted of two opposite open arms and two enclosed arms, elevated to a height of 80 cm above the floor. Animals were placed in the junction area of the four arms (central zone) and were allowed to explore the maze for 5 min. Their behaviour was video recorded using an overhead camera. On the elevated plus-maze, time spent in the open arms represents anxiety, whereas number of entries in the closed arms is characteristic for locomotion.

Open-field test

The open-field test is another conventional test for the measurement of anxiety and locomotion. The apparatus was a round wooden area surrounded by a metal wall. Animals were placed in the open-field and their behaviour was recorded for 10 min by an overhead camera. The time spent in the central area of the apparatus represented anxiety, whereas the distance moved during testing measured locomotion.

Social interaction test

This test of anxiety is based on the assessment of social contacts between animals. During the10 minutes long testing period, unfamiliar animals exposed previously to the same treatment were faced. The following behavioural parameters were analysed: social sniffing, offensive behaviour, biting attacks, dominant posture, defensive behaviour, submissive posture, resting, exploration, self-grooming. Aggressive

behaviour is usually characterised by the sum of all agonistic behaviours. The increase in the time spent with social interaction indicates an anxiolytic effect.

Resident-intruder test

Resident rats were isolated in big territorial cages. Three days later, an intruder male of smaller size was placed in the resident's cage and their behaviour was video recorded. The behavioural variables used in this test are similar to the variables assessed in the social interaction test.

Fear conditioning test

On the first day of the experiment, rats were either shocked or exposed to the shocking cage as shown above. After a certain time, rats were returned to the shock-box for 5 min. Shocks were not delivered and behaviour was video recorded. The following behavioural parameters were analysed: resting, grooming, exploration, escape jump and freezing. The duration of freezing – absence of all observable movements except breathing– was used as an index of conditioned fear.

Conditioned place preference test

The conditioned place preference paradigm provides information on the rewarding properties of drugs, which is at the core of substance use disorders. On the first day of the experiment, rats were either shocked or exposed to the shocking cage as shown above. 23 days later, the conditioned place preference paradigm was started. Note that the first treatments were administered on the 27th day. Four groups were studied: non-shocked rats conditioned with saline, shocked rats conditioned with saline; non-shocked rats conditioned with morphine, and shocked rats conditioned with morphine. The apparatus consisted of three compartments: a black rectangular neutral ('central') area and two test compartments. The central area was connected to the test compartments by doors. The test compartments were differently marked but same sized. The experiment included a habituation phase, a conditioning phase, and a test phase. Two habituation trials were run on the 23rd and on 7 the 24th post-shock day. Rats were placed into the central area, and were allowed to freely explore the apparatus for 10 min. The conditioning phase started on the 27th post-shocking day, and consisted of 6 trials that were performed on consecutive days; 3 morphine and 3

saline conditioning trials were run on alternate days. Morphine injections were consistently associated with the compartment that was less preferred during the 2nd habituation (baseline) trial. The more preferred compartment was associated with saline. Both arms were associated with saline in saline-conditioned controls. The test phase consisted of six daily test trials that were run on consecutive days or on randomized in other experiment. During test trials, rats were placed in the central area and were allowed to explore the apparatus freely for 10 min. Time spent in the morphine-associated compartment served as the index of conditioned place preference.

Blood sampling, hormone measurements and adrenalectomy

Blood was sampled by tail incision and plasma corticosterone was measured by radioimmunoassay. 125I-labelled corticosterone-carboximethyloxime-tyrosine-methyl ester derivative was used as tracer. The interference with plasma transcortin was eliminated by inactivating transcortin at low pH. The sensitivity of the assay was 1 pmol/ml. Intra- and inter-assay coefficient of variation was 9.6 and 16.6%, respectively. Adrenalectomy was performed *post mortem* by the dorsal approach.

Biotelemetric measurements

Body temperature of rats was monitored by our biotelemetric system. Telemetric e-mitters (Mini Mitter Company, Bend, OR, USA) were implanted into the abdominal cavity. Receivers were placed under the rats' cages and the software (VitalView Data Aquisition System, Mini Mitter Company, USA) registered each animal's body temperature data minute by minute.

Statistics

For statistical analysis, the Statistica software 6.0 (StatSoft, Inc.; Tulsa, OK, USA) as used. Elevated plus-maze, open field, social interaction test, resident-intruder test and fear conditioning data were analysed by nonparametric Kruskal-Wallis or by ANOVA (one-way, factorial and repeated measures analysis of variance). Pair wise comparisons were carried out using *post hoc* Mann-Whitney U tests. Significance levels were corrected by Holm's method in case of multiple comparisons.

RESULTS AND DISCUSSION

Context specificity of different types of stressors

Context specificity of social and non-social

The aim was to evaluate whether the anxiety-increasing effects of a chronic social, a chronic non-social and a traumatic stressor generalize to heterotypic or homotypic stressful situations. Chronic stressors caused chronic stress-reaction confirmed with adrenal hypertrophy. However, the acute stress-reaction (elevated plasma corticosterone level) was manifested only in homotypic situations. Trauma evoked unconventionally long acute stress-response (longer than 24 hours); however, it resulted in only an acutely increased corticosterone level. In this case, the presence of chronic stress reaction was not manifested in adrenal weight. Traumatic experience did not affect anxiety in elevated plus-maze and social interaction tests, but markedly increased in conditioned fear test (homotypic situation).

Conditioned fear test

Shocked rats showed robust freezing behaviour when re-exposed to the shock-associated environment 28 days after shocks. Shock exposure induced long-lasting conditioned fear, a response which is frequently used to model PTSD in laboratory rodents. Although the conditioned fear response is usually studied shortly after shock exposure, it can be detected up to 200 days after shocks. Consistently with these earlier observations, our rats robustly expressed freezing in the shock-associated environment on the 28th post-shock day.

Effect of foot-shocks on plasma corticosterone level before and after novelty stress

The effect of trauma on HPA-axis is controversial and several mismatching results were published in the last few years. To clarify this, we investigated the plasma corticosterone level in different time points. 24 hours after foot-shock, the baseline level of corticosterone was elevated caused by the acute stress. However, corticosterone levels of shocked subjects were not different from controls in the latter time points. These findings are similar to most of the published result, in which shock procedure did not chronically increase corticosterone level compare to non shocked rats. However, the acutely increased corticosterone level may play role in the long

term effect of trauma. In the first 24 hours, glucocorticoids – modulating gene expression – commence genetic changes, which may cause the latter (behavioural) effects.

Effect of foot-shocks on drug-seeking behaviour

Conditioned place preference testing in regular intervals

During habituation trial 1, compartment preference was stronger in shocked rats and these animals also showed a decreased number of compartment entries. These shock-induced changes in the magnitude of compartment preference disappeared in the second (baseline) habituation trial, when compartment entries also normalized.

The test phase of this experiment included 6 consecutive days. As compared to the baseline, non-shocked rats trained with morphine spent significantly more time in the morphine-associated compartment during only the first testing day, but not during the later trials. This rapid disappearance of conditioned place preference was somewhat surprising. There are reports, however, where conditioned place preference disappeared as quickly as in our experiment. A close look at the literature shows that extinction depends on a multitude of factors: the duration of conditioning sessions, the dose of the drug, the number of compartments in the testing apparatus, and contextual cues. Shocked rats, however, showed compartment preference in 1-5th testing days, but not in the 6^{th} day.

Conditioned place preference testing in irregular intervals

Shocked rats used in these experiments also showed a very strong preference towards one of the compartments during the first habituation trial. This strong compartment preference of the shocked group rapidly disappeared during the second habituation trial. Conditioned place preference was tested in randomized time-points (on 1st, 2nd, 3th, 5th, 8th, 12th and 19th days). During this test trial, morphine conditioning significantly increased the preference towards the drug-associated compartment. Extinction occurred already on the 8th day in the non-shocked group, but only on the 19th day in the shocked group.

We must note that non-shocked rats spent somewhat more time in the morphine-associated compartment than shocked rats; however, the difference from baseline was

statistically similar in the two groups in both cases. Thus, the magnitude of morphine-induced place preference was not affected by shocks, but the extinction of place preference was considerably delayed. The latter indicates that the shocked rats were more sensitive towards the rewarding properties of morphine.

Effect of foot-shock on morphine tolerance

In vehicle-injected controls, the injection per se rapidly and transiently increased body temperature. This response was slightly but not significantly reduced over time. Surprisingly, morphine significantly reduced the injection-induced increase in body temperature in the first 20 min. At later time-points, however, morphine robustly increased body temperature and this effect remained significant throughout in non-shocked rats. In contrast, shocked rats rapidly habituated to the hyperthermic effect of morphine. Although non-shocked and shocked rats were similar on the first day, hyperthermia rapidly disappeared in the latter group. In shocked rats, the increase in body temperature was only transient on the second day, whereas no significant effects were noticed on the following days. Virtually no increase was noticed on the last day of injections, when morphine still increased body temperature in the non-shocked group. Although the hyperthermic effect of morphine was significant on all days, a slight tolerance was noticed in non-shocked rats as well. Here we show that a single exposure to electric shocks markedly accelerates tolerance to the hyperthermic effect of morphine.

SUMMARY

Stress is part of our everyday life. The response to a stressor is normally to maintain the homeostatic balance. However, if the stress remains on for a long time or its intensity is too high, this balance may collapse and anxiety could be developed due to a stressful context. In the present dissertation, our aim was to investigate the context-dependence of different stressors [a chronic non-social (immobilisation), a chronic social (aggressive encounter) and an acute traumatic stressor (electrical footshock)] via their anxiolytic-like effects in animal models. Our results clearly demonstrated that the formation of anxiety in rats was highly depended on the test situations: whether it was homolog to the conditions of the stress situation or not. Chronic social stress caused anxiety only under social behavioural challenges but not in a non-social condition, and its chronic effect was shown by the increased adrenal weight. In contrast, a non-social stressor caused anxiety-like behaviour only in non-social environment. Traumatic experience (which is a special form of stressor in a way that it is mostly a single but extreme event) did not provoke abnormal behaviour in any experimental situations except in trauma-like condition. However, incidental effects of trauma can be developed in heterogeneous condition, which is indicated by its co-morbidity with drug abuse. Thus, we also investigated the effect of traumatic stress on carving and tolerance. Our data showed that shock exposure dramatically prolonged the drug-seeking behaviour of rats in morphine-induced place preference. Furthermore, using biotelemtrical approach we found that electrical footshock accelerated the tolerance to the effects of morphine.

Taken together, our results indicated that anxiety-related effects of distinct stressors were context-dependent, however, non-anxiolytic effects – i.e. drug seeking behaviour – could be increased, caused by traumatic stress exposure. The latter suggests that traumatic experience has a major impact on drug abuse in conjunction with the development of post-traumatic stress disorder-like behavioural dysfunctions.

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LIST OF PUBLICATIONS

Publications related to the dissertation:

1. **Barsy B**, Mikics É, Barsvári B, Haller J. (2011) The long-term impact of footshock stress on addiction-related behaviors in rats.
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