The underlying mechanisms of pathological aggression induced by early social disturbances and glucocorticoid hypofunction

Ph.D. theses

Máté Tóth

Semmelweis University
School of Ph.D. Studies
Szentágothai János Program in Neuroscience

Supervisor: Dr. József Haller scientific advisor, D.Sc.

Opponents: Dr. Júlia Timár associate professor, Ph.D.
Dr. György Lévay scientific advisor, Ph.D.

Final Exam Committee
Head: Dr. Béla Halász professor, D.Sc.
Members: Dr. György Nagy professor, D.Sc.
Dr. György Bárdos associate professor, Ph.D.

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INTRODUCTION

Our results in male rats show that glucocorticoid hypofunction has a crucial, causal role in the etiology of cold-blooded violence and its altered autonomic profile (hypoarousal, low stress reaction). In order to clarify the underlying mechanisms, we investigated the neural correlates of these behavioral changes by using immunohistochemical methods. Our results show that glucocorticoid hypofunction induces functional deficits rather than global changes in neuronal activation, except for the central amygdala and PVN. While normal forms of aggression were inhibited by serotonergic and prefrontal activity, abnormal forms were not influenced by the above mentioned inhibitory mechanisms or even facilitated (in the case of prefrontal activity). In addition, the increased activity of the central nucleus of amygdala was accompanied by medial amygdaloid facilitation of abnormal attacks. In the latter case, substance-P neurotransmisson plays a crucial role because substance-P receptor (NK1) antagonist can abolish the abnormal character of aggressive behavior. Our findings may provide new therapeutic implications for these agents, especially in violence associated disorders that are resistant to the available pharmacotherapies.

The second part of our investigations aimed at modeling the impulsive type of abnormal aggression and at clarifying its autonomic, endocrine and neural background. Prompted by earlier human findings, we studied the effects of early social deficits. Male rats reared in social isolation from weaning showed a disorganized and hypervigilant aggressive behavior which was accompanied by exacerbated stress responses as shown by increased glucocorticoid and autonomic activation during aggressive encounters. In parallel, we found a hyperactivation of the medial amygdala and certain prefrontal subregions, which were associated with disinhibited, violent attack patterns.

AIMS

I. Abnormal aggression induced by glucocorticoid hypofunction

1. To clarify changes in the serotonergic system following glucocorticoid hypofunction and its role in behavioral alterations.

Human violent psychopathologies associated with low cortisol levels (cold-blooded, hypoarousal-driven) show serotonergic deficits and resistance for - otherwise effective - serotonergic pharmacotherapies.

2. To elucidate involvement of prefrontal cortex in mediation of abnormal aggression.

Hypoarousal–driven violence is often accompanied by prefrontal deficits (volume reduction and hypoactivity) but the alterations in cortical network systems are unknown.

3. To investigate whether substance-P has a role in abnormal aggression.

Substance-P and its receptor (NK1) are implicated in facilitation of aggressive behavior in cats. Anatomical and pharmacological studies suggest its crucial role in affective disorders but there is no data about involvement in abnormal aggression.

II. Abnormal aggression induced by early social deprivation

1. To clarify the impact of early social deprivation on aggressive behavior in adulthood.

There is growing number of studies reporting behavioral consequences of social deprivation but its impact on aggressive behavior (especially on qualitative changes) is undiscovered.

2. To clarify the impact of early social deprivation on the activity of HPA-axis and sympathetic nervous system.

Early social experiences influence the activity of HPA-axis resulting in hyper- or hypoactivity depending on the type of the stressor. Alterations in stress reaction following social deprivation is poorly known.

3. To study neuronal activation patterns induced by aggressive encounter in socially deprived rats.

There is growing body of evidence showing marked changes in brain anatomy and neurochemistry following social deprivation but there are few data about functional alterations in brain circuits controlling social (e.g. aggressive) behavior.

METHODS

Animals

In all experiments adult male Wistar rats (Charles-River Laboratories) were used. Rats were weaned on the 19th-21st postnatal days and housed socially (4-6 rats per cage) or individually (socially deprived) for 7-8 weeks under reverted day-night schedule. On 8-9th week - after one week isolation - resident-intruder tests were run. Resident rats weighed 400-450g and intruder rats weighed 250-300g. Standard laboratory food and tap water were available *ad libitum*. Experiments were always carried out in the first 4 hours of the dark (active) phase. Behavior 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.

Behavioral testing

In our experiments we measured territorial aggression using resident-intruder paradigm. Rats were isolated in big territorial cages and one week later, an intruder male of smaller size was placed in the resident's cage and their behavior was video recorded for 20 minutes. The following behavioral parameters were analysed: social sniffing, offensive behavior, biting attacks, dominant posture, defensive behavior, submissive posture, resting, exploration, self-grooming. Biting attacks were analysed in a more detailed manner (frame by frame) to identify the context (social signalling) and target (body parts of the opponent, its vulnerability) of attacks, so thus to differentiate between normal and abnormal aggressive acts.

Open-field test measuring locomotion was also run to clarify whether applied drug (NK1 antagonist) had a sedative effect. NK1 antagonist was injected intraperitoneally 30 minutes before tests.

Blood sampling and hormone measurements

Blood was sampled by tail incision and plasma corticosterone was measured by radioimmunoassay. ^{125}I-labelled corticosterone-carboximethyloxime-tyrosine-methyl ester derivative was used as tracer. The interference with plasma transcortin was eliminated by inactivating transcortin at low pH.

Surgical techniques

Biotelemetric measurements

Heart rate changes and locomotion of resident rats were monitored by biotelemetric system. Telemetric e-mitters (2-3 cm, Mini Mitter Company, Bend, OR, USA) were implanted into the abdominal cavity and its leads were fixed to the surface of pectoral muscles. Receivers were placed under the rats' cages and the software (VitalView Data Aquisition System, Mini Mitter Company, USA) registered each animal's heart rate, temperature and activity data minute by minute. Basal and reactive heart rates were considered as markers of autonomic activity.

Adrenamectomy and corticosterone substitution

Adrenalectomy (ADX) and sham operation were performed under ketamine–xilazine–pipolphen anesthesia by the dorsal approach. In ADX rats we ensured low and stable plasma levels of glucocorticoids (glucocorticoid hypofunction) by implanting subcutaneous corticosterone pellets which weighed 100 mg and contained 25% corticosterone and 75% cholesterol (both from SIGMA). Such pellets maintain approximately 100 nM/l plasma corticosterone concentrations for at least 3 weeks. Recovery from surgery lasted 1 week followed by behavioral testing. During this period 0,9% saline solution was available for all animals.

Selective lesion of NK1 expressing cells in the hypothalamus

Rats were anaesthetized with ketamine-xylazine-promethazine mixture (50–10–5 mg/kg i.p.), and their hypothalamic attack area was bilaterally infused with 6,25 ng SP-sap dissolved in 0,5 µl saline. Controls received 0,5 µl saline. The infusions were administered via fused-silica capillaries (outer diameter: 100 µm), and lasted 3 minutes. Recovery period lasted one week. Injection sites and lesion were verified by immunocytochemistry (see below).

Histological techniques

Perfusion

90 minutes after aggressive encounters, animals were anesthetized with sodium pentobarbital (Nembutal) and perfused through the ascending aorta with 150 ml ice-cold phosphate-buffered saline followed by 300 ml 4% paraformaldehyde. The brains were removed, post-fixed for 3 h and cryoprotected by 20% sucrose in phosphate-buffered saline at 4 °C. 30 µm frozen sections were cut in the frontal plane on a sliding microtome.

Immunocytochemistry and quantification of neuronal activation

For quantification of neuronal activity we labeled c-Fos protein with a rabbit polyclonal antibody raised against c-Fos p62 (Santa Cruz Biotechnology, USA, sc-52). To specify the cell-types of activated neurons we applied double labelling for c-Fos and the following cell-specific markers: tryptophan hydroxylase (serotonergic cells), neurokinin-1 receptor, parvalbumin and cholecystokinin (prefrontal interneurons), rat brain pyramidal antigen (prefrontal pyramidal cells) and NeuN (all neurons). Briefly, the primary antibodies were detected by biotinylated secondary antibodies (IgG, 1:1000) and streptavidin conjugated horse radish peroxidase (1:1000) (Jackson Laboratories, USA). The peroxidase reaction was developed in the presence of diaminobenzidine tetrahydrochloride (0.2 mg/ml), nickel–ammonium sulphate (0.1%) (in the case of single labelling for c-fos and NeuN) and hydrogen peroxide (0.003%) dissolved in Tris buffer. Microscopic images were digitized by a Sony CCD camera and the number of positive profiles in the case of single labeling was counted bilaterally by means of ImageJ software (NIH, USA). In the case of double labeling standard circular ocular fields (r=220µm) were counted bilaterally directly in the microscope at 500-fold magnifications. Activity of brain regions were expressed by average number of positive profiles in two representative sections (180µm apart).

Statistics

For statistical analysis, the Statistica software (StatSoft, Inc.; Tusla, OK, USA) was used. Data were analysed by nonparametric Kruskall-Wallis test or by ANOVA (one-way, factorial and repeated measures analysis of variance). Pair wise comparisons were carried out using *post hoc* Mann-Whitney U and Newman-Keuls tests. For normalization we used square root transformations. Spearman- and multiple regression analysis were performed to assess correlations.

RESULTS AND DISCUSSION

Abnormal aggression induced by glucocorticoid hypofunction

1. Glucocorticoids play a crucial role in the formation of abnormal aggression. Male rats with stable, low glucocorticoid levels show disrupted species-specific behavioral pattern, namely they attack the vulnerable body parts of the opponents in a territorial context (resident-intruder test) which can be considered an inadequate reaction. Such violence occurs only in life-threatening situations in natural settings. In addition, their attack signalling - a ritualized form of aggression - is decreased resulting in a shift toward more violent attacks. This behavioral deviance can be ameliorated by acute glucocorticoid injections which supports the causal role of glucocorticoids in hypoarousal-driven violence.

Immunocytochemical labelling pointed out that the number and activity of serotonergic cells (c-Fos/tryptophan hydroxylase double positive cell numbers) in the dorsal raphe is unchanged in glucocorticoid hypofunction. This serotonergic activation with a normal glucocorticoid background (sham-operated animals) could provide an inhibitory control over the expression of normal aggressive attacks (negative correlation). However, this control was not present in glucocorticoid hypofunction resulting in dissociation between central serotonergic activity and behavioral consequences. Therefore, we hypothesize that postsynaptic changes are responsible for the functional alterations. This assumption is supported by human findings showing a low efficacy of serotonergic treatments in hypoarousal-driven violent psychopathologies. Our group also showed that 5-HT_{1A} partial agonist buspirone did not lower aggression but increased the number of abnormal attacks.

2. Prefrontal cortex is also well-known for its control role over expressed behavior. Supporting this idea, in our experiments total prefrontal activity was strongly correlated with offensive behavior in sham-operated rats while the pyramidal activation of this region showed a negative correlation with the number of aggressive attacks. It means that prefrontal activity shifts aggressive acts from the more robust, direct attacks toward the more ritualized, inhibited forms (offensive threats). Contrarily, prefrontal cortex did not show any correlation with offensive behavior in animals with glucocorticoid hypofunction and the attack/offense ratio was shifted, indicating decreased intention signaling. A more detailed analysis clarified that in the prefrontal cortex, the number and activation of interneurons decreased, which resulted in the disinhibition of local pyramidal cells. The activation of the latter cell population showed a positive correlation with the number of abnormal attacks. These data

suggest that low glucocorticoid levels do not simply decrease the inhibitory effect of the prefrontal cortex on aggression, but changes its functional effect on aggressive behavior (facilitation of violent acts).

3. Enhanced central amygdala activation suggests that glucocorticoid hypofunction induced a "threatened state" in subjects. In addition to the increase activation of the central nucleus, medial amygdaloid neurons expressing substance-P receptors (neurokinin-1, NK1) showed an increased activity in glucocorticoid hypofunction. This latter cell population also showed a positive correlation with number of abnormal attacks which could be lowered by NK1 antagonist treatment in a dose-dependent manner (0,1 and 1 mg/kg, intraperitoneally). The NK1 antagonist (1 mg/kg) was also able to restore the normal heart rate reaction during aggressive encounter without any sedative effect. Our investigations suggest that the central target of this intervention is the intermediate hypothalamus because selective lesion of NK1 expressing cells in this region can effectively lower aggressive behavior. These findings indicate new therapeutic implications of NK1 antagonists beyond their well-known anxiolytic and antidepressant effect. It is especially promising for cases resistant to serotonergic therapies.

Early social deprivation induced abnormal aggression

4. Early social environment has a deep impact on social behavior in adulthood. Interacting with genetic and physiological predispositions (e.g. stress reactivity and serotonergic activity); deficient social interactions play a crucial role in the etiology of abnormal aggression. Rats housed individually right after weaning showed a dramatic increase in aggression. In addition, these animals attacked more the vulnerable body parts of the opponent (similar to glucocorticoid hypofunction) and showed less offensive threats (signalling attacks). Thus, social deprivation was accompanied by the loss of ritualized sequences before attacks. Noteworthy, socially deprived rats started attacks from unusual situations (e.g. submissive posture), as well. However, this violent phenotype was associated with anxious states, as shown by increased defense and frequent shifts from one behavior to another (hypervigilance, behavioral fragmentation). Defensive, fragmented behavioral profile alternating with unpredictable violent acts resulted in an ambiguous behavioral pattern. Deprived rats, regrouped in social colonies, showed a marked avoidance of conspecifics indicating a chronic outcome (social anxiety) of early social events. While socially housed

rats are in direct contacts during inactive phase (nearly 100%), deprived rats show rare contacts (20%) which is still slightly present after one week.

5. In line with human findings, behavioral consequences of early social deprivation suggest that these animals are hyperaroused. Our results confirm this hypothesis: both the heart rate and the glucocorticoid response during aggressive encounters showed an enhanced reactivity of the stress system (hyperarousal-driven violence). Noteworthy, glucocorticoid levels and heart rates did not change under basal conditions. This suggests that social deprivation induces hyper-reactivity but not a general hyperfunction of the HPA-axis and sympathetic nervous system. Deprived animals also showed a blunted adaptation of heart rate reactivity for repeated aggressive encounters compared to socially housed animals. These changes were not due to increased locomotion.

6. Social deprivation enhanced the fight-induced activation of many aggression-relevant brain regions such as the medial orbitofrontal- and anterior cingular cortex. The former directly projects to the hypothalamic attack area and shows marked changes in hypoarousal-driven violence as well. The latter area is believed to organize the temporal and sequential order of different behavioral patterns. Its hyperactivity may explain the disorganized and context-independent attack patterns of deprived rats.
Central amygdala did not show any changes but the medial nucleus was over-activated in deprived rats and showed a positive correlation with the rate of abnormal attacks. The data obtained in hypo- and hyperaroused rats together suggest that the enhancement of central amygdala activation is special for hypoarousal-driven aggression, the hyperactivity of the medial amygdala contributes to abnormal aggression in a more general manner. To understand the subtle changes underlying social deprivation induced violence, cell-type specific functional analysis are needed.

CONCLUSIONS

1) Chronic, non-reactive glucocorticoid levels result in abnormal aggression and marked functional changes of aggression-relevant brain regions:

    a. The aggression lowering effect of the serotonergic system is disturbed which may explain the low efficacy of serotonergic treatments in hypoarousal-driven violence.

    b. Disinhibition of prefrontal pyramidal system shifts aggressive behavioral profile toward a more violent pattern.

    c. Substance-P facilitates abnormal aggression via excitation NK1 expressing neurons in medial amygdala and intermedier hypothalamus. NK1 antagonists can effectively lower abnormal aggression which may provide new therapeutic targets in these human psychopathologies.

2) Early social deprivation induces abnormal aggressive behavior in adulthood. Mechanisms mediating these behavioral alterations are the following:

    a. Abnormal aggression induced by early social deprivation is associated with heightened anxiety states and disturbed social communication.

    b. Deprived animals show an exaggerated stress response (heart rate- and glucocorticoid increase) during aggressive encounters.

    c. Aggressive encounters are accompanied by enhanced prefrontal and amygdalar activity in socially deprived rats.

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LIST OF PUBLICATIONS

Publications related to the dissertation

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