The role of the prefrontal cortex in the regulation of abnormal aggression

Doctoral Thesis

László Biró

Semmelweis University
János Szentágothai Doctoral School of Neurosciences

Supervisors: Dr. József Haller, D.Sc.
Dr. Éva Mikics, Ph.D.

Official reviewers: István Gyertyán, Ph.D.
Ottó Pintér, Ph.D.

Comprehensive exam committee:
Chairman: Zoltán Rihmer, D.Sc.
Members: Gergely Zachar, Ph.D.
István Tarnawa, Ph.D.

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INTRODUCTION

Abnormal aggression is a central element of several psychiatric disorders such as antisocial personality disorder, juvenile behavioral disorders, borderline personality disorder, and intermittent explosive disorder. Recently, psychopathologies associated with abnormal aggression in the literature are considered as developmental disorders that can be caused by the interaction of environmental and biological (genetic, neurobiological) factors. Children growing up in the aversive social environment are more likely to show psychopathological traits in their adult life, with symptoms such as anxiety, increased stress reactivity, antisocial behavior, and increased predisposition to aggression. In order to understand the neuronal mechanisms behind abnormal aggression, the exploration of pathways and the affected brain areas that modulate natural aggression is of paramount importance. Aggression-related disorders are characterized by structural and functional deficits of the medial prefrontal cortex (mPFC), which is considered to exert inhibitory control over aggression. It has a particularly long-term developmental trajectory, resulting in enhanced sensitivity towards juvenile aversive environmental influences. Following such adversities (eg. childhood neglect), mPFC shows structural deficits in humans. In apparent contradiction with aggression-related mPFC deficits, studies employing assessment of neuronal activity marker expression revealed that aggression increases PFC activation; moreover, PFC hyperactivation was observed in animals submitted to models of escalated, abnormal aggression. Likewise, human studies in which the impact of actually performed aggression was studied (e.g., subjects played aggressive video games or retaliated aggressively in competitive situations), the PFC was also found to be activated by aggressive actions; moreover, the activation was stronger in subjects with aggression-related psychopathologies.

Although the mPFC sends widespread projections to different subcortical targets, it remains unclear how particular prefrontal projections modulate aggressive qualitative and quantitative aspects of aggressive behavior.

In the first part of our work, we examined the role of the hypothalamic projections of mPFC in aggression, more specifically the mPFC afferents to the mediobasal hypothalamus (MBH) and the lateral hypothalamus (LH). The former brain area is the brain center of intraspecific aggression in rodents while the latter plays a key role in predatory aggression, but there is also evidence for its role in certain types of intraspecific aggression. Although the mPFC input of these hypothalamic regions suggests that mPFC directly modulates aggression, it was not previously supported by experimental evidence.

In the second part of our work, we aimed to investigate structural and functional characteristics of the mPFC in an animal model of abnormal aggression. We submitted rats to post-weaning social isolation (PWSI), an established model of early social neglect in humans, which is a significant contributor to adult violence and criminality. PWSI replicates many detrimental effects of human early social neglect in rats, including escalated aggression as shown by enhanced bite counts and enhanced shares of bites directed towards vulnerable body parts of opponents (head, throat, belly). We studied putative structural deficits induced by PWSI in the mPFC, in order to investigate whether changes observed in abnormally aggressive humans are reproduced by the model. We also investigated neuronal activation patterns induced by fighting in the same subjects.

AIMS

1. Anatomical and behavioral characterization of prefrontal-hypothalamic projections

1.1. Assessment of the overlap between MBH and LH projecting mPFC neurons.

1.2. Characterization of neurochemical features of mPFC-MBH and mPFC-LH projections.

1.3. Assessment of the functional role of mPFC-MBH and mPFC-LH projections on aggressive and other social behaviors during aggressive interaction.

2. Prefrontal cortical structural and functional changes associated with PWSI-induced abnormal aggression

2.1. Assessment of structural alterations of the mPFC induced by PWSI.

2.2. Examination of changes in neural activity related to PWSI-induced abnormal aggression in the mPFC.

2.3. Neurochemical characterization of activated mPFC neurons during aggressive interaction.

METHODS

Animals

We studied adult male Wistar rats (Charles River Laboratories), which were group housed until surgery, and thereafter housed individually until the end of the experiment. Food and water were available *ad libitum*, while temperature and relative humidity were kept at 22 ± 2°C and 60 ± 10%, respectively. Rats were maintained in a reversed 12 h

light/dark cycle with lights off at 10:00 A.M. Opponents used in aggressive encounters were obtained from the same source and weighed ~30% less than residents at the time of aggressive interactions. Experiments were performed in accordance with the European Communities Council Directive of 2010 (2010/ 63/EU) and were reviewed and approved by the Animal Welfare Committee of the Institute of Experimental Medicine.

Post-weaing social isolation (PWSI)

Pups were weaned on the 21st postnatal day. During the following 7 weeks, littermates were randomly assigned either to individual housing (PWSI treatment), or to social housing in groups of 4 (socially reared) until adulthood.

Behavioral procedures

Resident-intruder (RI) test

Territorial aggressive behavior was studied in the RI test performed in Plexiglas cages measuring 40 x 25 x 25 cm. Test rats (residents) were individually transferred into test cages three days prior to RI test to faciliate the appearance of territorial behavior. RI test consisted of placing a smaller intruder conspecific into the cage for 20 min. Behavioral analysis focused on the patterns of biting attacks, namely on attack targeting and the relationship between offensive threats and attacks. Attack episodes were analyzed at low speed (frame-by-frame if necessary) for identifying attack targets and the context of attack. An attack was considered 'vulnerable' if it targeted the head, throat, belly or paws. Dorsal and lateral areas were considered non-vulnerable targets. An attack was considered 'non-signaled' if it was not preceded by an offensive threat, and signaled if it was performed in the context of an offensive threat. Vulnerable and non-signaled attacks, respectively, were expressed as the percentage of total attacks. The frequency and duration of the following, non-aggressive behavioral variables were also

assessed: exploration; social investigation; grooming; offense; defense; dominant posture; and submissive posture.

Resident-intruder test paired with photostimulation

Three days before the RI test, resident animals were transferred into test cages designed for photostimulation. The 10 min-long RI test started with the placement of a smaller conspecific into the test cage. Concomitantly, photostimulation was initiated and maintained for 3 min (473 nm light delivery, 20 mW output power, 20 Hz with 10 ms pulses). The same rats underwent the test four times in 2 day intervals. Photostimulation was applied in two of the trials. In the other two trials, rats were connected to optic fibers, but no light was delivered. Photostimulation and no-photostimulation trials followed according to a randomized crossover design.

Sociability test

Subjects were placed in an arena measuring 100 x 100 x 40 cm. After a 5 min-long habituation period, during which animals could explore the arena freely, a smaller male conspecific was presented in a plastic perforated cylinder in the corner of the arena, whereas an empty cylinder was placed in the opposite corner. Immediately after placement of the cylinders, the photostimulation protocol described for the RI tests was initiated. The time spent with cylinder exploration was assessed by means of time percentage of total test time by H77 event-recorder software.

Surgeries

All surgeries were performed in a stereotaxic frame (Kopf Instruments) under deep ketamine (50 mg/kg; Medicus Partner), xylazine (20 mg/kg; Medicus Partner), and pipolphen (0.2 ml/kg; Egis) anesthesia.

Retrograde tracer injections

We unilaterally injected either 50 nl of 1% cholerotoxin β (CTB, List Biological Laboratories) into the MBH (anteroposterior (AP), -1.6 mm; ML, 1.2 mm; DV, -9.2 mm) and 20 nl of 2% Fluorogold into the LH (AP, -2,3 mm; ML, -2,1 mm; DV, -8,5 mm; FG, Fluorochrome).

Virus injections and optic fibers implantation

For optogenetic manipulations of mPFC terminals in the hypothalamus, AAV2.5.-hSyn.hChR2-(H134R)eYFP.-WPRE.h (10^{13} GC/ml titer; catalog #26973, Addgene; Penn Vector Core) and AAV-hSyn-EYFP (titer, 3 x 10^{12}; Addgene; University of North Carolina, Chapel Hill, NC) were delivered into the mPFC (AP, 2.8 mm; ML, 0.6 mm; DV, 4.4 and 5.0mm from bregma) and optic fibers were placed on top of the MBH (AP, -1.8 mm; ML, 1 mm; DV, -8.8 mm) or LH (AP, -2.3 mm; ML, 2 mm; DV, -7.4 mm).

Histology

Perfusion and brain slicing

Rats were anesthetized and transcardially perfused with 150 ml of ice-cold saline, followed by 300 ml of 4% paraformaldehyde in 0.1 M PBS, pH 7.4. Brains were removed and postfixed in the same fixative, and cryoprotected in 30% sucrose in PBS before sectioning on a sliding microtome.

Assessment of structural changes in the mPFC

The thickness of the mPFC was measured bilaterally on dark-field images. We drew two lines perpendicular to the medial longitudinal fissure; one through the upper, whereas the other through the lower end of the forceps minor of the corpus callosum. The thickness of the mPFC was characterized by the length of these lines. To investigate whether the observed changes were due to an overall change in brain volume, we also estimated the size of the

periaqueductal gray (PAG) by delineating the whole central gray area on dark-field images.

Immunohistochemistry

Free-floating sections were processed for c-Fos, NeuN, CTB, FG, GFP, vGAT, vGLUT1, GFAP, lectin, SMI-32, GABA, CaMKIIα staining. Tissue sections were blocked at room temperature in PBS-T (0.1% - 0.5% Triton X-100) with either normal donkey, horse or goat serum, and stained with primary antibodies (24-72 hrs at 4°C or room temperature) and secondary antibodies (2 hrs at room temperature).

Microscopy

Microscopic images were digitalized by an Olympus CCD camera using a 10x magnification lens. Relative optical density was assessed in the same area delineated above for cortical thickness analysis, i.e. between the upper and lower end of the forceps minor of corpus callosum, using Image J software with standardized settings across experimental subjects. Fluorescent images were captured by a C2 confocal laser scanning microscope (Nikon Europe, Amsterdam, The Netherlands). For excitation of antibody-conjugated fluorophores we used 488, 561 and 642 nm lasers (CVI Melles Griot), respectively, while scanning was performed in channel series mode through Plan-Apochromat VC 20x and 60x objectives. A background level (threshold) was obtained from each section and subtracted from measured optical density for statistical analysis.

***In vitro* electrophysiology**

Brains were removed rapidly and immersed in ice-cold sodium-free solution, then acute coronal slices containing the MBH were prepared. Following preparation a neuron was patch clamped in the close vicinity (in 200–300 μm) of the end of the glass fiber at the MBH. Whole-cell patch-clamp measurements were performed to record postsynaptic currents

(PSCs). The neurons were voltage clamped at a pipette holding potential of -70 mV. A train of laser pulses was applied at 0.2 Hz (60 pulses totally). For analysis, records of the 60 pulses were averaged. In total, analysis contained 12 recorded cells from three animals.

Staistical analysis

Data were expressed as mean ± SEM. Behavioral data were analyzed by Kruskal–Wallis ANOVA or two-factor ANOVA. Histological data were analyzed by repeated measure ANOVA, followed by Tukey post-hoc analysis when appropriate. P values underwent Bonferroni–Holmes correction in case of c-Fos data. When necessary, data were square root transformed to fulfill ANOVA criteria. Significance level was set at $p<0.05$

RESULTS

1. Anatomical and behavioral characterization of prefrontal-hypothalamic projections

1.1. Largely distinct mPFC neuron populations innervate the MBH and LH

For detailed investigation of the pathways connecting the mPFC to hypothalamic attack areas, we injected the retrograde tracers CTB and FG into the MBH and LH, respectively. Our findings revealed a large share of mPFC neurons that send projections to the relatively small hypothalamic attack areas. The distribution of neurons projecting to the MBH and LH was different across mPFC layers. Dual CTB/FG retrograde tracing revealed three distinct types of projection neurons in the mPFC. Of the 864 neurons counted in three rats, 598 were retrogradely labeled from the LH and 168 were labeled from the MBH, whereas 98 were labeled from both nuclei. MBH-projecting neurons were localized in layers III/V, whereas LH-projecting neurons showed prominent expression in both layers III/V and VI. In

all layers, projection neurons were intermingled with other neurons.

1.2. The mPFC densely innervates hypothalamic centers of attack, which respond to photostimulation

Projection areas were investigated by injecting an AAV vector carrying the gene of ChR2 fused to eYFP into the mPFC as an anterograde tracer. This study revealed a rich network of mPFC fibers in both the LH and MBH. Co-expression of eYFP with vGLUT1 and vGAT showed minimal mPFC GABAergic afferents in the hypothalamus, but numerous glutamatergic release sites. Finally, our electrophysiological study in acute hypothalamic brain slices showed that the photostimulation of ChR2-expressing terminals originating from the mPFC is able to generate postsynaptic responses in the hypothalamus. These findings demonstrate robust and relatively distinct excitatory projections from the mPFC to the two hypothalamic attack areas, and show that the terminals are responsive to photostimulation.

1.3. Photostimulation of mPFC–MBH pathway selectively increases bite counts

The study aimed at investigating the functional role of the mPFC–MBH projection during aggressive interactions. Rats delivered significantly more bites under photostimulation compared with non-photostimulation trials. This effect was also highly selective, as bite latencies, the shares of abnormal attacks, as well as behaviors regularly performed in RI tests were all unresponsive to photostimulation. A within-trial analysis of events showed that the frequency distribution of bites was markedly changed by photostimulation. The increasing phase of bite delivery was similar in the two trial types, but rats did not diminish biting behavior over time when mPFC afferents were photostimulated in the MBH.

1.4. Photostimulation of mPFC–LH pathway selectively promotes abnormal patterns of attack

The study aimed at investigating the functional role of the mPFC–LH projection during aggressive interactions. Photostimulation did not affect the bite counts or the latency of bite delivery. However, the vast majority of bites either targeted vulnerable body parts of opponents or were not signaled socially in photostimulation trials. Such attacks are considered abnormal in the context of regularly performed resident/intruder tests but are frequently observed in models of abnormal aggression with LH stimulation as no similar bites were present when the MBH was stimulated. Photostimulation affected no other behavior in the RB test, demonstrating the high behavioral specificity of the effects. The temporal distribution of biting attacks followed the typical inverted U-shaped curve in both types of trials, but context typical bites were replaced by abnormal bites over the whole trial.

1.5. Control experiments with light-insensitive eYFP virus constructs

To exclude that the behavioral effects of photostimulation were due to the physical effects of light rather than to the stimulation of hypothalamic mPFC afferents, we performed a study similar to the previous ones except that the virus carried the light-insensitive eYFP gene alone. Under these conditions, photostimulation of the MBH did not affect behavior in the resident/intruder test. Furthermore, we investigated whether axonal discharges elicited by photostimulation propagate backward, thus affecting the mPFC in addition to the MBH and LH. The viral vector carried either eYFP alone or together with ChR2 in these studies, whereas light was administered into the LH or MBH. According to our results, c-Fos expression in the mPFC was highly similar in the two groups.

Finally, we assessed whether the effects of photostimulation were secondary to changes in sociability, which would have rendered them consequences of general changes in social motivation rather than real behavioral effects. Photostimulation, however, affected neither social preference nor locomotion in the sociability test. These findings show that behavioral effects observed in the previous studies were due to the selective activation of prefrontal–hypothalamic projections and underline the behavioral specificity of the effects.

2. Prefrontal cortical structural and functional changes associated with PWSI-induced abnormal aggression

2.1. PWSI resulted in abnormal aggression

As expected based on earlier studies, PWSI increased aggression in quantitative terms, and also induced the expression of abnormal forms of aggression: rats submitted to the model started attacks sooner and delivered more biting attacks than SR rats in the resident/intruder test. They also exhibited more attacks on vulnerable targets of the opponents, and were less prone to signal their attacks by offensive threats.

2.2 PWSI induced structural alterations in the mPFC

The thickness of the mPFC was significantly reduced by PWSI at both the upper and lower end of the forceps minor in the right hemisphere. The dorso-ventral extension of the forceps minor did not change significantly. The size of the PAG did not change either, suggesting that the thinning of the mPFC was not due to an overall reduction in brain size. Despite the thinning of the right mPFC, the total number of neurons in the whole area was unaltered on either side as shown by NeuN staining. By contrast, dendritic density indexed by SMI-32 staining was reduced bilaterally in PWSI rats. A similar decrease was observed in glial cell counts as indexed by reduced GFAP density in both hemispheres. The

vascularization of the investigated mPFC area was also reduced by PWSI specifically on the right hemisphere as shown by reduced staining for lectin.

2.3. Aggressive interaction evoked neuronal activation within the mPFC

Exposure to the RI test increased c-Fos expression in both SR and PSWI rats, but the increase was larger in the latter group. Our initial analysis suggested that the increase was distributed relatively uniformly over the whole area, without outstanding increases in particular subregions. By contrast, a detailed layer-specific analysis revealed the activation of a clearly circumscribable area in PWSI rats. The area roughly corresponded to layers III/V and VI, covered both the PrL and IL. In summary, these data show that the activation of mPFC was greater in isolated animals and this effect was concentrated in a well-defined subregion of mPFC.

2.4. Neurochemical characterization of activated neurons in the mPFC

c-Fos counts were increased by fights, and was further increased by PWSI. Surprisingly, similar situation was observed when CaMKII-expressing and GABA-expressing neurons were considered separately. Fights increased the number of activated glutamatergic and GABAergic neurons in socially-reared controls, and activation of both types of neurons was significantly higher in PWSI rats.

CONCLUSIONS

Based on our experimental results, the following conclusions can be drawn:

1. Distinct mPFC neuron populations modulate different aspects of aggressive behavior at the level of the hypothalamus:

a. Separated neuronal populations of mPFC project into MBH and LH, where they form dense excitatory synaptic vesicle release sites.

b. The mPFC-MBH projection modulates quantitative measures of aggression, while the mPFC-LH projection modulates qualitative features of aggression.

c. Activation of both mPFC-MBH and mPFC-LH projections selectively influences aggressive behavior without impact on other forms of social behavior.

2. PWSI induces structural deficits and functional hyperactivity of mPFC in association with abnormal forms of aggression:

a. PWSI induces a decrease in mPFC volume, accompanied by lower expression of dendritic, glial and vascular components.

b. PWSI rats show increased neuronal activity within deep layers of the mPFC where LH- and MBH- projecting neurons are localized, presumably contributing to the occurrence of enhanced and abnormal forms of aggression.

c. Neuronal hyperactivity accompanied by aggressive interaction is associated with increased activity of both glutamatergic and GABAergic neurons.

SUMMARY

Social neglect and early-life stress constitute a serious risk factor in the development of abnormal aggression-related psychopathologies and also influence the development and maturation of the PFC. Indeed, structural and functional deficits of PFC have been observed in aggression-related psychiatric disorders. However, preclinical and clinical research has shown *enhanced* activation of the PFC during aggressive behavior, therefore the role of the PFC in the modulation of aggression is still unclear.

In the first experiment, we optogenetically photostimulated excitatory mPFC efferents projecting to MBH and LH regions in male rats. Photostimulation of the mPFC-MBH projection increased attack frequency without qualitative changes. On the other hand, activation of the mPFC-LH projection influenced the qualitative aspects of aggression, i.e. reduced social signaling before attacks and increased the ratio of attacks aiming vulnerable body parts of the opponent (abnormal patterns). In summary, our results show that the mPFC modulates qualitative and quantitative aspects of aggression *via* two distinct hypothalamic projections.

In a second set of experiments, we investigated the effects of PWSI on aggressive behavior and associated structural and functional changes in the mPFC. Our results show that social isolation induces abnormal aggression in adulthood characterized by increased attack bouts, and robust increases of abnormal attacks aiming vulnerable body parts, and significant reduction of social signaling before attacks. These behavioral changes were associated with reduced thickness and vascularization of the right mPFC, and bilateral decrease in dendritic and glial density in isolated animals. Isolation-induced abnormal aggression was also accompanied by increased activation of two subregions of the mPFC, the infralimbic (IL) and prelimbic (PrL) cortices. These regions

are anatomically connected to the hypothalamic centers of aggression, i.e. MBH and LH. In summary, our results confirmed the important role of early-life social disturbances in the development of prefrontal structural deficits resulting in abnormal aggression. Moreover, we revealed a direct prefronto-hypothalamic circuits involved in aggression modulation.

PUBLICATIONS OF THE AUTHOR

Publications that form the basis of the Ph.D. dissertation:

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