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ORIGINAL INVESTIGATION

Prefrontal cortex—nucleus reuniens—hippocampus network exhibits sex-differentiated responses to stress and antidepressant treatment in rats

V. Kafetzopoulos[1,2] · N. Kokras[3] · Filippos Katsaitis[4] · N. Sousa[5,6,7] · H. Leite-Almeida[5,6] · I. Sotiropoulos[4,5,6] · C. Dalla[1,8]

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Abstract

Rationale Depression is a serious psychiatric disease, which is diagnosed twice as frequently in women than men. We have recently shown that lesioning or inactivation of the nucleus reuniens (RE), which interconnects the prefrontal cortex (PFC) and hippocampus, promoted resilience to stress in males, exerts an antidepressant effect in the Forced Swim Test (FST) and prevents the development of behavioral and neurobiological alterations induced by the chronic mild stress model of depression.

Objectives In this study, we expand our findings on the FST in female rats and we investigate whether RE lesion presents sex differences following treatment with two distinct antidepressants, a selective serotonin reuptake inhibitor, i.e. sertraline and a tricyclic antidepressant, i.e. clomipramine.

Methods Male and female rats received either a surgical lesion of the RE or sham operation, then treated with vehicle, sertraline (10mg/kg) or clomipramine (10mg/kg) and were subjected to the FST. Activation of key brain areas of interest (PFC, Hippocampus and RE) were measured by c-Fos immunoreactivity.

Results RE lesion induced an antidepressant-like phenotype in both female and male rats, confirming its crucial role in the stress response. Similarly to RE lesion, sertraline treatment resulted in increased swimming and decreased immobility duration, as well as enhanced head shake frequency, in both sexes. Notably, climbing behavior was increased only following clomipramine treatment. RE area was less active in females compared to male rats and in clomipramine-treated males compared to their corresponding vehicle-group. Activation of the PFC and the CA1 hippocampal area was reduced in clomipramine-treated females, in comparison to vehicle-treated female rats. This effect was not evident in males, which exhibited less activation in the PFC and the hippocampus than females.

Conclusion Re lesion proves equally effective in female and male rats, but sex is highlighted as a pivotal factor in behavioral and treatment response in FST, as well as in related circuit connectivity and activation.

Keywords Forced swim test · Depression · Sex differences · Nucleus reuniens · SSRI · Tricyclic antidepressant · Rat

Introduction

Depression, a serious mental disease, is recognized as one of the leading causes of disability and one of the costliest diseases in the western world (Roehrig 2016; Theis et al. 2018; Wittchen et al. 2011). Treatment of depression substantially relies on compounds that are able to alleviate depressed mood, but this is apparent over the course of some weeks (Harmer et al. 2017). Most of the available compounds target the monoaminergic neurotransmitter systems (Lopez-Munoz and Alamo 2009). Specifically, the widely used selective serotonin reuptake inhibitors (SSRI) almost exclusively target the reuptake of serotonin, while others, like the previous generation of tricyclic antidepressants, exert a less specific action on more neurotransmitter systems, like the noradrenergic and dopaminergic neurotransmission (Hamon and Blier 2013). However, pharmacological treatment is effective only in some patients, and up to a third of patients do not respond to such treatments (Chung et al. 2023). Therefore,

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there is a significant need for faster and better acting antidepressants. Recent developments in the field of rapid acting antidepressants, focus on glutamatergic agents, such as esketamine, as well as classical psychedelics, such as lysergic acid diethylamide (LSD) that has been shown to alter connectivity between the thalamus and cortical areas (Avram et al. 2024; Pavlidi et al. 2021a). Therefore, the quest for more effective, safe and rapid novel antidepressant treatments requires a better understanding of the neurocircuits involved in depression, as well as its underlying neurobiology (Brady et al. 2023).

In this context, we have recently found in experimental models of depression, such as the chronic mild stress and in a test of antidepressant activity, such as the Forced Swim Test (FST), that the nucleus reuniens (RE) is crucially involved in behavioral responses, as well as in the development of neurobiological alterations linked with the emergence of depression and stress response (Kafetzopoulos et al. 2018, 2021). RE interconnects the PFC and the hippocampus relaying signals from the PFC to the hippocampus (Cassel et al. 2013) mainly via glutamatergic activity. The PFC receives a monosynaptic innervation from the ventral CA1 and subiculum of the hippocampus and there is a directionality in this communication because hippocampal activity leads the activity in the PFC (Oliveira et al. 2013; Siapas et al. 2005). In contrast, the reciprocal PFC output to the hippocampus is not monosynaptic, but relayed via the nucleus reuniens (RE), a thalamic midline nucleus (Vertes 2002). Additionally, RE modulates oscillatory patterns of both structures (Bertram et al. 2001; Thorn et al. 2022; Zhang et al. 2012). Abnormal connectivity, among other correlates, is a prominent characteristic in mental disorders and the thalamus plays a key role in the dysconnectivity exhibited in depression (Greicius et al. 2007; Veer et al. 2010). Our recent results have shown that a lesion of the RE prevents the establishment of stress-induced brain pathology and promotes resilience to depressive pathology (Kafetzopoulos et al. 2018, 2021).

However, our previous studies were performed in male experimental animals. Several clinical and preclinical studies confirm the existence of various sex differences in neurobiological mechanisms of depression and anxiety, along with sex differences in antidepressant treatment response (Kokras and Dalla 2014; 2017; Pavlidi et al. 2023) and interestingly there are sex differences in the pharmacokinetics of antidepressants, as well (Kokras et al. 2011). As a result, it has been demonstrated that both sexes should be incorporated in preclinical studies (Clayton and Collins 2014; Dalla et al. 2024; Miller et al. 2017) and that animal models should be developed or validated for both sexes (Becker and Koob 2016; Butlen-Ducuing et al. 2021; Eck et al. 2022; Hodes et al. 2024; Kokras and Dalla 2017; Palanza and Parmigiani 2017). This is of particular importance in depression research, as women are twice more likely to experience depression than men (Weissman and Klerman 1977) and they experience greater symptom severity, more past suicide attempts, and more sleep, appetite, and energy disturbances (Altemus et al. 2014; Blanco et al. 2012). Moreover, women with depression are more likely to suffer from anxiety, whereas men have higher rates of comorbid substance use disorders, such as alcoholism, as well as higher rates of suicide (Marcus et al. 2005; Pavlidi et al. 2023; Schuch et al. 2014).

Therefore, in this study we aim to replicate our previous findings regarding the role of the RE in stress and extend our findings of an antidepressant effect of RE-lesioning to female rats, as well. In particular, we are using antidepressants from two different classes on male and female animals that have a disrupted PFC-Hippocampus interconnection, and we investigate the antidepressant response and the activation of these brain areas, as well as the activation of the RE.

Materials and methods

Animals

Adult male and female Wistar rats (3 months old, 250–350 g) were used. All animals were housed under controlled light/dark cycle (12:12 h, lights on at 08:00 h) and constant temperature/humidity (22 °C/40–60%) with ad libitum access to food and water. Animals were single-housed post-surgery. Behavioral testing was carried out during the light phase. Procedures on animal experiments were reviewed and approved by the relevant local ethics committee and studies were carried out in accordance with European Union Directives 86/609/EEC and 2010/63/EU.

Surgical procedure for the RE lesion

Surgical procedures were carried out as previously described (Kafetzopoulos et al. 2018). Briefly, animals were anesthetized by i.p. injection of a mixture of ketamine and xylazine (100 mg/kg and 10 mg/kg, respectively) and placed in a stereotaxic frame (David Kopf Instruments). An infusion of 0.6 µl of 100 mM NMDA (in 0.1 M PBS, pH = 7.4; 0.1 µl/min) or vehicle (0.1 M PBS, pH = 7.4) was performed directly into the RE (+ 2.3 mm AP, ± 1.7 mm ML, and -6.2 mm DV from bregma); the syringe was left in place for an additional 5 min to ensure adequate diffusion. To avoid damage to midline brain structures and vessels, the infusions were performed with a mediolateral angle of 15° and alternating between left and right angle of access. Animals were given 1 week to recover before further testing.

Histological verification of RE targeting

The site and extent of RE lesions was evaluated in cresyl violet stained brain sections as previously described (Kafetzopoulos et al. 2018). Specifically, rats with lesion ratios < 50% of the RE and animals with a lesion covering ⩾10% of any other brain area in the vicinity of the RE were excluded from the analysis (13% of total animals).

Forced swim test and treatments

The FST was carried out as previously described (Kokras et al. 2018, 2014). In brief, rats were given one week to recover from the surgical procedure, and then placed in a cylindrical tank (60cm × 19cm, filled with 40 cm of water at a temperature of 24 ± 1 °C) and were forced to swim for 15 min during a pretest session. After 24 h, animals were subjected to a 5-min swimming session (test session). All animals were given an i.p injection of sertraline (an SSRI) at 10 mg/kg), clomipramine (a TCA) 10 mg/kg) or a vehicle solution at 23, 5 and 1 h before the FST test session (n = 6–10/group). Therefore, the following groups were tested: male sham-operated, vehicle-treated (n = 7), male RE lesion, vehicle-treated (n = 6), male sham-operated, sertraline-treated (n = 5), male RE lesion, sertraline- treated (n = 5), male sham-operated, clomipramine (n = 5), male Re lesion, clomipramine-treated (n = 5), female sham-operated, vehicle-treated (n = 6), female RE lesion vehicle-treated (n = 5), female sham-operated, sertraline-treated (n = 5), female RE lesion sertraline-treated (n = 4), female sham-operated clomipramine-treated (n = 5), female RE lesion clomipramine-treated (n = 4). Immobility (floating), swimming, climbing and head shaking behaviors were scored from the videotaped 5-min FST sessions, using Kinoscope (Kokras et al. 2017a). A detailed explanation of these behaviors has been described previously (Kokras et al. 2015). Especially, regarding head shakes in the FST, a video has been previously published (Kokras et al. 2017b).

c-FOS immunostaining

c-FOS immunostaining was performed on brain sections from rats exposed to the FST. Briefly, 90 min after the last FST session, animals (n = 5/group) were anesthetized and perfused with 4% PFA (in 0.1 M PBS) before careful excision of the brain, postfixation (4% PFA), and transfer to 30% sucrose (in PBS 0.1M). 50 µm sections were cut on a vibratome and after incubation in 0.3% Triton X-100/0.1 M glycine/10% fetal bovine serum, they were incubated with c-FOS antibody (1:10,000; overnight; cat no. PC05, Calbiochem, Darmstadt, Germany). Sections were then incubated in biotinylated goat anti-rabbit antibody (cat no. E0432, Dako, Glostrup, Denmark) and Avidin/Biotin Complex (ABC solution; Vectorstain Elite, Burlingame, CA, USA). Neurons in the PFC, hippocampus and RE that were c-FOS-immunoreactive were counted using StereoInvestigator software (MicroBrightField). Immunoreactive c-FOS was visualized with diaminobenzidine before light counterstaining with hematoxylin. The prelimbic and the infralimbic part of the PFC were initially analyzed separately, but the two regions were not differentiated, so data were combined. The experimenter was blind to the experimental groups during evaluation (Leite-Almeida et al. 2014; Sotiropoulos et al. 2014).

Statistical analysis

Results were analyzed with SPSS v.29 (IBM SPSS Inc, USA) using three-way analysis of variance (ANOVA) with sex (male:female), antidepressant treatment (vehicle:sertraline:clomipramine) and surgery (sham:lesion) as independent factors. Specifically, for cFOS analysis on nucleus reuniens, which was performed only in sham operated animals, a two-way analysis was performed with sex and antidepressant treatment as independent factors. Significant two- and three-way interactions were further tested with post-hoc pairwise comparisons using Bonferonni's type I error correction method. All data met ANOVA assumptions for normality and homogeneity of variance. Significance level was set at p = 0.05. All results are expressed and depicted as mean ± s.e.m. A power analysis indicated that for an effect size of 0.4, α set to 0.05 and 1-β to 0.8, a total number of 64 animals would be required. However, a limitation of the current study is that a complex 3-way ANOVA was needed to be performed, which was powered accordingly, but due to exclusion of animals, because of surgical operations, the ANOVA had unbalanced groups. In two groups out of the 12 groups in total, only 4 instead of at least 5 animals were present and this may have limited the power of the involved post-hoc pair comparisons.

Results

Behavioral analysis of the FST

Immobility duration

A three-way ANOVA indicated a significant sex * drug interaction [$F_{(2,50)} = 4.284$ $p = 0.019$] and a significant lesion x drug interaction [$F_{(2,50)} = 17{,}079$ $p < 0.001$]. Subsequent post-hoc testing showed that vehicle-treated female rats displayed more immobility time than males, irrespectively of sham or RE lesion surgery.

As expected, in sham-operated rats both sertraline ($p < 0.001$) and clomipramine ($p < 0.001$) reduced

immobility duration in males and females alike. Moreover, RE lesion reduced immobility in vehicle-treated male and female rats in comparison to their sham-operated counterparts. Interestingly, RE lesion further reduced immobility in sertraline-treated male and female rats ($p < 0.001$ and $p = 0.008$ respectively). However, RE lesion did not cause any further decreases in immobility duration in clomipramine-treated male and female rats in comparison to their sham-operated counterparts (Fig. 1).

Swimming duration

The three-way ANOVA for swimming duration indicated a significant sex * drug interaction [$F_{(1,50]} = 5{,}479$ $p = 0.007$] and a significant lesion * drug interaction [$F_{(2,50)} = 5{,}465$ $p = 0.007$]. Regarding the sex * drug interaction, post-hoc testing showed that vehicle-treated female rats displayed less swimming that males ($p < 0.001$), irrespectively of sham or RE lesion. However, this sex difference was not evident in sertraline- and clomipramine-treated rats. Moreover, sertraline increased swimming duration in both male and female rats in comparison to their vehicle-treated counterparts ($p < 0.001$; $p < 0.001$ respectively). Finally, post-hoc testing showed that irrespectively of treatment (vehicle, sertraline, clomipramine), R E lesion elongated swimming ($p < 0.001$; $p = 0.018$; $p = 0.019$ respectively), (Fig. 2).

Climbing duration

A three-way ANOVA for climbing behavior showed a marginally significant lesion * drug interaction [$F_{(2,50)} = 3.032$ $p = 0.057$] and a highly significant drug main effect [$F_{(2,50)} = 11.944$ $p < 0.001$]. Strictly interpreting the statistical results, male and female sham-operated and RE lesion rats treated with clomipramine had increased climbing behavior. However, it should be noted that the marginally significant lesion * drug interaction, on top of the significant drug main effect, was mainly driven by the steep increase of climbing duration primarily in male sham-operated clomipramine-treated rats and secondarily in their female counterparts ($p < 0.001$; $p < 0.001$), while differences in RE lesioned rats were very moderate comparatively (Fig. 3).

Head shaking frequency

A three-way ANOVA for head shaking showed a significant lesion * drug interaction [$F_{(2,50)} = 33.659$ $p = 0.033$] and a significant sex x lesion interaction [$F_{(1,50)} = 5.604$ $p = 0.022$]. Pairwise comparisons upon post-hoc testing showed that RE lesion females had lower head shakes than RE lesioned males ($p < 0.001$) while this sex difference was not found in sham-operated females vs. males. RE lesion increased head shaking in all male groups vs. their sham-operated counterparts, irrespectively of vehicle, sertraline or clomipramine treatment ($p = 0.001$). However, in females RE lesion increased head shakes only in vehicle-treated rats. Finally,

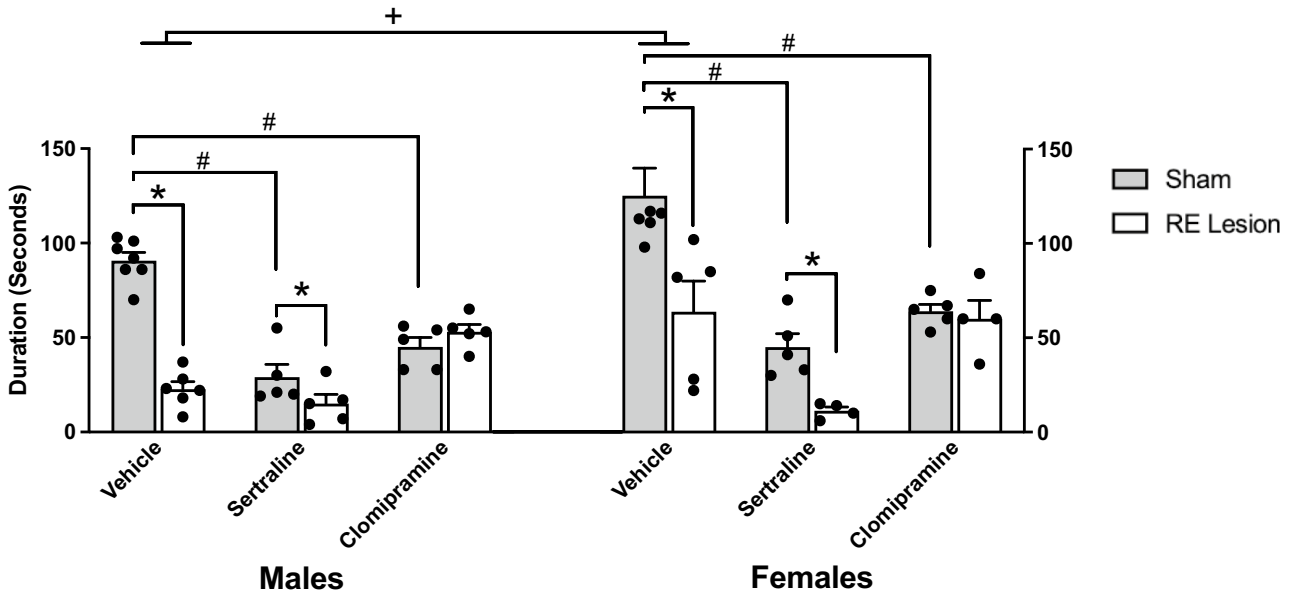

Fig. 1 Immobility duration during the Forced Swim Test (FST). Both sertraline and clomipramine (#=$p < 0{,}05$), as well as nucleus reuniens (RE) lesion (*=$p < 0{,}05$) reduced immobility duration compared to respective controls for both sexes. Vehicle-treated female rats had higher immobility levels (+=$p < 0{,}05$) than respective males, as previously reported

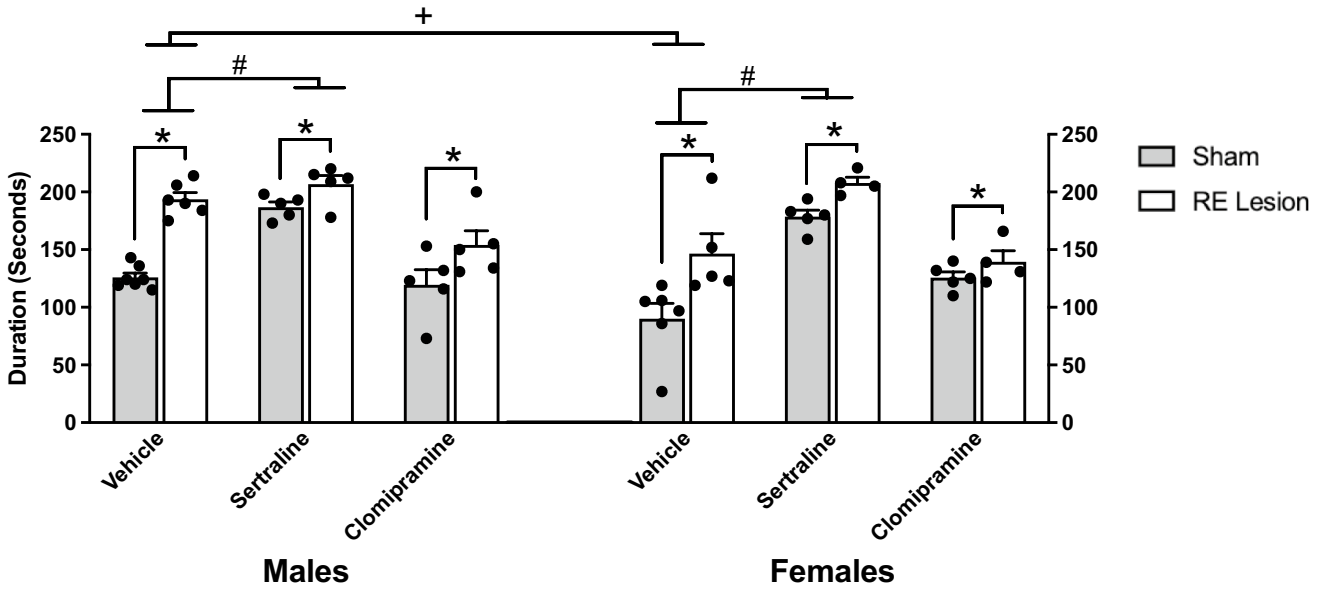

Fig. 2 Swimming duration during the Forced Swim Test (FST). Sertraline (#=$p<0{,}05$) and RE lesion (*=$p<0{,}05$), but not clomipramine, increased swimming duration, compared to respective controls for both sexes. Vehicle-treated females had a decreased swimming duration, compared to respective males (+=$p<0{,}05$)

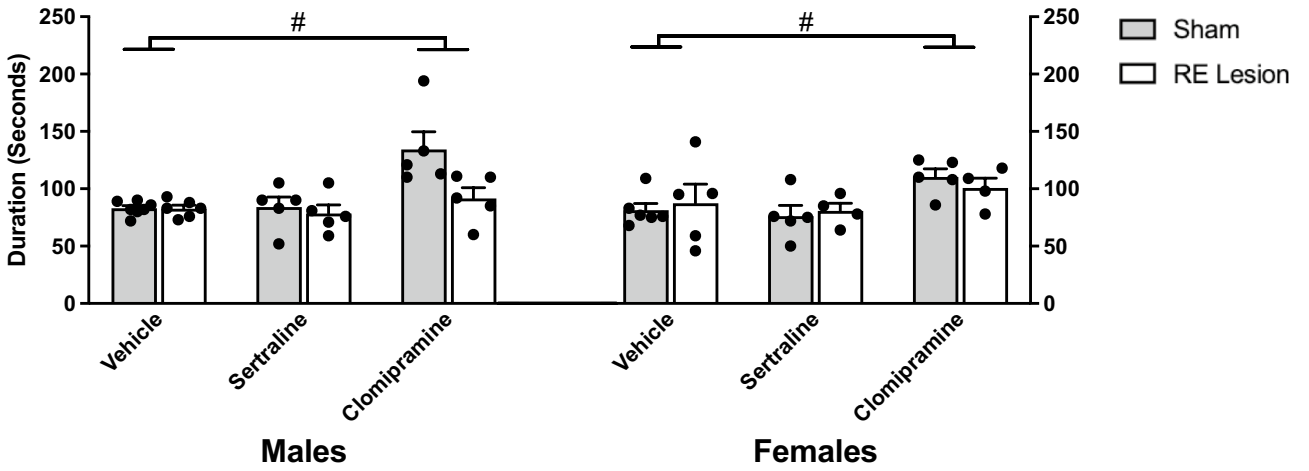

Fig. 3 Climbing duration during the Forced Swim Test (FST). Climbing duration was increased in male and female sham-operated and RE-lesioned rats, that received clomipramine compared to vehicle-treated, respective groups (#=$p<0{,}05$)

sertraline treatment increased head shakes in sham-operated only male and female rats (p = 0.001), (Fig. 4).

c-Fos activity results

PFC c-Fos activity

The three-way ANOVA for prefrontal cortex c-Fos revealed a sex * drug interaction [$F_{(2,50)} = 6.680$ $p = 0.003$]. Post-hoc testing showed that sham-operated and RE lesion female rats treated with either vehicle or sertraline displayed more c-Fos activity than their corresponding male counterparts [$p < 0.001$ for the vehicle-treated pairwise comparisons and p = 0.003 for the sertraline-treated pairwise comparisons]. This sex difference was not observed in clomipramine-treated rats. In fact, post-hoc testing showed that clomipramine-treatment reduced c-Fos activity in female sham-operated and RE-lesion rats in comparison to their vehicle-treated counterparts (p = 0.008), (Figs. 5 and 8 a,b).

Hippocampus c-Fos activity

Similarly, a three-way ANOVA for hippocampal CA1 c-Fos activity revealed a significant sex * drug interaction

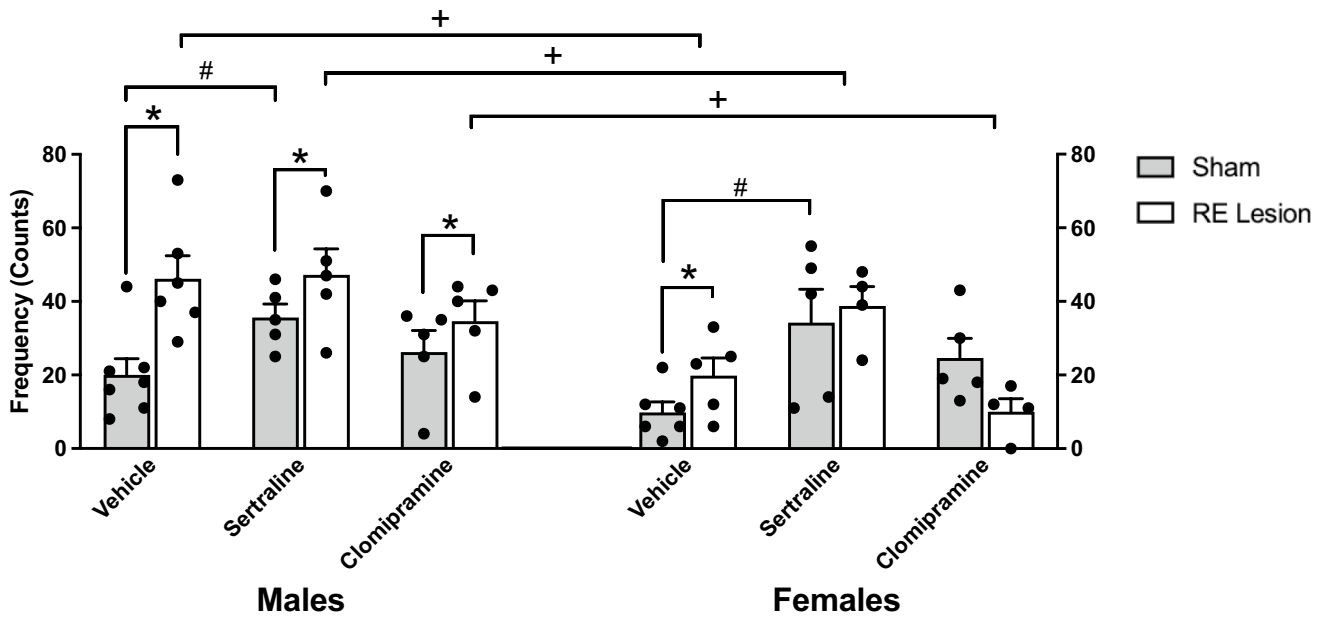

Fig. 4 Head shakes frequency during the Forced Swim Test. Head shakes were enhanced by RE-lesion in all male rats (*=$p<0,05$), irrespectively of their treatment. Head shakes were also enhanced by RE-lesion in vehicle-treated females only (*=$p<0,05$). Sertraline enhanced head shakes in male and female sham-operated rats only (#=$p<0,05$). Male RE-lesioned rats had a higher head shake frequency than RE-lesioned females (+=$p<0,05$), irrespectively of their treatment

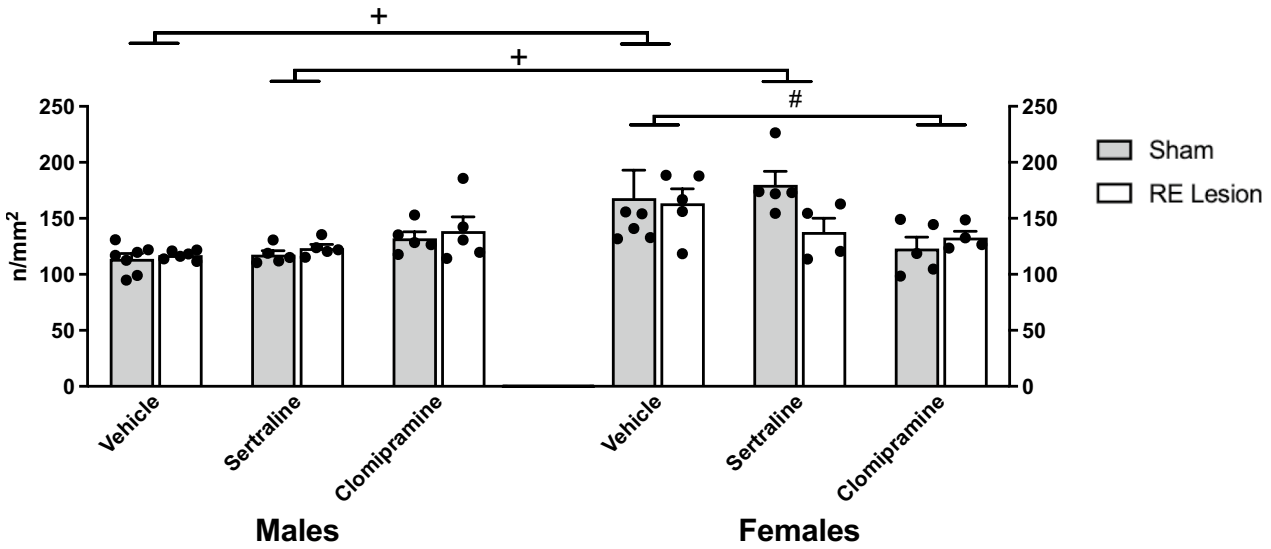

Fig. 5 Activation of the prefrontal cortex (PFC; including prelimbic and infralimbic regions) after the Forced Swim Test (FST). Vehicle- and sertraline- treated females had higher c-Fos activation than respective males (+=$p<0,05$). Clomipramine treatment decreased c-Fos activation in the PFC of sham-operated and nucleus reuniens (RE)-lesioned females only

[$F_{(2,50)} = 14.290$ $p < 0.001$]. According to post-hoc pairwise comparisons, female sham-operated and RE lesion rats treated with either vehicle or sertraline displayed more CA1 c-Fos activity than their corresponding male counterparts ($p < 0.001$; $p < 0.001$ respectively). As was the case for the prefrontal cortex c-Fos activity, female sham-operated and RE lesion rats treated with clomipramine had lower activity in comparison to the corresponding vehicle-treated females ($p < 0.001$), and because of that, the sex difference mentioned previously was not observed between male and female clomipramine-treated rats, (Figs. 6 and 8 c,d).

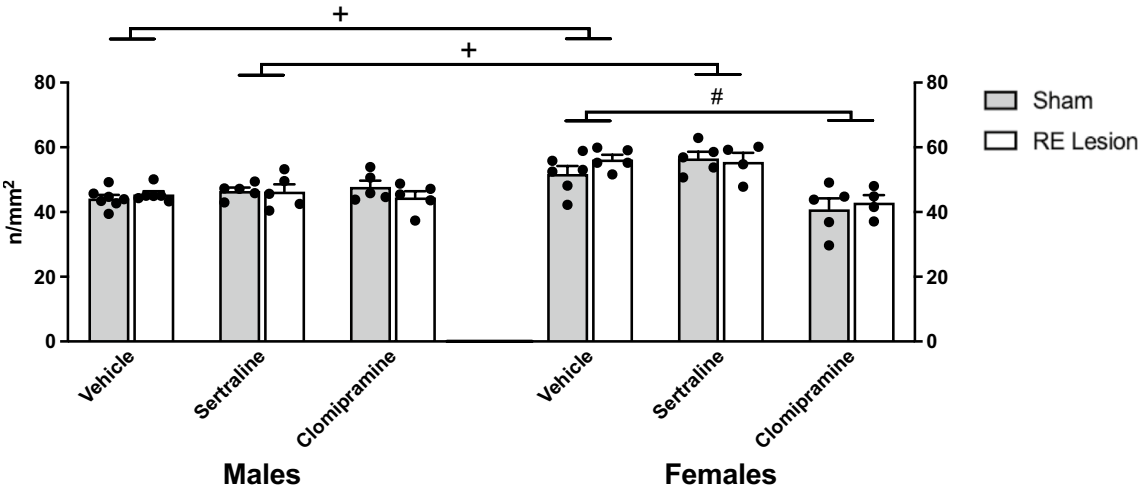

Fig. 6 Activation of the CA1 of the hippocampus after the Forced Swim Test (FST). Similarly to the prefrontal cortex, activation of CA1 of the hippocampus after the Forced Swim Test, was higher in vehicle- and sertraline- treated females than respective males (+ = $p < 0{,}05$). Clomipramine treatment decreased c-Fos activation in the CA1 area of the hippocampus of sham-operated and RE-lesioned females only

Nucleus reuniens c-Fos

A two-way ANOVA for RE c-Fos showed a significant sex main effect [$F_{(1,25)} = 16.158$ $p < 0.001$] and a significant sex * drug interaction [$F_{(2,25)} = 13.272$ $p < 0.001$]. Subsequent post-hoc testing showed that vehicle-treated females had lower c-Fos activity than males ($p < 0.001$). Likewise, sertraline-treated females also had lower c-Fos activity than their male counterparts ($p < 0.001$). However, clomipramine-treated male and female rats had no differences in c-Fos activity. Moreover, clomipramine but not sertraline treatment lowered c-Fos activity in RE in male rats only ($p = 0.005$). No differences were detected following sertraline or clomipramine treatment in female rats, (Figs. 7 and 8 e,f).

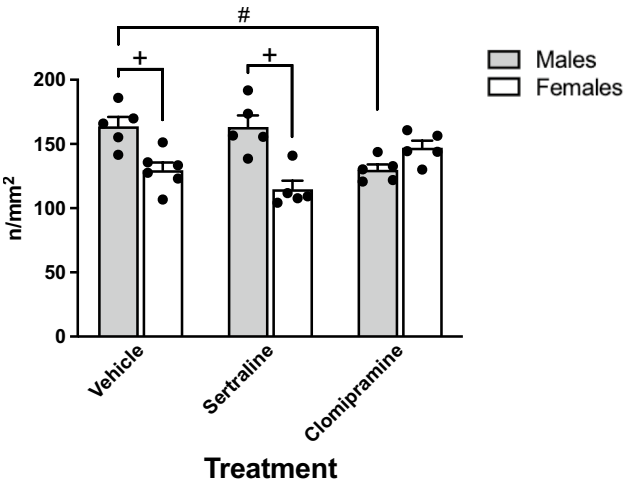

Fig. 7 Activation of the nucleus reuniens (RE) after the Forced Swim Test (FST). Neuronal activation, measured by the density of c-Fos expressing neurons in RE, after the Forced Swim Test (FST). Vehicle- and sertraline-treated females had lower activation compared to vehicle- and sertraline-treated males (+ = $p < 0{,}05$). Clomipramine, but not sertraline, reduced the density of active neurons in the RE after the FST only in male rats, in comparison to vehicle-treated males (# = $p < 0{,}05$)

Correlation of behavioral and c-Fos activity data

As seen in Table 1, a correlation analysis showed that cortical c-Fos activity correlated well with immobility duration in both male and female vehicle-treated sham-operated rats, essentially the "control" groups for both sexes. Swimming behavior was inversely correlated with cortical c-Fos activity, again in both sexes. Interestingly, head shaking frequency correlated with cortical c-Fos activity only in males. No correlations were found between CA1 and RE c-Fos activity and any behavioral parameter in sham-operated vehicle-treated male and female rats. The only exception was that climbing duration was inversely correlated with CA1 activity in sham-operated vehicle-treated female rats (Table 1, part A). Moreover, sertraline and clomipramine treatment abolished the correlation between cortical cFOS activity and behavioral indices, which was observed in "control" male and female rats. Interestingly, a new correlation emerged, with RE c-Fos activity correlating to immobility behavior following clomipramine treatment in both sexes. Sertraline treatment correlated to immobility behavior only in female sham-operated rats. In all groups of rats having undergo the RE lesion surgery (Table 1, part B), the above-mentioned correlations were no more evidenced, possible

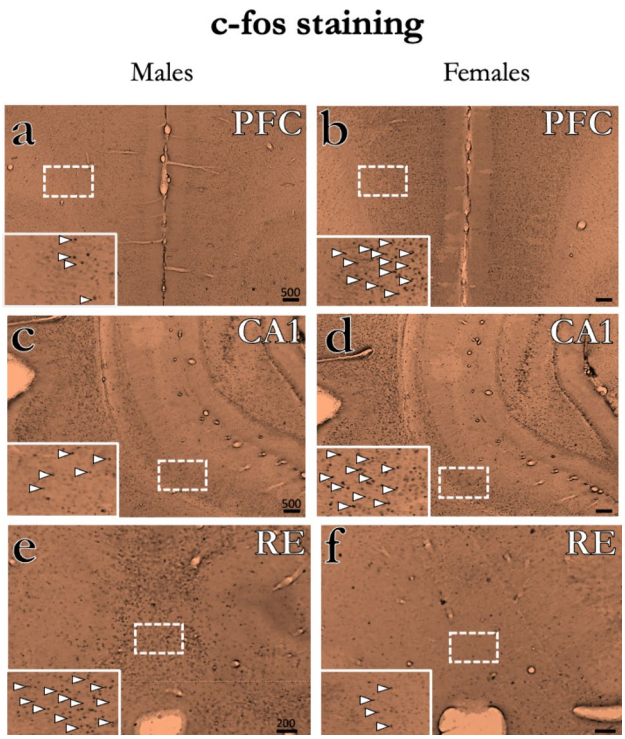

Fig. 8 Representative photomicrographs of c-Fos staining in the prefrontal cortex (PFC; a, b) the CA1 hippocampal area (c, d) and nucleus reuniens (RE; e, f). In the PFC and CA1 hippocampal area, females showed higher c-Fos staining than males (a,b and c,d, respectively), whereas in RE males exhibited an increased c-Fos density compared to females (e,f). The scale bar indicates 500μm of length (a,b,c,d) and 200μm of length (e,f). White arrows are used to point to representative immunoreactive cells from the magnified area

due to the disruption incurred by the RE lesion itself. Interestingly, in male sertraline-treated rats head shaking correlated well with PFC c-Fos activity.

Discussion

The present study aimed to identify sex differences in the behavioral and neuronal indices induced by the disruption of the PFC-hippocampus circuit and compare it with treatment with two antidepressants with different mechanism of action. In the FST, RE lesion proved effective in producing an antidepressant effect in both females and males. Specifically, RE lesion increased swimming and decreased immobility duration, enhanced head shake frequency, but had no effect on climbing duration. Moreover, both antidepressants produced the well-known phenotype at the FST characterized by increased active and decreased passive behavior in both sexes. In particular, similarly to RE lesion, sertraline treatment resulted in increased swimming and decreased immobility duration, as well as enhanced head shake frequency, in both sexes. Notably, climbing behavior was increased only following clomipramine treatment. Interestingly, RE was less active in female compared to male rats and in clomipramine-treated males compared to their vehicle-group. On the other hand, activation of the PFC and CA1 was reduced in clomipramine-treated females, compared to vehicle-treated rats, an effect that was not observed in males. Present results show the main role of RE in shaping the antidepressant response in males and females, but sex is highlighted as a crucial factor in behavioral and treatment response in FST paradigm, as well as in RE related circuit activation.

We firstly examined the effect of sex, antidepressant treatment and RE lesion in behavioral indices of the FST, which is a behavioral paradigm, primarily used for screening and studying substances for antidepressant potential (Cryan et al. 2005; Detke et al. 1995). Specifically, immobility is a passive behavior, while swimming and climbing are active behavioral responses linked to serotonergic and noradrenergic activation, respectively (Detke and Lucki 1995). Female rats, regardless of the disruption of the PFC-Hippocampus circuit, had a higher immobility and a lower swimming time, as repeatedly reported by our group (Dalla et al. 2009a; Kokras et al. 2018) although conflicting results have been reported (for review see (Kokras et al. 2015)). Climbing duration was unaffected by sex, lesion or sertraline, while only clomipramine increased its duration, as previously found, because of its noradrenergic action (Detke and Lucki 1995). Importantly, in this study we demonstrate that the disruption of the PFC-Hippocampus circuit by lesioning the RE nucleus results in a clear antidepressant effect in female rats as well, in agreement with our previous observations in male rats (Kafetzopoulos et al. 2018, 2021). Regarding immobility duration this effect is comparable in terms of size to that of established antidepressants like sertraline and clomipramine. Notably, the behavioral effect of RE- lesioning was similar to that of the SSRI sertraline, whereas it was differentiated from that of clomipramine, which is a tricyclic antidepressant, it is non-selective and acts through serotonergic and noradrenergic mechanisms. This may be explained in part by the fact that RE receives heavy innervation of serotonergic fibers (Vertes et al. 2010), whereas there is no evidence of existence of noradrenergic or dopaminergic fibers in the RE.

Next, we examined whether these behavioral findings could be linked to specific brain region activation and elucidate the role of each region and their circuit. The patterns of brain activation in FST has been well characterized in the rat (Cullinan et al. 1995; Duncan et al. 1993; Silva et al. 2012) and the activation of PFC and hippocampus is prominent, among other limbic and cortical areas (Duncan et al. 1996; Kawahara et al. 2013; Silva et al. 2012). Specifically, FST has been shown to elevate activation via increased c-Fos activity in all brain regions in question (PFC, RE, hippocampus) (Cullinan et al. 1995). Moreover, it has been

Table 1 Correlations between FST behaviors and c-Fos activation in the PFC, CA1 and the RE

		Males			Females		
		PFC	RE	CA1	PFC	RE	CA1
A. Sham	Vehicle						
	Immobility	0.778 p=0.04	*ns*	*ns*	0.964 p=0.002	*ns*	*ns*
	Swimming	-0.932 p=0.002	*ns*	*ns*	-0.898 p=0.015	*ns*	*ns*
	Climbing	*ns*	*ns*	*ns*	*ns*	*ns*	-0.816 p=0.048
	Head Shaking	0.857 p=0.014	*ns*	*ns*	*ns*	*ns*	*ns*
	Sertraline						
	Immobility	*ns*	*ns*	*ns*	*ns*	0.896 p=0.04	*ns*
	Swimming	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
	Climbing	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
	Head Shaking	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
	Clomipramine						
	Immobility	*ns*	0.883 p=0.047	*ns*	*ns*	0.954 p=0.012	*ns*
	Swimming	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
	Climbing	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
	Head Shaking	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
B. Lesion	Vehicle						
	Immobility	*ns*	lesioned	*ns*	*ns*	lesioned	*ns*
	Swimming	*ns*	lesioned	*ns*	*ns*	lesioned	*ns*
	Climbing	*ns*	lesioned	*ns*	*ns*	lesioned	*ns*
	Head Shaking	*ns*	lesioned	*ns*	*ns*	lesioned	*ns*
	Sertraline						
	Immobility	*ns*	lesioned	*ns*	*ns*	lesioned	*ns*
	Swimming	*ns*	lesioned	*ns*	*ns*	lesioned	*ns*
	Climbing	*ns*	lesioned	*ns*	*ns*	lesioned	*ns*
	Head Shaking	0.964 p=0.008	lesioned	*ns*	*ns*	lesioned	*ns*
	Clomipramine						
	Immobility	*ns*	lesioned	*ns*	*ns*	lesioned	*ns*
	Swimming	*ns*	lesioned	*ns*	*ns*	lesioned	*ns*
	Climbing	*ns*	lesioned	*ns*	*ns*	lesioned	*ns*
	Head Shaking	*ns*	lesioned	*ns*	*ns*	lesioned	*ns*

PFC: Prefrontal cortex, *RE:* Nucleus reuniens, *CA1:* Cornu ammonis 1 area of the hippocampus, *Minus sign (-):* Negative correlation, *Plus sign (+):* Positive correlation, *ns:* Not statistically significant, *lesioned*: analysis not performed in the concerned area due to lesion

demonstrated that antidepressant treatment elicits c-Fos expression, a marker for neuronal activation, in brain areas implicated in stress response and depression, including the PFC and the hippocampus (Beck 1995; Duncan et al. 1996). Tricyclic antidepressants and SSRIs activate, PFC while only tricyclic antidepressants activate PFC neurons that project to limbic areas (Chang et al. 2015). Additionally, antidepressant treatment can abate the activation of brain regions induced by FST (Jama et al. 2008). Interestingly, in the present study, FST behaviors, such as immobility and swimming duration were correlated with c-Fos activation in vehicle-treated, sham-operated rats, indicating an effect of swim stress on c-Fos activation, which is prevented by RE lesion or antidepressant treatment. This correlation was significant in the PFC and not in the hippocampus, as we have previously shown, that in the FST in males, behavioral response is linked to PFC rather than hippocampal serotonergic activity (Mikail et al. 2012).

Sex is emerging to be an important player in understanding behavioral models and tests (Kokras and Dalla 2017) and there have been reports about sex differences following other stressors in c-Fos activation (Bland et al. 2005; Girard-Joyal et al. 2015; Moench et al. 2019; Ter Horst et al. 2009). Notably, FST neurobiology is fairly characterized in male experimental animals, but less is known about whether male and female rats differ in terms of neuronal activation. To our knowledge, studies that examine activation of brain regions for both sexes in FST or any similar paradigm are scarce. What is known is that females exhibit higher levels of activation in the PFC (Perkins et al. 2017). Stress effects

on the hippocampus have been shown to be influenced by sex (Dalla et al. 2009b; Galea et al. 1997; McLaughlin et al. 2005), and this differential response is comparable to hippocampus-mediated behaviors (Bowman et al. 2009; Luine 2002). Similarly, FST-induced neurochemical changes in the PFC are influenced by sex (Dalla et al. 2008; Kokras et al. 2018; Mikail et al. 2012).

In the present study neuronal activation in the PFC was higher after the FST in female rats compared to males, a finding relevant to recent reports in rats exposed to social interaction (Perkins et al. 2017). However, herein clomipramine attenuated this sex difference, by reducing c-Fos activation, while sertraline had no effect. This is of interest, as aberrant PFC activity, and in turn its normalization, seem to mediate depression and its remission (Diener et al. 2012; Herrington et al. 2010). The efficacy of SSRIs and tricyclic antidepressants in males and females is another emergent point. It has been debated that SSRIs may be more efficient in women (Keers and Aitchison 2010; Pavlidi et al. 2023). Moreover, serotonergic drugs, but not noradrenergic, enhance activation in the PFC, and this enhancement correlates with treatment response (Gyurak et al. 2016). Hence, sex may be interacting at a fundamental neuronal level with serotonergic drugs. Pertinent to this, sex hormones and estradiol is known to modulate serotonin receptors and serotonin transporter, but not noradrenalin receptors (Pitychoutis et al. 2012; Sell et al. 2008). Similarly, to PFC activation, females exhibited an increased activation of the hippocampus following FST compared to males. This pattern was preserved when females were treated with sertraline. However, activation was decreased after clomipramine administration.

Therefore, in the present study, two different antidepressants exerted a sex- and brain-region specific effect on c-Fos expression, in broader agreement with similar previous findings (Chang et al. 2015; Ionov et al. 2019; Miyata et al. 2005), further highlighting the need to validate results on both sexes. However, several other possible explanations can be offered regarding the differential effects of sertraline and clomipramine in the present study. We used the same dose in both males and females, however there are known sex differences in the pharmacokinetics of SSRIs and tricyclic antidepressants (Kokras et al. 2011). Therefore, this differential effect of sertraline and clomipramine could be attributed to different brain and serum levels of antidepressants. Moreover, we have recently discussed that antidepressants by themselves may exert a direct action on hormone levels, thus modifying in turn c-Fos activity in a sex- and brain-region specific manner (Pavlidi et al. 2021b). This could also be related to the finding that head shakes frequency during the FST was enhanced by RE lesioning and by sertraline, but not by clomipramine treatment. Also, all RE-lesioned male rats had higher head shake frequency than respective females. Head shake frequency in the FST is a behavior that has been shown to correlate with testosterone levels and is influenced by some antidepressant treatments (Kokras et al. 2017b). It remains to be elucidated in future studies, whether head shake frequency in the FST is also influenced by 5-HT and the activation of its receptors, as it is the case with head twitching behavior in the open field (Essman et al. 1994; Freo et al. 2010; Wettstein et al. 1999).

Regarding nucleus reuniens (RE), this is a thalamic structure that connects PFC and hippocampus and is activated in FST (Cullinan et al. 1995). As mentioned, we have recently shown that it is crucially involved in preventing the detrimental effects of stress and promoting resilience in models of depression (Kafetzopoulos et al. 2018, 2021). In this study, female rats exhibited increased immobility in the FST, but interestingly lower activation of the RE than males. Moreover, sertraline treatment reduced passive FST behaviors, but did not affect the RE activation. On the contrary, clomipramine treatment reduced c-Fos activation in males and there was a positive correlation between immobility levels and RE c-Fos activation. Hence, this nucleus might not be a direct target of serotonergic modulation, but could be a target for monoaminergic modulation of the entire circuit in general. Since none of the drugs altered PFC or CA1 activation in males, it can be postulated that this circuit is involved in the FST in terms of overall activation mainly in females, but not in males. It has been shown that activation alone only partly describes behaviors seen in the FST, with circuit synchrony and communication playing a pivotal role, among others (Kafetzopoulos et al. 2018). More interestingly, it has been demonstrated that depressive-like behavior is connected to increased delta and reduced theta power only in the hippocampus in males, but globally in females (Theriault et al. 2021). As a result, it can be speculated that females employ more readily and more widely circuits such as the one described here.

This study is limited by the fact that only FST and not chronic mild stress, was applied in female rats. FST is not a model of depression and the behaviors exhibited have been interpreted, either as despair and helplessness, but also as coping behaviors to stress (Molendijk and de Kloet 2022). However, it is a useful tool and in the present study, it was used as a test of antidepressant activity.

Moreover, future studies can shed light on the contribution of different neurotrasmitter's pathways, i.e. glutamatergic, serotonergic, noradrenergic, on the role of the RE in depression and stress response. In conclusion, this study highlights the importance of sex as a factor when interpreting findings pertaining to circuits and their contribution to depressive-like phenotypes. Indeed, sex differences can emerge not only in behavioral tests and treatment response, but also in the contribution of infralimbic structures in depression. Importantly, we demonstrate that the RE is indeed crucially involved in the stress response and in

continuation of previous studies (Kafetzopoulos et al. 2018, 2021) we report that our findings, regarding the antidepressant effect of RE lesioning, apply to female animals, as well.

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Declarations

Conflict of interest The authors declare no conflicts of interest.

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Authors and Affiliations

V. Kafetzopoulos[1,2] · N. Kokras[3] · Filippos Katsaitis[4] · N. Sousa[5,6,7] · H. Leite-Almeida[5,6] · I. Sotiropoulos[4,5,6] · C. Dalla[1,8]

✉ C. Dalla
cdalla@med.uoa.gr

[1] Department of Pharmacology, Medical School, National and Kapodistrian University of Athens, 75 Mikras Asias St, Athens 11527, Greece

[2] Department of Psychiatry, Medical School, University of Cyprus, Nicosia, Cyprus

[3] First Department of Psychiatry, Medical School, National and Kapodistrian University of Athens, Athens, Greece

[4] Laboratory of Brain Exosomes & Pathology, Institute of Biosciences & Applications, NCSR Demokritos, Athens, Greece

[5] Life and Health Sciences Research Institute (ICVS), School of Medicine, University of Minho, Campus de Gualtar, Braga, Portugal

[6] ICVS/3B's - PT Government Associate Laboratory, Braga, Guimarães, Portugal

[7] Clinical Academic Center (2CA), Braga, Portugal

[8] Second Department of Obstetrics – Gynecology, Aretaieio Hospital, School of Medicine, National and Kapodistrian University of Athens, Athens, Greece