The African striped mouse *Lemniscomys barbarus* as a model for aggression. Brain areas activated by agonistic encounters

Nisrin El Mlili¹, Rachid Boutoual², Ana M. Sanchez-Perez³, Ali Ouarour², Ibtissam Chakir², Mohamed Errami², Francisco E. Olucha-Bordonau³

¹Institut Supérieur de Profession Infirmières et Techniques de Santé, Rabat. Morocco, ²Faculté des Sciences, Université Abdelmalek Essaâdi, Tétouan. Morocco, ³Unidad Predepartamental de Medicina, Facultad de Ciencias de la Salud, Universitat Jaume I, Castellón de la Plana 12071 (Spain), ⁴present address, Faculté de Médecine et de Pharmacie. Université Sultan Moulay Slimane, Fez Morocco

**SUMMARY**

During agonistic behavior several brain areas became differentially activated depending on the role the subject is taking. Several areas are mostly activated during the offender role and several others are activated if the subject plays a defensive role. The main goal of this work is to study in detail the anatomic areas involved in agonistic behavior using a novel animal model, the striped mouse *Lemniscomys barbarus*, a North African diurnal rodent well known by its natural high aggressiveness toward conspecifics. After social encounters, neural activation in brain areas related to agonistic behavior was measured by c-fos immunostaining. The encounters were recorded and behaviors related to the encounter were analyzed. We differentiated between the aggressive behavior (offender) and escape behavior (defender or defeated). Our results showed that conspecific confrontation induced general c-fos activation in both offender and defender in all measured areas in comparison with non-confronted control. Differences in neural activity between offender and defender were observed specifically in the lateral, cortical and medial amygdala, suprachiasmatic nucleus and the nucleus incertus, suggesting a potential role of these areas in displaying different kinds of behavior during conspecific confrontation. We found that, while in the lateral, medial and cortical amygdala defenders express significantly more c-fos than offenders, in the nucleus incertus of the brainstem the differential activation is just the opposite. Additionally, defenders display significantly more freezing than offenders. This work provides data showing that *Lemniscomys barbarus* is a widely useful model to study the anatomic background supporting agonistic behavior.

**Key words:** Amygdala – Social behavior – c-fos – Immediate early genes – Fear – Emotion – Aggression

**Abbreviations:** Acc – Nucleus Accumbens, AHA – Anterior Hypothalamic Area, APT – Anterior pretectum, BLA – Basolateral Amygdala, BMA – Basomedial Amygdala, C – Control, CeA – Central Amygdala, CoA – Cortical Amygdala, Def – Defender, DK – Nucleus of Darkschwitz, DL – Dorsolateral column of the periaqueductal gray, DM – Dorsomedial column of the periaqueductal gray, DTg – Dorsal tegmental nucleus, EW – Nucleus of Edinger Westfal, ICj – Insula of Calleja, IEG – Immediate early genes, IL – Infralimbic cortex, IP – Interpeduncular nucleus, ITC – Intercalat-

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**Corresponding author:** Francisco E. Olucha-Bordonau. Unidad Predepartamental de Medicina, Facultad de Ciencias de la Salud, Universitat Jaume I, Av Vicent Sos Baynat, Castellón de la Plana 12071, Spain. Phone: +34964387460; Fax: +34 964 72 90 16. E-mail: folucha@uji.es


### INTRODUCTION

Aggression or agonistic behavior was originally defined as a strategy of adaptation to situations involving physical conflict (Scott, 1966). Aggressive behavior may increase the opportunities of an individual to obtain resources like food and water. But not all types of aggression have positive outputs and, when it becomes escalated, it could lead to serious injuries or death.

During a dyadic encounter, one of the subjects (the dominant-offender) obtains a positive outcome while the opponent (the subordinate-defender) displays a default reaction rather than escalation. After the encounter, the dominant subject only needs to approach or threaten the subordinate to obtain the resource (Drews, 1993). According to this view, the stability of the relationships depend on submissive and escape kinds of behavior of the subordinates that have behaved as defenders in the encounter (Kaufmann, 1983). In humans, escalated aggressiveness is itself a pathological personality disorder as typified by DSM5 (DSM5, 2013). In addition, repeated defeat leads to serious psychological trauma, decrease of self-esteem, feeling inferior, PTSD and depression (Carvalho et al., 2013). Subordination is also a source of organic and cerebral affecting organs like testes, thymus or spleen in addition to serotonin metabolism (Blanchard et al., 1993). In addition, even the resilience from social defeat may lead to changes in fear and extinction (Meduri et al., 2013). In this context it is of particular interest to have animal models to study the brain areas differentially activated when displaying offensive or defensive behavior during co/specific encounters.

It has been also postulated that species-specific defensive reactions like freezing and flight are triggered by all kinds of threats including natural predators, conspecific opponents or electrical foot-shocks (Bolles, 1970). However, it has been found that separated hypothalamic circuits support defensive responses to a predator, a dominant conspecific or subordinated conspecific (Motta et al., 2009).

Several animal models have been used to explore the neuroanatomical, neurochemical and behavioral profiles of aggression (Adams, 2006; Ramirez, 2006) including artificially inbred (Nehrenberg et al., 2013); genetically modified strains (Miczek et al., 2001) or naturally aggressive rodent species displaying higher belligerent behavior (Takahashi and Miczek, 2014). Selective breeding of highly aggressive and nonaggressive offspring results in artificially generated strains displaying different kinds of agonistic behavior like pronounced inter-male aggression (van Oortmerssen and Bakker, 1981) or isolation-induced aggression (Sandnabba, 1996). Within the naturally occurring aggressive strains, male hostility may occur directed towards other males (NZB/BINJ strain) or towards females (FVB/NtacBR strain). By contrast, other strains are naturally tame (A/J mice), and do not display aggressive behavior (Canastar and Maxson, 2003; Roubertoux et al., 2005). Interestingly, these different aggression pattern behaving strains respond to ambient factors like defeating, maternal separation and stress in a variety of ways indicating diverse genetic susceptibility to environmental challenges (Schneider et al., 1992).

In rodents, males that are confronted display either an aggressive pattern, (i.e., the offender) or a submissive pattern (i.e. the defender) (Blanchard and Blanchard, 1977; Adams, 2006). The offender behavior is characterized by movement towards the opponent, and attacks or bites on body flanks or the back of the opponent, in general not vulnerable areas; and by contrast, the defender behavior includes escape movements or freezing and, if any, attacks to vulnerable areas as the face, (Adams, 1980).

Studies on the anatomical circuits involved in aggression during conspecific confrontation have been done with rodent models by analyzing the immediate early genes (IEG) activation in different brain areas following encounters between conspecifics (Veene and Neumann, 2007; Konoshenko et al., 2013). Areas and centers participating in agonistic behavior include prefrontal cortex, amygdala, septum, different hypothalamic regions and the periaqueductal grey area (Kollack-Walker et al., 1997; Siever, 2008; Toth et al., 2010; Konoshenko et al., 2013). Konoshenko and co-workers showed that neuronal activation in the anterior hypothalamic area (AHA) was significantly higher in the offender males compared to their defeated counterparts using the resident-intruder test. Thus, they proposed that lower activation of the AHA is therefore associated with attenuation of inter-male aggression (Konoshenko et al., 2013).

However, up to date, the anatomical areas involved in one or the other pattern of behavior, and the reasons why a subject follows one or the other, are not completely understood. A major drawback
in this type of studies comparing both patterns of behavior is the lack of an animal model that clearly displays both behavioral prototypes in order to carry out a comprehensive analysis of anatomical areas involved.

In our present study, we used a new animal model for aggression studies, the striped grass mouse (Lemniscomys barbarus L) (Lb), a wild diurnal rodent. This rodent displays a natural aggressiveness toward other individuals of its own species and has already been described as a valuable model to study circadian activity (Lahmam et al., 2008; Chakir et al., 2015). Another African striped mouse, Rhabdomys pumilio, has been used to study reproductive suppression when shifted from solitary living to group living males and changes in hormones involving this transit (Schoepf and Schrading, 2013). Thus, having different models of rodent behavior may provide a better inside into the neural circuits commonly affected in social behavior.

The main goal of this study is to identify brain areas activated during confrontation and to analyze which anatomical areas are differentially activated in the offending (attacker) or defending (subordinate) subject. To this end, subjects underwent a conspecific confrontation test and the expression of c-fos was measured in brain areas. We found that specific regions show different pattern of activity in offenders and defenders.

MATERIALS AND METHODS

Animals

Lemniscomys barbarus is a rodent (suborder: Sciurognathi; family: Muridae) with a body length of 9 to 12 cm, plus a fine tail of 10 to 15 cm. The coat of its back and flanks is lined longitudinally with 11 brown lines. This species has a strictly African distribution, and can be commonly found in North and sub-Saharan Africa in dry and semi-arid zones. It is frequent in local biotopes relatively wet and rich in plants. Animals used in the present study were trapped in the region of Tetouan (north of Morocco) around the village of Benkarrich (approx. 35830 N, 5825 W). Captures were made throughout the summer (June 15th to September 15th) with traps checked and baited daily with fresh food (bread and olive oil). After transportation to the laboratory, animals were housed individually in transparent cages (22 x 16 x 14 cm) under controlled photoperiodic conditions 12h light - 12h dark (LD) cycles and controlled temperature of 23±3C for at least 4 weeks before any experimental manipulation. Water and regular rodent lab food were supplied ad libitum. All animal manipulations were made in Morocco in agreement with local legislation. The Moroccan Ministry of Agriculture has authorized the use of these animals for research in the laboratory conditions of the Faculty of Sciences of the University of Tetouan. Moreover, all procedures used in animal experimentation complied with the European Communities Council Directive 86/609/EEC. All efforts were made to minimize the number of animals used and their suffering.

Conspecific confrontation test

Twenty-five animals were used in the present study, with body weight ranging between 33 and 49 g. Each couple of experimental animals has been placed in a new neutral cage to be confronted; their behavior was registered during 25 min. During the first fifteen minutes the animals were placed in the same cage but separated by a grill to avoid physical contact, permitting only olfactory recognition. Then, the grill was removed and the animals may come into physical contact with each other during ten minutes. The encounter was videotaped and two observers, blind to the experimental conditions, measured the behavioral interactions and confrontation between each couple of animals. In case of strong fight, the experiment was stopped and retired from the analysis. This happened in one case.

The control group is composed of naïve animals that have not been exposed to any confrontation test or experimental manipulation.

Behavioral analysis

For each pair, a subject was considered as offender when it chased the conspecific and hit it mostly to the flanks; and defender when escaping away from the offender’s attack, and defending himself from opponent attack by hitting the opponent’s face (Adams, 1980). Once categorized as offender or defender, behavioral data were collected for each animal, which included latency before the first attack, number of attacks with bites, number of attacks without bites (the offending animal displays an approaching movement to its partner in the cage without biting), time of freezing and number of freezing episodes (Table 1). We considered it a freezing behavior when animals remained totally immobile (except for respiratory movement) during at least 2 seconds.

Brain fixation and sectioning

One hour after the behavioral test of confrontation, subjects from each experimental group were deeply anaesthetized with an intraperitoneal injection of 5% chloral hydrate at a dosage of 40 mg/kg and transcardially perfused with saline (250 ml) followed by fixative solution (4% paraformaldehyde in 0.1M PB, pH 7.4) for 10 min (~250 ml). Brains were removed from the skull and immersed in the same fixative for 24 h at 4°C. Then, brains were cryoprotected in 30% sucrose in 0.01 M phosphate-buffered saline (PBS), pH 7.4, for 48 h at 4°C. Coronal brain sections were obtained (40 µm) using a freezing slide microtome (Leica SM2010R, Leica Microsystems, Heidelberg, Germany). For
each brain, 6 series of sections were collected in 30% sucrose in 0.01M PBS. One series was used for Nissl stain, another one was directly used for c-fos immunohistochemistry and the rest were frozen at -40°C.

**c-fos immunohistochemistry**

For measuring c-fos activity, immunohistochemistry was carried out on free-floating sections. Sections were rinsed 2 x 10 min and immersed in a blocking media containing 4% normal donkey serum (NDS, Jackson Immunoresearch, West Grove, PA USA) and 2% bovine serum albumin (BSA) (Sigma, St Louis Mo) in Tris (pH 8, 0.05 M)buffered saline (TBS) containing 0.1% Triton X-100 (Sigma, St Louis Mo) (TBS-Tx) for 1 h at room temperature. Sections were then incubated overnight in primary antibody solution containing 1:10.000 rabbit anti-c-fos (PC38 Anti-c-Fos (Ab-5, Calbiochem, St Louis Mo), 2% BSA and 2% NDS in TBS-Tx. Slices were rinsed 3 times in TBS-Tx and incubated in 1:200 dilution of biotin-donkey anti-rabbit (cat 711-065-152, Jackson Immunoresearch) in TBS-Tx during 2 hours. Sections were then rinsed and transferred to 1:50 ABC (Vectastain-Elite, Cat No. PK-6100; Vector Laboratories, Burlingame, CA, USA) in TBSTx. After rinsing (3 x 10 min) in 0.05 M TBS and (2 x 10 min) in TB, the peroxidase activity was revealed by the use of the chromogen 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma) (0.025%) and 0.25% ammonium nickel (II) sulfate hexahydrate (A1827 Aldrich St Louis Mo) dissolved in TB in the presence of H2O2 (0.003%) for 15 minutes. The colorimetric reaction was stopped by successive rinsing of sections in TB followed by 3x10 mins in PBS 0.01M. Finally, sections were mounted in chrome alum coated slides and overnight air dried. Finally, sections were re-hydrated, cleared with graded ethanol and xylene and coverslipped in DPX (Sigma).

**Giemsa staining**

For the cytoarchitectonic study, Giemsa’s stain was used according to a commonly used standard protocol (Iñiguez et al., 1985). Briefly, sections were mounted on chrome-alum coated slides, rinsed 2x5 min in 0.06 M KH2PO4 (pH 4.5) at 60°C and were dipped in a 1/10 solution of Giemsa stock solution from Sigma (cat # GS-500) at 60°C for 12 min. Then slides were rinsed in the 0.06 M KH2PO4 at room temperature in a shaker table 3x5 min. Finally, sections were dehydrated in ethanol, cleared with xylene and coverslipped with DPX.

**Image analysis and c-fos positive cells quantification**

Images were acquired using a Nikon Eclipse E600 (Nikon, Tokyo, Japan), equipped with a Nikon D-MX-2000 camera connected to a PC with ACT-1 acquisition software (Nikon, Tokyo, Japan). We used the stereotaxic mice Atlas (Paxinos and Franklin, 2012) to delineate the analyzed areas. The Geimsa series were also used to assess the boundaries of the analyzed nuclei. For c-fos quantification, we used the 20x objective and measure c-fos activity as described (Perez-Villalba et al., 2005). Briefly, using Image J software, the background of the images was automatically removed with a rolling ball radius of 50.0 pixels, only labeled areas of more than 12 pixels were considered to be positive. Three sections per case were analyzed and the mean value of these three sections was considered as a single value for this case and nucleus. Data were expressed as the number of c-fos positive cells by mm2 of the analyzed area (Table 2). An observer blind to experimental conditions conducted all analyses.

**Table1. Behavior analysis of Lemniscomys barbarus conspecific confrontation**

<table>
<thead>
<tr>
<th>Sex of confronted animals</th>
<th>Number of freezing episodes</th>
<th>Total time of freezing from 600 sec</th>
<th>Latency before the first attack (second)</th>
<th>Total number of attacks</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Offender (n=9)</strong></td>
<td><strong>Defender (n=9)</strong></td>
<td><strong>Offender</strong></td>
<td><strong>Defender</strong></td>
<td><strong>Offender</strong></td>
</tr>
<tr>
<td>Male 1</td>
<td>Male 2</td>
<td>12 13</td>
<td>252 287</td>
<td>62 10</td>
</tr>
<tr>
<td>Male 4</td>
<td>Male 3</td>
<td>9 13</td>
<td>342 440</td>
<td>3 6</td>
</tr>
<tr>
<td>Male 5</td>
<td>Male 6</td>
<td>4 14</td>
<td>68 390</td>
<td>186 59</td>
</tr>
<tr>
<td>Male 7</td>
<td>Female 1</td>
<td>2 6</td>
<td>8 56</td>
<td>124 57</td>
</tr>
<tr>
<td>Female 2</td>
<td>Male 8</td>
<td>4 7</td>
<td>73 418</td>
<td>94 1</td>
</tr>
<tr>
<td>Male 10</td>
<td>Female 4</td>
<td>0 8</td>
<td>0 497</td>
<td>11 35</td>
</tr>
<tr>
<td>Female 6</td>
<td>Female 5</td>
<td>4 8</td>
<td>88 248</td>
<td>5 9</td>
</tr>
<tr>
<td>Female 7</td>
<td>Female 8</td>
<td>3 6</td>
<td>41 376</td>
<td>195 2</td>
</tr>
</tbody>
</table>
defenders. One-way analyses of variance (ANOVA) followed by Newman-Keuls post hoc test were used to compare the number of c-fos positive cells between the three experimental groups (offenders, defenders and control animals) in all areas studied.

RESULTS

Behavior

During the confrontation test we found that in most cases one of the animals took an offender role while the other displayed a defensive behavior. In one case, the two subjects took the role of offender and defender alternatively and this couple was removed from the study. We measured the first attack latency, which ranged from 3 sec to 19.5 sec (Table 1). In addition to offensive or defensive behavior parameters we measured freezing time, for both offenders and defenders. On average, freezing time for defenders (327 ± 45.01 n=8) was significantly higher than freezing time for offenders (115.4 ± 38.7 n=8; Student t-test t=3.57 p=0.0025). All behavioral parameters followed a normal distribution for both behavioral patterns according to normality Shapiro Wilk test.

c-fos immunohistochemistry in particular anatomic areas

Nickel intensified immunohistochemistry (IHC) reaction to c-fos rendered blue-black labeling corresponding to activated neuronal nuclei. The outlining of each nucleus was followed by comparison with alternate Giemsa stained sections. In the present study, we found that conspecific confrontation induced general c-fos activation in both offenders and defenders in all measured cerebral areas in comparison with naïve control. The studied areas included the prefrontal cortex, septum, amygdala, anterior hypothalamus, suprachiasmatic nucleus, periaqueductal grey matter and nucleus incertus (Table 2).

Prefrontal cortex (PFC). The prefrontal cortex that we have analyzed in the present study corresponded approximately to level Bregma 1.54 mm of the mouse stereotaxic atlas (Paxinos and Franklin, 2012). At this level the prelimbic cortex occupied the dorsal part and contained a clear granular layer II and a wider layer III while the infralimbic cortex was composed of a dispersed layer II and a thinner layer III (Fig. 1A). However, it was difficult to delineate PrL and IL areas in c-fos reacted sections and we have considered both areas in the study of c-fos quantification. Conspecific confrontation induced a significant increase (F(3,6-7) = 40.66, Newman Keuls post-hoc p<0.05) in c-fos expression in offenders (633.5±35.03 n=8) and defenders (603.3±55.08 n=8) vs controls (227.03±20.56 n=7) (Fig. 1B). No significant differences were reached between offenders and defenders. Representative images are shown (Figs. 1 C-E).

Amygdala. The amygdala appeared to be larger in Lb than other mice strains, and the lateral nucleus more developed at caudal levels (Fig. 2A). For this work, we considered Bregma ~ –1.34 (Paxinos and Franklin, 2012). At that level, lateral amygdala (LA) is encapsulated between the external and intermediate capsule. Ventral to LA, the basolateral amygdala (BLA) characteristically contained large cells. Just inside the intermediate capsule a dense band of cells corresponded to one of the

Table 2. Statistical analysis of cfos expression in brain areas of Lemniscomys barbarus after conspecific confrontation test

<table>
<thead>
<tr>
<th>Brain area</th>
<th>Subarea</th>
<th>Control Mean ± SE</th>
<th>Offender Mean ± SE; statistical significance</th>
<th>Defender Mean ± SE; statistical significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prefrontal cortex</td>
<td>Whole area</td>
<td>227.3 ± 20.56</td>
<td>633.5 ± 35.03, *</td>
<td>603.3 ± 55.08, **</td>
</tr>
<tr>
<td></td>
<td>BL</td>
<td>106.8 ± 23.19</td>
<td>310.9 ± 27.4; ***</td>
<td>255.6 ± 35.9; ***</td>
</tr>
<tr>
<td></td>
<td>BMA</td>
<td>86.53±15.68</td>
<td>275.6±38.91 ***</td>
<td>222.1±20.79; ***</td>
</tr>
<tr>
<td>Amygdala</td>
<td>CeA</td>
<td>109.1±19.91</td>
<td>186±23.15 *</td>
<td>205.7±20.58 *</td>
</tr>
<tr>
<td></td>
<td>La</td>
<td>108.6±20.55</td>
<td>304.8±25.39 ***</td>
<td>465.9±25.84 ***</td>
</tr>
<tr>
<td></td>
<td>MeA</td>
<td>159.8±33.23</td>
<td>464.6±65.14 ***</td>
<td>795.9±57.72 ***; ***#</td>
</tr>
<tr>
<td></td>
<td>CoA</td>
<td>93.81±19.04</td>
<td>403.6±35.85 ***</td>
<td>524.3±34.54 ***; ***#</td>
</tr>
<tr>
<td>Septum</td>
<td>LSD</td>
<td>69.42±17.92</td>
<td>170.1±28.3,*</td>
<td>184.6±37.41 *</td>
</tr>
<tr>
<td></td>
<td>LSI</td>
<td>147 ± 36.61</td>
<td>341.1±29.45 *, ***</td>
<td>313.2±54.65, *</td>
</tr>
<tr>
<td></td>
<td>LSV</td>
<td>238.7±39.13</td>
<td>499.7±32.51, ***</td>
<td>599.9±54.21, ***</td>
</tr>
<tr>
<td></td>
<td>MS</td>
<td>111.9±20.01</td>
<td>272.3±22.02, ***</td>
<td>238.3±23.11, ***</td>
</tr>
<tr>
<td>Suprachiasmatic</td>
<td>Whole area</td>
<td>178 ± 17.69</td>
<td>710 ± 115.7, **</td>
<td>498.6±32.38, **; **#</td>
</tr>
<tr>
<td>nucleus</td>
<td>Anterior Hypothalamus</td>
<td>210.1 ± 18.91</td>
<td>540.1 ± 29.2, *</td>
<td>555 ± 23.75, **</td>
</tr>
<tr>
<td></td>
<td>Whole area</td>
<td>177.9 ± 11.45</td>
<td>386.7 ± 19.78, ***</td>
<td>334.3 ± 27.78, ***</td>
</tr>
<tr>
<td>Gray substance of</td>
<td>DM</td>
<td>187.7±15.41</td>
<td>452.6±35.29, ***</td>
<td>378.4±21.4, ***</td>
</tr>
<tr>
<td>aqueduct</td>
<td>DL</td>
<td>173.2±12.16</td>
<td>430 ± 31.44, ***</td>
<td>349.7±16.11, ***</td>
</tr>
<tr>
<td></td>
<td>VL</td>
<td>175.9±16.75</td>
<td>423 ± 41.73, ***</td>
<td>344.6±30.51, ***</td>
</tr>
<tr>
<td>Nucleus Incertus</td>
<td>Whole area</td>
<td>496.8±37.71</td>
<td>1443 ± 111.3, *</td>
<td>1053 ± 154, **; #</td>
</tr>
</tbody>
</table>

(*) Symbol is used when values of offenders or defenders were significantly different comparing with controls (#) Symbol is used when values of offenders were significantly different comparing with defenders. (*) and (#): p < 0.05; (**) and (#): p < 0.01 and (***) and (###): p < 0.001.
Brain areas activated by aggression in African striped mouse

clusters of the intercalated nuclei (ITC), this band is more prominent in this species than in the rat or mouse. Medial to the BLA and lateral to the medial nucleus, a central nucleus (CeA) appeared in a roughly spherical shape. The medial amygdala (MeA) located in the medial border of the amygdaloid complex and it was composed of densely packed neurons. Laterally, the cortical nuclei (CoA) continued with the piriform cortex. The basomedial nucleus (BMA) laid between CoA, BLA and MeA.

Conspecific confrontation induced c-fos expression that was quantified in all amygdala nuclei (Table 2). We observed that confrontation increased c-fos expression in all amygdala nuclei in both offenders and defenders compared to naïve controls (Table 2). Comparing the density of c-fos expression between offenders and defenders no significant differences were observed in BLA, BMA nor CeA nuclei (Table 2). However, c-fos density was found significantly increased ($F_{3,6-7}=39.23; 28.66$ and $49.2$ respectively, Newman Keuls post-hoc $p<0.05$) in LA ($465.9\pm25.9$ $n=8$), CoA ($524.3\pm34.54$ $n=8$) and MeA ($795.9 \pm 57.2$ $n=8$) in defender subjects compared to c-fos density in the LA ($394.8 \pm 25.4$ $n=8$), CoA ($403.6 \pm 35.85$ $n=8$) and MeA ($464.6 \pm 65.1$ $n=8$) from offenders (Fig. 2B). Representative images from LA (Figs. 2C-E), MeA (Figs. 2F-H) and CoA (Figs. 2I-K) show the differences in c-fos expression in naïve, offenders and defenders.

**Septum.** The general organization of the septal nuclei resembled that of the mouse brain. The medial septum was characterized as displaying large neurons in a continuous band containing two main divisions, the medial septum which was continuous to the vertical limb of the diagonal band and the horizontal limb of the diagonal band. For c-fos quantification we took samples from the dorsal aspects of this division which contained some medial septum and some parts of the vertical limb of the diagonal band. The lateral septum was composed of the ventral lateral septum (LSV) over the ventral sulcus of the lateral ventricle, the intermediate lateral septum (LSI) over the medial ependymal of the lateral ventricle and the dorsal lateral septum (LSD), which was ventral to the corpus callosum (Fig. 3A). For quantification we used Bregma ~ +1 to -0.5. Conspecific confrontation increased c-fos expression in all septum subareas analyzed (Table 2). Quantification and statistical analysis of the density of the activated cells for
each nucleus was carried out (Fig. 3B). In LSD c-fos density in offender (170.1±28.3 n=8) and defender (184.6±37.4 n=8) was increased (F_{3,67}=4.65, Newman-Keuls post-hoc p<0.05) in respect to control (69.4±17.9 n=7). Similarly, c-fos density was higher in the LSI, offender (341.1±29.5 n=8) and defender (313.2±54.6 n=9) (F_{3,67}=7.5, Newman Keuls post-hoc p<0.05) compared to controls (147±36.6 n=7). Also c-fos positive cells density was higher in the LSV, offender (499.7±32.5 n=8) and defender (559.5±54.2 n=8) than naive (238.7±39.1 n=7) (Newman Keuls post-hoc p<0.05). Finally, an increase (F_{3,67}=17.43, Newman Keuls post-hoc p<0.05) in activity was found in the MS in offender (272.3±22.2 n=9) and defender (238.3±23.11 n=9) vs. control (111.9±20 n=7). Representative images of c-fos staining are shown for LSD (Figs. 3 C-E); LSI (Figs. 3F-H); LSV (Figs. 3I-K) and MS (Figs. 3L-N).

**Hypothalamus.** Within the hypothalamus, we studied the suprachiasmatic nucleus (Sch), a round-shaped nucleus located dorsal to the optic

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**Fig. 2. Activation of c-fos in the Amygdala of Lemniscomys barbarus.** A) Giemsa stained section representing the general cytoarchitectonic structures of the amygdala. Representative photomicrographs of c-fos-immunoreactivity in La, MeA and CoA of normal controls (C, F and I), offenders (D, G and J) and defenders (E, H and K). B) Graph representing the density of c-fos positive cells in La, MeA and CoA of control, offender and defender after intraspecific confrontation. (*) symbol is used when values of offenders or defenders were significantly different compared with controls. (#) symbol is used when values of offenders were significantly different when compared to defenders. (##) p < 0.01 and (***): p < 0.001. Calibration bar in (A) 500 µm in (K) 100 µm.
Brain areas activated by aggression in African striped mouse

Fig. 3. Activation of c-fos in the Septum of Lemniscomys barbarus. A) Giemsa stained section representing the general cytoarchitectonic structures of the Septum and related areas. B) Graph representing the density of c-fos positive cells in LSD, LSI, LSV and MS of control, offender and defender after confrontation. C) Representative photomicrographs of c-fos-immunoreactivity LSD, LSI, LSV and MS of control (C, F, I and L), offender (D, G, J and M) and defender (E, H, K and N). (*) symbol is used when values of offenders or defenders were significantly different comparing with controls. (*): p < 0.05; (**) p < 0.01 and (***) p < 0.001. Calibration bar in (A) 500 µm in (N) 100 µm.
chiasm at both sides of the rostroventral tip of the 3rd ventricle (Fig. 4A) and the anterior hypothalamic area (AHA) which extended from Bregma -0.4 to -1.06 (Paxinos and Franklin, 2012).

In Sch, c-fos density was significantly higher ($F_{3,6} = 59.13$, student test $P=0.022$) in offenders ($710.5 \pm 115.7$ n=8) than in defenders ($498.6 \pm 32.9$ n=8). In contrast, no differences were found in AHA between offenders ($540.1 \pm 29.3$ n=8) and defenders ($555 \pm 23.7$ n=8) (Fig. 4B). Representative images are shown for Sch (Figs. 4 C-F) and AHA (Figs. 4 G-I).

Periaqueductal gray (PAG). The basic structure of the Lb PAG was fairly similar to that of the mouse (Paxinos and Franklin, 2012) and rat (Ruiz-Torner et al., 2001). The PAG consisted of four longitudinally arranged columns along the mesencephalon surrounding the aqueduct (Fig. 5A). The inner part of the PAG was composed of a poor celled area. The outer ring contained the columns. The dorsomedial (DM) column was present all along the PAG and was composed of comparatively large cells. The dorsolateral (DL) column was arranged from the lateral tip of the aqueduct and contained small cells. The lateral (L) extended from the lateral part of the aqueduct all along the PAG. Finally, the ventrolateral column was only present at caudal levels.

Conspecific confrontation increased c-fos expression in all PAG analyzed columns (Table 2). Quantification and statistical analysis of the density of the activated cells for each column was carried...
Fig. 5. Activation of c-fos in the Periaqueductal Gray (PAG) of Lemniscomys barbarus. A) Giemsa stained section representing the general cytoarchitectonic structures of the PAG. B) Graph representing the density of c-Fos positive cells in the L, DM, DL and VL parts of the PAG in control, offender and defender mice after intraspecific confrontation. Representative photomicrographs of cFos-immunoreactivity in the L, DM, DL and VL parts of the PAG in control (C, F, I and L) offenders (D, G, J and M) and defenders (E, H, K and N). (*) symbol is used when values of offenders or defenders were significantly different compared to controls. (*) p < 0.05 and (***) p < 0.001. Calibration bar in (A) 500 µm in (L) 100 µm.
In DM, c-fos density in offender (452.6 ± 35.29 n=8) and defender (378.4 ± 21.4 n=8) was increased (F\textsubscript{3,6-7}=4.65, Newman Keuls p<0.05) respect to control (187.7 ± 15.41 n=7). Similarly, in the DL, offender (430 ± 31.44 n=8) and defender (349.7 ± 16.11 n=8) c-fos density was higher (F\textsubscript{3,6-7}=28.51 Newman kels post-hoc p<0.05) compared to controls (173.2 ± 12.16 n=7). Also in the L offender (368.7±19.78 n=8) and defender (334.3±27.78 n=8) c-fos positive cells density was higher (Newman Keuls post-hoc p<0.05) than naive (177.9±11.45 n=7). Finally, an increase (F\textsubscript{3,6-7}=15.76 Newman Keuls post-hoc p<0.05) in activity was found in the VL in offender (423±41.73 n=8) and defender (344±30.51 n=8) vs. control (175.9±16.75 n=7). Representative images of c-fos staining are shown for PAG DM (Figs. 5C-E); DL (Figs. 5F-H); L (Figs. 5I-K) and VL (Figs. 5L-N).

**DISCUSSION**

In this paper, we report a novel animal model, *Lemniscomys barbarus*, to study aggressive behavior. Individuals of this rodent species show naturally high levels of aggressiveness to conspecifics of the same or different sexual category. In this work, we have observed that freezing is not a particular feature of the defensive behavior, as offenders also display sustained freezing from longer periods of times, although in a significantly shorter period than in defender subjects. Freezing has been traditionally viewed as a defensive reaction (Fanselow, 1994). According to this view, freezing is present in both moderate and strong....
levels of fear. In moderate levels of fear, freezing is promoted from amygdala projections to the ventrolateral PAG. Strong levels of fear, those resulting from physical contact are shifted by the superior colliculus and dorsolateral PAG. It is also proposed that inhibitory interactions allow for a quick switching between different modes of freezing. In our samples, different responses like freezing, pursuing or hitting are continuously switching during encounters. This general activation is being reflected by the fact that encounters evoke strong c-fos activation in all areas studied and the behavioral outcome is very similar in any case, with stereotyped behaviors like freezing, hitting, or running. In addition, freezing is also viewed not as a passive reaction but an active form being ready to respond (Misslin, 2003). The fact that during encounters, offenders also display freezing reinforces the idea that freezing is a status to keep ready for a response that has been also observed in humans (Gladwin et al., 2016).

Many rodent models of aggressiveness have been used to describe different brain areas involved in agonistic behavior; as tame and aggressive selected rats (Konoshenko et al., 2013); genetically selected aggressive rodents strains (Veennema and Neumann, 2007); or in inbred mice strain with high or lower aggressive phenotype (Nehrenberg et al., 2013).

We placed the animals to encounters and recorded their behavior. For each couple, a subject was categorized as offender or defender according to previous works (Adams, 2006). A first conclusion we can extract from our observations is that, in most cases (8 from 9) the role that a particular subject takes depend on the role taken by the opponent and/or vice versa. The second conclusion is that, for this model, gender is one of the factors to take the offender or the defender role, but not the only one. In some cases, females attacked males (one up to three cases).

Brain activity was studied by c-fos IHC. In both, offender and defender cases, confrontation resulted in strong c-fos activation in many brain areas such as prefrontal cortex, amygdala, septum, different hypothalamic regions, nucleus incertus, suprachiasmatic nucleus and the periaqueductal grey area.

We did not find differences between offender and defender in the septal area, suggesting, from our results in Lb, that the septum is not the central core to shift between offensive and defensive behavior. The septum is considered an area involved in telencephalic modulation, via its projections to and from prefrontal cortex, hippocampus, amygdala and hypothalamus (Zaborszky et al., 2015).

Activation of c-fos in the septal area is a particular feature of the agonistic behavior vs other forms of social behavior such as mating as it has been observed in the Syrian hamster for the lateral septum (Haller et al., 2006). Also, inhibition of the lateral septum increases aggression in Syrian hamster (McDonald et al., 2012). In our samples, all divisions of the septum show increased c-fos activity in both offenders and defenders.

Lateral septum and the hypothalamus communicate together to modulate aggression behavior. Wong and coworkers have shown that Lateral septum modulate aggression behavior via its projections to ventromedial hypothalamic area. They found that the inputs from the LS inhibited the attack-excited cells but surprisingly increased the overall activity of attack-inhibited cells (Wong et al., 2016).

Several studies have demonstrated that anterior hypothalamus is also implicated in aggression behavior in mammals (Ferris et al., 1997; Delville et al., 2000; Bertoglio and Zangrossi Jr, 2005; Haller et al., 2006; Gobrogge et al., 2009; Pan et al., 2010). A study shows increased expression of pCREB in anterior hypothalamic area, central amygdala, medial amygdala, lateral septum and preoptic area in male high aggressive Golden hamster; this study found a positive correlation in labeling density between the lateral septum and the anterior hypothalamus (David et al., 2004). In this case, individuals are also characterized by a de-synchronization between the inhibitory output of the septum and the aggression areas of the hypothalamus (David et al., 2004). Our results have shown an increase in c-fos expression in both anterior hypothalamic area and all septum nuclei after confrontation test, without any difference between offenders and defenders. However, in the long-tailed hamster, it was found an increase in c-fos expression in the anterior hypothalamus of the defender subjects in respect to the offender ones (Pan et al., 2010).

On the other hand, we found significant differences in c-fos expression between attacker and defensive subjects in distinct subnuclei within the amygdala; lateral (LA), medial (MeA), and cortical (CoA). No differences were found between offender and defender in the c-fos expression in the CoA and MeA of the long tailed hamster (Pan et al., 2010). By contrast, no distinction was found in Basolateral (BLA), Basomedial (BMA) and central (CeA) amygdala c-fos expression. Interspecific differences in the anatomical structures that manage co-specific encounters could explain differences between hamsters and the Lb striped mice. Again, the amygdala may play a role in shifting from different fear and agonistic responses (Fanselow, 1994).

The amygdala plays a central role in processing adaptive social and emotional behaviour (Aggleton, 1993; Phelps, 2006; Adolphs, 2008). Typically amygdala can mediate Pavlovian-like fear conditioning (LeDoux, 2000), decision-making (Bechara et al., 2003) and recognition of emotional facial expressions in humans (Benarroch, 2015).

From the different amygdala divisions, the medial...
amygdala (MeA) is active during social behaviors such as fighting and mating (Hong et al., 2014) and it is anatomically distinct from lateral, basal and central amygdala nuclei involved in conditioned fear (Nader et al., 2001; Amano et al., 2016). Although the role of the MeA is clear in male mating (Kondo, 1992), in the case of aggression, the MeA effect is not clear: in some studies MeA lesions decreased aggression (Kemble et al., 1984; Takahashi and Gladstone, 1988), or direct electrical stimulation would increase aggressiveness (Potegal et al., 1996) while in others they increased it, or had no effect (Vochteloo and Koelhaas, 1987; Hong et al., 2014).

Our results showed higher c-fos activity in the MeA from subjects that showed defensive behavioral pattern (escape and defensive attacks to the opponent face) compared to offenders, so they would be in accordance with a role of MeA in decreasing aggressiveness in Lb. Recent studies have demonstrated that different neuron populations within the MeA may have opposite function i.e. GABAergic neurons promote aggressiveness and social behavior whereas excitatory neurons promote repetitive and asocial behavior (Hong et al., 2014). It has been also found that defeat induces a up to ten fold c-fos expression in the medial amygdala and most of these neurons expressing c-fos also express the receptor 2 of corticotropin releasing hormone (Fcetke et al., 2009). It has been found that lesions in the medial amygdala prevented c-fos activation in oxytocin and vasopressin neurons of the hypothalamus during a resident intruder paradigm (Wang et al., 2013). In our model we do not know the phenotype of the c-fos positive neurons, but according to the results by Hong and co-workers, it is plausible that the excitatory neurons are active in defenders, thus inhibiting aggressive attacks.

The ability of CeA and extended amygdala to quickly integrate and respond to intimidation and/or threats from conspecific or allospecifics is suggested to improve survival (Fox et al., 2015). Our results show that c-fos CoA activity, similar to MeA, is higher in defenders. This suggests that together with MeA, CoA may antagonize aggressiveness, or promotes escape and defensive behavior.

The medial prefrontal cortex (mPFC) and orbitofrontal cortex (OFC) are involved in aggression, and these brain regions are highly activated during aggressive behavior (Halász et al., 2006; Wang et al., 2011). In agreement with those studies, we have observed a strong activation of the mPFC after conspecific encounters, but no differences were observed between offensive and defensive behaving individuals, suggesting that mPFC may not be involved in the distinction between the two behavioral patterns. In a recent work, it has been proposed an inhibitory role of the mPFC in reducing aggressive bursts and the intensity of aggressive behavior (Takahashi et al., 2014).

It is necessary to consider the fact of a differential effect of aggression defensive display in the SCh nucleus. However, it is need to point out that the retino-hypothalamic tract that targets mainly the SCh is composed of other tracts and one of them targets the anterior hypothalamic nucleus responsible for defensive mechanisms (Canteras et al., 2011).

Most of research in agonistic behavior has been centered in telencephalic and diencephalic circuits, however, brainstem projections may also be involved in agonistic behavior (Walterschek and Raab, 1982). We have also observed that the medial amygdala receives a strong projection from the nucleus incertus mediated by the peptide relaxin 3 (Santos et al., 2016). The nucleus incertus projects to, among others, septum, PAG, hypothalamus, suprachiasmatic nucleus, hippocampus, amygdala and prefrontal cortex (Goto et al., 2001; Olucha-Bordonau et al., 2003). Our results show that offenders present higher neuronal activation of NI in respect to defenders during confrontation test. Moreover, we observed that neuronal activation in this area correlates negatively with fear behavior measured by freezing time. The fact that the activation in the nucleus incertus is higher in the offenders than defenders while in the medial and cortical amygdala the effect is just the opposite lead us to postulate that an inhibitory effect of the nucleus incertus over the amygdala. In this sense, the G protein coupled receptor RXFP3 is the cognate receptor of the peptide relaxin3 and is coupled with the inhibitory subunit G\(_{\text{ia}}\) (Bathgate et al., 2002; Van der Westhuizen et al., 2005). Supported that view, we have observed that relaxin3 projections arising from the NI and targeting the amygdala, specifically concentrate in the medial amygdala (Santos et al., 2016). Considering another system, relaxin3 infusion disrupts the natural spontaneous alternation indicating a potential inhibitory role over the septal area (Albert-Gascó et al., 2016).

As a general conclusion of this paper, we introduce a new model for studying agonistic behavior in the stripped mouse. This model is characterized by displaying strong aggressiveness independently of the gender. When confronted, subjects assume a role of offender or defender and the main feature to differentiate them is by studying approaching and attack or flying. Episodes of attacks are separated by freezing episodes that appear in both offenders and defenders, although in defenders they appear in significantly higher levels. Freezing is also a good measure of the role that each subject is taking. In this model, confrontation produces a general increase of c-fos expression. In addition, in several anatomical structures, namely the lateral, medial, and cortical amygdala on one site and the nucleus incertus on the other side, there was significant differences between offenders and defend-
ers. While offenders displayed more c-fos activation in the nucleus incertus, the defenders displayed more c-fos activation in the medial and cortical amygdala.

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