The Swiss sublines of Roman High- (RHA/Verh) and Low- (RLA/Verh) Avoidance rats, derived from the original Roman stock (Bignami, 1965), have been psychogenetically selected (and outbred) for good vs extremely poor acquisition of two-way, active avoidance. Inbred strains (RHA-I and RLA-I), derived from those two lines, have been maintained at our laboratory since 1997. The RLA line/strain shows increased stress-induced endocrine responses and enhanced anxiety/fearfulness in a variety of unconditioned behavioural variables and tests. Thus far, however, the Roman rat strains have not been compared in procedures involving classical fear conditioning to cues or contexts. Therefore, the present work was aimed at comparing RHA-I and RLA-I rats in 1) two different procedures of fear-potentiated startle and 2) in a classical fear conditioning (i.e., conditioned freezing) paradigm. The results indicate that, compared to RHA-I rats, RLA-I animals display higher levels of conditioned fear (as measured either by startle responses or freezing behavior) across those different tasks.

Las cepas de ratas Roman de alta y baja evitación difieren en respuesta de sobresalto potenciada por miedo y en condicionamiento clásico aversivo. Las sublíneas suizas de ratas Romanas «High»-(RHA/Verh) y «Low»-(RLA/Verh) «Avoidance» han sido seleccionadas genéticamente, desde 1972, en función de su excelente (RHA) o extremadamente pobre adquisición de la tarea de evitación activa en dos sentidos. Cepas consangüíneas (RHA-I y RLA-I), derivadas de las dos líneas anteriores, se mantienen en nuestro laboratorio desde 1997. En comparación con la cepa RHA-I, la cepa RLA-I muestra incrementos en las respuestas hormonales al estrés, así como en conductas de ansiedad/miedo en una variedad de pruebas y variables conductuales incondicionadas. Hasta la fecha, las cepas de ratas Romanas no han sido comparadas en procedimientos de condicionamiento clásico de miedo a contextos o estímulos discretos. El presente trabajo tuvo como objetivo comparar ambas en 1) dos procedimientos de medida de la respuesta de sobresalto potenciada por miedo; y, 2) en un procedimiento de condicionamiento clásico de miedo (petrificación condicionada). Los resultados indican que las ratas RLA-I muestran niveles mayores de condicionamiento de miedo (respuesta de sobresalto y respuesta de petrificación) que las RHA-I, reforzando así los perfiles diferenciales de ansiedad/miedo de las dos cepas.
treatment with morphine, cocaine and amphetamine (for reviews see Giorgi, Piras, & Corda, 2007; Guitart-Masip, Johansson, Cañete, Fernández-Teruel, Toheña, Terenius, & Giménez-Llort, 2008). In contrast, as concerns to responses to aversive stimuli, RLA rats have shown increased hormonal (ACTH, corticosterone, prolactin) and behavioural stress-induced responses (for reviews see Carrasco, Márquez, Nadal, Toheña, Fernández-Teruel, & Armario, 2008; Castanon, Dulluc, LeMoal, & Mormede, 1994; Driscoll et al., 1998; Fernández-Teruel et al., 1997; Steimer & Driscoll, 2003), as well as enhanced anxiety/fear responses in a variety of novelty- and conflict-based anxiety models (including the elevated «zero» maze; López-Aumatell, 2008; for reviews see Escorihuela et al., 1999; Fernández-Teruel et al., 1997; Fernández-Teruel, Giménez-Llort, Escorihuela, Gil, Aguilar, Steimer, & Toheña, 2002c; Steimer & Driscoll, 2003, 2005), in the Vogel’s punishment test (Corda, Piras, Valentini, Scano, & Giorgi, 1998; Ferré, Fernández-Teruel, Escorihuela, Driscoll, Corda, Giorgi, & Toheña, 1995), in baseline and stress-enhanced acoustic startle response (Aguilar, Gil, Toheña, Escorihuela, & Fernández-Teruel, 2000; Yilmazer-Hanke, Faber-Zuschratter, Linke, & Schwegler, 2002), and in several procedures of frustrative non-reward (Rosas, Callejas-Aguilera, Escarabajal, Gómez, de la Torre, Agüero, Toheña, Fernández-Teruel, & Torres, 2007; Torres, Cándido, Escarabajal, de la Torre, Maldonado, Toheña, & Fernández-Teruel, 2005).

However, although stress- and sensitization-enhanced acoustic startle responses have been reported in inbred RLA-I rats (Aguilar et al., 2000; Yilmazer-Hanke et al., 2002), a systematic between-strain comparison of the levels of fear-conditioning to cues or contexts (i.e. fear conditioning to conditioned stimuli —CS—), either measuring increases of startle or freezing responses, has not been carried out thus far. Therefore, with the aim of further characterizing the RHA-I and RLA-I rat strains in regard to their respective proneness for fear conditioning, we have evaluated their performance in both the acoustic fear-potentiated startle (FPS) and in a classical fear (freezing) conditioning (CFC) test, as these models have been considered essential to disentangle the detailed neuroanatomy of anxiety and fear, particularly the role of amygdala regions and its related limbic circuitry (for review see Carrasco, Márquez, Nadal, Toheña, Fernández-Teruel, & Armario, 2008; Castanon, Dulluc, LeMoal, & Mormede, 1994; Driscoll et al., 1998; Fernández-Teruel et al., 1997; Steimer & Driscoll, 2003).

Methods

Subjects

The animals used in the present experiments were males (Exps. 1, 2 and 3) and females (only in Exp. 3) of the inbred Roman High- (RHA-I) and Low-Avoidance (RLA-I) rat strains maintained at our laboratory. They were approximately 5 months old (weight 300-400 g), and were housed in same-sexed pairs in standard (50 × 25 × 14 cm) macrolon cages. They were maintained under a 12:12h light-dark cycle (lights on at 08:00 a.m.), with controlled temperature (22 ± 2 ºC) and humidity (50-70 %) and with free access to food and water. Rats from each experimental group belonged to at least 8 different litters.

Fifty rats (20/strain) were initially used for baseline acoustic startle testing (according to the procedure described below) in Exp. 1. Mean ± SD values were 204.9 ± 140.8 (SE= 34.2) for RHA-I rats and 623.3 ± 352.6 (SE= 78.2) for RLA-I rats. After a matching process for similarity of response (selecting those with the highest startle values from the RHA-I strain and those with the lowest values from the RLA-I strain) two strain groups (n= 8 rats/group) with similar baseline ASR-1 values were obtained, as shown in Fig. 1-A.

Sixteen rats (8/strain) were used for experiment 2, and 35 rats (n= 17-18 / strain) were used for experiment 3.

The experiments were performed from 9:00 to 18:00 h. and were approved by the committee of Ethics of the Autonomous University of Barcelona in accordance with the European Communities Council Directive (86/609/EEC) regarding the care and use of animals for experimental procedures.

Baseline acoustic startle response (Habituation —ASR-1— session): Experiments 1-2

Two sound-attenuated boxes (San Diego Instruments, USA) were used and each box housed a plexiglas cylinder with a grid placed in the bottom. For any test session each animal was placed in the cylinder, and movements of the cylinder resulting from startle responses were transduced by an accelerometer into a voltage which was amplified, digitized and served into a computer for analysis. A white noise generator provided background noise of 55 dB in the unit chambers. For the ASR-1 session (i.e. baseline startle), and after 5 min of familiarization to the startle chamber, each rat was exposed to 30 acoustic stimuli of 105 dB (50 ms duration) with a 30-s intertrial interval (ITI).

Fear-potentiated startle (FPS): Experiments 1-2

The procedure involved 1-2 conditioning sessions (depending on the experiment; see below), followed by an ASR-2 phase (i.e. measurement of acoustic startle in absence of the CS but in the context where the rats were conditioned) and by a FPS test phase (see below). Each of these sessions was always preceded by 5 min of familiarization to the startle chambers.

Conditioning sessions

Following ASR-1 measurement, each animal was given 10 conditioning trials, each of which consisted of presentation of an acoustic stimulus (70 dB; conditioned stimulus —CS—) of 3.2 s after which a 0.6-mA shock was delivered through the grid, which continued with the acoustic stimulus for a further 0.5 s. Every 2 consecutive trials were separated by a 30-s ITI.

In Experiment 1 the animals were first matched (see ASR-1 session of Exp. 1, Fig. 1-A) and given two 10-trial conditioning sessions (spaced 24h apart), the first one being administered one week after the ASR-1 session.

In Experiment 2 the animals received only one 10-trial conditioning session which was administered immediately following the ASR-1 session.

FPS test session

In the FPS test session, administered 24 h after the last conditioning session, the rats were placed in the boxes and after a 5 min acclimatization period they received 40 acoustic stimuli of 105 dB (50 ms) to habituate them partially (ASR-2 phase). This phase was immediately followed by administration, in a pseudorandom order, of 20 acoustic stimuli (105 dB, 50 ms) alone...
and 20 of these stimuli preceded by the CS (70 dB, 3.7 s). ITI was 30 s during the whole FPS test session.

The average response difference between those 20 «alone» trials and those 20 trials preceded by the CS is considered the measure of cue-conditioned fear-potentiated startle.

**Classical fear conditioning (CFC): Experiment 3**

The apparatus was a white chamber divided into two equal compartments (23 × 12 × 20 cm). A 1-mA scrambled electric footshock (0.5 s; unconditioned stimulus, US) was administered through the grid floor (Shocker Leticia, L1 100-26). A 15-s light from a 20-W bulb in the upper part of a wall was the conditioned stimulus (CS). Training consisted of five CS-US pairings and started with the onset of the CS. US and CS terminated simultaneously. A 120-s (mean) pseudorandom intertrial (resting) interval was used. After 24 h, the rats were placed in the training chamber and freezing behaviour was monitored for 10 min. For the first 5-min period the light was absent (to evaluate contextual fear conditioning). The light was then switched on for 5 min to measure fear conditioning to the CS. Freezing behaviour was scored by direct observation and considered as the complete absence of movement except for breathing. Agreement between the two blind (to the «rat strain» condition) observers was higher than 0.98 (reliability/correlation score).

There were (approximately) equal numbers of rats from each sex for each strain in Exp. 3, but sexes were pooled for analysis because ANOVA did not show any significant «sex» or «sex X strain» interaction effects.

**Data analysis**

Multivariate analyses of variance (MANOVA) were first applied to data from ASR-1 and ASR-2 sessions (factors: 2 «strain» × 3 or 4 «trial blocks»). Student’s t-tests were then applied to data of different 10-trial blocks of those to phases, as well as to the averaged difference between the 20 «potentiated» and the 20 «startle alone» trials of the FPS testing session. Covariance analysis (with ASR-2 values as covariates) were also applied to test whether or not between-strain FPS scores and differences were influenced by baseline (ASR-2) measures.

Repeated measures ANOVA (with 2 «strain» × 2 «phases») and Student’s t-tests were also applied to data from the context-conditioned and cue (CS)-conditioned freezing results of Exp. 3.

**Results**

MANOVA analyses of the ASR-2 session from Exp. 1 (Figure 1B), and for ASR-1 and ASR-2 sessions from Exp. 2 (Figure 2A-B), showed no significant effects of «trial block» (within subject factor) nor «strain × trial block» interactions (all Fs<1.7, p>0.2). Strain effects were significant in ASR-2 session from Exp. 1 [F(1,14)= 6.3, p<0.03], as well as in ASR-1 [F(1,14)= 28.9, p<0.001] and ASR-2 sessions [F(1,14)= 5.4, p<0.04] from Exp. 2.

Student’s t-tests applied to data from the ASR-2 session in Exp. 1 (Figure 1B) confirmed the results of MANOVA analyses, by showing that RLA-I rats displayed higher startle responses than their RHA-I counterparts in the first and fourth 10-trial blocks [t (14)>2.5, p<0.05 in both cases). RLA-I rats also showed higher fear-potentiated startle than RHA-I rats as seen by the average

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**Figure 1.** Fear-potentiated startle following two conditioning sessions in RHA-I and RLA-I rats.
(A) Twenty RHA-I and twenty RLA-I male rats underwent an ASR session (30 acoustic stimuli 105 dB, 50 ms). Eight rats from each strain were then matched according to their similar ASR scores (ASR-1 SESSION) and used for the experiment. They were submitted to two conditioning sessions (see «Methods»). Twenty-four hours later they underwent another (B) ASR session (ASR-2, 40 trials of startle stimulus alone) which was immediately followed by a FPS session (C). Data are means ± SEM of maximum startle amplitude averaged for trial blocks.

*p ≤ 0.05, **p ≤ 0.01 vs the RHA-I group (Student’s t-test)
The «difference» between acoustic startle stimulus preceded by the CS (i.e. «potentiated») and acoustic «startle stimulus alone» $[t (14) = 3.0, p < 0.01]$; Figure 1C).

In Exp. 2 Student’s t-tests also confirmed significant differences between both strains in the ASR-1 phase (the three 10-trial blocks) as well as in the fourth 10-trial block of the ASR-2 phase $[all t (14) > 3.1, p < 0.01]$; Figure 2A-B]. Again, RLA-I rats showed higher fear-potentiated startle (Figure 2C) than RHA-I animals $[t (14) = 3.23, p < 0.01]$.

Covariance analysis of fear-potentiated startle responses taking ASR-2 values (averaged for the 4 10-trial blocks) as covariates showed significant «Strain» effects in both experiments $[both F(1, 14) > 6.1, p = 0.03]$ while the covariate was not significant $[in both experiments F(1, 14) ^{\leq} 3.6, p \geq 0.08]$.

As «2 (strain) × 2 (sex)» factorial ANOVAs separately applied to contextual and cue-conditioned freezing results (exp. 3) showed no significant sex nor «strain × sex» interaction effects $[both F(1, 34) < 3.5, p > 0.1]$, the data from experiment 3 were pooled by sex and a repeated measures $2 —strain— × 2 —context and cue phases—$ ANOVA, followed by between-strain Student’s t-tests, were applied. Results from the repeated measures ANOVA analysis showed significant «Strains» $[F(1, 33) = 10.8, p = 0.002]$ and «Phase» $[F(1, 33) = 6.7, p = 0.014]$, but no interaction $[F(1, 33) = 1.1, p = 0.3]$ effects, thus showing that RLA-I rats displayed a significantly greater (two-fold) amount of freezing in both context and cue conditioning tests than their RHA-I counterparts and that freezing levels in the «CS» (cue) phase were overall higher. Between-strain Student’s t-tests applied to each phase confirm these ANOVA results (both $t(33) > 2.91, p < 0.01$) (see Figure 3).

**Discussion**

In agreement with previous results (Aguilar et al., 2000; Yilmazer-Hanke et al., 2002) the present work reports that RLA-I rats showed higher baseline acoustic startle responses than RHA-I animals during both the noise-alone —ASR-1 and ASR-2— phases (i.e. unconditioned startle stimulus alone) in Exps. 1-2. It is worth pointing out that when both strain groups were matched as a function of their ASRs during the first session (experiment 1;
AR -1 phase), RLA-I rats also showed increased startle responses during the habituation/postconditioning phase of the test (ASR-2 phase) session, thus indicating a higher degree of context-conditioned fear as compared to RHA-I rats. Moreover, as indicated by comparison of experiments 1 and 2, a main finding of the present study was the observation that, regardless of whether the animals were matched or not according to their ASRs (in ASR-1 phase), RLA-I rats displayed a markedly enhanced (CS-induced) fear-potentiated startle response as compared to the RHA-I strain in the FPS phase of both experiments. In fact, the potentiation (i.e. the evidence of fear (cue-conditioning) of startle observed in that phase was about 6-11 times more pronounced in RLA-I rats than in their RHA-I counterparts, as the latter did not show any evidence of startle potentiation. It is also remarkable that such an enhanced FPS in RLA-I rats, relative to their RHA-I counterparts, was observed regardless of whether the procedure involved either one or two fear-conditioning sessions (i.e. 10 or 20 CS-shock pairings, respectively). This is a relevant issue, as it points out that prominent FPS, and the observed between-strain differences, can be obtained after 10 (rather than 20, as in exp.1) CS-shock pairings and by using a 2-day (rather than 4-day, as in exp.1) experimental procedure.

On the other hand, and in line with the data of these two FPS studies, RLA-I rats also showed elevated fear responses (relative to RHA-I rats) in the CFC study (exp. 3), as indicated by their enhanced levels of learned freezing in both the contextual phase and in the presence of the cue stimulus (i.e. the light —CS—).

While being partly in line with data from Yilmazer-Hanke et al. (2002), who used a procedure of shock-induced context-sensitization of startle in a single session, the present results represent the first demonstration of differences between the RHA-I and RLA-I rat strains in two cue-induced fear-conditioning procedures also involving context conditioning, thus allowing differentiation among overall anxiety responses (to contexts) and fear conditioned to discrete/phasic stimuli.

Between-strain differences in fear-potentiated startle and/or classical fear conditioning are an important prerequisite for comparative morphological and functional studies on the neuroanatomy of fear, as both procedures have been essential cornerstones in the study of the role played by the amygdala and its associated circuitry in regard to these emotional responses (e.g., Davis, Falls, Campeau, & Kim, 1993; Gray & McNaughton, 2000; LeDoux, 1996). In that context, studies with the Roman rat lines/strains have shown that: (i) low doses of arginine-8-vasopressin administered into the central amygdala enhanced shock-induced bradycardia and immobility towards contexts in RLA rats while not affecting RHA rats (Roozendaal, Wiersma, Driscoll, Koolhaas, & Bohus, 1992); (ii) posttraining injections of corticotropin-releasing hormone, or norepinephrine, into the central amygdala also induced distinct behavioural and neurochemical (FOS induction) effects in both Roman rat lines when tested in stressful situations involving aversive conditioning (Roozendaal, Koolhaas, & Bohus, 1993; Wiersma, Konsman, Knollem, Bohus, & Koolhaas, 1998); (iii) inbred RLA-I rats have a greater number of CRF-expressing neurons in the central nucleus of the amygdala as compared to RHA-I rats (Carrasco et al., 2008; Yilmazer-Hanke et al., 2002); (iv) RLA-I rats also have an increased neuronal density (Torres, Morón, Esteban, Gómez, de la Torre, Cándido, Maldonado, Tobeña, & Fernández-Teruel, 2006) as well as higher number of GABAergic neurons expressing PARV (i.e. parvalbumin) and the «anxiolytic» peptide NPY (i.e. neuropeptide Y) in the basolateral complex of the amygdala (Yilmazer-Hanke et al., 2002); (v) NGF-A, which is induced in the amygdala as a consequence of fear, is strongly activated by acute amphetamine in the central nucleus of the amygdala in RLA-I rats, but not in RHA-I animals (Guitart-Masip et al., 2008); and, (vi) we have recently found that RLA-I rats also show enhanced CRF mRNA in the dorsal aspect of the bed nucleus of the stria terminalis (BNST) (Carrasco et al., 2008).

It appears relevant, at this point, to compare RHA/RLA rats with other rat lines which have been psychogenetically-selected for divergent anxious behavior on the basis of different criteria, as it is the case of HAB («high anxious») and LAB («low anxious») rats, bidirectionally selected and bred for divergent behavior in the elevated plus-maze test for anxiety (EPM; e.g., Landgraf & Wigger, 2002, 2003). The similarities between RHAs and LABs (both «low anxious»), as compared to RLAAs and HABs (both «high anxious»), respectively, are remarkable in most rat anxiety models based on conflict or activity/exploration of novel spaces as well as regarding stress-induced neuroendocrine responses (Landgraf & Wigger, 2002, 2003). But, contrary to what is seen between RHA-I and RLA-I rats, LAB rats show an enhanced (baseline and fear-potentiated) startle response, as compared to HAB rats (Yilmazer-Hanke, Wigger, Faber-Zuschratter, Linke, & Schwegler, 2004).

In conclusion, the present results and the reviewed (behavioural, neuroendocrine and neuroanatomical) phenotypic characteristics of RLA vs RHA rats provide compelling evidence for considering these lines/strains of rats as a well-validated behavioral and neurobiological model of trait anxiety/fearfulness and for proposing them as a particularly suitable tool to disentangle the behavioural and molecular mechanisms of fear-related responses (Driscoll et al., 1998; Fernández-Teruel, Escorihuela, Gray, Aguilar, Gil, Giménez-Llort, Tobeña, Bhomra, Nicod, Mott, Driscoll, Dawson, & Flint, 2002b; Steimer & Driscoll, 2003, 2005).

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