MAIN PAPERS

ADDITIONAL RESULTS

With the aim of rounding out prior findings, other results obtained from additional analyses of the same F_2 data base are presented. The observation of a similar 3-fold structure of emotionality between males and females in our test battery is reported, which is taken as evidence for the consistency of that factor solution. Further evidence for the robustness of the 3-fold solution is suggested by the finding of consistent sex differences across tests, males being more anxious/fearful than females in a host of measures. In addition, encouraging results of the molecular analysis of multiple genetic markers for anxiety are shown, suggesting that one locus, at chromosome 5, influences behaviour in a way that parallels the effects of anxiolytic drugs.

SEX DIFFERENCES ANALYSES

Sex differences in fearful behaviour have been observed in many experimental situations suggesting, in general, that male rats are more fearful than females[12]. For example, it has been reported that males, as opposed to females, defecate more and ambulate less in the OF (Gray 1971; 1987), exhibit higher avoidance of the "anxiogenic" open arms of the PM (Johnston and File 1991), show enhanced responding in the startle probe (Lehmann et al. 1999), show poor performance in the bi-directional SAC task (Saavedra et al. 1990), as well as a more pronounced impairment of shuttlebox acquisition as a consequence of inescapable footshocks (Steenbergen et al. 1989). Pharmacologically-induced states of anxiety seem to be more marked as well in males than females, as shown by the administration of the beta-carboline FG 7142 (Meng and Drugan 1993). Because our general experimental design was based on an F_2 population composed of males and females (almost equally divided) we had the opportunity of testing Gray's hypothesis on sex differences in fearfulness (Aguilar et al. submitted). We therefore expected a systematic pattern of differences in fearful responding with males being more fearful than females, as infered from a higher frequency of defecation, more time engaged in self-grooming, less ambulation/exploration and heightened startle in response to unconditioned fear stimuli (OF, PM, HB, A and ASR), as well as a greater susceptibility for developing passive coping behaviours when confronted with conditioned fear stimuli (i.e. enhanced Pavlovian fear conditioning and poor shuttlebox avoidance). As can be seen in **Table 6**, the results showed unidirectional sex effects in the expected direction in all the tests investigated.

Regardless of the existence of sex differences in fearfulness in the F_2 animals, we expected to find similar factor structures to that found in the total sample in our test battery, which divided fearful responding into two principal categories, i.e.

learned vs unlearned fear. We obtained 3-factor solutions for males and females (n = 400, for each group), each of which suggested, from visual examination of the allocation of loadings, close resemblances to the global factor structure as well as between the two sexes (**Tables 7 and 8**). In order to confirm this inference, a congruency coefficient was applyied to the factor structures. The results showed that the Learned Fear and Fear of Heights factors were practically indentical for both sexes, with the Emotional Reactivity Factor being slightly different. In addition, we also compared the degree of congruency for each sex with respect to the global 3-factor structure from the total sample. Once again the factor structures were very similar. Therefore, these results strongly suggest that the 3-fold solution for the behaviour of the F_2 progeny reported here is robust, in spite of the existence of unidirectional sex effects.

TABLE 6

MEAN ± SEM SCORES FOR SELECTED VARIABLES
TAKEN ACROSS THE BATTERY OF TESTS,
SHOWING CONSISTENT MALE-FEMALE DIFFERENCES

Type of test	Type of response	Mean ± SEM Males	Mean ± SEM Females	F	Sig.
OF	# Defecations	3.4 ± 0.1	1.7 ± 0.1	101.3	**
	Distance (cm)	2255.3 ± 21.0	2480.2 ± 23.6	50.7	**
PM	# Enclosed arm entries	8.0 ± 0.1	8.5 ± 0.1	7.7	*
	% Open Arm Entries	34.7 ± 0.7	38.9 ± 0.7	17.5	**
	% Time Spent in the Open Arms	25.4 ± 0.8	30.0 ± 0.8	17.6	**
HB	# Head-dips	5.4 ± 0.1	9.5 ± 0.2	298.3	**
A	Mean Activity Counts (30 min)	1738.9 ± 19.2	1897.4 ± 22.1	29.4	**
ASR	Mean 1-20 trials (mV)	402.1 ± 25.2	228.6 ± 14.3	35.8	**
CFC	# Defecations in the Test Phase	5.1 ± 0.1	3.8 ± 0.2	35.0	**
	Freezing to the CS during 3 min (sec)	58.1 ± 2.1	68.6 ± 2.3	11.3	**
SAC	# Avoidances at 40 trials	9.3 ± 0.4	12.2 ± 0.5	21.6	**

*$P < 0.01$ **$P < 0.001$ (Adapted from Aguilar et al. submitted).

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[12]Though strong evidence exists supporting Gray's hypothesis (1971; 1987) of heightened fearfulness in male rats, a number of studies showing the reverse pattern of sex differences have been reported. For example, two of the experimental preparations in which females seem to behave more fearfully are the Vogel conflict test (Johnston and File 1991) and the anxiety-provoking (A/DTB) burrow system (Blanchard et al. 1991; Shepherd et al. 1992).

TABLE 7

THREE-FOLD SOLUTION FOR THE 14 TARGET MEASURES
FOR MALES

Factor	Fear test battery measures
Learned fear	**Avoidances** (0.80); **intertrial crossings** (0.80); crossings during habituation period (0.48); **freezing to context** (- 0.23); **freezing to CS** (- 0.23); enclosed arm entries (0.23); self-grooming (- 0.31); distance (0.44); activity counts (0.48).
Emotional reactivity	**Crossings during habituation period** (- 0.23); **freezing to context** (0.74); **freezing to CS** (0.78); **defecations** (- 0.36); **self-grooming** (- 0.35); **distance** (0.33).
Fear of heights	Crossings during habituation period (0.33); enclosed arm entries (0.46); **% of open arm entries** (0.80); **% of time in the open arms** (0.89); distance (0.48); head-dipping duration (0.27); startle (0.21).

Numbers in parentheses indicate factor loadings > 0.20. Main variables for the interpretation of the factors in bold print. The correlations among factors were as follows: $r_{1,2} = 0.20$; $r_{1,3} = 0.05$; $r_{2,3} = - 0.08$. (Adapted from Aguilar et al. submitted).

TABLE 8

THREE-FOLD SOLUTION FOR THE 14 TARGET MEASURES
FOR FEMALES

Factor	Fear test battery measures
Learned fear	**Avoidances** (0.75); **intertrial crossings** (0.75); crossings during habituation period (0.61); **freezing to context** (- 0.59); **freezing to CS** (- 0.62); activity counts (0.27).
Emotional reactivity	**Crossings during habituation period** (0.28); **enclosed arm entries** (0.68); **defecations** (- 0.49); **self-grooming** (- 0.57); **distance** (0.69); **activity counts** (0.37).
Fear of heights	Freezing to context (- 0.20); freezing to CS (- 0.20); **% of open arm entries** (0.90); **% of time in the open arms** (0.90).

Numbers in parentheses indicate factor loadings > 0.20. Main variables for the interpretation of the factors in bold print. The correlations among factors were as follows: $r_{1,2} = 0.10$; $r_{1,3} = 0.05$; $r_{2,3} = 0.02$. (Adapted from Aguilar et al. submitted).

MOLECULAR GENETIC (QTL) ANALYSIS

There is much evidence, especially from quantitative genetic research, that genetic factors influence psychological traits (Loehlin et al., 1988; Plomin et al. 1994). The designs commonly used, however, merely permit a rough estimation of the phenotypic variance accounted for by genetic determinants, i.e. " 'the bottom line' of transmissible genetic effects on behaviour, regardless of the number of genes involved, the complexity of their interactions, or the influence of nongenetic factors" (Plomin et al. 1994). Neither family, twin or adoption studies in humans, nor selective breeding or research done with inbred strains in rodents has been able to clarify the nature of that influence, i.e. to identify the genes responsible

for individual differences in a given trait. These answers belong to the realm of molecular biology. Because complex psychological traits are continuously distributed, they are thought to be controlled by a number of genes (perhaps with pleiotropic action) with small effects disseminated across the genome (Plomin 1990), rather than just a few major genes with large effects. Another empirical finding that has led to that notion is the failure of linkage mapping approaches to detect loci (containing few genes with large influence) for vulnerability to psychiatric disorders (Flint et al. 1995). One of the most fruitful approaches toward disentangling these sort of genetic influences is the quantitative trait loci (QTL) mapping strategy (Mott et al. 2000; Talbot et al. 1999).

Using the same F_2 phenotypic (behavioural) data base employed in the Main Study, we sought to establish by means of univariate and multivariate analyses (Fernández-Teruel et al. submitted) chromosome locus candidates for fearfulness. The genome of the rats was covered (75 per cent) at a resolution of 15 cM or less. In **Table 9** we show the LOD scores for all behavioural measures on chromosomes where at least one chromosome (Chr) exceeded a 5 % significance level as determined by a permutation test (shown in bold print). On Chr 1, the QTL appeared to influence rearing only, while a QTL on Chr 5 influenced nine measures: avoidances, escape latencies, intertrial crossings, freezing to CS, freezing to context, % of time in the open arms, ambulation in the periphery, self-grooming and rearings. **Figure 9** shows plots of the LOD curves for several of the traits on chromosomes 5, 10 and 15. For each QTL, the direction of effect of the allele from the RHA strain was examined. The direction of allelic effects is given by the sign associated with the effect size for each phenotype in **Table 9**.
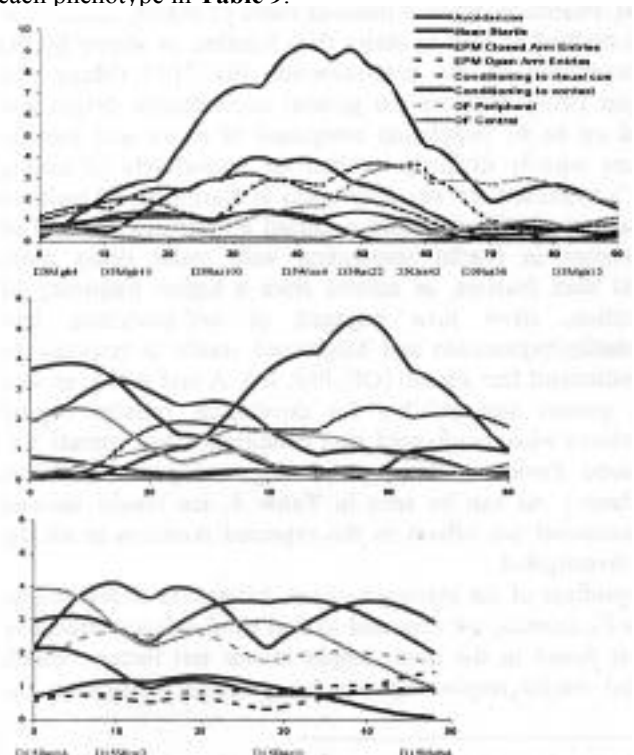

Figure 9. LOD plots for single measures on chromosomes 5, 10 and 15. The horizontal distance shows the distance along the chromosome in centimorgans (cM) and the markers used in the study are shown. (From Fernández-Teruel et al. submitted).

TABLE 9

LOD SCORES, EFFECT SIZES AND STANDARD DEVIATIONS
FOR UNIVARIATE ANALYSES

Chr		Shuttle Box			Fear Conditioning		Elevated Plus Maze		Open Field		Acoustic Startle Response	Spontaneous Activity	Grooming	Rearing	Defecation
		Avoidances	Latency	Intertrial Crossing	Cue	Context	Pct Open Arm Time	Closed Arm Entries	Activity in Periphery	Activity in Centre					
1	LOD	1.190	1.250	0.890	1.950	1.830	0.530	2.270	0.720	0.890	1.190	2.890	0.600	**3.510**	1.400
	Effect Size	0.120	-0.188	0.100	-0.142	-0.181	-0.052	0.200	0.198	-0.095	-0.225	-0.273	-0.035	**0.368**	-0.055
	Stand. Dev.	0.259	0.229	0.233	0.142	0.146	0.202	0.246	0.187	0.174	0.162	0.236	0.169	0.117	0.204
3	LOD	1.590	1.440	1.850	2.040	1.520	2.520	0.650	**5.040**	0.870	0.820	0.240	1.400	1.970	**3.180**
	Effect Size	0.367	-0.203	0.314	0.071	-0.202	-0.281	-0.019	**0.341**	-0.163	-0.078	0.006	-0.205	0.216	**-0.272**
	Stand. Dev.	0.219	0.116	0.269	0.177	0.153	0.128	0.227	0.118	0.151	0.203	0.149	0.107	0.104	0.072
5	LOD	**9.470**	**6.120**	**6.460**	**3.490**	**4.460**	**4.080**	1.650	**3.140**	1.110	1.500	2.490	**3.050**	**4.630**	1.090
	Effect Size	**0.664**	**-0.539**	**0.544**	**-0.374**	**-0.415**	**0.342**	0.173	**0.365**	-0.044	0.239	0.246	**-0.304**	**0.376**	0.079
	Stand. Dev.	0.100	0.110	0.120	0.160	0.134	0.180	0.158	0.149	0.187	0.146	0.175	0.111	0.095	0.113
6	LOD	2.970	2.190	1.380	1.690	1.820	0.710	1.400	0.800	1.030	2.560	**7.450**	1.140	1.330	**3.360**
	Effect Size	-0.422	0.268	-0.054	0.246	-0.029	0.159	0.207	-0.034	-0.030	0.333	**-0.743**	-0.079	-0.146	**0.187**
	Stand. Dev.	0.181	0.174	0.192	0.141	0.163	0.171	0.152	0.179	0.179	0.170	0.210	0.168	0.108	0.091
10	LOD	**4.130**	**3.130**	**4.000**	1.590	**5.950**	0.420	0.670	2.270	2.420	**3.530**	**3.260**	1.340	0.430	1.670
	Effect Size	**0.282**	**-0.353**	**0.386**	0.350	**0.575**	-0.060	-0.021	-0.360	-0.077	**-0.437**	**-0.458**	0.167	0.006	0.143
	Stand. Dev.	0.389	0.239	0.320	0.200	0.131	0.182	0.192	0.142	0.130	0.187	0.216	0.171	0.134	0.158
15	LOD	1.050	0.450	2.230	1.520	2.200	1.360	**3.430**	2.650	**3.450**	**4.830**	1.070	**3.060**	1.480	0.970
	Effect Size	0.243	-0.141	0.340	-0.105	0.220	0.157	**0.440**	0.368	**-0.377**	**0.450**	0.158	**0.304**	0.229	-0.040
	Stand. Dev.	0.136	0.134	0.127	0.171	0.159	0.125	0.113	0.099	0.124	0.111	0.126	0.081	0.085	0.076
19	LOD	2.680	2.860	1.190	0.700	0.040	2.440	0.430	2.220	1.570	0.520	0.910	**3.950**	0.950	**3.310**
	Effect Size	-0.337	0.374	-0.023	0.205	0.242	0.325	0.187	0.291	-0.039	-0.161	0.130	**-0.384**	0.184	**-0.275**
	Stand. Dev.	0.126	0.155	0.215	0.168	0.154	0.145	0.135	0.170	0.142	0.180	0.170	0.097	0.114	0.097
X	LOD	2.170	1.680	2.040	2.770	1.040	1.100	1.980	2.010	0.310	1.010	**3.900**	1.920	0.700	**6.180**
	Effect Size	0.144	-0.023	0.200	-0.339	-0.207	-0.149	-0.269	-0.287	0.067	0.017	**0.349**	0.122	-0.051	**-0.091**
	Stand. Dev.	0.109	0.127	0.107	0.108	0.117	0.126	0.124	0.084	0.134	0.153	0.097	0.086	0.110	0.083

Scores significant at the 5% threshold are shown in bold. The LOD scores, and associated effect sizes, are the maximum across the chromosomes. (From Fernández-Teruel et al. submitted).

On Chr 5 the allele from the RHA rats increased avoidance responses and intertrial crossing, while decreasing escape latency, consistent with the allele's origin from that strain and with a role in determining variation in fear. The allele's influence on other measures was also consistent with the hypothesis that it influences fear. It decreased conditioned freezing in response to both context and cue, and increased the time spent in, and number of entries into, the open arms of the PM. It increased rearing and activity in the periphery of the OF whilst decreasing time spent grooming in novel environments. Hence, these results indicated that a locus on Chr 5 could be a major candidate to shape common neural pathways underlying fearfulness, as measured by this array of responses in the Roman rats. As there seemed to be a parallelism between the effects of this locus and anxiolytic drugs (e.g. neither of which influence defecation or startle), it could be expected that the neural systems potentially influenced by that locus would be those mediating anxiolytic action. Thereby, we could predict that a locus on Chr 5 may play a role in determining some crucial aspect of brain development of the septo-hippocampal system, the amygdala, the cingulate cortex and/or the ascending noradrenergic projections connecting these regions of the limbic system.

When these genetic results were coupled with the factor analytic description of the same data, a striking pattern of relationships emerged. All the measures loading on the first factor (Learned Fear) were linked with this segment of Chr 5, whereas measures of the other two factors (Emotional Reactivity and Fear of Heights) were associated to this QTL plus a variety of other QTL candidates. The fact that the CFC and SAC paradigms provoked, presumably, stronger fear reactions than did the tests of spontaneous responding to novelty, could indicate that factor analysis grouped the different types of behaviours as a function of the net intensity of fear (experienced by the rats) in each particular test of the battery. That is, it is as if the rats had expressed acute and intense fear in the two aversive learning paradigms studied (factor 1), whilst in the other tests, involving diffuse and weak emotional stimuli (accompanied by less fear; especially factor 2), the rats had engaged in a more flexible and varied behavioural repertoire[13]. That the size effect of the locus on Chr 5, as well as the defecation scores, were generally greater in SAC and CFC measures than in the rest of the battery, is congruent with this explanation.

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[13]Our factor solution seems to fit with genetic data once again if we consider the fact that the startle reflex (also involving an intense stressful experience due to inmobilization in the cylinder) was not reflected in the three-fold structure, showing a parallelism with the lack of effect of anxiotytic compounds on that reflex.

ADDITIONAL PAPERS

SEX DIFFERENCES IN A BATTERY OF TESTS OF FEARFULNESS USING F2 ROMAN RATS: A FACTOR ANALYTIC STUDY

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ABSTRACT. The pattern of sex differences in a large sample (n = +/- 800; sexes equally divided) of F2-generation rats, derived from inbred Roman high- and low-avoidance strains differing in fearfulness, was investigated. We obtained measures from responses to a battery of novel/threatening stimuli tests [open field (OF), plus-maze (PM), hole-board (HB), activity (A), and acoustic startle reflex (ASR)] as well as learned fear paradigms [classical fear conditioning (CFC) and shuttlebox avoidance conditioning (SAC)]. The results showed that almost all behaviours assessed fitted with a pattern of unidirectional sex effects characterised by male rats as being more emotional (fearful) than females: males defecated more than females in the OF, PM, HB, ASR and CFC; ambulated less in the OF, PM, A, and SAC; showed more self-grooming in PM and HB; explored the open arms of the PM and the holes of the HB less; displayed enhanced acoustic startle reflex; and showed poorer performance in the SAC task. We applied two factor analyses to each sex showing that, in general, they shared a common three-factor structure: a Learned fear Factor comprising SAC and CFC responding, a Fear of Heights Factor with the highest loadings for open arm behaviour in the PM and an Emotional Reactivity Factor, mainly grouping defecations, ambulation and self-grooming. These results indicate that the factor structure describing anxious behaviour is similar for both sexes, regardless of the fact that we demonstrated systematic sex differences across the majority of measures for fearfulness.

The existence of sex differences in fearfulness has been widely documented in rodents, though it is still unclear which sex is more emotional. Gray (1971; 1987) concluded that male rats are more fearful than females, mainly based on the observation that they defecate more and ambulate less in the open field arena (OF). Although this conclusion has been criticised, primarily due to the narrow sample of fear-inducing stimuli on which it was based (Shepherd et al. 1992), studies using other aversive situations have provided further support for the contention that males are more fearful than females: they display a lower frequency of open arm visits in the PM (Johnston & File 1991), stronger reactions to acute stress (Steenbergen et al. 1990, 1991; Leret et al. 1994), an enhanced startle reflex (Lehmann et al. 1999), a stronger freezing response in classical fear conditioning (in some strains of rats; Pryce et al. 1999), impaired escape shuttlebox performance after inescapable shock administration (Steenbergen et al. 1989), and poor avoidance behaviour in shuttlebox

conditioning (Saavedra et al. 1990). Using factor analysis, Fernandes et al. (1999) studied the behaviour of male and female rats in various tests of fearfulness, and concluded that anxiety was one of the main factors accounting for males' behaviour, whereas in females activity was predominant. Moreover, by administering the beta-carboline FG 7142, Meng and Drugan (1993) found that males were more responsive to the anxiogenic properties of the drug than females.

Conversely, some studies appear to indicate that males can be less fearful, as inferred from the observation that they drink more in the Vogel punishment procedure (Johnston & File 1991), and show a pattern of responses indicative of a less pronounced reactivity in terms of the anxiety/defense test battery (Blanchard et al. 1991; Shepherd et al. 1992). Some authors have suggested that male rats are more responsive (fearful) to acute stress, whilst females would be more vulnerable to chronic stress (Paré et al. 1999). It appears, therefore, that the type of anxiety-provoking situations can be critical for differential sex responses of fearfulness. If threatening stimuli have the capacity to evoke characteristic fearful or fearless reactions in male vs. female rats, then attending to ecologically salient conditions for the rat might be useful for understanding sex differences in fearfulness, as has been successfully shown in other fields of animal research (Spear & Kucharski 1984).

In the present paper we sought to determine whether systematic sex differences appeared in a wide range of behaviours measured in seven tests of emotionality applied to a large F2 sample of male and female rats derived from parental lines which have been selected for anxiety-prone behaviours. The approach was two-fold: 1) to compare mean scores of males and females across tests, and 2) to apply factor analytic techniques on the behavioural data for each sex, using the fourteen target measures employed in a previous analysis of the data (for a description of the selection criteria of the variables, see Aguilar et al. 2001). Rats were derived from an F2-generation intercross of inbred Roman high- (RHA/Verh) and low-avoidance (RLA/Verh) strains in the context of a study on genetic markers (i.e. quantitative trait loci) for anxiety (unpublished data, A. Fernández-Teruel, R. M. Escorihuela, J.A. Gray, R. Aguilar, L. Gil, L. Giménez-Llort, A. Tobeña, A. Bomhra, A. Nicod, P. Driscoll, G.R. Dawson, & J. Flint). These Swiss substrains of the original Roman rat lines have been psychogenetically selected for, respectively, good vs extremely poor performance in a shuttlebox avoidance task. Due to this selection they also exhibit marked differences in fearfulness. Thus, RLA/Verh do not acquire shuttlebox avoidance behaviour, show enhanced freezing to fear conditioned stimuli and pronounced acoustic startle responses (basal, stress-induced, and fear-conditioned; Schwegler et al. 1997; Aguilar et al. 2000; and unpublished data), display a greater amount of self-grooming, and explore the open arms of the plus maze, the holes of the hole-board, and the central area of the open field less (Escorihuela et al. 1999), in addition to showing enhanced hormonal responses to stressors (Steimer et al. 1997; Driscoll et al. 1998). As previous evidence indicates that sex differences in emotional behaviour are reduced in inbred strains (Gray 1971), there could be advantages to studying such differences in a genetically more heterogeneous population, such as the present F2 cross.

The battery of tests that we have used in the present study was conceived to evaluate anxiety-related behaviours, and consisted of an open field (OF), elevated plus-maze (PM), hole-board (HB), actimeter in a novel cage (A), and acoustic startle reflex (ASR), as well as classical fear conditioning (CFC) and shuttlebox avoidance conditioning (SAC).

Based on previous studies using similar behavioural procedures, we expected that males would exhibit a behavioural profile congruent with higher fearfulness relative to females, though the strength and regularity of that difference was open to study in our particular sample. With respect to factor analysis we aimed to determine whether the factor structure obtained from the total sample, with male and female F2 rats pooled (Aguilar et al. 2001), remained consistent across sexes, or if different traits account for fearfulness in the two sexes.

METHOD

Subjects
About four hundred F2-generation rats of each sex were used (see Table 1 for n's), derived from the inbred RHA/Verh and RLA/Verh strains, and bred in three batches over an 18 month period. Behavioural testing was carried out separately for each batch. Rats were maintained under controlled conditions of humidity (60 ± 10 %) and temperature (22 ± 2 ºC), a 12 h light cycle (lights on at 8:00 h and off at 20:00 h), and with free access to food and water. They were housed in groups of two (males) or three (females). Testing started at the age of 4 months, and males and females were evaluated simultaneously in a counterbalanced manner. A testing-free period of 10 to 20 days was allowed between consecutive tests. Behavioural testing took place between 9:30-19:00 h of the lighted phase. The experimental order of testing was as follows.

Apparati and procedures

Open field (OF). The apparatus, placed in a quiet room, was a beige circular arena (diameter, 83 cm) enclosed by white walls (height, 34 cm) and divided into 19 equal sectors by lines drawn on the floor. It was illuminated by a white 200-W bulb placed 90 cm above the centre of the arena. 20-30 rats were evaluated daily until the completion of a given batch. Rats were first weighed and then transported in a carrying box (aprox. 2 min) to the experimental room. Once the rats were placed in the centre of the OF arena, the 5-min recording period began. Defecations, rearings against the wall and "free" rearings (rearings without contact with the wall), latency to start self-grooming and time spent self-grooming were scored by two trained observers, in addition to video camera measurement of distance covered.

Elevated plus-maze (PM). The apparatus was made of black wood. It consisted of two opposed open arms, each 50 x 10 cm, and two opposed enclosed arms, each 50 x 10 x 40 cm, with an open 10 x 10-cm square in the centre. The maze was elevated at a height of 50 cm. The room was lighted normally. 20-30 rats/day were evaluated following the same routines (weighing, carrying the rats to the experimental room and videocamera recording) as that performed for the previous test.

Rats were placed in the centre of the PM facing an enclosed arm and their behaviour was then measured during a 5-min period. The number of entries into the arms (open and enclosed) and the time spent in them, self-grooming latency and duration, and defecations were scored.

Hole-board (HB). The apparatus was a white 66 x 66 x 47 cm wooden box, which had four equidistant holes (3.7 cm diam., 18 cm deep) in the floor. The floor was divided into 16 equal squares with red lines. The four holes contained identical objects (i.e. a miniature car and a little plastic ball in a metal container), strange to the animals. The room was lighted normally (i.e. fluorescent light). 20-30 rats/day were evaluated following the same routines as that performed for the previous tests. The number of head-dips, head-dipping duration, self-grooming latency, self-grooming duration, and defecations were scored during 5 min.

Activity meter (A). Motor activity was measured by means of a multicage activity meter system (three cages, 35 x 35 x 25 cm, each; Interface PANLAB 40035, Sensor Unit PANLAB 0603). 12 rats/day were evaluated in a regularly illuminated (fluorescent lights) experimental room. A computerized recording of the sum of vertical and horizontal rat movements was automatically registered during 30 min. Defecations were measured at the end of the session. The same room was used for the acoustic startle test and classical fear conditioning (see below).

Acoustic startle reflex (ASR). An SR-Lab Startle Response System (San Diego Inst.) was used. Each animal was first placed in a plexiglas cylinder (located within a 35 x 33 x 39 cm sound-attenuated chamber, lighted by a 20 W bulb). Movements of the cylinder resulting from startle responses were transduced by an accelerometer into voltage which was amplified, digitised and saved in a computer for analysis. Acoustic stimuli of 110 dB and 50 ms were delivered by a loudspeaker, mounted at a distance of 23 cm above the plexiglas cylinder. Background noise was continuously provided by a fan located inside the sound-attenuated chamber. Startle response amplitude was defined as the maximum accelerometer voltage during the first 200ms following the startle stimulus onset. Animals were tested pairwise, using two identical startle response chambers, allowing 30-40 rats to be tested daily. Each animal was given a 5 min acclimatisation period before the onset of the first acoustic startle stimulus. Each testing session consisted of 20 startle stimuli, with an interstimulus interval of 30s. The mean startle response amplitude for the 20 trials (and for separate blocks of 5 trials) was calculated for each animal and used, with defecations at the end of the session, as the dependent variables.

Classical fear conditioning (CFC). The apparatus was a white chamber divided into two equal compartments (23 x 12 x 20 cm). A 1mA scrambled electric footshock (0.5s) (unconditioned stimulus (US)) was administered through the grid floor (Shocker Letica SA, LI 100-26). A 15 s duration light from a 20 W bulb located in the upper part (16 cm from the grid floor) of a wall was the conditioned stimulus (CS). Training consisted of five CS-US pairings that started with the

onset of the CS. US and CS terminated simultaneously. A 120s (mean) pseudorandom intertrial interval was used, with a shock-free interlude of one minute. After 24 hours, 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 five minutes to measure fear conditioning to the CS. Defecations were scored at the end of the two phases (i.e. training and test) of the procedure.

Shuttlebox avoidance conditioning (SAC). The experiment was carried out with three identical shuttleboxes (Letica Inst.) each placed within independent, sound-attenuating boxes constructed of plywood. A dim and diffuse illumination was provided by a fluorescent bulb placed behind the opaque wall of the shuttleboxes. The experimental room was kept dark. The shuttleboxes consisted of two equally sized compartments (25 x 25 cm, 28 cm high), connected by an opening (8 x 10 cm). A 2400 Hz, 63-dB tone plus a light (from a small, 7-W lamp) functioned as the CS. The US, which commenced at the end of the CS, was a scrambled electric shock of 0.7 mA delivered through the grid floor. Once rats were placed into the shuttlebox, a 4-min familiarisation period elapsed before training commenced. Each trial consisted of a 10-s CS, followed by a 20-s US. The CS or US were terminated when the animal crossed to the other compartment, with crossing during the CS being considered as an avoidance response and during the intertrial interval (ITI) as an intertrial crossing (ITC). Once a crossing had been made and/or the shock (US) discontinued, a 1 min fixed ITI was presented (to facilitate a high occurrence of ITC's). Defecations were measured at the end of the training, which consisted of a single 40-trial session.

Data Analysis
In order to evaluate possible sex-linked differences in fearfulness, we applied one-way ANOVAs on a wide range of behavioural measures taken across the battery of tests, using "sex" as the independent variable (see Table 1). In addition, we analysed the behavioural data by means of factor analytic techniques, in order to compare the structure of behaviour between males and females of the F2-intercross of the Roman rats. The behavioural variables used here were chosen following a step by step analysis of the total sample of about 800 subjects carried out in a previous study (Aguilar et al. 2001). The rationale was based on a three-step strategy (see Box 1). We began by applying the Pearson's correlation coefficient to a large number of measures (more than fifty) in order to see whether the pattern of correlations could indicate which variables were appropriate to be entered into the factor analyses. We established additional criteria to ensure a suitable selection of target measures: i.e., avoidance of redundancy among measures, and maintenance of consistency with the previous literature. We thereby obtained 33 measures representing the seven tests. Many of the measures were closely related, forming linear combinations that could obscure the emergence of meaningful factors. To overcome this we first analysed each test, and then the battery of tests. The separate factor analysis of each test determined the factor structure accounting for behaviour in the test; from each of the resulting factors we then selected one or two variables. Thus,

14 target variables were chosen to enter into a general factor analysis (i.e. a description of the whole test battery), which grouped them around six factors. This 6-fold structure strongly resembled the tests used, so we performed a 3-fold solution (based on Cattell's Scree test) to search for a smaller number of meaningful factors. The resulting solution grouped the 14 measures in a coherent and robust manner giving the following three main factors: 1) a "Learned Fear" Factor containing the measures of aversive/fear conditioning; 2) an "Emotional Reactivity" Factor with 11 out of the 14 variables entered; and 3) a "Fear of Heights" Factor with high loadings on open arm behaviour in the PM. In order to evaluate whether sex-associated differences exist in the general three-fold structure of fearfulness (based on the entire sample of 800 rats of both sexes), we applied a three-factor solution separately to data from male and female rats, using the same 14 variables. The resemblance in the factor structure between sexes was calculated by means of the congruency index. We used the SPSS statistical package for both the ANOVAs to evaluate sex differences in mean scores and the factor analyses (Direct Oblimin rotations).

PUT BOX 1 ABOUT HERE

RESULTS

ANOVAs comparing male and female rats
Male rats showed scores indicative of higher emotionality than females in almost all fearful behaviours measured (see Table 1 for mean, SEM, F, and p values). In the OF they defecated more, travelled a lesser distance, and reared less ("free rearings" and "wall rearings"). In addition, male rats exhibited a tendency to spend more time in the inner area of the OF arena at the start of the test, presumably reflecting the tendency of fearful rats to remain (freezing accompanied by risk assessment movements) in the place where they were initially placed by the experimenter. In the PM test males also showed a pattern of heightened anxiety, as revealed by consistent sex differences across the main variables of that test: they displayed less open arm behaviour (number of entries and time spent therein), and spent more time in the closed arms. With respect to the HB, the females explored the holes more than the males. Defecations and self-grooming were consistently more frequent among males in both the PM and HB tests. Male rats were less active than females in the A test, in the ASR they showed stronger startle across all 5-trial blocks, and in the SAC they were poorer avoiders. Finally (in contrast to the rest of the measures), in the CFC the males displayed less freezing, although they showed more defecation.

PUT TABLE 1 ABOUT HERE

Factor analysis for male rats
As can be seen in Table 2A, the highest loadings on Factor 1 corresponded to avoidances and intertrial crossings, whilst slight loadings were observed (with opposite sign) for conditioned fear to the CS and to the context, so that the lower the performance in the SAC task, the greater the freezing response in the CFC procedure. Crossings during habituation to the shuttlebox, activity in the A test and distance travelled in

the OF test also had moderate loadings, in the same direction as the SAC variables, on Factor 1. We termed this the "Learned Fear" Factor. On Factor 2 the highest loadings were for % of entries in the open arms and for % of time spent in them in the PM, with moderate loadings for distance covered in the OF and enclosed arm entries in the PM, and slight loadings for crossings during habituation to the shuttlebox, time spent head-dipping in the HB test and (negative) amplitude of the acoustic startle reflex. As this factor strongly loaded on open arm behaviour, we labelled it the "Fear of Heights" Factor. Finally, in Factor 3 the highest loadings were for freezing to the CS and to the context in the CFC paradigm, with slight loadings for defecation and self-grooming (of negative sign), and for distance travelled in the OF test (of positive sign). This factor was labelled the "Emotional Reactivity" Factor. For correlations between factors and the % of variance they accounted for, see Table 2B.

PUT TABLES 2A AND 2B ABOUT HERE

Factor analysis for female rats
Table 3A shows the results of the factor analysis for female rats. The highest loadings on Factor 1 were for avoidances and intertrial crossings, closely followed by loadings of crossings during habituation to the shuttlebox and freezing to the context and to the CS (negative sign), and with a marginal loading of activity (in the activity meter). This first factor clearly reflected a learned fear dimension, so we again termed this the "Learned Fear" Factor. Factor 2 mainly contained open arm behaviour in the elevated plus-maze (both % entries and % time spent in them), thought to be a pure anxiety index in that test, so this is again the "Fear of Heights" Factor. Finally, in Factor 3 the highest loadings were for distance covered in the OF and enclosed arm entries in the PM, closely followed by self-grooming and defecation (with opposite sign) in the OF test, with a slight loading for the A measure. This third factor appeared to represent a blend of activity/visceral responses to novel and threatening stimuli labelled as the "Emotional Reactivity" Factor. To judge the independence between factors and the % of variance they account for, see Table 3B.

PUT TABLES 3A AND 3B ABOUT HERE

Congruency index
In order to evaluate similarities among factor structures, the congruency index (C.I.) was calculated (see Tables 2A and 3A). When comparing the three-fold solutions for each sex, the corresponding Learned Fear and the Fear of Heights factors seemed to be practically identical (C.I. = .97 and C.I. = .94, respectively), with the Emotional Reactivity Factor showing the lowest score (C.I. = .75). Both three-fold structures showed a high degree of factor similarity (C.I. = .89). With respect to the total sample, the factor structures of males and females were almost identical, especially in relation to Learned Fear (males, C.I. = .97; females, C.I. = .99) and Fear of Heights (males, C.I. = .98; females, C.I. = .97) factors, whilst similarities concerning the Emotional Reactivity Factor were again lower (males, C.I. = .93; females, C.I. = .81). Globally, the males' three-fold structure was practically identical to that

of the total sample (C.I. = .97), with the females' factor structure being very similar, as well (C.I. = .92).

DISCUSSION
Using a large F2 population derived from inbred Roman rats, and several test situations, we evaluated whether males and females present a consistent pattern of differences in fearfulness. The results showed that there were systematic differences in emotional behaviour in the predicted direction (males more fearful than females) in almost all the tests used. They may be summarised as follows: 1) male rats defecated more than females in the OF, PM, HB, ASR and CFC; 2) ambulated less in the OF, PM, A, and SAC; 3) displayed more self-grooming in PM and HB; 4) explored the open arms of the PM and the holes of the HB less; 5) displayed enhanced acoustic startle reflex; and 6) showed poorer performance in the SAC task.

Males regularly showed higher defecation scores than females: five out of seven emotionality tests reliably distinguished sex-linked, individual differences in that parameter. That difference did not reach statistical significance in the SAC task and in the A measure. Given the similarities between the A and the rat's homecage, this test presumably evoked a low emotional response, so a floor effect might explain the lack of a sex difference. The opposite appeared to be the case in the SAC task: the footshocks that rats commonly receive during the acquisition of shuttlebox behaviour increase the fear-inducing properties of the situation, and a ceiling effect probably masked male-female differences (the defecation scores for both sexes were maximal in that task).

Activity-related behaviours were also sex-dependent throughout the tests. Female rats exhibited a higher OF ambulation and an increased number of enclosed arm entries in the PM, when compared to males. They were more active as well in terms of vertical and horizontal movements in the A test. Due to the reduced anxiogenic potential of this test, the existence of sex-linked differences might reflect a net differentiation in basal activity (not anxiety). Finally, females differed from males in the number of intertrial crossings during shuttlebox acquisition, with females changing compartments between trials much more than males. Taken together, the consistent sex differences across these multiple sorts of movement-related measures strongly suggest that the two sexes differ in the level of basal and situation-induced activity.

Reliable sex differences in self-grooming emerged for two out of three parameters measured in the PM (i.e. "latency" and "duration"), and on the three aspects of self-grooming evaluated in the HB (i.e. "latency", "duration", and "episodies"). Similarly, Thor et al (1988) found that adult male rats, when exposed to an unknown juvenile conspecific (under a "lights on" condition), exhibited more self-grooming (i.e. dorsal, ventral and genital; not facial) than females, as seen here for time spent in self-gooming in the PM and HB, a variable that comprises all varieties of that behaviour. The (inbred) parental strains of this sample of F2 rats (as well as outbred) Roman high- and low-avoidance rats also differ in that parameter, with RLA/Verh (the more fearful animals) also showing more self-grooming across tests (Escorihuela et al 1999). The duration of self-grooming in male rats became increasingly persistent with successive exposures to the

different tests (an increase of 36 per cent when comparing OF and HB tests). This progressive increase in self-grooming across tests could have been the result of a cumulative effect, due to repeated exposure to novelty. In addition, there was a linear increase in time spent self-grooming as well as a linear decrease in self-grooming latency throughout the OF, PM, and HB testing, suggesting the presence of such cumulative effects. Differences between males and females were found in exploratory behaviour as well: females were more prone to explore the unknown objects under the holes of the HB test, as compared to male rats, and to spend more time exploring the open/unprotected arms of the PM.

In the acoustic startle reflex (ASR) paradigm, male rats showed stronger startle responses than females, replicating the findings of Lehmann et al. (1999), who used Wistar rats, and the results of Aguilar et al. (2000) with inbred RLA/Verh rats. It is worth pointing out, in regard to the ASR test, that this task is independent of locomotor activity. This was confirmed in the present study by the fact that the (relatively less active) males showed more pronounced startle responses than females, thus again supporting a relationship of basal ASRs with emotionality/anxiety processes (e.g. Davis et al. 1993; Liang et al. 1996; Frankland et al. 1997; Pelton et al. 1997; Aguilar et al 2000). With respect to the SAC task, we have found that females perform better than males, in line with the previous literature. For example, Saavedra et al. (1990) reported that gray rats and albino rats of the Sprague-Dawley strain showed marked sex differences in the two-way shuttlebox task, whereas in the one-way version of this task, which lacks a conflict between active and passive avoidance components (Gray 1971; 1987), male-female differences were not observed.

The two parameters measured in the CFC procedure (i.e. defecation and freezing) revealed discordant sex differences: whereas male rats showed a greater amount of conditioned defecation, they spent less time freezing than females. Interestingly, previous results on sex differences in Pavlovian fear conditioning are far from conclusive: Pryce et al. (1999) reported that males did not show stronger freezing behaviour than females (neither to the training context nor to the CS) in a CFC paradigm, in two out of three strains studied by them. With the Fischer strain providing the exception, Lewis and Wistar rats exhibited a lack of sex effects on the expression of fear in the test phase, suggesting that fear conditioning is not unequivocally sex-dependent. The absence of male-female differences in that measure in the Wistar rat strain could be relevant to our results, as this was the original stock from which the Roman rats were derived (Bignami 1965). In addition, Johnston and File (1991) found, by training hooded Lister rats in the Vogel test, that male rats exhibited more licking (i.e. lower anxiety) during the punishment period than females. During unpunished responding they did not differ. This finding may also be pertinent for our data, as the Vogel test presumably encompasses similar fear conditioning mechanisms to those governing CFC procedures. Finally, Brush et al (1988) observed that females of the Syracuse high- and low-avoidance rat strains acquired CER more rapidly than males did.

Turning to factor-analytic studies, Fernandes et al. (1999) recently investigated the emotional behaviour of male and female rats in a PM, a HB and a sexual "orientation" test. They found that in the case of females variables typically reflecting activity tended to load onto Factor 1, whereas in males the first factor contained anxiety-related behaviours. They concluded that "female rat behaviour is characterised primarily by activity, whereas male rats are driven by sex and anxiety". In our seven tests, which included a wide variety of fear-, anxiety- and activity-related measures, factor analyses did not reveal a pattern consistent with Fernandes et al.'s conclusion. To begin with, the first factor for both males and females was Learned Fear, with loadings on CFC and SAC measures, the second factor was PM anxiety (Fear of Heights Factor), and the third one contained a mixture/blend of activity- and anxiety-related indices (Emotional Reactivity). Neither the male's nor the female's factor structure support the hypothesis proposed by Fernandes and colleagues. Additionally, the results of these 3-factor solutions for each sex were similar to the previous analysis applied to the total sample (i.e. the pooled males and females; Aguilar et al. 2001). In that study we found a clear dissociation between Learned Fear, Emotional Reactivity and Fear of Heights, as seen here for males and females. So, despite the existence of systematic sex-linked differences in fearfulness across the test measures, the factor structure for both sexes was very similar, suggesting that it is a robust solution.

We also applied factor analyses to the PM (data not shown), as this test seems to distinguish between anxiety- and activity-related behaviour. We found that the order of factors was again the same for males and females, with PM anxiety being first (with loadings between 0.93-0.94 on % of open-arm entries and on % of time spent in the open arms), and PM activity being second for both sexes (with loadings between 0.98-0.99 on entries into the enclosed arms). Although our results are not in agreement with those of Fernandes et al., the fact that in our global factor structure the measures of locomotor activity in females appear to be more discriminative (i.e. high loadings only on the third factor) than in males (i.e. loadings of 0.4-0.5 on all three factors), provides some support for the contention that the relative importance of activity-related measures is different for both sexes.

The present study has also shown that two different forms of fear-related conditioning tasks (i.e. SAC and CFC paradigms), although involving opposite, predominant responses (movement vs inmobility, respectively) shared a common component beyond activity: i.e. fear (Weiss et al. 1968; Gray 1971; Wilcock & Fulker 1973; Gray 1987; Fernández-Teruel et al. 1991). This finding may shed light on the controversy concerning the role that activity plays in animal models of anxiety (Ramos & Mormède 1998). Another controversial issue concerning rodent analogues of anxiety refers to the validity and reliability of defecation as an index of fear (Walsh & Cummins 1976; Royce 1977; Ramos & Mormède 1998). As already discussed, "fearful" male rats in our test battery had higher defecation scores, displayed less activity and exhibited more persistent self-grooming behaviour, as compared to "fearless" females. These findings thus confirm the consistency of these measures (i.e. across different tests), especially activity and defecation, using a large number of animals. Convergent support for the relationship among these measures comes from factor analysis. The three-

fold solutions for each sex revealed a third factor that contained these three variables, and which correlated with that factor in the expected manner: that is, the higher the defecation score, the greater the persistence of self-grooming and the lower the amount of activity.

We have reported here a systematic pattern of sex effects in a large battery of fear-related tests that confirms Gray's (1971, 1987) view of male rats as being more fearful than females, extending it to a sizeable F2 generation derived from animals selected for differences in fearfulness. But further work is needed to settle the inconsistences in the literature concerning sex differences in anxiety/fearfulness. Behavioural procedures using individual measures in artificial settings may not be sufficiently sensitive to reproduce ecological conditions to which laboratory rats could be genetically prepared to respond. It may be, for instance, that when stimuli with ecological salience are present in the test situation, male-female differences might change direction, with females being more fearful than males, as observed by Blanchard et al. (1991). Those authors developed the anxiety/defense test battery (A/DTB), which involves exposure of rodents to natural predators (i.e. cats or their smell) in a lab burrow system that simulates the corridor-like places in which wild rodents commonly live. They found that female rats consistently exhibit marked anxiety responses, as compared to males, in the majority of variables taken from the A/DTB (Shepherd et al. 1992). Congruent with these observations are the findings of Johnston and File (1991), who studied sex differences in a social interaction test. Confronted with social stimuli, male rats showed increased approach toward a conspecific partner (social interaction) compared to females. This would be indicative of lower anxiety, because it suggests disinhibition in response to social novelty.

From a genetic standpoint, it should be noted that studies cited here have used various stocks of non-selected pigmented/nonpigmented rats obtained from various sources, so it is not surprising that they also obtained various results. In the present study, a genetically heterogeneous population was used, an F2 cross which was, in addition, derived from inbred strains selected for highly divergent emotional profiles. Sex differences were not greater in the F2 rats used here than in the parental strains they were bred from (data -not shown-obtained from a sample of inbred RHA and RLA rats run in parallel to the last batch of F2s). We would propose, therefore, that the favorable genetic material used here, as well as the large battery of tests employed, argue affirmatively for the validity of these results.

An otherwise, potentially useful approach toward more integrated research on sex differences could be based on the notion of the hierarchical organization of the brain systems governing defensive behaviour, as outlined by some authors (see Graeff, 1994; LeDoux 1996; Gray & McNaughton 2000). For instance, Gray and McNaughton (2000) state that the various systems governing anxious response functionally depend upon distinctive, but interconnected, neuronal sites and systems, with each type of behaviour differentially activated by threat stimuli (actual or potential) as a function of the coping options, i.e. avoidable vs. unavoidable threat. Within this framework, sex-linked differences in fearfulness would be the result of a specific neuronal organization (for each sex) of the defensive system: whilst the female brain may favour fear reactions to certain emotional stimuli, male brains may react to other kinds of fear-inducing situations as a function of the environmental requirements.

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BOX 1. Rationale of the factor analyses

Study of the structure of the battery of tests of fearfulness

with the total sample of 800 rats (Aguilar et al. 2001)

1) Selection of the 14 target variables
   Correlation matrix plus criteria (see the text)
   Factor analysis of each test plus criteria
2) Factor analysis with the 14 target variables: 6-fold structure
   Factor 1: SAC; Factor 2: CFC; Factor 3: PM anxiety; Factor 4: PM and OF activity;
   Factor 5: ASR anxiety; and Factor 6: OF and A anxiety/activity
3) Factor analysis with the 14 target variables: 3-fold structure
   Factor 1: Learned Fear; Factor 2: Emotional Reactivity; and Factor 3: Fear of Heights

Study of the structure of the battery of tests of fearfulness

for male and female rats (400 by sex)

1) Factor analysis with the 14 target variables: 3-fold structure

LEGENDS

Table 1. Behavioural scores of males and females of the F2-generation rats across the battery of tests (mean ± SEM, F, and p values). The variables for the factor analyses are highlighted in bold print. The df were 1 for the number of experimental groups, and 389 (at least) for the number of subjects used. The *n* in each test was as follows: OF, male (m) = 459, female (f) = 455; PM, m = 413, f = 414; HB, m = 411, f = 400; A, m = 399, f = 394; ASR, m = 411, f = 412; CFC, m = 390, f = 395; SAC, m = 405, f = 411.

Table 2. A) Oblique three-factor solution (Direct Oblimin) with fourteen variables in male rats. Loadings < 0.20 are not shown. Each factor (with eigenvalues greater than one) of the unrotated factor solutions accounted for the following % of variance: Factor 1 = 18%, Factor 2 = 14 %; and Factor 3 = 12 %. B) Correlations between factors.

Table 3. A) Oblique three-factor solution (Direct Oblimin) with fourteen variables in female rats. Loadings < 0.20 are not shown. Each factor (with eigenvalues greater than one) of the unrotated factor solutions accounted for the following % of variance: Factor 1 = 18 %, Factor 2 = 13 %; Factor 3 = 12 %.

TABLE 1: Sex-linked differences in fearful behaviour

F2-generation rats	Mean ± SEM		F	Sig.
	Males	Females		
OPEN FIELD				
# Defecations	**3.4 ± 0.1**	**1.7 ± 0.1**	**101.3**	***P < 0.001***
# Free Rearings	5.2 ± 0.2	7.0 ± 0.2	30.5	*P < 0.001*
# Wall Rearings	13.7 ± 0.3	17.0 ± 0.3	63.7	*P < 0.001*
Grooming Latency (sec)	127.0 ± 2.6	131.7 ± 2.7	1.5	*n.s.*
Grooming Duration (sec)	**25.1 ± 0.9**	**23.6 ± 0.7**	**1.9**	***n.s.***
Distance Covered in the Inner Area (cm)	386.2 ± 10.6	433.1 ± 11.7	8.8	*P < 0.01*
Distance Covered in the Outer Area (cm)	1869.1 ± 21.3	2047.0 ± 21.6	34.3	*P < 0.001*
Total Distance (cm)	**2255.3 ± 21.0**	**2480.2 ± 23.6**	**50.7**	***P < 0.001***
Latency to Arrive to the Outer Area (sec)	19.9 ± 1.3	16.7 ± 1.1	3.4	*P < 0.07*
PLUS-MAZE				
# Defecations	1.3 ± 0.0	0.5 ± 0.0	44.9	*P < 0.001*
# Total Entries	12.9 ± 0.2	14.4 ± 0.2	20.1	*P < 0.001*
# Open Arm Entries	4.9 ± 0.1	5.9 ± 0.1	22.0	*P < 0.001*
# Enclosed Arm Entries	**8.0 ± 0.1**	**8.5 ± 0.1**	**7.7**	***P < 0.01***
Time Spent in the Open Arms (sec)	68.8 ± 2.1	80.4 ± 2.1	15.6	*P < 0.001*
Time Spent in the Center (sec)	30.0 ± 1.1	31.0 ± 1.0	0.5	*n.s.*
Time Spent in the Enclosed Arms (sec)	201.5 ± 2.2	188.7 ± 2.2	16.3	*P < 0.001*
Grooming Latency (sec)	116.0 ± 3.1	124.8 ± 3.3	3.8	*P < 0.051*
Grooming Duration (sec)	30.3 ± 1.1	26.6 ± 1.0	6.4	*P < 0.05*
% Open Arm Entries	**34.7 ± 0.7**	**38.9 ± 0.7**	**17.5**	***P < 0.001***
% Time Spent in the Open Arms	**25.4 ± 0.8**	**30.0 ± 0.8**	**17.6**	***P < 0.001***
HOLE-BOARD				
# Defecations	2.6 ± 0.1	1.2 ± 0.1	71.2	*P < 0.001*
# Head-dips	5.4 ± 0.1	9.5 ± 0.2	298.3	*P < 0.001*
Time Spent Head-dipping (sec)	**12.9 ± 0.3**	**30.6 ± 1.0**	**271.8**	***P < 0.001***
Grooming Latency (sec)	67.1 ± 2.2	96.3 ± 3.2	55.2	*P < 0.001*
Grooming Duration (sec)	39.1 ± 1.2	26.7 ± 0.9	66.7	*P < 0.001*
ACTIVITY METER				
# Defecations	2.7 ± 0.1	2.5 ± 0.1	1.1	n.s.
Mean Activity Counts (30 min)	**1738.9 ± 19.2**	**1897.4 ± 22.1**	**29.4**	***P < 0.001***
ACOUSTIC STARTLE REFLEX				
# Defecations	4.4 ± 0.1	3.2 ± 0.1	34.1	*P < 0.001*
Mean 1-5 trials (mV)	660.0 ± 38.0	300.4 ± 19.0	71.7	*P < 0.001*
Mean 6-10 trials (mV)	383.8 ± 30.3	238.2 ± 16.5	17.8	*P < 0.001*
Mean 11-15 trials (mV)	308.8 ± 24.9	196.1 ± 14.7	15.2	*P < 0.001*
Mean 16-20 trials (mV)	253.8 ± 17.8	178.6 ± 12.3	12.1	*P < 0.001*
Mean 1-20 trials (mV)	**402.1 ± 25.2**	**228.6 ± 14.3**	**35.8**	***P < 0.001***
CLASSICAL FEAR CONDITIONING				
# Defecations in the Training Phase	5.2 ± 0.1	4.5 ± 0.1	11.2	*P < 0.001*
# Defecations in the Test Phase	5.1 ± 0.1	3.8 ± 0.2	35.0	*P < 0.001*
Freezing to the Context during 3 min (sec)	**56.9 ± 2.5**	**70.0 ± 2.6**	**13.2**	***P < 0.001***
Freezing to the CS during 3 min (sec)	**58.1 ± 2.1**	**68.6 ± 2.3**	**11.3**	***P < 0.001***
SHUTTLEBOX AVOIDANCE CONDITIONING				
# Crossings During Habituation Period (3 min)	**6.3 ± 0.1**	**6.1 ± 0.1**	**1.4**	**n.s.**
# Defecations at 40 trials	5.9 ± 0.1	5.6 ± 0.1	2.1	n.s.
# Avoidances at 40 trials	**9.3 ± 0.4**	**12.2 ± 0.5**	**21.6**	***P < 0.001***
# Intertrial Crossings at 40 trials	**14.9 ± 0.8**	**23.1 ± 1.2**	**33.3**	***P < 0.001***

TABLE 2A

Three-fold factor solution for male rats

	Factor		
	1	2	3
SHUTTLEBOX CONDITIONING			
# Avoidances at 40 trials	.80	---	---
# Intertrial Crossings at 40 trials	.80	---	---
# Crossings During Habituation Period	.48	.33	-.23
CLASSICAL FEAR CONDITIONING			
Freezing to the Context during 3 min (sec)	-.23	---	.74
Freezing to the CS during 3 min (sec)	-.23	---	.78
PLUS-MAZE TEST			
# Enclosed Arm Entries (5 min)	.23	.46	---
% Open Arm Entries (5 min)	---	.80	---
% Time in the Open Arms (5 min)	---	.89	---
OPEN-FIELD TEST			
# Defecations (5 min)	---	---	-.36
Self-grooming Duration during 5 min (sec)	-.31	---	-.35
Distance Covered during 5 min (cm)	.44	.48	.33
HOLE-BOARD TEST			
Head-dipping Duration during 5 min (sec)	---	.27	---
STARTLE REFLEX TEST			
Startle Amplitude (mV) (mean 20 trials)	---	.21	.20
ACTIMETER			
Activity Counts during 30 min	.48	---	---
Congruency indices			
Relative to the total sample (C.I. = .97)	.97	.93	.98
Relative to females (C.I. = .89)	.97	.75	.94

TABLE 2B

Correlations between factors

	1	2	3
1	1		
2	.20	1	
3	.05	-.08	1

TABLE 3A

Three-fold factor solution for female rats

	Factor		
	1	**2**	**3**
SHUTTLEBOX CONDITIONING			
# Avoidances at 40 trials	.75	---	---
# Intertrial Crossings at 40 trials	.75	---	---
# Crossings During Habituation Period	.61	---	.28
CLASSICAL FEAR CONDITIONING			
Freezing to the Context during 3 min (sec)	-.59	-.20	---
Freezing to the CS during 3 min (sec)	-.62	-.20	---
PLUS-MAZE TEST			
# Enclosed Arm Entries (5 min)	---	---	.68
% Open Arm Entries (5 min)	---	.90	---
% Time in the Open Arms (5 min)	---	.90	---
OPEN-FIELD TEST			
# Defecations (5 min)	---	---	-.49
Self-grooming Duration during 5 min (sec)	---	---	-.57
Distance Covered during 5 min (cm)	---	---	.69
HOLE-BOARD TEST			
Head-dipping Duration during 5 min (sec)	---	---	---
STARTLE REFLEX TEST			
Startle Amplitude (mV) (mean 20 trials)	---	-.20	---
ACTIMETER			
Activity Counts during 30 min	.27	---	.37
Congruency indices			
Relative to the total sample (C.I. = .92)	.99	.81	.97
Relative to males (C.I. = .89)	.97	.75	.94

TABLE 3B

Correlations between factors

	1	2	3
1	1		
2	.10	1	
3	.05	.02	1

A QUANTITATIVE TRAIT LOCUS INFLUENCING ANXIETY IN THE LABORATORY RAT

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ABSTRACT. A critical test for a gene that influences susceptibility to fear in animals is that it should have a consistent pattern of effects across a broad range of conditioned and unconditioned models of anxiety. Despite many years of research, definitive evidence that genetic effects operate in this way is lacking. The limited behavioural test regimes so far used in genetic mapping experiments and the lack of suitable multivariate methodologies have made it impossible to determine whether the quantitative trait loci (QTL) detected to date specifically influence fear-related traits. Here we report the first multivariate analysis to explore the genetic architecture of rodent behaviour in a battery of animal models of anxiety. We have mapped QTLs in an F2 intercross of two rat strains, the Roman high and low avoidance rats, that have been selectively bred for differential response to fear. Multivariate analyses demonstrate that one locus, on rat chromosome 5, influences behaviour in different models of anxiety. The QTL influences two-way active avoidance, conditioned fear, elevated plus maze and open field activity, but not acoustic startle response or defecation in a novel environment. The direction of effects of the QTL alleles, and a coincidence between the behavioural profiles of anxiolytic drug and genetic action, are consistent with the QTL containing at least one gene with a pleiotropic action on fear responses. As the neural basis of fear is conserved across species, we suggest that the QTL may have relevance to trait anxiety in humans.

Introduction

Both pharmacological and genetic studies suggest that the neural basis of fear in animals underpins anxiety in humans. Thus, major advances in our understanding of the neuronal basis of anxiety in humans followed the successful development of behavioural tests for investigating fear responses in rodents (Lang, et al. 2000; McNaughton and Gray 2000; Gray and McNaughton 2000; LeDoux 2000). As a first step towards identifying the genetic basis of individual differences in response to fear-provoking stimuli in rodents, we, and others, have shown that using crosses between inbred rodents, it is possible to map genetic loci that influence behaviour in rodent models of anxiety (Flint, et al. 1995; Wehner, et al. 1997; Caldarone, et al. 1997; Gershenfeld and Paul 1997; Moisan, et al. 1996; Turri, et al. 2001). However in every genetic mapping experiment carried out to date, variation in rodent fear responses has been inferred from a limited number of behavioural tests.

While it is often assumed that genetic effects on fear have a broad influence, and that the loci so far detected will account for variation in conditioned responses such as the fear potentiated startle and conditioned avoidance paradigms favoured in neurobiological investigation of emotion (McNaughton and Gray 2000; LeDoux 2000), this hypothesis has been difficult to test, for a number of reasons. First, genetic mapping in rodents has, until recently, been easiest to carry out in the mouse, while investigation of the neuronal basis of fear and anxiety is based primarily on behavioural tests developed in the rat. Equivalent behavioural tests in the mouse can be found (Falls, et al. 1997), but they are in general time consuming to carry out and not suited to the genetic mapping of fear, which requires analyzing large numbers of animals to detect the small genetic effects involved (Darvasi 1998). Tests of an animal's response to a novel, hence potentially threatening, environment (the open field, elevated plus maze and light dark box) can be relatively easily carried out on hundreds of mice so most available genetic mapping data is for tests of this type. Mapping results derived from these tests alone may have limited applicability, as anxiety disorders in humans consists of more than pathological responses to fear of the unknown.

Second, it has been difficult to determine whether the quantitative trait loci (QTL) so far detected specifically influence fear and anxiety, or other, unrelated, traits. For example, measures taken in the open field and elevated plus maze rely on differences in locomotor activity, so that they reflect individual variation in both fear responses and spontaneous activity (Turri, et al. 2001). The requisite multivariate analytical techniques have not been available to disentangle the genetic architecture of the traits and to test whether, as predicted for a genetic effect on fear responses (Ramos and Mormede 1998), a locus has a joint action on several behavioural measures or whether fear is multidimensional, consisting of independent traits, each with a limited domain of action.

With the development of dense genetic markers for the rat (McCarthy, et al. 2000) and of appropriate multivariate tools (Knott and Haley 2000; Korol, et al. 2001) it is now possible to ask whether the same genetic loci contribute to variation in different behavioural models of anxiety, including both conditioned fear and tests of novelty. We therefore set out to map quantitative trait loci influencing fear related behaviours in one of the most thoroughly documented animal models of anxiety, the Roman high and low avoidance rats (RHA/Verh and RLA/Verh respectively), the product of bi-directional selection for two-way active avoidance acquisition in a shuttle box (Bignami 1965).

The behavioural differences of the Roman rat strains are consistent with an inter-strain variation in responses to fear stimuli. In the shuttle-box, RHA/Verh rats quickly acquire the active avoidance response, whereas RLA/Verh rats display much freezing and escape responses during the acquisition phase (Driscoll and Battig 1982; Fernandez-Teruel, et al. 1997; Escorihuela, et al. 1995). Results from other models of anxiety (the open field, elevated plus maze and light-dark box and freezing to a conditioned stimulus) concur: both inbred and outbred RHA/Verh rats are less anxious than their inbred or

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outbred RLA/Verh counterparts (Driscoll, et al. 1998; Escorihuela, et al. 1999; Steimer, et al. 1997). Furthermore, differences in neuroendocrine responses support the view that the RLA/Verh animals are more susceptible to environmental stressors than the RHA/Verh rats, as would be expected from the strain that is more responsive to fear-provoking stimuli (Driscoll, et al. 1998).

We used two approaches to define genetic influences on fear. First, we expect genetic effects on fear responses to work in a theoretically predictable fashion. Thus a QTL that influences two-way active avoidance should not only influence variation in conditioned fear as well, but the allele that decreases avoidance response in the shuttle box should also increase freezing to the conditioned stimulus. Additionally, the same QTL should increase fear-related behaviour in the open field and elevated plus maze. Within these tests, the animal is presented with a choice between threatening and non-threatening environments and the allelic effects of the QTL are expected to reflect this distinction. In the elevated plus maze, rats have a choice between two relatively fear-provoking regions (the open arms) and two relatively safe regions (the closed arms) (Pellow, et al. 1985; Hogg 1996; Rodgers and Dalvi 1997). Within the open field, there are thought to be distinctions in the level of threat the exposed area provides: the periphery is safer than the centre. Consequently an allele that decreases the number of entries and time spent on the open arms of the elevated-plus maze should decrease activity in the centre of the open field.

Second, we expect the genetic effects to be specific to fear responses and not to other behaviours. For instance, in the elevated plus maze, a QTL with a putative effect on fear responses is expected to have little or no influence on entries into the closed arms of the apparatus, a measure of activity. Nor should this QTL have an influence on spontaneous activity, which we measured in the home cage.

Thus we aimed to measure responses to fear-provoking stimuli from different perspectives and to employ multivariate techniques to determine whether loci act pleiotropically across all or a subset of the animal models of anxiety. We set out to determine specificity of action by including measures of spontaneous activity and by using multiple measures within each apparatus. Our experiment sought to identify common genetic effects that could be interpreted as influencing fear in rodents and, consequently, fear and anxiety in humans.

Results
Univariate analyses
We found that correlations between measures taken in the same test are, in almost all cases, highly significant and exceed 0.4, while correlations between tests are low, never exceeding 0.4 (see table 1). These results, consistent with previous reports, indicate that common genetic effects, if present, are likely to be small. Because of the very high intratest correlations, for subsequent multivariate analyses we chose a subset of measures from the elevated plus maze (percentage of time spent on the open arms and numbers of entries into the closed arms) and shuttle box (avoidance responses).

A total of 908 rats were genotyped. From 436 markers that amplified DNA from the parental strains, 82 polymorphic markers were obtained. The distribution of polymorphic

markers across the genome was not random: only 10% of 49 markers tested on chromosome 1 were polymorphic, compared to a third of those on chromosomes 5, and half of those tested on chromosomes 12 and 13. Despite screening all available markers, only two were polymorphic on chromosomes 11 and 18. Overall 75% of the genome was covered at a resolution of 15 cM or less.

In table 2 we show the LOD scores for all behavioural measures on chromosomes where at least one chromosome exceeded a 5% significance level as determined by a permutation test (shown in bold). The 5% threshold corresponds to the 95%, 99.29% and 99.69% quantiles of the estimated permutation of the LOD score, derived from 1,000 permutations (Churchill and Doerge 1994). Eight loci were identified, with a variety of effects across tests. On chromosome 1, the QTL appears to influence rearing time only, while a QTL on chromosomes 5 influences nine measures in six tests. Figure 1 shows plots of the LOD curves for several of the traits on chromosomes 5, 10 and 15.

The apparent specificity of QTL action may reflect our inability to detect small effect loci. We estimated the effect of each locus on each phenotypic measure and their associated standard deviations (table 2). The estimated effect sizes of non-significant QTL are small, but the standard deviations are large. These results are consistent with the argument that genetic action is relatively specific, but do not prove it to be the case.

Multivariate analyses and a test of pleiotropy
We next mapped the traits jointly, using Multi-QTL (http://www.multiqtl.com). We used methods that combine multivariate analysis with permutation techniques to assess the significance of a locus's contribution to the detection of a QTL and to test the significance of the QTL effect for each of the traits(Korol, et al. 2001). The individual values of each phenotype are reshuffled relative to the other traits and genotypes, and the resulting data set is re-analysed. Then, over a large number of permuted data sets (10,000 in our case) for each phenotype, the proportion of analyses is calculated where the estimated QTL effect is greater than or equal to the QTL effect obtained with un-permuted data. The procedure is applied in a stepwise fashion, excluding the insignificant traits by creating a new data set without them, and repeating the permutation.

Using this procedure, we took each QTL identified by the univariate analysis and estimated the probability that each measure contributes to the LOD score. For example on chromosome 1, when all measures are analysed together, activity in the periphery of the open field had the lowest probability (p = 0.84) (see table 3). Following the procedure of Korol et al (Korol, et al. 2001), a new trait complex was constructed without this measure, and the permutation test repeated until the only remaining traits made significant contributions to the LOD score (at a 0.05 level). Table 3 shows the *P*-values for these analyses for all eight chromosomes bearing a QTL, but omits the intermediate steps. Two columns are shown for each chromosome: the first displays the results when all traits are included in the analysis and the second when all but traits making a significant contribution have been removed (Korol, et al. 2001).

The multivariate analysis indicates that only three loci (on chromosomes 5, 10 and 15) have broad effects across different test measures. At other locations significant contributions to the LOD scores derive from a single (chromosome 19) or two phenotypes (chromosomes 1, 3 and 6). Both defecation and activity in a novel environment contribute to the LOD score on the X chromosome, but there is no significant contribution from the other measures of fear. Of the three potential candidates as loci influencing fear, that on chromosome 15 has the most circumscribed effect. The evidence is strongest for an effect on grooming and there is no significant contribution from shuttle box, fear conditioning or elevated plus maze to the LOD score.

The analyses do not distinguish a joint effect due to physical linkage of two quantitative trait loci from the pleiotropic action of a single locus. Therefore we sought evidence for pleiotropic action on chromosomes 5, 10 and 15 using the multivariate regression method of Knott and Haley (Knott and Haley 2000). We chose those traits known to make a significant contribution to the LOD score for loci on chromosomes 5, 10 and 15, and tested the hypothesis of one QTL for each trait versus one QTL influencing all traits. The test statistic is based on the ratio of the determinants of the residual sum of squares matrix from the best pleiotropic QTL model, to the residual sum of squares matrix from fitting a model in which a QTL affects each trait individually. In this test the null hypothesis is a single pleiotropic QTL. The estimates obtained from the best pleiotropic QTL model were used as parameters for replicate simulations. A test statistic was calculated from each of 1,000 replicates and a significance threshold obtained. The test statistic from the original data set was compared with this threshold to determine significance. Table 4 gives the results of these analyses. We were able to reject the hypothesis of a single pleiotropic QTL on chromosome 10 at the 5% threshold.

Direction of allelic effects

The multivariate analyses indicated that the loci on chromosomes 5 and 15 have pleiotropic effects on fear responses. We next asked whether the alleles of these loci act in a manner consistent with this interpretation across all tests. For each QTL we looked at the direction of effect of the allele from the RHA/Verh strain. The direction of allelic effects is given by the sign associated with the effect size for each phenotype in table 2.

On chromosome 5 the allele from the RHA/Verh rats increases avoidance responses and inter-trial crossing, while decreasing escape latency, consistent with the allele's origin from that strain and with a role in determining variation in fear. The allele's influence on other measures is also consistent with the hypothesis that it influences fear. It decreases conditioned freezing in response to both context and cue, and increases the time spent in and number of entries into the open arms of the elevated plus maze. It increases rearing and activity in the periphery of the open field arena while decreasing time spent grooming in novel environments.

On chromosome 15 the allele increases acoustic startle responses, time spent grooming and entries into the closed arms of the elevated plus maze, but decreases activity in the centre of the open field. The QTL therefore influences one measure of activity (entry into the closed arms of the elevated plus maze) as well as three measures of fear. It has little effect on other measures in the shuttle box or fear conditioning.

The QTL on chromosome 10, which, by a test of pleiotropy consists of two linked QTL, has alleles whose direction of action are inconsistent with a role in fear. The same allele that increases two-way active avoidances increases contextual conditioning and decreases the startle response. Presumably, the allele operating to increase avoidance responses belongs to a different QTL from that which influences fear conditioning. However we cannot determine how many individual loci are operating and if any have pleiotropic action.

Discussion

Our study is the first to exploit multivariate analyses to explore the genetic architecture of a battery of behavioural tests, all of which are used as animal models of anxiety. We have identified eight quantitative trait loci, of which three, on chromosomes 5, 10 and 15, influence more than one behavioural measure of fear. Multivariate approaches were used to establish the significance of the contribution to the LOD score of each trait. These analyses provide evidence that loci on chromosomes 1, 3, 6, 19 and X have effects inconsistent with an influence on fear responses (for example the QTL on chromosome 6 affects spontaneous activity and defecation in a novel environment) while the loci on chromosomes 5, 10 and 15 influence a broad range of measures of fear, as would be expected if they contain genes involved in determining a response to fear-provoking stimuli.

At each of the loci we have detected, multivariate analyses has been used to set the significance of the contribution from each trait (Korol, et al. 2001) and show that, with the exception of the loci on chromosomes 5, 10 and 15, QTL effects are relatively specific. However, our analysis cannot exclude the existence of other quantitative trait loci that have pleiotropic influences on fear, but with such small effects that they are undetectable with the number of animals we have used.

A more difficult problem is to decide whether a joint genetic effect is due to the presence of a single pleiotropic QTL or to multiple linked genes. However, again using a novel multivariate statistic (Knott and Haley 2000), we have been able to demonstrate that multiple linked genes are more likely than pleiotropy on chromosome 10. At the other loci, on chromosomes 5 and 15, the test could not rule out pleiotropy.

Examination of the direction of QTL effects provides additional support for a QTL's influence on fear. The direction of allelic effects can be interpreted as indicating the presence of a gene that determines variation in fear responses at only one locus, on chromosome 5. Here, the allele that increases two-way active avoidance also decreases cue and contextual fear conditioning and grooming, while increasing time in the open arms of the elevated plus maze and activity in the open field, as well as rearing. It has no discernible influence on spontaneous activity, the acoustic-startle response, or defecation. This pattern is consistent with the action of a gene influencing an animal's reaction to a fear stimulus, and parallels the effects of drugs used to treat anxiety disorders in humans (reviewed in (Simon and Soubrie 1979; Gray 1977; Fernandez-Teruel, et al. 1991)), which improve two-way active avoidance, block the acquisition of conditioned freezing,

increase the time spent on and number of entries into the open arms of the elevated plus maze and increase activity in the open field. Neither anxiolytic drugs nor the QTL affect ASR and defecation. The finding that genetic effects on defecation can be dissociated from other tests of fear is supported by our analysis of Maudsley rat strains, derived by selection for differences in open-field defecation(Paterson, et al. 2001).

How can the influence of other QTL be explained? Some of the inconsistencies of action are likely to be due to linked genes, as we have shown for chromosome 10 where the presence of at least two loci is required to explain an effect that decreases ASR and increases contextual fear conditioning. Multivariate analysis supports such a division, but does not say how many genes there might be at this locus. Our analysis failed to rule out a pleiotropic locus on chromosome 15, but the fact that the QTL influences measures that do not cohere in any way predicted by current theories of the neuropsychology of anxiety suggests that the genetic effect may be due to multiple linked genes. At other locations, the QTL's influences are far too restricted to fit expectations. Using the results of the multivariate analyses, we find a QTL on chromosome 6 that influences defecation and spontaneous activity and loci on chromosome 1 and 19 specific for rearing and grooming respectively.

In summary, our results give rise to two conclusions. First, it is possible to detect quantitative trait loci that have a consistent pattern of effects across a broad range of relevant tests of animal behaviour. This is important because, despite many years of research, evidence that genetic effects operate in this way has been lacking. Based on the phenotypes that the QTL influences, the direction of effects of the QTL alleles, and a coincidence between the behavioural profiles of anxiolytic drug and genetic action, we argue that the QTL on rat chromosome 5 harbours a gene that influences fear behaviour and that identification of the homologous gene in humans may lead to a better understanding of the neural basis of human anxiety. Second, our results are important for showing that many loci have narrow, often test-specific, ranges of influence, precluding ready functional interpretation. Our data demonstrate that a limited behavioural repertoire cannot be used reliably to infer a genetic action as an effect on fear, whether that gene is a transgene or is contained within a QTL.

Methods

F2 intercross. The F2 generation Roman rats, derived from inbred RHA/Verh and RLA/Verh, and equally divided between males and females, were bred in three batches over an eighteen-month period. Behavioural testing was carried out separately for each batch. Rats were maintained under controlled conditions of humidity (60 ± 10 %) and temperature (22 ± 2 ºC), a 12 h cycle (lights on at 8:00 h and light off at 20:00 h), with free access to food and water. They were housed in groups of two (males) or three (females). Rats were tested at the age of 4 months and male and females were evaluated together in a counterbalanced manner. A period of 10 to 20 days was allowed between consecutive tests. The experimental sequence was as follows:

Open field. The apparatus was a beige circular arena (diameter, 83 cm), enclosed by white wood walls (height, 34 cm) and divided into 19 equal sectors. It was illuminated by a white 200 W bulb placed 90 cm over the center of the arena. Rats were placed in the center of the open field arena for a 5-min recording period. A computerized image analysis system (SMART, Panlab) was used to record distance covered in the centre and the periphery of the open field and the latency to leave the centre. Defecation was scored manually.

Elevated plus-maze. The apparatus, made of black wood, consisted of two opposing open arms (50 x 10 cm), two opposing enclosed arms, (50 x 10 x 40 cm), and an open 10 x 10-cm square in the center, the whole being set 50 cm above the ground. Testing was carried out in ambient light. Rats were placed in the center of the plus-maze facing an enclosed arm and behaviour measured for a 5-min period. The number of entries and time spent in the arms (open and enclosed) and defecations were scored.

Spontaneous activity. Motor activity was measured in a multi-cage actimeter system (three cages simultaneously, Interface PANLAB 40035, Sensor Unit PANLAB 0603). Testing cages (transparent Plexiglas, 35 x 35 x 25 cm) were slightly different from the home cage and contained clean sawdust. Activity was automatically scored over a 30-minute period.

Acoustic startle response. A Startle Response System (San Diego Inst.) was used. Each animal was first placed in a Plexiglas cylinder (located within a 35 x 33 x 39 cm sound-attenuated chamber lit by a 20 W bulb). Cylinder movements resulting from startle responses were detected by an accelerometer. Acoustic stimuli of 110 dB for 50 ms were delivered by a loudspeaker, mounted at a distance of 23 cm above the Plexiglas cylinder. A fan located inside the sound-attenuated chamber provided background noise. Startle response amplitude was defined as the maximum accelerometer voltage during the first 200ms following the startle stimulus onset. Animals were tested pairwise, using two identical startle response chambers. Each animal was given a 5 min acclimatization period before the first acoustic startle stimulus. Each testing session consisted of 20 startle stimuli, with an interstimulus interval of 30s. The mean startle response amplitude for the 20 trials was calculated for each animal.

Classical fear conditioning. The apparatus was a white chamber divided into two equal compartments (23 x 12 x 20 cm). A 1mA scrambled electric footshock (0.5s) (the unconditioned stimulus (US)) was administered through a grid floor. A 15 s duration light from a 20 W bulb located in the upper part of a wall was the conditioned stimulus (CS). Training consisted of five CS-US pairings that started with the onset of the CS. US and CS terminated simultaneously. A 120s pseudorandom intertrial interval was used, with a shock-free interlude of one minute. After 24 hours, 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 five minutes to measure fear conditioning to the CS.

Two-way active avoidance conditioning. The experiment was carried out with three identical shuttle boxes (Letica Inst.) each one of them placed in independent, sound-attenuating boxes constructed of plywood. A fluorescent lamp provided dim and diffuse illumination. The shuttle boxes consisted of two equally sized compartments (25 x 25 cm, 28 cm), connected by an opening (8 x 10 cm). A 2400 Hz, 63-dB tone plus a light (from a small, 7 W lamp) functioned as the conditioned stimuli (CS). The unconditioned stimulus (US), which started at the end of the CS, was a scrambled electric footshock of 0.7 mA delivered through the grid floor. Once rats were placed into the shuttle box a 4-min familiarization period elapsed before starting training. After this period, 40 acquisition trials were administered. Each trial consisted of a 10 s CS, followed by a 20 s US. The CS or US were terminated when the animal crossed to the other compartment, with crossings during the CS being considered avoidance responses and crossings during the inter-trial interval (ITI) considered as inter-trial crossings. Once a crossing had been made and/or the shock (US) discontinued, a 1 min fixed ITI was presented.

Defecation, rearing and grooming time. Time spent self grooming and number of fecal boli were recorded in the open field, elevated plus maze and during habituation to the shuttle box. Time spent rearing (both free and against the wall) was recorded for the duration of the open-field test and in the habituation phase of the shuttle box. Mean scores for each of the three were used in subsequent analyses.

Genotyping. DNA was extracted from tails and genotyped using standard techniques (5). We chose markers from the radiation hybrid (RH) map (Watanabe, et al. 1999) aiming for intervals of between 20 and 30 centimorgan (cM) intervals. The order of all markers was determined using the MAPMAKER software package (Lincoln, et al. 1992) and results compared with radiation hybrid maps (Watanabe, et al. 1999).

Statistics. Data were analysed by regression to assess mean differences as a function of sex and weight. Data were corrected for weight and sex by multiple regression: standardized residuals were used in all subsequent analyses. We performed univariate analyses on each measure using the map distances derived from the MAPMAKER software by interval mapping (Lander and Botstein 1989) in QTL-MAPMAKER (Lincoln, et al. 1992) and composite interval mapping (Zeng 1994) in QTL-CARTOGRAPHER (Basten, et al. 1994). Significance levels were evaluated by permutation using the method of Churchill and Doerge (Churchill and Doerge 1994).

Multivariate analyses were performed using Multi-QTL (http://www.multiqtl.com) (Korol, et al. 2001). Significance levels were evaluated by permutation, carried out in the Multi-

QTL package (http://www.multiqtl.com)(Korol, et al. 2001). In order to test between two linked quantitative trait loci or one pleiotropic QTL we used the method of Knott and Haley(Knott and Haley 2000). The test was carried out in a Fortran programme kindly provided by Dr Sara Knott. Traits for testing were chosen on the basis of whether they made a significant contribution to the LOD score, as determined by the Multi-QTL results. Genotype probabilities were generated using the program HAPPY(Mott, et al. 2000).

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Table 1: Correlations between phenotypes

	Open Field Activity in Centre	Open Field Activity in Periphery	Elevated Plus Maze Pct Open Arm Entries	Elevated Plus Maze Pct Open Arm Time	Elevated Plus Maze Closed Arm Entries	Elevated Plus Maze Closed Arm Time	Acoustic Startle Response	Fear Conditioning to Context	Fear Conditioning to Cue	Shuttle Box Avoidances	Shuttle Box Intertrial Crosses	Shuttle Box Latency	Defecation	Time Grooming	Time Rearing
Open Field Activity in Periphery	-0.42														
Elevated Plus Maze Pct Open Arm Entries	0.08	0.02													
Elevated Plus Maze Pct Open Arm Time	0.16	0.02	0.83												
Elevated Plus Maze Closed Arm Entries	0.27	-0.02	-0.06	0.30											
Elevated Plus Maze Closed Arm Time	-0.08	-0.07	-0.67	-0.79	-0.21										
Acoustic Startle Response	0.05	0.00	0.05	0.01	0.01	0.00									
Fear Conditioning to Context	-0.07	0.02	-0.06	-0.06	-0.05	0.03	0.10								
Fear Conditioning to Cue	-0.06	0.05	-0.09	-0.09	-0.04	0.07	0.01	0.62							
Shuttle Box Avoidances	0.06	-0.03	-0.02	-0.05	0.03	0.01	0.03	-0.15	-0.17						
Shuttle Box Intertrial Crosses	0.08	-0.05	0.03	0.02	0.04	-0.06	-0.01	-0.10	-0.17	0.75					
Shuttle Box Latency	-0.04	0.01	0.02	0.05	-0.02	-0.02	-0.04	0.14	0.17	-0.87	-0.72				
Defecation	-0.10	0.09	-0.02	-0.10	-0.13	0.08	0.09	0.14	0.15	-0.05	-0.12	0.07			
Time Grooming	-0.06	-0.01	0.03	-0.10	-0.22	0.25	0.07	-0.09	-0.09	0.07	-0.05	-0.07	0.11		
Time Rearing	0.35	-0.05	0.11	0.15	0.20	-0.06	0.04	-0.23	-0.20	0.14	0.17	-0.15	-0.12	-0.04	
Spontaneous Activity	0.00	0.03	-0.01	0.00	0.03	0.01	0.05	-0.12	-0.13	0.20	0.19	-0.18	-0.23	-0.12	0.14

Table 2: LOD scores, effect sizes and standard deviations for univariate analyses. Scores significant at the 5% threshold are shown in bold. The LOD scores, and associated effect sizes, are the maximum across the chromosomes.

Chr		Shuttle Box			Fear Conditioning		Elevated Plus Maze		Open Field		Acoustic Startle Response	Spontaneous Activity	Grooming	Rearing	Defecation
		Avoidances	Latency	Intertrial Crossing	Cue	Context	Pct Open Arm Time	Closed Arm Entries	Activity in Periphery	Activity in Centre					
1	LOD	1.190	1.250	0.890	1.950	1.830	0.530	2.270	0.720	0.890	1.190	2.890	0.600	**3.510**	1.400
	Effect Size	0.120	-0.188	0.100	-0.142	-0.181	-0.052	0.200	0.198	-0.095	-0.225	-0.273	-0.035	**0.368**	-0.055
	Stand. Dev.	0.259	0.229	0.233	0.142	0.146	0.202	0.246	0.187	0.174	0.162	0.236	0.169	0.117	0.204
3	LOD	1.590	1.440	1.850	2.040	1.520	2.520	0.650	**5.040**	0.870	0.820	0.240	1.400	1.970	**3.180**
	Effect Size	0.367	-0.203	0.314	0.071	-0.202	-0.281	-0.019	**0.341**	-0.163	-0.078	0.006	-0.205	0.216	**-0.272**
	Stand. Dev.	0.219	0.116	0.269	0.177	0.153	0.128	0.227	0.118	0.151	0.203	0.149	0.107	0.104	0.072
5	LOD	**9.470**	**6.120**	**6.460**	**3.490**	**4.460**	**4.080**	1.650	**3.140**	1.110	1.500	2.490	**3.050**	**4.630**	1.090
	Effect Size	**0.664**	**-0.539**	**0.544**	**-0.374**	**-0.415**	**0.342**	0.173	**0.365**	-0.044	0.239	0.246	**-0.304**	**0.376**	0.079
	Stand. Dev.	0.100	0.110	0.120	0.160	0.134	0.180	0.158	0.149	0.187	0.146	0.175	0.111	0.095	0.113
6	LOD	2.970	2.190	1.380	1.690	1.820	0.710	1.400	0.800	1.030	2.560	**7.450**	1.140	1.330	**3.360**
	Effect Size	-0.422	0.268	-0.054	0.246	-0.029	0.159	0.207	-0.034	-0.030	0.333	**-0.743**	-0.079	-0.146	**0.187**
	Stand. Dev.	0.181	0.174	0.192	0.141	0.163	0.171	0.152	0.179	0.179	0.170	0.210	0.168	0.108	0.091
10	LOD	**4.130**	**3.130**	**4.000**	1.590	**5.950**	0.420	0.670	2.270	2.420	**3.530**	**3.260**	1.340	0.430	1.670
	Effect Size	**0.282**	**-0.353**	**0.386**	0.350	**0.575**	-0.060	-0.021	-0.360	-0.077	**-0.437**	**-0.458**	0.167	0.006	0.143
	Stand. Dev.	0.389	0.239	0.320	0.200	0.131	0.182	0.192	0.142	0.130	0.187	0.216	0.171	0.134	0.158
15	LOD	1.050	0.450	2.230	1.520	2.200	1.360	**3.430**	2.650	**3.450**	**4.830**	1.070	**3.060**	1.480	0.970
	Effect Size	0.243	-0.141	0.340	-0.105	0.220	0.157	**0.440**	0.368	**-0.377**	**0.450**	0.158	**0.304**	0.229	-0.040
	Stand. Dev.	0.136	0.134	0.127	0.171	0.159	0.125	0.113	0.099	0.124	0.111	0.126	0.081	0.085	0.076
19	LOD	2.680	2.860	1.190	0.700	0.040	2.440	0.430	2.220	1.570	0.520	0.910	**3.950**	0.950	**3.310**
	Effect Size	-0.337	0.374	-0.023	0.205	0.242	0.325	0.187	0.291	-0.039	-0.161	0.130	**-0.384**	0.184	**-0.275**
	Stand. Dev.	0.126	0.155	0.215	0.168	0.154	0.145	0.135	0.170	0.142	0.180	0.170	0.097	0.114	0.097
X	LOD	2.170	1.680	2.040	2.770	1.040	1.100	1.980	2.010	0.310	1.010	**3.900**	1.920	0.700	**6.180**
	Effect Size	0.144	-0.023	0.200	-0.339	-0.207	-0.149	-0.269	-0.287	0.067	0.017	**0.349**	0.122	-0.051	**-0.091**
	Stand. Dev.	0.109	0.127	0.107	0.108	0.117	0.126	0.124	0.084	0.134	0.153	0.097	0.086	0.110	0.083

Table 3. Permutation tests of significance of the contribution to a multitrait LOD score of individual measures. Two columns are shown for each QTL. In the first, the probabilities of the contribution when all measures are included in the analysis are shown. The second displays the probabilities after all non-significant contributors have been excluded in stepwise fashion, as explained in the text. The results are based on 10,000 permutations.

		CHR 1		CHR3		CHR 5		CHR 6	
Shuttle Box	Avoidances	0.662		0.888		**0.021**	**0.000**	0.561	
Fear Conditioning	Cue	0.800		0.731		0.121	**0.000**	0.732	
	Context	0.682		0.661		0.228	**0.002**	0.790	
Elevated Plus Maze	Pct Open Arm Time	0.552		0.551		0.203	**0.000**	0.832	
	Closed Arm Entries	0.202		0.981		0.711		0.411	
Open Field	Activity in Periphery	0.842		0.222	**0.000**	0.129	**0.000**	0.953	
	Activity in Centre	0.732		0.718		0.841		0.289	
Acoustic Startle Response		0.478		0.732		0.879		0.154	
Spontaneous Activity		**0.017**	**0.030**	0.863		0.861		**0.014**	**0.000**
Grooming		0.427		0.232		0.093	**0.000**	0.143	
Rearing		**0.000**	**0.000**	0.920		0.678		0.920	
Defecation		0.118		**0.000**	**0.000**	0.456		**0.000**	**0.000**
		CHR 10		CHR 15		CHR 19		CHR X	
Shuttle Box	Avoidances	**0.000**	**0.000**	0.747		0.765		0.621	
Fear Conditioning	Cue	0.471		0.562		0.881		0.881	
	Context	0.555	**0.000**	0.444		0.831		0.923	
Elevated Plus Maze	Pct Open Arm Time	0.647		0.447		0.518		0.921	
	Closed Arm Entries	0.522		0.161		0.759		0.929	
Open Field	Activity in Periphery	0.059		0.645		0.800		**0.038**	**0.000**
	Activity in Centre	0.133		0.060	**0.021**	0.561		0.677	
Acoustic Startle Response		**0.027**	**0.000**	0.412	**0.011**	0.627		0.621	
Spontaneous Activity		0.237		0.761		0.929		0.510	**0.019**
Grooming		0.521		**0.000**	**0.000**	**0.033**	**0.000**	1.000	
Rearing		0.242	**0.012**	0.145		0.666		0.321	
Defecation		**0.027**	**0.000**	0.432		0.479		**0.000**	**0.000**

Table 4. A test of pleiotropy compared to close linkage. The 5% threshold is the likelihood ratio for fitting one QTL explaining variation in all traits used in the analysis compared to individual QTL influencing variation in each trait separately. A ratio below the threshold implies that pleiotropy cannot be excluded at the 5% level.

	Chromosome 5		Chromosome 10		Chromosome 15	
	Likelihood ratio	5% Threshold	Likelihood ratio	5% Threshold	Likelihood ratio	5% Threshold
One QTL for each trait versus one QTL influencing all traits	9.38	23.24	20.81	18.53	12.72	16.72

Figure Legend

LOD plots for single measures on chromosomes 5, 10 and 15. The horizontal distance shows the distance along the chromosome in centimorgans (cM) and the markers used in the study are shown.
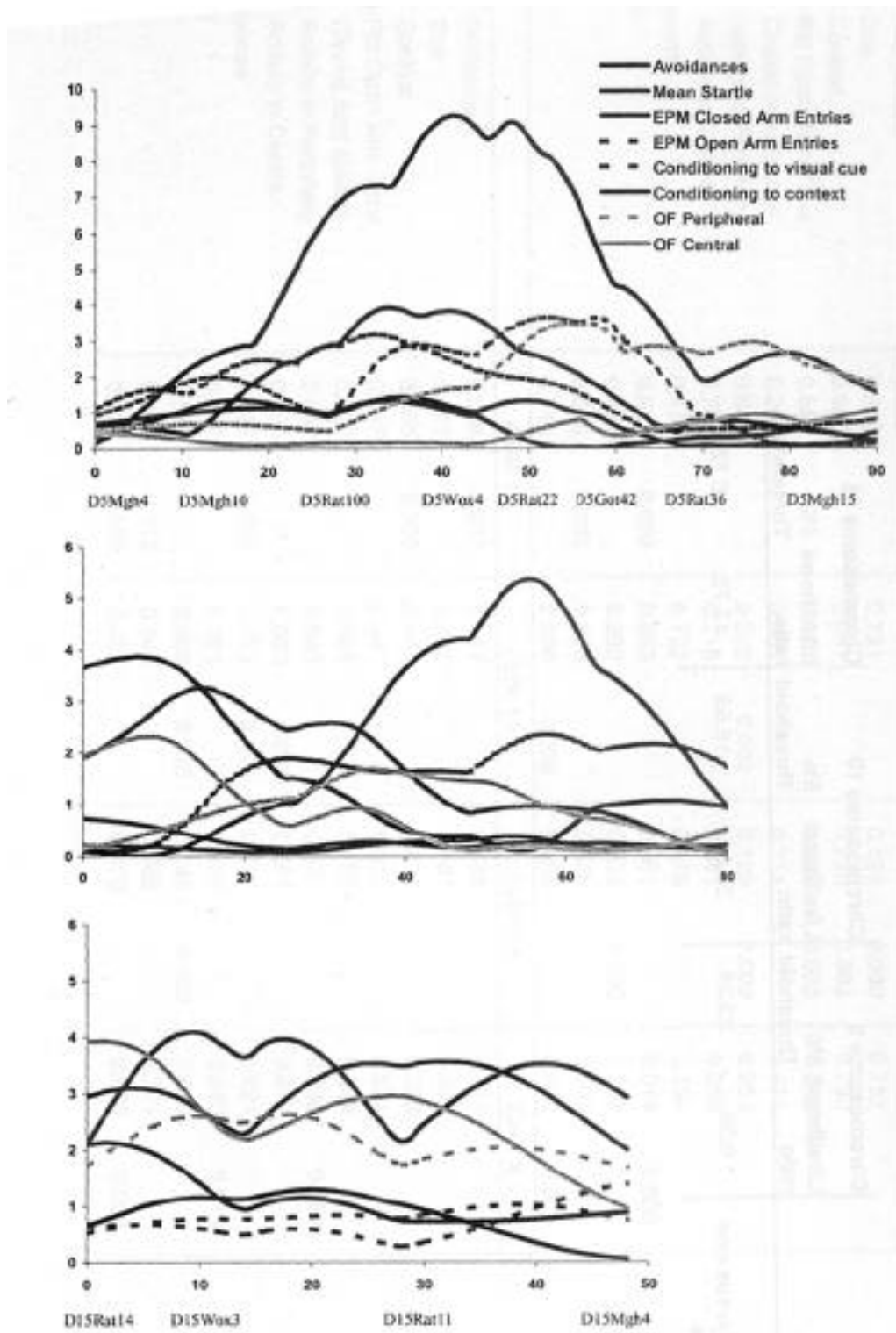
DISCUSSION

USEFULNESS OF THE ROMAN STRAINS

Almost forty years ago, Bignami (1965) published the first successful experiment on selective breeding for avoidance learning (see Brush 1991, for a comprehensive review), from which the Roman rat strains, used here, were derived: the fearful RLA and fearless RHA strains arising from extreme differences exhibited in shuttlebox performance. Since 1965, many experiments on the psychogenetics of fearfulness using these and other related strains (e.g. the Syracuse's, selected for avoidance behaviour as well, and the Maudsley's, selected for defecation in an open field) have been carried out. Rat psychogenetic models of susceptibility to anxiety have a better validity than other animal analogues, which only simulate a few simple symptoms of a syndrome or psychopathology (e.g. PTZ-induced convulsions for epilepsy and latent inhibition for schizophrenia). Because of their well-defined fearful/fearless repertoires, these strains of rats, and especially the Roman's (with a high degree of construct validity), have been widely investigated in order to establish underlying differences in neurobiological and endocrine parameters which could account for fearfulness.

It has been shown, for example, that the hypothalamic-pituitary-adrenal axis of the RLA's is more sensitive to stressful stimuli than that of the RHA's and that they have a GABAergic system which functions less efficiently than the latter (apparent validity). Pharmacological (e.g. benzodiazepines) and environmental (e.g. postnatal handling[14]) treatments seem to be more effective in reducing the anxious temperament of the RLA rats, as opposed to the RHA's (predictive validity). As the Roman rat strains fulfil the major validity criteria, they can therefore be considered to be an excellent animal analogue of human fearfulness. In favour of homology, rather than analogy, are the following two, additional points: 1) the brain mechanisms underlying defensive behaviour and related primitive emotional states (e.g. fear and anxiety) are common across species; and 2) selective breeding performed in other species (e.g. mice, birds and primates) have given rise as well to divergent progenies differing in fearfulness (see Introduction). None of the existing animal models of psychopathology meets these requirements.

Some of these arguments are equally valid when considering these rat strains as models for investigating other behavioural processes, such as impulsiveness and incentive-seeking (e.g. novelty-seeking and drug-taking behaviour). When compared to RLA rats (and other control strains), the RHA's are more explorative in novel environments (e.g. open spaces and mazes), exhibit problems in delay reinforcement in the DRL-20 bar-pressing task for food-reward, expose themselves to situations involving physical risk (open arms of the PM), show an enhanced tendency to taste and consume unknown substances (saccharin and quinine) and drugs of abuse (e.g.

alcohol), have an hyperactive mesolimbic-dopaminergic system and show, as well, augmented visual evoked potentials as do human and cat "sensation-seekers" (Driscoll et al. 1990; Fernández-Teruel 1997; Razafimanalina et al. 1996; Siegel 1997). This set of divergent behavioural and neurobiological characteristics projects them as a suitable tool for modelling human sensation-seeking and genetic susceptibility to drug addiction. Additionally, the Roman rat strains have provided a useful model for the study of genetic-environment interactions. During the last 10 years our laboratory has investigated the effects of environmental treatments on fearfulness (and more recently in sensation-seeking behaviour). We have found, for example, that postnatal handling drastically reduces the anxious temperament of RLA rats, with the effects being permanent (e.g. Fernández-Teruel et al. 1997).

Considering the extensive literature documenting differences among behavioural, hormonal and neurochemical indices in the Roman rat strains (see Fernández-Teruel et al. 1997 and Driscoll et al. 1998, for recent reviews), their heuristic value for testing current psychobiological theories of fear and anxiety (e.g. Blanchard et al. 1990, 1993; Gray and McNaughton 2000, LeDoux 1996) seems warranted. It would be interesting, for example, to compare them in regard to the different stages of the double neural pathway mediating fear conditioning (LeDoux 1995, 1996), as well as to delineate potential differences in the organization of the brain structures underlying defence systems, in terms of the hierarchical framework recently proposed by Gray and McNaughton (2000). One can imagine, in this context, how these animals would behave in the burrow system developed by the Blanchards. The RLA rats would probably exhibit exaggerated freezing and defensive threat when confronted with an anaesthetised cat in the F/DTB (regardless of defensive distance). In contrast, the RHAs would escape and eventually attack the predator. In the A/DTB, the RLAs would once again be engaged in strong freezing behaviour if exposed to a cat odor (maybe omitting defensive threat). After a long delay a timid (not active) pattern of risk assessment might emerge. In contrast, RHAs would initially show a marked active risk assessment followed, within a brief period, by active exploration of the open arena.

Given these hypothetical behavioural baselines, differential predictions (based on the Blanchard's pharmacological findings) concerning anxiolytic action could be made, as a function of strain and type of burrow system. A lack of consistent effects of drugs on RLA's and RHA's behaviour in the F/DTB could be expected. With respect to the A/DTB, however, anxiolysis could occur in both strains, with RHAs spending less time in risk assessment behaviour and with the RLA's repertoire being deeply influenced, changing from initial, persistent freezing to a timid risk assessment pattern and then to an eventual progressively increasing exploration of the open zone. In summary, fear (as defined by the Blanchards) would be insensitve to anxiolytic action (F/DTB) and anxiety (A/DTB) would be attenuated in a strain-dependent manner, the effects exerted upon the fearful animals being much more pronounced. If that proved to be the case, animals with an anxious temperament would not be sensitive to anxiolysis when exposed to fear stimuli (as ocurrs in

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[14]This is a treatment administered to rats during their infancy (i.e. 1-21 day old) which consists of isolating each pup from its litter and mother every day being gently handled for a few minutes. It has, among other effects, anxiolytic-like properties.

humans suffering from phobia), but they would show marked behavioural desinhibition in an anxiety-inducing situation as a result of drug effects (as when an anxious person is suffering from a situation over which he/she has no control). Taking into account the well-defined psychobiological profile of the Roman strains (after more than three decades of experimental work), the usual strategy of searching for strain differences in simple parameters should give place to disciplined, theoretically-oriented lines of research.

FEAR, ANXIETY AND STARTLE

Davis and colleagues (e.g. Boulis and Davis 1989; Davis 1989) developed the shock sensitization startle paradigm to study what they thought to be a form of unlearned fear, hypothetically related to generalized states of anxiety. One important advantage of such a procedure was the possibility of dissociating the neuroanatomical bases of fear and anxiety. Contrary to this proposal, it has been demonstrated that learned fear (contextual fear conditioning) also plays a role in the shock sensitization of the startle response (e.g. Richardson 2000), so that the two processes (unlearned anxiety and learned fear) could explain a part of the potentiation effect. An interesting alternative has been recently proposed, based on the premise that rodents are nocturnal animals and that they fear intense illumination. Walker and Davis (1997) studied the effect of a bright light on the startle response. One encouraging result using this procedure was the neuroanatomical dissociation of the "light potentiation of startle" from "fear-potentiated startle". By administering an AMPA receptor antagonist, Walker and Davis (1997) found that fear-potentiated startle seemed to depend critically upon the central nucleus of the amygdala, whilst the enhancer effect of the light on the startle probe, which presumably simulates an anxiety-potentiated state, relied on the bed nucleus of the stria terminalis (**Figure 10**).

Another paradigm of startle that may be potentially useful in experimentally distinguishing between fear and anxiety is the cohort removal procedure investigated here, which could trigger anxiety in rats in a specific way (e.g. without activating the -presumably different- brain mechanisms of fear). This manipulation involves the removal of the homecage partner during an unpredictable time period. In other words, this treatment deprives the subject of social companionship, leading to an internal state which is closer to anxiety than fear. What is distressing for the animal in this situation is not a specific/phasic stimulus as occurs in the fear-potentiated paradigm, but the possibility that something potentially unpleasant may occur. The rat's partner has been removed from the homecage in which they shared their lives, without disturbance, since weaning.

We tested the effects of this form of anxiety on the acoustic startle reflex of the inbred Roman rats. The principal finding was that startle was much stronger in male RLA rats, the most emotional of all groups, an observation that demonstrates the well-known tenet that stressful experiences interact with individual differences to determine the magnitude of anxious responding. In line with the work of Walker and Davis (1997), mentioned earlier, it would be interesting to investigate in further studies whether the infusion of an AMPA blocker would permit the discrimination of unconditioned anxiety (cohort removal procedure) vs learned fear (fear-potentiated startle) in the Roman low-avoidance strain. The observation of a differential involvement of the central nucleus of the amygdala (Roozendaal et al. 1992, 1993) and of the bed nucleus of the stria terminalis in these paradigms would be a result consistent with the hypothesis that fear and anxiety are neuroanatomically distinguishable.
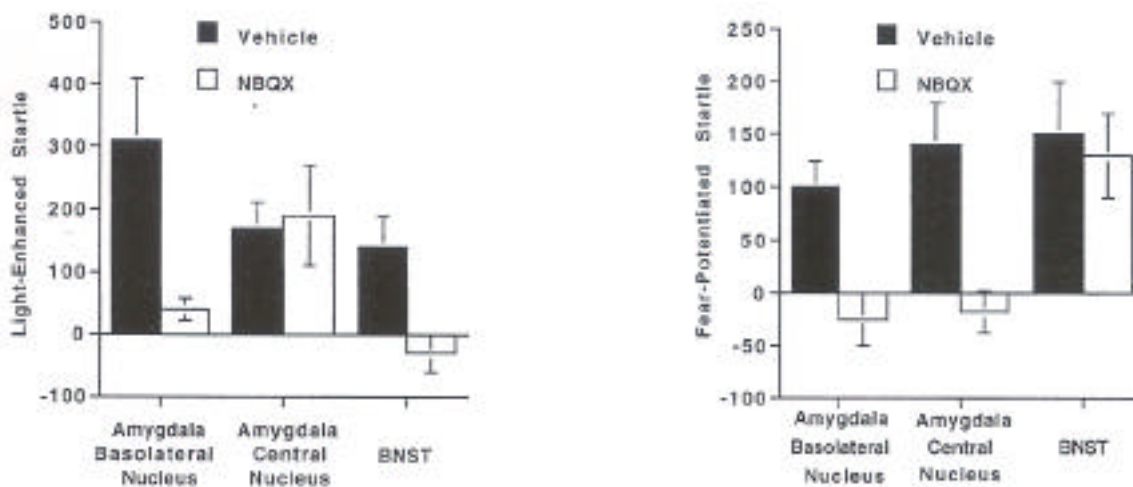

Figure 10. To the left, it can be seen that glutamate inactivation of the basolateral amydala or bed nucleus of the stria terminalis (BNST), but not central amygdala, blocks light-enhanced startle. A mean change occurs in startle amplitude from the dark phase to the light phase (light-enhanced startle) after infusion of the glutamate antagonist NBQX or its vehicle into either the basolateral nucleus of the amygdala, the central nucleus of the amygdala, or the lateral bed nucleus of the stria terminalis. To the right, it can be seen that glutamate inactivation of basolateral or central amygdala, but not BNST, blocks fear-potentiated startle. A mean change occurs in startle amplitude on the light-noise versus the noise alone trials (fear-potentiated startle) after infusion of the glutamate antagonist NBQX or its vehicle into either the basolateral nucleus of the amygdala, the central nucleus of the amygdala, or the lateral nucleus of the stria terminalis. (Adapted from Davis and Shi 1999).

A FACTOR ANALYTIC MAP

A number of potential advantages and limitations of our factor analytic approach will first be outlined, some of which are bound up with the particular behavioural experimental design employed here. The potential advantages include the following: Are the results of the factor analyses simple in structure? Did the three-factor solution distinguish between two general categories of fearful responding? Is it reasonable to conclude that a fear of heights could be part of the innate repertoire of rats? To what extent does the three-factor solution fit with genetic markers for fearfulness? At least six potential limitations should also be considered: Why did we use this battery of tests? Were the optimal parameters selected for each test? Did the given order of test administration influence the findings? Could the fact of performing three successive experimental series have affected our results? Is it possible that an alternative selection of variables could have given rise to a different factor structure? Is the restriction of factors a legitimate procedure in factor analysis research? The limitations will be considered first.

The main question to be considered in the present experimental design is why we used a battery composed of these particular tests. The aim of this genetically-oriented project was to map the genome of the rat in a search for QTLs related to fear and anxiety; we therefore evaluated a large F_2 cross relative to the two principal categories of animal models of anxiety, i.e. unconditioned and conditioned response paradigms. We based the selection of tests on three principal tenets: 1) to utilise procedures commonly employed in animal experimental psychology and behavioural neuroscience which displayed a good combination of construct, apparent and predictive validity; 2) to obtain from them a wide sample of indices representing the two general types of fearful responding in order to cover, if necessary, detailed aspects of behaviour in each test; 3) to be familiar with the experimental preparations with respect to the way in which the Roman rats behave in them.

Within the tests of unconditioned or spontaneous responding, the OF was selected because of it's traditional utilisation in the field of emotionality testing as a test for developing various psychogenetically selected strains of rodents. We included the PM due to it's being widely studied by means of pharmacological, ethological and factor analytic approaches, thus being well-characterised in terms of reliability and validity. The HB was applied in order to measure a type of exploratory behaviour thought to be independent of locomotion, including as well a component of novelty that allows the additional measurement of typical indices of fearfulness (e.g. defecation and self-grooming). With the A we intended to establish a behavioural baseline (i.e. as control) for activity under low stressful conditions. As startle is a (neuroanatomically described) simple reflex, independent of the aversive motivational system but sensitive to (primed by) negative affective states (e.g. anxiety), it was also evaluated. Within the paradigm of learned fear we trained rats in CFC because it is a simple procedure to induce aversive learning whose genetic (in mice: e.g. Caldarone et al. 1997; Wehner et al. 1997) and neurobiological (in rats: LeDoux 1995, 1996) bases have begun to be established in recent years,

and SAC, as this task constitutes the bi-directional selection criterion of the parental Roman stocks (Bignami 1965). One additional reason to administer ASR, CFC and SAC tests relates to the fact that these procedures have potential for generalisation across species, so the findings arising from them may presumably be relevant for anxiety-related psychopathology.

As to the parameters chosen for testing, a close look at the experimental procedures is warranted. Some striking features may be noted: e.g. in the OF test the use of loud noise is lacking; in the HB novel objects were placed under the holes; the startle test was designed to obtain an habituation curve; relatively few CS-US pairings were applied in the fear conditioning procedure; and in the shuttlebox acquisition task a single-training session was administered. In the OF we had previously found, in many studies and pilot experiments under these conditions, that strain differences in the Roman rats continued to be reliable for the majority of measures, so we decided to administer the OF in the absence of background noise. The reason for placing novel objects in the HB is illustrated in Study 1 (Exp. 2), in which we demonstrated that strain differences in the explorative behaviour of the parental rats only arose when strange objects were present under the holes. The startle procedure employed in our test battery consisted of a simple sequence of 20 startling stimuli at 30-sec intervals, from which an habituation curve was obtained. Providing that the startling stimuli are themselves aversive, differences in the magnitude of the startle response would reflect individual differences in anxious behaviour. Though alternative procedures for measuring fear- and anxiety-related states by means of the ASR could have been used (e.g. fear-potentiated startle), we chose this experimental regime on the basis of its procedural simplicity. Fear-conditioning training consisted of only five CS-US pairings with a 20-W light as the CS in an illuminated room, so that a capacity for acquiring control of freezing responses (over contextual background) was presumably diminished. Thereby, the chance for obtaining reliable individual differences to both contextual fear stimuli and discrete CS was increased. Finally, a 40-trial, single-training session in shuttlebox acquisition was administered because there is extensive evidence to show that it is enough to distinguish between poor and good avoiding animals[15], thereby facilitating the collection of relevant data for the two processes governing avoidance performance. As can be seen, we were interested in the measurement of individual differences in temperament, so the general rule in all experimental preparations was to administer "soft" fear-inducing stimuli in order to avoid ceiling effects obscuring them. Additionally, the extension of some of the procedures in order to provoke strong fearful/anxious reactions would have been time-consuming, an important concern when considering that almost a thousand rats were used as subjects.

Why wasn't the sequence of tests counterbalanced? One of the principal generalisations in psychology is that previous experience influences subsequent behaviour, so that if we want to investigate a given phenomenon we must take into account the potential presence of so-called carry-over effects. The best

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[15]Marked strain differences in the Roman rats are commonly found within the first 10 trials.

way to rule out this kind of confusing variable is to vary the order of (i.e. to counterbalance) prior experiences with the aim of seeing whether the final output (the phenomenon of interest) remains consistent across possible permutations, or not. For example, if a train of inescapable footshocks is administered to a group of rats, further 2-way shuttlebox acquisition will be impaired (e.g. Steenbergen et al. 1990). Carry-over effects can appear as well when the previous task does not imply a traumatic footshock experience, as can be seen in the fact that the mere exposure to the PM during 5 min changes the behavioural expression of a second exposure on the following day (e.g. File et al. 1993), so that the rat becomes reluctant to again visit the open arms: an effect interpreted by some authors as reflecting fear acquisition to the unprotected space. Therefore, since our F_2 sample of rats were sequentially tested across OF, PM, HB, A, ASR, CFC, SH and SAC, fearful responses to each successive test were likely affected by the exposure to the prior tests.

Obviously, to repeat the same study 49 times (each time with 800 animals) in order to control for this effect, widely exceeds the possibilities of any laboratory. In regard to the initial objectives of the study, however, we are not a) investigating the effect of an acute treatment, such as a pharmacological challenge, in which a consideration of past experience can be important (e.g. to have been subjected to another active pharmacologically substance, or b) interested in isolating the small effect of a particular parametric manipulation as, for example, the influence of the duration of the CS-US interval in the strength of conditioning (e.g. to have administered prior training in Pavlovian conditioning). In both of these cases, slight variations in previous experience can deeply affect the results. In contrast, our aim was to evaluate anxious temperament, a construct that implies the presence of a stable pattern of lifelong behaviour which has it's biological roots in ancient brain nuclei and which is expressed through species-typical defensive responses. It may therefore be presumed that chronically fearful animals will always behave in an anxious manner and that typically fearless animals will show a consistent pattern of less fearful responding than the former, regardless of the order of the tests applied. In conclusion, we think that the results reported in the present work would be replicated in further studies, even with a change in the order of testing, with respect to QTLs and factor structure analyses, even though the behavioural expression of fearfulness in each particular test might possibly vary, depending upon the particular experimental sequence.

Could the utilization of three independent series of experiments for collecting the global data matrix (800 subjects) have affected the 3-factor structure reported here? Notwithstanding that each F_2 batch came from a different generation of Roman rats, separate factor analyses revealed that in two out of three batches the allocation of loadings was practically identical: Learned Fear (Factor 1), Fear of Heights (Factor 2) and Emotional Reactivity (Factor 3). In the first batch used, learned fear was also differentiated from unlearned fear, although the first factor corresponded with Emotional Reactivity (including open arm behaviour in the PM), the second factor mainly reflected freezing to context and to CS and the third factor grouped avoidance behaviour with intertrial crossings in the SAC task, sharing these two learned

fear factors with a moderate loading from crossings during habituation to the shuttlebox. Taking into account the additional fact that each of the experimental series was evaluated during different seasons of the year, presumably providing "background noise" for the behavioural output, it seems reasonable to conclude that the global 3-factor structure of our large matrix can be considered to be reliable and robust.

Can the selection of variables entering factor analysis influence the factor solution? The answer is yes. We intended to follow the rationale of selecting an appropriate sample of target measures from the test battery, although an alternative combination, selected through another logical route, may have possibly led to a different factor structure. For example, a "bottom-up" approach could have been to keep just those variables associated with known genes. If different combinations of such genes seem to be related to different sets of correlated measures, then we could apply factor analysis to such measures to confirm whether emerging factors are coincident with the various patterns of gene-behaviour associations. The factor solution resulting from this approach could have been different to that reported here, as expected from the observation that factor analysis in rodents appears to be very sensitive to the number and type of measures included (**Table 4**). Confronted with the dilemma of choosing between one of these rival options, one should take into account the relative importance of the objectives originally formulated: i.e. of seeking a simple, robust and coherent structure of the anxiety test battery while recognising, at the same time, that certain blends of genes can influence learned fear, anxiety or activity measures in various ways.

Is it legitimate to restrict factor solutions? When compared to previous factor analyses in the animal literature, one of the most striking features of our factor analytic approach is the restriction of factors from an initial 6-fold solution to a simpler 3-fold one, which is viewed as the reflex of a supra-ordinate structure differentiating two general categories of emotion related to defensive responding. As noted by Kline (1994), factor reduction is a matter of discussion among researchers, although it is generally agreed that the lower the number of factors the broader their meaning. Computers commonly have statistical packages which automatically select the number of factors to be extracted on the basis of whether the rotated factors have eigenvalues greater than 1. It is known, however, that large matrices tend to produce many separate factors, thus overestimating the differences among them (Kline 1994). One way of limiting the emergence of split-up factors is applying Cattell's Scree test, which is based on a graph in which the principal components and the eigenvalues are represented. The researcher's task consists of judging at what point the slope of the curve changes. Using this method we can obtain a factor structure akin to that derived by means of second-order factor analysis, which yields broad factors comprising general, hypothetical constructs. It can be argued, therefore, that our three-fold solution distinguished two main types of fearful responding (learned vs unlearned), due to the fact, presumably, that we performed an empirically-based (legitimate) factor restriction.

Among the virtues of any factor solution must be the accomplishment of obtaining simple structure for the position of factors. Does our factor solution meet this criterion?

Thurstone (1947; cited by Kline 1994) was the first to propose criteria for establishing whether simple structure was present, or not, in a given rotation of factors. When we inspected the allocation of loadings in the obliquely rotated factor analyses, we could see that all of these criteria were fulfilled rather well for the 6-fold factor structure and almost all of them for the 3-fold one.

Are two general forms of fear empirically distinguishable in the test battery? We have repeatedly stated that the three-fold structure for fearfulness was able to distinguish between two main categories of responding, namely learned and unlearned fear. Freezing to context and to CS plus avoidance behaviour, intertrial crossings and crossings during habituation were the principal variables loading onto the Learned Fear Factor. An array of diverse unconditioned and conditioned responses to fear stimuli correlated along a general second factor of Emotional Reactivity. Finally, avoidance of the anxiogenic properties of the open arms of the PM was grouped around the third factor (Fear of Heights). Hence, learned fear (first factor) was differentiated from unlearned fear (Emotional Reactivity and Fear of Heights factors) by means of factor analysis, which is consistent with conceptual and neurobiological distinctions between both (e.g. Davis and Shi 1999; Richardson 2000; Walker and Davis 1997).

However, one may argue that what is reflected by this three-fold solution is, in reality, a distinction between unconditioned responses to electric footshock versus unconditioned responses to non-electric (threatening/novel) stimuli. Although this line of reasoning would be plausible concerning shuttlebox performance at the early stages of training (i.e. when the animals receive footshocks during testing), two additional observations would seem to be inconsistent. Neither crossings during habituation to the shuttlebox (SAC) nor freezing in the test phase (CFC) are acompanied by the administration of footshocks. The process involved in these behaviours has to do, presumably, with a stored internal representation of the US, which would trigger (by association with the CS) a conditioned (freezing) response. Though an interpretation alluding to the unconditioned effects of the electric footshock seems to be questionable, another related hypothesis could be, in a broad sense, one based on the effects of high intensity stimuli. It is not doubted that CFC and SAC paradigms were the tests of the battery that involved the strongest aversive effects. If it is assumed to be irrelevant that the fear CSs in both paradigms have acquired their aversive properties by association with USs, then the factor becoming crucial is the extent to which the CS is capable of provoking acute fear. According to this hypothesis the distinction between learned and unlearned fear factors would be the result of a classification of fearful behaviour as a function of the net intensity of the fear experience.

This last hypothesis can be viewed as a forceful interpretation competing with the principal description of the 3-factor structure in terms of learned and unlearned fear. As we were searching for a meaningful map of our test battery we highlighted the empirical distinction between both general types of tests (i.e. CFC and SAC vs the rest of tests) in line with previous theoretical and empirical classifications. Nevertheless, this hypothesis does not preclude the potential importance that intense, painful USs can have for these kinds of aversively learned behaviours, which may be mediated by particular neural pathways activated by acute fear. In other words, it is as if factor analysis would have detected two, difficult to distinguish, interacting components (i.e. acute fear provoked by highly intense stimuli and aversive learning mechanisms), grouping them together. As our data do not permit a choice of either of these two hypotheses we then assume a provisional coexistence between them. Therefore, with respect to a particular factor analytic map for this battery of tests, the difference between learned and unlearned fear paradigms is emphasized, whereas the "intensity of fear" hypothesis will be used in relation to QTL findings, providing a more parsimonious fit.

Is the fear of heights a legitimate, phylogenetic response in the rat repertoire? Previous factor analytical studies using the PM alone, or combined with other tests, have shown that open arm behaviour tends to separate itself from activity indices (e.g. enclosed arm entries) as well as from a number of measures encountered in other tests (e.g. Fernandes et al. 1999; Flaherty et al. 1998; Rodgers and Dalvi 1997; Rodgers and Johnson 1995). In the present studies we have confirmed this tendency in two, complementary factor analyses: 1) the 6-fold structure which clearly distinguished between PM-anxiety and PM/OF-activity components; and 2) the restricted 3-fold structure which surprisingly maintained open arm behaviour (PM-anxiety) as a relevant, and independent, factor. This factor is frequently interpreted as an isolated (distinctive) anxiety component, in relation to other forms of activity- and fear-related behaviours. When one contemplates the PM literature, the main impression that emerges is that it is generally unknown what the test actually measures. From an evolutionary point of view, the Blanchard's (1993) have argued that the pressures of natural selection not only favored defensive responses that improved fitness, but also the link between those responses related to overriding threat stimuli. Just as rats rapidly learn that certain tastes can provoke illness (García and Koellin 1966), so do rats rapidly learn to avoid elevated and unprotected spaces. One of these "phylogenetic" relationships may be the avoidance of elevated/unprotected spaces, such as cliffs and precipices. In this context, the PM can be thought of as an experimental situation which activates this kind of specific, self-protection behaviour. We would propose that "fear of heights" is legitimately responsible for the frequent dissociation of open arm behaviour in factor analyses of the elevated PM, either used alone or as one test used among others.

This hypothesis could be explored by testing for the presence of correlations between open arm behaviour and similar types of responses involving confrontation with heights. For example, an "Elevated Table Test" would be the simplest experimental preparation for evaluating behaviours related to a fear of heights. The apparatus could consist of an elevated, circular table with concentric lines located at different distances, forming rings. Each ring would represent progressive degrees of risk tolerated by the animal, so that a greater proximity to the edge of the table would represent a lower degree of fear of heights. In addition, complementary behaviours could be measured, such as the typical indices of fearfulness (e.g. defecations and self-grooming) as well as, and especially, the number of times that rats dipped their head over

the edge of the table, the time spent in that activity, and time spent in the external ring. These last three measures would be the target variables in relation to factor analysis. Our prediction would be that these indices would load onto the same factor as open arm behaviour in the PM, thus providing evidence for a general interpretation of fear of heights as a legitimate construct in the stimulus-response relationship.

GENETIC RESULTS

One assumption of factor analytical techniques is that they can reduce the relationships among a number of behaviours, which posess something in common, to a minimun of hypothetical, underlying factors. As the nature of factor analysis is merely descriptive, the search for external evidence through experimental design, in order to strengthen the consistency of a given structure, is needed. The goal of both descriptive and experimental approaches would be to locate connections between factors which have emerged and the differential biological substrates accounting for them. In this direction a meaningful fit between the 3-factor structure described here and a genetic marker appears to have been revealed in these studies, as a locus on chromosome 5 seems to affect anxious responding, influencing nine measures in the expected fashion, as well as paralleling the effects of anxiolytic drugs.

It is tempting to speculate, in relation to the factorial findings, that the cluster of behaviours loading onto the first factor could be reflecting an effect of this locus on a susceptibility to intense fear, as measured by the CFC and SAC paradigms (**Figure 11**). The means by which the strength of conditioned fear was acquired could therefore be irrelevant for the basic genetic architecture of fearfulness (e.g. associative mechanisms involved in CFC and SAC). On the other hand, the key element for determining the involvement of a locus on Chr 5 in fearful responding may rather be the net intensity of emotional stimulation[16] (and not the nature of threat stimuli). Supposing that only acute and intense threat stimuli were capable of triggering exaggerated fear reactions, neuronatomically mediated by circumscribed neural pathways, this kind of limited (core) state of fear would be preferentially activated as a response to strong emotional inputs. However, if fear stimuli were more diffuse and less aversive, other neural sites could contribute to the final activation. This latter line of reasoning seems to be congruent with the following three observations: First, a blend of multiple chromosomal loci were found to influence many of the responses (a few of them being associated with Chr 5), judging by test situations in which the aversive component was possibly less intense and/or confined. Second, the size effect of the Chr 5 locus on conditioned responding in CFC and SAC paradigms was generally greater than its effect on unconditioned responses. Third, our three-factor solution distinguished between a few responses to intense emotional stimuli (Learned Fear) and a varied behavioural repertoire displayed in response to weaker and

more diffuse threats (the Emotional Reactivity and Fear of Height factors[17]). Taken together, these features of the fit attribute an important role to the intensity of the state of fear, which could emerge from genomic mechanisms to be found on Chr 5. It seems reasonable, therefore, to expect that this particular locus is related to the functioning of regions within, or connected to, the limbic system (i.e. neural systems for fear[18]).
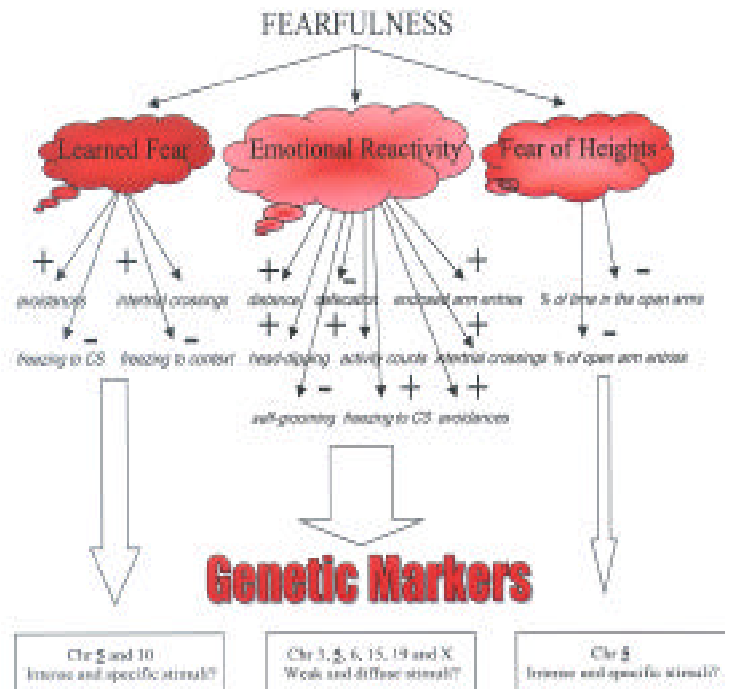

Figure 11. Schematic diagram of the fit among the genetic markers and the three-fold factor solution. The "clouds" represent three simple main psychological constructs infered from direct observation of the behaviours displayed in the fear test battery, which are dripping as indicated by the arrows. These indices of fearfulness are in turn bound up to chromosomal locus candidates in a differentiated way, especially when the Learned Fear (Chr 5 and 10) and Emotional Reactivity factors (Chr 3, 5, 6, 15, 19 and X) are compared. In addition, one common locus at rat chromosome 5 (in bold print) is present over the three factors influencing multiple behavioural phenotypes, suggesting a simple genetic basis for individual differences in fearfulness. The intensity of correlations among behaviours with factors is reflected by a closer proximity to the "clouds". The signs +/- represent the direction of the correlation of the loadings to each of the factors.

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[16]Though we have no external evidence for stating that differential levels of fear were present across tests, one relevant clue in this direction could be the observation that animals defecated more in the two aversive learning tasks than in the other experimental situations.

[17]The emergence of Fear of Heights as an independent and "general" factor could be indicating that rat's behaviour in the open arms of the PM is primed by strong fear (the vision of the abyss). If this *ad hoc* interpretation were correct it would then also be consistent with the apparent importance that the QTL contained in Chr 5 seems to have for the genetic basis of fearfulness in our test battery, as this chromosomic segment influenced open arm behaviour in a specific fashion.

[18]Because the inherent difficulty of dissociating behaviourally independent traits in the tests here investigated, the possibility exists that alternative processes other than fearfulness may have been linked to genetic markers. Our interpretation of the data has been entirely based on the assumption that this test battery measured fear- and anxiety-related responses. If it was presumed, however, that the behavioural output could be accounted for some additional trait, the most reasonable candidate would be impulsiveness, as in terms of impulsiveness the expected pattern of behaviours would be very similar to that observed in the present work.

The relevance of animal research for psychology ultimately rests in the generalisation of findings to human behaviour. The endeavour of building a bridge between animals and humans in the biological urderpinnings of fearfulness has produced, in recent years, excellent dividends suggesting, for example, that specific amygdaloid and septo-hippocampal systems seem to be crucial routes for fear and anxiety. Recent evidence coming from linkage genetic analysis (Gratacòs et al. 2001) has shown that one potential alteration on human chromosome 15 could be implied in the aetiology of anxiety-related conditions such as panic and phobic disorders. Those authors found that an interstitial duplication at 15q24-26 (called DUP25) was present in 90 % of patients with anxiety disorders (one or more gnosologic entities), whilst the same duplication arose in only 7 % of individuals without any kind of disturbance in anxious behaviour. When considering these findings in the context of what is known about QTLs for fearfulness in mice (e.g. Caldarone et al. 1997; Flint et al. 1995; Wehner et al. 1997) and (now) in rats, coupled with the recent successful sequentation of the human genome, it opens a challenging panorama to the frontiers of the genetic and neurobiological determinants of fear-based emotions, notwithstanding that the expected fit among chromosomic loci across species has, thus far, not been established. Preliminary evidence suggests a wide spectrum of loci for fearfulness (**Table 10**), which is consistent with current views of fear and anxiety as emotional states embedded within entangled, neural tracks which are presumably the result of multiple genetic effects compounded by developmental and environmental factors. The nature of the interactions between those states of mind and molecular biology awaits further clarification.

TABLE 10

SOME EXAMPLES OF GENETIC MARKERS (QTLs)
FOR FEAR-RELATED BEHAVIOURS IN MICE

Fear-related measures	Map position
OF and PM tests (Flint et al. 1995)	D1*Mit*150 (0 cM)
	D12*Mit*47 (6 cM)
	D15*Mit*63 (3 cM)
Contextual fear conditioning (Wehner et al. 1997)	
	D1*Mit*60 (8 cM)
	D2*Mit*52 (4 cM)
	D3*Mit*151 (12cM)
	D10*Mit*28 (12 cM)
	D16*Mit*105 (14 cM)
Contextual fear conditioning (Caldarone et al. 1997)	
	D1*Mit*123 (9 cM)
	D3*Mit*106 (4 cM)
	D7*Mit*238 (3 cM)
	D8*Mit*4 (4 cM)
	D9*Mit*113 (6 cM)
	D18*Mit*94 (0 cM)

The number after the "D" indicates the localisation of the genetic marker (QTL) within a particular chromosome (e.g. D1*Mit*60 = Chr 1). cM = centimorgan.

CONCLUSIONS

I) *Selective outbreeding, as well as inbreeding, has effectively produced stocks of Roman low- and high-avoidance rats which radically differ in 2-way, active avoidance acquisition. Beyond this criterion of selection, the Roman rats also differ in a host of behaviours which have been measured here in eight tests (NC, HB, OF, PM, HNP, SH, SAC and CFC) related to fear and anxiety. We take such generalised effects of selective breeding as evidence of their pervasiveness in the organisation of the rats' defence system.*

II) *The acoustic startle reflex is sensitive to several sources of variation in fearfulness: the magnitude of the startle is higher in hyperemotional RLA's than in hypoemotional RHA's (strain effect); male rats display an enhanced startle response relative to females (sex effect); and startle can be potentiated by means of a state of induced anxiety through cage partner removal (cohort removal effect), when fearful animals are used as subjects (i.e. male RLA rats).*

III) *Our test battery is capable of being described by three simple principal factors: Learned Fear, Emotional Reactivity and Fear of Heights. This 3-fold solution is taken as evidence for a dissociation between learned and unlearned fear on behavioural grounds, suggesting that brain mechanisms underlying these kinds of defensive responding could also be different. When genetic markers were revealed, a connection emerged between the factor analyses and QTLs for anxiety. The fact that a locus on Chr 5 influenced an array of fearful responses to both conditioned and unconditioned emotional stimuli, the size of its effects being greater for responding to the former (the most aversive tests), suggests that our factor analytic map may have also captured the differential intensity of emotional inputs, thereby classifying the behavioural output as a function of net intensity of the fear experienced in each test of the battery.*

IV) *The analysis of sex differences in the test battery revealed that the aforementioned 3-fold structure is simple and robust, as males' and females' behaviour were practically identical, even though male rats tended to be more fearful/anxious than females.*

V) *The QTL analysis conducted in conjunction with the test battery detected one locus, on chromosome 5, which had effects on emotional behaviour which parallel the action of anxiolytic drugs, thereby providing a potential candidate in the search of genes for anxiety.*

VI) *We have extended the well-known, divergent behavioural profile of outbred Roman rats to inbred Roman rats, as well as having demonstrated the existence of QTLs for anxiety, using a second generation cross of the latter. Therefore, the Roman lines/strains can be considered to be an excellent rat model of emotionality/anxiety.*

VII) *Given the conservation across species of the main rudiments of fear responses, we believe that our genetic findings are potentially relevant for understanding anxiety-related conditions in humans.*

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