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Medical Psychology Unit

FEARFULNESS IN A LARGE F_2 PROGENY OF THE ROMAN RATS: PAVING THE WAY FOR QTL'S GENES LOCALISATION

Doctoral thesis presented by
Raúl Aguilar Heras
in order to opt for the degree of Doctor in Psychology,
under the supervision of the Doctors
Adolfo Tobeña Pallarés and Alberto Fernández Teruel.

Barcelona, 12 December 2001.

ADOLFO TOBEÑA PALLARÉS, Professor of Psychiatry at the Department of Psychiatry and Forensic Medicine (Autonomous University of Barcelona), and ALBERTO FERNÁNDEZ TERUEL, Associate Professor of Medical Psychology at the same department,

CERTIFY: that the work entitled FEARFULNESS IN A LARGE F_2 PROGENY OF THE ROMAN RATS: PAVING THE WAY FOR QTL'S GENES LOCALISATION, has been carried out by Raúl Aguilar Heras under their supervision at the Department of Psychiatry and Forensic Medicine (Autonomous University of Barcelona), with the aim of opting for the degree of Doctor in Psychology.

Adolfo Tobeña Pallarés

Alberto Fernández Teruel

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Se dice que quien lleva a cabo una tesis doctoral debe ser cortés, a la hora de redactar los agredecimientos, con todo aquel que directa o indirectamente tiene algo que ver con el producto final. Siguiendo esta tradición oral me dispongo sin más a disparar mi particular retahíla. Agredezco a mis compañeros de la Unidad de Psicología Médica, Paula Queralt, Yolanda Pardo, Beatriz Molinuevo, Montserrat Albertos, Rosa Escorihuela, Lidia Giménez, Alberto Fernández, y muy especialmente, a Magdalena Buenaño, Antoño Cañete, Juan Pereira, Rafael Torrubia, Javier Caseras, Luis García y Adolfo Tobeña, su disponibilidad incondicional para resolver los pequeños contratiempos de la vida cotidiana en el lugar de trabajo y hacer posible que algunos días hayan sido verdaderos divertimentos. También quiero agradacer a Ignacio Morgado (y a parte de su equipo de "biopsicólogos") de la Facultad de Psicología de esta universidad, su atención y seguimiento desinteresado de mis andaduras investigadoras, a pesar de encontrarme discretamente apartado del mundanal ruido bajo el cobijo que proporciona la Facultad de Medicina. Y reconocer la influencia que tuvieron durante mi formación los profesores Fernando Díaz, Francisco Esmorís y Alberto Fernández en la Facultad de Psicología de la Universidad de Santiago de Compostela (A.F., concretamente, la temporada que estuvo trabajando en esta universidad gallega y durante los primeros pasos del proyecto genético que sobrevuela esta tesis -una vez retornó a tierras catalanas). Aunque pueda parecer algo anómalo, me voy a permitir la licencia, además, de anticipar aquí unos "agradecimientos orientados al futuro" hacia el grupo de investigación animal que se geste el próximo año, en particular a Lidia Giménez, a Antoño Cañete y a Alberto Fernández, en quienes veo (si los astros se alinean apropiadamente) a unos excelentes colaboradores. No me cabe la menor duda de que juntos (incluida la gente que se quiera sumar a esta empresa) podremos sacarle mucho jugo a nuestros roedores romanos.

Quiero dar las gracias a Rafael Torrubia y Luis García por haberme invitado a asistir a unos seminarios sobre personalidad vibrantes e inolvidables, que me han agrandado un poquito más el sentido común con el que tiene que mirar el psicólogo a su objeto de estudio. El ritmo de fondo de los análisis factoriales llevados a cabo en esta tesis son herencia suya. Además he podido adivinar en ellos unas personas en realidad sencillas y cercanas, y aquí incluyo también a Adolfo Tobeña, con el don para detectar en el comportamiento del personal aquello a lo que B. F. Skinner llamaba "un vago sentido de orden". Este puede ser el regalo más valioso que me lleve de la Unidad de Psicología Médica. Por eso me siento afortunado de seguir compartiendo de momento este espacio, puerta con puerta, con estos ilustres señores, hasta que las circunstancias me obliguen a emprender nuevas andaduras.

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verdadera naturaleza de los animales que moran en estos nichos ecológicos. Después de unas cuantas adaptaciones necesarias nos sentimos en nuestros respectivos trabajos, por fin, como pez en el agua.

En cuanto a mi querida Montse he de decir, comprimiendo lo incomprimible (los sentimientos), que compartí momentos mágicos, como aquellas tardes y noches que pasamos en Galicia, oliendo juntos el viento y la lluvia azotando los bosques de eucaliptos de limón y mentolados, o aquel escenario nocturno, al pie del Pirineo leridano, de paredes ingentes de tosca piedra azulada y fluorescente, una medicina sin la cual este último tramo hubiera sido un auténtico sindiós. Ella fue mi cómplice durante este trocito de vida, acompañándome "desde dentro" en las últimas compras de formas de pensar y, sobre todo, de sentir. Con esta señorita me salieron "en el cerebro de la cabeza" varias capas de cebolla nuevas (muy importantes) "de quererse" las personas. De casi todo íbamos bien servidos, excepto de ansiedad y distancia (que en realidad nos sobró bastante).

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Lo que valoro de estas cuatro personas (Luis, Montse, Adolfo y Jose) es, en definitiva, la facilidad y el confort para hablar con franqueza de absolutamente de todo, sin guardar la ropa (i.e. honestamente y con respeto) y predicando con el ejemplo (i.e. con coherencia). La prueba de que una gran sensibilidad no está para nada reñida con el buen entendimiento.

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como pretendía,
un premio a la constancia.

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CONTENTS

FEARFULNESS IN A LARGE F_2 PROGENY OF THE ROMAN RATS: PAVING THE WAY FOR QTL'S GENES LOCALISATION

A Elia

ABSTRACT

In the present doctoral dissertation a series of studies dealing with the psychogenetics of fearfulness are reported. In Study 1, the correlative effects of selective inbreeding on an array of fearful behaviours in the Roman rat strains were investigated. In Study 2, we determined whether a brief removal from the homecage partner (i.e. cohort removal procedure) could differentially potentiate the startle reflex of these rats. In the Main Study, we sought to establish, by means of factor analytic tools, the behavioural profile of fearfulness for a large sample (n = 800) of F_2 rats in a battery of eight tests. The results were the following: First, Roman low-avoidance (RLA/Verh) rats were markedly more anxious/fearful than their high-avoidance (RHA/Verh) counterparts. Second, the magnitude of the startle was higher in fearful RLA/Verh's than in fearless RHA/Verh's, male rats displayed enhanced startle response relative to females and the startle was potentiated by cage partner removal in male RLA/Verh rats. The analysis of the behaviour of the F_2 progeny in the fear test battery revealed, by using factor analytic techniques, that three main factors accounted for more than 40 per cent of the variance: a "Learned Fear" Factor containing the measures of aversive/fear conditioning, an "Emotional Reactivity" Factor with 11 out of the 14 variables analyzed and a "Fear of Heights" Factor with high loadings on open arm behaviour in the elevated plus-maze. Additional results on sex differences in, and QTL's (genetic markers) for, anxiety, derived from the analyses of the same F_2 data base, are presented. We conclude by proposing a biobehavioural fit between our factor analytic map and the genetic markers explored, mainly based on the role that a locus, at chromosome 5, may have in mediating intense responses of fear to emotional stimulation. Given the conservation across species of the main rudiments of fear responses, we believe that our psychogenetic findings are potentially relevant for understanding anxiety-related conditions in humans.

RESUMEN

En esta tesis doctoral se presentan una serie de estudios sobre psicogenética del miedo. En el Estudio 1, se investigaron los efectos correlativos de la crianza selectiva (consanguínea) en varias respuestas de miedo en las cepas de ratas Romanas. En el Estudio 2, determinamos si una separación de corta duración del compañero de caja-hogar (i.e. procedimiento de retirada de la cohorte) podría potenciar diferencialmente el reflejo de sobresalto de estas ratas. En el Estudio Principal, buscamos establecer, por medio de herramientas analítico-factoriales, el perfil conductual de temerosidad de una gran muestra de ratas F_2 (n = 800) en una batería de ocho tests. Los resultados fueron los siguientes: Primero, las ratas Romanas de baja evitación (RLA/Verh) fueron marcadamente más ansiosas/miedosas que las Romanas de alta evitación (RHA/Verh). Segundo, la magnitud de la respuesta de sobresalto fue mayor en las miedosas RLA que en las no-miedosas RHA, los machos desplegaron una respuesta de sobresalto aumentada en relación a las hembras y este reflejo fue potenciado en los machos RLA por la retirada de su compañero. El análisis de la conducta de la progenie F_2 en la batería de pruebas de temerosidad reveló que tres factores principales explicaban más del 40 % de la varianza: un factor de "Miedo Aprendido" que contenía las medidas de condicionamiento de miedo/aversivo, un factor de "Reactividad Emocional" con 11 de las 14 variables analizadas y un factor de "Miedo a las Alturas" con cargas altas para la conducta en los brazos abiertos del laberinto elevado en cruz. Se presentan aquí también resultados derivados del análisis de la misma base de datos F_2, sobre diferencias sexuales en, y marcadores genéticos de, ansiedad. Concluimos proponiendo una correspondencia psicobiológica entre nuestro mapa analítico-factorial y los marcadores genéticos explorados, basada principalmente en el papel que puede tener un locus en el cromosoma 5 como mediador génico de las respuestas de miedo intenso ante estímulos emocionales. Dada la conservación a través de las especies de los principales rudimentos del miedo, creemos que nuestros hallazgos psicogenéticos son relevantes, potencialmente, para la comprensión de los cuadros psicopatológicos relacionados con la ansiedad en humanos.

INTRODUCTION

PSYCHOBIOLOGICAL UNDERPINNINGS OF FEARFULNESS

A long tradition exists among animal psychologists of measuring observable behaviours under rigourous laboratory conditions with the aim of investigating hypothetical, underlying internal states such as fear and anxiety (Boakes 1984). Although a large body of research relies on the measurement of arbitrary responses (e.g. bar-pressing), researchers have increasingly focused their attention on a small number of species-typical[1] defensive responses (e.g. flight and freezing); i.e. behaviours of the mammalian defence system which can be detected in many species ranging from infra-human animals (e.g. birds, rodents and primates) to Man. This impetus toward studying more genuine repertoires emerged from the growing recognition of the importance of the biological underpinnings of fearful behaviours (Gray 1987; LeDoux 1996). Enormous progress has been made in recent years in the behavioural neuroscience of fear. For example: 1) the neurobiological bases of a simple form of learned fear termed Pavlovian fear conditioning[2] have been delimited in rats as well as in primates and humans, using freezing behaviour and startle reflex as target measures (Fendt and Fanselow 1999; Lang et al. 2000; LeDoux 1996); 2) the analysis of two-way, active avoidance behaviour (i.e. "flight") in rats and dogs trained in shuttleboxes constitutes a cornerstone for the construction of the principal theories of fear and avoidance learning (Levis 1989; 1991; Mineka 1979); and 3), our current knowledge of the genetic and molecular basis of anxiety is primarily based on rodent's measures of defecation and ambulation/exploration under stressful conditions (e.g. Flint et al. 1995).

The behavioural defence system[3] has endowed Man with a flexible and adaptative emotional machinery to confront threatening challenges. Given the variety and complexity of these challenges and the peremptory value for survival of responding appropriately to them, it is not surprising that particular parts of our brain are devoted to the specialized processing of threat. A good example of these brain systems is the double neural pathway described for fear conditioning (LeDoux 1995, 1996; Phillips and LeDoux 1992). LeDoux and colleagues have been able to differentiate one circuit in which visual information at the retina travels to the amygdala through the thalamus providing a rapid and rough processing of threat stimuli, as well as affording an immediate defensive reaction: this pathway is succinctly described by LeDoux as "quick and dirty" (**Figure 1**). The second circuit conveys impulses from the thalamus to the multiple layers of the visual cortex where the threat stimuli are processed in detail, giving rise to a precise definition. This elaborate information is then sent to the amygdala to permit an efficient and adjusted emotional response: this pathway is viewed as being "slow and sophisticated". Suppose, for example, that you are taking a walk in a lonely street at night and suddenly a loud noise occurs behind your back. Before you know the exact cause of the noise, a psychophysiological cascade (e.g. startle reaction, tachycardia, muscular tension and enhanced vigilance), prepares you for a fast and steady defensive response, triggered by the "quick and dirty" neural pathway. Meanwhile, the "slow and sophisticated" circuit will be working in parallel in order to identify the source of the noise as well as to assess if it is dangerous, or not. As a function of this evaluation the behavioural defence cascade will be vigorously maintained or abruptly cancelled.

The emotional states that accompany defensive behaviours, i.e. fear and anxiety, can be easily distinguished in animals in terms of the physical presence or absence of the eliciting stimulus (e.g. Blanchard et al. 1990, 1993; Gray and McNaughton 2000). For example, Blanchard and colleagues (1993) differentiate between fear and anxiety responses by confronting rats with a predator, i.e. an anaesthetised domestic cat (Fear/Defence Test Battery: F/DTB), or by exposing them to a situation associated with that predator, i.e. brief exposure to places or odors linked with cats (Anxiety/Defence Test Battery: A/DTB)[4]. Those authors have observed that wild rats confronted with a cat (i.e. actual threat) in the F/DTB exhibit four predominant varieties of defensive responding: flight, freezing, defensive threat and defensive attack. The transition from one to another mainly depends on distance between prey and predator, increasing fear as defensive distance decreases (**Figure 2**). Flight is the dominant response when a route of escape is available and the defensive distance lies between 1-5 m. The following, predominant, fearful response is freezing or motionlessness (crouched posture) which replaces flight if the option of fleeing does not exist. When distance from the predator lies between 0.5-1 m, then sonic vocalisations plus exposure of the teeth serves as defensive threat which can arrest an eventual attack. Finally, rats engage in defensive attack at distances close to contact (zero-0.5 m), which can be preceded at greater distances by jump attack oriented to the head of the predator (a possible form of fear-potentiated startle[5]). In contrast, anxiety is prompted in the A/DTB by exposing rats to cat odor as well as to the place where the cat was previously viewed, i.e. stimuli associated with the predator which can predict its eventual presence (i.e. potential threat). Here, wild rats inmediately flee to a protected place where they

[1]This term, coined by the Blanchard's (1993), is a modified version of the Bollesian concept of *species-specific defensive responses*. Whilst Bolles (1972) intended to highlight the fact that different species were endowed with particular behavioural repertoires which facilitated or interfered with the learning of arbitrary responses (theorizing about aversive learning theories), the Blanchards extended the concept by indicating that such behaviours were, in reality, present across many species, thus more aptly designated *typical*.

[2]This is an experimental procedure whereby a previously neutral stimulus (e.g. a light) becomes capable of prompting a response of fear due to its pairing with an aversive event (e.g. electric footshock).

[3]There are alternative ways of self-protection in infra-mammalian species (e.g. hedgehog and crabs) which can rely on defence systems more simple than those behaviourally-based, such as hard, specialised structures covering the animal's surface (e.g. spines or armor; e.g. Blanchard et al. 1993).

[4]The F/DTB and A/DTB are two procedures of a lab burrow system with tunnels and chambers which is considered to be a simulation of the ecological niche of the wild rat.

[5]The fear-potentiated startle is a laboratory phenomenon which consists of an increase in the startle reflex in response to a previously neutral stimulus now endowed with fear-inducing properties acquired by its pairing with an aversive event (usually an electric footshock)

may remain motionless during hours or days. Their main activity consists of displaying a risk assessment pattern (e.g. stretch-attend/stretch-approach postures), as if they were anxiously evaluating the potential threat of the eventual attack of the predator. Under these conditions, nondefensive behaviours such as eating, drinking and sex are temporarily abandoned (Blanchard et al. 1990).

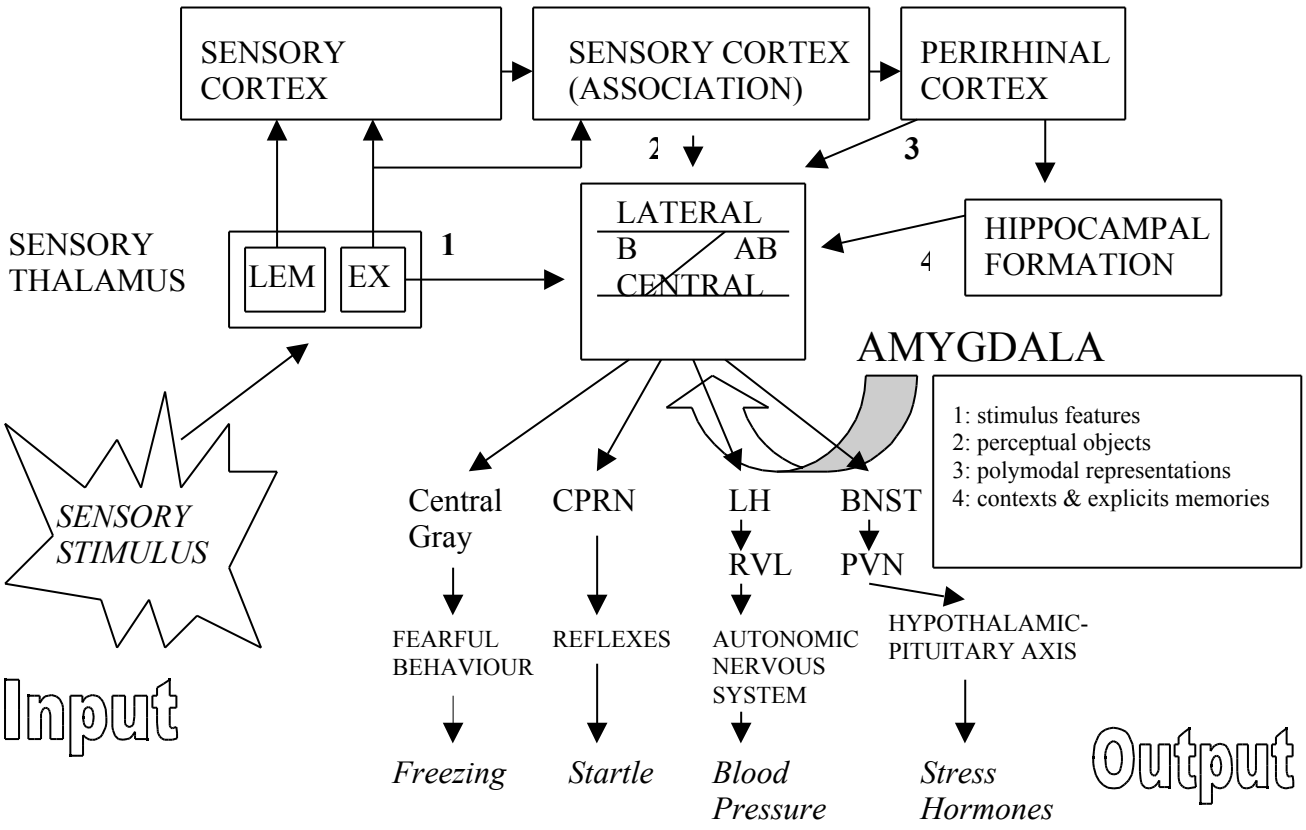


Figure 1. Neural circuits of fear conditioning. The neural pathways by which a sensory CS elicits emotional responses involve the relay of sensory inputs to the thalamus. While the lemniscal nuclei (LEM) transmit only to the primary sensory cortex, the extralemniscal areas (EX) transmit to the primary sensory and association regions of the cortex, as well as to the basolateral nucleus of the amygdala. This region of the amygdala also receives inputs from sensory association areas of the neocortex, as well as from polymodal areas such as the perirhinal cortex and the hippocampal formation. The thalamo-amygdala sensory projection has been implicated (1) in simple fear conditioning [one conditioned stimulus (CS) paired with an unconditioned stimulus (US)]; the cortico-amygdala sensory projection (2) in differential fear conditioning (one CS paired with US, another not paired); and the hippocampo-amygdala projection (4) in contextual conditioning (conditioning to situational cues other than the CS). The hippocampal projection may also be involved in conditioning of fear to explicit or declarative memories that occur in the presence of an US, but this has not been studied. The role of the perirhinal projection to amygdala (3) is not known, but it may have something to do with the elicitation of fear by complex polymodal stimulus representations. The central nucleus of the amygdala is the interface with motor systems, as it connects with various brainstem areas involved in the regulation of specific defense response networks. Projections to the central gray control freezing and other defensive behaviors; projections to the lateral hypothalamus (LH) and from there to the rostral ventral lateral medulla (RVL) control sympathetic autonomic nervous system responses; projections to the bed nucleus of the stria terminalis (BNST) and paraventricular hypothalamus control stress reactions involving the pituitary-adrenal-axis; and projection to caudal pontine reticular nucleus (CPRN) controls startle reflex. The amygdala nuclei are the sensory- and motor-independent parts of the circuitry and are likely to play important integrative roles in fear conditioning. (Adapted from LeDoux 1995).

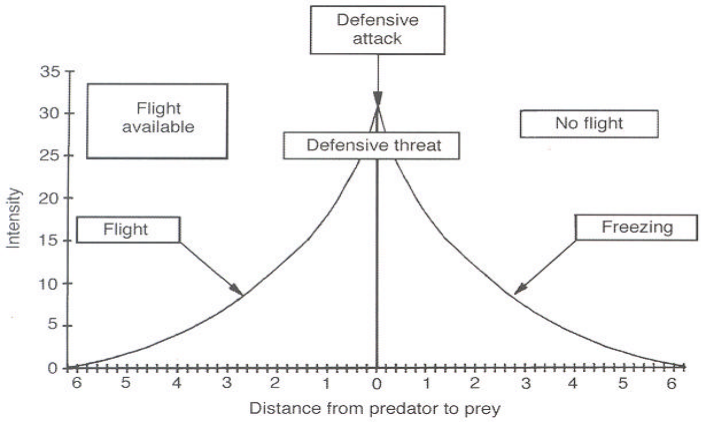


Figure 2. Defensive distance. As distance from a predator decreases, the intensity of fear is held to increase in an accelerating fashion. This intensity controls, very tightly, a progression from flight or freezing (depending on whether flight is available) through defensive threat to defensive attack. (From Gray and McNaughton 2000).

These behavioural definitions of fear and anxiety have been considerably supported by pharmacological and neurophysiological findings. For example, Blanchard et al. (1993) have found, in a series of ethopharmacological studies using the burrow system, that the pattern of behaviours that rats display to potential threat (risk assessment activities) is affected by anxiolytic drugs, whilst that related to actual threat is not influenced in the same fashion. Walker and Davis (1997) have reported, working with the startle reflex probe, that a double dissociation exists for the neural basis of fear (fear-potentiated startle) and anxiety (light potentiation of the startle): by blocking the AMPA receptors they have seen that the central nucleus of the amygdala is responsible, in part, for conditioned fear, whilst the bed nucleus of the stria terminalis seems to play a specific role in anxiety. Gray and McNaughton (2000) have used these distinctions to elaborate an integrative neuropsychological theory in which fear and anxiety are considered to closely resemble one another in some respects (e.g. similar psychophysiological arousal), whilst being essentially distinguishable from a hierarchical viewpoint on the basis of the brain structures involved, as well as the nature of their particular eliciting stimuli (following Blanchard's analysis). Those authors argue that fear is primarily provoked by actual threat stimuli for which the option of being avoidable (fleeing) exists (**Figure 3**). The area subserving fear would be the amygdala and its hyperactivity could be related to phobias. On the other hand, potential threat stimuli (also avoidable) would induce assessment maneuvers bound up with anxiety. The neural site for anxiety would be the septo-hipocampal system and its hyperactivation could lead to generalised anxiety disorder. These psychobiological definitions of fear and anxiety fit well, therefore, with the general inclination among psychologists and psychiatrists to consider these negative affective states as distinctive entities, since the stimuli provoking fear tend to be phasic (well-defined onset/offset) and specific (e.g. an environmental object, such as a snake), with associated patterns of behaviour "seemingly" adaptative (e.g. escape from the existing threat), whilst those stimuli prompting anxiety are generalized and imprecise (i.e. uncertain), inducing responses with unclear, inmediate functional consequences (e.g. risk assessment and behavioural inhibition).

PSYCHOGENETICS OF FEARFULNESS

The field of psychogenetics has accumulated a great deal of evidence showing that individual differences in fearfulness[6] are, in part, genetically-based. The method of selective breeding has provided excellent examples of this. The rationale of this method is to simulate, under controlled laboratory conditions and in an accelerated manner, *the evolution of adaptative traits by means of natural selection*, i.e. to select individuals by imposing a specific criterion (e.g. subjects with extreme scores in a given trait; artificial selection), and to interbreed them. If the trait of interest is under genetic control then it would be expected that after a number of generations of selective breeding those lines of individuals should differentiate from one another. Multiple research programs of selective breeding using rats as subjects have been successfully carried out, taking as the selection criterion diverse emotional behaviours such as high vs low defecations in open field (e.g. Broadhurst 1975; Gray 1987), high rates vs low rates of conditioned avoidance performance in 2-way shuttlebox task (see **Figure 4**; Bignami 1965; Brush 1991), and (more recently) high vs low % of time in the open arms of the elevated plus-maze (Liebsch et al. 1998). The main result of these experiments was that an array of related phenotypes was also co-selected, showing that the rats lines differ in multiple behaviours in a largely predictable fashion. The emergence of this set of additional behaviours (unintentionally selected), which was bound up with the original criterion, suggests that an underlying trait (a general endophenotype) of fearfulness may have been entwined with selective breeding, thus providing indirect evidence for the existence of a heritable, complex trait.

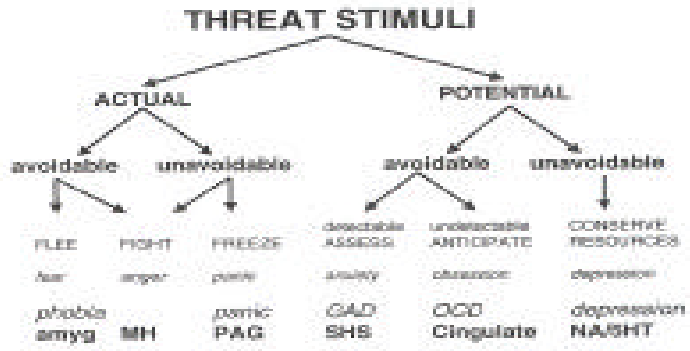


Figure 3. Nature of stimuli (top three rows) and their relation to function (fourth row), emotion (small italics), psychological disorder (large italics), and principal neural system involved (bottom row). GAD, generalized anxiety disorder; OCD, obsessive-compulsive disorder; amyg, amygdala; MH, medial hypothalamus; PAG, periacueductal grey; SHS, septo-hippocampal system; NA, noradrenaline; 5HT, 5-hydroxytryptamine. (From Gray and McNaughton 2000).

[6] *Fearfulness* (synonymous with emotionality) is a term often employed in animal literature as an analogue of neuroticism or anxiety trait in humans. Calvin S. Hall (1934) defined it "as the state of being emotional. This state consists of a group of organic, experiential and expressive reactions and denotes a general upset or excited condition of the animal. Emotionality can be thought of as a trait since animals and men differ in the intensity of emotional reactions displayed". When I henceforth refer to humans, primates, rats or birds which are said to have profiles of high fearfulness I will be meaning that they are especially susceptible to stressful stimuli, i.e. are chronically fearful/anxious.

That a constellation of behavioural indices tend to co-vary to shape a trait of high fearfulness has been shown in species other than the laboratory rat. For example, Kalin and colleagues (2001) have characterised the behavioural and physiological profile of a group of rhesus monkeys (*M. mulatta*) as being chronically fearful or anxious. These primates seem to be shy and behaviourally inhibited, displaying strong freezing responses in the presence of a human. Physiologically, they exhibit pronounced plasma level responses of cortisol and CSF corticotropin-releasing factor, as well as a marked right asymmetry in the electrical activity of the frontal cortex[7]. In consonance with that, Suomi (1991) has reported that 20 % of rhesus monkeys living in a particular colony showed a pattern of exaggerated fearfulness, accompanied by physiological and behavioural disturbances, in response to social challenges. Infant monkeys which were born from these fearful parents had a proneness to inherit the same psychophysiological responding profile to threats.

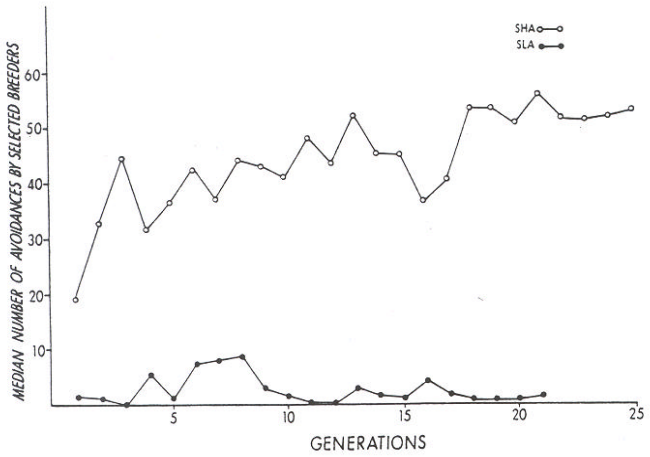


Figure 4. Median number of avoidance responses made by selected breeders as a function of generations of original selection. (From Brush 1991).

These observations are not limited to mammalian species (primates and rats). The heredity of anxious temperament has also been documented in birds, as illustrated by Jones and colleagues (1991) who worked with two strains of quail chicks (*Coturnix coturnix japonica*) psychogenetically selected for showing short vs long periods of tonic inmobility (STI's and LTI's, respectively) when trapped by the experimenter. As a result of this selection, LTI quail chicks show stronger freezing responses, higher defecation scores and less exploratory behaviour in novel situations, as compared to the STI's, a pattern of enhanced fearfulness akin to that found in rodents and primates.

Three major conclusions can be drawn, therefore, from this sample of studies: First, the grouping in extreme populations of a set of behaviours indicative of high vs low fearfulness is a common phenomenon in several species. Second, the consistency of these profiles, revealed by multiple selective breeding experiments, strongly suggests that they can be influenced by genetic factors. Third, from points 1) and 2) it appears reasonable to assume that fearfulness is associated with a particular typology of the central nervous system, as hypothesized by Ivan Pavlov (1927) in relation to individual differences in the temperaments of dogs.

TOWARD A GENETIC ARCHITECTURE OF FEARFULNESS IN RATS: A BEHAVIOURAL STRATEGY

Developmental differences in fearfulness, presumably of genetic origins, have been observed in human infants. For example, Kagan and colleagues (1989, 1991) have observed that 4 month old infants (23 % of a sample of 94 children) showing high motor activity (limb movement, protrusion of the tongue, and arching of the back) and irritability (fretting or crying) in response to unknown visual and auditory stimuli were more fearful[8] when assessed at 9, 14, and 21 months, than those with low scores in such behaviours (37 %). They also found that both fearful and fearless temperaments of 2 year-old-children were relatively stable over time, as measured at 9 years. In addition, these divergent profiles (labelled as *inhibited* and *uninhibited to the unfamiliar*, respectively) were accompanied by a set of distinctive physical and physiological characteristics (e.g. eye color, body build, susceptibility to atopic allergies, heart rate, voice, cortisol and pupillary dilation), suggesting that genetic factors can partly determine both types of temperament. Given that most of these differences have been found in early days of development when environmental stimulation is just beginning to shape the brain, the biological roots of anxious temperament in children makes the endeavour of searching for a simple genetic basis in infra-human animals even more reasonable.

Molecular candidates for the genetic architecture of fearfulness in laboratory animals, using quantitative trait loci (QTL) analysis, were found for first time by Flint and colleagues (1995). They mapped the genome of inbred mouse strains (n = 879) derived from animals selected for high and low ambulation in the open field. Fearful mice were defined as those defecating more and ambulating less in the open field, showing lower activity scores in a Y maze, and exploring the open arms of the plus maze less frequently. The authors expected that the potential effects of QTLs on behaviour were in the same direction, i.e. a chromosomic segment increasing ambulation and open arm behaviour should, in turn, decrease defecation. The results showed that loci contained in mouse chromosomes 1, 12 and 15 acted in the predicted fashion, accounting for a bulk of genetic variance in fearfulness. Two years after that publication, two studies carried out in independent laboratories reported that chromosome 1 was implied as well in individual differences in susceptibility to fear conditioning (Caldarone et al. 1997; Wehner et al. 1997),

[7]Interestingly, lesions of the amygdala of these primates were unable to abolish their anxious temperament suggesting, in congruence with the aforementioned ideas, that the "seat" of anxiety (state or trait) must be in other brain structures.

[8]"Fear was operationally defined as fretting or crying to an unfamiliar event or procedure (placement of electrodes, placement of a blood pressure cuff, facial disapproval from an examiner or the mother, a noisy rotating wheel, request to taste liquid from a dropper) or failure to approach an unfamiliar object (a robot) or unfamiliar adults, despite a friendly invitation to do so" (Kagan 1991).

suggesting that it could be especially important for the general trait of fearfulness. As concluded by Flint (1997), "perhaps the work may eventually explain variations in fearfulness in our own children".

In 1995 the Animal Laboratory of the Unit of Medical Psychology, UAB, initiated a research program in collaboration with leading teams in the research of the genetic and neurobiological basis of anxiety: Jonathan Flint at the Wellcome Trust Centre for Human Genetics (Roosevelt Drive, Oxford, UK), Jeffrey A. Gray at the Institute of Psychiatry (DeCrespigny Park, London, UK), Peter Driscoll at the Institut Fuer Nutztierwissenschaften (ETH, Schwerzenbach, Switzerland) and Gerard R. Dawson at the Merck Sharp and Dohme Research Laboratories (The Neuroscience Research Centre, Terlings Park, Essex, UK). The aim of the project was to scan the genome of the rat in the search of genetic markers for anxiety, using an F_2 population derived from intercrossing inbred Roman high- and low-avoidance rats. One core assumption of this approach is that complex psychological traits, which are usually continuously distributed, depend on many genes (or perhaps a few with pleiotropic action) with small effects spread out through the genome. In order to detect these small influences by mean of QTL analysis in a cross between two inbred strains, the use of a large number of animals is mandatory (Talbot et al. 1999). The experimental animals (the so-called F_2 generation) must be obtained from successive crossings originating from parental inbred strains (Gora-Maslak et al. 1991; Wimer and Wimer, 1985). By brother-sister mating of the parental strains the first filial generation (F_1) is produced which is also crossed to give place to the second filial generation (F_2). This F_2 intercross constitutes the population of target animals for the factor analytic and QTL studies compiled in the present work (**Figure 5**).

The Swiss sublines of Roman high- and low-avoidance rats had been selected, and outbred, for high rates (RHA/Verh) or low rates (RLA/Verh) of 2-way shuttlebox avoidance, using stock from the original RHA's and RLA's developed by Broadhurst and Bignami (1965). Convergent evidence suggests that two different processes contribute to shuttlebox performance acquisition (i.e. inmobility: conditioned fear; and active crossing between compartments: instrumental learning), in which fear conditioning plays an important role at the early stages of training[9] (Aguilar et al. in press; Fernández-Teruel et al. 1991; Weiss et al. 1968; Wilcock and Fulker 1973). Bi-directional selection for this task has presumably favoured prevailing coping styles to aversive events in the Roman rats, RLA/Verh's being passive copers (prone to freeze: conditioned freezing) and RHA/Verh's active copers (prone to flee: conditioned avoidance) (Steimer et al. 1997). These marked passive vs active (i.e. freeze vs flee) coping responses have a general effect on their defensive repertoires, RLA/Verh rats being more fearful across aversive situations than RHA/Verh (for reviews, see Driscoll and Bättig, 1982; Driscoll et al. 1998; Fernández-Teruel et al. 1997). The thorough and extensive evaluation of these rat strains across a wide range of fear-inducing situations, plus convergent findings coming from neuroendocrine and neurobiological (and now molecular genetic) experiments, has established them as one of the best existing rat models of emotionality to date (**Tables 1and 2**).

TABLE 1

BEHAVIOURAL PROFILE OF FEARFULNESS OF THE OUTBRED ROMAN HIGH- (RHA) AND LOW-AVOIDANCE (RLA) RATS STRAINS

Type of test	Type of response	Strain with the overall higher score
Novel cage	Defecations	RLA
Open field	Activity	RHA
	Defecations	RLA
Plus-maze	Total arm entries	RHA
	Open arm entries	RHA
Holeboard	Defecations	RLA
	Self-grooming	RLA
	Head-dipping duration	RHA
Black and white box	Crossing latency	RLA
	Self-grooming latency	RHA
	Defecations	*n.s.*
Social interaction	Active social interaction	RHA
Hyponeophagia	Latency to start eating	RLA
	Self-grooming duration	RLA
	Defecations	RLA
Fear conditioning	Defecations	RLA
Shuttlebox conditioning	Avoidances	RHA

n.s. = non statistically significant difference. (Adapted from Fernández-Teruel et al. 1997).

TABLE 2

HORMONAL AND NEUROCHEMICAL PROFILES RELATED TO FEARFULNESS OF THE OUTBRED ROMAN HIGH- AND LOW-AVOIDANCE RATS STRAINS

HORMONAL INDICES
RLAs show increased levels of ACTH and corticosterone in response to stress
RLAs show increased levels of ACTH after CRF administration
RLAs show increased levels of aldosterone in response to stress
RLAs show increased freezing and decreased hear rate in response to stress after amygdaloid injection of vasopressin
NEUROCHEMICAL INDICES
RLAs show a less efficient GABAergic functioning
RHAs show enhanced dopaminergic activation in response to stress

(Adapted from Fernández-Teruel et al. 2000).

[9]One complementary way of conceptualizing these strain differences can be the following: 2-way shuttlebox acquisition implies a conflict between two antagonistic responses, i.e. freeze vs flee, in which the former retards avoidance acquisition whereas the latter favours it. Theoretically, the Roman low-avoidance rats fail to acquire shuttlebox behaviour because conditioned fear provoked by the initial CS-US pairings is expressed through exaggerated freezing, which sabotages the resolution of the active/passive conflict.

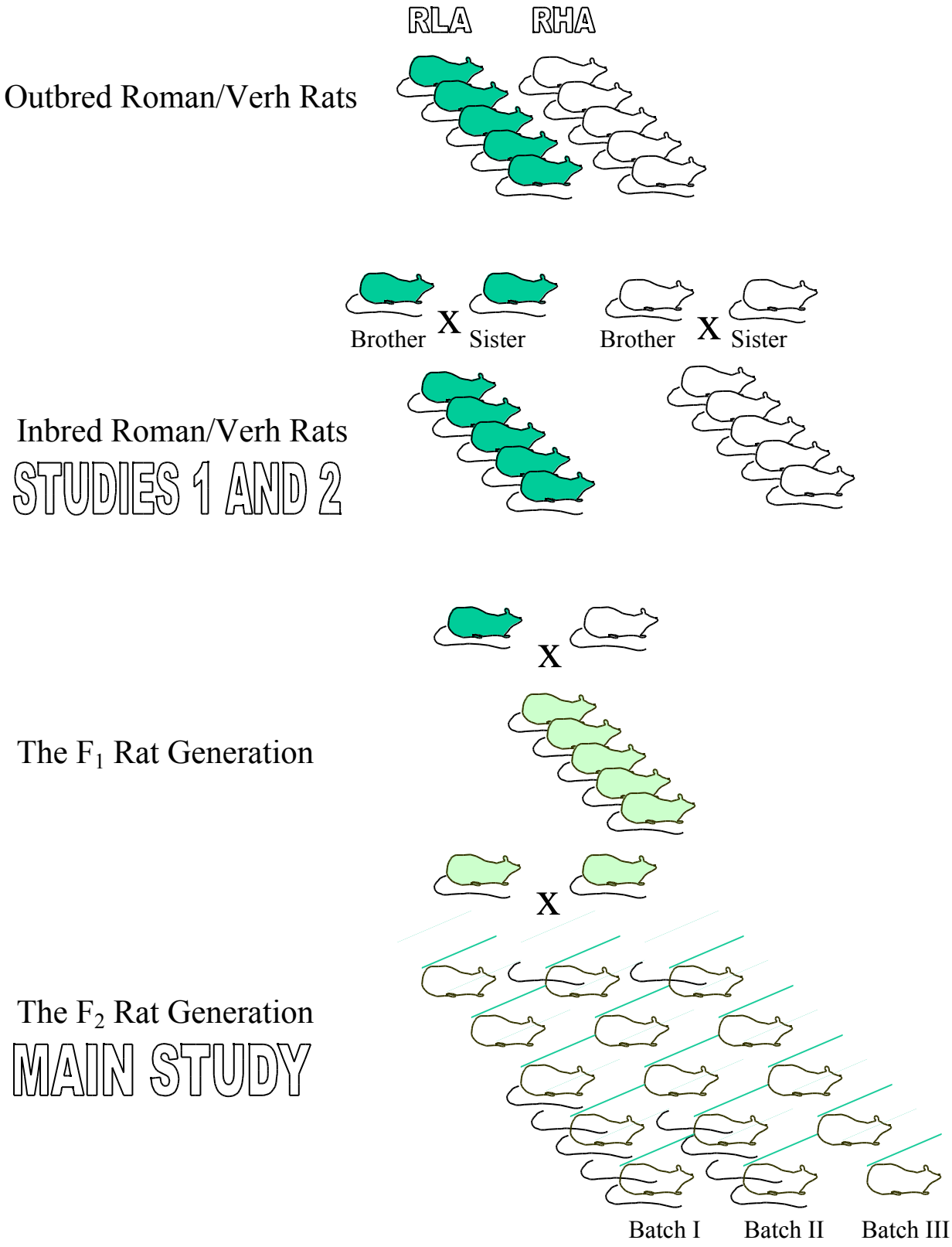


Figure 5. Genetic background of the rats used as subjects in the present series of studies. The subjects used in Study 1 (Escorihuela et al. 1999) were rats of the 10th generation of inbreeding (except in Pilot on Pavlovian fear conditioning in which rats were of the 15th generation; Fernández-Teruel et al. 2000). Those employed in Study 2 (Aguilar et al. 2000) came from the 15th generation. The rats used in the Main Study were derived from intercrossing inbred Roman high- and low-avoidance rats.

As a prerequisite for the genetic mapping experiment, a thorough study of the anxiousness profile of the rats was carried out. This involved the assessment of the parental inbred strains and of the F_2 progeny in multiple tests, involving novel/threatening stimuli and learned fear paradigms. At the time of this doctoral dissertation most of the relevant data have been published (or are in press). The most remarkable findings of this endeavor can be summarised as follows. First, the inbred(I) Roman/Verh rats seemed to behave like their outbred predecessors, RLA-I/Verh`s being markedly more anxious/fearful than their RHA-I/Verh's counterparts. Second, the analysis of the behaviour of the F_2 progeny in the battery of tests revealed, by using factor analytic techniques, that three main factors accounted for more than 40 per cent of the variance: a) a "Learned Fear" Factor containing the measures of aversive/fear conditioning, b) an "Emotional Reactivity" Factor with 11 out of the 14 variables analysed and c) a "Fear of Heights" Factor with high loadings on open arm behaviour in the elevated plus-maze. Third, the genetic mapping experiment demonstrated that one locus, on rat chromosome 5, influences anxious behaviour across the battery of tests in a manner that parallels the effects of anxiolytic drugs, which is consistent with the QTL containing at least one gene with a pleiotropic action on anxious responses.

The particular objectives of the present doctoral dissertation, within the scope of the aforementioned project, were three-fold. 1) In Study 1, to demonstrate that correlative effects of selective breeding on fearfulness are also present in inbred Roman rats. 2) In Study 2, to investigate if a brief removal from the homecage partner could differentially potentiate the startle reflex of these strains. 3) In the Main Study, our purpose was to establish, using factor analytic tools, the behavioural profile of fearfulness for a large sample (n = 800) of F_2 rats in a battery of eight tests, i.e. to create a simple and meaningful map of the rat's emotional repertoire in the test battery. Additional results on sex differences, as well as QTLs for anxiety, coming from the analyses of the same F_2 data base, are also introduced.

EXPERIMENTS

STUDY 1: SELECTIVE INBREEDING FOR AVOIDANCE LEARNING: EFFECTS ON FEARFULNESS

In his review on genetic determinants of individual differences in avoidance learning, Brush (1991) compared three stocks of rats bi-directionally bred for active, shuttlebox performance: the Roman high- (RHA) and low-avoidance (RLA) lines derived from Wistar rats; the Syracuse high- (SHA) and low-avoidance (SLA) strains derived from Long-Evans rats; and the Australian high- (AHA) and low-avoidance (ALA) strains derived from Sprague-Dawley rats. An additional strain was uni-directionally selected for high-active avoidance from Jcl-Wistar rats: the so-called Tokai high avoider (THA) strain. The selection for individual differences in response to this kind of aversive stimulation was accompanied by a host of additional behavioural differences. For example, as compared to SHA rats, the SLA's defecate more, exhibit a greater effect in a succesive negative contrast procedure, and more rapidly acquire conditioned fear (CER), as well as passive-avoidance conditioning. Similarly, as compared to RHA rats, RLA's also show higher defecation scores, display stronger Pavlovian aversive conditioning (e.g. conditioned taste aversion and shock-induced suppression of drinking), and exhibit enhanced passive avoidance (Brush 1991). These resemblances in the way the poor active avoiders (SLA/RLA) and their high avoiding counterparts (SHA/RHA) differ from each other fit rather well with the hypothesis that the former are more fearful/anxious than the latter.

In the present study we aimed to delineate the main behavioural characteristics of the inbred Roman strains (derived from the outbred Swiss sublines; see Introduction). As selective breeding was able to generate pronounced divergences in avoidance performance, as well as in an array of fearful/anxious behaviours (i.e. fearfulness), we sought to establish if such a distinctive pattern held true after selective inbreeding. The inbreeding program (brother-sister mating) was initiated in 1993 and the experiments reported here were conducted using animals of the 10th generation of that inbreeding (Escorihuela et al. 1999) and of the 15th generation (Fernández-Teruel et al. 2000). In order to evaluate whether inbred RLA/I-Verh rats were more fearful than RHA/I-Verh's (henceforth shortened to RLA and RHA, respectively), in accordance with the widely studied, outbred Roman/Verh rat sublines (for reviews, see Driscoll and Bättig, 1982; Driscoll et al. 1998; Fernández-Teruel et al. 1997), we carried out a series of experiments conceived to characterize their behavioural profile. To do this, we examined their behaviour in multiple tests which consisted of novel cage (NC), hole-board (HB), open-field (OF), elevated plus-maze (PM), hyponeophagia (HNP), shuttlebox habituation (SH), shuttlebox avoidance conditioning (SAC), and classical fear conditioning (CFC). (NC, HB, OF, PM, HNP, SNE and SAC experiments [for additional methodology] were reported by Escorihuela et al. 1999, and CFC experiments by Escorihuela et al. 1997 and Fernández-Teruel et al. 2000).

In Experiment 1, the timidity/emotionality of the rats was evaluated by simultaneously exposing two of them to the NC procedure during a 1-min period, consisting of two uncovered cages (identical to their homecages, but without sawdust) with a 15-cm separation between them. Timidity was measured, utilising the following parameters: the distance traveled, frequency of rearings and defecations, and self-grooming latency/duration. One month later, in Experiment 2, exploratory behaviour was evaluated in the HB test, a white wooden box (66 x 66 x 47 cm) containing four holes in the floor (divided into 16 equal squares), in which we also differentiated the effects of the presence/absence of novel objects (under the holes) on exploration in the Roman rats. The measurement of ambulation (crossings across squares), frequency of rearings, number of head-dips and time spent head-dipping was carried out during 5 min. In Experiment 3 (one month after HB testing), the rats were sequentially assessed in two tests (OF-PM, 5 min each), one half during the lighted phase of the light-dark cycle with normal fluorescent

illumination and the other half during the dark phase with red light. The OF (first test) was a brown wooden box (116 x 116 x 40 cm) divided into 25 equal squares and the PM (second test; immediately administered) was an elevated black apparatus (height = 50 cm) with two opposed open arms (50 x 10 cm each) plus two opposed enclosed arms (50 x 10 x 40) all connected by a 10 x 10-cm open area in the middle. In Experiment 4, the animals were submitted to an HNP test (2 weeks after Exp. 3) which necessitated a 15-day food-deprivation schedule, resulting at the end in 1 h daily of free access to food. A prior "baseline test" was performed in which each cage was slightly pulled out from the rack (15 cm). Under these conditions the latency to start eating and the time spent eating were scored. The HNP apparatus was a raised, brown wooden box (57 x 28 x 32 cm) containing eight holes in the floor, each with food pellets inside. Eating latency/duration, distance traveled and the number of rearings were measured.

Three weeks after the HNP test, Experiment 5 on shuttlebox acquisition (SAC task; the selection criterion of the Roman strains) was performed. A "10-min familiarization period" (SH test) was introduced just before shuttlebox training, in which activity (i.e. crossings between compartments), self-grooming and defecations were measured. The conditioned stimulus (CS) for SAC was a light–tone combination (7-W lamp plus 2400 Hz, 63-dB), and a 0.7-mA electric footshock was used as the unconditioned stimulus (US). Three 50-trial sessions were administered, each trial composed of the following sequence of events: a 10-s CS followed by a 20-s US in such a manner that when a crossing was already made during the CS, the US was not administered (i.e. avoidance response), whilst if the animal failed to avoid then it received a footshock (escape response). Once the animal had crossed to the other compartment a 60-s intertrial interval was administered. Finally, a pilot experiment on Pavlovian fear conditioning was carried out in a white chamber divided into two equal compartments (23 x 12 x 20 cm). A 20-W light (15 sec) functioned as the CS and an electric footshock (0.8-1 mA) delivered through the grid floor acted as the US. Training consisted of eight CS-US pairings that started with the onset of the CS. US and CS terminated simultaneously. A 120-sec (mean) pseudorandom intertrial interval was used, with a shock-free interlude of 60 sec. After a retention interval of 24 h, freezing to the training context (contextual fear conditioning) was measured during 5 min. The light was then switched on for five minutes to measure fear conditioning to the CS.

Our predictions were based on the hypothesis that RLA rats are more emotionally reactive to threatening events than are RHA's, as well as more susceptible to develop learned fear responses, and that they would show higher scores in the measures of emotionality (e.g. defecation and self-grooming), faster acquisition of fear responses (e.g. conditioned freezing) and decreased activity/exploration in novelty situations (e.g. ambulation and rearing). It can be seen in **Table 3** that the results confirmed this general pattern of differential responses between the two strains, with RLA rats showing more defecations, more self-grooming, less ambulation and less rearings in the tests of novelty, less exploration (i.e. head-dipping duration) in HB, poorer avoidance performance in SAC and stronger freezing responses in CFC. When inbred

TABLE 3

SUMMARY OF THE RESULTS OF STUDY 1, CONCERNED WITH SELECTIVE INBREEDING EFFECTS ON FEARFULNESS

Experiments	Type of test	Type of measure	Strain with the overall higher score
Exp. 1	Novel cage	Distance traveled	RHA
		Rearings	RHA
		Self-grooming latency	RHA
		Self-grooming duration	RLA
		Defecations	RLA
Exp. 2	Holeboard: without objects	Squares crossed	RHA
		Rearings	RHA
		Head-dips	*n.s.*
		Head-dipping duration	*n.s.*
	Holeboard: with objects	Squares crossed	RHA
		Rearings	RHA
		Head-dips	RHA
		Head-dipping duration	RHA
Exp. 3	Open field: lighted	Distance traveled	*n.s.*
		Rearings	RHA
		Self-grooming latency	RHA
		Self-grooming duration	RLA
		Defecations	*n.s.*
	Open field: dark	Distance traveled	RHA
		Rearings	RHA
		Self-grooming latency	RHA
		Self-grooming duration	RLA
		Defecations	*n.s.*
	Plus-maze: lighted	Total arm entries	RHA
		Rearings	*n.s.*
		Open-arm entries	RHA
		Time spent in the open arms	*n.s.*
		Distance traveled in the open arms	RHA
		Self-grooming latency	RHA
		Self-grooming duration	RLA
	Plus-maze: dark	Total arm entries	RHA
		Rearings	RHA
		Open-arm entries	RHA
		Time spent in the open arms	*n.s.*
		Distance traveled in the open arms	RHA
		Self-grooming latency	RHA
		Self-grooming duration	RLA
Exp. 4	Hyponeophagia: baseline	Latency to start eating	RLA
		Time spent eating	RHA
	Hyponeophagia: test	Latency to start eating	*n.s.*
		Time spent eating	*n.s.*
Exp. 5	Shuttlebox as a novel environment	Crossings	RHA
		Self-grooming latency	RHA
		Self-grooming duration	RLA
		Defecations	RLA
	Shuttlebox avoidance conditioning	Avoidances	RHA
		Escape latencies	RLA
Pilot	Pavlovian fear conditioning	Freezing to context	RLA
		Freezing to CS	RLA

n.s. = non-statistically significant difference

strains are used all variation in their behaviour can be attributed to genetic background if environmental conditions for all animals are identical (as is the case in laboratory settings): that is, the magnitude of the observed differences among individuals and between strains is genetic in origin (Wimer and Wimer 1985). As we used animals of the 10th and 15th generation of inbreeding, they should have been close to becoming genetically homogeneous. Our results have shown that inbred RLA rats behave as if they were more anxious/fearful than RHA's across eight different experimental situations, thus extending the consistency of the behavioural profile of the outbred Roman rats to the inbred ones. We have therefore demonstrated the effects of selective inbreeding in multiple tests of fearfulness, constituting further evidence for genetic differences in a complex psychological trait, using two strains of psychogenetically selected rats.

STUDY 2: EMOTIONALLY-PRIMED STARTLE: STRAIN, SEX AND COHORT REMOVAL EFFECTS

Major advances in our understanding of the brain mechanisms of fear come from studies with the startle reflex paradigm, which has permitted an analysis of the neural sites, and their interconnections, involved in fear responses (Davis et al. 1993; Gray and McNaughton 2000; Koch 1999; Richardson 2000). The startle response is a short-latency reflex (i.e. 5-10 ms; Fendt and Fanselow 1999) that occurs immediately after the presentation of an unexpected, intense stimulus that can be of a visual, acoustic or tactile sensory nature. It consists of a sequential muscle contraction initiated around the facial area and then expanding through the skeletal muscles. It is a simple reflex whose neural pathway mainly involves the caudal pontine reticular nucleus in the lower brainstem. In rat studies it is usually evaluated as the whole-body jump response of the animal, whereas in human experiments just the eye-blind component is taken as the index of the startle response **(Figure 6)**.

In spite of its apparent simplicity the startle reflex can be bi-directionally modulated by internal states and external stimuli, that is, it has a non-zero baseline that can be either augmented (e.g. potentiated by an internal state of fear: "negative affect") or reduced (e.g. attenuated by a pleasure experience: "positive affect"), providing a useful tool for the neurobiological study of emotion in both animals and humans (Fendt and Fanselow 1999; Koch 1999; Lang et al. 1990; Lang et al. 2000). Another important aspect of the startle reflex has been noted by Lang et al. (2000), that "the reflex is not a specific component of the fear [*emotional*] state, but rather a response to a probe event that is primed when the state is present". This is relevant because it is then possible to differentiate neuroanatomically what is a reflex from what is not, thus facilitating the dissection of related, more complex hypothetical central states, such as fear. When primed by aversive events startle is accompanied by a set of responses, such as tachycardia, inmobility and enhanced vigilance, that suggest a primary defensive function designed to protect the organism against damage. These negative events can be natural stimuli ocurring in the wild (e.g. a mixture of odors and noises signaling the

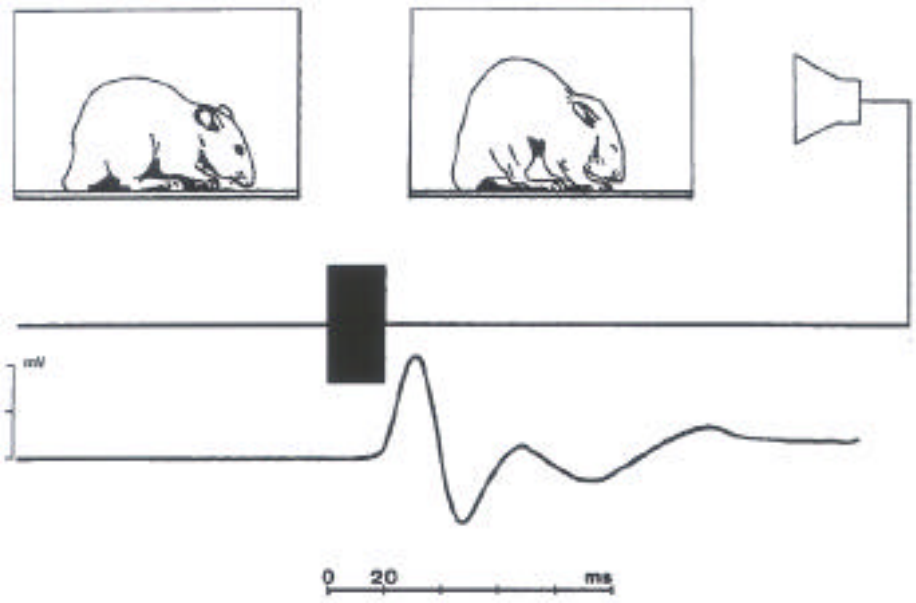


Figure 6. The acoustic startle response in a rat *ca* 30 ms after stimulus onset. The pictures are redrawn from a film taken by Carsten Spiekermann (Unpublished Diploma-thesis at the University of Tübingen) with a high-speed camera (150 frames sec^{-1}). The trace at the botton of the figure shows the ballistogram of the whole-body ASR. The ASR is usually expressed as arbitrary units or millivolts (mV) of the accelerometer output. (From Koch 1999).

presence of a predator) as well as simple laboratory stimuli scheduled to be precisely administered (e.g. lights or tones paired with electric footshocks). Given its short latency, the startle reflex can be considered to be an early component of emotional behaviour, when the organism is recruiting its defensive resources to confront a threat (Koch 1999).

In summary, the startle reflex is a good index of fear/emotionality, due to at least the following five reasons: First, it is a test in which locomotion does not interfere with the measurement of emotionality. Second, this reflex is triggered by neutral stimuli as well as primed by emotional events, so that it serves as its own control, providing an appropriate baseline. Third, this reflex has been observed in a host of species across phyla, ranging from fish to infra-human and human mammals, thus permitting cross-species generalisation of experimental findings. Fourth, the startle reflex has a non-zero baseline, i.e. it can be bi-directionally modulated (i.e. potentiated or attenuated) as a function of the valence of the emotional experience (i.e. negative or positive affective states). Fifth, because it is a simple reflex, it's basic circuitry presumably involves a relatively small number of neural connections, thereby making it easier to establish a precise starting point for the neuroanatomical study of related complex states such as fear.

The differential "up/down"modulation of the startle can be seen in several paradigms. From those procedures in which an enhancement of the startle has been observed, sensitization and fear-conditioning have been the most widely studied. Sensitization is an increase in the magnitude of responding as a result of prior presentation of a strong stimulus. Davis and colleagues (e.g. Boulis and Davis 1989) developed the footshock sensitization paradigm of startle as a device for studying the effects of aversive, unconditioned stimuli on anxiety. That same laboratory experimentally exploited the phenomenon of Pavlovian fear conditioning in a procedure based on the measurement of this reflex, by calculating the difference in amplitude between two types of trials, i.e. startling stimuli acompanied by the presence of a fear-provoking CS minus startling stimuli alone: this procedure was called fear-potentiated startle (Brown et al. 1951; Davis et al. 1993). With respect to the paradigms in which an attenuation of the startle magnitude can be registered, habituation and prepulse inhibition are most frequently encountered in the literature. Habituation consists of a reduction in startle response as a consequence of repeated presentations of the startling stimulus. Taking into account that the startling stimulus itself is in reality an aversive event, then the resulting habituation curve can reflect an attenuation in the rat's emotional state. Prepulse inhibition is a phenomenon in which a reduction in startle responding to a strong (i.e. startling) stimulus occurs, due to the immediately prior presentation (30-500 msec) of a weaker (i.e. non-startling) stimulus (Koch 1999).

As the evidence of divergent emotionality in the Roman rats can be attributed in part to the role that activity plays in most animal models of anxiety in which they have been tested (e.g. Brush 1991), by using the paradigm of the acoustic startle reflex (ASR) we could unambiguously elucidate whether such strain differences are the result of emotionality, rather than activity. Previous work in this direction investigated the existence of strain and footshock sensitization effects on the acoustic startle response in inbred, male Roman rats (Schwegler et al. 1997). As expected, the results convincingly showed that RLA animals displayed much more pronounced acoustic startle than RHA's, that difference being further increased by shock sensitisation. To replicate and extend those findings, we designed a similar experiment, employing rats of both sexes. We wanted to evaluate further whether a brief isolation experience (i.e. cohort removal during 30 min) could differentially affect the acoustic startle response of inbred Roman rats. In reference to Schwegler's results, we expected that RLA rats would have an stronger ASR than RHAs, and that the isolation effects would be more pronounced in the emotional strain. Based on Gray's hypothesis (1971; 1987), that male rats are more fearful than females, sex-linked differences on the acoustic startle reflex were expected as well.

The results of our study showed strain, sex and cohort removal effects on the startle reflex, mediated by differences in fearfulness. **Figure 7** shows a schematic representation of the size of the effect of these three variables on the startle reflex (each bar is the result of the combination of four experimental groups, i.e. mixing rats from different experimental conditions; arbitrary units). As can be seen, the manipulation responsible for the effect of greater magnitude (regardless of the influence of sex plus cohort removal) was selective inbreeding, the method by which the divergent emotional profiles of the Roman rats was created. The size effect of sex was smaller (regardless of the influence of strain plus cohort removal). Finally, the smallest effect was for cohort removal (regardless of the influence of strain plus sex), an entirely environmental source of variation. Therefore, there was a gradation in the amplitude of the emotionally-primed startle related to the relative involvement of biological and environmental factors (strain > sex > cohort removal).

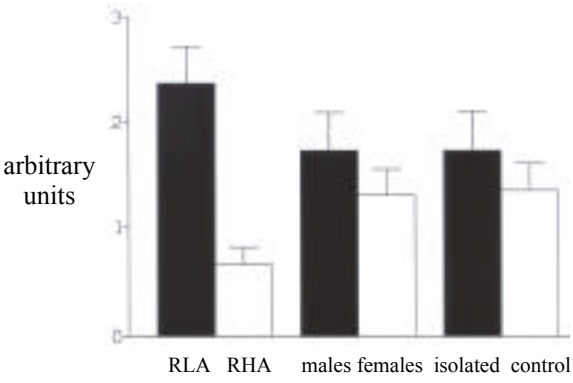


Figure 7. Schematic representation of the size effect of strain, sex and cohort removal.

It is important to note that the cohort removal manipulation used here was capable of substantially enhancing startle by increasing anxiety in the strain that manifests a greater susceptibility to stressful stimuli (see male RLA group in Fig. 1 of the paper by Aguilar et al. 2000, page 73). At least three mechanisms could account for this hypothetical, internal state of increased anxiety in male RLA rats. One possibility would be fear of the unfamiliar, a source of anxiety across species, as the rat that remains in the homecage does not know what is going to happen. Another mechanism that may underly this

effect is the removal of safety signals, which could prompt anxiety (e.g. Gray 1987): in this case, that would be the withdrawal of reinforcement coming from social interaction, which is also associated with well-being or safety. The third possible explanation could be related to the concept of anticipatory anxiety: if the rat has been briefly isolated on prior occasions, plus being submitted afterwards to some kind of aversive experience (e.g. prior exposure to novelty-tests such as OF or PM), the prediction of a potential aversive consequence, signaled by the momentary absence of the partner, could also trigger a state of anxiety.

MAIN STUDY: THE F_2 GENERATION BEHAVIOURAL PROFILE: FEAR TESTING

The main objective of the present doctoral dissertation was the assessment of a large F_2 progeny of Roman rats (n = +/- 800) in a test battery designed to measure their fearful profile, thus setting up an investigation, using the same animals, of putative QTLs for anxiety. Eight tests were utilised (i.e. OF, PM, HB, A, ASR, CFC, SH and SAC), encompassing unlearned and learned fear as well as indices of locomotor activity (as a control). The statistical method employed for the description of the rats' profile was factor analysis, a set of tools which is useful in simplifying complex patterns of correlations among variables. We sought to establish a simple map for the behavioural output of this large F_2 population, i.e. a small number of meaningful factors grouping the different types of measures in a coherent and robust manner. From a theoretical and empirical viewpoint, a reasonable factor solution would be one differentiating between unlearned and learned fear. Our factorial search, though explorative, was designed with this in mind.

In animal research there is no consensus about what the structure of fearfulness is, that is, whether a common trait underlies different forms of anxious responding (e.g. Gray and McNaughton 2000), or whether distinctive types of responses depend on a number of independent traits (Ramos and Mormède 1994). The fact that separate fear systems can be anatomically distinguished, e.g. a neural pathway for freezing vs another for defecation, does not preferentially support either hypothesis. One may argue that a common state of fear underlies the two responses through a quest for general mechanisms of defensive behaviour, but it may also be argued that when evidence for a distinction in an emotional component exists (e.g. different neural circuitry), separate systems are indicated. How can we decide whether freezing and defecation are the expression of different emotional states, or two different routes by which a common state of fear is expressed? The answer is a theoretical matter, rather than empirical.

Factor analytic techniques seem to be a potentially good approach to solve this dilemma, separating into independent factors those variables that are hardly related (perhaps with different neural bases). So, if freezing and defecation are grouped within the same factor, this could be taken as evidence for the unidimensionality of fear, but if these variables load to distinctive factors, it could indicate separate emotional systems for both. In any case, the problem is that the application of factor analytic tools is also theoretically-oriented (e.g. the researcher decides what variables are entered into the analysis, as well as the number of factors that will explain the correlation matrix) (Kline 1994). This is the reason why factor analysis is primarily considered to be a descriptive technique, rather than an explanatory one.

In recent years, several factor analytic studies have been carried out with rodents in order to describe the structure of anxious behaviour (e.g. Belzung and LePape 1994; Fernandes et al. 1999; Flaherty et al. 1998; Griebel et al. 1996; Maier et al. 1988; Ramos and Mormède 1998; Rodgers and Johnson 1995). The usual finding has been that factor solutions consistently differentiate multiple factors, regardless of the number of tests investigated (**Table 4**). Moreover, there is no clear agreement among studies, neither conceptual nor empirical, with respect to the factor structures resulting from analyses of different tests or combinations of them. Based on these observations, most authors have arrived at one of the following two conclusions: 1) anxiety seems to be multifaceted (the higher the number of emerging factors, the greater the multidimensionality of anxiety; Ramos and Mormède 1998), and/or 2) common animal models of anxiety do not measure the same type of phenomena (e.g. Belzung and LePape 1994; Flaherty et al. 1998; see **Table 4**).

This lack of consistency can be attributed, at least in part, to the way in which each author used factor analysis. Rodgers and Johnson's work (1995) may provide a good example of this. Those authors studied the structure of the PM in a sequential manner, first applying factor analysis to the typical variables, and then to those plus a number of ethological measures. The analysis of the standard measures (i.e. open and closed arm behaviour) yielded a two-fold solution distinguishing between anxiety and activity components. The addition of time spent in the center led to the emergence of a third factor that the authors interpreted as decision making. When the ethological measures were entered into the analysis, a six-fold solution arose: anxiety, locomotor activity, decision making, risk assessment, vertical activity, and exploratory behaviour. There seemed to be, therefore, a positive relationship between the number of variables included and the number of emerging factors.

If they had included all the measures in the analysis, they would had found two factors (e.g. with anxiety/risk assessment/decision making grouped in the first one, and locomotor activity/vertical activity/exploratory behaviour in the second one), a simple and easy way to interpret a map of rodent's behaviour in the PM test, i.e. one factor reflecting "emotionality" and another "activity". This hypothetical structure could had been approached by applying second-order factor analysis to the correlation matrix among factors, as well as by forcing the structure of the first-order solution onto a reduced number of factors (two, in this case), a method that gives similar results to the former (Kline 1994). Although some rules exist in selecting the number of factors (Kline 1994), the researcher always makes the final decision whether to maintain or to eliminate them, on the basis of the particular objectives envisaged.

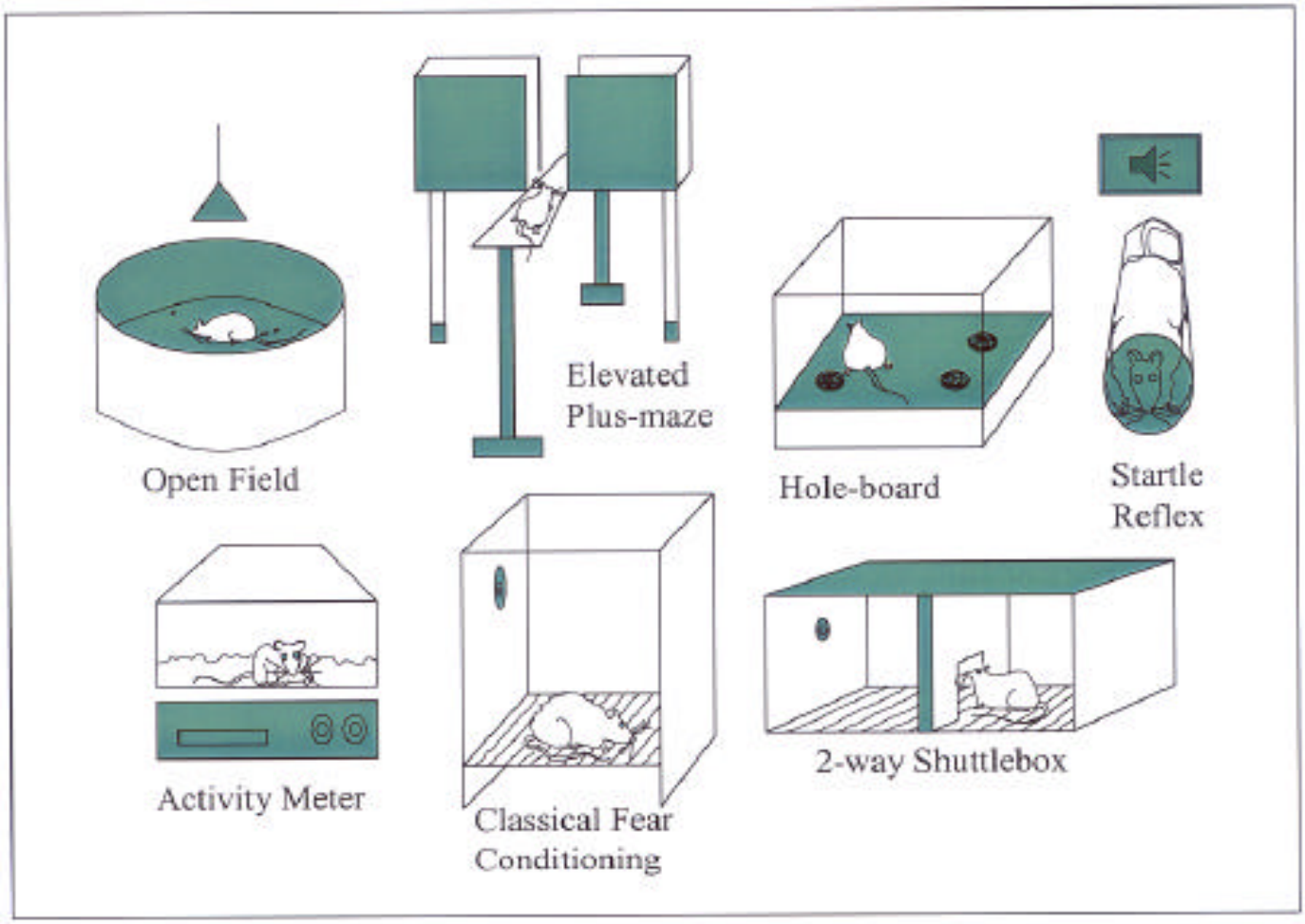
TABLE 4

SUMMARY OF A SAMPLE OF STUDIES ON FACTOR ANALYSIS WITH RODENTS

N	# of tests	Type of test	# of factors	Interpretation	Author
800	8	OF, PM, HB, A, ASR, CFC, SH, SAC.	3	F1: Learned fear; F2: Emotional reactivity; F3: Fear of heights.	Aguilar et al. 2001(*)
80(**)	1	MDTB.	7	F1: Anxiety (BZ); F2: Anxiety (5-HT); F3: Anxiety ("Affective"-orientated defense); F4: Anxiety (Terminal defenses; F5: Anxiety (EADRE); F6: *No label;* F7: *No label.*	Blanchard et al. 1996
30	1	PM.	4	F1: Anxiety; F2: Motor activity; F3: Waiting capacity/Decision making F4: *No label.*	Cruz et al. 1994
42	3	HB, PM, SOT.	3	F1: Sexual preference/Social interest; F2: Plus-maze Anxiety/Activity; F3: Holeboard Exploration/Activity.	Fernandes et al. 1999
60	4	NCP, PM, OF, CFC.	4	F1: Learned fear of the open arms; F2: Generalised anxiety state; F3: Timidity/Fear; F4: Disappointment.	Flaherty et al. 1998
30	4	OF, RC, SH, SAC.	3	F1: Avoidance learning; F2: Exploration/Activity; F3: Shuttle defecation.	Gomà and Tobeña 1985
65	7	ER, RW, OFd, OFl, OFnod, WR, WM.	5	*None label.*	Maier et al. 1988
144	4	OF, PM, BW, SI.	3	F1: Approach/Avoidance; F2: Locomotion; F3: *No label.*	Ramos et al. 1997
267	2	PM, OF.	3	F1: Anxiety; F2: Locomotion; F3: *No label.*	Ramos et al. 1998
90(**)	1	PM.	6	F1: Anxiety; F2: Locomotor activity; F3: Decision making; F4: Risk assessment; F5: Vertical activity; F6: Exploratory behaviour.	Rodgers and Johnson 1995
96	1	OF.	2	F1: Exploratory (Locomotor). F2: Emotional reactivity.	Whimbey and Denenberg 1967

This table summarises a sample of factor analytic studies carried out with rodents. (*) Our study constitutes an exception for all the works compiled here, as we restricted the first-order six-fold solution to three simple main factors. (**) The work was carried out using mice as subjects. BW = black and white box; BZ = related to benzodiazepine receptor ligands; EADRE = escape from an area in which danger has been recently encountered; ER = emotionality rating; MDTB = mouse defense test battery; NCP = negative contrast procedure; OFd = open-field in the dark; OFl = open-field in the light; OFnod = novel-object detection in the open-field; RC = rearing-cage; RW = activity running-wheel; SH = shuttlebox habituation; SI = social interaction; SOT = sexual orientation test; WM = water maze; WR = water rotation; 5-HT = related to serotonin neurotransmision.

In the present study we sought to establish a parsimonious description of our test battery which was comprised, as mentioned above, of unlearned (OF, PM, HB, A, ASR) and learned fear (CFC, SAC) paradigms, using a large sample of subjects (**Box 1**). We therefore expected to find a reliable correlation matrix, as well as a robust factor solution, for measures of anxiety. In order to carry out an exhaustive measurement of the different manifestations of emotional reactivity across tests, more than fifty dependent variables were initially considered. Our first attempts (not shown) to establish an adequate global picture of the correlation matrix were unsuccessful. We obtained a solution with many factors (more than twenty): the first ones grouped the same variables measured in various tests (e.g. one factor for defecation, another for self-grooming) as well as measurements of a given variable at different moments within a test (e.g. a factor grouping freezing at one minute, at two minutes and so on), whilst the last factors accounting for the smallest amount of variance were difficult to interpret. This lack of meaningful clustering between variables can be attributed in part to the large number of closely, related variables entered. As most of them formed linear combinations (the same variable measured in several tests or at different moments within a single test), the emergence of meaningful factors was presumably prevented. Although it was reassuring to confirm that, for example, the measures of defecation loaded onto the same factor, thereby probably having the same meaning, our principal objective was to determine whether different responses (e.g. defecation, avoidance behaviour or conditioned freezing) shared something in common, or not.



Box 1. About four hundred F2-generation rats of each sex were used, 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: open field (OF); elevated plus-maze (PM); hole-board (HB); activity meter (A); acoustic startle reflex (ASR); classical fear conditioning (CFC); shuttlebox avoidance conditioning (SAC).

In order to preclude the proliferation of redundant and nonsense factors, a substantial reduction of variables was carried out before applying factor analyses, on the basis of two principal criteria: 1) an avoidance of linear combinations among variables which could give rise to spurious factors; and 2) the maintenance of consistency with previous literature. By means of the Pearson correlation coefficient, the relationships among 33 measures were calculated, serving as the starting point for the factor analytic decription of the test battery. The correlation matrix permitted the notation of some significant relationship patterns among different measures which were indicative of potential underlying factors (**Table 5**), but too complex to be appropriately interpreted. Moreover, from the examination of the correlation matrix it was difficult to rule out redundant variables. One way to avoid the selection of almost equivalent measures (forming linear combinations) might be to perform separate factor analyses on each test yielding a simple structure (i.e. independence among factors) and from each of the resulting factors accounting for behaviour in a given test, then to choose just one or two variables as a subset of the selected measures representative of the eight tests (see **Box 2** for a schematic representation of the plan of the data analyses). Following this strategy, we considered 14 target variables: defecation (in the OF test), self-grooming duration (OF), distance (OF), enclosed arm entries (PM), % of open arm entries (PM), % time spent in the open arms (PM), head-dipping duration (HB), activity counts (A), startle amplitude (ASR), freezing to context (CFC), freezing to CS (CFC), crossings during habituation (SAC), avoidances (SAC), and intertrial crossings[10] (SAC).

	OFDis	PMEAE	%TOA	SHCROSS	CFCCTX	CFCCUE
OFDis	1					
PMEAE	0.38	1				
%TOA	0.16	0.17	1			
SHCROSS	0.20	0.25	0.12	1		
CFCCTX	- 0.02	- 0.03	- 0.01	- 0.27	1	
CFCCUE	0.04	- 0.01	- 0.06	- 0.29	0.59	1

Table 5. This correlation matrix with selected variables illustrates the extent to which underlying factors to be extracted by factor analysis could be advanced from the inspection of the patterns of correlations. It must be noted that these variables have been chosen with the help of the 6-fold solution reported in the present work. The factors to be extracted are Classical Fear Conditioning (Factor 2, mainly grouping CFCCTX, CFCCUE, and SHCROSS measures) and PM/OF activity (Factor 4, mainly grouping PMEAE, OFDis, and SHCROSS measures). OFDis = distance; PMEAE = enclosed arm entries; %TOA = % of time in the open arms; SHCROSS = crossings during habituation to shuttlebox; CFCCTX = freezing to context; CFCCUE = freezing to CS.

[10]We consider intertrial crossings in 2-way shuttlebox conditioning as a fear-mediated (ritualistic) behaviour. There is compelling evidence suggesting a direct relationship between intertrial crossings and the level of fear to the background cues, so that the lower the fear to the training context the lower the frequency of intertrial responding (Callen, 1986; Mowrer 1947; Thompson, Sachson, and Higgins, 1969). For example, we have demonstrated, using RHA rats, that intertrial crossings can be partially extinguished after long-lasting exposure to the background cues (Aguilar et al. submitted).

Rationale of the factor analyses:
Study of the structure of the battery of tests

1) Reduction of the number of initial variables
   Criteria: avoidance of redundancy; consistency with previous literature (see Aguilar et al. 2001)
2) Selection of the 14 target variables
   Correlation matrix plus criteria
   Factor analysis of each test plus criteria
3) 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
4) Factor analysis with the 14 target variables: 3-fold structure
   Factor 1: Learned fear; Factor 2: Emotional reactivity; and Factor 3: Fear of heights

Box 2. Plan of the data analyses.

A global factor analysis onto the 14 target variables, representing all tests of the battery, extracted six independent factors (see **Table 6**). Factor 1 contained SAC behaviour; Factor 2, freezing in CFC; Factor 3, PM anxiety; Factor 4, PM and OF activity; Factor 5, ASR anxiety; and Factor 6, a blend of activity and anxiety measures. This was a good summary of the correlation matrix: the six factors fostered a noticeable simplification of the 33 initial variables, and was easier to interpret. The fact that SAC and CFC training were dissociated into two distinctive factors is interesting because the former seems to involve a learning mechanism more complex than the latter, that is, whilst freezing in CFC is generally seen as the expression of stimulus-stimulus associations, avoidance behaviour in the SAC task is thought to be the result of stimulus-stimulus plus response-consequence associations (Dickinson 1980). What is reflected in the first factor is presumably the response-consequence component involved in this aversively-motivated instrumental task (SAC). In addition, that PM anxiety and PM activity factors could be distinguished is also relevant, as it reinforces the large body of evidence suggesting the existence of both components in that test. Finally, the additional emergence of an ASR-anxiety factor plus a factor mainly containing defecation, self-grooming, distance in the OF and activity in the A, suggests that in this six-fold solution we could have detected fear response systems in the rat's repertoire, perhaps connected with particular neural pathways.

By inspecting the quantity of variance explained for each factor of the nonrotated solution we could see that the first three factors (especially Factors 1 and 2) accounted for the major part (43 % vs 24 %): an ocular examination of the eigenvalues curve (Cattell's Scree test; **Figure 8**) confirmed this observation. Hence, we applied a three-factor solution to the same 14 target variables to see whether the six "subtraits" obtained in the first-order solution could be coherently organised around a small number of principal traits. We expected SAC plus CFC paradigms to load onto the same factor, thus being distinguishable from unlearned emotionality measures. There are both theoretical and empirical reasons behind this hypothesis. Because both SAC and CFC procedures theoretically provoke a state of fear (learned by means of simple CS-US associations; McAllister et al. 1983; Mowrer 1947; Weiss et al. 1968), this common aspect could be

TABLE 6

SIX-FOLD SOLUTION
FOR THE 14 SELECTED MEASURES OF FEARFULNESS

Factor	Fear test battery measures
SAC	Avoidances (0.91); intertrial crossings (0.91); crossings during habituation period (0.38).
CFC	Freezing to context (0.85); freezing to CS (0.85); crossings during habituation period (- 0.51).
PM anxiety	% of open arm entries (- 0.94); % of time in the open arms (- 0.93).
PM and OF activity	Enclosed arm entries (- 0.84); distance (- 0.73); crossings during habituation period (- 0.38).
ASR anxiety	Startle (0.75); head-dipping duration (- 0.66); defecations (0.37).
Blend of fear and activity	Activity counts (- 0.72); self-grooming (0.68); defecations (0.51); distance (- 0.37).

Numbers in parentheses indicate factor loadings > 0.30. The correlations among factors were as follows: correlation between Factor 1 and Factor 2 ($r_{1,2}$) = - 0.16; $r_{1,3}$ = - 0.09; $r_{1,4}$ = - 0.11; $r_{1,5}$ = - 0.06; $r_{1,6}$ = - 0.13; $r_{2,3}$ = 0.02; $r_{2,4}$ = 0.02; $r_{2,5}$ = - 0.08; $r_{2,6}$ = - 0.03; $r_{3,4}$ = 0.18; $r_{3,5}$ = 0.09; $r_{3,6}$ = 0.06; $r_{4,5}$ = 0.04; $r_{4,6}$ = 0.18; $r_{5,6}$ = 0.08. (Adapted from Aguilar et al. 2001).

reflected in a higher-order factor solution. Empirically, the emergence of an independent, learned fear factor is predictable on neurobiological grounds, as recent evidence has begun to suggest that some key neural nuclei have specific roles in fear responses when learning is involved (Davis and Shi 1999; Lang et al. 2000; Richardson 2000).

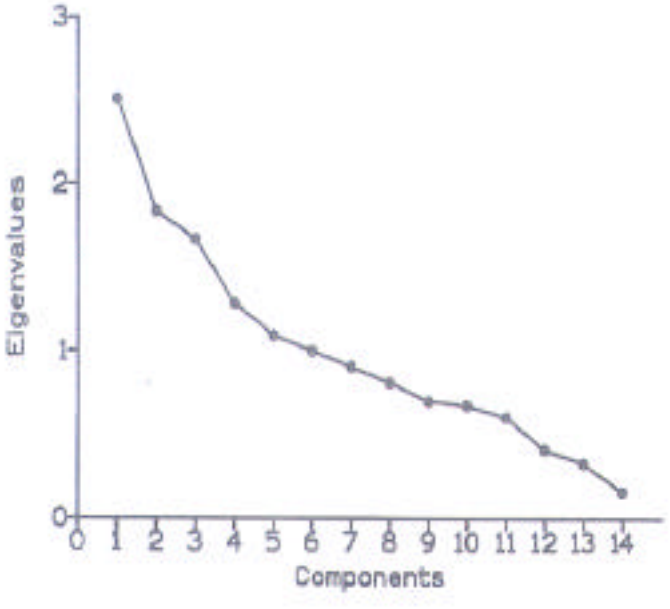


Figure 8. Cattell's Scree test of the unrotated factor solution with the 14 selected measures of fearfulness. (From Aguilar et al. 2001).

In concordance with that, our three-fold structure was composed of a Learning Fear Factor (the first factor) plus two additional factors of fearfulness termed Emotional Reactivity (the second one) and Fear of Heights (the third one; **Table 7**). That the three factors were orthogonal (hardly related to one another), empirically confirms the expected differentiation between learned and unlearned fear indices of emotionality[11]. The measures from SAC and CFC paradigms were also grouped in Factor 2 (but with slight to moderate loadings), as if factor analysis had also detected, beyond basic differences, a common role shared by learned and unlearned fear responses: i.e. two main varieties of defensive responding tailored for natural selection from an old motivation system (e.g. Lang et al. 2000) with the function of protecting organisms against damage and threat of damage. Therefore, we named the second factor Emotional Reactivity (in a broad sense) to reinforce the fact that all types of fearful/anxious responses entered into the analysis (except ASR) loaded onto it. Finally, another form of unlearned fear was distinguished by factor analysis around the third factor, termed Fear of Heights (for representing open arm behaviour in the PM), a specific "phobia" (seemingly) of the rat's defence repertoire. It is worth noting that the Fear of Heights Factor seemed to have, as well, something in common (reflected by slight loadings) with behaviours falling into the category of learned fear, thus hypothetically sharing basic properties of the same brain mechanism underlying the defence system.

TABLE 7

THREE-FOLD SOLUTION
FOR THE 14 SELECTED MEASURES OF FEARFULNESS

Factor	Fear test battery measures
Learned fear	Avoidances (0.69); intertrial crossings (0.68); crossings during habituation period (0.61); freezing to CS (- 0.64); freezing to context (- 0.60); activity counts in A (0.35).
Emotional reactivity	Distance (0.62); defecations (- 0.53); enclosed arm entries (0.45); intertrial crossings (0.42); head-dipping duration (0.41); activity counts (0.37); freezing to CS (0.37); freezing to context (0.36); avoidances (0.33).
Fear of heights	% of time in the open arms (- 0.88); % of open arm entries (- 0.86).

Numbers in parentheses indicate factor loadings > 0.30. The correlations among factors were as follows: $r_{1,2}$ = 0.10; $r_{1,3}$ = - 0.07; $r_{2,3}$ = - 0.01. (Adapted from Aguilar et al. 2001).

[11]SAC and CFC are two forms of aversive learning that mainly differ in that the former involves instrumental conditioning, i.e. response-consequence associations, as hypothetically detected in the six-factor solution. The first-order factor analysis could have separated a complex learning mechanism (bi-process: SAC) from a more simple one (uni-process: CFC) on the basis of the type of contingency in which they differ.