

Marker-assisted selection of a neuro-behavioural trait related to behavioural inhibition in the SHR strain, an animal model of ADHD

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The search for the molecular bases of neuro-behavioural traits in Spontaneously Hypertensive Rats (SHR), an animal model of Attention Deficit Hyperactivity Disorder (ADHD), led to the discovery of two quantitative trait loci related to the locomotor activity in the centre of the open field. In the present study, rats from an F2 intercross between the SHR and Lewis strains were selected with markers on the basis of their genotype at these two loci. We obtained a 'high line' in which rats have the alleles increasing the trait, and a 'low line' with the lowering alleles. In activity cages with a dim light, the low line was more active than the high line. The reverse was found in the open field, and the inhibition of locomotor activity in the low line (as compared to the high line) was directly related to the aversiveness of the situation (larger in the centre than in the periphery, and in high light than in low light), and was more intense in males than in females. This inhibition is not attributable to a classical 'anxiety' factor as measured in the elevated plus maze, in which the open arms behaviours were not different between the lines. The high line also showed a deficit in prepulse inhibition of the acoustic startle reflex. The present data show that the two loci previously described in a SHR × Lewis intercross as related to the activity in the centre of the open field are indeed involved in a behavioural inhibition trait. The marker-based selected lines described here are unique tools for the study of the neurobiological bases of this trait and the molecular foundations of its variability of genetic origin.

Keywords: Acoustic startle response, attention deficit disorder with hyperactivity, elevated plus maze, Lewis rat strain, marker-assisted selection, open field, prepulse inhibition, Spontaneously Hypertensive Rat strain

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Attention Deficit Hyperactivity Disorder (ADHD), a condition characterized by inattention, impulsivity and hyperkinesis in different combinations, is most common among children (Taylor 1998). Numerous family and twin studies suggest that ADHD has a substantial genetic component (Thapar *et al.*

1999), and the search for the molecular bases of the disorder is very active (Comings *et al.* 2000; Faraone *et al.* 2001).

The Spontaneously Hypertensive Rat (SHR) strain is considered as a good animal model for the study of ADHD (Sagvolden & Sargeant 1998). This strain was selected from outbred Wistar Kyoto (WKY) rats for high blood pressure (Okamoto & Aoki 1963). These rats are also hyperactive in novel environments, show low levels of behavioural inhibition, altered responding in a number of operant procedures suggestive of inattention/impulsivity, and display more aggressive behaviours (Portegal & Myers 1989; Hendley *et al.* 1992; Sagvolden *et al.* 1992a & b, 1993, 1998; Castanon *et al.* 1993; Berton *et al.* 1997; Ramos *et al.* 1997; Durand *et al.* 1999). Multifactorial analyses of behavioural reactivity in six rat strains studied in a number of tests designed to evaluate the emotional reactivity of experimental animals disclosed distinct dimensions describing the overall behavioural response, namely the general locomotor activity, the approach/avoidance tendency, the aggressiveness in social situations (Berton *et al.* 1997; Ramos *et al.* 1997; Ramos & Mormède 1998). In this test panel the SHR is at the extreme of all three dimensions. Our work is directed towards the search for the molecular mechanisms involved in these behavioural traits by molecular genetic analysis.

Genetic studies by Hendley and collaborators (1983) showed that hypertension and hyperactivity are not genetically linked traits in the SHR, and these authors could derive from a WKY × SHR intercross two new strains with isolated hypertension, the Wistar Kyoto Hypertensive (WKHT) strain, or with isolated hyperactivity, the Wistar Kyoto Hyperactive (WKHA) strain (Hendley *et al.* 1986; Hendley & Ohlsson 1991). We have previously described in a WKY × WKHA intercross a quantitative trait locus (QTL) on chromosome 8 linked to locomotor activity in a novel environment (Moisan *et al.* 1996).

In order to investigate the second factor related to the inability to refrain motor activity (high approach tendency, low emotionality), we studied a Lewis × SHR intercross and found two QTL linked to the locomotor activity in the centre of the open field (OF) (Ramos *et al.* 1998, 1999). These studies revealed a complex inheritance of this trait because one locus, *Ofi1* on chromosome 4, is transgressive, the Lewis allele increasing the phenotypic value of the trait, while the other locus, *Ofi2* on chromosome 7, acts in the expected direction, the SHR allele increasing the trait. In order to investigate further the neuro-behavioural influence of these loci, two lines of rats were genetically selected from an F2 population (Lewis × SHR) on the basis of their genotype at these two loci. We obtained a 'high' line (homozygous for the alleles

increasing activity in the centre of the OF) and a 'low' line (homozygous for the alleles decreasing activity in the centre of the OF). These animals were tested in activity cages, OF and elevated plus maze (EPM) to evaluate their behavioural reactivity. Because they were shown to diverge by the degree of inhibition of their locomotor activity with increasing environmental aversiveness, we also measured the prepulse inhibition of the startle response, classically used to study sensorimotor gating mechanisms (Koch 1999). Altogether, these data demonstrate the existence of several neuro-behavioural dimensions characterizing the SHR rats that can be analyzed by molecular genetics to ultimately point out the neural pathways responsible for these genetic variations of neuro-behavioural traits. These data should open new avenues to the understanding of ADHD.

Animals and Methods

Animals

Male and female Lewis/Nico (LEW) and SHR/Nico (SHR) breeders were received from IFFA CREDO (L'Arbresle, France). One hundred and sixty-eight rats were produced from an F2 intercross and selected with polymorphic markers of *Ofi1* (D4Rat59 and D4Rat124) and *Ofi2* (D7Mgh11 and D7Rat35) loci (Ramos *et al.* 1999). Animals with the two extreme genotypes were used as founders of a 'high' line (Lewis/Lewis at *Ofi1*, SHR/SHR at *Ofi2*) and a 'low' line (SHR/SHR at *Ofi1*, LEW/LEW at *Ofi2*), respectively. Two successive generations (F3 and F4) were necessary to obtain enough experimental animals of the expected genotypes. Rats belonging to the F4 generation, 29 from the high line (14 males and 15 females from 4 litters) and 43 from the low line (21 males and 22 females from 6 litters) were tested successively in activity cages, low-light open field, elevated plus maze, and high-light open field. Behavioural testing started when the animals were 10 weeks old and successive tests were run 1 week apart. At the end of the experiments, the animals were bred to obtain an F5 generation, of which 31 rats (16 females and 15 males from 4 litters) from the 'high' line and 24 (12 females and 12 males from 3 litters) from the 'low' line were tested for the prepulse inhibition of the startle reflex, when they were 9 months old.

The litters were culled at 8 pups (4 males and 4 females) and the rats were weaned and separated by sex at 4 weeks of age and, thereafter, kept in collective plastic cages (4 rats/cage) with food and water available ad libitum, under a 12-h light/dark schedule with lights on at 7 AM.

Methods

Behavioural testing

Several tests were used to evaluate the two main dimensions of emotional reactivity as previously described (Courvoisier *et al.* 1996; Ramos *et al.* 1997). Activity cages are used to assess locomotor reactivity to novelty in a minimally challenging environment. The OF and EPM tests give indices of locomotor reactivity (activity in the periphery of the OF, entries in the closed arms of the EPM) and anxiety/behavioural inhi-

bition (activity in the centre of the OF, time spent on the open arms of the EPM). Because the two selected lines were found to diverge mostly by the level of inhibition of locomotor activity, animals were tested in the prepulse inhibition (PPI) procedure that is classically used to evaluate inhibitory processes.

Activity cages

Rats were placed individually for 1 h in wire-mesh cages with transparent plastic sides (23 × 23 × 36 cm, 1 × h × L) located inside a rack equipped with infra-red lights and photocell detectors connected to a computer which recorded horizontal activity in 10-min bins (Imetronic, Pessac, France). The light intensity in the centre of the cages was 6 lux.

The OF apparatus was made out of laminated wood with a white floor of 100 × 100 cm divided into 25 squares of 20 × 20 cm, and 40-cm high white walls. Animals were tested at two different light intensities, 7 and 70 lux, as measured at the centre of the OF. Each rat was placed in the centre of the OF and the number of squares crossed (outer squares adjacent to the walls and inner squares) were recorded for 5 min using a video-camera.

The EPM apparatus, made of opaque Perspex, with black floor and gray walls, was located 66 cm from the floor. The four arms, 45 cm long and 10 cm wide, were arranged in a cross-like disposition, with two opposite arms being enclosed by 50-cm high walls and two being open. At their intersection, a central square platform (10 × 10 cm) gave access to any of the four arms. Light intensity was 70 lux on the central platform. Each rat was placed on the central platform of the EPM facing an open arm and its behaviour was video-recorded for 5 min. The number of entries and the time spent (with all four paws) inside each arm were recorded.

The acoustic startle chamber (Imetronic) consisted of a transparent plexiglass tube (diameter 24 cm) enclosing a plate mounted on a force sensor connected to a computer which recorded the weight of the animal located on the plate. Acoustic pulses and prepulses were presented via a speaker mounted 31 cm above the plate. The chamber was located into an insulating enclosure. Animals were individually tested for the prepulse inhibition of an acoustic startle response. After a 5-min acclimatization period, the subjects were exposed to nine blocks of three trials presented pseudo-randomly: a pulse only trial, a prepulse + pulse trial, and a no-stimulus trial. The pulse characteristics were 93 dB intensity, 2000 Hz frequency, 30 ms duration. The prepulses (1000 Hz frequency, 10 ms duration) were presented in the following order on successive blocks: 73 dB, 77 dB, 80 dB, 83 dB, 83 dB, 80 dB, 77 dB, 73 dB, 70 dB. The time interval between the prepulse offset and the pulse onset was 100 ms. The time interval between successive trials was random between 14 and 16 s and between successive blocks was 20 s. Startle amplitude (in grams) was measured by a force sensor mounted below the plate where the animal stays, during 300 ms after startle pulse onset. Analysis was performed on the startle amplitude and on the percentage of inhibition induced by each prepulse, calculated as $[100 - (100 \times \text{startle amplitude at prepulse trial}) / (\text{startle amplitude at startle pulse alone})]$.

Genotyping

A drop of blood was collected from a nick of the tail in 400 μ l of sterile water and heated to 100 °C for 10 min. After centrifugation (5 min, 12 000 *g*), the supernatant was collected and kept at 4 °C until used for genotyping. Rat microsatellite markers were purchased from Research Genetics Inc. (Huntsville, AL). Genotype determinations were performed by polymerase chain reaction (PCR) in microtiter plate in a Hybaid Omnigene apparatus (Hybaid Ltd, Teddington, UK). In a 20- μ l reaction volume 5 μ l of the boiled blood sample supernatant was mixed with 5 pmol of each primer and 0.4 unit of Taq polymerase (Promega, Charbonnières, France) in Promega type A buffer. The PCR program was: (i) 96 °C 4 min; (ii) 35 cycles 92 °C 40 s, 55 °C 1 min, 72 °C 30 s, and (iii) 72 °C 2 min. Alleles were visualized on ethidium bromide-stained 3% agarose gels.

Statistics

Results are shown as means \pm standard errors. Data were analyzed by ANOVA with sex and lines as main factors. For activity data, the six successive 10-min time bins were introduced as a third factor and for the PPI experiment, the repeated measures for the nine trials were introduced as a third factor.

Results

Activity cages (Fig. 1)

ANOVA showed major effects of sex ($F_{1,68} = 9.86, P < 0.01$), the females being more active than the males, line ($F_{1,68} = 7.61, P < 0.01$), the low line being more active than the high line, and time ($F_{5,340} = 71.74, P < 0.0001$), with a significant line-time interaction ($F_{5,360} = 4.49, P < 0.001$), owing to the more rapid decline of locomotion in the low line over time. During the first 10 min of this novel environment test, the difference in activity between the two lines was 23.2% (LL > HL, $P < 0.05$) for the males and 47.6% for the females (LL > HL, $P < 0.0001$). During

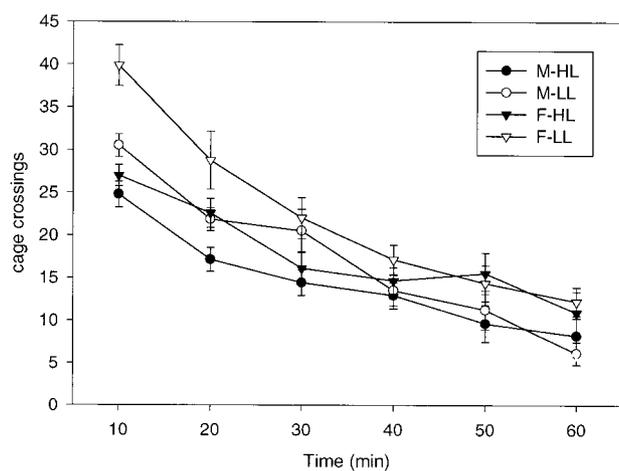


Figure 1: Number of cage crossings in activity cages located in a dim light room (6 lux). Results are given by 10-min bins over a 1-h period. M = males, F = females, HL = high line, LL = low line. See text for statistical significance.

the last 10 min of the test, these differences were no longer significant.

Open field activity

Open field (Fig. 2)

At low light intensity (7 lux), males were less active than females in the periphery of the OF ($F_{1,68} = 50.20, P < 0.0001$) and rats from the low line were less active than rats from the high line ($F_{1,68} = 5.11, P < 0.05$). However, the differences between the lines were rather small, -16.5% for males (HL > LL, $P < 0.05$), and -4.6% for females (HL > LL, NS). Females entered the centre of the OF more than males ($F_{1,68} = 7.99, P = 0.0002$) and rats from the high line more than rats from the low line ($F_{1,68} = 17.02, P = 0.0002$). The line difference was significant in both sexes (-36.8% in males and -27.4% in females, HL > LL and $P < 0.01$ in both sexes). At higher light intensity (70 lux), the pattern was similar but the difference between the lines was increased, the low line being less active in the periphery ($F_{1,68} = 24.12, P < 0.0001$) as well as in the centre ($F_{1,68} = 35.24, P < 0.0001$). The line difference was again slightly larger in males (periphery -34.5%, $P < 0.001$; centre -52.0%, $P < 0.01$) than in females (periphery -14.7%, $P < 0.05$; centre -46.8%, $P < 0.001$), and larger in the centre than in the periphery.

Elevated plus maze (Fig. 3)

The total number of arm entries was lower in males ($F_{1,68} = 25.60, P < 0.001$) and in the low line ($F_{1,68} = 6.65, P < 0.05$),

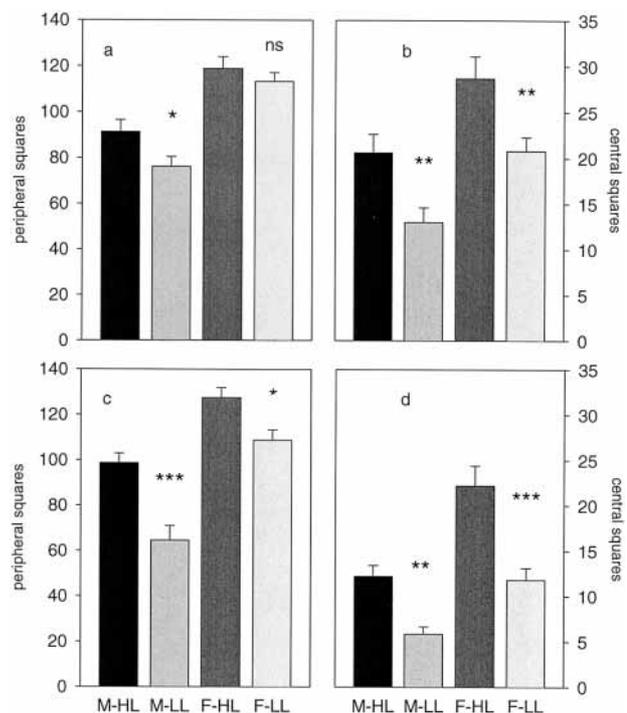


Figure 2: The number of peripheral (a and c) and central (b and d) squares entered, under two light intensities, 7 lux (a and b) and 70 lux (c and d). M = males, F = females, HL = high line, LL = low line. LL vs. HL: NS = non-significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

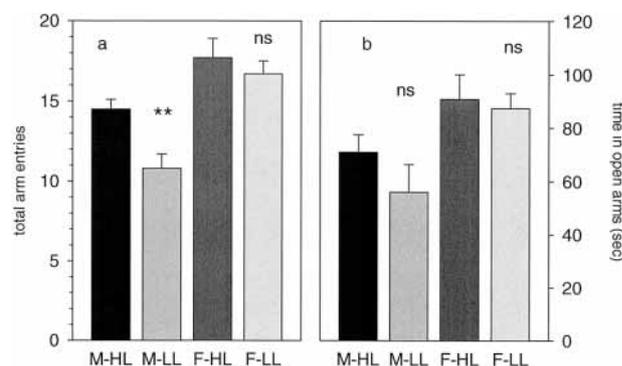


Figure 3: Elevated plus maze. Total number of arm entries (panel a) and time spent in open arms (panel b) during a 5-min test. M = males, F = females, HL = high line, LL = low line. LL vs. HL: NS = non-significant, ** $P < 0.01$.

compared to their respective counterparts. Again the line difference was larger in males (LL vs. HL, -25.5% , $P < 0.01$) than in females (-5.3% , NS). The number of closed arm entries followed the same pattern ($F_{1,68} = 10.22$, $P < 0.01$ and $F_{1,68} = 4.25$, $P < 0.05$ for the sex and line factor, respectively, data not shown). The number of open arm entries, the percent of open arm entries and the time spent in open arms were larger in females ($F_{1,68} = 22.72$, $P < 0.001$; 4.57 , $P < 0.05$ and 9.04 , $P < 0.01$, respectively) but did not differ between lines ($F_{1,68} = 3.48$, 0.27 and 1.17 , respectively).

Acoustic startle response (ASR) and PPI

The ANOVA did not reveal any significant effect of sex ($F_{1,54} = 0.003$) or line ($F_{1,54} = 2.651$, $P = 0.11$) on the mean startle amplitude across trials. If anything, the ASR was higher in the low line (80.76 ± 4.51 g, 279 measures) than in the high line (65.95 ± 4.66 g, 216 measures). For the prepulse inhibition, the ANOVA revealed a significant main effect of line ($F_{1,54} = 10.073$, $P < 0.01$) reflecting a higher PPI in the low line ($50.36 \pm 2.58\%$) than in the high line ($32.16 \pm 3.77\%$). The main effect of sex ($F_{1,51} = 0.385$) and the trial-line interaction ($F_{8,440} = 1.037$) were not significant.

Discussion

The SHR strain combines different neuro-behavioural traits such as locomotor hyperactivity/reactivity, low inhibition of motor behaviour in aversive places (centre of the open field, open arms of the elevated plus maze) or in various operant procedures, aggressive behaviour in social settings. Therefore, this rat strain is considered as an interesting model for the study of hyperactivity disorder with attention deficit (Sagvolden & Sargeant 1998; Bull *et al.* 2000). We have previously shown by multidimensional analysis of behavioural reactivity that these different traits may be inherited independently (see Ramos & Mormède 1998). In order to investigate the molecular mechanisms of the approach/avoidance factor, we searched for QTL in a SHR \times LEW intercross. These two strains do not differ in their general locomotor activity in novel

environments (locomotion in low-light activity cages, total locomotion in the OF, entries in the closed arms of the EPM). However, the SHR is more active than the LEW rat in more stressful environments, such as the centre of the OF, the open arms of the EPM and the white compartment of the black and white box (Ramos *et al.* 1998). Two QTLs were shown to be linked to the activity score in the centre of the open field, located on rat chromosomes 4 and 7, the LEW alleles on chromosome 4 (*Ofi1*) and the SHR alleles on chromosome 7 (*Ofi2*) increasing the trait (Ramos *et al.* 1999). The present experiments were designed to confirm the existence of these QTL and analyze further their influence on the emotional reactivity of the animals. Two lines were selected from an F2 population (Lewis \times SHR) on the basis of their genotype at these two loci to produce a 'high' line (homozygous for the alleles increasing activity in the centre of the OF) and a 'low' line (homozygous for the alleles decreasing activity in the centre of the OF).

Paradoxically, the basal locomotor activity/reactivity of the low line, when measured in a low stress environment (activity cages with dim light), is higher than in the high line. However, the decrease of locomotor activity with time is also faster in the low line, suggesting that habituation processes are stronger in these animals. On the other hand, OF studies show that the low line is less active in the centre of the OF, confirming that these chromosomal loci influence this trait, as found in the initial QTL studies (Ramos *et al.* 1999). Open field data also show that the inhibition of locomotor activity in the low line (as compared to the high line) is related to the aversiveness of the situation (larger in the centre than in the periphery, and in high light than in low light) and is more intense in males than in females. This inhibition is not attributable to a classical 'anxiety' factor as measured in the elevated plus maze, in which the open arms behaviours were not different between lines. Only activity-related measures (total number of arm entries or closed arms entries) were lower in the low line. These data show that the different behavioural traits measured in these tests must be interpreted with caution in terms of psychobiological significance. Indeed, different aggregation profiles of these traits were already observed in different genetic populations. In a comparison of six inbred rat strains, activity in the centre of the OF and in the open arms of the EPM were shown to be related to the same factor through principal component analysis (Ramos *et al.* 1997). Conversely, these two traits are not correlated in the SHR or WKY strains (Durand *et al.* 1999) and segregate independently in an F2 population between the WKY and WKHA strains (Courvoisier *et al.* 1996). Altogether, these data suggest that anxious behaviour and behavioural inhibition in aversive places are not superimposable, and the two selected lines described here allow the neuro-behavioural study of this 'inhibition' trait.

Prepulse inhibition of startle is a classical approach to the neuro-behavioural study of this trait (Koch 1999). Large differences were found among rat strains in the amplitude of the startle response (Glowa & Hansen 1994), the intensity of prepulse inhibition (Palmer *et al.* 2000) and the influence of drugs upon it (Rigdon 1990; Farid *et al.* 2000; Swerdlow

et al. 2000). In Wistar rats, females show a lower amplitude of the startle response and less prepulse inhibition (Lehmann *et al.* 1999). In the present study, the two lines did not differ in their basal startle response to an acoustic stimulus, but the degree of inhibition of the response by a prepulse stimulus was larger in the low line. This result confirms that behavioural inhibition processes are more efficient in the high line than in the low line. The two lines will allow a more detailed study of these inhibitory processes and their neuro-behavioural bases. These phenotypic approaches are complementary to the molecular genetic studies aiming at the understanding of the respective influence of the two loci on the traits under investigation, and at the finding of the genes located in these loci and the mutation of which is responsible for the behavioural differences.

The present results clearly show that in the SHR model of ADHD, behavioural hyperactivity and inhibition processes are independent traits that can be analyzed separately via the genetic approach. Indeed, although the WKHA strain has retained the hyperactivity displayed by the SHR strain, it does not show the lack of behavioural inhibition in the centre of the OF that is characteristic of the SHR strain (Castanon *et al.* 1993), and it has no deficit in PPI as compared to the outbred Wistar rat (Deschepper *et al.* 1998). Therefore, the SHR rat has a unique combination of locomotor hyperactivity/reactivity and lack of inhibition in aversive environmental conditions. We have previously described in a WKY × WKHA intercross a chromosomal locus related to the hyperactivity trait (Moisan *et al.* 1996), and the present data show that the loci previously described in a SHR × Lewis intercross as related to the activity in the centre of the OF (Ramos *et al.* 1999) are indeed involved in a behavioural inhibition trait. These marker-based selected lines are a unique tool for the study of the neurobiological bases of this trait and the molecular foundations of its variability of genetic origin.

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