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# Attack behaviors in mice: From factorial structure to quantitative trait loci mapping

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**Abstract**

The emergence or non-emergence of attack behavior results from interaction between the genotype and the conditions under which the mice are tested. Inbred mice of the same strain reared or housed under conditions do not react the same way; reactions also vary according to the place selected for testing and the different opponents. A factor analysis showed that the attack behavior in non-isolated males, tested in neutral area covaried with high testosterone and steroid sulfatase and low brain 5-hydroxytriptamine (5-HT), β-endorphin and Adrenocorticotropic Hormone (ACTH) concentration, whereas, for isolated males tested in their own housing cage, it covaried with high testosterone activity and low brain 5-HT concentration. A wide genome scan was performed with two independent populations derived from C57BL/6J and NZB/BlNJ, each being reared, housed and tested under highly contrasting conditions, as described above, and confronted with A/J standard males. Common Quantitative Trait Loci emerged for two rearing/testing conditions. For rattling latency we detected Quantitative Trait Loci on *Mus musculus* chromosome 8 (MMU8) (at 44, LOD score=3.51 and 47 cM, LOD score=6.22, for the first and the second conditions) and on MMU12 (at 39 cM, LOD score=3.69 and at 41 cM, LOD score=2.99, respectively). For the number of attacks, Quantitative Trait Loci were common: on MMU11 at 39 cM LOD score=4.51 and 45 cM, LOD score=3.05, respectively, and on MMU12 (17 cM, LOD score=2.71 and 24 cM, LOD score=3.10). The steroid sulfatase gene (*Sts*), located on the X–Y pairing region, was linked, but only in non-isolated males, tested in neutral area for rattling latency, first attack latency, and number of attacks (LOD scores=4.9, 4.79 and 3.57, respectively). We found also that the Quantitative Trait Locus encompassing *Sts* region interacted with other Quantitative Trait Loci. These results indicate that attack behavior measured in different rearing and testing conditions have different biological and genetic correlates. This suggests that further explorations should be done with standardized tests and, in addition, with a wide range of tests, so as to gain an understanding of the true impact of genes or pharmacological treatments on specific categories of aggressive behavior.


*Keywords:* Aggression; Quantitative trait locus; Steroid sulfatase; Pseudo-autosomal region; Y-chromosome

**Contents**



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## 1. Introduction

Aggression is an event and not a behavior and, for this reason, is suited to genetic analysis. An aggressive act may result from aptitudes for reacting by violence to frustrating, dangerous or unpleasant circumstances. Not all organisms that possess these aptitudes to a high degree will display in the course of their life, but a violent act may occur if actual or virtual threatening events occur. Individuals with potential for violence may not perform aggressive act during their life if, by chance, their environment is free of violence inducers (Roubertoux et al., 1999). The art of the behavior specialists is to detect these hidden potentialities, in humans, and demonstrate them in laboratory animals, providing estimates of scales and degree.

The development of new protocols to measure ability to develop aggressive behavior gained importance with the development of experimental genetics in mice. Transgenic and gene targeted mice are routinely examined for attack behavior (Maxson, 1992, 1996, 1998). Most of the studies use "home made protocols" which show how ingenious the researchers are, but despite claims that they measure "aggressiveness", they do not measure necessarily the same traits (Guillot et al., 1995; Roubertoux et al., 1999). The procedures that are used for initiating attack behavior do not refer to the same triggers.

Half a century of research on aggression has seen an accumulation of procedures for inducing or measuring attack behavior in mice, since the seminal papers by Ginsburg and Allee (1942) and Scott (1942). Most refer to the dyadic encounter protocol where the male to be observed (tested male) is confronted with a conspecific male, considered as a reagent or opponent. An analysis of published literature on dyadic encounters suggested that the protocols could be described according to three dimensions (Roubertoux et al., 1999). We presented an updated version of three-dimensional description of attack behavior induction in Fig. 1.

The *X*-axis dimension corresponds to the tested male. Reactivity to conditions depends on the male's physiological status. The effect of weight (Scott, 1992), state of development (Selmanoff et al., 1976; Maxson et al., 1979; Suzuki et al., 1983; Mann et al., 1983; Shrenker and Maxson, 1983), hormonal concentration (vom Saal, 1983; van Oortmerssen, 1984; Carlier et al., 1990; Michard-Vanhee and Roubertoux, 1990; Sluyter et al., 1996a) or neurotransmitters functioning (Miczek et al.,

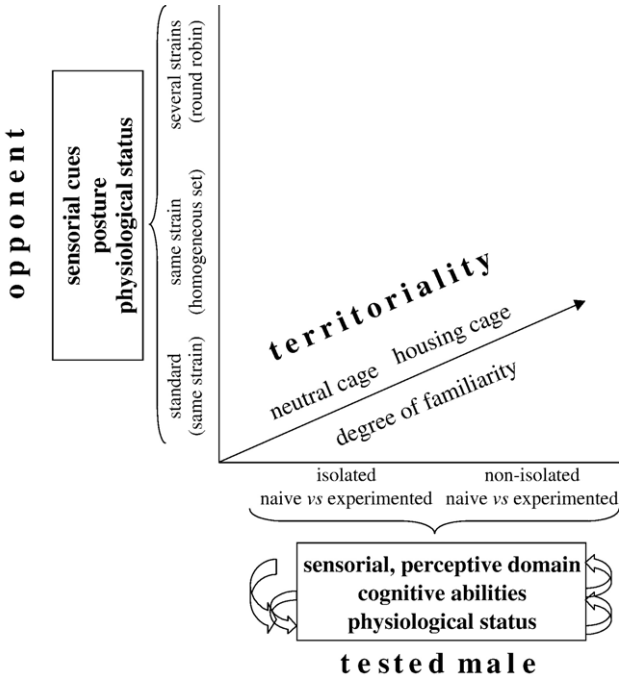


Fig. 1. Dimensions affecting attack behavior recorded during dyadic encounter.

2001) are well documented among other indicators of the physiological status of the tested male. Cognitive skills may be decisive in attack behavior (Blanchard and Blanchard, 1988). Cognition may be involved in risk assessment when confronted with a new opponent or it may benefit from previous experience. Male mice `ttermates having experience of deterrent signals (Michard and Carlier, 1983; Roubertoux et al., 1999). Sensorial and perceptive abilities have a crucial impact on attack behavior as the triggering of the attack depends on visual, auditory or olfactory signals from the opponent (Ropatz, 1967; Ginsburg and Allee, 1942; Cohen-Salmon, 1988; Yamazaki et al., 1982; Novikov, 1993). Physiological state, sensorial and cognitive abilities cannot be considered as independent categories as they interact. A newly acquired element of information induces structural modification in the synapse, while the hormonal status changes the perception of sensorial cues. The strains of mice used in experimental genetics differ by their genotypes. Genotypic differences contribute not only to individual variation but also to differential reactivity to similar events. Thus, males from two different strains can react in opposite ways to an identical situation (Scott, 1960, 1992; Roubertoux et al., 1999). The popular protocol for inducing attack behavior consists of isolation but isolated C57BL/6 mice, unlike CBA mice, do not display more frequent attack behavior than their non-isolated counterparts. Both C57BL/6J and BALB/cJ mice which won a first encounter exhibited more signs of aggressiveness during the second encounter but aggressiveness decreased in C57BL/10Bg (see Fig. 2) providing additional proof that the individual (or its genotype) interacts with the situation. Subtle events also modify, moreover, the social status of the male. Position of the fetuses in utero (between two males or between two females) modifies testosterone concentration and the propensity to attack (vom Saal, 1983; Sluyter et al., 1996b).

The *Y*-axis dimension corresponds to the effect of the opponent. Males from the same strain display different patterns and record different scores of aggression with opponents belonging to different strains (François et al., 1990). Several factors can explain this effect. The opponent produces visual cues (movements, postures), auditory cues (ultrasounds and clicks) (Cohen-Salmon, 1988) or olfactory cues (Del Punta et al., 2002). Cues differ between strains in number, changing in number, intensity and qualities (Roubertoux et al., 1999). The tested male can perceive or ignore these cues. Several lines of evidence indicated that a sensorial channel could be of crucial importance as shown by Monahan and Maxson (1998) reporting that the number of attacks by one strain depended on the origin of urine with which the opponent was coated. The difficulty arose when the comparison of two strains was made on the basis of dyadic encounter with an opponent with a different genotype. The dyadic situation implies the cues produced by the emitter, the signal perceived by the receptor and the analysis of the signal by the receptor. Let us consider a mouse that does not attack. Is it placid or sensorialy impaired? Is it unable to analyze the signal?

The oblique axis represents the ability to perceive the place where the encounter occurs as the animal's own territory. We know that dominance across *Mus musculus domesticus* and wild mice (*M. spretus* or *M. spicilegus*) or between mice with different t-haplotypes differs according to the size of the area

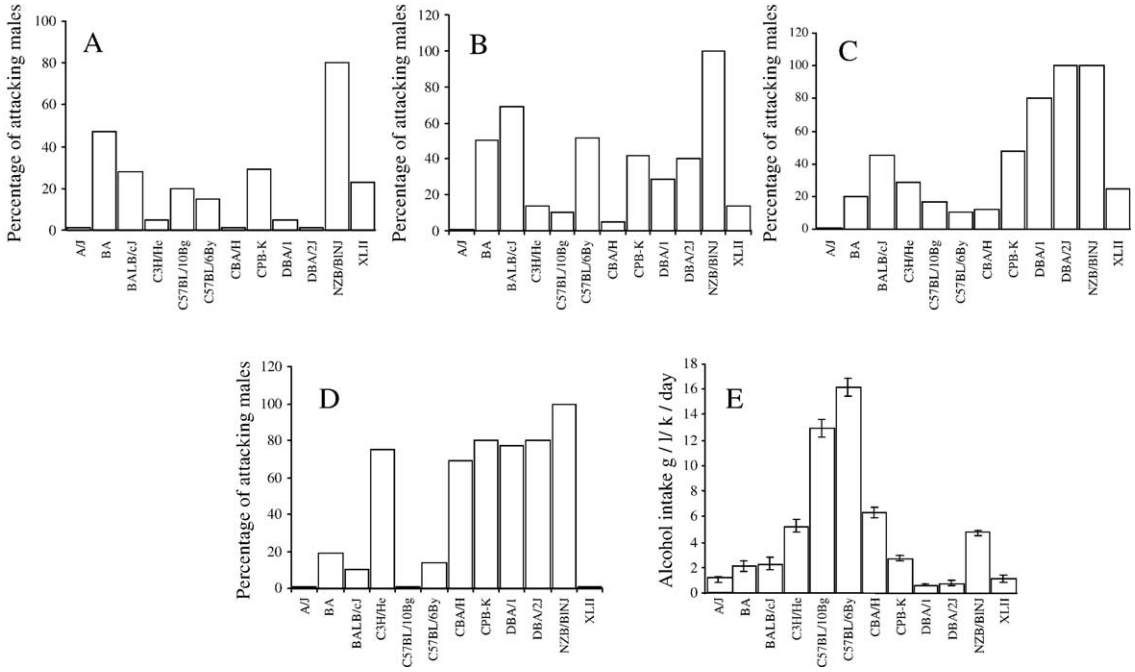


Fig. 2. Percentage of attacking males, in twelve inbred strains of mice, under four conditions: A) non-isolated, tested in neutral area with a standard opponent (A/J), tested once; B) non-isolated, tested in neutral area with a standard opponent (A/J), tested 24 h after previous dyadic encounter; C) isolated for 13 days, tested in the resident cage with a standard opponent (A/J); D) isolated for 13 days, tested in the resident cage with an opponent from the same strain. E) Alcohol intake expressed as alcohol intake g/l/k/day (Mean±S.E.M.) (partially adapted from Roubertoux et al., 1999).

that is used for testing (Thaler, 1981; Lenington et al., 1996). The size of the area used for the encounter, the number of markings and the familiarity of the odors in different situations produce different conditions and different results are expected. Two areas are commonly used: neutral area (clean new cage) or housing cage ("resident intruder" procedure).

Most authors select only one condition assuming implicitly that the other conditions would provide similar results. We selected representative conditions to test this hypothesis and reached a negative conclusion, demonstrating that attack scores obtained under different conditions did not correlate (Roubertoux et al., 1999).

"Do the dimensions express apparent associations or do they imply biological causation?" In the second section, we addressed the question via a meta-analysis designed to approximate genetic correlations.

"Do the different dimensions of aggressive behavior, measured under different conditions, have different genetic bases? " A wide genome scan approach was used to find a direct answer to the question. The third section reported comparative genome scans for two populations for which we recorded attack behavior under highly contrasting conditions.

## 2. Inter-strains biological correlates of attack behavior

Several years ago (Roubertoux et al., 1999) we selected several of the conditions corresponding to the three dimension graph shown in Fig. 1. The selection fulfilled two criteria: first, the conditions had to include the most diverse characteristics on the three axes, secondly, they had to cover the conditions commonly used in studies performed with gene-targeted mice. Testing was performed under four conditions: i) males were maintained until the test with a female and tested once in a neutral area with a standard opponent from A/J strain. ii) The same condition but the male had experienced a dyadic encounter the day before with an A/J male. iii) The male was isolated thirteen days before the dyadic encounter. An A/J male was then placed in the cage where the tested male was maintained (resident intruder procedure). iv) The same procedure was employed but the opponent and the tested male were of the same strain (homogeneous set test). Mice came from eleven strains, each mouse was used only once. A two factor structure emerged from a factor analysis (Roubertoux et al., 1999). The first factor was defined by the status of the tested male (non-isolated vs isolated) and the second by the origin of the opponent (same or different strain). What are the biological bases of these dimensions? We addressed the question using approximates for genetic correlation (Hegmann and Possidente, 1981). A set of inbred strains can be considered as a pool of recombinant inbred strains. Additive effect, epistasis between homozygous loci, plus maternal pre- and post-natal effects were components of the correlation observed. The phenotypic correlation is thus the upper limit of the genetic correlation.

### 2.1. Behavioral and biological measures

Estimates were made of the upper limit of the genetic correlations between attack-behavior-related variables and several biological measures. Most of the values had been previously published; only the meta-analysis was original.

#### 2.1.1. Strains

Previous experience of dyadic encounter produces, generally, dramatic modifications in a new social behavior in a new encounter. For this reason, we selected an inbred strain strategy. The mice used for the behavioral tests were naïve. As the genotype survives the individual, in inbred strains, we considered the average value obtained by a strain as the unit of observation. We reanalyzed data from the following inbred strains: A/J, BA, BALB/cBy, C57BL/10Bg, C57BL/6, CBA/H, CPB-K, DBA/1Bg, DBA/1J, DBA/2j, NZB/BINj and XLII. The strains were selected on the basis of their intrinsic interest and the availability of all the behavioral and biological measurements. This is the sole reason for the slight difference in the strain panel compared to our previous study. Between 15 and 20 mice per one strain were observed for the behavioral measurements.

#### 2.1.2. Behavior

We used results previously published (Roubertoux et al., 1999) but excluded Cast/Ei which are difficult to bred and for which we could not obtain all the biological measurements. Two other strains were added: DBA/1J and A/J. The percentage of males initiating attacks under different conditions is reported in Fig. 2. A measurement of alcohol preference was introduced to test for a possible correlation with the initiation of attack behavior. We used the two-bottle preference test. The mice had free access to two glass pipettes (tap water and 10% alcohol

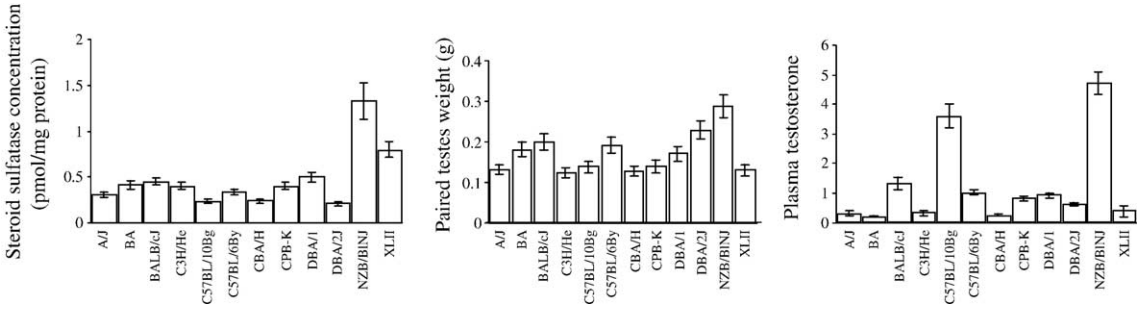


Fig. 3. Steroid sulfatase; paired tested weight and plasma testosterone concentration in twelve inbred strains of mice (Mean±S.E.M.).

solution). The quantity of liquid was recorded to the nearest 1 / 10 ml on each of two consecutive days. The results were expressed as g of alcohol per 1 kg of body mass, per 1 l of fluid intake.

### 2.1.3. Biochemical measurements

Testosterone contributes for attack behavior (Carlier et al., 1990). Testosterone action was estimated from paired testes weight (Le Roy et al., 2001) and plasma testosterone concentration was assayed directly by radioimmunoassay using a specific testosterone antibody ([$^{125}$I]-TESTOSTERONE COATRIA, kit Biomérieux). Bound radioactivity was counted for 1 min; the specific testosterone antibody produced only a small cross-reaction (7.5%) with other androgen steroids. Concentration was expressed as pmol/mg protein.

We reported a correlation between Steroid sulfatase concentration and attack behavior (non-isolated male tested in a neutral area) in a set of inbred strains (Le Roy et al., 1999). Could this positive correlation also be found with attack behavior under different conditions? We addressed the question measuring liver steroid sulfatase by Enzyme Linked Immunoabsorbent Assay (ELISA) after production of anti-steroid sulfatase antibodies (Mortaud et al., 1995). Protein measurement followed Schaffner and Weissmann (1973). Steroid sulfatase concentration was expressed as pmol/mg protein.

5-HT contributes to attack behavior (Saudou et al., 1994; Ramboz et al., 1998; Hen, 1996) but the general scope and size of the effect are still disputed (Balaban et al., 1996). We analyzed the correlation between plasma and brain 5-HT with attack behavior under the four conditions described above. The 5-HT concentration was measured using a radioenzymatic method (Walker et al., 1983) based on the conversion of 5-HT to tritiated melatonin measured radioenzymatically, after an ethanol–acetone extraction (Tordjman et al., 1995). 5-HT was measured in plasma and in the whole brain (expressed as pmol/mg protein).

The relationship between aggression and brain opioids has been studied in both animals and humans and it has been suggested that central opioids may inhibit attack behavior, although previous studies have produced contradictory results (Avis and Peeke, 1975; Kinsley and Bridges, 1986; Haney and Miczek, 1989; Spiga et al., 1990; Kjaer et al., 1992; Espert et al., 1993; König et al., 1996; Fischer et al., 2000; Dyakonova et al., 2000). More recently, Tordjman et al. (2003) demonstrated that the link varied according to the opioid and to the component of attack behavior measured. Here, we investigated the link between the same opioids and percentage of males attacking under different conditions. Concentration of Met$^5$-Enkephalin and Dynorphin A (1–8) was obtained by radioimmunological quantification (Pohl et al., 1990) in the brain (Tordjman et al., 2003). β-endorphin levels were determined using a radio-immunoassay procedure previously described (Colas-Linhart et al., 1982, 1986). Brain ACTH (ACTH 1–39) was measured using a solid phase two-site immunoradiometric assay. The obtained neurochemical values were correlated with several measures of behavior recorded during a dyadic encounter that corresponded to condition I.

## 2.2. Results

Figs. 2–5 indicate the percentages or the mean values for the twelve strains. We analyzed the correlations according to a factor analysis (principal component procedure) and then performed a varimax rotation. The results are shown in Table 1. The three factors contributed to 71% of the total variance.

The first factor showed a positive covariation between frequency of attacking males under the two first conditions (non-isolated, having experienced or not a dyadic situation, tested in neutral area with a standard opponent), and with testosterone, steroid sulfatase levels, and negative covariation with brain 5-HT, β-endorphin and ACTH. The second factor showed positive covariation between frequency of attacking males (isolated condition) and the effect of testosterone (testes weight) and negative covariation with brain 5-HT concentration. The percentage of attacking males with dyadic encounter experience had a moderated loading on the factor. For the third factor, we observed a positive covariation between the first condition (non-isolated, with no experience of a dyadic situation, tested in neutral area with a standard opponent) plasma testosterone concentration, β-endorphin and ACTH and negative covariation with 5-HT plasma concentration and Met$^5$-Enkephalin. Alcohol intake did not appear here to covary with attack behavior.

## 2.3. Discussion

We worked with a reduced sample of biological variables; we did not measure dopamine, substance P, adenosine, histamine, for instance, as contributing to attack behavior. It should have been desirable to have a more refined selection of the brain structures rather than the whole brain. However, using a reduced

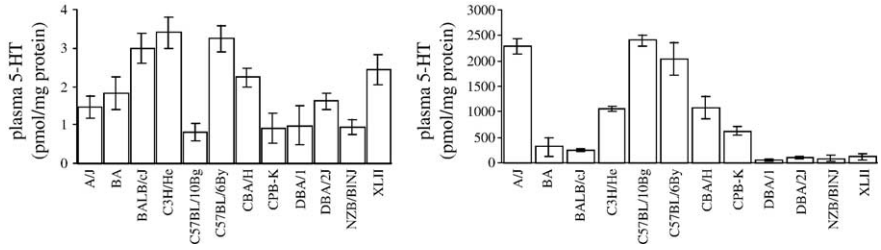


Fig. 4. Plasma and brain 5-HT in twelve inbred strains of mice (Mean±S.E.M.).

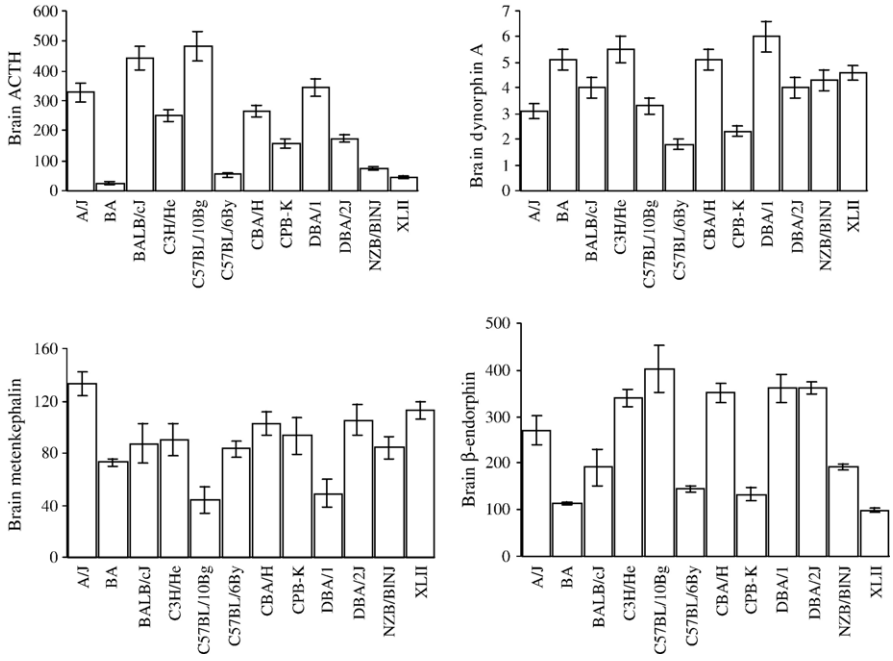


Fig. 5. Concentration of brain ACTH, Dynorphin A, Met[5]-Enkephalin and β-endorphin, in twelve inbred strains of mice (Mean±S.E.M.).

and arbitrary set of biological variables, we confirmed that attack behavior has different biological correlates depending on the conditions where it is induced or observed and, therefore, it should have different genetic correlates.

A basic analysis of the conditions shows how crucial isolation is. The first factor, defined by the percentage of attacking males under the first condition (non-isolated male) and the second condition (isolated for one day only) could be defined as spontaneous aggression. The second factor was determined by percentage of attacking males under conditions three and four, both in isolation. Isolation is particularly stressful for gregarious mammals and the conditions that were highly loaded on the second factor may reflect reactivity to stressful situations. Surprisingly, the use of different opponents (A/J vs a male from the same strain) did not oppose attack behavior and similar loadings were found under the two conditions. The third factor was defined by attack behavior observed under the first condition. Spontaneous attack behavior (factors I and III) covaried with high concentration of plasma testosterone and the effect of testosterone (paired testes weight). This might suggest a strain polymorphism of the gonadal receptor hormones (androgen and estrogen). The high loading of steroid sulfatase concentration on factor I confirmed previous results from our group (Le Roy et al., 1999) and was observed through all changes in sampling the strains. The present analysis did not find any steroid sulfatase involvement in the other forms of attack behavior. Low 5-HT concentrations also covaried with a high percentage of attacking males.

Table 1
Factor analysis of attack behavior, alcohol consumption and biological variables

| Variables | Factor I | Factor II | Factor III |
|---|---|---|---|
| % of attacking males non-isolated; standard opponent A/J; neutral area; tested once | .90 | | .34 |
| % of attacking males isolated (1 day); standard opponent A/J; neutral area; tested twice | .75 | .52 | |
| % of attacking males isolated (13 days); standard opponent A/J; resident intruder; tested once | | .86 | |
| % of attacking males isolated (13 days); homogeneous set test; resident intruder; tested once | | .83 | |
| Alcohol intake | | | |
| Paired testes weight | .56 | .69 | |
| Plasma testosterone concentration | .40 | | .76 |
| Steroid sulfatase concentration | .78 | | |
| 5-HT (plasma concentration) | | | −.62 |
| 5-HT (brain) | −.39 | −.46 | |
| Met[5]-enkephalin | | | −.76 |
| Dynorphin A | | | |
| β-endorphin | −.82 | | .42 |
| ACTH | −.64 | | .51 |

Saturations after varimax rotation (Kayser's normalization). Loadings > .30 are reported.

Given these results, a linkage should be expected with genes involved in the serotoninergic system. A high incidence of attack behavior covaried with low levels of ACTH and β-endorphin and not Met[5]-Enkephalin and Dynorphin. This finding tallied with the conclusions proposed by Tordjman et al. (2003) when studying sequences of attack behavior recorded under condition I; they found that a low level of brain ACTH and β-endorphin and to a lesser extent Met[5]-Enkephalin covaried with low latency and high frequency indicators of aggressiveness. This would suggest a linkage with genes involved with the opioid system.

The second factor, corresponding to attack behavior induced by isolation, indicated covariation with the action of testosterone

(paired testes weight). This was not surprising because several studies demonstrated that isolation induced an increased level of testosterone in different organs (Batty, 1978a,b). The low concentration of brain 5-HT seemed thus to be common to increase attack behavior frequency under every condition. The loadings that we found for testosterone concentration and 5-HT concentration may suggest common genetic linkages corresponding to mice examined under conditions corresponding to the first and second factor and involved in the gonadal hormone and to the serotoninergic systems.

The third factor specified frequency of attack behavior tested in condition I. It confirmed the results that we analyzed above for plasma testosterone and indicated an association of attack behavior with a low level of brain $Met^5$-Enkephalin associated with rising levels of ACTH and β-endorphin, confirming Tordjman et al. (2003).

## 3. Are different dimensions of aggressive behavior linked to different chromosomal regions or to different genes?

The incidence of attack behavior has the highest loadings for the first two factors obtained by factor analysis. The same factor analysis shows several biological measurements with high loadings on the first two factors, although some of the other measurements had significant loadings for one factor but not on the other. We previously demonstrated that across strain correlation approximated genetic correlation and that this correlation corresponded to the upper limit of genetic correlation. The loadings given in Table 1 might be therefore considered as "genetic loadings", or at least for a major part of their components. This observation led us to conduct more direct investigations of the chromosomal regions or genes linked to attack behavior observed under different conditions. A linkage study could lead to three different results: first, attack behaviors as measured under different conditions should be linked to the same chromosomal regions or genes suggesting that the apparently divergent phenotypes derive from identical genetic sources; secondly, different linkage regions should be expected, showing that attack behaviors do not have the same genetic origins when obtained from animals reared and tested under contrasting conditions; thirdly, an intermediate solution should emerge with common linkage to all the conditions, plus linkages specific to each condition, indicating common general genetic mechanisms and condition-dependent genetic modulators.

Here we selected behaviors occurring during the attack phase and induced under two contrasting conditions, in two independent populations. First we considered non-isolated males, opposed to conspecific A/J males; the dyadic encounter procedure took place in a neutral area. Under the second condition, we subjected the tested males to a stringent isolation period (13 days) and then confronted them with a conspecific A/J male, the dyadic encounter procedure taking place in the cage where the tested male was housed. The two conditions differed not only in their characteristics, but also in the corresponding loadings which appeared on independent factors (I and II), although they did share certain specific biological correlates. The "intermediate situation" mentioned above was to be expected given the initial observations on the two contrasted conditions.

### 3.1. Phenotyping

#### 3.1.1. Rearing conditions

The pregnant females were removed from the mating cages. The litters with fewer than four pups were discarded and those remaining were culled to six pups. At weaning, each male was housed with one female (generally a littermate) in an opaque cage until testing (see Carlier and Roubertoux, 1986; Carlier et al., 1991).

#### 3.1.2. Behavioral testing

The following conditions were kept constant: photoperiod 12 : 12 with lights on at 8:30 am; food (IM UAR) and tap water ad libitum; dust-free sawdust bedding; and weaning 30 ± 1 days after birth.

We defined a first situation referred to as "non-isolated condition" where the tested male was house with a female until testing (66 ± 4 days after birth). The conditions of observation and measurement were described by Roubertoux and Carlier (1988), Roubertoux et al. (1994, 1988). Briefly, the test was performed in a transparent cage with a transparent lid (Carlier and Roubertoux, 1986). Each test was a dyadic encounter with an A/J male as the opponent, this strain being chosen for its low scores of aggression. The A/J opponent came from group male cages. Behavioral recordings began when the tested male sniffed the A/J opponent for the first time; when no attack eventuated the test was terminated after 6 min.

The second situation referred to as "isolated intruder" was more complex. Rearing and recording conditions were identical as "non-isolated condition" but at 54 ± 2 days the males were removed and housed in a clean cage, and thirteen days later a standard opponent (A/J male) was introduced into the housing cage.

We recorded rattling episodes and attacks under the two conditions. Rattling behavior could occur at variable latencies, after the first contact. The tested male moved his tail in a rattling manner: we measured the latency of the first rattling and we counted the number of rattling episodes. The attack was defined as biting or an attempt to bite. We recorded the latency of the first bite and the number of bites. The difficulty in considering several measures during one dyadic encounter is that number and latencies of rattling and bite are not independent by construct. Males attacking registered a small number of attacks when the duration of the test was limited. To overcome this difficulty we developed a simple procedure (Roubertoux and Carlier, 1988). Behavioral recording started when the tested male sniffed the opponent for the first time, and the test lasted 2 min after the first attack or 6 min after initial sniffing when no attack occurred. When no attack occurred a latency corresponding to the maximal value was recorded for the individual. Under these conditions the frequency of different behaviors was measured for 2 min hence the risk of an artifactual correlation between the latency of the behavior and

its frequency was reduced. This limited duration is scientifically acceptable and takes into consideration animal welfare since with some strains, such as NZB/BlNJ, more than 2 min of fighting may result in mortal wounds.

### 3.1.3. The populations

We selected the C57BL/6 and the NZB/BlNJ strains that exhibited clearly contrasting behavior in both situations. In 1992 and 1993, we derived their reciprocal $F_1$s and $F_2$s. They were tested under the "isolated condition" protocol, in Paris. We reported sample size, percentage of attacking males, latency and frequency of rattling and attack in Table 2. In 1998, in Orléans, we derived an independent population from the same parental strains, following the same mating regimen and tested under the "non-isolated condition".

## 3.2. Quantitative trait loci mapping

### 3.2.1. Genotyping

Genotyping was performed at the CNRS (Paris, Orléans and Marseille) according to the same protocols. We selected 65 Short Sequences of Length Polymorphism on the 20 chromosomes, with an average interval length between two SSLPs of 22.5 cM. We selected also the Nsts primers for the X–Y pairing region (Mouse Genome Database (MGD), 2000) since we previously suspected a link between this region and attack behavior (Roubertoux et al., 1994). Short Sequences of Length Polymorphism differed generally by around 20 bp. DNA fragments were obtained from tails and individually amplified according to usual protocols. PCR products were visualized using 4% agarose gels and ethidium bromide. Three possible genotypes at each Short Sequences of Length Polymorphism locus were expected: B//B, N//N and B//N. We recorded the bands with a computer system. Discordant observations resulted in a second amplification.

Table 2
Measures of aggressive behavior during dyadic encounter under two conditions in NZB/BlNJ (N) and C57BL/6J (B6)

| | Condition 1<br>Non-isolated<br>Standard opponent (A/J)<br>Neutral area<br>Tested once | Condition 2<br>Isolated 13 days<br>Standard opponent (A/J)<br>Resident intruder<br>Tested once |
|---|---|---|
| % attacking males N | 68 [a] | 100 |
| % attacking males B6 | 9 [a] | 23 |
| First rattling latency N | 84.67±7.52 [a] | 15.12±6.97 |
| First rattling latency B6 | 118.54±9.45 | 116.51±10.25 |
| Number of rattling N | 19.83±5.61 [a] | 27.61±7.59 |
| Number of rattling B6 | 0.60±0.34 [a] | 8.12±1.04 |
| First attack latency N | 56.10±4.65 [a] | 18.20±5.58 |
| First attack latency B6 | 116.19±7.34 | 119.5±8.66 |
| Number of attack N | 14.41±1.51 [a] | 21.86±2.12 |
| Number of attack B6 | 0.35±0.21 | 0.17±0.11 |

Means±S.E.M. (untransformed values).
[a] Indicates a $P<.05$ difference between the conditions All the comparisons between N and B6 differed ($P<.05$).

### 3.2.2. Statistics and QTL localisation

Group differences were tested using a two-way ANOVA. Individual measures were transformed in logit to ensure homoscedasticity in the non-segregating generations (untransformed values were reported in Table 2). The origin of fathers or mothers (N vs B in the $F_1$ or $NBF_1$ vs $BNF_1$ in the $F_2$) was also examined. Heritabilities in the broad sense were computed as usual (Mather and Jinks, 1971) and were calculated after individual logit transformations.

To test, in $F_2$s, for allelic substitution effects, latency and the number of rattling episodes and attacks, we compared the phenotypic values of the three genotypes: N//N, N//B and B//B, using a non-parametric Kruskal–Wallis test (Lander and Kruglyak, 1995) with $P<.01$ as a level of significance. Hence, the Quantitative Trait Loci were investigated with simple interval mapping method (van Ooijen and Maliepaard, 1996) on the chromosomes where a significant difference had been previously detected. We considered a 4.3 threshold lod score value corresponding to "highly significant linkage" (Lander and Kruglyak, 1995) but we did not eliminate "significant" or "suggestive" linkages. The confidence interval has been computed as

$$\mathrm{CI} = 530/(N_{F_2} \times v)$$

where 530 is a constant obtained from the simulations, $N_{F2}$ is the number of $F_2$ mice and $v$ is the proportion of variance explained by the Quantitative Trait Loci (Darvasi and Soller, 1997). We used a simple interval mapping method (MapQTL program version 3.0).

## 3.3. Results: wide genome scan for attack behavior recorded under two conditions

Tables 2 and 3 give the untransformed mean values for the parental strains, the reciprocal $F_1$s and $F_2$s. NZB/BlNJ mice attacked more than the C57BL/6J under the two conditions and both strains attacked more under the second condition.

The results obtained here were similar to those reported for the strain comparison (Fig. 2). Measures obtained for latency or number of rattling episodes and attacks followed the same trend. The examination of the reciprocal $F_1$s showed that $B6.NF_1$s attacked more than $N.B6F_1$s, under the first condition whereas this was the case for first rattling or attack latency under the second condition. The increased attack behavior of $B6.NF_1$s males compared to $N.B6F_1$s tallied with possible mitochondrial contribution or with a MMUY effect, but the contribution of mitochondrial DNA had already been rejected for aggressive behavior (Roubertoux et al., 2003), leaving support for the second hypothesis.

The next question was therefore "What part of the MMUY is implicated in increased attack behavior?" We had previously demonstrated that there was an association with attack behavior and X–Y pairing region (Roubertoux et al., 1994) and with steroid sulfatase(Le Roy et al., 1999). MMUY is composed of two parts, one transmitted from father to son and said to be "specific", the other being common to MMUX and MMUY

Table 3
Measures of aggressive behavior during dyadic encounter under two conditions in reciprocal crosses B6.NF$_1$ and N.B6F$_1$ (upper part), and in F$_2$ (lower part)

| | Non-isolated Standard opponent (A/J) Neutral area | Isolated 13 days Standard opponent (A/J) Resident intruder |
|---|---|---|
| Reciprocal F$_1$s | (N.B6F$_1$=27, B6.NF$_1$=22) | (N.B6F$_1$=19, B6.NF$_1$=25) |
| % attacking males N.B6F$_1$ | 21[a,b] | 64 |
| % attacking males B6.NF$_1$ | 57[a] | 71 |
| First rattling latency N.B6F$_1$ | 104.67±8.54[a,b] | 72.12±6.54[b] |
| First rattling latency B6.NF$_1$ | 91.22±6.87[a] | 58.23±7.51 |
| Number of rattling N.B6F$_1$ | 1.89±1.71[a,b] | 7.83±2.59 |
| Number of rattling B6.NF$_1$ | 9.63±2.17[a] | 8.09±1.11 |
| First attack latency N.B6F$_1$ | 116.10±5.67[a,b] | 61.23±6.51[b] |
| First attack latency B6.NF$_1$ | 82.13±6.36[a] | 54.69±5.71 |
| Number of attack N.B6F$_1$ | 1.46±1.77[a,b] | 11.71±2.47 |
| Number of attack B6.NF$_1$ | 9.43±2.21[a] | 12.91±2.38 |
| Reciprocal F$_2$ | $n$=158 | $n$=317 |
| % attacking males F$_2$s | 57[a] | 68 |
| First rattling latency F$_2$ | 122.76±21.16[a] | 87.11±9.63 |
| Number of rattling F$_2$ | 7.79±2.85[a] | 19.63±7.51 |
| First attack latency F$_2$ | 113.47±13.71[a] | 64.19±8.57 |
| Number of attack F$_2$ | 11.95±2.56[a] | 17.43±3.26 |

[a] and [b] indicate $P$<.05 difference between the two conditions and between reciprocal F$_1$s, respectively.
Means±S.E.M. (untransformed values).

recombines at male meiosis. The X–Y recombining region behaves as an autosomal region and for this reason is said to be "pseudo-autosomal" (Ellis and Goodfellow, 1989). It is thus transmitted from father to daughter (Simmler et al., 1985; Petit et al., 1987). This region is located on the telomeric part of the long arm in mice it carries the steroid sulfatase gene (*Sts*), which is the only functional gene known on the recombining X and Y region (Keitges et al., 1985). The wide genome scan strategy which we used here, and the subsequent detection of Quantitative Trait Loci should therefore help clarify the question as to whether the specific or recombining region of MMUY is linked to attack behavior.

Table 4 shows the chromosomal regions linked with the four measures of attack behavior recorded under two contrasted conditions, in independent populations.

We estimated heritability in the broad sense and computed it after transforming raw data to meet the requirement of homoscedasticity in non-segregating generations. The usefulness of this statistics was in the comparison with the sum total of the genetic variance attributable to the Quantitative Trait Loci. Heritability reached 62% and 78% for latency of the first rattling in non-isolated males and isolated males respectively. The deviation from heritability to the total contribution of Quantitative Trait Loci to the phenotypic variance could result from genes with too small effects to be detected. The apices of the Quantitative Trait Loci found on MMU8 at 44 and 47 cM, in non-isolated and isolated, respectively, were encompassed in their mutual confidence intervals, suggesting that it may be the same Quantitative Trait Loci common to first rattling latency recorded under both conditions. The second Quantitative Trait Loci that emerged, on MMU9 at 39 and 41 cM, were also included in the same confidence interval. A last linkage was found between first rattling latency and the pairing region of MMUX and MMUY, for non-isolated males only.

We detected Quantitative Trait Loci only in an isolated population for a number of rattling episodes even though heritability was 30% in non isolated males. In isolated males, two Quantitative Trait Loci, on MMU11 and MMU12,

Table 4
Quantitative Trait Loci (QTL) for attack behaviors recorded under two conditions

| Condition | Non-isolated; standard opponent (A/J); neutral area | Isolated 13 days; standard opponent (A/J); resident intruder |
|---|---|---|
| | Closest marker chromosomal location (cM); QTL location±CI (cM); LOD score value | Closest marker chromosomal location (cM); QTL location±CI (cM); LOD score value |
| First rattling latency | *D8MIT8* (32 cM)<br>44±26.36 cM; LOD=3.51;<br>% of variance=15<br>*D9MIT12* (55 cM)<br>39±12.90 cM; LOD=3.69;<br>% of variance=26<br>*MSts*<br>75±12.42 cM; LOD=4.9;<br>% of variance=27<br>Total % of phenotypic variance: 68 | *D8MIT8* (32 cM):<br>47±2.83 cM; LOD=6.22;<br>% of variance=59<br>*D9MIT12* (55 cM)<br>41±27.86 cM; LOD=2.99;<br>% of variance=6<br>Total % of phenotypic variance: 65 |
| Number of rattling | | *D11MIT39* (44 cM)<br>24±13.93 cM; LOD=4.02;<br>% of variance=12<br>*D12MIT2* (19 cM)<br>26±18.60 cM; LOD=2.8;<br>% of variance=9<br>Total % of phenotypic variance: 21 |
| First attack latency | *D9MIT12* (55 cM)<br>47±37.27 cM; LOD=2.70;<br>% of variance=9 | |
| | | *D12MIT2* (19 cM)<br>19±11.94 cM; LOD=3.9;<br>% of variance=14<br>*DxMIT25* (27.8 cM)<br>28±4.52 cM; LOD=4.68;<br>% of variance=37 |
| | *MSts*<br>75±5.15 cM; LOD=4.79;<br>% of variance=43<br>% of phenotypic variance: 52 | % of phenotypic variance: 51 |
| Number of attack | *D8MIT8* (32 cM)<br>45±15.97 cM; LOD=3.85;<br>% of variance=21<br>*D11MIT39* (44 cM)<br>39±12.42 cM; LOD=4.51;<br>% of variance=17<br>*D12MIT2* (19 cM)<br>17±27.95 cM; LOD=2.71;<br>% of variance=12<br>*MSts*<br>75±9 cM; LOD=3.57;<br>% of variance=37<br>% of phenotypic variance: 87 | *D11MIT39* (44 cM)<br>45±9.83 cM; LOD=3.05;<br>% of variance=17<br>*D12MIT2* (19 cM)<br>24±15.14 cM; LOD=3.10;<br>% of variance=11<br>% of phenotypic variance: 28 |

accounted for 21% of the variance leaving 13% of the phenotypic variance uncovered.

The heritability of the first attack latency was 49% and 57% in non-isolated and isolated populations, respectively. We found Quantitative Trait Loci contributing to 52% of the phenotypic variance in the first population and 51% in the second one. The linked chromosomal regions differed under the two conditions: MMU9 and X–Y pairing region for non-isolated males and MMUX and MMU12 for isolated males.

The estimated genetic variation contributed greatly to the number of attacks in the non-isolated population (80%), but to a lesser extent (43%) in the isolated population. The Quantitative Trait Loci, on MMU11 and MMU12, were common to the two populations since the confidence intervals of each Quantitative Trait Loci included the apex of the other one. The other regions differed according to the rearing and testing conditions.

Wide genome scan confirmed that the behaviors that we recorded during dyadic encounters were linked with partially different genetic mechanisms. Estimated heritabilities in the broad sense were not the same size. Rattling and attack behaviors shared common Quantitative Trait Loci when recorded in an isolated, neutral area and non-isolated, resident intruder test conditions. One striking observation was that under the non-isolated condition, the linkage with the X–Y region accounted for a substantial part of variance for three of the four measurements. Another important finding was the presence of epistatic effects on attack behavior between the markers that were the closest to the Quantitative Trait Loci. The effect reached the $P<.05$ threshold for *Msts* and *D9MIT12*, *Msts* and *D11MIT39* and Msts and *D12MIT2*. This result indicated that the groups of regions linked with different types of attack behavior interacted. Only one wide genome scan has been previously performed with aggressive behavior in mice (Brodkin et al., 2002): the animals were isolated and tested over three consecutive days with standard opponents. We were unable to replicate the results either in the first or second population and as we were testing different behaviors, we reached different genes.

### *3.4. Discussion: from quantitative trait loci to neurobiological mechanisms*

The investigation of candidate genes offers the possibility to discover the neurobiological process behind the chromosomal locations, although the size of the confidence interval may preclude such an approach, as 1 cM encompasses approximately 15 genes, depending on the region, usual confidence intervals may cover hundred genes. The difficulty can be circumvented (Roubertoux and Le Roy-Duflos, 2001) when genes that had been previously shown as relevant for the behavior are present in the region and this is the case for genes modulating attack behavior.

The putative candidates and their links with variables we used for measuring attack behavior were represented in Fig. 6.

The group common to both isolated and non-isolated condition include *Esr1* (estrogen receptor 2), *Got2* (glutamate oxaloacetate transaminases 2), *Gabra3* (γ-amino butyric acid receptor, subunit α3) and *Htr1b* (5-hydroxytriptamine receptor 1b). A specific group for isolated-resident intruder condition included *Gabra1* (γ-amino butyric acid receptor, subunit α1), *Gri1a* (glutamate receptor, ionotropic, $AMPA_1$) and *Ar* (androgen receptor). The linkage for non-isolated mice was specified by a link to *Sts* (steroid sulfatase gene). *Sts* gene was seen to play a key role in attack behavior being linked to rattling latency, attack latency and the number of rattling episodes and attacks.

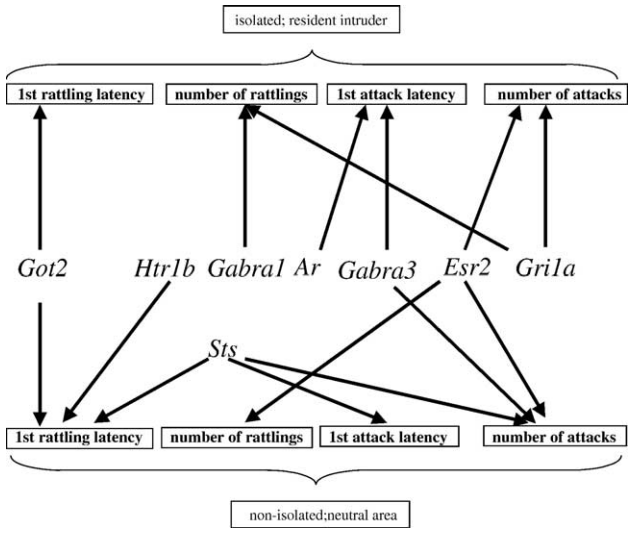


Fig. 6. Candidate genes linked with four measures of attack behavior under two conditions (non-isolated, A/J as standard opponent, tested in neutral area vs isolated, A/J as standard opponent, tested in housing cage). *Esr1* (estrogen receptor 2), *Got2* (glutamate oxaloacetate transaminases 2), *Gabra3* (γ-amino butyric acid receptor, subunit α3), *Htr1b* (5-hydroxytriptamine receptor 1b), *Gabra1* (γ-amino butyric acid receptor, subunit α1), *Gri1a* (glutamate receptor, ionotropic, $AMPA_1$), *Ar* (androgen receptor) and *Sts* (steroid sulfatase).

These results fit with the results of the factor analysis and data previously published on agonistic behavior. The common group of genes included *Htr1b*. *Htr1b* was the first targeted gene for which an increased attack behavior was reported (Saudou et al., 1994), initiating a stream of research confirming the implication of 5-HT in the propensity to attack behavior (see Maxson, for a review, 1996). The results from our factor analysis (see Table 1) confirmed the general correlation between reduced brain 5-HT and attack behavior. Factor I had a high positive loading for the percentage of attacking males under non-isolated condition and a negative loading for brain 5-HT. This opposition reappeared on factor III with plasma 5-HT. A negative correlation between the 5-HT level in olfactory bulb and the percentage of attacking males under the non-isolated condition followed the same trend (Tordjman, 1995).

The contribution of glutamatergic system to agonistic behavior that was detected via the present wide genome scan (i.e. possible involvement of *Got2* and *Gri1a*) fits with the conclusions of other experiments reporting the involvement of the glutamatergic/*N*-Methyl-D-Aspartate (NMDA) system in aggression, e.g. the injection of excitatory amino-acid in brain elicited the attack behavior whereas NMDA antagonist injections reduced the expression of attack behavior (Siegel et al., 1999; Sukhotina and Bespalov, 2000). Chen et al. (1994)

found aberrant agonistic behavior in *α-Calcium-Calmodulin Kinase-II* knock out mice, this being the gene that conditions the NMDA receptor functioning.

A linkage with *Gabra3* and *Gabra1*, which are sub-units of the γ-amino butyric acid $(GABA)_A$ receptor, suggested that this main inhibitory transmitter plays a role in attack behavior. The role played by the GABAergic system in the control of aggressive behavior in rodents was described for the first time by Mandel's research team (Molina et al., 1986): injections of a $GABA_A$ receptor agonist into the olfactory bulb in rats caused aggression to decrease. Similar studies reported that microinjections and microdialysis perfusions of $GABA_A$ receptor agonists or antagonists into rodent brains (e.g. the hypothalamic attack area—hypothalamic attack area, the amygdala or periaqueductal grey matter) caused, respectively, a decrease and an increase in aggressive behavior towards opponents (Molina et al., 1986).

Our findings, with the wide genome scan of attack behavior in mice housed and tested under contrasting conditions, challenged simple views. We initially concluded that there was a group of genes involved in the behavioral phenomena recorded under different conditions, and also specific groups of genes. Our results showed that several Quantitative Trait Loci linked with the variables comprising these groups were in epistasis. This finding was also confirmed by a survey of the literature.

## 4. General discussion

### *4.1. The factor analysis and quantitative trait locus mapping converge*

The conclusions drawn from the factor analysis approach tally with the conclusions deduced from the wide genome scan, pending further confirmation of probable candidate genes. Both the factor analysis and wide genome scan supported two sets of processes: one mainly covaried with the first testing condition (isolated male, tested in neutral area), while the other covaried with the third and fourth conditions which shared two key characteristics, i.e. the animals were isolated and tested in their home cage.

The loading common with the steroid sulfatase concentration seemed specific to the first testing condition and the wide genome scan confirmed this, showing a significant linkage with *Sts* gene for most behaviors observed under this condition. The testosterone activity and 5-HT loadings may be confirmed by the Quantitative Trait Loci encompassing respectively *Esr2* and *Htr1b*, and in their respective confidence intervals.

The second set of processes had no loadings with steroid sulfatase or opioid measurements, and there was no linkage with any genes involved in the opioid system. Two biological measurements presented loadings on factor II: brain 5-HT and "paired testes weight" were confirmed by the detection of Quantitative Trait Loci encompassing *Htr1b* and *Ar* in their respective confidence intervals.

Interactions between the two sets of processes were supported by both the factor analysis and wide genome scan. Brain 5-HT loadings were present on factors I and II and the behaviors covered by these factors showed linkages with the chromosomal region including the 5-hydroxytriptamine receptor 1b. Factors I and II had high loadings for the measurements, both direct and indirect, and the corresponding behaviors were linked to estrogen receptor 2 and the androgen receptor.

### *4.2. Role of the steroid sulfatase gene*

Paradoxically, the main difference between factors I and II was the high loading of steroid sulfatase on factor I. In non-isolated males tested in a neutral area, the linkage of behaviors observed with the *Sts* gene confirmed the results of the factor analysis but did not shed any light on the relationships between the processes involved in attack behavior either by non-isolated males tested in neutral area or by isolated males tested in their own cages.

One gene in the glutamatergic system (*Got2*, glutamate oxaloacetate transaminase 2), but not *Gri1a* (glutamate receptor, ionotropic, A), was linked with the processes operating under both conditions. One gene $MPA_1$ in the GABAergic system (*Gabra3*, γ-amino butyric acid receptor, subunit α3), but not *Gabra1* (γ-amino butyric acid receptor, subunit α1), was also linked to the processes operating under both conditions. In fact, different lines of evidence show that steroid sulfatase modulates both the glutamatergic and GABAergic systems.

The microsomal enzyme steroid sulfatase (STS, E.C.3.1.6.2) has a central function in the mode of action of neurosteroids, as steroid sulfatase is responsible for the conversion from sulfated to free forms. The enzyme is ubiquitous in mammalian tissues where it hydrolyses several steroid sulfates, and specifically 3β-hydroxysteroid sulfates (Akwa et al., 1991; Beaulieu, 1997; Compagnone and Mellon, 2001). The steroids are synthesized and accumulate in the CNS in the form of unconjugated, sulfated or fatty acid esters. The presence of unconjugated forms of steroids differentiates the gonadal steroid pathway (dehydroepiandrosterone, androstendione then testosterone and finally estradiol/estrogen). But several neuroactive steroids modulate the activity of the neurotransmitter receptors (Beaulieu, 1997; Compagnone and Mellon, 2000). Dehydroepiandrosterone sulfate and pregnenolone sulfate act as excitatory neurosteroids and are negative allosteric modulators of the $GABA_A$ receptors (Lan and Gee, 1994; Hauser et al., 1995; Hawkinson et al., 1994; Majewska et al., 1986; Majewska, 1992, Meyer et al., 1999; Park-Chung et al., 1999) and potentiate NMDA receptor activity (Fahey et al., 1995; Hauser et al., 1995; Mathis et al., 1994). Pregnenolone and dehydroepiandrosterone metabolites, such as allopregnanolone (3α-5α-THP, 3α-hydroxy-5α-pregnane-20-one), display $GABA_A$ receptor agonist properties.

The effect of the two receptors, for which *Got2* and *Gabra3* codes may be potentiated by the steroid sulfatase mechanisms, was described above. The action of dehydroepiandrosterone sulfate on the GABAergic system may be enhanced by its potent positive modulation of the NMDA receptors. Steroid sulfatase activity may provide the balance between the two types of receptor when controlling attack behavior via the levels of sulfated steroids in the brain. Steroid sulfatase is the

difference between the two biological systems underlying the two dimensions of attack behavior and modulates both systems. The complexity of the control of glutamatergic and GABAergic systems appeared, however, more complex since glutamatergic receptors modulate GABA signaling (Semyanov and Kullmann, 2000). Using pharmacological methods, Nicolas et al. (2001) demonstrated that steroid sulfatase modulated dehydroepiandrosterone sulfate in the brain and therefore aggressive behavior in mice.

### *4.3. The search for genes modulating attack behavior*

Rearing and testing conditions interact with genotypes, or at the very least with the strain. We demonstrated that identical replicated individuals and strains did not behave in the same way when reared and tested differently. The biological correlates were not the same and the chromosomal regions linked with the behavioral phenomena observed during the different dyadic encounters were partially different. We can draw several deductions from these observations.

We cannot expect to find the same results (the same Quantitative Trait Loci or the same effects from the over-expressed or targeted gene) for attack behavior when rearing and testing conditions are not identical. This was previously shown by Guillot et al. (1995) who observed that strains congenic for the specific part of MMUY displayed attack behavior only when they had been isolated. Strictly standardized procedures are needed to discover genes involved in attack behavior. Isolation induces reactivity to stress in gregarious animals such as rodents, while repeated encounters modify plasma hormone concentrations (Batty, 1978a; Carlier et al., 1990) and brain neurochemistry (Miczek and Thompson, 1984). One procedure alone is not sufficient. If we are to gain an understanding of the genetic mechanisms in aggressiveness, we need to investigate the general scope and effects of a gene under different conditions of rearing and testing.

While the same genotypes react differently under different testing conditions, different genotypes may react differently again to the same conditions. A protein-deficient mouse may attack in the resident intruder test, but not under other conditions. We therefore need multiple standardized tests covering a wide range of conditions where the species displays aggressiveness.

As different genotypes react differently to the same conditions, the study must include the isogenicity of the genetic background which is crucial for identifying the functions of a given gene. We now know that there are a large number of strains from the 129 family and that these strains have different genotypes (Simpson et al., 1997). We need to take greater care in selecting the origin of the 129 stem cells and the controls. We need to be stricter on the number of backcrosses which, quite often, falls short of the 14 or 16 generations required to approach isogenicity.

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