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ORIGINAL RESEARCH

Delineation of Violence from Functional Aggression in Mice: An Ethological Approach

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Abstract The present study aims at delineating violence from aggression, using genetically selected high (SAL, TA, NC900) and low (LAL, TNA NC100) aggressive mouse strains. Unlike aggression, violence lacks intrinsic control, environmental constraints as well as functional endpoints. Conventional measures namely latency, frequency and duration were used initially to accomplish the objective of delineation using the above strains. However, these quantitative measures fail to reveal further details beyond the magnitude of differential aggression, especially within the high aggressive mouse strains. Hence, it was necessary to analyze further, the behavioral sequences that make up the agonistic encounter. Novel measures such as *threat/(attack + chase)* (T/AC) and *offense/withdrawal* (O/W) ratios, context dependency and first-order Markov chain analysis were used for the above purpose. Our present analyses reveal clear qualitative behavioral differences between the three high aggressive selection strains based on the following facets namely *structure* and *context* in an agonistic interaction. *Structure* refers to a detailed study of the agonistic interaction components (ritualistic display, offense and sensitivity to the opponent submission cues) between any two subjects (inter-male interaction for the present study). *Context* refers to the capacity to identify an opponent by nature of its state (free moving/anesthetized), sex and the environment (home/neutral territory). NC900 displayed context dependency and structurally a rich repertoire of agonistic interaction components with an opponent. SAL failed to show discrimination and its inter-male agonistic behavior is restricted to a repetitive and an opponent-insensitive pattern of attack and chase. TA was comparable to SAL in terms of the *structure* but sensitive to *context* variables. Thus, SAL seems to display a violent form of aggressive behavior, while NC900 display 'functional' hyperaggression against a docile opponent in an inter-male agonistic interaction.

Keywords Functional aggression · Mouse models · SAL · TA · NC900 · LAL · Psychopathology · Violence · Genetic selection

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Introduction

Aggression research in rodents has reached a point where the distinction between functional and deviant forms culminating in violence is now being acknowledged (Haller and Kruk 2006; Haller et al. 2005). The general view in biology is that animals express aggression towards a functional endpoint, for example to acquire social ranking and resources from the environment (Collias 1944). However, in humans, aggression is often considered maladaptive and associated with socio-economic and health concerns,

suggestive of a loss of functional significance (Lorenz 1966; Krug et al. 2002). Violence is a heterogeneous phenomenon often characterized by uninhibited aggression; social and emotional dysfunction, leading to out of context behaviors; reduced individual and population fitness in a society (Krakowski 2003; Davidson et al. 2000; van Oortmerssen and Busser 1989; Vitiello and Stoff 1997). Violence and related behavioral anomalies have been observed in non-human primates and monkeys (Carpenter 1934; Higley 2003; Manson and Wrangham 1991; Schaller 1963). Southwick (1970) has reviewed the ubiquity of violence in other mammalian and non-mammalian species (including elephants, tigers, hippopotami, musk oxen, grizzly bears, rodents, lizards and social insects) in detail.

The earliest lab-based experiments focused on violent 'rage' behaviors in cats following neuro-physiological manipulations in the brain (Bard 1929; Kaada 1966). Several rodent models of violence were developed using lesions to pre-frontal cortical areas, models of Alzheimer's disease, epilepsy and electric shock models as summarized by Haller and Kruk (2006). Hyper-arousal-driven aggressiveness seen in human diseases, such as intermittent explosive disorder and post-traumatic stress disorder (PTSD), has been modeled in rodents by *frustration* (omission of a scheduled reward) or *instigation* (indirect sensory contact with an opponent) (Miczek et al. 2002).

Repeated exposures to an opponent also lead to the loss of ritualistic behaviors (Kudryavtseva 2000; Kudryavtseva et al. 2000) and a decline in sensitivity to the opponent's submission cues in highly aggressive rats (Benus et al. 1991; de Boer and Koolhaas 2005). Hypo-arousal-driven aggression salient in habitual violent offenders, antisocial personalities and those with conduct disorders are modeled by glucocorticoid-deficient rats (Kruk et al. 1990), the hypothalamic attack paradigm (Halasz et al. 2002; Haller et al. 2001; Koolhaas 1978; Kruk et al. 1979; Kruk 1991) and genetically selected aggressive (SAL) mice (van Oortmerssen and Bakker 1981).

Aggression in these animals is characterized by intensified offensive behavior as shown by a plethora of ethological measures, including attack bouts, bites at vulnerable parts of the body and a blunted sensitivity toward social signals (e.g. sex and hierarchical status) of the opponent (Miczek et al. 1994; Brain and Benton 1981, Brain and Hui 2003).

Despite the ready availability of the above-mentioned animal models, objective studies on violence and/or pathologically aggressive phenotypes are scarce. In fact, many studies consider merely high levels of aggression as being reflective of pathological aggression and/or violence (e.g. Miczek et al. 2002; Haller and Kruk 2006). Several studies focus on the magnitude of aggression in relation to socio-environmental (Sprott and Staats 1975), neurological and neuro-pharmacological manipulations (Nikulina 1991; Robertoux et al. 2005; Crawley et al. 1997).

Deficient serotonin function has often been correlated with impulsivity, suicide and escalated aggression in humans (Asberg et al. 1976; Brown et al. 1982). However, this finding has been difficult to prove in rodents. Recent evidence in rats and mice suggests that the above human correlation holds only for abnormal/violent conspecifics (de Boer et al. 2003, 2005). In another study, however, a positive correlation was found between functional aggression and central serotonin (5-HT) function (van der Vegt et al. 2003). Thus, a clear distinction between functionally relevant aggression and deviant, pathological forms of aggression at an ethological level, might explain the inconsistencies behind these contrary findings on the neurobiology of aggression. Hence, the present study aims at more objective behavioral criteria to delineate functional aggression from the deviant/violent forms of aggression.

The present study considers aggression as a form of social communication characterized by a pattern of constrained actions, reactions and social signals between partners in conflict. The term 'constraint' is used to describe *rules and rituals* of certain magnitude, expression and sequence, which makes aggression functional, dynamic yet *structured* behavior within inhibitory limits (Haller and Kruk 2006). Regardless of species-specific rules, the following components are considered essential for functionally driven aggression. When an unfamiliar conspecific is encountered, exploratory behaviors commence with social exploration and ano-genital inspection. Sustained presence of the intruder invites consequent offensive threat displays (Matthews 1964; Tinbergen 1951). Failure of compliance, or competition between equally ranked individuals, eventually leads to overt offense. Intra-sexual competition for access to a mate is a notable example of such goal-driven aggression, which is terminated once the competitor submitted or has fled (Scott 1962, 1963). Although speculative, functional aggression is not anticipated to target vulnerable body parts even in the midst of an agonistic interaction unless challenged as seen in defensive aggression (Matthews 1964). The conflict is terminated upon submission of one of the interacting partners, as shown in a number of animals including European hamsters, cichlids, cocks, gulls, jackdaws, wolves, and fallow deer (Lorenz 1966). Functional aggression amongst social animals is likely to be discriminatory towards the opponent and/or the environment in question, e.g. males should refrain from harming familiar healthy female partners (Lorenz 1966; Christian 1971) and dead/immobilized or unhealthy subordinates.

The objective conceptualization of violence must therefore consider the deviation of functional aggression in terms of these particular component patterns and sequential

structures, separately from magnitude of combat (Haccou et al. 1988; Haccou and Meelis 1992). Several behavioral aspects may reflect these components of deviance, for example: (1) the disappearance of the normal investigatory and threatening sequence of acts and postures from the agonistic behavioral repertoire, and early engagement in the ultimate consummate phase of aggression; (2) persistence in the aggressive attack-biting mode despite the intruder's submissive supine displays and crouching/defeat postures; (3) attack bites of high magnitude and directed to vulnerable areas, if not stopped by the experimenter, (4) a lack of discrimination between types of opponent (resulting in attacks on females and/or even anesthetized/dead conspecifics) or the current environment (unfamiliar/home).

The present study analyzes agonistic behavior in terms of three characteristics: *magnitude, structure* and *context*, which encompasses the above aspects. *Magnitude* is defined as the level of offensive behaviors, in terms of both duration and frequency. The first three components of deviance described above, are studied under *structure* of an agonistic interaction, in the following order namely the (pre-offensive) *entry* into the aggressive behavioral sequence (the ritualistic adherence and offensive display forewarnings), the offensive *event* per se and the *exit* out of the aggressive sequence (post-offensive). The final component of deviance is subsumed under *context* and was determined using different opponents and environments.

Genetic studies aimed at identifying genes, genotypes or aggressive loci (QTL) generally do not premise on these potential distinctions between aggression and violence (Mozhui et al. 2007; Brodkin 2005). This may be a serious confound in the study of the neurobiology and/genetics of violence in animals and their consequent comparison with human data. Hence, three strains of mice genetically selected for high and low aggression were used for the present study with a controlled environment having the same opportunities for food, mates, space and physical conditions.

Materials and methods

Animals and housing

Male mice aged 3–4 months from three different genetic selection lines (SAL, LAL; TA, TNA; NC 900, NC 100; $n = 8$ per strain tested) were considered for the behavior analysis. Short Attack Latency (SAL) and Long Attack Latency (LAL) are outbred strains selected artificially from a wild population in Groningen, the Netherlands (van Oortmerssen and Bakker 1981). Turku aggressive (TA) and non-aggressive (TNA) are outbred strains obtained through artificial selection from laboratory Swiss albino mice in Turku, Finland (Sandnabba 1996). NC900 (aggressive) and NC100 (non-aggressive) are outbred strains selected from laboratory ICR mice in North Carolina (Gariepy et al. 1996). These mice are hereafter referred to as 'residents'. The residents were bred and kept in familiar groups until weaning (3 weeks after birth), then co-housed with a female of the same line in Makrolon Type II cages (375 cm^2). The litters were culled periodically. The mice were fed ad libitum on standard pellets (AMII, ABDiets, Woerden, The Netherlands) and water with low chloride content. They were exposed to a reverse light:dark cycle of 12 h shifting at 0030 h. Each cage was provided with sawdust bedding, shredded paper (*envirodry,* the Netherlands), nesting and cardboard tubing enrichment materials. Room temperatures were maintained at 22 ± 2°C. The animal care complied with the Law on Animal Experimentation and was approved by Institutional Animal Care and Use Committee (IACUC), University of Groningen [D4328A].

Behavior test: resident–intruder paradigm

A simplified resident–intruder paradigm (van Oortmerssen and Bakker 1981) was employed. The experiments were carried out during the first half of the active (dark) phase. The test comprised three successive days of interactions in the resident cages and a final day of interaction in a neutral cage. Neutral cages were fresh cages, never used for holding any other mice during the experiment. The experimental design is depicted in Fig. 1. The test cages were 75 × 29 × 27 cm and were divided into two equal compartments by a perforated transparent sliding door. The front wall of the cage was a transparent Plexiglas sheet that allowed appropriate lighting and video recording during the experiments. Mice were allowed to habituate in their respective new cages for a period of 3 days. Behavioral testing comprised inter-male interactions in the residents' cages for 5 min followed by an interaction with their familiar female conspecific partner for 2 min. The male opponent used is the docile inbred albino Mas-Gro strain (van Oortmerssen 1989). Interactions in the neutral cage were the same, but with an added interaction with an anesthetized intruder for 2 min between the inter-male interaction and the familiar female-male interaction.

Inter-male interactions were preceded by separating the female of the pair, 1 h before the lights went off. The male mouse was allowed to retain one half of the cage by introducing a sliding door, which separated the cage into two halves without limiting access to food or water. Five minutes before the test, the male opponent was introduced into the unoccupied half of the cage. The perforated sliding door allowed only sensory contact and prevented any direct physical contact between the males. The sliding door was then removed and the direct inter-male interaction was

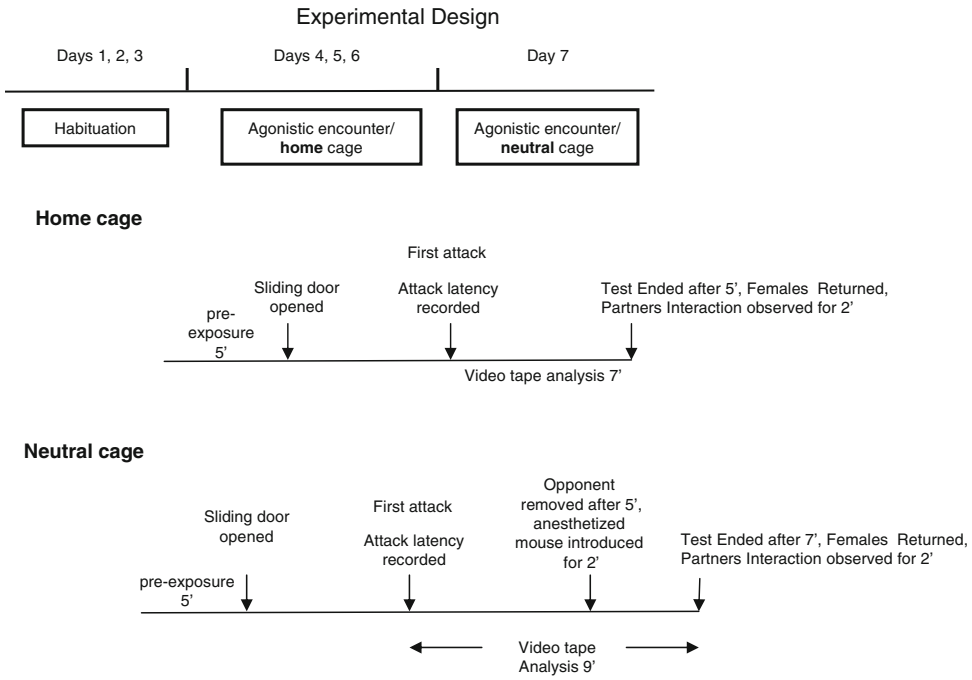


Fig. 1 Shows the experimental design for the present study

recorded. The interaction was allowed to last for 5 min after the first attack. When the resident failed to attack within the first 5 min of testing, the attack latency time (ALT) was recorded as 300 and the test was terminated. The opponent was then removed from the cage and the resident's female partner was reintroduced and observed for a period of 2 min.

The second interaction in the neutral cage involved a Mas-Gro male opponent that had been anesthetized 30 min prior to the test. This opponent was therefore in a semi-conscious state during the interaction. Anesthesia was induced in the opponent by intramuscular injection of 5 µl Ketanest-Rompun cocktail/gm body weight (Richardson and Flecknell 2005). The cocktail was 2% Ketanest-S® 25 Multidose (Pfizer, the Netherlands) and 0.3% Rompun® (Bayer, the Netherlands) in physiological saline.

Video analysis

All recorded inter-male agonistic encounters in the resident cages were analyzed using the software Observer Pro 5.0 (Noldus BV, Wageningen, the Netherlands) at low speeds (1/5th of regular speed) and the behavioral phenotypes were quantified (Koolhaas et al. 1980; Brain and Benton 1981). The following behaviors were quantified: *digging*, *non-social exploration* (explore the cage), *social exploration* (approach, investigation—crawl over, crawl under, follow, allo-groom, head groom, investigate, nose sniffing), *immobility*, *resting*, *body care* (self grooming, wash, shake, scratch), *feeding* (drink/eat), *attack* (lunge, attack), *chase* (charge), *threat* (aggressive groom, sideways, offensive, upright offensive, tail rattle), *defense*. The values for *attack, chase* and *threat we*re summed together to give a combined measure of *offense*.

Statistical analysis

Classic measures as the latencies, frequencies and duration of the offensive behaviors namely attack, chase and threat were used to analyze the differential aggressive phenotypes in all the mouse strains used. Attack latency time (ALT) and the mean duration and frequency of *offense*, obtained from the videotapes, were analyzed for the residents using a two-way ANOVA, with 'strain' (3 levels: Groningen, Finland and North Carolina) and 'type' (3 levels: high, intermediate and low aggression) as between-subjects factors. Post-hoc analyses were carried out by means of *t*-tests and Tukey tests for multiple comparisons. Effects of repeated interaction on the above parameters were analyzed with ANOVA for repeated measurements, with 'day' as a within-subject factor (3 levels: Days 1, 2 and 3) and the above-mentioned as between-subjects factors.

Since SAL was anticipated to be less ritualistic than any other mouse strain, we carried out a planned contrast between SAL and the intermediate aggressive aTNA strain (for details, refer to results section). Low-aggression mouse strains were not considered for this analysis owing to the scarcity of offensive behaviors. All statistical analyses were carried out using SPSS version 12.0. Outcomes in the neutral cage were analyzed using chi-square statistics.

Sequential analysis, using the pooled transition-frequency matrices for each mouse strain obtained from the

Observer software, was done using first-order Markov chain analysis with MatMan software, version 1.1.4 (Noldus Information Technology, Wageningen, the Netherlands; de Vries et al. 1993). The data from day 3 were used for this analysis. Again, owing to the scarcity of offensive behaviors, the low-aggression lines were not considered. The expected values and adjusted residuals were computed for each transition matrix with an undefined diagonal, by means of an iterative algorithm (de Vries et al. 1993) that is equivalent to the iterative proportional fitting method (Goodman 1968). The log-likelihood ratio test (G test) was used to evaluate whether the observed transition frequencies in the matrix as a whole, deviate significantly from the frequencies expected under independence. The significance of the individual residual values was adjusted to a table-wide level of 5% (two-tailed) with Hochberg's improved Bonferroni method (1988). Significant transition frequencies (alongside specific non-significant transition frequencies) were used for data interpretation. To facilitate this, behavioral kinetograms were constructed, focusing on the resident's behavioral transitions when interacting with an opponent. Behaviors such as *feeding*, *grooming and rest* were lumped together as 'other behaviors' for the sake of simplicity. Significant positive adjusted residuals were identified and displayed as '*P*' values less than 0.0001, 0.001 and 0.05. Negative residuals and selected non-significant residuals are indicated without the '*P*' value. Matrix-specific '*P*' values are also indicated along with G values.

Results

Ethogram and attack latency

Figure 2 shows a simple ethogram (% time) for mice strains selected for differential aggression. The category 'others' includes *grooming* and *feeding* (drink/eat). 'Inactivity' includes *rest* and *immobility*. Withdraw includes *approach–withdrawal* and *withdrawal* behaviors. Interestingly, a considerable number of animals in the Groningen and Turku low-aggression strains had attack latencies and offensive magnitudes intermediate, between the high- and low-aggression counterparts. These are referred to as the attacking low-aggression strains namely aLAL and aTNA in order to differentiate them from the non-attacking low-aggression nLAL and nTNA strains, respectively. Additionally, an unanticipated hesitation-like behavior was observed. This was termed *approach–withdrawal* since it was a mix of an approach-like and concomitant withdrawal-like behaviors, distinct from *social exploration* and *withdrawal* behaviors. This behavior is discussed in detail in the Discussion section.

All mice displayed extensive *social-* and *non-social exploratory* behaviors, almost up to 50% of the total time spent in the presence of the opponent, suggestive of normal activity in these animals. SAL mice displayed the least amount of these behaviors. When compared to the other lines, they spent most of the test period alternatively in offensive combat with the opponent. The nLAL mice showed extensive *non-social* behaviors and the NC100 mice showed extensive *social exploratory* behaviors. NC100 mice exhibited *allogrooming* behaviors more prominently than the other lines (personal observation).

The attack latency time (ALT) of each mouse line are shown separately as Fig. 3. The ALT was consistent with previous findings and in line with the genetic selection (Caramaschi et al. 2007). The high-aggression mouse strains launched their first attack within a few seconds. SAL showed the lowest ALT compared to the other strains on all 3 days of home cage testing. The intermediates attacked within the first 150 s while the low-aggression mice attacked, on average, over 150 s after the partition was removed. A two-way ANOVA revealed significant effects of 'strain' [$F_{(2,63)} = 4.700$; $P < 0.05$] and 'type' [$F_{(2,63)} = 110.056$; $P < 0.001$] (figures not shown). Tukey's post-hoc analysis revealed that the Turku mice differed significantly from the Groningen ($P < 0.01$) and the NC ($P < 0.001$) mouse lines. The 'strain × type'

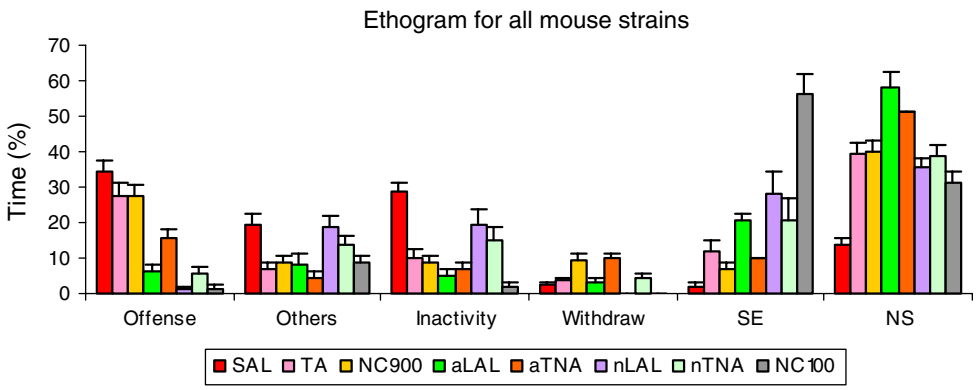


Fig. 2 Shows a time-based ethogram for all mouse lines investigated. 'Offense' is the sum of *attack*, *chase* and *threat* behaviors. 'Others' represents *feeding* and *grooming* behaviors. 'Inactivity' represents *rest* and *immobility*. 'Withdraw' represents *approach–withdrawal* and *withdrawal* behaviors. 'SE' = *social exploration* of the intruder. 'NS' = *Non-social exploration*

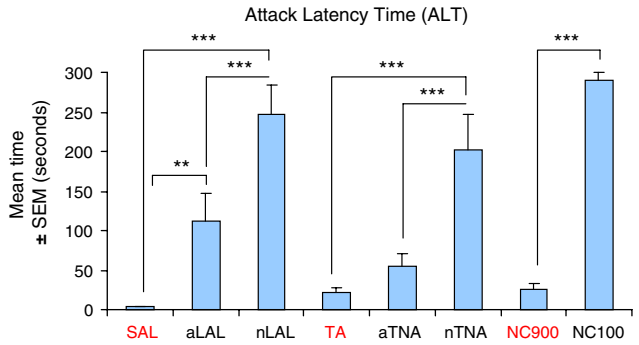


Fig. 3 Shows the attack latency time (ALT) for all mouse strains investigated. Data are plotted as mean ± SEM. Significant '*P*' values are represented as $*P < 0.05$; $**P < 0.01$; $***P < 0.001$

interaction was marginally non-significant [$F_{(3,62)} = 2.693$; $P = 0.054$]. Tukey's post-hoc analysis revealed the following. Within the Groningen strains, SAL attacked significantly earlier than aLAL ($P < 0.01$), both SAL and aLAL attacked significantly earlier than nLAL ($P < 0.001$). Thus, all three mouse strains were distinct from each other. Within the Turku mice, the TA strain attacked significantly earlier than the nTNA strain ($P < 0.001$), nevertheless TA was not distinct from aTNA. The nTNA mice were distinct from the aTNA ($P < 0.001$). Within the NC mice, NC900 attacked significantly earlier than NC100 ($P < 0.001$). In other words, all the high-aggression lines attacked earlier than their low-aggression counterparts did. The attack latencies of the different aggressive types were consistent across strains.

The effects of repeated agonistic interactions on the attack latencies (ALT) for all mouse strains were also investigated. A repeated-measures ANOVA revealed a significant 'day' effect [$F_{(2,62)} = 5.149$; $P < 0.01$] with repeated interactions across all 3 days of testing. Paired *t*-tests (two-tailed) revealed a significant reduction of the ALT on day 2 ($t_{70} = 3.132$, $P < 0.01$) and day 3 ($t_{70} = 2.835$, $P < 0.01$) compared to day 1. No differences were observed between days 2 and 3. No significant effects of 'selection' or 'type' were observed.

Offense

Figure 4 shows the percentage-based stack distribution of those direct behaviors between the resident and the opponent, namely *social exploration*, *offense*, *approach–withdrawal* and *withdrawal*, in terms of both duration and frequency. The high-aggression strains and the aTNA showed longer and more frequent agonistic interactions with an opponent.

Figure 5 shows the mean duration of offense behaviors for each mouse strain. A two-way ANOVA with mean offense revealed no 'strain' effect but a significant effect of 'type' [$F_{(2,55)} = 107.138$; $P < 0.001$] and 'strain' × 'type' effect [$F_{(3,55)} = 5.375$; $P < 0.05$]. Further post-hoc analysis with Tukey's revealed the following. The high-aggression mice were significantly more offensive than the intermediate-aggression ($P < 0.001$) and the low-aggression ($P < 0.001$) mice as seen in the inset 5a. The intermediate strains were also slightly (but significantly) more offensive than the low-aggression ones ($P < 0.05$) (Fig. 5b). The mean durations of offense behaviors for the different aggressive types were consistent across strains. Post-hoc analysis for the 'strain × type' interaction effect revealed a significantly higher degree of offense in the high-aggression strains than the intermediate and low-aggression ones [SAL/aLAL ($P < 0.001$) and SAL/nLAL ($P < 0.001$); TA/aTNA ($P < 0.001$) and TA/nTNA ($P < 0.001$); NC900/NC100 ($P < 0.001$)].

Further statistical analysis considered individual and combined offensive behaviors to assess if there was a significant bias toward specific offensive behaviors as a plausible strategy of strain-specific offense. *Attack+chase*

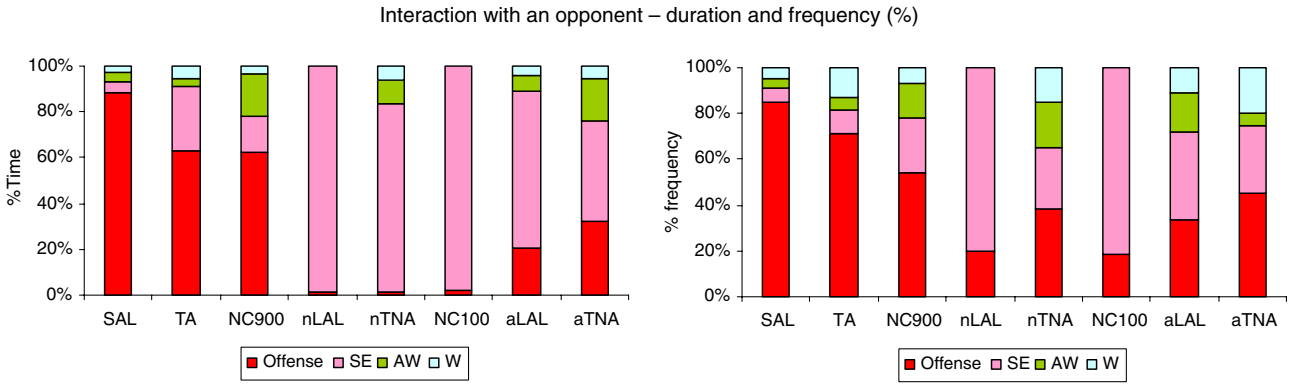


Fig. 4 Shows a distribution stack of the resident mouse behaviors, namely *social exploration* (SE), *offense* (O), *approach–withdrawal* (AW) and *withdrawal* (W). These behaviors are direct interactions of the resident mouse with an opponent, taking place in the resident's cage. Both duration and frequency of the behaviors are shown (in %)

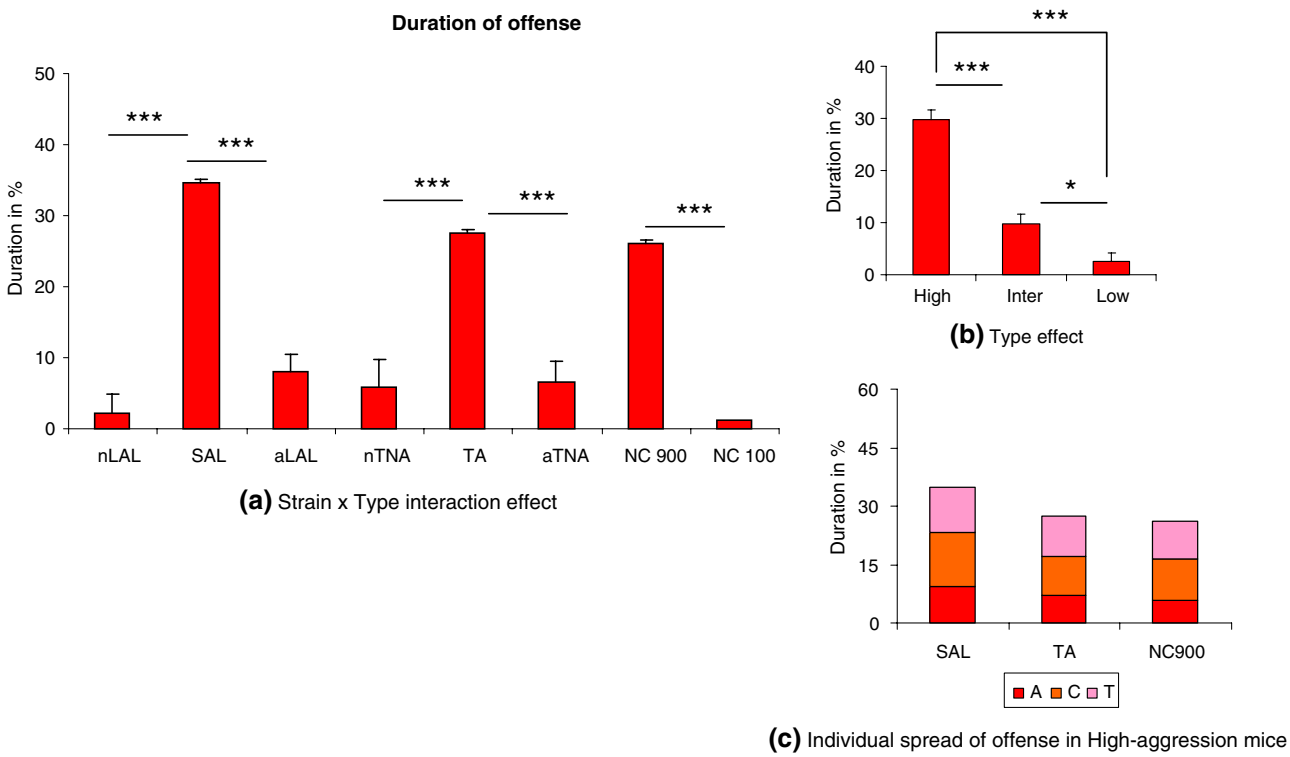


Fig. 5 Shows the following: (**a**) 'Strain × Type' interaction effects (**b**) 'Type' effects on the duration of the offensive behaviors of all the mouse strains. (**c**) The individual spread of offense to *attack*, *chase* and *threat* behaviors are shown exclusively for the high-aggression mouse strains. Data are plotted as mean ± SEM. Significant '*P*' values are represented as * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

(A + C) was used for the comparison of the high and the intermediate lines. (A + C) behavior showed a significant 'type' effect [$F_{(1,35)} = 61.58$; $P < 0.001$] and a significant 'strain × type' interaction [$F_{(1,34)} = 5.091$; $P < 0.05$]. Mice from the high-aggression strains attacked and chased their opponents for longer durations than their low- and intermediate-aggression counterparts. Further Tukey's post-hoc analysis showed the following. SAL differed significantly from aLAL ($P < 0.001$), and TA differed significantly from aTNA ($P < 0.001$, figure not shown). A similar trend was observed with the *threat* behavior: *threat* duration showed a significant 'type' effect [$F_{(1,35)} = 15.98$; $P < 0.001$] and a marginally non-significant 'strain × type' interaction [$F_{(1,34)} = 3.834$; $P = 0.058$].

Within the high-aggression strains, the offensive behaviors were analyzed individually. Although SAL showed longer periods of offense than TA and NC900 on average, none of them showed significant changes in the mean offensive behaviors (*attack, chase* and *threat*), as shown in Fig. 5c.

Repeated-measures ANOVA failed to reveal significant effects of 'day', 'day × strain', 'day × type' or 'day × strain × type', regardless of the overall/the individual offensive behaviors. The same was the case within the high-aggression lines.

With regards to the frequency of the offensive behaviors (figures not shown), a two-way ANOVA with mean offense revealed no 'strain' effect but a significant 'type' effect [$F_{(2,55)} = 112.31$; $P < 0.001$] and a significant 'strain × type' interaction effect [$F_{(3,55)} = 2.879$; $P = 0.044$]. High-aggression mouse strains attempted significantly more offensive behaviors than their intermediate counterparts ($P < 0.001$) as seen from post-hoc analysis. No differences were observed between the intermediate and low-aggression ones. Analysis within each strain revealed that offense frequencies in SAL were significantly higher than in aLAL ($P < 0.001$) and nLAL ($P < 0.001$), those in TA were significantly higher than in aTNA ($P < 0.001$) and nTNA ($P < 0.001$), and NC900 was significantly more offensive than NC100 ($P < 0.001$). No intra-type differences were observed.

Within the high and intermediate strains, *attack* + *chase* behavior showed only a significant 'type' effect [$F_{(1,35)} = 63.369$; $P < 0.001$]. *Threat* behavior also showed a significant 'type' effect [$F_{(1,34)} = 26.792$; $P < 0.001$]. No intra-type differences were observed within the high-aggression strains. In line with the duration data, a repeated-measures ANOVA failed to reveal significant effects of 'day', 'day × strain', 'day × type' and 'day × strain × type', either for the overall or for the individual offensive behaviors (*attack* + *chase* or *threat*).

Classic measures thus showed distinct high-, intermediate- and low-aggression phenotypes. However, they failed to show any intra-type variations, including those pertaining to those within the high- and intermediate-aggression strains. So additional measures (T/AC) ratio, offense/withdrawal (O/W) ratio and response to an anesthetized male intruder were analyzed to assess potential differences within the aggressive strains of moderate to high offensive magnitudes. T/AC and O/W ratios were used to assess the *structure*. The residents' responses to an immobilized intruder and to female conspecific were used to assess the discriminatory component of *context.*

Threat/(attack + chase) (T/AC) ratio

Mice following ritualistic agonistic interactions were predicted to show more *threat* behaviors than *attack* or *chase* behaviors by magnitude, so an additional measure namely the *threat/(attack + chase)* ratio was considered. The T/AC ratio, in terms of both duration and frequency, is presented in Fig. 6 for all high and intermediate strains. aLAL and the low-aggression mice had low magnitudes of the offensive behaviors and hence were not considered for this analysis. aTNA was considered along with the high-aggression strains. SAL and TA strains were comparable and had T/AC ratios less than 1 either by frequency or duration, suggesting that they were likely to launch more *attack* and *chase* than *threat*. The aTNA line showed T/AC ratios more than 1. A one-way ANOVA failed to reveal significant differences between the mouse strains considered.

SAL was hypothesized to possess a lower T/AC ratio than all other strains, notably the intermediate- and low-aggression ones. Hence, planned contrasts were carried out on the T/AC data, after square-root transforming them to correct for non-homogeneity across strains. The analysis revealed SAL to have a significantly lower ratio than aTNA, both for duration ($t_{9.70} = -2.56$; $P < 0.05$) and frequency ($t_{8.32} = -2.39$; $P < 0.05$; the variances were not assumed to be equal for this analysis). Other high-aggression mouse strains showed no differences when compared to the aTNA line. No differences were observed within the high-aggression strains. aTNA strain thus is anticipated to show more ritualistic adherence to threat behaviors than the actual attack, chase behaviors.

Offense/withdrawal ratio (O/W) ratio

The offense/withdrawal rate can be considered as a simplistic index of the *sensitivity* of the offensive resident male. Table 1 shows the total offense and withdrawal frequencies and the consequent offense/withdrawal rates summed over all 3 days in the high- and intermediate-aggression strains. SAL had the highest total frequency of offense, with TA and NC900 comparable to each other. The intermediate strains were roughly 3–4 times less offensive than the high-aggression strains. In terms of the offense/withdrawal ratio, SAL showed the least withdrawal compared to TA, NC900 and the other intermediate strains. TA withdrew at a frequency comparable to the intermediate strains. TA and NC900 were thus shown to differ in terms of withdrawal, despite having comparable offense frequencies.

Table 1 Offense/withdrawal (O/W) ratios in the high- and intermediate-aggression mouse lines

Mice line	Offense (O)	Withdrawal (W)[a]	O/W ratio
SAL	1978	52	38
TA	1608	146	11
NC900	1544	56	28
aLAL	436	31	14
aTNA	603	65	9

[a] Offense-specific withdrawal is not dealt in this section. Refer to the results section for *withdrawal*-related transitions

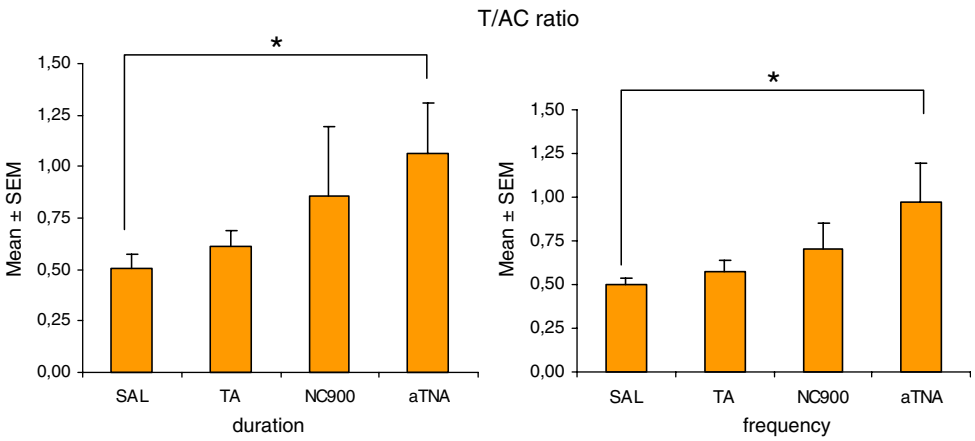


Fig. 6 Shows the T/AC ratio for the high aggression mouse strains and aTNA in terms of both duration and frequency. Data are plotted as mean ± SEM. Significant '*P*' values are represented as $*P < 0.05$

Table 2 Context dependency with respect to conscious/free-moving and anesthetized opponents in a neutral environment[a]

Intruder status	SAL	TA	NC900	aLAL	aTNA
Free-moving	8	8	8	1	5
Anesthetized	7	0	0	0	0

[a] The numbers represent the number of animals per mouse line that attacked a free-moving/anesthetized male opponent in the neutral cage

Context dependency

Context dependency is defined as the ability to discriminate between opponents according to their sex, state and the environment (neutral/home) where the agonistic interactions take place. None of the male mice attacked their female conspecific partners after the latter were returned to the cages.

Notable differences were observed, however, with respect to agonistic interactions with an immobilized opponent in a neutral environment (Table 2). All the high aggressive mice and most from the aTNA strain attacked a free-moving male conspecific in the neutral environment, while aLAL failed to. SAL was thus distinct from aLAL ($\chi^2 = 15.944$, df $= 1$, $P < 0.001$). The Turku mice showed no type differences.

With an anesthetized conspecific male, SAL showed no discrimination compared to any mouse strain and hence was distinct from TA, NC900 and aLAL ($\chi^2 = 15.944$, df $= 1$, $P < 0.001$).

In summary, SAL and TA showed the lowest T/AC ratio, although this was not significantly different from the other strains studied. SAL showed a lower O/W ratio than TA and NC900. SAL was the only strain, which failed to discriminate between free-moving and immobilized intruders. SAL showed the least opponent sensitivity as seen from the O/W ratio. SAL thus showed indications of a violent phenotype although the above measures were not sensitive enough to reveal such a distinction unequivocally. Therefore, higher-order complexities in the behavior were considered by investigating each mouse strain for specific sequential patterns and behavioral transitions, which probably holds the key in identifying different aggressive phenotypes, especially within the high-aggression mice. First-order Markov chain analysis was used for the sequential analysis.

Sequential analysis using first-order Markov chain analysis

First-order Markov analysis considers frequent transitions between pairs of behaviors (e.g. *social exploration* to *withdrawal* or vice versa) in an ethogram sequence. The data from day 3 was analyzed for this study since 'novelty' effects are expected to play a role during the first 2 days. The microstructure of offensive behaviors for each mouse line, and thereby the possibility of differential high-aggression phenotypes, were studied in detail using this statistical approach. Figures 7–11 represent the behavioral kinetograms of aLAL, aTNA, NC900, TA and SAL strains, respectively. Low-aggression mice strains were not considered for the analysis since they had very low offensive frequencies. Nevertheless, the kinetogram of aLAL is represented to illustrate the likely selection difference with SAL. Only the highly significant behavior transitions with positive residuals are discussed for the sake of simplicity. *P* values less than 0.0001 were considered the most significant and are represented as bold arrows. *P* values less than 0.001 and 0.05 are represented as blue and thin black arrows, respectively. Non-significant yet notable transitions are shown by broken arrows. Significant negative transitions for a few are shown with thick broken arrows. The box size give the frequency of occurrence of the concerned behavior. The higher the frequency, the bigger the size of these boxes. The numbers above the arrows represent the percentage of occurrence of that transition. The findings are described in terms of the resident's pre-offensive behaviors (entry) leading to offense (event) and its eventual release (exit).

Entry

SAL and TA clearly showed less social exploratory behavior (2% and 5%, respectively of the total behaviors in the inter-male interaction) than NC900 and the intermediate lines. NC900 showed 2- to 6-fold higher intruder exploration (13%) than the above strains, while aLAL explored the most (23%). aTNA was comparable to NC900 (12%).

The transitions from *social exploration* to *threat* or vice versa, *social exploration* to *withdrawal* are considered to reflect 'ritualistic' pre-offensive behaviors. The transition from *social exploration* to *threat* was 5- to 6-fold less frequent in SAL (58% = 14 transitions; $P < 0.0001$) than NC900 (45% = 79; $P < 0.0001$). TA and SAL showed less social exploration, which in SAL is a strong predictor of threat whereas in TA it significantly leads to withdrawal. Both TA and aTNA failed to show significant transitions from *social exploration* to *threat*, but did show significant transitions from *social exploration* to *withdrawal* (TA: 31% = 12; $P < 0.0001$; aTNA 24% = 28; $P < 0.0001$). Other strains failed to show any significance. The reverse transition from *threat* to *social exploration* was also investigated. TA alone showed this transition significantly (11% = 15 transitions; $P < 0.0001$). None of the strains showed significant transitions from *withdrawal* to *social exploration.* Thus, NC900 and Turku strains displayed more pre-offensive transitions than SAL, although qualitatively different as shown above.

Figure 7

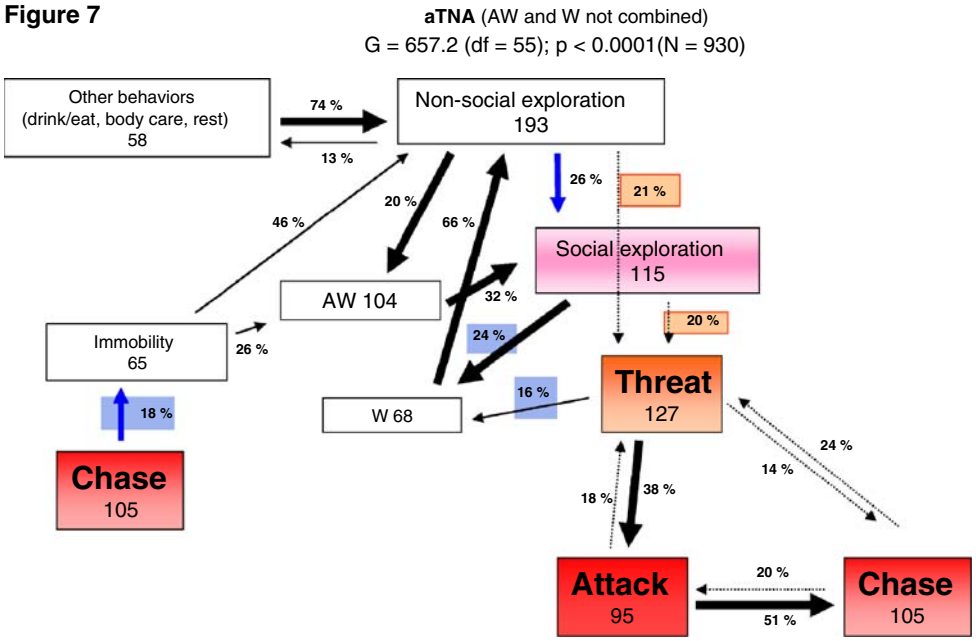
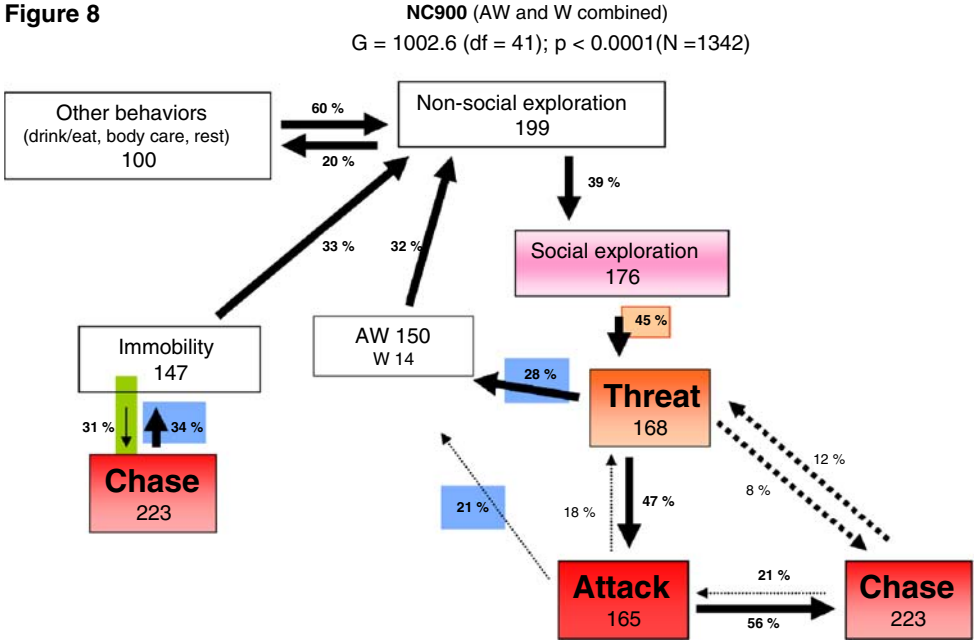


Figure 8



Figs. 7–11 Show the behavioral kinetograms for the high- and intermediate-aggression mouse strains considered for the sequential analysis. The boxes represent the behaviors of concern and their sizes denote the frequency of occurrence, relative to the total frequencies. Behavior transitions are represented as '*P*' values less than 0.0001, 0.001 and 0.05. Those transitions with $P < 0.0001$ are represented as thick arrows (▶). Those transitions with '*P*' < 0.001 are represented as blue arrows (⟶) and those with $P < 0.05$ are represented by thin black arrows (⟶). Selected non-significant behavioral transitions are represented as thin broken black arrows (·····▶). Negatively significant transitions are represented as thick broken arrows (▪ ▶). Those transition frequencies pertinent to the entry and exit to/from offense are represented as transparent red and blue boxes, respectively. The likelihood ratio statistic (G) values for the matrices are represented alongside their '*P*' values and total behavior transitions for each mouse line. The magnitude of each displayed transition is given as the % of the initiating behavior frequency alongside each arrow. Abnormal transitions are highlighted in green (as seen with NC900)

Figure 9

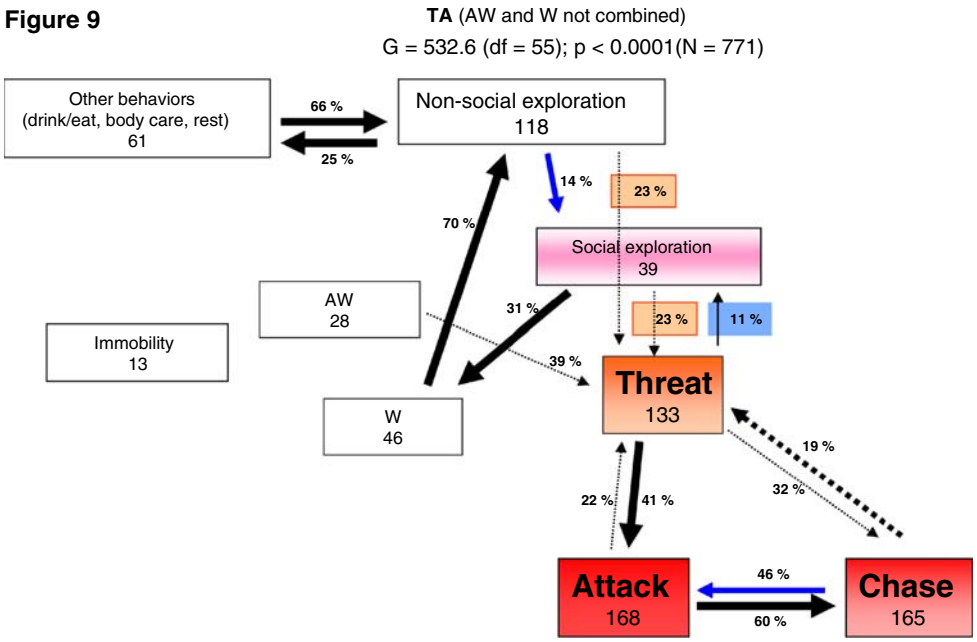
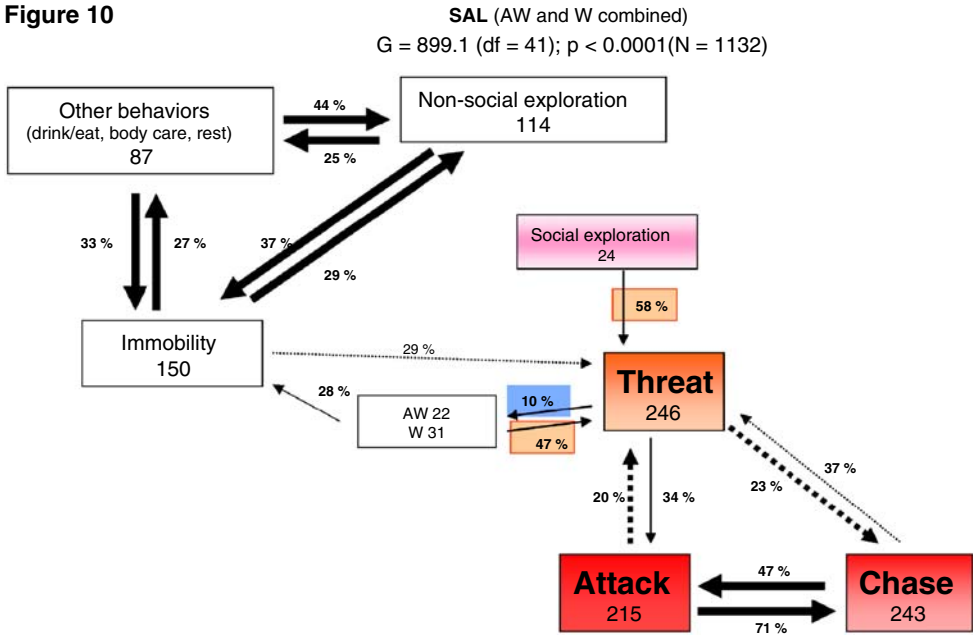


Figure 10



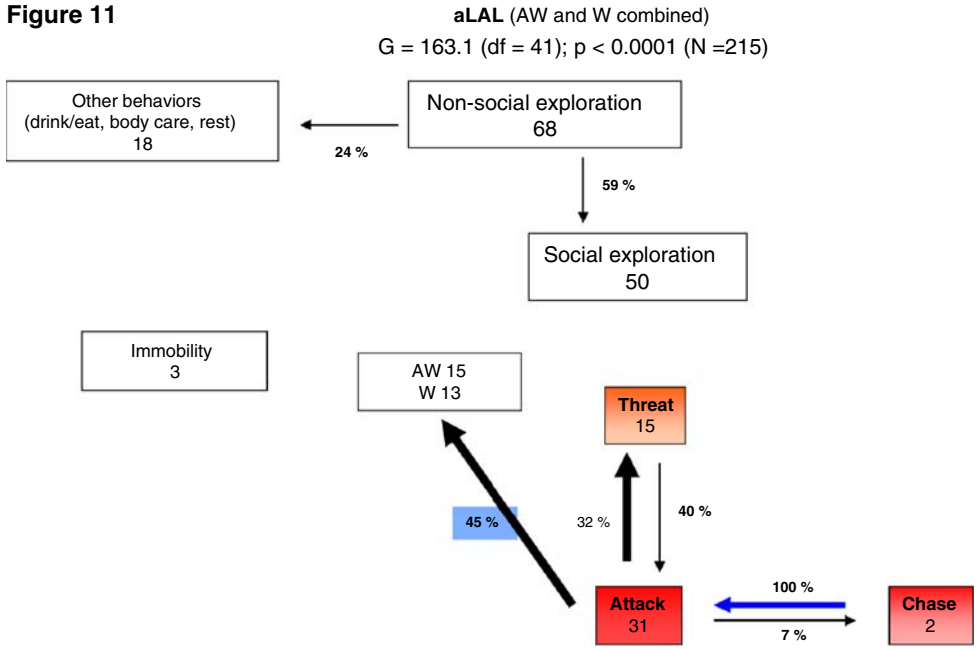
Figs. 7–11 continued

Offense

About 40% and 35% of the total transitions for SAL and TA, respectively were offense-oriented, while the corresponding figure was 16% for NC900, 15% for aTNA and about 10% for aLAL. Within the offensive transitions (*threat* to *attack, attack* to *chase*, *chase* to *threat* and their reverses), SAL showed almost double the number of significant transitions (429) than TA (230) and NC900 (245), and almost four times that of aTNA (117).

All strains showed the transition from *threat* to *attack*, with SAL (34% = 84; $P < 0.0001$) and NC900 (47% = 79; $P < 0.0001$) scoring highest. TA (41% = 54; $P < 0.0001$) and aTNA (38% = 48; $P < 0.0001$) strains showed comparable transitions in this regard, but less than SAL and NC900.

Figure 11



Figs. 7–11 continued

All strains showed a frequent attack-to-chase transition and its reverse. All strains except aLAL showed a tendency to be locked into the transitions pertaining to *chase* and *attack*. SAL showed the most locked-in attack–chase pattern, as seen from the sum of *chase* to *attack* transitions and vice-versa ($152 + 115 = 267$; $P < 0.0001$), compared to TA ($76 + 100 = 176$; $P < 0.0001$), NC900 ($46 + 93 = 139$; $P < 0.0001$ for *chase* to *attack* and $P < 0.05$ for *attack* to *chase*) and aTNA ($21 + 48 = 69$; $P < 0.05$ and $P < 0.0001$, respectively).

Further, SAL intensified offense by showing the highest number of transitions from *chase* to *threat* ($37\% = 91$; $P < 0.05$). Other strains did not show the same magnitude for this transition. While NC900 and TA displayed these transitions with a negative probability, aTNA did so non-significantly. This supports the notion of an offense-oriented agonistic interaction by SAL, as seen in Figs. 2 and 4. Thus, SAL shows the most diverse and intense offensive behavior transitions.

Exit

Transitions from offense to *approach–withdrawal* or *withdrawal* are likely to reflect the sensitivity of the resident to an opponent. The frequencies of the *approach–withdrawal* and *withdrawal* behaviors for SAL is the lowest amidst all the high-aggression strains compared (<5% of the total behaviors). Multiple direct/indirect exit routes (highlighted in blue) are observed in aTNA and NC900 strains. In the aTNA strain, offense is released from *threat* directly to *withdrawal* ($16\% = 20$; $P < 0.05$) and from *chase* indirectly to *immobility* ($18\% = 12$; $P < 0.001$). NC900 releases offense from *threat* to *approach–withdrawal/withdrawal* ($28\% = 47$; $P < 0.0001$) and *chase* to *immobility* ($34\% = 76$; $P < 0.0001$). *Immobility* is also significantly followed by *chase* in NC900 ($31\% = 46$; $P < 0.05$). This switching between chase and immobility will be discussed below. SAL showed the exit from offense via only one route, namely from *threat* to *withdrawal/approach–withdrawal* ($10\% = 25$; $P < 0.05$). TA failed to reveal any direct transitions from the offensive behaviors to withdrawal or approach–withdrawal behaviors.

Given the common transition from *social exploration* to *threat* (as discussed earlier under *entry*) in the Turku strains, the *approach–withdrawal* and *withdrawal* behaviors were however not lumped together. The analysis in SAL and NC900 were however done by pooling the transitions to *withdrawal* and *approach–withdrawal* together in the other lines, since such transitions were not observed significantly.

Further transitions from *withdrawal/approach–withdrawal* were seen to lead to *non-social exploration* in aTNA ($66\% = 45$; $P < 0.0001$); TA ($32\% = 52$; $P < 0.0001$) and NC900 ($70\% = 32$; $P < 0.0001$) mice. These transitions to *non-social exploration* do not lead back to offense. Thus, the *approach–withdrawal* behavior seems to favor release from offense in these strains. On the contrary, SAL loops back to offense (*threat)* feebly from *immobility* ($29\% = 44$; non-significant). SAL mice failed to show transitions from *withdrawal* to *non-social exploration* as

Table 3 Summary of behavioral profiles of mouse lines displaying high to moderate aggression

Features	SAL	TA	NC900	aLAL	aTNA	
Ritualistic adherence						STRUCTURE
1. Social exploration (frequency and transition)	Lowest	Low	High	Moderate	High	
2. T/AC ratio (duration, frequency)	Low	Low	Variable	NA	High	
Threat display—Duration, frequency	High[#]	High	High	Low	Low	
Immobility	High	Moderate	High	NA	Moderate	
Sensitivity to submission cues	Lowest	Moderate	Moderate	NA	High	
Free-moving conspecific male opponent/neutral cage—inhibition	Low	Low	Low	High	High	CONTEXT
Anesthetized male—inhibition	Low	High	High	High	High	
Female (observation)—inhibition	High[a]	High	High	High	High	
Abnormal behavior transitions	No	No	Yes[b]	No	No	
Offense-specific interaction	Highest	Higher	High	Moderate	No	
Magnitude	Highest	High	Moderate	Low	Moderate	
Arousal (attack latency time)[c]	Lowest	Low	Low	High	Moderate	

[#] SAL displayed uncontrolled post-agonistic *threat* behaviors even in the absence of the opponent proximity (personal observation)

[a] SAL displayed variable attacks beyond the experimental period (personal observation)

[b] NC900 showed transitions from immobility to chase

[c] Attack latency time was assessed over all 3 days of home cage testing

seen in other lines, suggestive of a qualitatively different post-offensive exit.

Thus, post-offensive transitions were found to be poor in SAL compared to the other strains. Although TA was comparable to SAL in terms of release of offense, its follow-up transitions from *withdrawal* to *non-social exploration* for instance, was at par with the other strains.

Table 3 summarizes the above findings for the high- and intermediate-aggression lines. SAL was the most noteworthy deviant of aggressive behavior with respect to both the *structure* and *context* components of agonistic interactions. Additionally, SAL ranked highest amongst all the strains in terms of *magnitude* of offense. TA followed SAL in terms of *magnitude* and *structure*.

Discussion

The present study is an ethological attempt toward identifying pathological/violent behavioral phenotypes in mice. Analysis of frequency and duration of aggressive behavior in the three selection lines clearly revealed high and low aggression levels, in line with the genetic selection. However, there are no major differences within the high aggressive selection strains in terms of duration and frequency of aggressive behaviors. Hence, a distinction between aggression and violence is not evident with these classic quantitative measures.

Among the different high-aggression strains analyzed, the behavior of the NC900 mice was rich by *structure*. In the home cage, the probabilities of transitions from *non-social exploration* to *social exploration*, from *social exploration* to *offense*, and from *offense* to *withdrawal* and related behaviors are the same, suggesting that these animals have a tendency to release from a once initiated offensive interaction. NC900 mice exit offense by *immobility* and *approach–withdrawal* behaviors leading ultimately away from offense. The transition from *immobility* to *chase* illustrates an 'ambush'-like behavior as a likely strategy of agonistic interaction in this selection line. Alternatively, these males also give an impression that they have difficulties pursuing unfamiliar conspecific males (personal observation). This may be due to a poor sensory capacity of albino strains as described by Adams et al. (2002). However, this is unlikely since the TA mice are also albinos but they failed to show the same phenomenon. The apparent difficulties of the NC900 mice to pursue opponents may also be due to their body weights. NC900 mice weigh considerably more than mice of any of the other high-aggression lines (by 5–15 g) considered for the present study, and hence are not as fast as SAL or TA mice. This can also account for high transitions from offense to *immobility* as seen from the Figs. 7–11. Moreover, these mice failed to attack immobilized opponents in the neutral cage. NC900 mice were thus clearly discriminatory by *context* and the *structure* of the behavior as characterized by a number of transitions toward and away from overt aggression with equal propensity.

SAL and TA had the highest frequency of offense-oriented behavior transitions although SAL was higher by

magnitude and showed more diverse offensive transitions than TA. On the contrary, both SAL and TA showed low T/AC ratio, suggestive of a lack of adherence to rituals during the entry phase. SAL and TA displayed repeated attack–chase transitions more than the other strains suggesting that these males are locked into an inescapable attack–chase sequence. However, SAL shows more signs of pathological behavior in that they displayed poor pre- and post-offensive transitions compared to TA. Moreover, SAL was the only strain, which consistently showed aggressive behavior out of context, as was evident in the neutral environment with immobilized intruders. Although none of the SAL males attacked their female partners for the present study, a majority of them was seen to attack their partners though after the experimental time and in general, during cage changes variably (personal observations). This study did not consider specific issues like the estrous state of the female. Caramaschi et al. (2008) has recently confirmed that SAL males do attack their female partners frequently. By contrast, the TA mice did not show any appreciable attacks towards their female counterparts even after 9 days of repeated aggressive encounters. Thus, SAL is the least inhibited strain by latency and *structure*, less discriminatory by *context* and highly aggressive by magnitude.

According to the arguments described in the introduction section, the SAL males can thus be considered violent, given the same environmental conditions experienced by all these mice strains. The above behavioral analyses using these mice strains thus conform to the definition of violence as the dysfunctional form of aggression, which is offense-oriented, uninhibited by *structure* and indiscriminate by *context*.

It is also evident that aLAL, nLAL, nTNA and NC100 are docile strains characterized by low levels of aggressive behavior, and high context dependence. aTNA is a moderately offensive animal characterized by high frequencies of approach–withdrawal and withdrawal behaviors away from offense as well as the failure to attack an immobilized opponent.

The present study made use of mouse strains artificially selected for differential aggression latencies and magnitudes. The frequency of occurrence of these differential aggressive phenotypes in a natural population is unknown. De Boer et al. (2003) demonstrated the existence of similar phenotypes however, in unselected feral rats. It might be possible that the difference between the selection lines may be related to their parental strains. The parental animals of the SAL/LAL selection lines were derived from a natural population of house mice at a stage of the population cycle with a high incidence of aggression (van Oortmerssen and Bakker 1981). The same does not apply to TA and NC900, since they were generated from lab strains.

Although field studies have proved extremely challenging, owing to complex relationships between the individuals and the environment in a given population, violence and/or intense agonistic interactions are observed to be common in dispersing societies, such as those seen in mice. Gerlach (1996) reported intense aggressive episodes amongst non-emigrating males competing for dominance, leading almost to death even within family members in a given colony. She observed that the male offspring do not wait until their fathers die before attempting to take over the dominant position in the hierarchy. Aggressive behaviors may also spiral toward violence under specific and/or unusual circumstances such as captivity (Carpenter 1934; Schaller 1963), crowding (Krebs 1970; Rowe et al. 1964; Southwick 1958), colonization, unfamiliar odors and appearances (Steiniger 1950; Calhoun 1948), limited shelter, breeding sites, mates or food (Southwick 1955), sexual conflict (Scott 1962, 1963) and skewed sex ratios (Galliard et al. 2005). Averting such circumstances has restored aggressive levels without individual mortality, for instance in fence lizards (Fitch 1941).

The current analysis describes limitations of a few methodologies including the conventional ones, below. Classic measures of aggression such as duration and frequency, although informative, fail to uncover the structural aspects of behavior. Previously, attack/threat (A/T) ratios were used by Haller et al. (2001). Given the high magnitude of attack behaviors in these highly aggressive mice lines in the present study, the ratio T/AC was used. *Threat/(attack + chase)* (T/AC) ratios are useful as rough estimates, but fall short for statistical reasons when applied to animals that fight sporadically. The offense/withdrawal (O/W) ratio used for the present paper failed to show direct transitions from offense to withdrawal. Most of these shortcomings with quantitative descriptive statistics have been surmounted using first-order Markov chain analysis. In this approach, frequency transitions between any two behaviors A and B are investigated in either direction. The use of frequency matrices for the Markov first-order sequential analysis applied well for most of the strains investigated for the present study, given their very high frequencies of attempts to interact with an opponent. aTNA, SAL, TA and NC900 all showed appreciable transitions which enabled their characterization using Markov first-order analysis. The exceptions were those lines with a low overall number of scored behaviors, for example aLAL. Markov first-order analysis has been previously applied in the study of aggression in American lobsters (Huber and Kravitz 1995) and fruit flies (Chen et al. 2002). Many ethological investigations have used the more complex continuous-time Markov chain model (CTMC) or its alternatives like the Proportional Hazards model (Haccou et al. 1988; Bressers et al. 1995; Puopolo et al. 2004).

The present study avoided the investigation of intruder behavior for several reasons. The behavior of the resident was analyzed in detail to see if violent phenotypes could be identified directly within the resident mouse itself. We cannot exclude the possibility that the violent nature of the SAL and TA mice is an artifact of the absence of escape routes within the resident–intruder paradigm. The present study shows that SAL exhibits maximal chase behaviors, suggesting that submissive behavior and immobility of the intruder does not inhibit the behavior of the 'violent' resident. Novel parameters like the intruder's behavior and proximity have been proposed by Blanchard and Blanchard (1988). Caramaschi et al. (2008) adopted these variables and found similar results.

Attack bites are an important parameter used to distinguish offensive and defensive aggressive behaviors (Blanchard and Blanchard 1984). Attack bites have not been considered in detail for the identification of violence in these mice for several reasons. Recent experiments have emphasized the complexity of attack-bite investigations, not only by including the areas attacked but also by the nature of attack, classified as either a pinch or a bruise or a wound (Litvin et al. 2007). This study also indicated a high occurrence of non-wounding pinch vocalization as the form of attack in a given conspecific agonistic interaction between the resident and the intruder. The video analysis and in particular the lighting conditions in our study did not allow a distinction between these various forms of attack bites. However, preliminary data failed to reveal specific target sites attacked by the high-aggression lines. This is in line with studies on rats that failed to discriminate in terms of both the areas attacked or the magnitude of the wound, as seen excessively over the posterior back or the ventrum of the intruder (Kruk et al. 1979). Further studies are required to assess whether these target specificities and the nature of the attack might serve to differentiate functional aggression from violence.

This study did not include a comparison with unselected control lines, owing to their unavailability. Although desirable, unselected controls could not be expected to show an intermediate offense. There are no standard reference mouse models, which could be used to make such studies control-compatible. Nevertheless, the intermediate lines (aTNA, aLAL) solve this dilemma in a loose sense with their moderate offensive behaviors and transitions. However, sufficient evidence is lacking as far as their genetic background and/or genetic drift with respect to the other extreme selected lines is concerned.

The strategy used to delineate aggression from violence used in this paper appears helpful for a fundamental understanding of aggressive behavior, whether innate or induced by environmental conditions and/or by genetic and/or pharmacological manipulations. The importance of structure and context for the distinction between deviant and functional forms of aggression is summarized pictorially in Fig. 12. The success of current pharmacological interventions targeting neurotransmitter homeostasis (including serotonin, GABA and dopamine) also relies heavily on this distinction between functional aggression and violence for clinical validation in rodents (de Almeida et al. 2005). Markov chain analysis can thus be a valuable tool that can be used in the identification of behavioral specificities of drugs at a pre-clinical level in violence research.

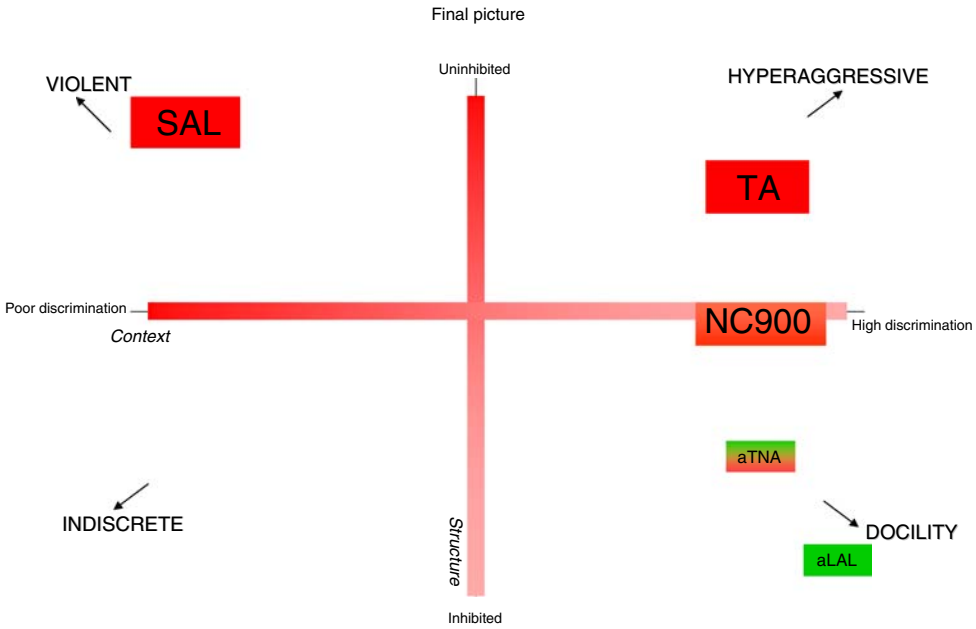


Fig. 12 Gives a behavioral overview of the distinctive delineation of violence from functional aggression and the spatial placement of the mouse strains investigated for this study. A two-tier *structure*- and *context*- based distinction was used for the identification of the phenotypes

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