Title

Social hierarchy and behavioral individuality in colonies of isogenic female mice

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Abstract

The mechanisms underlying social organization in mice have predominantly been studied in male colonies, where sociability predicts higher rank and high-ranked individuals show greater anxiety. Here, we demonstrate that groups of isogenic female mice also form stable social hierarchies. Our data indicate that females destined for high rank already exhibit greater sociability and possibly higher anxiety before group formation, and these traits remain consistent afterwards. We further investigated the influence of sex by creating mixed-sex colonies, which revealed a similar hierarchical structure, with both males and females having equal chances of becoming high- or low-ranked. We previously found reduced dopamine neuron activity in high-ranked males; in contrast, high-ranked females show the opposite pattern. Furthermore, while glucocorticoid receptor signaling in dopaminoceptive neurons restricts high rank in males, this effect is absent in females. Overall, these results highlight sex-specific mechanisms that contribute to social ranking and related behavioral traits in mice.

Introduction

The emergence of social organization is a widespread phenomenon in the animal kingdom, offering several evolutionary advantages, including the reduction of inter-individual conflicts, more efficient resource sharing, and task allocation [1, 2]. At the individual level, social status can contribute to the development of individual behavioral traits, reflecting diverse strategies for foraging, predator avoidance, and competition with conspecifics. Social hierarchy in mice is a flexible structure influenced by factors such as colony size, food availability, and territorial boundaries. In the wild, mice inhabiting large natural environments with limited resources tend to be less territorial and adopt a sedentary lifestyle only during reproduction and pup-rearing periods [3]. In contrast, commensal mice living alongside humans form smaller social groups of 4 to 8 adults, with one dominant male subordinate individuals that may be either males or females [4, 5, 6].

Over the past decade, laboratory research has intensified effort to understand the physiological mechanisms and brain circuits underlying the emergence of social organization and its impact on behavioral individuation [7-13]. In mice, social rank within a colony can be assessed through observations of antagonistic interactions, territorial marking, access to limited resources and precedence behaviors [9]. In colonies of four isogenic C57Bl/6 male mice, we recently demonstrated that highly ranked individuals exhibit enhanced anxiety and sociability compared to their lower ranked counterparts. While differences in anxiety emerged after colony formation, increased sociability in future high-ranking individuals was already evident prior to the establishment of social hierarchy. At the physiological level, we found that reducing dopamine neuron activity in the ventral tegmental area facilitates access to higher social rank, an effect that was mimicked by genetically inactivating the glucocorticoid receptor (GR) in dopamine-receiving neurons [13].

To date, most studies have focused primarily on characterizing social dominance in male mice, often excluding females from such assessments [14]. One reason may be the variability in female behavior caused by hormonal fluctuations associated with estrus [15]. Another reason is the relative absence in females of dominance-related behaviors typically used to assess hierarchy in males, such as urine marking, vocalization, and aggression [16]. The precedence tube-test frequently employed to study social hierarchy in mice, offers a way to overcome these

limitations and enables direct comparison of social organization between males and females. This test involves the encounter of two familiar individuals within a narrow plexiglass tube, which prevents turning or overlapping, the individual that forces the other to retreat is considered the winner. In males, the tube-test reveals stable social hierarchy over time and correlates with other status-related behaviors, including occupation of a warm spot in a cold environment, patterns of urine marking and outcome in food competition [7, 13]. So far only a few recent studies with variable designs, involving colonies of 2 to 5 individuals and differing genetic backgrounds (inbred *versus* outbred), have explored social organization in female colonies using the tube test [17, 18, 19, 20]. A further notable gap in the current literature, is the question concerning the influence of sex on social organization in mixed-sex colonies under laboratory conditions.

In this study, we adopted the same experimental design as in our previous work on male mice and found that C57Bl/6 female mice housed in groups of four also rapidly develop a stable social hierarchy. By assessing individual behavior before and after colony formation, we found that increased sociability, and possibly elevated anxiety, are pre-existing traits in females that ultimately attain the highest rank. To examine whether hierarchical ranking is influenced by sex, we formed mixed-sex colonies by housing a vasectomized male with three females. These groups established a hierarchical structure similar to that of unisex colonies, with both sexes showing an equal likelihood of attaining either the highest or lowest rank. In males, we previously observed reduced activity of dopamine neurons in the ventral tegmental area among high-ranking individuals, whereas females exhibited the opposite pattern. Finally, although glucocorticoid receptor signaling in dopaminoceptive neurons restricts access to the highest rank in males, this mechanism does not appear to operate in females.

Methods

Animals

C57BL/6JRj, female mice, 6 and 8 weeks old, and vasectomized 6-week-old male mice were purchased from Janvier Labs (Le Genest-Saint-Isle, France). Mice were housed under standard laboratory conditions (22°C, 55% to 65% humidity) with a 12:12h light/dark cycle (lights on at 07:00) and had *ad libitum* access to food and water. All procedures complied with the European Directive 2010/63/EU and Recommendation 2007/526/EC for animal care and use, and were approved by the Sorbonne Université animal ethics committee. *Nr3c1* (*GR*) gene inactivation was selectively targeted in dopaminoceptive neurons (*Nr3c1*$^{loxP/loxP}$;Tg:D1aCre, hereafter designed GR^{D1aCre}), as described in Ambroggi *et al.* 1999 (21). Experimental animals were obtained by crossing Nr3c1$^{loxP/loxP}$ females with Nr3c1$^{loxP/loxP}$;Tg:D1aCre males. Half of the progeny were mutant the other half littermate controls.

Constitution of tetrads

Upon arrival, mice were weighted and grouped into tetrads of four weighted-matched individuals. If behavioral testing preceded group formation, mice were singly housed for two weeks (see Fig. S1). In mixed-sex tetrads, one vasectomized male was housed with three weighted-matched females. To assess the role of GR in dopaminoceptive neurons on social rank, tetrads consisted of one GR^{D1aCre} mutant female and three age- and weight-matched control females (GR$^{loxP/loxP}$), from different litters, unfamiliar to one another.

Behavioral studies.

Prior to each experiment, mice were accustomed to the experimental room for one hour. Apparatuses were cleaned with 20% ethanol between subjects to eliminate olfactory cues.

Social rank assessment, Tube-test.

Following two weeks of group housing, mice were trained to walk through a transparent Plexiglas tube (diameter, 2.5 cm; length, 30 cm) for 2 consecutive days (8 trials on day 1, 4 trials on day 2). Each mouse entered from alternating ends and was allowed 30 seconds to traverse the tube; those failing were gently guided out. The diameter of the tube allowed only one mouse to pass and prevented turning.

Social rank was determined through all six pairwise combinations of the tetrad across repeated trials, with each trial consisting of three confrontations. Two mice were introduced simultaneously from opposite ends; the one that backed out first was considered the loser. The winner of at least two confrontations was ranked higher. Ranks were assigned from 1 (three wins) to 4 (zero wins). Trials longer than 2.5 minutes were aborted and repeated. The order of pairings followed a randomized round-robin schedule.

Ranks were initially assessed over a minimum of six days and considered stable if rank 1 and 4 remained consistent for three consecutive days. Tetrads not meeting this criterion were tested further, up to a maximum of 14 days. All 33 tetrads achieved stability. One cohort underwent repeated testing every four weeks for four months.

Anxiety-like behavior

Dark-light box. Mice were placed in a two-chamber box (45×20×25 cm) consisting of a lit (30 cm, 200–280 lux) and a dark compartment (15 cm, covered). A 5×5 cm opening connected the chambers. Mice started in the dark compartment, and exploration was recorded for 10 minutes.

Elevated O-maze. The circular black PVC maze (5.5 cm width, 56 cm outer diameter) was elevated 30 cm and divided into four sections—two open, two enclosed (15.5 cm walls). Mice started in a closed section, the head directed inward, under 200–300 lux, and behavior was recorded for 10 minutes. A mouse was considered in a section if all four paws were within it.

Despair, forced swim test

Mice were placed in a transparent cylinder (40 cm height, 12 cm diameter) filled with water (23°C, 10 cm depth) and filmed for 6 minutes. Behavior was scored as follows: i) **Escape behavior**: vigorous wall-climbing movements involving all four paws; ii) Balance movements: minimal locomotion using primarily the hind limbs; iii) Immobility: floating with no purposeful movement. The test was repeated 24 hours later if required by the experimental design.

Sociability, three-chambers test

The apparatus consisted of a rectangular box divided into three chambers (30×20×15 cm) connected via 5×5 cm doorways at the center of each partition, under 50-30 lux. Transparent perforated containers (10×7×7 cm) were placed in opposite side chambers, one containing an unfamiliar adult male (C57BL/6J), the other left empty. Mice were habituated for 5 minutes in

the central chamber with closed doors. The doors were then opened, and mice were allowed to explore freely for 7 minutes. Interaction time, defined as head-directed, close proximity contact, was recorded.

To assess **social memory**, the test was extended: the mouse was confined with the social stimulus for 5 minutes, then allowed to explore the arena again. Time spent interacting with the familiar versus a new unfamiliar mouse was scored during a second 7-minute session.

Locomotor activity

The mice were placed for 2 hours in a circular transparent PVC corridor (4.5 cm wide, 17 cm external diameter) equipped with four infrared sensors (1.5 cm above the base) at quarter intervals (Cyclotron, iMetronics, Bordeaux). Locomotor activity was recorded automatically

***In vivo* electrophysiological recordings**

Female R1 and R4 mice (3-5 months) were anesthetized with chloral hydrate (8%, 400 mg kg^{-1} *i.p.*) and placed in a stereotaxic frame. A hole was drilled in the skull above midbrain dopaminergic nuclei (coordinates: 3.0 ± 1.5 mm posterior to bregma, 1 ± 1 mm [VTA] lateral to the midline, Watson and Paxinos 2010). Recording electrodes were pulled from borosilicate glass capillaries with a Narishige electrode puller. The tips were broken under microscope control and filled with 0.5% sodium acetate. Electrodes had tip diameters of 1-2 µm and impedances of 20–50 MΩ. A reference electrode was placed in the subcutaneous tissue. The recording electrodes were lowered vertically through the hole with a microdrive. Electrical signals were amplified by a high-impedance amplifier and monitored with an oscilloscope and an audio monitor. The unit activity was digitized at 25 kHz and stored in the Spike2 software. The electrophysiological characteristics of dopamine neurons were analyzed in the active cells encountered when systematically passing the microelectrode in a stereotaxically defined block of brain tissue including the VTA (1). Its margins ranged from -2.9 to -3.5 mm posterior to bregma (AP), 0.3 to 0.6 mm (ML) and -3.9 to -5 mm ventral (DV) [22]. Sampling was initiated on the right side and then on the left side. Extracellular identification of dopamine neurons was based on their location as well as on the set of unique electrophysiological properties that distinguish dopamine from non-dopamine neurons *in vivo*: (i) a typical triphasic action potential with a marked negative deflection; (ii) a long duration (>2.0 msec); (iii) an action potential width from start to negative trough >1.1 msec; (iv) a slow firing rate (< 10 Hz and >

1 Hz). Electrophysiological recordings were analyzed using the R software[49]. Dopamine cell firing was analyzed for the average firing rate and the percentage of spikes within bursts (%SWB, the number of spikes within a burst divided by the total number of spikes). Bursts were identified as discrete events consisting of a sequence of spikes such that: their onset is defined by two consecutive spikes within an interval < 80 ms whenever and they terminate with an inter-spike interval > 160 ms. The firing rate and % of spikes within bursts were measured on successive windows of 60 seconds, with a 45 second overlapping period.

Statistical Analysis

Data are expressed as mean ± SEM. Statistical analyses included the Mann-Whitney non-parametric test, paired t-test, Wilcoxon test, Gehan-Breslow-Wilcoxon test, unpaired Kruskal-Wallis test and two-way repeated measures ANOVA. In this case, when primary effect was found to be significant, post hoc comparisons were performed using Bonferroni or Dunn correction. Statistical significance was set at $P \leq 0.05$. Analyses were performed using Prism software (GraphPad). For each panel, statistical parameters are detailed in the joined data set.

Results

Social hierarchy within a colony (tetrad) of female mice.

To determine whether female mice, like males, exhibit a social hierarchy, we formed colonies (tetrads) of four C57BL/6 females aged 6 to 9 weeks (Fig. 1 A, Fig. S1). Since body weight can influence social rank [23], we grouped together mice of similar weights. Two weeks later, we assessed the social rank of each individual using a tube test based on pairwise encounters within a plastic tube. In this test, the lower-ranked individual is identified by its retreat backward out of the tube [7] (Fig. 1A). All six possible pairwise combinations within a tetrad were tested nine times a day. The mouse with the highest number of forward exits was classified as the higher ranked. We tested each tetrad daily for at least six days, and testing continued until both top rank (rank 1 named R1) and bottom rank (rank 4 named R4) remained stable for three consecutive days. Among the 33 tetrads analyzed, the stability criterion was reached more rapidly for R1 and R4 individuals (Fig. 1B).

As previously reported in males [7, 13], social rank also influenced the duration of confrontations in females. Encounters between R1 and R2 individuals lasted an average of 9 seconds whereas confrontations involving R4 individuals were three times shorter (Fig. 1C). We next assessed the long-term stability of social organization within female colonies. The upper panel of Fig. 1D illustrates the social trajectories of individuals from 12 tetrads monitored across four sessions over a 4-month period. Among the 48 individuals, only 12 maintained a consistent rank throughout. Compared to male colonies from our previous study, the female hierarchy appeared significantly less stable (Fig. 1D, lower panel). Between sessions 1 and 2, 10 out of 12 R1 identified at the end of the first session, and 9 out of 12 R4 individuals retained their ranks (compared to 12 and 11, respectively in male colonies [13]). By session 3, the numbers declined to 9 R1 and 6 R4 (*vs*. 12 and 11 in males), and by the final session, only 4 R1 and 5 R4 individuals maintained their original ranks (*vs*. 12 and 10 in males). Notably, by the end of the 4-month period, we observed that 3 individuals initially identified as R1 had shifted to R4, and 1 R4 had moved to R1, highlighting a relatively high degree of instability in female social hierarchies. As in our study on males, female mice were weight-matched at the time of tetrad formation. However, in contrast, female initially ranked as R1 gained more weight over the four-month compared to R4 individuals, despite of the overall instability of social ranks (Fig S2).

Social rank correlates with behavioral differences.

We analyzed the relationship between social rank and behavioral phenotypes by comparing R1 and R4 individuals in assays measuring social, anxiety-like, and depression-like behaviors.

Sociability was assessed using the three-chamber test, in which mice choose between exploring a compartment containing a conspecific enclosed in a transparent box and an empty box (Fig. 2A, top left). As expected, isogenic C57BL/6 mice (pooled R1 and R4 mice) displayed a strong preference for the social stimulus, though with considerable interindividual variability (Fig. 2A, social preference, gray bars). Stratification by social rank revealed that R1 individuals exhibited a significant social preference (green bars), whereas R4 mice did not (red bars). Thus, part of the interindividual variability observed in female sociability can be attributed to social rank, as previously described in males [13].

Social memory was also assessed in the three-chamber test by comparing interaction times with a previously encountered (familiar) and a novel (unfamiliar) conspecific (Fig. 2A, top middle). In contrast to social preference, social rank did not influence social memory. Individuals from both R1 and R4 groups showed a clear preference for the unfamiliar conspecific (Fig. 2A, right panel).

We quantified anxiety-like behavior in two exploration and avoidance conflict tests, the elevated O-maze and the dark-light tests. These rely on the innate aversion of mice to open, elevated or lit environment (Fig. 2B). R1 individuals spent significantly less time in the open section of the O-maze (left green bars), and the illuminated compartment of the dark-light box (right, green bars), than R4 individuals, indicating increased anxiety levels. This pattern mirrors findings in males [13].

We also measured despair behavior in the forced-swim test. We quantified immobility and escape behaviors over a 6-minutes period. We observed no differences between R1 and R4 individuals. Both groups displayed increased immobility over the course of the session on day 1 and, as expected, a further increase on day 2 (Fig. 2C left part). Escape behavior showed the opposite (Fig. 2C right part). Finally, we did not observe differences in general locomotor activity between R1 and R4 mice (Fig. S2).

Preexisting differences in sociability and possibly anxiety-like behaviors influence social fate.

Behavioral differences between ranks may either arise as a consequence of social adaptation within the tetrad or reflect pre-existing individual traits that predispose certain individuals to

specific social outcomes, including ranking. To investigate this, we singly housed C57BL/6 females prior to group formation and assessed their sociability and anxiety-like behaviors. Following tetrad formation and subsequent rank assignment, these pre-tetrad behavioral profiles were retrospectively stratified according to the individuals' future social rank (Fig. 3A). Future R1 and R2/3 females displayed a marked sociability while future R4 females showed no significant preference for social interactions (Fig. 3B upper panel, green, blue and red bars, respectively). Social memory, however, was similar across all groups, with a consistent preference for the unfamiliar conspecific (Fig. 3B lower panel). Anxiety-like behaviors showed a more complex pattern. On one hand, in the elevated O-maze, future R1 individuals spent less time in the open sections of the maze compared to both future R2/R3 and future R4 ones, suggesting increased anxiety (Fig. 3C left panel). On the other hand, this difference was not observed in the dark-light test, where time spent in the lit compartment did not differ significantly between groups (Fig. 3C right panel). These findings indicate that social rank in female C57BL/6 mice is, at least in part, predicted by pre-existing behavioral traits, particularly sociability, and potentially anxiety-like behavior.

In mixed colonies, rank attainment is similar for males and females.

To determine whether access to a specific social rank is sex-dependent and whether a social hierarchy can be established in a mixed-sex colony, we formed colonies composed of one male C57BL/6 mouse and three female C57BL/6 mice of similar body weight (Fig. 4A, Sup. Fig. 4).

To avoid behavioral biases related to gestation or lactation, males were vasectomized. Two weeks later, we assessed social ranks using the tube test. In the 12 mixed-sex tetrads, male mice were evenly distributed across all ranks, indicating that sex does not influence position in the hierarchy (Fig.4C). As observed for female and male tetrads (this study and Battivelli *et al.* [13]), the extreme ranks in mixed colonies reached stability faster than intermediate ones (Fig. 4B). Half of the R1 and R4 individuals had a stabilized rank by day 5, whereas R2 and R3 individuals reached stability by day 10 (R1 *vs.* R2: $P < 0,05$ and R1 *vs*. R3: $P < 0,05$; R4 *vs*. R3: $P < 0,05$ and a tendency for R4 *vs*. R2: $P = 0,06$).

Notably, tube test contest between male and female mice were significantly shorter than those between two females (6,0 ± 0,3 s, n = 324 *vs.* 4,7 ± 0,3 s, n = 324, respectively; $P < 0,01$ T-test). This difference was particularly marked in R1-R3 and R2-R3 encounters (Fig S5). When repeated, one month later, 9 out of 12 R1 individuals remained at the highest rank, and 7, out

of 12 R4 individuals, kept the same ranking, somehow similar to the stability of females only tetrads (Fig. 1D).

Increased activity of dopamine neurons from the ventral tegmental area in highest ranked females.

We previously reported that R1 males exhibit reduced bursting activity of ventral tegmental area (VTA) dopamine neurons [13]. We therefore examined whether a similar difference could be observed in by comparing VTA dopamine neuron activity between R1 and R4 female mice. We performed juxtacellular single-unit recordings in anesthetized mice (Figure 5A, left). Analysis of 39 neurons from four R1 female and 40 neurons from four R4 females unexpectedly revealed that both the spontaneous firing frequency and the percentage of spikes occurring within bursts were significantly higher in R1 individuals (Figure 5, right graph). This pattern contrast sharply with what was observed in males, where R1 mice displayed similar firing frequencies but reduced bursting activity compared to R4 males.

In female mice, GR gene inactivation does not facilitate high rank attainment.

In male mice, genetic ablation of the stress-responsive GR gene in dopaminoceptive neurons promotes access to the highest social rank [13]. To assess whether the same applies to females, we housed one adult GR^{D1aCre} female with three unfamiliar control ($GR^{loxP/loxP}$) individuals (Figure 6, left panel) and evaluated social rank using the tube-test two weeks later.

Unlike in male groups, GR^{D1aCre} females did not exhibit a significantly higher average number of daily wins during the last three days of rank stabilization compared to controls (Figure 6, middle chart). A significant interaction between genotype and days even indicates that the number of wins in GR^{D1aCre} females rather decreases across the last 3 days of stabilization. Considering social rank, the absence of GR did not confer an advantage in attaining high rank. If anything, it may have slightly impaired rank acquisition, as only 4 mutant mice achieved ranks 1 or 2, whereas 8 attained ranks 3 or 4. However, this difference did not reach statistical significance.

Discussion.

The mechanisms underlying the emergence of social organization in mice have mostly been studied in male colonies. In this context, we recently reported in isogenic (C57BL/6) males that individual behavioral traits, such as sociability, facilitate the access to high social ranks, while anxiety appear to be shaped by the social environment. We also demonstrated that high-ranking males display reduced activity of VTA dopamine neurons, and that decreasing this can promote upward social mobility. Stress response also patterns male social fate: ablation of the GR gene in dopaminoceptive neurons leads to higher social ranking. In this study, we sought to determine whether the mechanisms shaping male social hierarchy also apply to females.

As reported by several authors [19, 24, 17] female mouse colonies establish social hierarchies that can be assessed through precedence-based test (e.g., tube-test) or resource competition (e.g., food or warmth) [19, 24]. Interestingly, the duration of contests in the tube test suggests that social organization may form more rapidly in females than in males, as stability is reached sooner [20]. Our data indicate, however, that female hierarchies are less stable than male ones over time since only 40% of females initially ranked 4 and 25% of those initially ranked 1 maintained their positions, compared to 100% and 80% for their male counterparts over a four-month period.

Weight has a known effect on social hierarchy, with heavier individuals often becoming dominant [23]. To control for this, we assembled weight-matched tetrads. While weight differences are unlikely to explain the sex-based differences in rank stability, we observed that initially ranked 1 females had a moderately higher average weight than rank 4 individuals, a disparity that emerged three to four weeks after group formation.

Similar to males, pre-existing behavioral traits promote rank ascension in females. Future rank 1 females were more sociable and possibly, in contrast to males, more anxious in the elevated O-maze test (but not in the dark-light box). Such divergence between anxiety tests is unusual in our experience. Increased sociability persisted after hierarchy was established, whereas increased anxiety was reinforced. An increased sociability and anxiety in highest ranked females has been reported in female dyads [19], although Varholick et al. [17] reported no anxiety differences between ranks in either sex, possibly due to limited sample size (5 pentads).

Given the behavioral and hierarchical similarities between only males and only females colonies, we questioned whether mixed-sex groups would organize hierarchically and whether rank would be sex-dependent. This is not the case, males and females had the same probability to reach each rank. Social organization in mixed colonies mirrored that of single-sex groups. However, male-female interactions in the tube test were significantly shorter than female-female interactions, suggesting that sex affects encounter dynamics.

In males, the mesocorticolimbic system has been implicated in social rank regulation. D1-receptor antagonism promotes dominance in mid-ranked mice, while D2-receptor antagonism decreases rank in both mice and macaques [25, 26]. In the prefrontal cortex, D2-receptor inactivation lowers social ranking, and *ex vivo* data show higher D1 receptor activity in top-ranked mice and increased D2 activity in lower ranks [27]. In the nucleus accumbens, D1-receptor activation is associated with social contests, and its blockade reduces dominance [28]. We previously found that VTA dopamine neuron activity is reduced in top-ranked males [13]. In line with our findings, a recent study using a similar precedence tube test reported denser mesocortical dopamine projections in highly ranked rodents (rat and mouse), while lower-ranked individuals exhibited increased dopaminergic function in the mesolimbic pathway. Interestingly this study failed to detect differences in the mesocorticolimbic dopamine system between social ranks in females. We also observed a similar sexual dimorphism in dopamine neuron activity across ranks [29]. Specifically, and in contrast to our findings in males, dopamine neurons in the VTA of rank 4 females displayed significantly lower firing rates and bursting activity compared to rank 1.

Sex-based differences in VTA activity are commonly reported in stress studies. For instance, oxytocin or its antagonists have opposing effects on social avoidance following defeat in stressed males versus females [30]. Subchronic stress reduces VTA firing rates in females but not males [31]. Estrogen may play a modulatory role in VTA excitability [32, 33], though studies in female rat triads, and large mouse colonies suggest estrus cycle does not influence social rank [34, 35]. Conversely, social rank may affect estrus cycling: dominant CD1 females exhibit longer cycles [15].

In many vertebrate species, elevated stress-induced glucocorticoid levels are affected by social rank [36, 37, 38, 39, 40, 41, 42]. Most of these studies focus on males, and those examining female

rodents reported association or absence of association [15, 43]. In male mice, we showed that GR gene inactivation in dopaminoceptive neurons promotes high rank [12, 13]. This effect was not replicated in females, where the trend may even be reversed.

Taken together, our results show that social organization emerges develops in female and male colonies. In genetically identical mice, individual pre-existing traits influences social fate. female hierarchies are more fluid and governed by distinct physiological mechanisms.

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Contributions.

F.T. and S.P conceived and designed the experiments. E.C., C.V., A.G., F.M, P.F., S.P. and F.T. acquired and analyzed the data. E.C., S.P. and F.T. wrote the manuscript, and all authors revised it.

Competing interests Statement.

The authors declare no competing interests.

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Figures

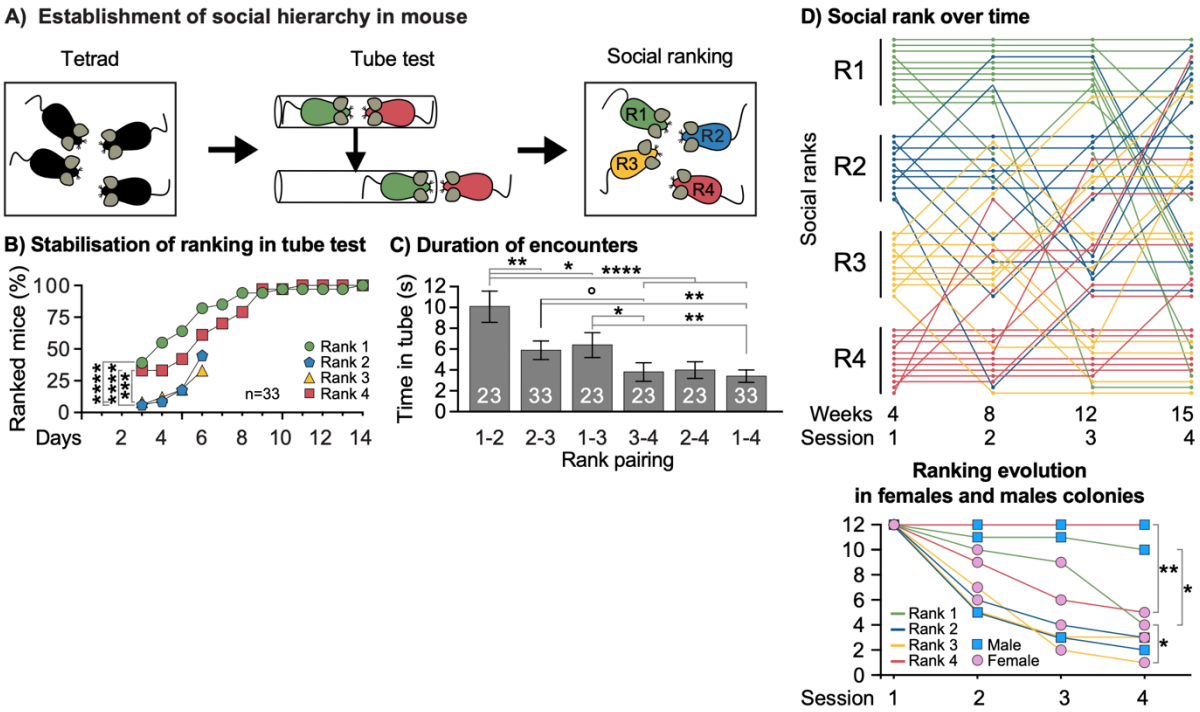


Figure 1: Establishment and stability of social hierarchy in female mice

(A) Schematic representation of social hierarchy formation and assessment. Unfamiliar female mice were grouped into tetrads (groups of four). After 2 weeks, their social rank was determined using the tube test. (**B)** Dynamics of rank identification in the tube test. The cumulative percentage of individuals with stable rank classification is plotted for each test day (n = 33 tetrads). Gehan-Breslow-Wilcoxon test, **** P < 0,0001, *** P < 0,001. Error bars represent ± SEM. (**C**) Average duration of confrontations in the tube test according to the rank of individuals, measured over the last three days of stabilization. (n = 33 tetrads). All possible rank pairings are shown. Kruskal-Wallis test (* P < 0,05; ** P < 0,01; **** P < 0,0001, ° P = 0,053). Error bars represent ± SEM. (**D)** Top panel: Longitudinal evolution of social ranks across 15 weeks in 12 tetrads. Each row represents one mouse. The position of the row indicates the tetrad it belongs to. The different colors indicate the rank: green (rank 1), blue (rank 2), orange (rank 3), red (rank 4). Bottom panel: Proportion of mice with stable ranks in female (blue dots) and male (blue squares, data from Battivelli *et al.* [13]) tetrads. The ranks are indicated by the line color. Gehan-Breslow-Wilcoxon test. * P < 0,05, ** P < 0,01. Error bars represent ± SEM.

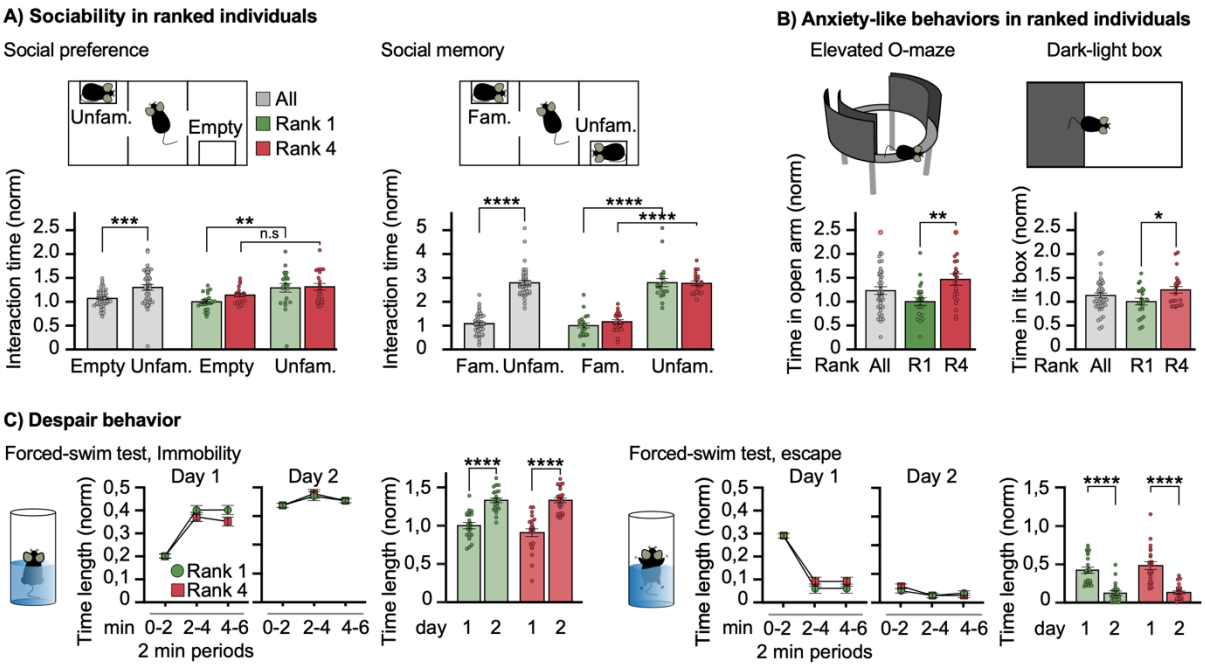


Figure 2: Behavioral differences are associated with social rank in genetically identical mice. **(A)** Higher-ranking mice show increased sociability but similar social memory compared to lower-ranking individuals. ***Social preference test*** (left panel, schematic above). Time spent interacting with an empty box (Empty) or a box containing an unfamiliar mouse (Unfam.) is shown for all C57BL/6 mice (grey, n = 42), including rank 1 (R1, green, n = 21), and rank 4 (R4, red, n = 21) individuals. Values were normalized to empty R1 mean. All mice: Wilcoxon matched pairs rank test. *** P < 0,001. R1 *vs*. R4 mice: Two-way repeated measures ANOVA, Bonferroni post hoc. No interaction effect, P = 0,38, F (1, 40) = 0,78; effect of social cue, ** P < 0,01, F (1, 40) = 11,75; no effect of social rank, P = 0,1945, F (1, 40) = 1,741. Empty box *vs.* social cue for R1 mice, ** P < 0,01. Empty box *vs*. social cue for R4 mice, P = 0,16. Error bars represent ± SEM. ***Social memory test*** (right panel). Time spent interacting with a familiar (Fam.) versus an unfamiliar (Unfam.) mouse is presented for all C57BL/6 mice (grey, n = 42), including R1 (green, n = 21) and R4 (red, n = 21) individuals. Values were normalized to familiar R1 mean. All mice: Wilcoxon matched pairs rank test. **** P < 0,0001. R1 *vs*. R4 mice: No interaction effect, P = 0,40, F (1, 40) = 0,71; familiarity effect, **** P < 0,0001, F (1, 40) = 224,9; no effect of social rank, P = 0,62, F (1, 40) = 0,25. R1: familiar *vs.* unfamiliar, **** P < 0,0001; R4: familiar *vs.* unfamiliar, **** P < 0,0001. Two-way Repeated Measures ANOVA, Bonferroni test. Error bars represent ± SEM.

(**B**) Rank 1 individuals show increased anxiety-like behavior. **Elevated O-maze test** (left): Time spent in the open sections for all C57BL/6 mice (*n* = 45), including R1 (green*, n* = 22) and R4 (red*, n* = 23) individuals. Time values were normalized to R1 mean time spent in open section. All mice: Mann Whitney test ** P < 0,01. Error bars represent ± SEM. Of note, for R4 mice a point out of scale (3,36) is indicated by a red circle. **Dark-light box test** (right): Time spent in the lit compartment for all C57B/L6 (n = 44), including R1 (green, n = 22) and R4 (red, n = 22) individuals. All mice: Mann Whitney test. * P < 0,05. Error bars represent ± SEM. (**C)** Despair-like behavior assessed by the **forced swim test** in R1 (n = 23) and R4 individuals (n = 23). Time values were normalized to the average immobility of R1 mice on Day 1. Left: Immobility duration course in 2-min blocks on Day 1 and Day 2. For Day 1, two-way repeated measures ANOVA. No interaction effect P = 0,37, F (2, 88) = 1,02, no effect of social rank P = 0,16, F (1, 44) = 2,02, effect of time: **** P < 0,0001, F (2, 88) = 103,0. For Day 2 two-way repeated measures ANOVA. No interaction effect P = 0,80, F (2, 88) = 0,23, no effect of social rank: P = 0,92, F (1, 44) = 0,01, effect of time *** P < 0,001, F (2, 88) = 8,08. Middle left: total immobility duration. Day 1 *vs*. Day 2 for R1 and R4. Two-way Repeated Measures ANOVA, Bonferroni test. **** P < 0,0001. Middle right: Escape duration course in 2-min blocks on Day 1 and Day 2. For Day 1, two-way repeated measures ANOVA. No interaction effect P = 0,47, F (2, 88) = 0,75, no effect of social rank P = 0,39, F (1, 44) = 0,75, effect of time: **** P < 0,0001, F (2, 88) = 169,1. For Day 2 two-way repeated measures ANOVA. No interaction effect P = 0,20, F (2, 88) = 1,63, no effect of social rank: P = 0,73, F (1, 44) = 0,12, effect of time ** P < 0,01, F (2, 88) = 6,09. Right: total escape duration. Day 1 *vs*. Day 2 for R1 and R4. Two-way Repeated Measures ANOVA, Bonferroni test. **** P < 0,0001. Error bars represent ± SEM.

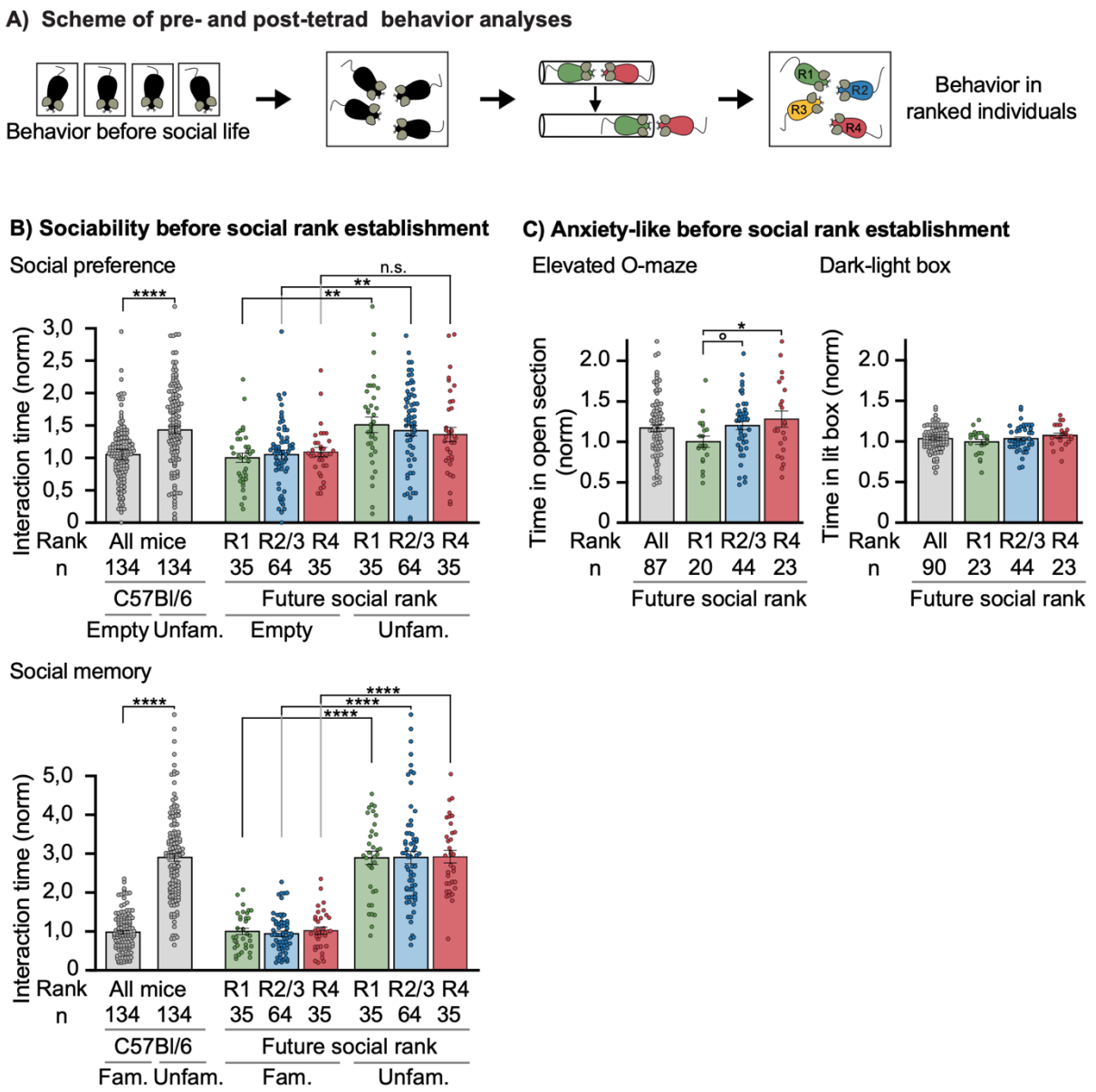


Figure 3: Differences in behavior between rank 1 and rank 4 individuals pre-exist the establishment of social hierarchies

(A) Prior to social housing, mice were singly housed and subjected to behavioral testing. (**B)** Social preference **test** (top panel): Future R4 mice did not exhibit social preference in contrast to future R1 individuals. Shown is time spent interacting with an unfamiliar conspecific (Unfam.) versus an empty box *vs*. (Empty) for 134 C57BL/6 mice from tetrads (grey bars) including the future R1 (green bars, n = 35), R2/3 (blue bars, n = 64) and R4 (red bars, n = 35) individuals. All mice: Wilcoxon matched pairs rank test. **** P < 0,0001. Ranked individuals: two-way Repeated Measures ANOVA, Bonferroni test. No effect of interaction, P = 0,55, F (2, 13) = 0,60; effect of social cue, **** P < 0,0001, F (1, 131) = 21,04; no effect of social rank, P =

0,94, F (2, 131) = 0,062. Empty box *vs*. social cue for R1 mice, ** P < 0,01; empty box *vs*. social cue for R2/3 mice, ** P < 0,01. Error bars represent ± SEM. **Social memory** (lower panel). Time spent interacting with a familiar (Fam.) mouse versus an unfamiliar (Unfam.) conspecific is presented. All mice: Wilcoxon matched pairs rank test. **** P < 0,0001. Ranked individuals: two-way Repeated Measures ANOVA, Bonferroni test. No effect of interaction, P = 0,98, F (2, 135) = 0,021; effect of familiarity, **** P < 0,0001, F (1, 135) = 314,7; no effect of social rank, P = 0,88, F (2, 135) = 0,13. Familiar *vs*. unfamiliar for R1 mice, **** P < 0,0001; Familiar *vs.* unfamiliar for R2/3 mice, **** P < 0,0001; Familiar *vs.* unfamiliar for R4 mice, **** P < 0,0001. Error bars represent ± SEM. (**C**) **Anxiety-like behavior**. **Elevated O-maze test** (left panel). Time spent in open-sections is presented. All mice (n = 87). Future rank 1 (green n = 20), Future rank 2-3 (blue, n = 44) and Future rank 4 (red, n = 23). Kruskal-Wallis test P < 0,05. Dunn's post-hoc. Differences in anxiety-like between Future R1 and Future R4: * P < 0,05; Future R1 and Future R2/3: ° P = 0,058. Error bars represent ± SEM. **Dark- light box test** (right panel). Time spent in the lit compartment is illustrated. No differences in anxiety different between ranks P = 0,23. Kruskal-Wallis test. Error bars represent ± SEM. Time values were normalized to the average value of Future R1 mice.

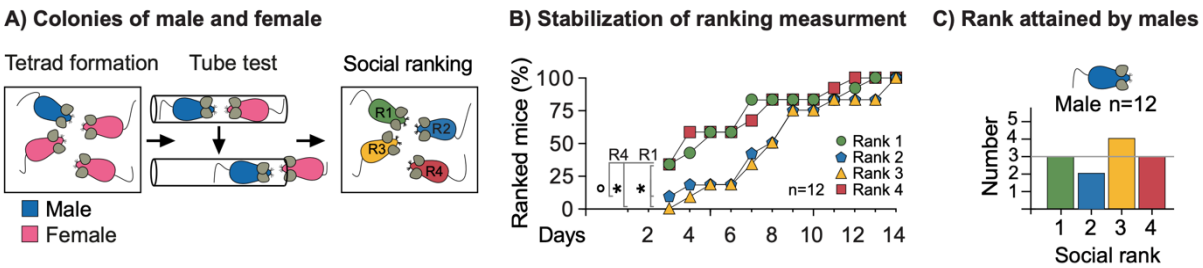


Figure 4: Social hierarchy analysis in mixed-sex tetrads

(A) Constitution of male and female tetrads and ranking analysis. (**B**) Stabilization of rank identification in the tube test. The cumulative percentage of individuals consistently classified at each rank is shown across successive days of tube testing (n = 12 tetrads). Gehan-Breslow-Wilcoxon test, R1 *vs.* R4, P = 0,93, R1 *vs.* R2, * P < 0,05, R1 *vs.* R3, * P < 0,05, R4 *vs.* R2, ° P = 0,06, R4 *vs.* R3, * P < 0,05, R3 *vs.* R2, P = 0,84. Error bars represents ± SEM. (**C**) Final social rank distribution of male mice from 12 mixed-sex tetrads.

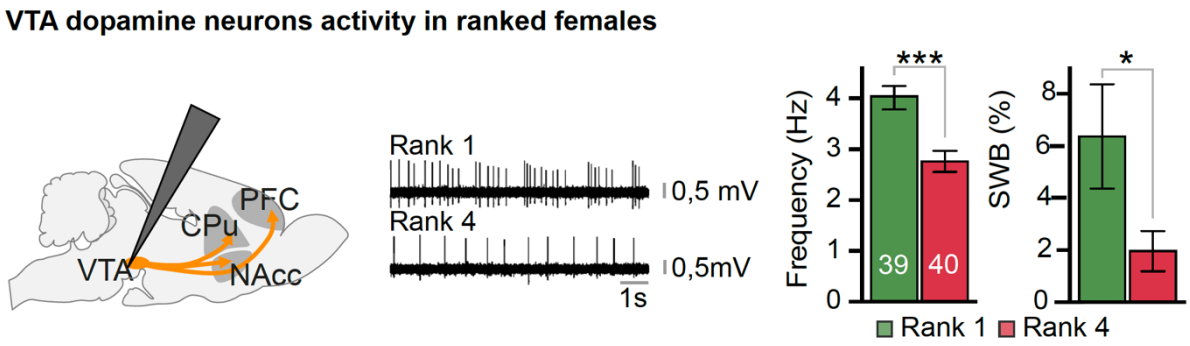


Figure 5: Dopamine neuron activity in the VTA is elevated in rank 1 females

Left: Schematic illustrating electrode placement for in vivo recordings. Middle: Representative electrophysiological traces from R1 and R4 mice. Right: Quantification of mean firing frequency (Hz) and percentage of spikes within bursts (SWB) from dopamine neurons in R1 (n = 39 cells, 4 mice) and R4 (n = 40 cells, 4 mice) animals. Frequency: R1 *vs.* R4: *** P < 0,001. R1. Percentage of spikes within bursts: R1 *vs.* R4 %SWB: * P < 0,05 (See Material and Method section). Error bars represent ± SEM.

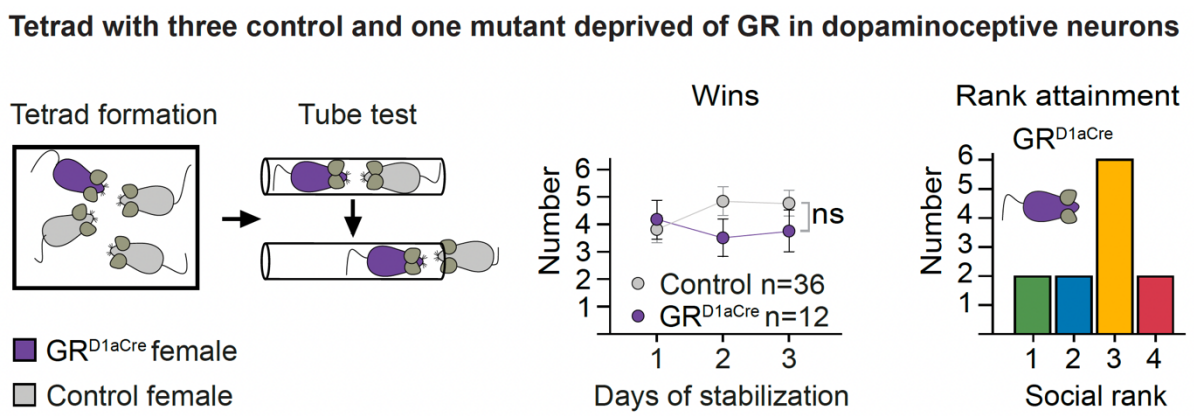
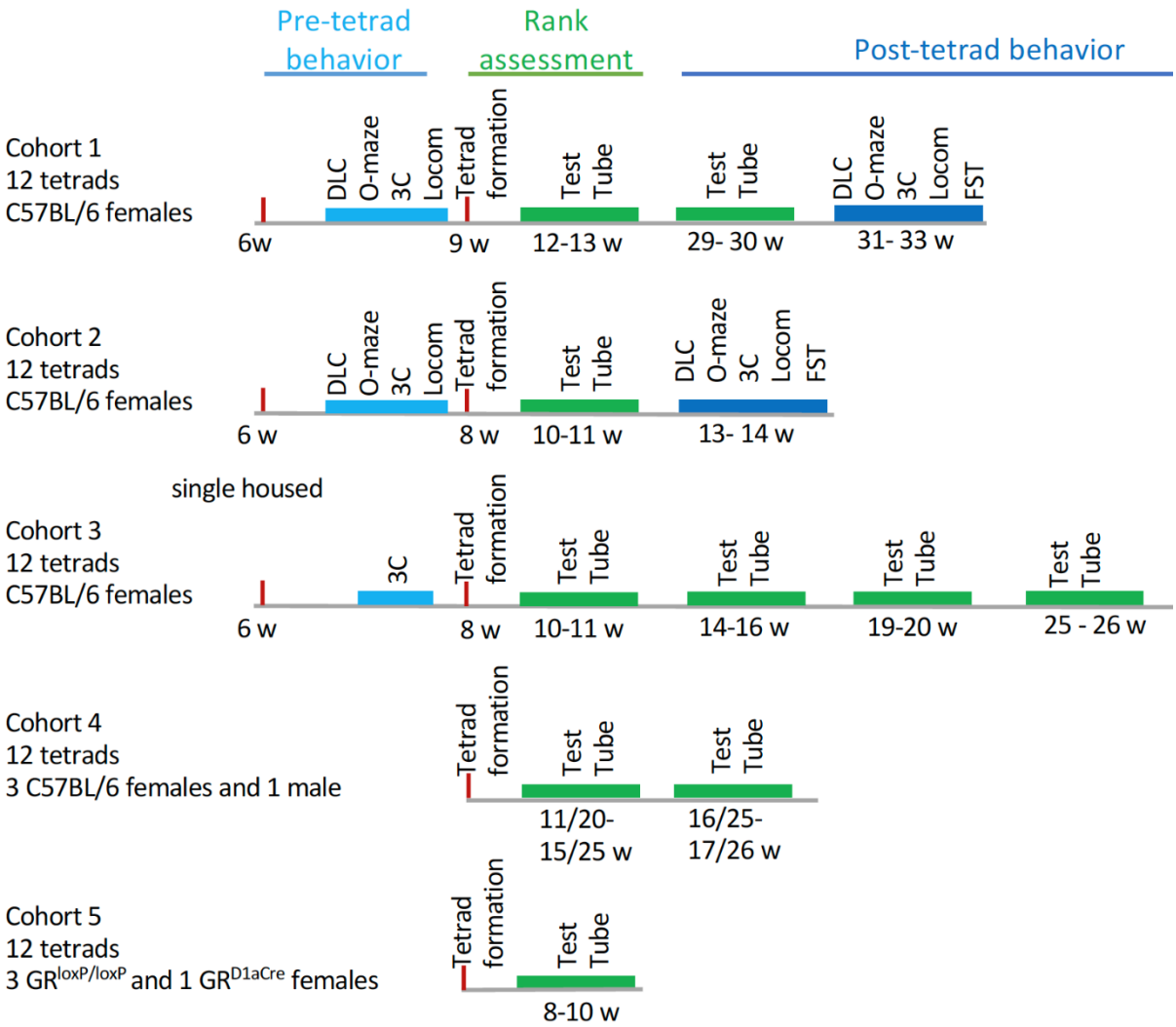


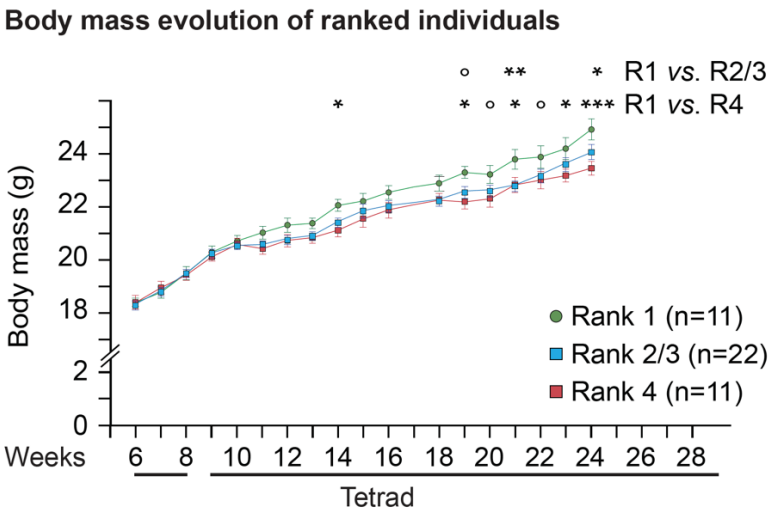
Figure 6: Disruption of GR signaling in dopaminoceptive neurons does not alter social rank attainment in female mice.

Left: Composition of tetrads containing GR^{D1aCre} mutant and control mice, and corresponding rank assignment following tube test. Middle panel: Number of cumulative wins during the last three days of stabilization period for control mice (grey) and GR^{D1aCre} mutant (purple) females. GR^{D1aCre} mutant *vs.* control mice: two-way repeated measured ANOVA, Bonferroni's test. Interaction effect, **** $P < 0{,}0001$, $F(2, 92) = 13{,}23$. Effect of time, * $P < 0{,}05$, $F(2, 92) = 3{,}308$. No effect of genotype, $P = 0{,}4773$, $F(1, 46) = 0{,}5133$. Error bars represent ± SEM. Right panel: Rank attainment of GR^{D1aCre} females from 12 tetrads.



Supplementary Figure 1. Experimental timeline for studies using ranked tetrads.

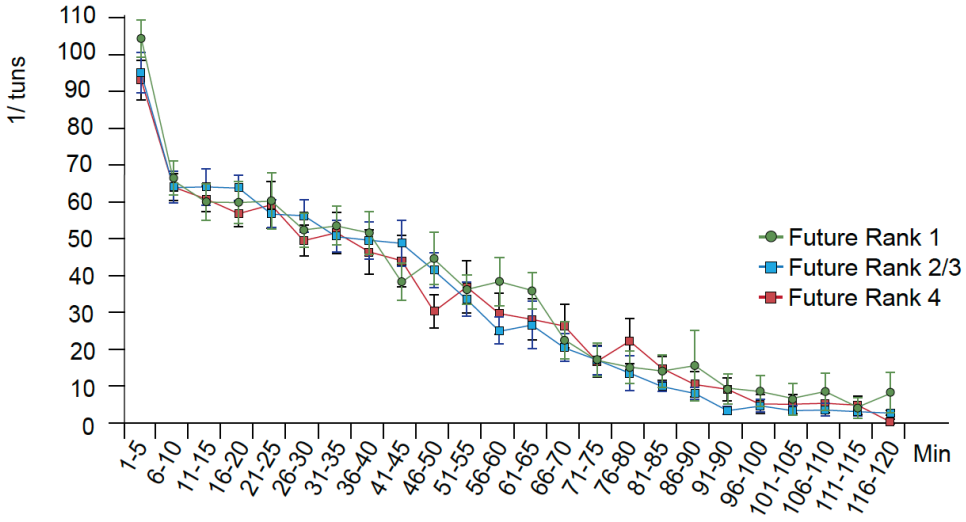
Schematic representation of the sequential experimental procedures conducted before and after social rank establishment in tetrads. Green boxes indicate ranking procedures (tube-tests), while blue boxes denote behavioral assays: DLB (Dark-Light Box), O-Maze (Elevated O-Maze), 3C (Three-Chamber Sociability Test), FST (Forced-Swim Test), and Locom (Locomotor Activity). Ages of the mice at each timepoint are indicated in weeks (w) below the boxes.



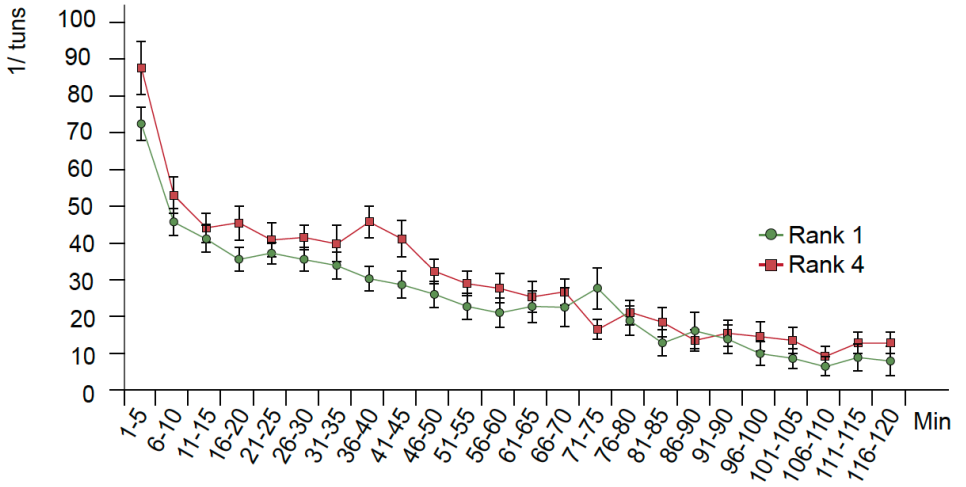
Supplementary Figure 2. Change in body mass according to rank at the first test tube.

Average weight of each rank measured over 24 weeks. Two-way repeated measured ANOVA, Bonferroni's test. No interaction effect, * P < 0,001, F (34, 697) = 2,03; effect of time, **** P < 0,0001, F (17, 697) = 359,4; no effect of rank, P = 0,12, F (2, 41) = 2,3. Error bars represent ± SEM.

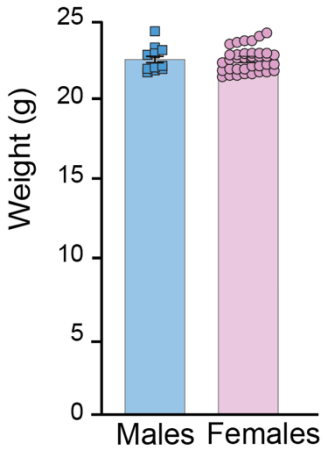
A) Locomotor activity of rank 1 and rank 4 female mice before tetrad formation



B) Locomotor activity of rank 1 and rank 4 female mice after tetrad formation



Supplementary Figure 3. Basal locomotor activity in female mice prior to social life and in ranked individuals. A) Time course of the locomotor activity of future rank 1 (green), future rank 2/3, and future rank 4 individuals prior to tetrad formation, measured as quarter turns in 5-minute intervals. Two-way repeated measured ANOVA, Bonferroni's test. No interaction effect, P = 0,62, F (46, 1587) = 0,93; effect of time, **** < 0,0001, F (23, 1587) = 144,9; no effect of rank, P = 0,76, F (2, 69) = 0,28. Error bars represent ± SEM. **B)** Time course of the locomotor activity of ranked individuals, measured as quarter turns in 5-minute intervals. Two-way repeated measured ANOVA, Bonferroni's test. Interaction effect, * < 0,05, F (23, 989) = 1,58; effect of time, **** < 0,0001, F (23, 989) = 59,0; no effect of rank, P = 0,19, F (1, 43) = 1,8. Error bars represent ± SEM.



Supplementary Figure 4. Body weight of male and female mice at the time of mixed tetrad formation. Males (blue, n = 12) and females (pink, n = 36) weights. P = 0.96. Mann-Whitney non-parametric test. Error bars represent ± SEM.