Published in final edited form as:

Behav Brain Res. 2007 January 10; 176(1): 27–39. doi:10.1016/j.bbr.2006.07.027.

Colony formation of C57BL/6J mice in visible burrow system: Identification of eusocial behaviors in a background strain for genetic animal models of autism

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Abstract

Deficits in social interaction are primary characteristics of autism, which has strong genetic components. Genetically-manipulated mouse models may provide a useful research tool to advance the investigation of genes associated with autism. To identify these genes using mouse models, behavioral assays for social relationships in the background strains must be developed. The present study examined colony formation in groups of one male and three female mice (Experiment 1) and, groups of three male mice (Experiment 2) of the C57BL/6J strain in a semi-natural visible burrow system. For adult mixed-sex colonies, four-hour observations during both the dark and light cycles for 15 days demonstrated day-dependent increases in huddling together in the chamber accompanied by decreased frequencies of active social behaviors. Sequential analyses of social interactions indicated that approaches to the back of the approached animal typically elicited flight, while approaches to the front of the approached animal failed to do so. This was seen for female to female, and for female to male approaches, as well as male to female approaches, strongly counter indicating a view that rear approach/flight specifically reflects female responsiveness to unwanted male sexual approach. For adult male colonies, similar protocols found that these social behaviors were similar to those of adult mixed-sex colonies. These findings suggest two potentially useful measures of eusocial behavior in mice, of possible value for genetic mouse models of autism; that is, huddling together and approaches to the front but not the back, of conspecifics.

Keywords

Autism model; Social behavior; Huddling; Colony formation; VBS; C57BL/6J mice

1. Introduction

Autism is a severe neurodevelopmental disorder defined by social and communication deficits and ritualistic-repetitive behaviors that are typically detectable in early childhood and continue throughout life [1–2,23]. In the original description of autism by Kanner [31],

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autistic children were characterized by the lack of interest in, and interactions with, others; whereas current DSM-IV criteria identify substantially more subtle deficits in reciprocal social interaction as sufficient for meeting criteria for this disorder [1]. Behavioral abnormalities in this domain in autism are varied and include such characteristics as deficits in non-verbal expression, abnormalities in the social use or understanding of language, and the presence of repetitive and stereotyped behaviors [2,41], as well as developmental deficits in social perception [56]. Although there is considerable evidence that autism has genetic components, possible genetic mechanisms of autism have not yet been identified [22–23,39,64–65].

Mouse models may provide a useful research tool to advance the investigation of genes associated with autism [16,27]. Recent advances in genetic technology have made it possible to create genetically-manipulated mice that harbor very specific alterations in single genes of interest [14,19,24,36]. When the targeted gene is expressed in the brain, the behavioral phenotype of the genetically-manipulated mice may reveal genetic mechanisms underlying behaviors. Mice are a relatively social species [25,37,63]. Suitable mouse models for autism would show some relationship to the types of social deficits that are considered core symptoms of autism, including very low levels of eusocial behaviors and deficits in maintaining peer relationships [23,41].

Genetic manipulation of mice often involves a congenic breeding strategy utilizing C57BL/6J and 129 substrains as backgrounds [16,57]. The characteristics of the background strain may have considerable relevance for interpreting results in genetically-altered animals [19,24], with the possibility that specific qualitative or quantitative aspects of behavior in such mice might be determined by their genetic background rather than by the loss or overexpression of gene function [16,54].

C57BL/6J mice are a standard, commercially available inbred strain, which have used in a number of studies with a focus on social components of behavior [15–16]. However, conspecific social behaviors include approach and interactive components based on sexual and aggressive, in addition to eusocial, motivations. As sexual and aggressive motivations are not notably deficient in autistic individuals [2,41], it is important to identify behaviors that are specifically eusocial, involving purely amicable motivations.

Mice are social animals that typically establish group or overlapping territories involving one male and several females [26,42,62]; often with shared nests that provide extensive underground burrow and tunnel complexes [11,40]. However, groups of adult mice that include multiple males plus females tend to produce high levels of male aggression with serious wounds, weight loss, and death [8,35,58]. In order to describe all gender combinations of social interactions in a semi-natural habitat, the present study investigated such behaviors during colony formation and maintenance in groups of one male and three female mice (Experiment 1) and in groups of three adult male mice of the C57BL/6J strain (Experiment 2). These groups were housed in large Visible Burrow Systems (VBS) enclosures providing multiple tunnels and burrows in addition to an open “surface” area [7,9].

2. Materials and methods

2.1. Animals

Animals were maintained in accordance with the Guide for the Care and Use of Laboratory Animals [NIH, 1996]. All protocols and animal handling and treatment were approved by the Institutional Animal Care and Use Committee at the University of Hawaii.

2.1.1. Experiment 1—Four male and twelve female C57BL/6J mice, aged 8 weeks at the day of colony formation, were used as the subjects. Upon arrival from Jackson Laboratories (San Diego, CA), they were housed in polypropylene cages, 26.5 × 17 × 11.5 (H) cm, in a temperature-controlled room (22±1 Celsius) for 1 week before the experiment. Female mice were group housed (each N = 3), while male mice were singly housed, with free access to food and water.

2.1.2. Experiment 2—Twenty one male mice of the C57BL/6J strain, aged 9–12 weeks at the day of colony formation, were used as the subjects. There were offspring of dams from Jackson Laboratories (San Diego, CA). The subjects had been born and reared in polypropylene cages, 26.5 × 17 × 11.5 (H) cm, in group of 2–3 male littermates after weaning at the 26 days of age, in a temperature-controlled room (22±1 Celsius).

2.2. Apparatus

Each colony (VBS) was housed in a rectangular, galvanized metal bin, 86 × 61 × 26 (H) cm (Fig. 1). Three chambers, each 12 × 7 × 6 (H) cm, were positioned behind a barrier wall extending across a short width (61 cm) of the bin, 25 cm from the end wall. This wall separated an open “surface” area (the larger area of this apparatus) from the chambers in the smaller area (see Fig.1). These chambers were connected to and opened through the wall via clear Plaxiglas tubes 5 cm in diameter. Two of the three chambers, each connected to the “surface” area via a “Z” shaped tube, were connected to each other via a straight clear Plaxiglas tube. The 3rd chamber was connected only to the surface, via a straight tube. The animals could pass freely between each chamber and the “surface” area, or between the 2 connected chambers, by these tubes. Food hoppers and water tubes were located in a far corner of the “surface”. All dividing walls and chambers were constructed of black Plexiglas, except the chamber tops which, along with the surface area top, were clear Plexiglas to permit videotaping. The floor was covered by a layer of sawdust bedding (1 cm) in all chambers as well as the surface. A video camera connected to a DVD recorder was mounted on the ceiling over the VBS.

The experiment room was illuminated on a 12:12 light-dark cycle with lights-on 07:00–19:00 hours. The colony was illuminated by fluorescent lamps during the light period of the daily light-dark cycle and by infrared light in the dark period. The temperature and humidity were maintained at 22 ± 1 Celsius and 70 % humidity.

2.3. Procedure

2.3.1. Observation of VBS colonies—Twenty-four hours prior to colony formation, subjects were marked for identification with a commercial crème-based hair dye (SALLY HANSEN DIV., DIST, Extra strength crème hair bleach). On Day 1, each group of four mice (1 male and 3 females) in Experiment 1 and three male mice in Experiment 2 were moved from the rearing room to the testing room and placed in a VBS at the beginning of the dark period of the daily light-dark cycle. Four VBS colonies were scored simultaneously in the testing room. VBS colony grouping was maintained for 15 days. There were four colonies in Experiment 1 and seven in Experiment 2. All animals of a particular colony were previously unfamiliar each other. Each colony was cleaned after recording on days 3, 7, and 11. After cleaning of VBS on days 3, 7, 11, and 15 in Experiment 2, male mice were weighed in order to check their health and social dominance-subordinate relationships [9].

Video recordings were made of each colony for 4 hours daily, on days 1, 2, 6, 10, and 14 in the dark cycle and days 2, 3, 7, 11, and 15 in the light cycle. Behaviors were analyzed by time sampling with an initial 30-sec sample being taken every 10 minutes for each mouse.

2.3.2. Definitions of behaviors—Social behaviors from the mouse ethogram [e.g. 29] included: Huddle, Being alone, Allogrooming, Self-grooming, Approach to the front or the back of the approached animal, Flight, Chasing, Following, Mounting (only Exp. 1), Struggling (only Exp. 2) and Contact. Huddle in chamber involved lying in contact with another animal for more than 10 sec of the 30-sec time sample. Being alone; when an animal was alone and at least three bodies away from the nearest neighbor, for more than 10 sec of the 30-sec time sample. Allogrooming; all licking that an animal gave to another. Approach to front; an oriented moving towards the front of another animal from a distance. Approach to back; an oriented moving towards the back of another animal from a distance. Flight; quick locomotion away from an approaching animal. Chasing; rapid locomotion towards a fleeing animal. Following; slow speed following of another animal. Mounting; the animal mounts another from the back and shakes its hip quickly. Struggling (rough-and-tumble); vigorous activity (rolling and biting) of two animals following approach of one towards the other. Contact; physical contact following oriented movement towards another animal; not counted in chambers.

Frequencies of behaviors were counted. Frequencies of huddling, being alone, allogrooming, and self-grooming were then calculated as percentages of total time within a session by dividing the frequency of each behavior by 24, the highest possible number of occurrence.

2.4. Statistical analysis

The behavioral data for females in Experiment 1 and males in Experiment 2 were analyzed by two-way analyses of variance (ANOVA) with day of testing and lighting period (dark or light period) as the within subjects factors. Because animals showed a preference for a particular chamber to stay in each colony (i.e. the chamber that later came to contain that group on at least 80% of observations during the light periods), the total scores for locations in chambers were also analyzed by three-way ANOVA with chamber (preferred, connected, or unconnected) and day of testing and lighting period as the within subjects factors. For sequential analysis of social behavior, especially the rate of flight response during the dark period, two-way ANOVAs with day of testing (DAY 1, 2, 6, 10, or 14) and type of approach (to the front or the back) as the within subjects factors were used. For multiple post hoc comparisons, Bonferroni's test was used. A probability level of $p < .05$ was used as indicating statistical significance in each test.

3. Results

3.1. Experiment 1: adult mixed-sex colony

3.1.1. Location within the VBS—The percentage of total observations in each chambers (preferred, connected, and unconnected) for each male and female are shown in Fig. 2 (Upper graphs). During the initial 4-hr (dark) period after colony formation, animals remained in the surface area during nearly 90% of observations. During the following light period, animals were usually found in chambers, and a preference for a particular chamber was already evident in each colony, as shown by over 80% of samples showing location in this chamber. During the day 1 light period, the preferred chamber was always one of the two interconnected chambers. Chamber 2, the middle chamber, was preferred by 3 colony groups, and chamber 3 by the remaining colony.

For females, a three-way ANOVA (chamber $\times$ day $\times$ lighting period) on scores for the preferred chamber, the chamber connected to it, and the unconnected chamber, was significant $F(8,88) = 11.20$, $p < .01$, as were all 3, 2-way interactions (chamber $\times$ day; chamber $\times$ lighting period; day $\times$ lighting period, $p < .05$ for each). Females spent more time in the preferred chamber during light than dark periods ($p < .05$ or less for each day), and

showed increasing preference for location in the same preferred chamber over days in dark period ($p < .05$ for each) and tended to show increasing preference in light period (Day 10, $p < .05$). While male scores were comparable to those of females, the number of males (4) did not permit a meaningful analysis of the male data.

3.1.2. Specific social behaviors—Fig. 2 (lower graphs) and Table 1-1 present the percentages of samples in which animals were engaged in specific, relatively long-duration, behaviors: huddle, being alone, allogrooming, received allogrooming, and self grooming) during dark and light periods. Fig. 3 and Table 1-2 present frequencies of other, social, behaviors during dark and light periods.

Most social behaviors followed a very clear pattern of response to lighting period and day in the VBS. For each of these behaviors -- huddle; approach (front); approach (back); contact; flight; and chase -- effects of lighting period and days, and the interaction of the two were all significant (see Table 1-3). Except for huddle, all of these behaviors were more common during the dark period; showed a reduction over days in the VBS system; and showed a greater reduction over days in the dark period compared to the light period ($p < .05$ for each)

The remaining social behavior, huddle, was higher during the light period, and increased over days within the system, with a proportionately greater increase during the dark period ($p < .05$). Although most social behaviors were more common during the dark period, huddling was so prepotent a behavior in the light period that the converse of all social behaviors, being alone, was nonetheless more common in the dark period ($p < .05$). Being alone did not change significantly over days in the system.

3.1.3. Sequential analysis—Table 1-4 (“total approach”) presents the total number of approaches to the front or the back of another animal, for males to females, females to males, and females to females during the dark period. This table also presents the responses to each such approach: i.e. flight, chase, follow, or no response. These are given as number of events, and (in parentheses) as a percentage of approaches. Approaches during the light period were so few that they did not permit a meaningful analysis of sequential data during this phase.

Flight scores by females to male approach during the dark (active) period, were analyzed by two-way ANOVA with day of testing and type of approach to the front or back as variables. ANOVA indicated significant main effects of day, $F(4,44) = 6.86$, $p < .01$, and type of approach, $F(1,11) = 25.15$, $p < .01$, as well as a significant interaction, $F(4,44) = 4.15$, $p < .01$. Males approaches to either the front or the back induced more female flight on day 1 than later; significant on days 2, 6, 10 and 14 ($p < .05$ for each). On days 1, 2, and 10, females showed greater percentage of flight to males’ approach to the back than to the front.

Two-way ANOVA on female flight scores following female approach during the dark period indicated a significant main effect of approach, $F(1,11) = 13.09$, $p < .01$ but not of day. The interaction between day and approach was not significant. Females showed more flight to female approach to the back than to the front.

The response of the 4 colony males to female approach was analyzed by a two-way ANOVA, which resulted in a significant main effect of day, $F(4,44) = 3.91$, $p < .05$, but not of type of approach. The interaction of the two was significant, $F(4,44) = 7.05$, $p < .01$. Female approach to the back produced proportionately more flight by males than frontal approach, on days 1 and 2. The proportion of male flight to female back approach was higher on day 1 than day 10.

3.2. Experiment 2: adult male colonies

3.2.1. Weight gain or loss—Because weight gain or loss provides a sensitive index of subordination stress in VBS rodent colonies [9], percentage weight gain or loss was calculated for each animal in the all-male colonies throughout the colony period [mean weight at before introduction in the VBS: 23.24 ± 0.37 (S.E.M.); weight gain at the final day of observation; Day 15: $105.89\% \pm 1.37$ (S.E.M.)]. Only a single animal (No. 3 of colony 1) showed any degree of weight loss during the grouping period (96.5% of weight gain), suggesting that these all-male colonies did not establish strong dominant-subordinate relationships. Therefore, colony males were not divided into dominant and subordinate categories, and analyses of social behaviors in the adult male colonies involved all animals.

3.2.2. Location within the VBS—The percentages of total observations in each chamber (preferred, connected, and unconnected) for male mice are presented in Fig. 4 (upper graphs). As with the mixed sex groups, male colony location scores showed a significant triple interaction (lighting period $\times$ chamber $\times$ day $F(8,160) = 8.655$, $p < .01$) with significant 2-way interactions of lighting period $\times$ chamber, lighting period $\times$ day, and chamber $\times$ day ($p < .05$ for each). After day 2 all groups showed a preference for a single chamber during both the light and the dark periods. The preferred chamber was always one of the two interconnected chambers, with chamber 2 preferred by 4 colony groups, and chamber 3 by the 3 remaining colony groups, coming to spend more time in this chamber as function of days during the both dark and light periods.

3.2.3. Specific social behaviors—Fig. 4 (lower graphs) and Table 2-1 present percentages of specific behaviors; huddle, being alone, allogrooming, and self grooming, during the dark and light periods. Fig. 5 and Table 2-2 presents frequencies of other social behaviors; approach (front), approach (back), contact, flight, chase, follow, and struggle, during the dark and light periods.

As with the mixed-sex colonies, social behaviors in these 3-male groups of mice tended to show a consistent pattern (see Table 2-3). Huddle; being alone; allogrooming; approach (front); approach (back); contact; flight; and chase, all responded significantly to lighting period, day in system, and their interaction ($p < .05$ or less for each). Self-grooming exhibited significant effects of lighting period and of the interaction of lighting period and day ($p < .05$ or less for each). Except for huddle and allogrooming (rare at all times), these behaviors were more prevalent during the dark period; and during the dark periods most were more frequent on day 1 than other days of grouping. Only chase failed to respond to any of these variables. During the light periods, social behaviors other than huddle did not show any changes over days.

Huddling was somewhat similar to that of the mixed-sex groups, higher during the light period; during the light period high even on day 1 and stable thereafter; but lower on day 1 during the dark period.

3.2.4. Sequential analysis—Table 2-4 (“total approach”) presents the total number of approaches to the front, or, to the back of another animal. This table also shows the responses to each such approach: i.e. flight, chase, follow, or no response. These are given as number of events, and (in parentheses) as a percentage of approaches. Flight scores to approach during the dark period were analyzed with type of approach (front or back) as variables in each day of testing. ANOVA indicated that the main effect of type of approach was significant, $F(1,20) = 49.41$, $p < .01$, but day, and the type $\times$ day interaction were not. Throughout the observation days, the percentage of flight to frontal approach was significantly lower than to back approach.

4. Discussion

4.1. Location within the VBS

In mixed-sex and all-male colonies animals showed an early and lasting preference for a chamber that connected to both the surface and to another chamber, a finding congruent with the tendencies of many fossorial rodents to favor tunnel/chamber systems with multiple entry/exits to the surface [7,58]. This preference appears to be based on the additional escape route possibility offered to both chambers by this dual tunnel system. With the exception of the day 1 dark (active) period, mice spent about 50–60% of their time in the open or surface area of the VBS, and virtually all the remaining time (about 40%) in the same preferred chamber in which they slept. Overall there were remarkably few sex differences in locations of animals within the VBS, with the slight exception that male colonies took about a day longer than the mixed sex groups to establish a clear pattern of chamber preference.

4.2. Specific social behaviors

In both mixed-sex and male groups, the most striking social behavior measured was “huddle”. During the light (inactive) period, this occupied about 60–80% of time for all animals. During the dark (active) period, there was no huddling on the initial grouping day, but on day 2 and thereafter, about 20–40% of time was spent huddling, typically in the same preferred chamber where inactive period huddling was concentrated.

Huddling together, in a nest if available, during the inactive phase has often been documented in mice [32–33,38]. In this study, huddling was strong even on the first day of grouping, when animals were relatively unfamiliar with each other, congruent with work indicating that C57BL/6 mice have strong tendencies to huddle with a novel animal compared to other strains, including BALB/cJ and FVB/NJ [47,52]. Huddling may function in thermoregulation [3,28]. However, the thermoregulatory capacities of C57BL/6J are in line with those of other strains such as A/Js and BALB/cLac [18,48,60], suggesting that it may be difficult to ascribe their high tendency to huddle only to thermoregulatory needs. In addition, we noted that huddling mice, particularly males in the mixed-sex groups, spent considerable time (between 10 and 20% of huddle time during the active period) in allogrooming. Allogrooming has been considered to be a component of eusocial behaviors [20,47,61], although allogrooming between adult males may include aggressive grooming, related to aggression [30]. As it was most notable here by males to females, a eusocial interpretation of allogrooming seems more likely, and in turn supports the concept that huddling has some eusocial components.

During the active period, front and back approaches were higher on day 1 of colony formation for both mixed-sex and all-male groups, declining to a slightly lower and steady level thereafter. Few approaches were made during the inactive periods. In the mixed-sex colonies, males made many more approaches to the back than did females, especially during the active period of day 1. This, along with high rates of male chasing of females, contacts with females and allogrooming of females likely constitutes part of a pattern of enhanced male sexual approach on day 1, an interpretation consonant with the mount data, also largely limited to the active period of the first day of grouping. This suggests (as do other VBS data: See [9]) that VBS grouping may bring female rodents into estrus. It is also congruent with findings that 10 of 12 females became pregnant during early stages of VBS housing, which may additionally account for a paucity of observations of copulation during succeeding test days: Male mice tend to mate promptly in the initial period of confrontation with female partners, and courtship is diminished quickly thereafter [21,49]. C57BL/6J males appear to be particularly prompt in this regard, showing lower latency to mount and intromit, longer

times of intromission, and shorter interintromission interval than BALB/cJ and DBA/2J or FVB/NT strains [13,43–44,49]. Subsequently, they showed less mounting to females than DBA/2 mice [45].

Nonetheless, both males and females showed substantially higher frequencies of social behaviors during the initial two days of grouping. This was accompanied by increased huddling together in the preferred chamber, suggesting the establishment of amicable (eusocial) relationships among males and females with increasing familiarity. The conjunction of decreases in specific social behaviors with increased huddling together over time also emphasizes that social behaviors may be part of a familiarization/information-gathering process that may be independent of eusocial motivations.

Findings of little weight loss, along with very few chases and struggles suggest that these male C57BL/6J mice did not have clear dominance-subordination relationships. This is somewhat at variance with a number of earlier findings that in established territories C57BL/6J mice display highly levels of aggression and stable dominance relationships (see [10] for review). Thus, in resident-intruder tests, C57BL/6J mice were found to be more aggressive toward an intruder compared to DBA/2J strain [51] and to additional 7 inbred mouse strains [34]. Mondragon et al [47] found that following formation of group, C57BL/6J subordinates showed more submissive to dominants and less aggression to other subordinate males, compared to BALB/cJ strain. These findings suggest that C57BL/6J mice have well-developed capacities for both offensive aggression and for the establishment and maintenance of dominance relationships. However, the VBS affords both a large living space and 3 individual chambers in addition to the “surface” area. It is possible that these opportunities for individual spacing reduced intermale aggression. Reduced aggression, specifically when opportunities for spacing are available, suggest higher levels of social fearfulness or defensiveness in this strain. This possible interpretation is supported by the Mondragon et al [47] findings of enhanced submissiveness in subordinates, and with reports [59] that C57BL/6J males show lower levels of attack to strange males.

4.3. Sequential analysis

In addition to huddling, the social behavior that was identified in this study as most promising as a measure of eusocial tendencies is, paradoxically, flight. However, it is the flight of the approached or recipient animal that may potentially serve as a marker of that animal's evaluation of the motives –eusocial or otherwise- of the approaching mouse. Approaches to the back elicited flight; those to the front did not do so.

Approaches to the back of another mouse are strongly associated with attempts to mount. As noted earlier, it is likely that the very high level of male approaches to the back for females on day 1 were associated with sexual activity. However, as all mounting observed was on that day and as females quickly became pregnant, male approaches to females from behind were unlikely to have resulted in successful mounting after the first several days of grouping. However, such approaches continued, and females continued to flee from them, a finding that does not support a view that flight by females to male back-approach represents a specific avoidance of male sexual overtures. Moreover, females reacted in the same fashion, by flight, to female approaches from behind, as did (initially) males to female approaches, again suggesting that these flight responses may have been part of a defensive response to such approach rather than a negative response to a sexual situation.

It is interesting that males initially (day 1 and 2) showed the same flight response to approach from behind by females, but that this enhanced flight to back as opposed to front approach completely disappeared later in the grouping period. This suggests enhanced habituation of defensiveness of males to female back approach with increasing familiarity,

compared to that of male to male (Exp. 2), female to male, or female to female; a characterization that is consonant with the view that males are generally less defensive to females than are females to conspecifics of either sex. In this context, the lack of female flight to frontal approach, by male or female conspecifics, suggests that such approaches are regarded as eusocial rather than threatening.

In sequential analyses of a variety of social interactions, including social play and aggressive behavior, many social interactions are initiated by approaches to the back of the approached animal [e.g. 5–6,29,46,53,62]. This angle of approach in adult mice (for whom play is vanishingly rare: [53]) is likely determined by either the role of anal gland odorants in identification of the social or reproductive status of (particularly, strange) conspecifics, or of the prevalence of back-attack targets for offensive aggression in rodents [5–6]. Both aggression and sex involve motives of the approaching animal that are more specific, and often less agreeable to the approached animal, than is approach for purely amiable or eusocial purposes. Accordingly, an approach that does not result in flight of the approached animal, regardless of its sex or the sex of the approacher, may provisionally be taken to reflect a eusocial action of the approaching animal.

4.4. Conclusion

Animal models of autistic-like behaviors have tended to emphasize conspecific social recognition and social bonding [e.g. 17,22,39,64], encompassing a variety of measures from pure observation of interactive situations through analyses of responses in tasks devised and developed to describe particular aspects of behavior. It is possible; indeed it seems likely, that many or most of these tasks will prove responsive to manipulations of genes influential in autism. The present approach was different largely in observing animals in seminatural situations designed specifically to mimic important features of the natural environments of rodents. An initially, purely descriptive, approach indicated that huddling is the most time-intensive aspect of social behavior in C57BL/6J mice. It is one that has no substantial relationship to motives such as sex and aggression, although its relationship to thermoregulation remains a possible confound. Moreover, the apparent association of huddling with allogrooming additionally suggests a eusocial component.

Sequential analysis of conspecific behaviors in response to actions initiated by the focal animal suggested a second possible index of eusocial behavior. Approaches by one animal to another were common across group formation and maintenance. If the response of the approached animal is capable of indicating what this individual regards as a eusocial approach, it is clearly the approach from the front, in contrast to those made to the back. The informational and strategic roles of back approach relative to sexual and aggressive behaviors, both of which are sometimes or often disagreeable to the approached animal, are fully in agreement with the conclusion of this sequential analysis that back approach is seldom aimed at initiating a eusocial interaction. In contrast, frontal approach does not elicit flight, in any combination of sex of the approached and approaching animal. It provides no obvious drive-reduction, homeostatic, or informational advantage. Yet it occurs, frequently and rather consistently, in groups of familiar mice. Insofar as the concept of eusocial interaction can be applied to adult mice, this would appear to be a likely candidate.

The primary symptoms of autism are the lack of social approach and maintenance of reciprocal social relationships. This suggests that detailed description of social behaviors in strains of mice specifically relevant to genetic models of autism are needed for both initial and maintenance phases of social relationships [12,15,50,55]. The present findings suggest two behavioral measures; huddling, and approach from the front, that are relatively stable across different stages of familiarization and may provide independent and useful measures of eusocial motivations in C57BL/6J mice.

Acknowledgments

This study was supported by SNRP grant #2U54NS039406-06 to DCB and JSPS Grant-in-Aid for Scientific Research No.16.1415 to HA.

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Fig. 1.

A schematic diagram of the visible burrow system using in the present experiments.

C: chamber, mesh pattern: a layer of sawdust bedding

Fig. 2.

The percentage of observations in each location of chambers (upper graphs) and spent for each huddle or being alone (lower graphs) for males (M; N= 4) and females (F; N= 12) during both the dark and light periods in Experiment 1. Data are expressed as mean percentage ($\pm$ S.E.M.). P: preferred chamber, C: connected chamber, and U: unconnected chamber. For females, * indicates significant differences between day of testing compared to day 1; $p < .05$. # indicates significant differences between chambers compared to preferred chamber; $p < .05$, in upper graphs, and between dark and light periods; $p < .05$, in lower graphs.

Fig. 3.

The mean numbers of counts that males (N= 4) and females (N= 12) displayed each behavior (frontal approach, back approach, or flight) during both the dark and light periods in Experiment 1. Data are expressed as mean counts ($\pm$ S.E.M.). For females, * indicates significant differences between day of testing compared to day 1; $p < .05$. # indicates significant differences between dark and light periods; $p < .05$.

Fig. 4.

The mean percentage of observations in each location of chambers (upper graphs) and spent for each huddle or being alone (lower graphs) for male C57BL/6J mice (N= 21) in Experiment 2. Data are expressed as the mean percentage ($\pm$ S.E.M.). * indicates significant differences between days compared to day 1; $p < .05$. # indicates significant differences between chamber compared to preferred chamber, $p < .05$, in upper graphs, while between dark and light periods, $p < .05$, in lower graphs.

Fig. 5.

The mean numbers of counts that male mice ($N=21$) displayed each behavior (frontal approach, back approach, or flight) during both the dark and light periods in Experiment 2. Data are expressed as mean counts ($\pm$ S.E.M.). * indicates significant differences between day of testing compared to day 1; $p<.05$. # indicates significant differences between dark and light periods; $p<.05$.

Table 1-1
Percentage of observations that animals spent for each behavior during the dark and light periods in Experiment 1.

Period	DAY	Allo-grooming				Self-grooming			
		Received		Received		Received		Received	
		Males	Females	Males	Females	Males	Females	Males	Females
Dark	1	3.13 (2.71)	1.74 (1.03)	3.13 (0.90)	1.74 (1.33)	10.42 (1.04)	11.46 (7.22)		
	2	10.42 (3.45)	3.47 (2.05)	4.17 (2.55)	5.56 (2.46)	9.38 (2.71)	15.97 (3.59)		
	6	8.33 (1.47)	3.47 (2.38)	3.13 (1.73)	4.17 (2.41)	12.50 (3.29)	14.93 (4.37)		
	10	3.13 (1.73)	1.04 (1.24)	0.00 (0.00)	2.08 (1.34)	4.17 (2.55)	10.76 (3.85)		
	14	5.21 (1.73)	1.74 (1.58)	1.04 (0.90)	3.47 (1.67)	4.17 (1.47)	9.38 (2.96)		
Light	2	6.25 (3.13)	0.35 (0.58)	0.00 (0.00)	2.43 (1.58)	7.29 (3.08)	8.33 (4.42) [#]		
	3	4.17 (1.47)	2.78 (1.96)	2.08 (1.04)	3.47 (1.67)	13.54 (3.08)	12.50 (4.17) [#]		
	7	4.17 (1.47)	1.39 (1.30)	1.04 (0.90)	2.43 (1.58)	2.08 (1.04)	6.60 (3.00) [#]		
	11	3.13 (1.73)	0.69 (0.78)	1.04 (0.90)	1.39 (0.98)	2.08 (1.80)	12.15 (4.46) [#]		
	15	3.13 (1.73)	0.00 (0.00)	0.00 (0.00)	1.04 (0.90)	4.17 (2.55)	7.99 (2.47) [#]		

Data are expressed as mean percentage ($\pm$ S.E.M.).

For females, * indicates significant differences between day of testing compared to day 1; $p < .05$.

[#] indicates significant differences between dark and light periods; $p < .05$.

Table 1-2

The mean numbers of counts that animals displayed each behavior during the dark and light periods in Experiment 1.

Dark period									
DAY	Received approach		Contact	Chase		Follow		Mount	
	Front	Back		Received	Received	Received	Received	Received	Received
1	13.50 (2.84)	16.50 (0.90)	25.50 (1.15)	17.50 (4.22)	2.50 (0.75)	2.75 (1.29)	4.00 (1.06)	1.75 (0.65)	0.00 (0.00)
2	9.25 (1.67)	9.00 (1.77)	17.50 (2.05)	1.25 (0.65)	1.25 (0.41)	1.00 (0.50)	1.75 (0.65)	0.25 (0.22)	0.00 (0.00)
Males	6	6.75 (1.34)	4.75 (1.19)	5.25 (1.63)	1.75 (0.89)	0.50 (0.43)	0.75 (0.41)	0.25 (0.22)	0.00 (0.00)
10	6.25 (1.71)	3.25 (0.54)	5.00 (1.84)	0.75 (0.41)	0.25 (0.22)	0.25 (0.22)	0.25 (0.22)	0.00 (0.00)	0.00 (0.00)
14	4.75 (1.67)	2.75 (0.89)	4.75 (1.47)	0.25 (0.22)	0.00 (0.00)	0.25 (0.22)	0.25 (0.22)	0.00 (0.00)	0.00 (0.00)
1	17.67 (2.17)	21.83 (2.69)	17.08 (2.23)	1.50 (0.56)	6.42 (1.66)	2.25 (0.96)	2.00 (1.04)	0.00 (0.00)	0.58 (0.38)
2	9.33 (1.94)*	7.50 (1.44)*	9.75 (1.25)	0.58 (0.38)	0.75 (0.41)*	0.83 (0.45)	0.75 (0.41)	0.00 (0.00)	0.08 (0.14)
Females	6	5.67 (0.82)*	3.33 (1.07)*	4.75 (0.72)*	0.33 (0.31)	0.75 (0.58)*	0.42 (0.25)	0.00 (0.00)	0.08 (0.14)
10	6.00 (1.37)*	3.92 (1.09)*	4.92 (0.72)*	0.58 (0.48)	0.75 (0.46)*	0.25 (0.30)	0.25 (0.30)	0.00 (0.00)	0.00 (0.00)
14	6.67 (1.70)*	3.25 (1.45)*	4.25 (0.52)*	0.33 (0.31)	0.42 (0.38)*	0.33 (0.24)	0.33 (0.24)	0.00 (0.00)	0.00 (0.00)
Light period									
DAY	Received approach		Contact	Chase		Follow		Mount	
	Front	Back		Received	Received	Received	Received	Received	Received
2	1.50 (0.25)	1.00 (0.00)	2.75 (0.65)	0.75 (0.41)	0.00 (0.00)	0.00 (0.00)	0.50 (0.25)	0.00 (0.00)	0.00 (0.00)
3	3.00 (0.94)	0.25 (0.22)	3.00 (0.79)	0.50 (0.25)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
Males	7	1.00 (0.35)	0.50 (0.43)	0.50 (0.43)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
11	0.25 (0.22)	0.75 (0.65)	1.25 (0.41)	0.00 (0.00)	0.25 (0.22)	0.00 (0.00)	0.25 (0.22)	0.00 (0.00)	0.00 (0.00)
15	0.75 (0.41)	0.75 (0.41)	1.25 (0.65)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
2	2.25 (0.87)#	1.92 (1.03)#	2.67 (0.53)#	0.00 (0.00)#	0.25 (0.30)#	0.17 (0.19)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
3	2.50 (0.52)#	0.58 (0.38)#	2.08 (0.28)#	0.08 (0.14)#	0.25 (0.30)	0.08 (0.14)	0.08 (0.14)	0.00 (0.00)	0.00 (0.00)
Females	7	1.67 (0.59)#	0.75 (0.62)#	0.08 (0.14)	0.25 (0.30)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
11	1.25 (0.71)#	0.08 (0.14)#	1.08 (0.30)#	0.08 (0.14)	0.00 (0.00)#	0.08 (0.14)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)

Dark period

DAY	Received approach		Contact	Chase	Follow		Mount	
	Front	Back			Received	Received	Received	Received
15	0.75 (0.46) [#]	0.33 (0.31) [#]	0.92 (0.28) ^{*#}	0.08 (0.14)	0.08 (0.14)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)

Data are expressed as mean counts (± S.E.M.).

For females, * indicates significant differences between day of testing compared to day 1; $p < .05$.

[#] indicates significant differences between dark and light periods; $p < .05$.

Table 1-3

Summary of the statistic results in Experiment 1.

	ANOVA results		Direction of the effects	
	Main effect		Interaction	Phase effect
	Light	Dark		
Huddle	**	**	**	↓
Being alone	**	n.s.	**	↑
Allogrooming	n.s.	n.s.	n.s.	=
Self-grooming	*	n.s.	n.s.	↑
Approach (Front)	**	**	**	↑
Received	**	**	**	↑
Approach (Back)	**	**	**	↑
Received	**	**	**	↑
Contact	**	**	**	↑
Flight	**	**	**	↑
Chase	**	*	**	↑
Received	**	**	**	↑

* $p < .05$,

** $p < .01$

↑ indicates, in period effect, behaviors were greater during the dark period than light period, while in day effect, behaviors were greater on day 2 and following days than on day 1.

↓ indicates, in period effect, behaviors were fewer during the dark period than light period, while in day effect, behaviors were fewer on day 2 and following days than on day 1.

= indicates no differences between the periods and among days of observation.

Table 1-4

The percentage of total responses to each approach to the front or back of each male or female during the dark period in Experiment 1.

DAY		Approach to front			Total approach	Approach to back			Total approach
		Flight	Chase	Follow		No response	Flight	Chase	
1	M to F	14 (15.73)	7 (7.87)	3 (3.37)	89	72 (80.90)	103 (69.13)**	62 (41.61)	149
	F to M	4 (7.55)	1 (1.89)	5 (9.43)	53	44 (83.02)	19 (29.23)*	7 (10.77)	65
	F to F	12 (8.89)	3 (2.22)	2 (1.48)	135	121 (89.63)	32 (27.59)**	5 (4.31)	116
2	M to F	5 (9.26)	0 (0.00)	2 (3.70)	54	47 (87.04)	15 (34.88)**#	8 (18.60)	43
	F to M	1 (2.70)	0 (0.00)	1 (2.70)	37	35 (94.59)	12 (33.33)*	5 (13.89)	36
	F to F	4 (6.67)	0 (0.00)	1 (1.67)	60	55 (91.67)	10 (21.74)**	2 (4.35)	46
6	M to F	1 (5.88)#	1 (5.88)	1 (5.88)	17	15 (88.24)	7 (33.33)#	6 (28.57)	21
	F to M	3 (10.71)	1 (3.57)	1 (3.57)	28	24 (85.71)	1 (5.26)	1 (5.26)	19
	F to F	2 (4.00)	1 (2.00)	0 (0.00)	50	48 (96.00)	4 (25.00)**	1 (6.25)	16
10	M to F	1 (3.13)#	1 (3.13)	0 (0.00)	32	31 (96.88)	9 (56.25)*	3 (18.75)	16
	F to M	4 (16.00)	1 (4.00)	1 (4.00)	25	20 (80.00)	1 (7.69)	0 (0.00)	13
	F to F	2 (4.88)	1 (2.44)	0 (0.00)	41	39 (95.12)	12 (41.38)**	5 (17.24)	29
14	M to F	2 (7.69)	0 (0.00)	0 (0.00)	26	24 (92.31)	3 (18.75)#	1 (6.25)	16
	F to M	0 (0.00)	0 (0.00)	0 (0.00)	19	19 (100.0)	0 (0.00)	0 (0.00)	11
	F to F	1 (1.85)	0 (0.00)	0 (0.00)	54	53 (98.15)	4 (17.39)**	4 (17.39)	23

Data are expressed as total number or events (percentage) following each approach, except or total approach which are expressed as total number or observations.

M to F: males' approach to front or back of females; F to M: Females' approach to front or back of males; and F to F: Females' approach to front or back of females.

* indicates significant differences between front and back,

* $p < .05$;

** $p < .01$.

indicates significant differences between day compared to day 1; $p < .05$.

Table 2-1
The percentage of observations that animals spent for each behavior during the dark and light periods in Experiment 2.

Dark period			Light period		
DAY	Allogrooming	Self-grooming	DAY	Allogrooming	Self-grooming
1	2.58 (0.91)	8.13 (1.67)	2	2.38 (0.72)	4.76 (0.94)
2	7.74 (1.52)	15.08 (1.95)*	3	3.97 (0.91)#	4.17 (1.35)#
6	10.52 (1.65)*	9.33 (1.22)	7	2.78 (0.76)#	3.97 (0.95)#
10	8.33 (1.25)*	12.30 (1.44)	11	3.57 (0.81)#	4.76 (1.23)#
14	7.74 (1.38)	10.52 (1.07)	15	2.58 (0.59)#	5.16 (1.01)#

Data are expressed as the mean percentage of observation ($\pm$ S.E.M.).

* indicates significant differences between days compared to day 1, $p < .05$.

indicates significant differences between light cycle, $p < .05$.

Table 2-2

The mean numbers of counts that animals displayed each behavior during the dark and light periods in Experiment 2.

Dark period					
DAY	Contact	Chase	Follow	Struggle	
1	15.29 (1.17)	4.67 (1.64)	1.05 (0.29)	0.76 (0.42)	
2	5.14 (0.77) *	0.95 (0.39)	0.62 (0.31)	0.52 (0.30)	
6	4.67 (0.88) *	1.19 (0.61)	0.76 (0.30)	0.00 (0.00)	
10	4.52 (0.68) *	0.62 (0.24)	0.62 (0.24)	0.24 (0.13)	
14	3.14 (0.57) *	0.86 (0.35)	0.48 (0.16)	0.05 (0.05)	
Light period					
DAY	Contact	Chase	Follow	Struggle	
2	1.71 (0.49) [#]	0.24 (0.16) [#]	0.43(0.17)	0.05(0.05)	
3	2.71 (0.64) [#]	0.52 (0.31)	0.38 (0.24)	0.38 (0.24)	
7	3.33 (0.48)	1.57 (0.79)	0.76 (0.23)	0.81 (0.45)	
11	0.90 (0.22) [#]	0.00 (0.00) [#]	0.10 (0.06)	0.00 (0.00)	
15	0.71 (0.17) [#]	0.00 (0.00) [#]	0.10 (0.06)	0.00 (0.00)	

Data are expressed as the mean number ($\pm$ S.E.M.).

* indicates significant differences between days compared to day 1, $p < .05$.

[#] indicates significant differences between dark and light periods, $p < .05$.

Table 2-3

Summary of the statistic results in Experiment 2.

	ANOVA results		Direction of the effects	
	Main effect		Interaction	Phase effect
	Light	Dark		
Huddle	**	**	**	↓
Being alone	**	**	*	↑
Allogrooming	**	**	**	↑
Self-grooming	*	n.s.	*	↑
Approach (Front)	**	**	**	↑
Approach (Back)	**	**	**	↑
Contact	**	**	**	↑
Flight	**	**	**	↑
Chase	**	**	**	↑

* $p < .05$,
** $p < .01$
↑ indicates, in period effect, behaviors were greater during the dark period than light period, while in day effect, behaviors were greater on following days than on day 1.
↓ indicates, in period effect, behaviors were fewer during the dark period than light period, while in day effect, behaviors were fewer on following days than on day 1.
= indicates no differences between the periods and among days of observation.

The percentage of total responses to each approach to the front or back of each male mouse during the dark period in Experiment 2.

Data are expressed as total number of events (percentage) following each approach which are expressed as total number of observations.

 $p < .01$.