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Commentary

The individuality of mice

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Mutant mice simulating human CNS disorders are used as models for therapeutic drug development. Drug evaluation requires a coherent correlation between behavioral phenotype and drug status. Variations in behavioral responses could mask such correlations, a problem highlighted by the three-site studies of Crabbe *et al.* (1999) and Wahlsten *et al.* (2003a). Factors contributing to variation are considered, focusing on differences between individual animals. Genetic differences due to minisatellite variation suggest that each mouse is genetically distinct. Effects during gestation, including maternal stress, influence later life behavior; while endocrine exchanges between fetus and parent, and between male and female fetuses dependent on intrauterine position, also contribute. Pre and perinatal nutrition and maternal attention also play a role. In adults, endocrine cyclicity in females is a recognized source of behavioral diversity. Notably, there is increasing recognition that groups of wild and laboratory mice have complex social structures, illustrated through consideration of Crowcroft (1966). Dominance status can markedly modify behavior in test paradigms addressing anxiety, locomotion and aggressiveness, to an extent comparable to mutation or drug status. Understanding how such effects amplify the behavioral spectrum displayed by otherwise identical animals will improve testing.

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Brain disorders are a challenge for drug development because of the paucity of animal models. A major thrust is to identify predisposing genes with a view to reproducing the disorder in transgenic mice (or rats). However, phenotypic variation between genetically identical animals is a complicating factor. The purpose of this Commentary is to extend an important debate on the origins of behavioral variation between inbred mice, highlighting the role of early genetic and environmental influences and later-life social status.

Variation in behavioral scores

Crabbe *et al.* (1999) reported a study in which genetically identical mice were transported to three different laboratories for parallel behavioral testing. Strikingly different results were obtained in the three laboratories, even though both the mice and the test apparatus were, as far as was practicable, exactly identical. Not all tests were subject to this variation, but some, particularly those assessing anxiety and exploratory activity, were especially susceptible.

A follow-up study extended the analysis (Wahlsten *et al.* 2003a). Some paradigms, such as ethanol preference, saw little or no test-site bias; other tests (particularly those noted earlier) confirmed that genetically identical mice appear to perform differently according to test site.

Subtle differences in test protocols may explain some of the bias, but other factors are likely to contribute. Differences in animal handling staff and worker movements are among the potential variables cited by Wahlsten *et al.*(2003) while diet and illumination might also contribute. Some of these have been reviewed previously (Würbel 2002).

However, these studies do not address a potentially important factor: individuality. Individuality is defined here as the collection of behavioral or physiological traits, both innate and acquired, that distinguish one animal from near relatives that, as far as possible, share the same genetic and environmental background. The importance of individuality is underlined by extreme differences sometimes seen between genetically identical cage-mates on standard behavioral tests (D. Wahlsten, personal communication; our unpublished observations). This parallels studies on human monozygotic (MZ) twins where, despite genetic identity, significant individual characteristics remain (see below).

Complex social and environmental factors including intertwin competition and parental attention may underlie the individuality of human MZ twins; for mice the influence of social context is often overlooked. Box 1 provides an introduction to the complexity of mouse society through consideration of Crowcroft (1966).

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Box 1. Background to mouse society

Mouse society

Given over 50 years of research in this area, systematic review would not be appropriate, though the contributions of Southwick (Southwick & Bland 1959), Davis and Christian (1957), Calhoun (1973) and Lloyd (1973) are to be noted. Instead, the complex society of the mouse is illustrated through the work of Peter Crowcroft (1922–96).

Crowcroft's diverse contributions (Lidicker & Pucek 1997) include his important book, *Mice All Over* (Crowcroft 1966) describing pest control research in the 1950s. During and after the war agricultural productivity was a high priority; significant losses in grain stores were due to infestation with mice. Conventional control methods were poorly effective; Crowcroft determined to investigate mouse populations in a near-natural setting; these experiments revealed the complexity of mouse society. Many of his observations are anecdotal; despite this they are informative for understanding the importance of social structure and how social status may reflect and contribute to diversification of behavioral phenotypes. One criticism levelled against Crowcroft's work is the attribution of human-style goals and motivations to another species. Nevertheless, this Commentary will follow Crowcroft (anthropomorphic terms are indicated in inverted commas).

Working with trapped wild mice, in a first experiment Crowcroft sequentially released male mice into a large sealed room with a rough floor and different kinds of obstacles. The first mouse explored the whole of the room and assumed 'possession'; the meaning of possession becoming clear on release of a second animal: mouse 1 chased 2 incessantly, threatening and attacking it. When mouse 3 was released, it was chased by both 1 and 2. When mice 4 and 5 followed, they took up positions at the bottom of the hierarchy. The top mice began to 'collaborate' in their domination of the other mice; 1 and 2 would sleep in the same box. The fiercely hierarchical social stratification apparent in these wild mice is reminiscent of the dominance hierarchy observed in some groups of primates (e.g. de Waal 1986).

A further study used a mixed male/female test population where nesting boxes were placed around the experimental room (Fig. 1) and the wild mice were introduced as a batch. According to Crowcroft, a mouse 'village' was quickly established: most boxes contained a single male and one or two females, though one box contained several males and several females. A final box contained only males (Table 1). To check this was not by chance, all the animals were marked *in situ*, collected together, and re-released in the area. Within a short time, with few rare exceptions, each animal made its way back to the same box and the same partner(s).

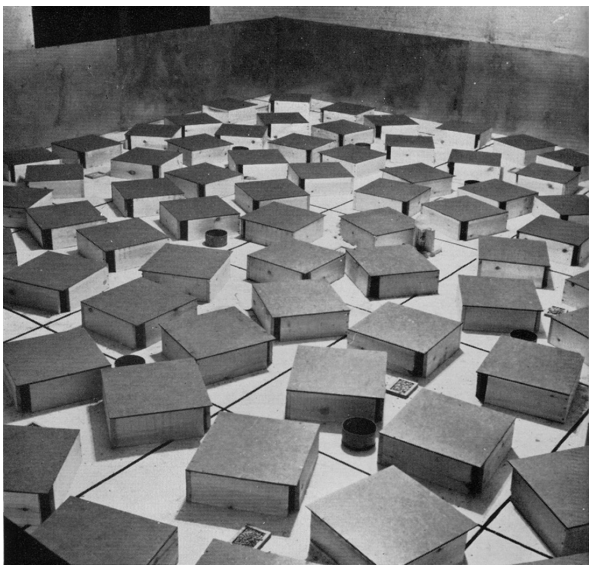

Figure 1: From Crowcroft (1966). 'Plate 5. A mouse paradise containing abundant cover and food sources'.

The mice were aggressively territorial: each home box was surrounded by an invisible line of demarcation beyond which an intruder would prompt attack by the resident male. Anecdotally, one mouse prowling around his home box would never look up. According to Crowcroft, the other mice appeared to 'take advantage' of this, and groomed themselves on top of this mouse's home box. The role of this behavior is not known; perhaps a display aimed at females.

Social exclusion is well-documented in primate society, including our own. In Crowcroft's village some males took two females, leaving a number of mate-less males. These were relegated to the bottom of the social hierarchy, were attacked when encountered by other males, and shared a home box, possibly for protection against aggressive marauders. Crowcroft noted that their coats were lustreless, pointing to endocrine changes and/or nutritional inadequacy.

Females have a distinct social behavior. Passive cooperation was the norm, though during pregnancy some became aggressive and fiercely territorial. Crowcroft recorded that females were diligent in mothering their offspring. They initiated their young into foraging, leading them cautiously but ushering them immediately back to the home box when danger threatens. Again risking anthropomorphism, in Crowcroft's words: 'Watching this female mouse ''mother'' her children and lead them about I felt that her

Table 1: Distribution of 56 mice in 14 nesting boxes (Crowcroft 1966)

BOX	a	b	c	d	e	f	g	h	k	m	n	p	q	r
Males	**8**	**13**	0	**1**	0	**1**	0	**1**	**1**	0	**1**	**1**	0	**1**
Females	0	**19**	0	**2**	0	**1**	0	**1**	**1**	0	**1**	**1**	0	**2**

behavior at this stage was as plastic and as relatively subject to her discretion and experience as that of a woman'. Crowcroft recorded how males also contribute to child-care, reporting that a male, encountering unsupervised pups away from the home box 'picked up the other three young and took them in tow behind him, as if by magnetism, and led them straight back to the nest box containing the mother' (Crowcroft 1966).

Aggressive territorial behavior in lab mice

Crowcroft's experiments predominantly employed trapped wild mice. In contrast, laboratory mice have been inbred for generations, with the risk of fixing deleterious alleles, perhaps producing mouse strains that are in some way disabled. Crowcroft addressed this by introducing lab mice into a fresh room. However, unfortunately he did not record the specific strains. Initially the lab animals were disinterested and unaggressive, suggesting that some typical behaviors are suppressed by cage-rearing, but after a while their behavior recovered, chasing and fighting like wild mice until a social hierarchy was established. Then, when a wild mouse chanced to invade this territory of lab mice, the incomer was overpowered by the strangely coloured residents, and was relegated to the bottom of the hierarchy. 'It seemed very odd to see a genuine mouse being chased around the room by an artificial one', Crowcroft wrote. Despite cage housing for generations, the lab mice had not lost the capacity for aggressive and effective territorial behavior. It remains to be seen whether this is generally true of inbred strains.

It would be inappropriate to summarize all Crowcroft's experiments, but his work demonstrates that mice in a near-natural environment are intensely social and hierarchical. Throughout, parallels with human behavior are noted, but Crowcroft was aware of this potential trap, stating: 'The more I observed mice, the more I came to recognize elements of the behavior of my fellow men, and the more I began to understand both species'. Attributes such as territoriality, cooperation and social exclusion appear evolutionarily ancient in the mammalian lineage: mice and humans share a common ancestor ~75 Myr ago (O'Brien & Woychik 2003).

Individuality: a combination of factors

This Commentary seeks to address three central questions. Firstly, what is the mechanistic origin of the variation (individuality) observed in otherwise identical animals? Secondly, to what extent do early individual differences contribute to later-life social status? Thirdly, does individuality (including social effects) influence the outcome of relevant behavioral or pharmacologic tests?

The individual phenotype of an adult mouse is influenced by multiple interacting factors. In addition to dominance/subordination status (Box 1), and acquired characteristics (including injuries and infection that can be controlled by the experimenter), other important factors may include temperament, endocrine status, possible genetic non-identity and early environmental effects, both *in utero* and in the postnatal period, that together contribute to long-lasting behavioral predispositions.

Temperament

This factor is considered first because, of all parameters influencing behavior, it is the least well documented. In human, a quality of behavior dubbed 'personality' or 'temperament' is widely recognized. A case for ascribing personality to experimental animals has been discussed, but not comprehensively (Gosling 2001; Vazire & Gosling 2003). Crowcroft (1966) recognized distinct behaviors in different animals; D. Wahlsten (personal communication) confirms that genetically identical littermates can behave very differently. We have observed individuals (from a genetically identical cohort) that can be distinguished by their behavior. As a pointer, singular behaviors were timidity; the converse, i.e. ignorance of risk; and failure to participate in the test procedure (unpublished data). These behaviors, while evident to the experimenter, were not specifically tabulated. The Wahlsten *et al*. (2003a) study notes that a fraction of (identical) animals on the elevated plus maze spent all the test period in the centre of the maze. Wahlsten *et al*. (2003b) discuss the failure of some mice to participate in tests.

Though systematic assessment may be warranted, in its absence the case for temperament as a specific component of individuality remains unproven. However, it does emphasize differences between individuals sharing (as far as possible) an identical genetic complement, and demands a mechanistic explanation.

Early origins of individuality

Minisatellite variation

Sporadic genetic changes in inbred mice potentially could add to both individuality and temperament. Although new point mutations are rare (Keightley & Hill 1992), the mammalian genome is peppered with repeat elements including minisatellites (or VNTRs; variable numbers of tandem repeats) and transposable elements. It is possible, if not likely, that minisatellite variation contributes to individuality. A single human chromosome contains over 14 000 tandem repeats (Dunham *et al*. 1999) of which one third meet the criteria for minisatellites (Vergnaud & Denoeud 2000). This would suggest up to 100 000 per genome. In human these are enriched towards chromosome ends; in rodents there is much less bias (Vergnaud & Denoeud 2000). For most loci, the frequency of spontaneous variation hovers very approximately around 10–4 per gamete per generation (Bois *et al*. 1998), pointing to ~10 changes per genome per generation. Moreover, a proportion of minisatellites (~3%) show a greatly increased rate of variation: in the 10 known human hypermutable sites reviewed by Vergnaud and Denoeud (2000), but excluding one massively unstable locus, the average change was 2.6% per gamete per generation. If minisatellite changes occur independently of one another, conservative figures (50 000 sites, 1% hypermutable, each at 2% per generation) predict a minimum of 10 changes per gamete, twice that in the diploid genome (overall, 30+ minisatellite changes from baseline per individual).

The extent to which these influence phenotype is not fully known, but variation at particular sites can have marked effects. A VNTR minisatellite upstream of the human insulin gene influences the development of type I diabetes (Bennett *et al*. 1995; reviewed by Stead & Jeffreys 2000) and juvenile obesity (Le Stunff *et al*. 2000). Regarding behavioral phenotypes, diabetes (type II) is associated with depression, and a second minisatellite at the tyrosine hydroxylase locus was associated with both insulin resistance and depressive symptoms (Chiba *et al*. 2000).

The situation in mice is less clear; minisatellite location is not generally conserved between species. Even so, the high rate of minisatellite variation is likely to contribute, at least in part, to the development of individual behavioral characteristics in otherwise genetically identical animals.

Intrauterine position

In rodents, multiple fetuses are arrayed linearly in the two horns of the uterus. Hormonal exchange takes place between adjacent fetuses. In consequence, a fetus flanked by two male fetuses (termed 2M) is in a subtly different hormonal milieu from one flanked by two females (0M or 2F) (Fig. 2). This influences development and later-life behavior (reviewed by Ryan & Vandenbergh 2002).

The development of male-type behavior is regulated, in part, by perinatal exposure to androgens, and particularly testosterone (vom Saal & Bronson 1980; MacLusky & Naftolin 1981) which, following aromatization to estrogen in the CNS, alter the development of brain nuclei concerned with reproductive and aggressive behaviors, among others. Testosterone (converted to estrogen) is not the only determinant; multiple hormones and receptors contribute to masculinization (Hutchison *et al*. 1999).

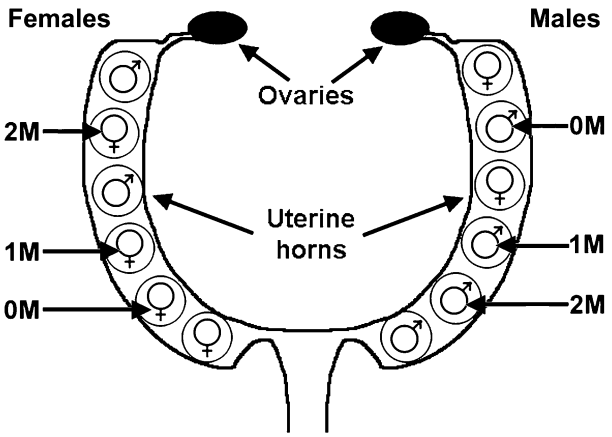

Figure 2: Intrauterine position in mice according to number of flanking males (0M to 2M). Adapted and redrawn from Figure 1 of Ryan and Vandenbergh (2002).

In mice, 2M fetuses are exposed to a higher level of androgens than 1M or 0M. Females adjacent to males become partially masculinized in their behavior, and 2M fetuses, both male and female, show increased body weight, greater aggression and territoriality, and enhanced male traits including reproductive behavior (Ryan & Vandenbergh 2002). In rat, overall litter male/female composition may be more important than precise intrauterine position (reviewed by Ryan & Vandenbergh 2002).

Conversely, proximity to female fetuses influences male physiological development: prostate glands of males flanked by two females were enlarged compared to controls (Timms *et al*. 1999); estradiol is implicated (vom Saal *et al*. 1997).

Imprinting errors

The laying down and maintenance of early developmental patterning (e.g. masculinization via androgens or aggressiveness changes with perinatal stress, see below) has been termed 'imprinting'. This operates via gene expression changes that are maintained by DNA methylation and chromatin condensation (Jaenisch & Bird 2003; Meehan 2003). As with all biochemical processes, stochastic errors may occur (Bestor 2003; Croteau *et al*. 2001). Petronis *et al*. (2003), studying phenotypic discordance for schizophrenia in human MZ twins, provided evidence that imprinting pattern might correlate with Sz phenotype; imprinting differences could therefore contribute to twin discordance (Bestor 2003; Hall 1996; Singh *et al*. 2002). In mice,

imprinting failure can lead to phenotypic variation. The agouti coat color mutation that produces variably penetrant switches between melanocyte pigment production is associated with insertion of a mouse retrovirus (Copeland *et al*. 1983). Cooney *et al*. (2002) found that maternal dietary methyl supplements markedly influence methylation-dependent agouti expression in offspring. Methyl supplements increased DNA methylation at the agouti locus, leading to a persistent alteration in phenotype. Though such effects have only been demonstrated at specific loci such as agouti, it is plausible to suggest that differential imprinting at other loci could contribute to behavioral diversification in inbred mice.

Nutrition in utero

Competition for nutrition during gestation has not been amply demonstrated, but fetal nutrition can affect later life behavior (Hilakivi-Clarke *et al*. 1997). Supplementation of maternal diet with unsaturated fatty acids affects the performance of offspring in open-field and swim-maze tests: male and female offspring of dams exposed to a diet high in polyunsaturated fatty acid were more aggressively territorial than controls (Raygada *et al*. 1998). Generally, offspring of food-deprived mothers are subordinate to controls in later life (Meikle & Westberg 2001).

Non-hormonal communication: twin effects

All mice within a litter are, in some sense, twins (products of multiple pregnancy). This would be purely circumstantial except that human twin studies suggest that multiple pregnancy may increase phenotypic variation.

Twinning *per se* in human is a risk factor for developmental symmetry disturbances (reviewed by Boklage in press). Early developmental interactions between monozyotic twins are suggested by behavioral disorders (e.g. schizophrenia and attention–deficit syndrome) where heritability is extremely high (generally above 80%), but where MZ twin concordance is no better than 50% (Davis *et al*. 1995; Farmer *et al*. 1987; Levy *et al*. 1997; Sharp *et al*. 2003). Phenotypic development of one embryo influences the cotwin, a concept dubbed 'mirror-imaging' (e.g. Golbin *et al*. 1993). Such effects may interact with hormonal masculinization: human homosexuality (possibly influenced by interuterine stress: Dorner *et al*. 1980), is discordant in MZ twins (Bailey & Pillard 1991; Bailey *et al*. 1993); cotwins of discordant phenotype ($n = 7$) were also discordant for a skeletal marker of masculinization (Hall & Love 2003).

It is not known how these considerations apply to experimental rodents. Artificial MZ mouse twins show reduced aggression, social interaction and activity (Baunack *et al*. 1984). However, the impact of twinning may depend on the frequency of natural MZs in experimental animals. Lack of genetic markers constrains studies in genetically inbred species; best estimates are that natural MZ twinning is very rare in mice (McLaren *et al*. 1995), as in rabbit and pig (Ashworth *et al*. 1998; Bomsel-Helmreich & Papiernik-Berkhauer 1976). McLaren *et al*. (1995) reported less than five identical pairs from over 2000 mice born.

However, this does not rule out early interfetus developmental communication over and above behavioral imprinting by steroids: human twin studies point to interactions between non-identical dizygotic (DZ) twins (Boklage in press; James 1992). Additional sibling interactions *in utero* may promote behavioral diversification in mice.

Maternal stress and infection

Maternal challenge can produce lifetime changes in offspring. Crowding pressures during gestation lead to reduced aggressive behavior in male progeny (Harvey & Chevins 1985); also maternal heat and restraint stress (Kinsley & Svare 1986). Female aggression was increased in some studies (Zielinski *et al*. 1991) implicated in population fecundity control (Cowell *et al*. 1998). Stress extends to infection: offspring of mothers infected with influenza display marked reductions in exploratory and open-field activity, with impaired social behavior (Shi *et al*. 2003). Note that lifetime behavioral changes following maternal stress may also occur in human (Lummaa 2003).

Prenatal stress effects are complex (see Ryan & Vandenbergh 2002), perhaps because in female mice (unlike males) both glucocorticoids and androgens are elevated by stress (Chapman *et al*. 1998). Paradoxical interplay between prenatal stress and intrauterine position has been noted (vom Saal *et al*. 1990): while androgens favor male-type behavior (above), coelevated glucocorticoids increase submissiveness (Leshner & Schwartz 1977).

A glimpse of non-genetic inheritance

Intrauterine position leaves traces in the next generation. In gerbils, females flanked by two males (2M fetuses) later, as adults, go on to produce a higher proportion of males in their own progeny (reviewed by Ryan & Vandenbergh 2002). These authors state: 'a 2M female will be more likely to produce 2M offspring than will other mothers' and 'a 0M female is likely to produce high numbers of 0M females' (p. 670). Some epigenetic phenotypes may be heritable: the variable phenotype of offspring carrying the agouti locus Ahyy mutation is influenced by maternal phenotype (Argeson *et al*. 1996). Keightley and Hill (1992) and Keightley *et al*. (1993) report that a genetically inbred line, when sequentially selected for high and low body weight, produces sublines with significantly different bodyweights: 'a surprisingly large response to selection occurred in the inbred control line' (Keightley *et al*. 1993, p. 1099).

Postnatal effects

Postnatal nutrition can also modify later-life behavior: assertive males tend to be those that gain weight more quickly during infancy (Barnard *et al*. 1998). Early life experiences, including social stress, impact on behavioral and physiological parameters and social status. Previous experience of

social settings can protect against glucocorticoid resistance, a stress marker (Avitsur *et al*. 2003). Social interactions with siblings and parents may predispose to particular temperaments: both in mouse and rat, adult brain development and social behaviors are heavily influenced by maternal attention during the postnatal period (Meaney 2001; Champagne *et al*. 2003). Positive maternal attention correlates with the aggressiveness of high-ranking males among the offspring (Barnard *et al*. 1998).

Endocrine factors

In females, marked changes occur in behavior during the estrus cycle and during pregnancy and lactation. Estrus changes are not reviewed here, but it is noteworthy that proestrus mice are less sensitive than estrus or diestrus mice to inhibition of exploration induced by social isolation (Palanza *et al*. 2001), paralleling changes in benzodiazepine responses (Carey *et al*. 1992) and hippocampal electrophysiology (Good *et al*. 1999) over the estrus cycle. A further spectrum of behavioral changes takes place during pregnancy and lactation. Increased aggression during pregnancy (Crowcroft 1966) has been confirmed (Ogawa & Makino 1984; Rosenson & Asheroff 1975). Females remain aggressive during lactation; males are the major but not exclusive targets of such aggression (Albert & Walsh 1995).

In males, significant increases in testosterone levels are seen in dominant males while subordinates show decline (Hardy *et al*. 2002); testosterone production (in males) is suppressed by stress (Armario & Castellanos 1984). Removal of testosterone by gonadectomy decreased attack rate against other males, but increased attacks against lactating females: both behaviors normalized with testosterone replacement (Whalen & Johnson 1987). Testosterone may thus mediate (and not merely respond to) some of the behavioral changes associated with dominance.

Social status

Prenatal and perinatal effects including intrauterine position have marked effects on later life physiology and behavior, and on animals of both sexes. Social experience and physical factors (e.g. nutrition and body size) contribute to social status, while body weight, aggression and territoriality are all factors influenced by early life events. Indeed, a case could be made for typing individual animals for behavioral predispositions or temperament (above).

The question arises of how individual differences contribute to later-life social status. One study suggests that there is no strict correlation between overt behavioral phenotypes and later social status. Hilakivi-Clarke & Lister (1992b) typed mice for exploration, anxiety and aggression before housing them in groups of four to five mice. Social hierarchies were quickly established in all of the cages, but there were no significant differences in any pre-existing behavioral parameter between the mice that later became dominant and those that became subordinate. This does not exclude a contribution of established behavioral proclivities, but suggests either that this is not the major determinant, or that the behavioral tests applied were not relevant for the end phenotype (social status).

Social status and behavioral phenotyping

Irrespective of origin, social status exerts a marked influence on behavior. Overall activity is significantly increased in dominant animals (D'Amato 1988; Hilakivi-Clarke & Lister 1992b). Socially dominant animals were more aggressive but showed a higher degree of anxiety in the elevated plus maze relative to subordinates (Ferrari *et al*. 1997).

Differences in pharmacologic response have been noted according to status. Benzodiazepine responses on intraspecific aggression differed between males according to social status (aggressive, counter-attacking or defeated) and housing conditions (individual vs. group housing): the lowest drug dose increased attacks mounted by the aggressive-dominant males but not by the other groups (Ferrari *et al*. 1997). Dominant animals appear less sensitive than subordinates to the effects of drugs such as amphetamine (Vekovishcheva & Zvartau 1999). In rats, social status affects the outcome of an alcohol test: only a few animals develop into extreme overconsumers of alcohol, and over-consumption correlates with low social status (Ellison & Potthoff 1984; Hilakivi-Clarke & Lister 1992a). Comparable results were obtained in mice (Kahn 1978).

Magnitude of the effect

Not all behavioral parameters appear to be subject to social influences. However, one measure: anxiety (as determined by the elevated plus-maze) is markedly affected. The magnitude of the bias is significant. In a review of recent papers measuring anxiety (not cited), control animals were recorded to make approximately ~15 open arm entries per 5 min test period (ranging from 2.5 to 24 according to strain and apparatus). Behavioral typing for decreased (or increased) anxiety involved increased (or reduced) entries by an average factor of 1.95 (0.51). From the data of Ferrari *et al*. (1997), dominants and subordinates, respectively, made ~13 and ~25 entries (differential factor = 1.92). Thus, the magnitude of the change elicited by social status is nearly identical to the extent of behavioral changes diagnosed as changes in anxiety.

Interdependence of activity and social status?

Increased activity (a correlate of status) enhances neurogenesis in the hippocampal dentate gyrus (Kempermann *et al*. 1998; van Praag *et al*. 1999b) and improves learning skills (van Praag *et al*. 1999a). Activity can therefore promote (and not merely reflect) social status; this may stabilize the dominant/subordinate status of individual animals.

Three site studies – sources of variation

At a most basic level, perinatal influences contribute to individuality, including bodyweight and stress responses. In turn,

these may bias, to a greater or lesser extent, behavioral measures including aggression and territoriality. It is possible that social stratification amplifies pre-existing differences in temperament: aggressive males becoming more aggressive on assuming dominance, while subordinate males become less assertive, but this has not been demonstrated.

Could such individual differences, including social status, contribute to variability in the three-sites studies? It is not known whether individual animals had established social positions in their home cages prior to dissemination to the three sites. However, dominant mice emerge within one week when groups of 4–5 male mice are co-housed; the alpha mice thereafter spend less time immobile in the swim-maze and display higher locomotor activity than subordinates (Hilakivi-Clarke & Lister 1992b). D. Wahlsten, co-author of the three-site study, is of the view that some of the observed variation could arise from such social factors (personal communication). Anxiety and activity, behaviors highly susceptible to perinatal and social factors, were the most variable behaviors in the Crabbe *et al*. (1999) study: arguing that early life influences combined with social effects could contribute to the variation. Nevertheless, the three-site studies employed large numbers of mice (>300) and, for this reason, individual differences including social status are unlikely to explain (though they may contribute to) test-site bias. Instead, local environmental effects are likely to underlie most of the recorded variation (Wahlsten *et al*. 2003).

A need for social context

Social status contributes to individuality, but removal from such a context risks introducing further variation. Mice in isolation behave very differently from mice raised in a social setting: Lipp (quoted in Bohannon 2002) states: 'After four weeks in isolation, mice become very strange' while Ferrari *et al*. (1997) report behavioral changes indicative of anxiety after only 24 h in isolation. Socially isolated mice have been employed as a model of depression (Guidotti *et al*. 2001; Katz 1981). The studies of Crabbe *et al*. (1999) avoided mice that had been housed individually.

Wolfer and Lipp (2000) argue for conservation of social context in experimental settings: IntelliCage (http://www.newbehavior.com) and Smartcube (http://www.rec.ri.cmu.edu/projects/smartcube) were designed to permit individual behavior to be monitored within a social group of mice. Crowcroft's (1996) mouse villages are beyond the reach of most research laboratories but test studies have been performed with transgenic mice in large arenas resembling Crowcroft's pen.

Vyssotski *et al*. (2002) reported the results of a study in which mice with a disrupted *trkB* neurotrophin receptor gene, were released into a large ($10\,m \times 10\,m$) outdoor pen. With a reliable food supply and suitable shelters, every third day food was dispensed inside the shelters. All the mice learned to patrol, but each third day the mutants continued to roam while their wild-type counterparts remained in the shelters. This may not address directly *trkB*'s role in behavior, but encourages the analysis of mutant mice in a complex social environment.

What happens if a gene mutation should affect social status? It is likely that some single gene changes will have dramatic effects. However, rather than being a complicating factor, this could be particularly revealing. Perturbed social interaction is a key feature of many psychiatric disorders including depression, schizophrenia, anxiety and autism. A mouse model accurately reproducing schizophrenia or depression might display no obvious phenotypical differences in an isolated cage, but could show impaired social interactions, emphasizing the need to analyze mouse behavior in a social context.

Consequences, recommendations

The variation discussed here would seem to operate over and above the site-effects observed by Crabbe *et al*. (1999) and Wahlsten *et al*. (2003). Individuality may complicate the analysis of mouse behavior when small experimental numbers are used. The genetic component ('g') of general cognitive ability is a case in point. Although for (outbred) mice there was a strong statistical correlation between performance achieved on a particular skill test and scores obtained across a battery of other tests (Matzel *et al*. 2003), a one-to-one correlation between genotype and ability has been challenged (Gould 1999). One study using rats (Anderson 1993) reported that general task scores showed a significant correlation with brain weight, a parameter probably sensitive to intrauterine position, nutrition, and neo-natal effects. Because brain weight is likely to correlate with body weight, itself a determinant of status, general task scores may vary according to social status.

A proportion of behavioral analyses risk artificial bias if small numbers of test and control animals are selected randomly from a social population. In a typical mutant mice experiment, multiple offspring of a heterozygote $\times$ heterozygote transgenic cross are sorted according to sex and transferred to cages (typically five mice per cage). Behavioral testing and genotyping for homozygous mutants is performed blind. Given that social stratification develops rapidly (within one week; Hilakivi-Clarke & Lister 1992a; Hilakivi-Clarke & Lister 1992b), if dominant and subordinate status develops independently of genotype, studies employing small numbers of animals may be biased if, in a particular experiment, dominants/subordinates are by chance unequally represented in the mutant/control groups.

Haller *et al*. (2002), using mutant mice on a largely CD1 background, sought to avoid possible confounds by housing mice individually for two weeks prior to experimentation, noting that social isolation in this mouse strain had no anxiogenic effects. Nevertheless, individual housing of a different strain (Swiss-Webster, males) reduced open-arm entries by a factor of $\sim$1.7 (Ferrari *et al*. 1997).

This Commentary draws attention to individual and social factors that influence behavior. The question inevitably

arises: what can the conscientious researcher do to minimize the effects of individuality and social status?

One option is to minimize social status effects through gender selection and housing. Overt endocrine changes in females are well recognized; effects may be minimized by restricting studies to males or by multiple sampling. Social effects may be greater in groups of males (only male groups were studied by Ferrari *et al*. (1997)) but status-bias in females cannot be excluded. The effect of stocking density is not known but, anecdotally, cohoused same-sex littermates are less rigidly hierarchical, while pairwise housing may also reduce social conflict. In Crowcroft's (1966) experiments the 'top' two mice co-operated in dominance. Small group housing (not more than 2–3 mice per cage) could lead to less strict dominance hierarchies, but this remains to be verified experimentally. Rats are typically housed pair-wise; this could contribute to their reputed reliability in behavioral experiments.

A second option is to normalize for precise social status. For large numbers of mice, nevertheless, patient observation of each cage over long periods is impractical.

Third, more pragmatically, it should be possible to address simple parameters that correlate with social status. At the very least, experimental data may be normalized to body weight: statistical comparison being made between the mean and standard deviations for (score/weight) values of individual animals within the control and experimental groups. Multifactorial analysis could improve accuracy, and parameters warranting consideration include (a) bodyweight, (b) overall activity including grooming/rearing and locomotor activity and (c) anxiety. Of these, anxiety requires specialized apparatus and may not be justified with large *n*-values. Specific passive activities may in principle be measured automatically as with SmartCube or Intellicage (see above) though weighting factors must be determined experimentally.

Concluding remarks

Indisputably, behavior is underpinned by genetics. Nevertheless, the mouse appears to exploit non-genetic mechanisms to increase the phenotypic diversity of progeny. Indeed, the success of the mouse as a species may reflect the evolutionary plasticity of its behavior (Bronson 1984): 'The defining characteristic of the species *M. musculus* is the decoupling of genetics and behavior' (Silver 1995). In mice, at least, early environmental and social influences may be as important as genetics in determining later life behavior.

This Commentary, far from a challenge to mouse behavioral analysis, argues that a full appreciation of the diversity of behavior will improve evaluation of drug and mutation data.

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