curso de posgrado

Análisis conductual de modelos animales de desórdenes psiquiátricos

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2016

Seminarios – Primera parte
**Cronograma de teóricas (de 9:30 a 13:30hs)**

**Primera parte: Estrategias de análisis conductual**

**Segunda parte: Algunos modelos animales de enfermedades psiquiátricas.**

**Cronograma de seminarios (de 14:30 a 17:30hs) Laboratorio N**

**Primera parte: Estrategias de análisis conductual**
- 14/3: Presentación y organización. AD, NW, JB
- 15/3: Funciones motoras y sensoriales. JB, AD
- 16/3: Recompensa y adicción. JB, NW
- 17/3: Comportamiento emocional: Modelos animales de enfermedades psiquiátricas. NW, AD
- 18/3: Comportamientos sociales y reproductivos AD, JB

**Segunda parte: Algunos modelos animales de enfermedades psiquiátricas.**
- 21/3: Modelos de ansiedad y depresión. NW, AD
- 22/3: Modelos de esquizofrenia. JB, NW
- 23/3: Modelos de autismo. Frágil X. AD, JB
- 29/3: Resumiendo: Elección de ensayos, orden de testeo, número de animales, equipamiento, ambiente de almacenamiento y de testeo. Explicación de práctico. AD, NW, JB
- 1/4: Modelos de ADHD. GP, AD

**Trabajo práctico**
30 y 31 de marzo de 9 a 18hs
5/4: 9:30-13 hs: Integración de los resultados obtenidos

**Examen escrito:** Martes 5 de abril, de 14:30 a 17:30hs.
Seminario 1: Funciones motoras y sensoriales

Seminario 2: Recompensa y adicción

Seminario 3: Comportamiento emocional

Seminario 4: Comportamientos sociales y reproductivos
Behavioural profiles of inbred mouse strains used as transgenic backgrounds. I: motor tests

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One of the characteristic manifestations in several neurodegenerative diseases is the loss of voluntary motor control and the development of involuntary movements. In order to determine the suitability of six mouse strains as transgenic background strains we investigated performance on a variety of tasks designed to identify subtle changes in motor control. On both the accelerating and the staggered speed rotarod all six mouse strains performed well. However, latency to fall from the rod was sensitive to both rotarod speed and repeated exposure to the apparatus. Performance of the DBA/2 mouse strain was highly variable across the time points used. On the acoustic startle test CBA mice showed the greatest degree of reactivity to the acoustic startle stimuli with both the C57 and DBA showing the least. Complex strain differences were also identified on measures of habituation to the startle stimuli and variations in the prepulse noise level, and prepulse/startle delay. Gait analysis using the footprint test did not reveal strain differences on measures of base width, overlap or stride length but the 129S2/Sv strain took significantly longer to traverse the runway than the other mouse strains. Finally, the swim tank test detected complex strain differences in swim speed, and the number of fore- and hindpaw paddles required to swim the length of the tank. These data taken together suggest that choice of background strain is a crucial consideration for the repeated behavioural assessment of motor deficits in transgenic mouse models of disease.

Keywords: Backgrounds, footprint, motor tests, PPI, rotarod, startle, strain comparison, swim

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Increasingly investigators are using transgenic (Tg) mouse models of diseases as a tool to probe the biochemical, cellular and behavioural aspects of the disease process and to screen novel therapeutics. In order to assess the linkage between, on the one hand, anatomical and morphological changes characteristic of the disorder, and on the other hand, how these changes affect the functionality of the whole organism, behavioural assessment of these mouse models becomes necessary. Consequently, choice of background strains for the Tg model is crucial for an accurate multifactorial assessment (see Crawley et al. 1997 for a review) of the disease state in these animals.

There have been many strain comparison studies that have focused on task performance on motor parameters. Strain comparisons on general locomotor activity employing an open field have been extensively studied (Cabib et al. 2002; Contet et al. 2001; Logue et al. 1997), resulting in clear strain specific differences in the underlying levels of spontaneous locomotor activity and open field behaviours. However, in many instances a more detailed cross strain analysis of motor deficits is required, especially when the investigator intends to use a Tg model, and maintain a Tg mouse line, whose phenotype is at least partly characterised by disordered or dysfunctional movements.

Several disease states are characterised by progressive changes in motor performance including Parkinson’s and Huntington’s disease (HD). Since HD is an autosomal dominant, genetically inherited disorder it is an ideal candidate for the development of mouse lines that express aberrant genes and indeed several models have been created using knock-in (for example Wheeler et al. 2000) or Tg (for example Mangiarini et al. 1996) technology (see Menalled & Chesselet 2002 for a review). In these models detailed analysis of several motor parameters is often necessary to capture accurately the essence of the neurodegenerative disease state through repeated testing at predefined time intervals over the animal’s life time.

To test for changes in the animal’s motor coordination and balance, the rotarod apparatus is typically employed at several time periods as it has been shown to be a sensitive tool for assessing progressive neurological deterioration in Tg HD animals (Carter et al. 1999). Strain comparison studies have found that some mouse strains perform significantly better than others on the accelerating version of this task (Rogers et al. 1999) but to date there is no data regarding strain specific changes in performance due to repeated usage and performance on the ‘staggered speed’ variation of the task.

To establish a more detailed analysis of motor deficits it is advantageous to employ several tests together as a battery. Gait analysis through the use of the pawprint test has been
used to determine lesion-induced neurological motor deficits (Fernagut et al. 2002), and neurodegeneration in Tg animals (Carter et al. 1999). Likewise, changes in swim speed and swim stroke patterns have also been found to be sensitive to the progressive nature of neurodegeneration in Tg mice (Carter et al. 1999). At present there is no strain comparison data regarding these particular tasks.

Acoustic startle and prepulse inhibition (PPI) tests are more readily associated with schizophrenia research as it has been proven that schizophrenic individuals show a deficit in their PPI response (Baker et al. 1987). However, several disease states have been found to be sensitive to these tests including Parkinson’s disease (Valdeoriola et al. 1996) and HD in people (Swerdlow et al. 1995) and an HD Tg model (Carter et al. 1999). There have been two extensive studies on startle/PPI strain comparisons in which several parameters were measured (Paylor & Crawley 1997; Willot et al. 2003). Of interest to our laboratory was to determine the optimum parameters for startle/PPI across six inbred mouse strains, whilst adding data from parameters that have not previously been examined (habituation and inter prepulse/startle interval) to the general data pool.

The aims of the present research were twofold. Initially the aim was to determine strain differences in performance on motor tests with a view to assessing their suitability as background strains for genetically modified mice in disorders. The strains used were 129S2/SvHsd, BALB/cOlaHsd, C3H/HeHsd, C57BL/6J, CBA/CaOlaHsd, DBA/2OlaHsd (all aquired from Harlan, Bicester, UK). Only male mice were used (n = 5–8 per group) and were obtained at 4 weeks of age and were used in an age-matched manner. All mice were housed in groups of between six and eight on a 12 h light/dark cycle with an ambient room temperature of 21°C. Animals had free access to food and water. All experiments were conducted under the regulations, licences and local ethical review of the UK Animals (Scientific Procedures) Act 1986.

Experimental procedures

Acoustic startle/prepulse inhibition (PPI)
The purpose of this study was to define the optimum parameters for the experimental assessment of potential startle/PPI deficits in Tg HD mice. The startle chambers (San Diego Instruments, San Diego, CA) were fitted with mouse enclosures and were then equilibrated using the standardisation unit to a give an average reading of 700. Throughout these studies the startle chambers were set to take 100 data samples at 1000 samples per second. Background white noise within the chambers was set at 70 dBs and an acclimatisation period where only background noise was employed lasted 5 min prior to the onset of the first trial.

Startle
The first protocol was designed to determine strain sensitivity to startle stimuli of 10 intensities of between 70 and 120 dBs (0–50 dBs above background) presented pseudorandomly in 50 msec pulses, with a variable intertrial interval (ITI) of 20–30 seconds. In this protocol each mouse experienced 8 trials at each noise level (70, 80, 85, 90, 95, 100, 105, 110, 115 and 120 dBs).

Habituation
This protocol was used to determine strain sensitivity to repeated noise stimuli (50 ms of 120 dBs). Each mouse received 80 trials of the stimuli with a 15 second ITI.

Pre-pulse interval
This study was designed to determine the optimum delay between prepulse and startle stimuli across the six mouse strains used. A 20 ms duration, 85 dB prepulse stimuli was delivered prior to one of 10 pseudorandomly presented intervals of between 0 and 1000 ms. Each delay was presented 8 times hence each mouse underwent 80 trials. The startle stimulus was a single 120 dB pulse of 50 ms duration.

Pre-pulse intensity
To determine how prepulse intensity may differentially affect mouse strain PPI, prepulses of differing magnitude (0, 2, 4, 8 and 16 dBs above background level) were presented in pseudorandom order. Pre-pulse stimuli were of 20 ms duration and were followed by a 20 ms delay prior to the primary startle stimuli. Startle stimuli were 120 dB and of 50 ms duration. ITI was of between 20 and 30 seconds and each mouse completed 12 trials for every prepulse noise level used.

Footprinting
Gait abnormalities were assessed using footprint pattern analysis (see Carter et al. 1999). Mouse fore- and hindpaws were dipped in blue and red non-toxic, water based paint, respectively. The mice were placed in a clear perspex runway (60 x 10 x 10 cm) that had a black goal box (10 x 12 x 10 cm) fixed to one of the distal ends. White paper was used to line the runway floor. The mice were permitted to walk to the goal box from the opposite end of the runway thus allowing their footprints to leave a pawprint pattern on the white paper. Five separate parameters were measured; stride length, hind- and forepaw base width, overlap between fore and hindpaws and latency to travel the 60 cm runway. For each set of pawprints three measurements (in cm) were taken for each of the parameters (except latency to traverse the runway). Stride length was assessed by measuring the distance of three strides and taking the average. Hind and forepaw width was measured by taking a straight line from

Materials and methods

Subjects
Six inbred mouse strains were used in the present experiments. The strains used were 129S2/SvHsd, BALB/cOlaHsd, C3H/HeHsd, C57BL/6J, CBA/CaOlaHsd, DBA/2OlaHsd (all acquired from Harlan, Bicester, UK). Only male mice were used (n = 5–8 per group) and were obtained at 4 weeks of age and were used in an age-matched manner. All mice were housed in groups of between six and eight on a 12 h light/dark cycle with an ambient room temperature of 21°C. Animals had free access to food and water. All experiments were conducted under the regulations, licences and local ethical review of the UK Animals (Scientific Procedures) Act 1986.
the centre of the hind/forepaw print to the centre of the preceding print from same paw. From this line, a perpendicular line to the corresponding print from the opposite side of the body gave the measurement of base width. Overlap was scored such that a positive or negative score could be produced. If the centre of the hind footprint fell directly on top of the front footprint a score of zero was given. If the hind-print fell short of the fore-print a negative value of the distance was recorded, and inversely if the hind-print overlapped with the fore-print a positive score of the distance was recorded. Footprints were always recorded mid-run to avoid taking measures when the animal was either accelerating or decelerating. For each parameter three measures were taken per animal.

**Rotarod**

The rotarod apparatus (Ugo Basile Biological Research Apparatus, Varese, Italy) is used to measure an animal’s balance and motor coordination. Latency to fall from the rotating beam (3.1 cm diameter) that is either accelerating slowly over a set time period (accelerating rotarod), or is locked at a specific speed for a short time period, the mouse being reintroduced to the apparatus several times at ever increasing locked speeds (staggered rotarod). The rotating rod of the apparatus was adapted by the addition of soft rubber covering (made from a cycle tyre inner tube). This covering had previously been found to prevent the animal from gripping the rotating rod and rotating with it rather than running on top, or falling from it, as the experiment required. The rare event of a mouse rotating with the rod was classed as a failure to maintain its balance/coordination and the mouse was subsequently removed form the rod and the time recorded.

**Staggered**

The rotarod apparatus was used to measure motor coordination and balance. The first day of rotarod training consisted of placing the animals on the rotating rod at a speed of 13 r.p.m. for 1 min. On completion the rotarod was accelerated to 19 r.p.m and the mouse was again placed on the rotating rod for 1 min. The second day of training employed a quicker speed setting of 23 r.p.m. Again the mice were placed on the rotating rod for 1 min. On this training day, 4 trials were completed by each animal. If a mouse fell from the rod at any point during training it was placed back on the rod and the 1 min was completed.

The first rotarod test day commenced on completion of the initial two day training phase. Mice received two trials (1 min/trial) on each of 10 different rotarod speeds (7–44 r.p.m.). The time spent on the rotating rod at each of the different speeds was recorded (latency to fall), consequently each mouse produced 10 scores for each trial. To assess behaviour change over time, mice were tested at weekly intervals over a five week period.

**Accelerating**

Mice were placed on the rotating rod which was rotating at 7 r.p.m. The rod increased in speed at roughly 3 r.p.m./30 seconds to a maximum velocity of 44 r.p.m. where the speed was maintained for 30 seconds. The time that the mouse remained on the rod was recorded. Mice that remained on the rod for 30 seconds at 44 r.p.m. were given a maximum score of 500 seconds.

**Swim analysis**

As an alternative approach to measuring motor abnormalities the swim tank was employed (see Carter et al. 1999; Perry et al. 1999). A perspex tank (100 x 6 x 40 cm) was filled with warm water (approx 21 °C) to a depth of 20 cm. A platform was placed at one of the distal ends so that it was clearly visible to the mouse form the opposite end of the tank. The task requires that the mouse, once placed at the starting end of the tank, swims to the platform at the opposite end of the tank. Initially the mouse requires a short training period during which the distance required for the mouse to reach the platform is increased until the mouse is able to swim the full length of the tank. In order to assess swimming parameters, the mouse is filmed in profile to provide video recordings for later analysis. Each mouse undergoes three swimming trials per session with only the two final trials being used for analysis. Three swimming sessions (1/week) were run. Swim speed was also recorded.

**Statistical analyses**

A 2, 3 or 4-way analysis of variance was applied to the data as required. For the post hoc analysis the Tukey HSD test was used to determine pairwise differences between groups and the Dunnett’s test was used to determine significant differences between a given data set and the control values of that data set.

**Results**

**Acoustic startle/prepulse inhibition (PPI)**

**Startle**

See Figs 1 (a,b) and 2 (a,b). Analysis revealed that the mouse strains were differentially sensitive to the primary acoustic startle stimuli (Strain x Stimuli: $F_{4,360} = 13.81$, $P < 0.0001$) with CBA/Ca mice being the most, and C57BL/6J the least sensitive of the strains. Peak startle response for all strains was either 115 or 120 dBs. For all strains startle effects became significant at between 95 and 105 dBs and remained significant for all higher stimuli levels.

**Habituation**

Data collected from the 80 trials used in this study was collapsed into 8 blocks of 10 trials for analysis. A significant two way interaction effect (Strain x Trial Block: $F_{35,280} = 2.47$, $P < 0.0001$) indicated that the strains were significantly affected by repeated exposure to startle stimuli. The strain differences for the habituation trials were comparable with the startle data in that the CBA/Ca mice exhibited
the greatest response to the 120dB stimuli and the C57BL/6J the least response. The strains most sensitive to habituation were the 129S2/Sv and the CBA/Ca, both of which showed significant reductions from baseline levels (trail block 1: trials 1–10) from trial block 2 (trials 11–20). Only the DBA/2 strain did not exhibit a significant habituation of the startle response although scores from trail block 3 (trials 21–30) were reduced from previous blocks. Post hoc analysis across strains with the Tukey HSD test confirmed that the CBA/Ca strain differed significantly from all of the other strains in the magnitude of the startle response across the eight blocks of trials, and the 129S2/Sv differed significantly from the C57BL/6J strain.

Pre-pulse interval
Varying the interval between prepulse and startle stimuli produced delay-dependent variations in startle reflex across the mouse strains (Strain × Interval: $F_{45,360} = 1.57$, $P < 0.013$). Dunnetts test found that two mouse strains, BALBc and C3H/He, showed significant inhibition at all delay intervals whereas the DBA/2 mice showed significant inhibition at only the 5 ms delay. Both the 129S2/Sv and and the C57BL/6J strains showed inhibition of the startle response at all delays with the exception of the longest interval (1000 ms). In the CBA/Ca mice a similar pattern of inhibited reactivity was found but no effect was seen at delays longer than 500 ms. Optimal response inhibition varied between the strains but was within a relatively narrow interval range (5–20 ms) beyond which the inhibition response degraded with increased interval (Delay: $F_{9,360} = 92.72$, $P < 0.013$). Tukey HSD test identified that the DBA mouse strain was significantly less flexible in response to varied delay intervals than all of the other strains (Strain: $F_{5,40} = 7.22$, $P < 0.0001$).

Prepulse intensity
Varying the prepulse stimulus intensity over a range of 0–16 dB above background level (70 dB) induced strain differences in the percentage of prepulse inhibition (Strain × Stimuli: $F_{20,160} = 6.61$, $P < 0.0001$). A 16 dB (above background) prepulse was optimal for every mouse strain and the C57BL/6J mice were the most sensitive to this prepulse manipulation displaying a 77.3% inhibition to the startle stimuli, in contrast with the DBA/2 mice that were the least sensitive displaying...
only a 27.5% inhibition. These differences are reflected in the highly significant main effect for strain differences (Strain: \( F_{5,40} = 9.82, P < 0.0004 \)). The DBA/2 mouse was the only strain not to show a significant reduction in startle at any of the stimuli intensities used, this was reflected in the post hoc analysis which identified that the DBA/2 mice differed significantly from all but the CBA/Ca mouse strain. All but the DBA/2 strain showed stimuli-dependent increases in the startle inhibition in response to increases in prepulse stimuli intensity, such that C3H/He strain exhibited significant inhibition with the minimum prepulse intensity (2dB) and C57BL/6J, BALBc, CBA/Ca and 129S2/Sv strains all showed significant reductions in startle (Strartle: \( F_{4,160} = 134.3, P < 0.0001 \)) with an 8 dB and higher stimuli, as indicated by Dunnetts test.

### Footprint analysis

See Fig. 3 (a-d). No significant differences were found between the age-matched mouse strains for stride length \( (P = 0.2) \), overlap \( (P = 0.7) \), forepaw base width \( (P = 0.3) \) and hindpaw base width \( (P = 0.1) \). However, the time that the different mouse strains took to traverse the runway differed markedly (Strain: \( F_{5,29} = 18.83, P < 0.0001 \)) with the 129S2/Sv mice taking longest and the DBA/2 mice taking the shortest time to reach the goal box. Tukey HSD test confirmed that the 129S2/Sv strain was significantly slower to traverse the runway than the other 5 mice mouse strains, and that the DBA/2 mice were also significantly quicker than the C3H/He and CBA/Ca strains.

### Rotarod

**Staggered**

See Fig. 4 (a-e). Performance on the rotorod differed between strains and was speed dependent (Strain × Speed: \( F_{4,270} = 1.97, P < 0.001 \)). Surprisingly, post hoc analysis with the Tukey HSD test identified that strain differences occurred at relatively low speed settings (within the range of 7–23 r.p.m) and that poor performance from the DBA/2 strain was exclusively responsible for these significant differences during weeks 6 and 10 at speeds of 19–33 r.p.m. and 13–23 r.p.m. respectively. During week 8 performance the CBA/Ca mouse performance differed significantly from both the C57BL/6J and C3H/He mice. The differing strain performance at different rotarod speeds changed over the five weeks.

Figure 2: Strain comparison \( (n = 8/\text{group}) \) of the sensitivity to a range of prepulse intervals (a) and prepulse intensities (b) were measured. Bars on the figures enclosed within or marked with the bars were significantly different from control values \( (*P < 0.05) \).
week test period (Strain × Speed × Week: F180,1080 = 1.441, \( P < 0.0005 \)) although post hoc analysis failed to clarify the precise cause of these variations in performance. It is also worth noting that strain performance between the two runs of each test session also changed over the four week test period (Strain × Run × Week: \( F_{20,120} = 1.97, P < 0.0003 \)). A four-way interaction effect (Strain × Speed × Run × Week) indicated that strain was an important factor in all aspects of rotarod performance (F180,1080 = 1.85, \( P < 0.0001 \)).

Accelerating
See Fig. 4(f). A single accelerating rotarod test session comprised of two trials produced significant performance differences between strains (Strain × RUN: \( F_{5,29} = 6.23, P < 0.001 \)) indicating that there were strain dependent changes in performance between the two trials. However, no significant main effects were found for strain (\( P = 0.39 \)) or between the two trials (\( P = 0.41 \)).

Swimming analysis
See Fig. 5 (a-c). There were significant differences in swimming speed between the mouse strains (Strain: \( F_{5,30} = 4.02, P < 0.01 \), post hoc Tukey HSD test indicating that the differences were largely due to the slow swimming speed of the C3H/He mice which contrasted with the relatively fast BALBc and C57BL/6J strains. With regards to the number

Figure 3: Strain comparison (n = 5–6/group) on the footprint test. Overlap (a), base width (b) and stride length (c) did not differ between strains for age-matched mice, but the latency to travel the length of the runway (d) differed significantly across strains (\( ^* P < 0.05; {}^\dagger P < 0.01 \)).
of paddles that each strain produced, significant main effects were found for the number of forepaw paddles (Strain: $F_{5,30} = 3.18, P < 0.05$) and number of hindpaw paddles (Strain: $F_{5,30} = 4.56, P < 0.05$) but in both cases there was no significant interaction effect for strain related effects over time. With regards to strain variations when the three-week data was collapsed to a single mean, Tukey HSD test indicated significant differences in the number of forepaw paddles that were found between the CBA/Ca and C57BL/6J strains, whereas with hindpaw paddling both CBA/Ca and C3H/He strains showed a significantly greater number of paddles than the BALB/c strain.

Discussion
The results from the present paper show that there are considerable strain differences across a variety of behavioural tasks that are designed to quantify specific aspects of motor control in the mouse. These results also demonstrate that even with tests that are in routine use (for example the rotarod), strain specific changes in performance can vary with repeated usage.

DBA/2 performance on the ‘staggered’ rotarod task was significantly worse than the performance of the other mouse strains used and somewhat surprisingly the detriment in performance occurred at relatively low speeds (7–23 r.p.m) rather than at higher speeds as would be expected if the deficit were motor related. Interestingly, CBA/Ca performance varied over time most noticeably during week 8 when performance appeared to be more resilient than during the other weeks. Rotarod performance also changed between successive weeks in a strain-dependent manner. Strain differences between the two separate daily runs on the ‘accelerating’ rotarod test were also found.

Analysis of reactivity to the primary startle stimuli found large and differential strain differences in response to a range of startle stimuli with the CBA/Ca mice displaying a large degree of reactivity and the C57BL/6J and DBA/2 mice

Figure 4: Accelerating rotarod trials over a five-week period (a-e) displaying the variation in performance over this period. Due to the complex nature of the strain differences, significance markers have been omitted for clarity (see Results). Continuous rotarod trial (f) taken on week 10. No main effect of strain was recorded. ($n = 6$/group).

Behavioural profiles of inbred mouse strains

Figure 5: Strain comparison (n = 6/group) on the swim tank task over a three-week period. Swim speed was found to differ across strains (a), as did the number of forepaw (b) and hindpaw (c) paddles (*P < 0.05).

showing relatively little. Interestingly, while C57BL/6J mice showed least reactivity to the primary startle, in subsequent tests comparing multiple prepulse intervals and delays the C57BL/6J mice showed the greatest inhibition of the primary startle response with the most inhibitory of prepulse stimuli (16 dB) and good inhibition to all but the longest of prepulse delays when scores where expressed as proportions of baseline (primary) startle. DBA/2 mice continued to exhibit poor adaptability to changes in both prepulse interval and delays compared to the other five strains. The DBA/2 mice also failed to exhibit a high level of habituation to the startle response, again in contrast to the other mouse strains.

The footprint test did not show significant differences between mouse strains on fore- and hindprint base width, stride length or overlap scores, but did find significant differences in the time that the animals took to traverse the runway to the goal box. It is of interest to note that the DBA/2 mice were the only strain that exhibited a natural degree of overlap, whereas the BALBc mice showed exact paw-planting and the remaining four strains all exhibited a hindpaw shortfall. It is also noticeable that the slowest mice exhibited the greatest shortfall (129Sv/S2 and CBA/Ca) whilst the quickest mice displayed the only positive overlap score (DBA/2).

With regards to the swim tank test there were significant differences in swim speeds suggesting that two mouse strains, C57BL/6J and BALBc, were the strongest swimmers, and the C3H/He mice were weak swimmers.

The rotarod is one of the most widely used behavioural tests for assessing motor deficits in both mice and rats. The findings from this paper suggest that the staggered speed rotarod method is more sensitive to motor changes in the mouse than the more commonly used accelerating method, which failed to identify significant main effects of either strain or within session trials. In agreement with a comparable strain comparison study, both the C57BL/6J and CBA/Ca mouse strains performed well on the accelerating rotarod with the DBA mice performing less well (McFadyen et al. 2003) suggesting that the accelerating rotarod is a reliable method for assessing motor coordination. Both the present and the latter study are generally in agreement with a previous study by Tarantino et al. (2000). Of interest, the most striking difference between the Tarantino et al. (2000) study and the McFadyen et al. (2003) study is that they present contrasting data regarding the rotarod performance of the DBA/2 mouse strain, with the Tarantino study showing evidence of improvement over time which was absent in the McFadyen study. These differences may be representative of the findings in the present study which showed variable performance of the DBA strain over time.

Of particular interest was the finding that there are strain specific changes in performance with repeated usage of the apparatus. As a general rule, performance remained stable over time for 5 of the 6 mouse strains used. However, it was noticeable that with repeated usage the DBA/2 mice learned that there were beneficial consequences (returned to the home cage) from falling off the rod and consequently were not motivated to remain on the apparatus. This behavioural adaptation resulted in large error bars for this particular mouse strain and contributed to the overall significant effects that were found.

Considerable strain differences were found with the acoustic startle apparatus. The CBA/Ca mice showed the greatest reactivity and the C57BL/6J mice the least. Comparisons with previous reports on this parameter are made difficult due to differing sensitivity settings of the equipment between laboratories and the different substrains that have been used between the papers. However, our results seem to contrast with comparable previous studies in that our C57BL/6J mice exhibited particularly low levels of startle reactivity, whereas the C57BL/6J mouse exhibited greater startle than the other strains used (Paylor & Crawley 1997; McCaughran et al. 2000). In contrast, a recent paper by Willett et al. (2003) reported similar results as presented in
the present study, in that comparison of the six strains used in our study with the same strains used in theirs, found that the lowest reactivity to a 100 dB stimuli was produced by the C57BL/6 and DBA/2 strains, respectively. Whereas the two most reactive strains common to both studies were the BALBc and the CBA mice, as found in the present paper.

What may be of importance is that the mice used in our studies had been used in several other (different) tests previously and may show markedly less reactivity to novel events. However, a previous report examining the effects of training history in the C57BL/6J mouse found that whilst training history can affect the performance of these mice on some tests, PPI scores in previously used mice were no different to those produced by naive animals (McIlwain et al. 2001). The same paper also showed that the order of tests can differentially affect mouse strain performance (McIlwain et al. 2001).

Some mice strains are susceptible to a progressive sensorineural hearing loss. These mice strains include C57BL/6J, 129/SvJ and the DBA/2J strain, and all of these strains show marked elevation of their auditory brainstem response prior to 3 months of age (Zheng et al. 1999). In addition, the BALB/c mouse has also been separately identified as having a progressive sensorineural hearing loss (Ralls 1967). In our experiments the worst performing mice in the startle tests were the C57BL/6J, DBA/2 and C3H/HeJ strains which have been found to have similar levels of reactivity in a normalized acoustic startle test (McCaughran et al. 2000). Both the C57BL/6J and the DBA/2J strains are used as primary mouse models for progressive hearing loss (see Willott et al. 1998), whereas the mouse strain that produced the highest startle reactivity in our study (CBA/Ca) was the strain used as the control in the Zheng et al. (1999) strain comparison study and exhibited good hearing.

It is also possible that some of the within-strain differences found between laboratories could be due to genetic drift caused by repeated breeding at different suppliers. The mice in the present paper were from well-established breeding colonies from a UK supplier and it may be that the same mouse strain obtained from, for example, a US supplier may have subtly different behavioural profiles.

Gait analysis with the footprint test has been used to determine gait abnormalities not only in transgenic mouse models of neurodegeneration, for example Parkinson’s disease (Gomez-Isla et al. 2003) or ataxia telangiectasia (Eilam et al. 1998), but also in models where the cause of the disease is viral and the prognosis acute, such as rabies (Taharaguchi et al. 2003). Normal mice have been shown to have a gait that remains consistent from postnatal day 24 throughout their adult lives (Clarke & Still 2001), suggesting that this test should be robust when comparing control with motor impaired animals longitudinally. Whereas neurodegenerative disorders typically manifest a debilitating motor deficit, it is feasible that some modelled disorders are prone to swings in levels of locomotor activity resulting in the necessity of measuring both increases and decreases in the animal’s gait. A prime example of this would be the dopamine transporter knockout mouse (Giros et al. 1996), which was designed to model disorders where abarent dopamine transport activity has been found, such as attention-deficit/hyperactivity disorder, bipolar disorder and schizophrenia. These mice are hyperactive in a novel environment, but are hypoactive in response to psychostimulants (Gainetdinov et al. 1999). This is particularly relevant if the effects of potentially novel therapeutic agents are being investigated and the motor effects are unknown.

We are not aware of any other papers comparing mouse strain performance on the swim tank task. As with the previous tests, performance was strain specific. Analysis of hindpaw paddling rather than forepaw paddling may be a more reliable measure of motor dysfunction in animal disease models due to a relatively low level of variance, although past papers have shown that both forepaw and hindpaw paddling can be sensitive measures of motor dysfunction (Carter et al. 1999; Perry et al. 1995). Indeed, only the hindpaw analysis found a significant effect over the three week test period although there was no interaction with strain. Of interest the C3H/HeJ mouse has a well reported visual deficit that is highlighted in tasks where visual acuity is required for task completion. For example, in water maze tasks this mouse typically exhibits highly thigmotaxic behaviour and a frequent inability to find visually cued platforms (Rogers et al. 1999). In the present paper, the C3H/HeJ mice produced the longest swim latencies but these latencies were only significantly longer than the quickest two mouse strains, suggesting that the C3H/HeJ mouse is not impaired at the current swimming task when compared with several other mouse strains with normal vision. The reason for the lack of impairment in this task was in the construct of the swim tank which was long and narrow and did not permit animals to turn with ease. Consequently, when placed at one extreme end of the tank the mice swam in the only available direction until they landed on the platform at the opposite end.

The findings of the present paper show marked strain variations in the tasks employed. The evidence presented suggests that there are strain-specific changes in behaviour that have resulted from repeated exposure to the test. We suggest that great care must be taken in choosing a potential background strain on which to maintain a Tg mouse line if this Tg mouse is to be employed in behavioural testing. In our hands, the 129S2/SvJ strain and the DBA/2 strain are unsuitable for behavioural testing when used as pure strains due to the apathetic nature of the former strain and the variability in performance of the latter.

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Commentary

Psychiatric endophenotypes and the development of valid animal models

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Endophenotypes are quantifiable components in the genes-to-behaviors pathways, distinct from psychiatric symptoms, which make genetic and biological studies of etiologies for disease categories more manageable. The endophenotype concept has emerged as a strategic tool in neuropsychiatric research. This emergence is due to many factors, including the modest reproducibility of results from studies directed toward etiologies and appreciation for the complex relationships between genes and behavior. Disease heterogeneity is often guaranteed, rather than simplified, through the current diagnostic system; inherent benefits of endophenotypes include more specific disease concepts and process definitions. Endophenotypes can be neurophysiological, biochemical, endocrine, neuroanatomical, cognitive or neuropsychological. Heritability and stability (state independence) represent key components of any useful endophenotype. Importantly, they characterize an approach that reduces the complexity of symptoms and multifaceted behaviors, resulting in units of analysis that are more amenable to being modeled in animals. We discuss the benefits of more direct interpretation of clinical endophenotypes by basic behavioral scientists. With the advent of important findings regarding the genes that predispose to psychiatric illness, we are at an important crossroads where, without anthropomorphizing, animal models may provide homologous components of psychiatric illness, rather than simply equating to similar (loosely analogized) behaviors, validators of the efficacy of current medications or models of symptoms. We conclude that there exists a need for increased collaboration between clinicians and basic scientists, the result of which should be to improve diagnosis, classification and treatment on one end and to increase the construct relevance of model organisms on the other.

Keywords: Anxiety, biological markers, bipolar disorder, construct validity, depression, endophenotypic, endophenotyping, genotype, intermediate phenotype, mania, mice, mouse, phenotype, rat, schizophrenia

Psychiatry endures a diagnostic and classification system that is not based upon etiology, neurobiology, epidemiology, genetics or response to medications but rather on gross behaviors that have imprecise similarity and/or correlation with each other within and between individuals [see (Charney et al. 2002; Krishnan 2005; Sadler 2004) for extensive discussions]. A harsh critique of the current system could elicit comparisons between fictitious and real entities, the former being difficult to define experimentally and having limited construct validity (Cronbach & Meehl 1955; Maher & Gottesman in press). Diagnostic and Statistical Manual (DSM) approaches provide a partially validated mechanism whereby physicians can provide reliable diagnoses, communicate amongst themselves and report their findings to insurance providers. However, disease heterogeneity implicit in the current classification schema, and the imprecise quantification of the behaviors being described, makes it difficult to even partially deconstruct such ‘diseases’ within model organisms. This heterogeneity arises not only because of lack of foresight in the diagnostic system but also, in part, from true deficits in basic knowledge. With limited input from scientific progress, it is not surprising that there has been inconsistency in clinical studies (albeit much less than before DSM-III, which preceded the current version IV), at the level of both neurobiology and genetics in the study of psychiatric illness.

There is an imperfect relationship between genes and behaviors so that different combinations of genes (and resultant changes in neurobiology) contribute to any complex behavior (normal or abnormal). Similarly, while the pathways, beginning with genes that are later expressed through biological processes, do not necessarily have a single quantifiable endpoint (i.e. behavior), it may be possible to assay the result of aberrant genes through more biologically ‘simple’ approaches. Simpler neurological processes reached through
optimizing reductionism (at a level lower than behavior) are – in most cases – controlled by similar biological processes as behavior (just not as many) and hence lend themselves to study.

In psychiatry, reducing complex behaviors into components, whether they are neurophysiological, biochemical, endocrine, neuroanatomical, cognitive or neuropsychological, is described as an endophenotype strategy or approach (Gottesman & Shields 1972; Gottesman & Shields 1973). Symptoms and clinical subtyping (i.e. depression with or without psychosis) generally are not considered endophenotypes. Subtyping in this manner amounts to little more than altering the defining observations of a complex behavior. While it may result in some constraints on heterogeneity, the difference is marginal: decades of applying this approach have resulted in only slightly greater reproducibility than with the broad definition disorders themselves. In other fields of medicine, ‘endophenotyping’ is considered routine; for example, such disease predictors as blood glucose assays are used for diabetes and stress-electrocardiogram (EKG) interpretations for heart disease.

Without question, the term endophenotype represents a current ‘buzz word’ in neuropsychiatric research, and it is envisioned that endophenotypes will assist in clarifying many relevant issues (Gottesman & Gould 2003). Among clinician-scientists in psychiatry, the approach has received immense interest in such diverse disorders such as schizophrenia (Braff & Freedman 2002; Gottesman & Erlenmeyer-Kimling 2001; Hariri & Weinberger 2003; Heinrichs 2005; Lenzenweger 1999; Weinberger et al. 2001), bipolar disorder (Ahearn et al. 2002; Glahn et al. 2004; Hasler et al. in press; Lenox et al. 2002), depression (Berman et al. 1999; Hasler et al. 2004; Niculescu & Akiskal 2001), Alzheimer’s disease (Kurz et al. 2002; Neugroschl & Davis 2002), attention-deficit hyperactivity disorder (Castellanos & Tannock 2002; Doyle et al. 2005; Gould et al. 2001; Waldman 2005), obsessive compulsive disorder (Chamberlain et al. 2005; Miguel et al. 2005), autism (Belmonte et al. 2004), alcoholism (Dick et al. in press; Porjesz et al. 2005) and personality disorders (Siever 2005). It has become increasingly obvious that among studies of psychiatric diseases, there exists an overwhelming number of biological markers. However, these often solitary findings frequently have limited reproducibility, both among and within patients, and may represent state-dependent results. This has led to current criteria for an endophenotype, distinguished from markers or biomarkers generally, in the hope of reaching genetically and biologically meaningful conclusions (Gottesman & Gould 2003) (Fig. 1).

1 An endophenotype is associated with illness, in the population.
2 An endophenotype is heritable.
3 An endophenotype is state independent (manifests in an individual whether or not illness is active) but age-normed and may need to be elicited by a challenge, e.g. glucose tolerance test in relatives of diabetics.
4 Within families, endophenotype and illness cosegregate.
5 An endophenotype identified in probands is found in their unaffected relatives at a higher rate than in the general population.

Additional considerations have been suggested, including the importance of good psychometric properties such as test-retest reliability and normal distribution (Waldman 2005). Following confirmation of these criteria, endophenotypes should share specific regions and gene association with the disorder (Waldman 2005). Importantly, not all

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**Figure 1:** Biological markers (a.k.a. subclinical traits and vulnerability markers) may be primarily environmental, epigenetic or multifactorial in origin. For this reason, criteria useful for the identification of markers to study psychiatric genetics have been proposed, adapted and refined over time (see Gershon & Goldin 1986; Gottesman & Gould 2003; Hasler et al. in press; Leboyer et al. 1998; Lenox et al. 2002; Shields & Gottesman 1973). Current criteria for an endophenotype, to be distinguished from biological markers, are designed to direct clinical research in psychiatry toward genetically and biologically meaningful conclusions. © 2005 I.I. Gottesman, and used with permission.
patients are expected to show endophenotypes, and not all persons with these impairments are expected to demonstrate symptoms of the psychiatric disease (probabilism vs. determinism).

What is the primary goal of utilizing endophenotypes? The central hypothesis begins with what is now wholly accepted – that genetics of psychiatrics disorders is complex and further complicated by epigenetic and stochastic contributors and various gene-by-gene and gene-by-environment interactions/coactions. Many genes interact at many levels, leading to activation of multiple neuronal circuits, which results in behavioral variations (Fig. 2). This is complicated by the knowledge that there can be more than one pathway to a given behavior. Endophenotypes represent more defined and quantifiable measures that are envisioned to involve fewer genes, fewer interacting levels and ultimately activation of a single set of neuronal circuits (Fig. 2). The fewer the pathways that give rise to an endophenotype, the better the chances of efficiently discovering its genetic and neurobiological underpinnings.

It is hoped that clinical research on the level of genetics, neurobiology or gross behavioral analysis will benefit from an endophenotype approach. This certainly has been the case thus far (Gottesman & Gould 2003). Ultimately, endophenotypes are envisioned to aid in diagnosis, classification, treatment, clinical research and the development of preclinical models. Indeed, this reductionist approach implicit in endophenotypes has clear parallels with the general mechanisms employed in preclinical research, where the concept of studying more than one variable simultaneously would rarely be warranted. In fact, animal modeling in psychiatry has relied almost exclusively upon simpler phenotypes. Future major advancements in biological modeling of psychiatric disease will likely be derived extensively from clinical

Figure 2: Endophenotypes are characterized by simpler neurobiological and genetic antecedents than exophenotypic psychiatric disorders, thereby employing optimal reductionism. The bipolar disorder phenotype, as an example, is associated with a number of candidate genes and chromosomal regions, the influence of which can be observed at either the levels of behavior or endophenotypes. Endophenotypes, located closer to genes in the pathway from genes to behaviors, have fewer genes associated and thus are more amenable to genetic investigations and studies in model systems. This skeleton (genes to endophenotypes to behaviors), which allows for epigenetic, ‘environmental’ and purely stochastic influences upon clinical observations, can be applied to other diseases of complex genetics with the input of disease-specific candidate genes/regions and endophenotypes (Gottesman 1997; Gottesman & Gould 2003; Hasler et al. in press; Manji et al. 2003; Sing et al. 1994; Sing et al. 1996). © 2005 I. I. Gottesman and used by permission.
endophenotypes, where acknowledgment, enthusiasm and development of valid endophenotypes are increasing. Novel clinically validated endophenotypes, with distinct gene-endophenotype relationships, have major implications for influencing preclinically oriented research in psychiatry, with ramifications for both in vitro and in vivo investigations.

It is well established that the development of better validated, and more appropriate, animal models is a task representing major importance for psychiatry. For most psychiatric disorders, this deficiency of suitable models for in-depth biochemical, histological and behavioral analysis has greatly hindered progress in understanding neurobiology and in developing novel medications (Einat in press; Flint et al. 2005; Gould & Manji 2004; Nestler et al. 2002; Seong et al. 2002; Spedding et al. 2005; Tecott 2003). While it is often considered (and certainly a view we support) an impossible task to fully model the diverse constellation of behavioral observations observed in psychiatric diseases in laboratory animals, endophenotypes are proving more amenable to the task (Petryshen et al. 2005; Seong et al. 2002).

Animal models of human symptoms do not necessarily have simpler genetics or biological mechanisms than the human disorders. This is not to say that genetic and biological studies of human symptoms, such as models of anxiety, do not advance our understanding of the neurobiology of behavior tremendously and may in fact provide important leads (Crusio 2001; Henderson et al. 2004; Yalcin et al. 2004). However, multiple genes and multiple pathways contribute – even in the mouse (Phillips et al. 2002; Seong et al. 2002). Thus, we may not be studying specific genes or biological pathways that are relevant to human anxiety but instead those most relevant to the mouse behavior in question. Symptom-based models rely upon observable signs and symptoms of the disorders often represented by DSM-IV criteria and can be useful in deciphering the general genetics and neurobiology of behavior. However, we believe that the future development of animal models for psychiatric disorders (not necessarily for the actions of medications) will require a greater focus on validated endophenotypes rather than on symptom-based models. This is especially true when attempting to find genes and validate neurobiological mechanisms in model organisms, which are derived from studies of the human condition (Harrson & Weinberger 2005; Kirov et al. 2005). Therefore, a major advantage of an endophenotype approach to understanding psychiatric illnesses, given their complexity, is that animal models using endophenotypes are generally more straightforward and congruent with the human condition both on the level of biology and genetics, thus facilitating the use of such approaches.

Although progress in defining endophenotypes for some disorders (such as mood disorders) has been slow in both clinical and preclinical research, the endophenotype concept has had success in schizophrenia, where endophenotypes including prepulse inhibition (PPI, a measure of sensory motor gating deficits), eye-tacking dysfunction and working memory deficits are already connected to genetic polymorphisms and have enough support to be considered true endophenotypes of the disorder. There are extensive efforts underway to study, in model organisms, the neurobiological antecedents of various schizophrenia endophenotypes including PPI deficits (Geyer et al. 2002; Joobor et al. 2002; Petryshen et al. 2005; Robbins 2005; Seong et al. 2002; Swerdlow et al. 2001). The cause of putative neuroanatomical and neurodevelopmental endophenotypes in schizophrenia (Harrison & Weinberger 2005; Weinberger 1995) may soon be explained as the functional consequences of susceptibility genes are elucidated (e.g. Brandon et al. 2004; Schurov et al. 2004). Furthermore, multiple cognitive domains deficient in schizophrenia, in addition to working memory, represent possible endophenotypes (Green et al. 2004). Many of these are quite amenable to rodent studies. Neuropsychological findings have been correlated with in vivo imaging measures (Weinberger et al. 2001). However, similar imaging technology in rodents is far from ideal and will require significant technical advances to become a feasible approach.

Current animal models for bipolar disorder or depression mostly attempt to observe naturalistic behaviors or the results of pharmacological manipulation (Crawley 2000; Crowley & Lucki 2005; Cryan & Holmes 2005; Einat et al. 2003; Einat in press). Evolving theories suggest that endophenotypes for these disorders, based upon genetic and biological contributions, include attention deficits, circadian rhythm instability, dysmodulation of motivation and reward, brain structural changes and increased sensitivity to stress and stimulant medications (Glahn et al. 2004; Hasler et al. 2004; Hasler et al. in press; Lenox et al. 2002) (Fig. 2). Some of these are more amenable to modeling in animals than others. For example, a polymorphism in the putative bipolar disorder susceptibility gene, brain-derived neurotrophic factor (BDNF), has been linked to anatomic variations in the hippocampus and prefrontal cortex (Pezawas et al. 2004), as well as to hippocampal and prefrontal cortex cognitive performance (Egan et al. 2003; Hariri et al. 2003; Rybakowski et al. 2005) in humans. There exists significant preclinical support for the involvement of BDNF in both learning and memory and neuronal growth/plasticity (Egan et al. 2003; Lu 2003). Ultimately, the endophenotypes most valuable for affective disorders may be specific structural, functional and neuropsychological deficits, which at face value have poor correlation with overt phenotype-based models (Glahn et al. 2004; Hasler et al. 2004; Hasler et al. in press; Lenox et al. 2002; McDonald et al. 2004). Limbic-hypothalamic-pituitary-adrenocortical axis malfunction represents another viable putative endophenotype for mood disorders, and extensive animal models are already in existence (Akil 2005; Gould et al. 2003; Hasler et al. 2004; Seong et al. 2002).

As ongoing genetic studies advance, a full complement of validated animal models will be necessary to pinpoint the relevance of each individual single nucleotide polymorphism.
Given appropriate caveats, transgenic mouse technology has become commonplace, allowing for relatively rapid modification of gene expression (Crusio 2004; Gerlai 2001; Phillips et al. 2002). Clinical linkage studies continue to provide leads, which will inform the study of syntenic regions on the mouse and human chromosomes. These are mostly established, and recent research has already taken advantage of the available knowledge (Bogani et al. 2005; Wang et al. 2005).

Current attempts to model human disease in model organisms often report multiple deficits in many behaviors. However, the present field of psychiatric genetics was initiated with the understanding that human diseases are complex in nature—needing multiple genes working in dis-harmony with non-genetic contributors for the human syndrome (Gottesman & Shields 1967). The current standard of 6+ rodent phenotypes to make a high-impact paper is questionable given the nature of the genetics of these disorders. In these instances, a single gene disruption results in widespread “human-related” psychiatric phenotypes, which is in contrast to the predictions of the field. An improved expectation, even in a best case scenario, may be one or two endophenotypes being modulated by a true susceptibility gene acting improperly, as in the parallel human condition. Thus, it is conceivable that an endophenotype approach to genetic studies, when applied to preclinical studies in rodents, will not run the gamut of fulfilling multiple, diverse animal models of bipolar disorder; instead it may singularly affect one biological process that will only be present in tests of that single arena of focus (endophenotype) in laboratory animals. These may have limited (or no) face validity vis-à-vis symptom-based models. Clearly, for the development of improved therapeutics, animal models of medication efficacy (e.g. the tail suspension test and approach-avoidance situations) have tremendous utility; however, as gene exploration studies continue, we may find that current models have limited utility in discerning the relevant contribution of susceptibility genes. Continued improvements in valid animals models will depend on well-defined endophenotypes, downstream of gene expression and upstream of clinical symptoms, that necessitate increased collaborations between clinical and bench scientists.

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Systematic, standardized and comprehensive neurological phenotyping of inbred mice strains in the German Mouse Clinic

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Abstract

Neurological and psychiatric disorders are among the most common and most serious health problems in developed countries. Transgenic mouse models mimicking human neurological diseases have provided new insights into development and function of the nervous system. One of the prominent goals of the German National Genome Research Network is the understanding of the in vivo function of single genes and the pathophysiological and clinical consequences of respective mutations. The German Mouse Clinic (GMC) offers a high-throughput primary screen of genetically modified mouse models as well as an in-depth analysis in secondary and tertiary screens covering various fields of mouse physiology. Here we describe the phenotyping methods of the Neurological Screen in the GMC, exemplified in the four inbred mouse lines C57BL/6J, C3HeB/FeJ, BALB/cByJ, and 129S2/SvPas. For our primary screen, we generated “standard operating procedures” that were validated between different laboratories. The phenotyping of inbred strains already showed significant differences in various parameters, thus being a prerequisite for the examination of mutant mouse lines.

Keywords: Neurological phenotyping; Inbred mouse strains; SHIRPA; Grip strength; Rotarod; EEG

1. Introduction

Studying the neurobehavioural phenotype of transgenic mice and their inbred background strains is a powerful tool to understand the neural basis of behaviour and the pathophysiology of neurological and psychiatric disorders. Hundreds of different genes expressed in the central nervous system have been targeted in transgenic and knockout mice (Hafezparast et al., 2002; Watase and Zoghbi, 2003). Comparison of the mouse and human brain transcriptomes shows a good correlation for highly expressed genes in both transcript identity and abundance (Fougerousse et al., 2000). Therefore, screening of mice with respect to neurological disorders potentially offers an understanding of aetiology and pathogenesis of the human nervous system. For the comparison of neurological phenotyping data, standardized protocols were developed only recently, including a neurodevelopmental screening (Dierssen et al., 2002) and a behavioural phenotyping of mice in pharmacological and toxicological research (Karl et al., 2003). The Mouse Phenome Project (http://aretha.jax.org/pub-cgi/phenome/mpdcgi?rrtn=docs/home) promotes the quantitative phenotypic characterization of a defined set of inbred mouse strains.
strains under standardized conditions (Paigen and Eppig, 2000; Bogue, 2003; Bogue and Grubb, 2004; Grubb et al., 2004). Several inbred strain comparisons have been published (Balogh et al., 1999; Tarantino et al., 2000; Volkar et al., 2002). Transgenic and knockout databases with behavioural profiles of mouse mutants are described by Anagnostopoulos et al. (2001).

The German Mouse Clinic (GMC, www.mouseclinic.de) has the goal to develop and optimize a battery of different test parameters in different disciplines. The GMC was established as a part of the German NGFN (National Genome Research Network) and in collaboration with the pan-European Eumorphia consortium (www.eumorphia.org) and offers a systematic, standardized and comprehensive phenotyping of mutant mice to identify and characterize mouse models for human diseases. The GMC comprises thirteen screens covering neurology, behaviour, nociception, dysmorphology, immunology, clinical chemistry, allergy, steroid metabolism, energy metabolism, lung function, expression profiling, cardio-vascular function and pathology (Gailus-Durner et al., 2005). A primary screening of a mutant mouse line (MML) includes all screens; each mouse is tested consecutively in several laboratories.

The neurological screen of the GMC established a standardized primary screening including validation of the data. This comprises the comparison of results of testing on selected inbred mouse strains and/or selected mutants at more than two Eumorphia laboratories (Green et al., 2005). In the primary neurological screening a modified SHIRPA protocol (Rafael et al., 2000; Rogers et al., 1997, 2001) and a grip strength analysis is used that allows for a high-throughput screening of MMLs. Dependant upon results of this primary screen and due to specific questions, additional tests can be carried out for further assessment of neurological functions in a hierarchical way (van der Staay and Steckler, 2001). The secondary screen comprises a rotarod test, and the tertiary test a staircase test and, if applicable, electroencephalography (EEG). We will further broaden this arsenal of methods in the future. Behavioural tests such as the modified hole board test, the open field test and the Morris–Water–Maze test are performed in the complementing Behavioural Screen of the GMC (www.mouseclinic.de). The main aim of the neurological screen is to provide well-characterized mouse models for known neurological diseases with known gene defects (generated by transgenic, knockout and gene trap approaches), to investigate the in vivo consequences of the mutations, and to allow for therapeutic trials. In this paper we present our large spectrum of methods using inbred mouse strains C57BL/6J (C57) from Charles River, Germany, C3HeB/FeJ (C3H) from GSF Munich, Germany, BALB/cByJ (BALB) from Jackson Lab, USA, and 129S2/SvPasIco (129/SvP) from Charles River, France. All tests of the primary (SHIRPA, grip strength) and secondary (rotarod) neurological screen were performed in 10-week-old mice. The mice were caged in an animal colony maintained on a 12:12 h regular light–dark cycle. All experiments were done according to the German laws on animal protection and with permission from the “Regierung von Oberbayern”.

2. Material and methods

2.1. Animals

Four inbred strains of male mice were used: C57BL/6J (C57) from Charles River, Germany, C3HeB/FeJ (C3H) from GSF Munich, Germany, BALB/cByJ (BALB) from Jackson Lab, USA, and 129S2/SvPasIco (129/SvP) from Charles River, France. All tests of the primary (SHIRPA, grip strength) and secondary (rotarod) neurological screen were performed in 10-week-old mice. The mice were caged in an animal colony maintained on a 12:12 h regular light–dark cycle. All experiments were done according to the German laws on animal protection and with permission from the “Regierung von Oberbayern”.

2.2. Primary screening: SHIRPA protocol

The SHIRPA (Smithkline Beecham, MRC Harwell, Imperial College, the Royal London hospital phenotype assessment) protocol (Irwin, 1968) is a rapid, comprehensive, and semi-quantitative screening method for qualitative analysis of abnormal phenotypes in mice. In the neurological screen within the GMC, it is used in a modified form (Rafael et al., 2000; Rogers et al., 1997, 2001). We carried out 23 test parameters that contribute to an overall assessment of muscle, lower motor neuron, spinocerebellar, sensory and autonomic function (Online supplement Table S I). A “standard operating procedure” (SOP) for the SHIRPA test was generated, tested in the four inbred mouse lines and validated between different laboratories.

2.2.1. Behaviour in the viewing jar

After weighing of the mouse, neurological assessment starts with the observation of the undisturbed animal’s behaviour in a glass-viewing jar (Ø 11 cm) for 3 min. Items of interest are body position, tremor, palpebral closure, coat appearance, whiskers, and excessive lacrimation or defecation.

2.2.2. Behaviour in the arena

After transfer into an arena (36 cm × 20 cm), each mouse is tested for transfer arousal, locomotor activity and gait along with any bizarre or stereotyped motor behaviour. For locomotor activity a grid (3 × 5 squares) on the floor of the arena is used. During 30 s the number of squares are counted that the mouse enters with all four paws. Additionally, tail elevation, touch escape, positional passivity, and skin colour are scored.

2.2.3. Behaviour in or above the arena

Then, each mouse is lifted vertically up at mid-tail for 15 cm and curling of the trunk as well as possible grasping of the hind paws is observed. Pinna and corneal reflexes are tested by approaching the pinna and the eye, respectively, with the tip of a clean cotton swab. For the air-righting reflex, the animals are held horizontally in an inverted position 30 cm above a 10-cm foam bed, and are then released. If the mice show any obvious movement abnormalities before and at the transfer to the arena,
however, the righting reflex is done in the arena by turning the mouse on its back. For the contact-righting reflex, each mouse is placed into a glass cylinder (Ø 5 cm) that is turned upside down. Biting and vocalisation are scored during the entire handling.

2.3. Primary screening: grip strength

A grip strength meter (TSE; Bad Homburg, Germany) is used to measure the muscle strength in the forelimbs of the mice. The animals grasp a horizontal metal bar while being pulled by their tail. A sensor allows measurements of up to 600 ponds. Five trials within 1 min are performed for each mouse and their values are used as statistical variables in subsequent analysis. All experimental equipment is thoroughly cleaned with Pursept-A and dried prior to subsequent tests. Again, a SOP was generated for the grip strength analysis, tested in the four inbred mouse lines and validated between different laboratories. The SOP was examined by the Eumorphia administration team for accuracy and consistency and finally approved by a Eumorphia scientist outside the working group (http://www.eumorphia.org/EMPReSS).

2.4. Secondary screening: rotarod

A rotarod apparatus (TSE, Bad Homburg, Germany) is used to measure motor coordination, balance and motor learning ability (Bogo et al., 1981; Carter et al., 1999; Crawley, 1999). Rotarod performance requires a high degree of sensorimotor coordination and is especially sensitive to damage in the basal ganglia and the cerebellum (Lalonde et al., 1995, 1996; Mason and Sotelo, 1997). The machine is set up in an environment with minimal stimuli such as noise and movement. The rotarod unit consists of a computer-controlled motor-driven rotating spindle and four lanes for four mice. Infrared beams are used to detect the falling of the mice.

On the first day, the mice are habituated to the apparatus in two 180 s sessions at constant speeds of 12 and 20 rpm. In the motor coordination performance test on the second day, mice exert four trials with accelerating speed from 4 to 40 rpm. The mean latency to fall off the rotarod is recorded. Mice that rotate 600 ponds. Five trials within 1 min are performed for each mouse and their values are used as statistical variables in subsequent analysis. All experimental equipment is thoroughly cleaned with Pursept-A and dried prior to subsequent tests. Again, a SOP was generated for the grip strength analysis, tested in the four inbred mouse lines and validated between different laboratories. The SOP was examined by the Eumorphia administration team for accuracy and consistency and finally approved by a Eumorphia scientist outside the working group (http://www.eumorphia.org/EMPReSS).

2.5. Tertiary screening: staircase test

The staircase test measures forelimb reaching and grasping abilities in mice but also depends on the tendency of rodents to explore a novel environment. This investigator-independent method measures side-specific deficits in coordinated paw reaching and shows impairments due to contralateral lesions in the motor pathways of the brain, e.g., in motor cortex, striatum, nigrostriatal tract, and subthalamic nucleus (Abrous et al., 1993; Baird et al., 2001; Dunbar et al., 1992; Fricker et al., 1996; Henderson et al., 1999; Montoya et al., 1991; Whishaw et al., 1997). The device (Campden Instruments Ltd.) is a plexiglas box with a removable double staircase. Food pellets (BIOSERV) are placed on both sides of the staircase and present eight stages of reaching difficulty, which provide an objective measure of side bias, maximum forelimb extension, and grasping skills. Before testing, fasted mice are accustomed to the food pellets in their home cage for 3 days. Then, they are familiarized to the test boxes by placing food pellets on the staircase steps for a further 2 days. On subsequent days, one pellet per step (eight on each side) is placed in the box and the mouse is set in the start compartment. After 15 min training sessions, the number of pellets remaining on the steps and fallen down to the floor are counted. From the raw data, two experimental variables are calculated for each side and used in subsequent statistical analysis: (i) number of pellets grasped by the mouse on each side (i.e., eight minus the number of pellets remaining); (ii) maximum distance reached: the lowest step (numbered 1–8 from the top) with a remaining pellet.

2.6. Tertiary screening: electroencephalography (EEG)

Monitoring of EEG has become increasingly important in the neurological examination of certain mouse models. In our tertiary screening, we offer telemetric EEG of mice. This is a very laborious and time-consuming method and is only applied in selected mouse strains with epileptic seizures. To establish the method and to give a proof of principle, we performed EEG in twenty-six 22–36 weeks old C57 mice. With the DSI PhysioTel® telemetry system we can monitor mice while they freely move in their cages. A miniaturized implant transmits the digitized data via radio frequency signals to a receiver. The data are collected with the Dataquest® software. For the EEG, the transmitter is placed subcutaneously in the lower lateral trunk with the leads routed subcutaneously to an incision accessing the cranium. Trepanations (1 mm deep in the skull) are done with a microdrill on each side of the midline and 1 mm anterior of the lambda fissure. Microscrews placed in the drill holes lie directly above the dura and act as electrodes for the EEG lead which is wrapped round the screws. The screws are held in place with dental acrylic. EEG recordings are collected with an RPC-1 telemetry receiver (Data Science International), which is placed beneath the mouse’s cage at a sampling rate of 250 Hz. Particular stress is laid on the detection of epileptic discharges such as spike-wave complexes and seizure patterns.

2.7. Statistical analysis

2.7.1. SHIRPA results

Values for body weight and locomotor activity are presented as means ± S.E.M. One-way ANOVA is used to test for strain effects in quantitative parameters. If there is a rough deviation from the normal distribution (checked by inspecting a nor-

Fig. 1. Two-minute trace of EEG activity filtered by a 1–40Hz bandpass. Recording of a surface EEG derived from an inbred C57 mouse in vigilance state ‘NREM’.
mal probability plot), the nonparametric Kruskal–Wallis test is applied. The $\chi^2$-test is used for qualitative data.

2.7.2. Grip strength and rotarod test results

The grip strength and rotarod trials produce repeated measurements, which are analyzed by linear mixed effects models (Pinheiro and Bates, 2000). A linear mixed effects model is a modified analysis of variance/covariance (ANOVA/ANCOVA), which allows for the analysis of dependencies in the data. The strain variable and the covariate weight are modelled as fixed effects, mouse-specific intercepts are allowed by including the intercept as random effect. A dependency on the trial number can be checked by including the trial number as fixed and/or random effect. Interaction effects between the independent variables are tested for and excluded if their contribution is not significant. Covariates, which are not involved in an interaction and have no significant effect are also excluded. The statistic of interest is the strain effect in the final model. A significant strain effect in presence of the covariates means evidence of a difference between the strains. In this case, post hoc pairwise comparisons between strains are calculated with the covariates as included in the final model; $p$-values are not adjusted for multiple testing. Model fitting was performed by the nlme-package in R/SPlus (Project for Statistical Computing, 2004).

2.7.3. Staircase test

The staircase test is evaluated with a likewise analysis of variance with repeated measurements as mentioned above, considering strain effects as well as session length and side preferences on the number of pellets collected and maximum distance reached.

2.7.4. Extended statistical analysis routine phenotyping when both genders are present

In routine phenotyping, mice of both genders will have to be analyzed. In this type of analysis, gender is included as a factor in the ANOVA and mixed-effects models. Interactions involving gender will be tested for. If there is a significant interaction between gender and strain, male and female mice are analyzed separately.

2.7.5. EEG data

Spectral analysis was performed using 24 consecutive 10-s EEG periods derived from each mouse. Each 10-s period was divided into eight segments of 2.56 s duration overlapping each other by 1.28 s. After band-pass filtering (1–40 Hz), elimination of linear trends and tapering, each segment was submitted to Fast Fourier transformation (FFT) resulting in spectral power values [$\mu$V$^2$] with a frequency resolution of 0.488 Hz. The distribution of the power values over the frequency is known as the power spectra. The consecutive spectral evaluation of EEG signals was described by Tirsch et al. (1988). For feature extraction the peak-frequency in Hz and the sharpness of the peak were calculated from each normalised power spectrum and averaged over 24 periods.

3. Results

3.1. SHIRPA

All SHIRPA results are shown in Table 1. Only male mice were used for analysis. Body weight was significantly different ($p<0.001$). C57 mice had a lower weight as compared to C3H, BALB and 129/SvP mice (Table 1). During observation in the viewing jar, no obvious differences could be found between the strains with the exception of the whiskers presence ($p<0.01$): in contrast to C3H, BALB, and 129/SvP mice, four C57 mice had no whiskers.

After transfer to the arena, there were significant differences in locomotor activity ($p<0.0001$). BALB mice showed the highest locomotor activity, followed by C57 mice, while C3H and 129/SvP mice had much lower locomotor activity. 129/SvP and C3H mice had a different tail elevation and touch escape behaviour as compared to C57 and BALB mice.

Behaviour recorded in or above the arena revealed biting and vocal reactions in all 10 C57 mice, but in only one BALB mouse and none C3H or 129/SvP mouse.

3.2. Grip strength

There was a clear effect of strain ($p<0.0001$) and weight ($p<0.05$) on grip strength. Trial number had no significant influence, and there was no significant interaction effect between any pair of independent variables. Heavier mice tend to have stronger grip strength. In pairwise comparisons by fitting mixed-effects models with covariable weight for each pair of strains, C3H mice had a significantly higher forelimb strength than C57, BALB, and 129/SvP mice ($p<0.001$; see Fig. 1). In addition, BALB mice performed significantly better than C57 mice ($p<0.001$).

3.3. Rotarod

On the accelerating rotarod, strain had a significant effect on the latency ($p<0.05$). There was a significant interaction effect between weight and trial number ($p<0.05$) in the way
Table 1
Results of modified SHIRPA-analysis of four inbred strains

<table>
<thead>
<tr>
<th></th>
<th>C57 (n = 10)</th>
<th>C3H (n = 10)</th>
<th>BALB (n = 10)</th>
<th>129/SvP (n = 10)</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Body weight [g]</td>
<td>24.5 ± 0.5</td>
<td>27.2 ± 0.8</td>
<td>26.6 ± 0.4</td>
<td>27.8 ± 0.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>2. Body position</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inactive</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>n.s.</td>
</tr>
<tr>
<td>Active</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Excessive active</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>3. Tremor</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>n.s.</td>
</tr>
<tr>
<td>Present</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>4. Palpebral closure</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eyes open</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>n.s.</td>
</tr>
<tr>
<td>Eyes closed</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>5. Coat appearance</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tidy and well groomed</td>
<td>10</td>
<td>10</td>
<td>9</td>
<td>10</td>
<td>n.s.</td>
</tr>
<tr>
<td>Irregularities</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>6. Whiskers</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>6</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Absent</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>7. Lacrimation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>n.s.</td>
</tr>
<tr>
<td>Present</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>8. Defecation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>4</td>
<td>5</td>
<td>6</td>
<td>7</td>
<td>n.s.</td>
</tr>
<tr>
<td>Absent</td>
<td>6</td>
<td>5</td>
<td>4</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>9. Transfer arousal</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extended freeze</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>n.s.</td>
</tr>
<tr>
<td>Brief freeze</td>
<td>7</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Immediate movement</td>
<td>3</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>10. Locomotor activity</td>
<td>23.9 ± 1.7</td>
<td>17.7 ± 2.2</td>
<td>30.7 ± 2.4</td>
<td>8.2 ± 1.9</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>[number of squares crossed]</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11. Gait</td>
<td>Fluid movement</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>9</td>
</tr>
<tr>
<td>Lack fluidity</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>12. Tail elevation</td>
<td>Dragging</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Horizontal extension</td>
<td>10</td>
<td>6</td>
<td>10</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Elevated tail</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>13. Touch escape</td>
<td>No response</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Response to touch</td>
<td>8</td>
<td>7</td>
<td>7</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Flees prior to touch</td>
<td>2</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>14. Positional passivity</td>
<td>Struggles when held by tail</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>No struggle</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>15. Skin colour</td>
<td>Blanched</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Pink</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Bright, deep red</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>16. Trunk curl</td>
<td>Absent</td>
<td>8</td>
<td>4</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>Present</td>
<td>2</td>
<td>6</td>
<td>4</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>17. Limb grasping</td>
<td>Absent</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Present</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>18. Pinna reflex</td>
<td>Present</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Absent</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>19. Corneal reflex</td>
<td>Present</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Absent</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>20. Righting reflex</td>
<td>Rights itself</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Fails to right</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>21. Contact righting reflex</td>
<td>Present</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Absent</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>22. Evidence of biting</td>
<td>Biting in response to handling</td>
<td>0</td>
<td>10</td>
<td>9</td>
<td>10</td>
</tr>
<tr>
<td>None</td>
<td>10</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>23. Vocalisation</td>
<td>None</td>
<td>0</td>
<td>10</td>
<td>9</td>
<td>10</td>
</tr>
<tr>
<td>Vocal</td>
<td>10</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>
Fig. 3. Rotarod performance test. The data points represent the means over the retention period of the mice on the rotarod in four consecutive trials. C3H mice performed better than C57 and Balb mice ($p < 0.05$).

that the performance improved with the number of trials, but the improvement was less pronounced in heavier animals. The interaction of weight with strain and of trial number with strain was not significant; in fact all groups were able to improve their performance over the training course (Fig. 2). For pairwise comparisons, the strain effect in mixed-effect models with covariables trial number and weight and their interaction was calculated for each combination of strains. C3H mice performed significantly better than C57 and BALB mice ($p < 0.05$).

3.4. Staircase

There were no significant differences between the inbred strains in the staircase test (Fig. 3). However, C57 mice performed better in the collection of pellets with ongoing time (30 and 60 min) and in the distance reached. All four inbred strains showed altered performance with increasing session duration. Maximal performance was reached after 30 min except for the BALB strain. This line also showed an increase in performance in the last 30 min. There was no significant effect of side preference.

3.5. EEG analysis

An example of an EEG trace is given in Fig. 4. The time course of relative power spectra derived from 24 consecutive periods of the same EEG is shown in Fig. 5. As it can be seen the spectra differ within the whole 4-min EEG-recording and show a clear variance in time. The main peaks of the spectra are mostly located about 3 Hz with a mean peak frequency of 3.6 Hz.

Fig. 4. Performance of inbred mouse strains in the staircase test. Session length, varied between 5 and 60 min. Each mouse performance was analyzed both as the number of pellets collected (a) and as the maximum distance reached (b). Data for the inbred strains are presented separately for each variable; results did not differ between the inbred strains.
The main result of the spectral analysis obtained from the sample set of \( n = 26 \) inbred C57 mice-EEGs is shown in Fig. 6. This scatter diagram illustrates the distribution of mean spectral peak frequency and steepness of the peak in the two-dimensional feature space calculated from each 4-min EEG. Each case is marked by a cross. An intra-individual variance is evident. The range of the spectral peak frequency is between 3.5 and 5.5 Hz, whereas, the complexity ranges from 7.4 to 8.7 corresponding to the relatively broad spectra of high-complex and noisy EEGs in NREM state.

4. Discussion

The neurological performance of four inbred mouse strains was evaluated and compared in a battery of different tests. The tests were standardized for the neurological screen of the GMC and were chosen to gain insight into motor performance, forelimb grip strength, skilled reaching and spatial motor learning. The need for standardization in phenotyping is obvious. As data of behavioural and neurological phenotypes accumulate from large-scale mutagenesis screens (Sayah et al., 2000), there is discussion about the most appropriate neurological tests for evaluating abnormal behaviour (Tarantino et al., 2000). Such tests need to cover a wide range of neuronal and neuromuscular functions as it is shown here. It was shown already that the modified SHIRPA protocol is an adequate method for successful screening of large cohorts of mice (Masuya et al., 2005). In addition, several targeted mouse mutants have been characterized (Burne et al., 2005; Lalonde et al., 2005). Previous studies have shown that inbred mouse strains used to generate mutant mice already display marked differences in neurobehavioural tasks (Crawley and Pavlory, 1997; Dierssen et al., 2002). This is confirmed by the marked differences between strains in the present work.

These data emphasize the importance of genetic background of mutant mice in order to evaluate neurological data.

In our primary observational screen, we show that there are marked differences between C57 and C3H mice, in particular regarding body weight, grip strength and locomotor activity. C57 were markedly more aggressive than the other inbred strains. Several genes appear to influence mouse aggression, and strain-related differences are reported (Miczek et al., 2001a,b). Absent whiskers of C57 mice can be explained by the overgrooming of mice that barber the whiskers of their cage mates, a phenomenon often seen in C57 mice but also in other strains (Kalueff et al., 2006; Sarna et al., 2000).

BALB and C57 mice exhibited a higher locomotor activity as C3H and 129/SvP mice. Differences in locomotor activity in mice can be due to differences in the dopaminergic system, which plays an important role in locomotor function and motivational processes (Robbins and Everitt, 1996; Wise, 1996). In contrast to the large difference between BALB and 129/SvP mice, C3H mice may be an intermediate between these strains. C57 and BALB mice were relatively more active than the C3H and 129/SvP mice in the viewing jar in contrast to published data (Rogers et al., 1999). In contrast to 129/SvP, C3H mice have marked visual defects due to retinal degeneration. This may influence locomotor activity in this strain.

Grip strength was highest in C3H as already described (Rogers et al., 2001) and in the range also seen in other laboratories (http://www.jax.org/phenome; Bogue and Grubb, 2004; Grubb et al., 2004).

In the rotarod test, C57 mice showed more often than the other inbred strains passive rotation behaviour. This leads to the significantly shorter latency on the rotarod as compared to C3H mice, whereas in other publications it had been described that C57 mice performed rather better than C3H mice (Brooks et al., 2004; McFadyen et al., 2003). In the inbred strains, we tested here, a significant influence of body weight on the improvement of rotarod performance over trials was detected. A similar effect of weight on performance was also described elsewhere (McFadyen et al., 2003).

In conclusion, strain-related differences as we found here with standardized, comprehensive SOPs in the GMC need to be taken into account when performing and interpreting results from genetically modified mice, particularly those of mixed background. Our results clearly demonstrate significant differences between the strains. Therefore, the screening of MMLs in the GMC is performed with wildtype littermates as control mice to minimize the influence of the genetic background.

In addition, the spectrum of neurological tests also includes EEG to detect differences in bioelectrical activity in brains of mutant mice. Benefits of using implanted telemetry monitors are: (i) stress-induced artefacts compared to restrictive monitoring are significantly reduced, (ii) measurements are free from the effect of anaesthesia, and (iii) animal handling is minimized. Differences in the specific rhythmic pattern of the waveforms are indicators of functional disorders in the cerebral cortex. This method is applied for the analysis of paroxysmal neurological disorders and allows the characterisation of mouse models for epilepsy. The facilities of the automatic EEG analysis are shown.

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Fig. 6. Scatter diagram of mean spectral peak frequency and steepness of the peak calculated from each 4-min EEGs of \( n = 26 \) inbred C57-mice.
in this paper for the C57 mouse strain. These features can be used to disclose differences between genotypes and to complete our neurological examinations of MMLs.

The results presented here could be complemented with an overall examination of a mouse in the GMC with data from behavioural, clinical-chemical, hematological, nociceptive, metabolic (energy, steroids), cardiovascular, immunological, lung function, expression, dysmorphological, and pathological data. Thus, the GMC is capable in performing an overall phenotyping of various MMLs. This leads to the standardized quantification of known phenotypes as well as the detection of new phenotypes in mutant mice covering a wide range of methods in a variety of screening categories. This approach contributes to the understanding of underlying mechanisms that may be affected by a transgene and offers a valuable tool in the expanding field of the generation and analysis of genetically modified mice as models for human diseases.

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Appendix A. Supplementary data


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Glucocorticoid receptor gene inactivation in dopamine-innervated areas selectively decreases behavioral responses to amphetamine

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The meso-cortico-limbic system, via dopamine release, encodes the rewarding and reinforcing properties of natural rewards. It is also activated in response to abused substances and is believed to support drug-related behaviors. Dysfunctions of this system lead to several psychiatric conditions including feeding disorders and drug addiction. These disorders are also largely influenced by environmental factors and in particular stress exposure. Stressors activate the corticotrope axis ultimately leading to glucocorticoid hormone (GCs) release. GCs bind the glucocorticoid receptor (GR) a transcription factor ubiquitously expressed including within the meso-cortico-limbic tract. While GR within dopamine-innervated areas drives cocaine’s behavioral responses, its implication in responses to other psychostimulants such as amphetamine has never been clearly established. Moreover, while extensive work has been made to uncover the role of this receptor in addicted behaviors, its contribution to the rewarding and reinforcing properties of food has yet to be investigated. Using mouse models carrying GR gene inactivation in either dopamine neurons or in dopamine-innervated areas, we found that GR in dopamine responsive neurons is essential to properly build amphetamine-induced conditioned place preference and locomotor sensitization. c-Fos quantification in the nucleus accumbens further confirmed defective neuronal activation following amphetamine injection. These diminished neuronal and behavioral responses to amphetamine may involve alterations in glutamate transmission as suggested by the decreased MK801-elicited hyperlocomotion and by the hyporeactivity to glutamate of a subpopulation of medium spiny neurons. In contrast, GR inactivation did not affect rewarding and reinforcing properties of food suggesting that responding for natural reward under basal conditions is preserved in these mice.

Keywords: glucocorticoid receptor, dopamine pathway, glutamate, amphetamine, food reward, motivation
INTRODUCTION
Reward processing involves the meso-cortico-limbic system, which includes dopamine midbrain neurons and their projections to the caudate putamen (CPUs), the nucleus accumbens (NAC), and the prefrontal cortex (PFC). Both addictive drugs and natural rewards act on these brain circuits that are likely to have evolved to motivate vital behaviors, including eating (Kelley and Berridge, 2002). Indeed, most drugs of abuse food rewards elicit an increase in DA release within the NAC (Di Chiara and Imperato, 1988; Hernandez and Hoebel, 1988) thought to participate to the encoding of rewarding and reinforcing properties of food rewards and addictive substances.

Vulnerability to abused drugs varies from one person to another. This interindividual variability most probably relies on both genetic and environmental factors, including stress exposure (Sinha, 2001). Similarly, stress exposure has also been shown to affect food intake and has been associated with feeding disorders (Torres and Nowson, 2007). Stress response triggers a large set of physiological reactions, including the activation of the hypothalamo-pituitary-adrenal (HPA) axis, ultimately leading to the secretion of glucocorticoids (GCs) by the adrenal gland in the blood flow. GCs activate two related nuclear receptors, the glucocorticoid receptor (GR) ubiquitously expressed, including within neurons of the reward circuitry, and the mineralocorticoid receptor (MR) restricted to more discrete brain regions. Both act as transcription factors, in the nucleus, to control gene expression and, at the membrane, participate to the rapid modulation of neuronal excitability and intracellular signaling cascades. During stress response MR is involved in the appraisal of novel situations whereas GR facilitates the consolidation of stress-related information (Groeneweg et al., 2011).

Clinical studies, supported by compelling animal data, underlie the central role of GCs in modulating responses to abused drugs and feeding behaviors (Marinelli and Piazza, 2002; Dallman et al., 2004; Sinha et al., 2006; Adam and Epel, 2007). For example, surgical suppression of circulating GCs in rats decreases locomotor responses to psychostimulants, an effect rescued by hormone replacement (Marinelli et al., 1997). Similarly, adrenalectomy have been shown to block the increase of fat intake observed after fasting in rat and this behavior is restored after corticosterone treatment (Castonguay, 1991). In addition, chronic GCs treatments in rats have been shown to impair goal-directed behavior as well as motivation to obtain food reward (Gourley et al., 2012).

We previously developed GRloxP/loxP and DATloxP/loxP mouse models. The GRloxP/loxP mice are deprived of GR in most of medium spiny neurons and neurons of the basal layers of the cortex (hereafter described as dopaminocinceptive neurons) while DATloxP/loxP mice are deprived of GR in dopamine neurons (Ambroggi et al., 2009; Barik et al., 2013). The absence of GR in dopaminocinceptive but not dopamine-releasing neurons diminished sensitizing, rewarding, and reinforcing effects of cocaine (Ambroggi et al., 2009; Barik et al., 2010). In striking contrast, we showed that morphine responses in both models remained unaltered (Barik et al., 2010) although stress facilitates opiates effects (Deroche et al., 1995). While these results suggest a GR-dependent dichotomy for the regulation of psychostimulant and opiate responses, such hypothesis still needs to be validated, as GR involvement in responses to other psychostimulant drugs such as amphetamine has never been clearly established. In addition, while extensive work has been made to uncover the role of this receptor in drugs of abuse-related behaviors, its potential contribution in responses to food rewards and the neuronal population that may be involved have yet to be investigated. We therefore, examined the ability of GR along the DA pathway to modulate behavioral responses to amphetamine and food rewards. We demonstrated that GR in dopaminocinceptive neurons selectively modulated behavioral and molecular responses to amphetamine without altering rewarding and reinforcing properties of food rewards.

MATERIALS AND METHODS
ANIMAL BREEDING AND DRUG TREATMENTS
Nr3c1 (GR) gene inactivation was selectively targeted in dopaminocinceptive (Nr3c1loxP/loxP;TgD1aCre) (Lemberger et al., 2007), hereafter designed GRloxP/loxP) or dopamine (Nr3c1loxP/loxP;TgBAC-DATiCrefo) (Turiault et al., 2007), hereafter designed GRloxP/loxP) neurons as described in Ambroggi et al. (2009). Experimental animals were obtained by mating Nr3c1loxP/loxP females with either Nr3c1loxP/loxP;TgD1aCre or Nr3c1loxP/loxP;TgBAC-DATiCrefo mice. Half of the progeny were mutant animals, the other half were control littermates. Animals were bred and raised under standard animal housing conditions, at 22°C, 55–65% humidity, with a 12-h light/dark cycle (7 am/7 pm) and free access to water and a rodent diet. All experiments were performed in accordance with French (Ministère de l’Agriculture et de la Forêt, 87-848) and the European Directive 2010/63/UE and the recommendation 2007/526/EC for care of laboratory animals. Mice were 2–4 month old males and backcrossed for more than 8 generations on C57BL/6 genetic background. All the experiments have been performed within the hours preceding or encompassing the beginning of dark phase (7 pm), when corticosterone levels are elevated (Le Minh et al., 2001). The behavioral sensitization experiments have been carried out from 6 pm to 9 pm; The CPP experiments from 7 pm to 11 pm and the food progressive ratio (PR) experiment were performed from 4 pm to 6 pm. All drugs were dissolved in saline 0.9%. D-amphetamine (freebase; Sigma-Aldrich, Saint-Quentin Fallavier, France), SKF81297 (salt; Tocris Cookson, Bristol, UK), and MK801 (salt; Tocris Cookson, Bristol, UK) were administered intraperitoneally (ip).

LOCOMOTOR ACTIVITY AND SENSITIZATION
Locomotor activity and sensitization were performed as described in Barik et al. (2010). Briefly, locomotor activity was assessed in circular chamber (4.5-cm width, 17-cm external diameter) crossed by four infrared capsors (1.5 cm above the base) placed at every 90° (Imetronic, Bordeaux, France). The locomotor activity was counted when animals interrupted two successive beams and thus, had travelled a quarter of the circular corridor. Mice were habituated to the apparatus for 3 h, for 3 consecutive days, and received a saline injection on days 2 and 3. To assess the response to SKF81297 or MK801, on day 4 mice were placed in the apparatus for 90 min before receiving an acute injection of SKF81297 (1.5 or 3 mg/kg) or MK801 (0.2 mg/kg). In the case of amphetamine-induced locomotor sensitization, from day 4 to 8,
mice were daily treated with amphetamine or saline after a 90 min habituation to the apparatus. Following 8 days of withdrawal, mice received an acute challenge of amphetamine. The locomotor activity post-injection was acquired for 1 h. For acute drug responses data are recorded as ½ turn per 5 min. For clarity reason, data are presented every 10 min. For locomotor sensitization, data are presented as the sum of activity over 1 h.

**CONDITIONED PLACE PREFERENCE**

The conditioned place preference (CPP) apparatus consisted of two chambers (20 × 20 × 25 cm) with distinct visual and tactile cues connected by a neutral area. On day 1 (pre-conditioning), mice were placed in the neutral area allowed to freely explore the apparatus for 18 min. The time spent in each chamber was measured. On days 2, 4, 6, and 8, amphetamine-paired mice received an amphetamine injection (1 or 2 mg/kg) and were confined to one chamber for 25 min. On days 3, 5, 7, and 9, amphetamine-paired mice received saline in the opposite chamber and were also confined for 25 min. Saline-paired animals received saline in both chambers. To examine food-induced CPP, mice had limited access to chow pellet in their home-cage for a week, to stabilize their bodyweight to 85% of their original weight. Conditioning for food was similar to that of amphetamine. Food-paired mice received a chow pellet (1 g; standard food CPP) or chocolate with cereals (“chocapic,” Nestlé, 0.5 g; palatable food CPP) in the paired chamber on days 2, 4, 6, 8 and confined for 30 min. On days 3, 5, 7, 9, mice were confined to the other, unpaired, chamber but had no access to food. No food-paired mice were alternatively placed in each chamber with no access to food at any time. During the post-conditioning (day 10), mice, in absence of any reward, were allowed to freely explore both chambers for 18 min. The CPP scores were expressed as the increase of time spent in the paired chamber between the post- and the pre-conditioning sessions.

**PROGRESSIVE RATIO FOR FOOD**

**Apparatus**

The PR experiment took place in 12 home cages containing an operant conditioning wall (24 × 28 × 28 cm, Operant Behavior System, TSE, Bad Homburg, Germany). The operant wall had two retractable levers, a food pellet dispenser delivering 20 mg sucrose pellets with peanut butter flavor (GlaxoSmithKline, TestDiet, Richmond, IN, USA) and white light bulbs above the levers and in the dispenser. The operant walls were covered outside periods of training and testing. The boxes were covered with a layer of corn cob bedding and enriched with cotton nest pads. Water was available ad libitum. During the period of habituation food (chow, SAFE, Augy, France) was also available ad libitum.

**Habituation, Training, and Testing**

Mice were maintained at 85% of their initial body weight during training and testing. We tested 6 control and 6 GR<sub>D1Cre</sub> mice. During the habituation period (2 days) mice were placed in the operant boxes with ad libitum access to food. The mice had continuously access to the operant wall and learnt to lever press for sucrose pellets under a fixed ratio 1 (FR1) schedule (i.e., a single press on the active lever resulted in the delivery of one sucrose pellet). Mice were trained on a FR1 schedule overnight. for 4 days. The FR1 schedule was followed by 6 days of PR schedule during which the cost of a reward is progressively increased for each following reward in order to determine the amount of work the mouse is willing to put into obtaining the reward. The response requirement increases incrementally according to a non-arithmetic progression: 1, 2, 3, 4, 4, 4, 5, etc. and forms the following series: 1, 2, 4, 6, 9, 12, 15, 19, 23, 27, 31, etc. PR sessions were carried out once a day. One non-responding control mouse was excluded from the analysis. Breaking point values were defined as the last ratio completed by the animal followed by 15 min during which no additional reward was earned.

**IMMUNOHISTOCHEMISTRY**

Immunohistochemistry was performed as described in Barik et al. (2010). Briefly, mice were deeply anesthetized with pentobarbital (Centravet, France) and transcardially perfused with cold phosphate buffer (PB: 0.1 M Na<sub>2</sub>HPO<sub>4</sub>/NaH<sub>2</sub>PO<sub>4</sub>, pH 7.4), followed by 4% PFA in PB. Brains were post-fixed overnight in 4% PFA-PB. Free-floating vibratome sections (30 μm) were rinsed twice with PBS (20 min) and incubated (30 min) in PBS-BT (PBS 0.5% BSA, 0.1% Triton X-100) with 10% normal goat serum (NGS). Sections were incubated (4°C) in PBS-BT, 1% NGS, with rabbit anti-c-Fos (1:500, Abcam, Cambridge, MA) for 36 h. Sections were rinsed in PBS and incubated (2 h) in goat anti-rabbit biotinylated secondary antibody (1:1000, Vector Laboratories, Burlingame, CA) in PBS-BT, 1% NGS. PBS-rinsed sections were incubated in avidin-biotin-peroxidase complex (ABC reagent; Vector Laboratories, 1:1000) for 1 h. Signal was revealed using the peroxidase-substrate-kit-DAB, as recommended by Vector Laboratories. Quantification of c-Fos immunopositive cells was done semi-automatically using Mercator Explora-Nova software (La-Rochelle, France). CPU and NAc regions were delineated according to Paxino’s mouse brain atlas. For, drug-induced c-Fos expression, mice received an acute challenge of saline or amphetamine, and were perfused 1 h later.

**MICRO-IONTOPHORESIS AND IN VIVO RECORDINGS**

Electrophysiological recordings of NAc medium spiny neurons were performed during the diurnal phase. The experimenter was blind to the genotype during recordings. Mice were anesthetized with chloral hydrate (5.0 mg/kg, i.p.) and mounted in a stereotaxic apparatus. The lateral tail vein was catheterized to administer additional anesthetic or drugs. Body temperature was monitored and maintained with a heating pad at 36.5–37.0°C. Standard electrophysiological procedures were employed. The electrode signal was amplified 2000 times with an AC high impedance amplifier, band pass filtered at 0.4–20 kHz (Digidata 1440A, Axon Instruments Inc., Foster City, CA) and fed to a computer for offline analysis.

For single unit recordings of NAc medium spiny neurons, five barrels manufactured electrodes (ASI instruments, Warren, MI) were pulled and broken to a tip diameter of 8–15 μm. The center barrel was filled with 2 M NaCl containing 1% Fast Green dye (impedance 2–6 MΩ) and was used to record neuronal activity. One side barrel (impedance 20–60 MΩ) was
FIGURE 1 | Impaired molecular but not locomotor responses following an acute amphetamine challenge in GR\textsuperscript{D1Cre} mice. (A) Representative example of c-Fos induction in the NAc core of a control and a GR\textsuperscript{D1Cre} mouse in response to saline, amphetamine 1 and 2 mg/kg. (B) Amphetamine-induced c-Fos expression in the caudate-putamen (left panel), the nucleus accumbens core (middle panel), and shell (right panel) of control and GR\textsuperscript{D1Cre} mice. n = 4-8 animals per group; saline vs. drug: *P < 0.05; **P < 0.01; control vs. mutant: *P < 0.01. Locomotor activity is expressed as the sum of \(\frac{1}{4}\) turns in a circular cylinder per 5 min following acute drug (gray or black) or saline (white) injections in control (circles), GR\textsuperscript{DATCre} (diamonds), and GR\textsuperscript{D1Cre} (squares) mice. (C) Similar locomotor response to a single injection of saline and amphetamine (1 mg/kg) in control and GR\textsuperscript{DATCre} mice. Interaction Drug \times \text{Time} F(29, 840) = 7.9, P < 0.001, with no genotype effect \(F(1, 420) = 1.3, P > 0.05\). (D) Control and GR\textsuperscript{D1Cre} mice equally respond to an acute 1 mg/kg of amphetamine. Interaction Drug \times \text{Time} F(87, 780) = 1.7, P < 0.001, with no genotype effect \(F(1, 637) = 0.1, P > 0.05\). (E,F) Amphetamine (2 mg/kg) induced a robust increase in locomotor response regardless of the genotype in control and GR\textsuperscript{DATCre} mice [(E), no genotype effect \(F(1, 656) = 0.3, P > 0.05\)] and control and GR\textsuperscript{D1Cre} mice [(F), no genotype effect \(F(1, 686) = 0.8, P > 0.05\)].
filled with 150 mM NaCl for automatic current balancing. The other barrels were filled with L-glutamate (100 mM, pH 8), which was ejected as an anion. A retaining current (5–10 nA) was applied during non-ejection periods to minimize passive diffusion.

Electrodes were lowered in the NAc as followed: AP +1.1/+1.7, L+0.6/1.2 and DV −3.9/−5.0 mm from the cortical surface. Because most NAc neurons are quiescent in the basal state, glutamate was ejected by micro-iontophoresis while searching for neurons. Once a neuron was detected, the stability of the signal to noise ratio and waveform characteristics were assessed. Recorded neurons were identified as medium spiny neurons NAc neurons by their anatomical location and waveform durations comprised between 1.1 and 1.8 ms (White, 1996; Kish et al., 1999; Mallet et al., 2005). To generate current-response curves, glutamate was ejected by micro-iontophoresis using escalating currents applied in 15 s pulses interspersed with 15 s of non-ejection periods.

STATISTICS
Data are presented as means ± s.e.m. Statistical analysis was carried out using Two-Way analysis of variance (ANOVA) for CPP, drug-elicited c-Fos induction. Acute locomotor responses and locomotor sensitization experiments were analyzed with Three-Way ANOVA with repeated measures. Recorded neurons were identified as medium spiny neurons NAc neurons by their anatomical location and waveform durations comprised between 1.1 and 1.8 ms (White, 1996; Kish et al., 1999; Mallet et al., 2005). To generate current-response curves, glutamate was ejected by micro-iontophoresis using escalating currents applied in 15 s pulses interspersed with 15 s of non-ejection periods.

RESULTS
ACUTE NEURONAL AND BEHAVIORAL RESPONSES TO AMPHETAMINE IN MICE DEPRIVED OF GR GENE WITHIN THE MESO-CORTICO-LIMBIC DOPAMINE SYSTEM

We studied neuronal activation upon amphetamine response by quantifying c-Fos expression in mutant and control littermates. Consistent with previous findings (Moratalla et al., 1996), amphetamine (1 and 2 mg/kg) elicited a significant increase in the number of c-Fos-positive cells within the CPu and the NAc core and shell of control animals (Figures 1A,B). This effect was significantly diminished within the NAc subdivisions and displayed a trend toward a decrease in the CPu when GR<sup>D1Cre</sup> mice were administered 1 mg/kg of the drug (Figures 1A,B). No significant genotype difference was observed when animals were administered a higher dose (2 mg/kg) of amphetamine (Figures 1A,B). These results indicate a hyporesponsiveness of the NAc of GR<sup>D1Cre</sup> mice to low doses of amphetamine.

In many species including rodents, psychostimulant injection triggers a typical increase in locomotor responses. Thus, locomotor activity of GR<sup>D1Cre</sup> mice and their control littermates was measured following acute amphetamine administration. To ascertain the lack of involvement of GR in dopamine-releasing neurons we also examined responses in GR<sup>DATCre</sup> mice and their respective controls. While saline injection failed to produce any locomotor hyperactivity, amphetamine increased locomotor activity in control mice with a stronger response at 2 mg/kg compared to 1 mg/kg (Figures 1C–F). The locomotor response to single amphetamine injection was the same in both GR<sup>DATCre</sup> (Figures 1C,D) and GR<sup>D1Cre</sup> mice (Figures 1E,F) compared to their respective control littermates, for both doses tested. Thus, the absence of GR in dopaminceptive neurons does not alter the acute behavioral response to amphetamine.

THE ABSENCE OF GR IN DOPAMINOCEPTIVE NEURONS DECREASES THE SENSITIVITY TO LOCOMOTOR SENSITIZING PROPERTIES OF AMPHETAMINE

One of the key features of abused drugs is their ability to trigger locomotor sensitization (Vanderschuren and Pierce, 2010), i.e., a progressive and enduring augmentation in locomotor activity following repeated drug injection. We assessed the sensitizing properties of amphetamine in GR<sup>D1Cre</sup> and GR<sup>DATCre</sup> mice, and respective control littermates. Five consecutive daily injections...
of amphetamine (1 mg/kg), but not saline, induced significant locomotor sensitization in control mice, which was still persistent following an 8-day withdrawal period (Figures 2A,B). In contrast GR\textsuperscript{D1Cre} mice failed to develop locomotor sensitization (Figure 2A) whereas the absence of GR in dopamine neurons (GR\textsuperscript{D1ATCre} mice) had no effect (Figure 2B). When tested at a higher dose (2 mg/kg), amphetamine induced a more robust locomotor sensitization that was similar in both mutant lines and their respective control littermates (Figures 2C,D). Hence, GR within dopaminceptive neurons is selectively required for enabling locomotor sensitization to low doses of amphetamine. This suggests that elevated doses of amphetamine are likely to provoke stronger molecular activations that hence may overcome GR’s modulatory effects.

THE ABSENCE OF GR IN DOPAMINOCEPTIVE NEURONS DECREASES THE SENSITIVITY TO REWARDING PROPERTIES OF AMPHETAMINE

Repeated pairings of abused drugs in a specific environment triggers reward-associated memories (Kelley, 2004) thought to reflect changes in the motivational state of the subject (Bardo and Bevins, 2000). We next studied amphetamine CPP, a commonly employed context-dependent paradigm, to measure the effects of rewarding stimuli in GR mutant animals. On the pre-conditioning day, all four groups of animals spent similar amount of time in the two distinct chambers (Figure 3A). Pairing injections of 1 or 2 mg/kg of amphetamine produced a significant CPP in control mice (Figures 3B,C). Mirroring the results obtained for locomotor sensitization, these rewarding effects were abolished in GR\textsuperscript{D1Cre} mice at the lowest dose of amphetamine tested, but were not significantly different from controls when the dose was increased up to 2 mg/kg (Figure 3B). The absence of GR in pre-synaptic dopamine neurons had no effect as GR\textsuperscript{D1ATCre} mice displayed normal CPP to 1 mg/kg amphetamine (Figure 3C).

ABNORMAL LOCOMOTOR RESPONSE TO NMDA ANTAGONIST IN ABSENCE OF GR IN DOPAMINOCEPTIVE NEURONS

In response to abused drugs, the increase of dopamine release within the CPu and NAc is thought to filter and selectively reinforce connections arising from excitatory corticostriatal projections (Barnford et al., 2004). Hence this dopamine/glutamate interaction is key to shape medium spiny neurons responsiveness at both electrophysiological and molecular levels, with a central implication of D1 dopamine receptors and NMDA glutamate receptors in these processes (Nicola et al., 2000; Pascoli et al., 2011). We therefore, examined whether GR gene inactivation within dopaminceptive neurons could impact on dopamine and glutamate receptor functions that may explain the observed phenotype. To challenge D1 dopamine receptor, we injected SKF81297, a selective D1-like receptor agonist and measured subsequent locomotor responses. As previously reported (Corvol et al., 2007), acute systemic SKF81297 injection elicited hyperlocomotion in control animals (Figure 4A). At the 2 doses examined (1.5 and 3 mg/kg), GR\textsuperscript{D1Cre} mice did not differ from their respective control littermates (Figure 4A) ruling out an impaired functionality of D1 dopamine receptors. To determine the state of glutamate transmission in GR\textsuperscript{D1Cre} mice, we then assessed the ability of MK801, a non-competitive NMDA antagonist, to elicit hyperlocomotion (Qi et al., 2008). Systemic injection of MK801 (0.2 mg/kg) triggered a robust hyperlocomotion in controls that was significantly decreased in mutant mice (Figure 4B). Therefore, this set of experiments suggests that the impaired glutamate response may play a role in the diminished response to amphetamine.

DECREASED RESPONSIVENESS TO GLUTAMATE IN A SUBPOPULATION OF MEDIUM SPINY NEURONS WITHIN THE NUCLEUS ACCUMBENS OF GR\textsuperscript{D1Cre} MICE

Altered glutamatergic neurotransmission within the NAc might contribute to the impaired behavioral responses to amphetamine and MK801 as well as to the decrease of accumbal c-Fos induction observed in GR\textsuperscript{D1Cre} mice. To investigate the functional effects of GR inactivation on glutamatergic neurotransmission within the NAc, we analyzed the reactivity to glutamate of NAc medium spiny neurons. We performed in-vivo recordings of NAc medium spiny neurons coupled to glutamate micro-iontophoresis in control and GR\textsuperscript{D1Cre} mice. For each neuron, incremental glutamate ejection currents were applied until the neuron reached its maximal firing frequency. In control mice, the maximal frequencies observed were normally-distributed, ranging from 7 to 17 Hz (Figures 5A–C). In contrast in GR\textsuperscript{D1Cre} mice, the distribution was bimodal: one population had maximal frequencies in a range similar to that observed in control mice, whereas another population was shifted toward lower frequencies; these neurons were unable to fire above 6 Hz. The analysis of the dose-response functions revealed that the EC\textsubscript{50} was similar between controls, fast and slow neurons (Figure 5D). We found no evidence of anatomical segregation of fast and slow neurons; in particular, they were found equally in the core and the shell (P > 0.05). This experiment shows that GR positively controls the reactivity to glutamate of a subset of NAc medium spiny neurons.

UNALTERED RESPONSES TO FOOD REWARDS IN GR\textsuperscript{D1Cre} MICE

As the processing of natural rewards and addictive drugs activate overlapping pathways, we sought to determine whether GR gene inactivation in dopaminceptive neurons resulted in a general impairment of natural reward-seeking. As we did for amphetamine, we first tested control and mutant mice in two CPP experiments in response to normal (chow pellets) or palatable (chocolate) food. Mice were exposed for 30 min to either food in the paired chamber and spent the same amount of time, without food, in the opposite (unpaired) chamber on alternate days. Following 8 days of conditioning, the time increase in the paired chamber was used as an index of place preference. Both normal and palatable food elicited significant CPP in control animals. However, unlike our results with amphetamine, CPP remained unaltered in GR\textsuperscript{D1Cre} mice (Figures 6A,B respectively).

Next, as GR inactivation within dopaminceptive neurons has been reported to decrease motivation for cocaine in a PR schedule (Ambroggi et al., 2009), we thus, tested motivation of GR\textsuperscript{D1Cre} mice to respond instrumentally for food rewards. During initial instrumental training, under a fixed-ratio\textsubscript{1} schedule, control and GR\textsuperscript{D1Cre} mice did not show significant differences in the number of responses (Figure 6C) and ate similar amount of pellets (control: 6.04 ± 0.29 g, GR\textsuperscript{D1Cre}: 6.3 ± 0.23 g). During
FIGURE 3 | GR
\textsuperscript{D1Cre} mice show a decreased sensitivity to amphetamine rewarding properties. CPP scores represent the time difference between post-conditioning and pre-conditioning phases that mice spent in the reward-paired chamber. (A) Time spent in each chamber of the CPP apparatus by control (gray bars) and GR
\textsuperscript{D1Cre} mice (black bars), during the pre-conditioning phase. (B) CPP to amphetamine 1 and 2 mg/kg in control and GR
\textsuperscript{D1Cre} mice. Interaction Drug × Genotype \(F_{(1, 39)} = 4.5, P < 0.05\). (C) Amphetamine (1 mg/kg)-induced comparable CPP in both control and GR
\textsuperscript{D1Cre} mice. No interaction Drug × Genotype \(F_{(1, 35)} = 1.1, P > 0.05\). ns: non-significant, *\( P < 0.05\); **\( P < 0.01\); control vs. mutant: *\*\( P < 0.01\). \(n = 8\)–12 mice per group.

FIGURE 4 | Normal D1-like dopamine receptor agonist induced locomotor activity, but impaired MK801-elicited hyperlocomotion in GR
\textsuperscript{D1Cre} mice. Locomotor responses are presented as \(1/4\) turn per 5 min. (A) Locomotor response to saline and SKF81297 1.5 mg/kg (left panel) and 3 mg/kg (right panel) in control and GR
\textsuperscript{D1Cre} mice. Interaction Drug × Time for SKF81297 at 1.5 mg/kg \(F_{(17, 714)} = 10.3, P < 0.001\) and 3 mg/kg \(F_{(17, 714)} = 9.4, P < 0.001\), but no interaction Drug × Time × Genotype, \(F_{(17, 714)} = 1.2, P > 0.05\) and \(F_{(17, 714)} = 0.8, P > 0.05\), respectively. (B) MK801 elicited a stronger hyperlocomotion in control than GR
\textsuperscript{D1Cre} mice, interaction Drug × Time × Genotype, \(F_{(18, 756)} = 1.9, P < 0.01\). **\( P < 0.01\), control vs. mutant. \(n = 8\)–14 mice per group.

the learning phase of the PR schedule (where the response-requirement increased after each reward obtained), control and mutant mice exhibited a comparable increase in their responding for food (Figure 6D left panel). Analysis of the breaking points (defined as the last ratio completed by the animal followed by 15 min during which no additional reward was earned) revealed no difference between controls and GR
\textsuperscript{D1Cre} mice (Figure 6D right panel). While during initial instrumental training GR
\textsuperscript{D1Cre} mice showed a trend toward a decrease in instrumental responses compared to controls, the opposite was rather observed during the last two sessions of PR. This set of data suggests that GR in dopaminceptive neurons does not modulate food reward responses.

DISCUSSION
In this study, we aimed at dissecting the modulatory role of GR within the meso-cortico-limbic dopamine system, on responses to amphetamine and food rewards. We showed that inactivation of GR gene in dopaminceptive cells, but not in dopamine cells, decrease amphetamine-mediated locomotor sensitization and CPP, two behavioral features of psychostimulants. Along with these behavioral deficits, absence of GR in dopaminceptive neurons does not modulate food reward responses.
cells decreased the post-synaptic response to amphetamine within the NAc as assessed by c-Fos immunostaining. These changes in behavioral and post-synaptic neuronal activation to amphetamine may involve abnormal glutamate transmission as mice deprived of GR in dopaminceptive neurons showed a decrease in locomotor response to NMDA receptor antagonist MK801 and a decrease in neuronal response to intra-accumbal glutamate administration. These results extend our previous findings which showed that inactivation of GR in the same cell population dampens behavioral and molecular responses to cocaine, another psychostimulant drug (Ambroggi et al., 2009; Barik et al., 2010). These modulatory effects of GR appear to be selective to neurons as neither morphine (Ambroggi et al., 2009; Barik et al., 2010) nor food reward responses (the present study) are affected by the inactivation of GR gene in the meso-cortico-limbic dopamine system.

While a body of evidence suggest that stress reaction, as well as GCs, facilitate behavioral responses to amphetamine, the brain regions targeted by GCs actions remained to be identified. Furthermore, the determination of the receptor type involved is still a matter of debate. Although pharmacological antagonism of GR, using the antagonist RU486, has been shown to decrease amphetamine-induced locomotor sensitization without changing the acute locomotor response to the drug (De Vries et al., 1996), systemic administration of GR agonist dexamethasone decreased amphetamine induced hyperactivity (Capasso et al., 1996). These confounding results are however difficult to interpret as the RU486 is also a potent progesterone receptor antagonist (Cadepond et al., 1997), and dexamethasone, when injected systemically, is actively expelled from the brain compartment, hence substantially limiting its effects (Meijer et al., 1998). Systemic dexamethasone may have resulted in a depletion of endogenous GCs levels via the negative feedback exerted by activation of GR in the pituitary gland. The response to amphetamine has also been studied in a transgenic mouse model expressing a neurofilament promoter-driven antisense RNA complementary to a fragment of cDNA that codes for the mouse GR. In this model, GR mRNA levels are decreased by 50% on average in GRD1Cre mice deprived of GR in dopaminoceptive neurons. Indeed, although expression of D1...
receptor have been reported in peripheral tissues, (Ozono et al., 1997) the potential GR gene recombination in the periphery in GR^{D1Cre} mice does not alter HPA-axis activity (Ambroggi et al., 2009) and is unlikely to alter amphetamine metabolism.

Strikingly, GR in dopaminergic neurons appears to modulate behavioral responses to low (1 mg/kg) but not high (2 mg/kg) doses of amphetamine. This effect has been observed for both locomotor sensitization and CPP. In rats, it was reported that the environmental changes in housing conditions, which differently shape the HPA axis, only affected the reinforcing properties of low doses of amphetamine, suggesting that the environment modifies the threshold for positive hedonic effects of amphetamine (Bardo et al., 2001; Green et al., 2002; Stairs et al., 2011). Altogether, these data suggest that stress-induced GCs release increases the sensitivity to reinforcing, rewarding, and sensitizing properties of amphetamine for moderate doses and this effect could be at least partially mediated through activation of GR within dopamine-targeted areas. In addition to these behavioral effects, we also observed a decrease in the induction of c-Fos by amphetamine specifically within the NAc of GR^{D1Cre} mice. An acute cocaine injection has been previously shown to predominantly (but not exclusively) induce c-Fos in D1-expressing medium spiny neurons (Bertran-Gonzalez et al., 2008). We might expect a similar pattern of induction in response to amphetamine. Despite the decreased neuronal response to amphetamine, their acute locomotor response was unaltered compared to controls. Such apparent contradiction can however be partially resolved. A previous study showed that complete absence of c-Fos in D1-expressing neurons, obtained by conditional gene inactivation, does not alter the marked locomotor response to an acute injection of the D1-like agonist SKF81297. It also has no effect on the acute locomotor response to a moderate (10 mg/kg) dose of cocaine but does impair locomotor sensitization (Zhang et al., 2006). Thus, c-Fos induction in dopamine-innervated areas is not necessary to build an acute locomotor response to moderate doses of psychostimulant drugs but seems crucial for the development of sensitizing effects. In the present study we did not investigate for c-Fos induction after repeated injection of amphetamine. However, we...
have previously shown that repeated administration of cocaine leads to a sensitization of c-Fos mRNA induction specifically in the CPu and motor cortex and that this sensitization was abolished in mice lacking GR in the whole central nervous system (Deroche-Gamonet et al., 2003).

Decreased behavioral and post-synaptic responses to amphetamine observed in GR<sup>D1Cre</sup> mice were unlikely due to alterations in post-synaptic dopamine D1-mediated signaling as these mice showed similar responses to dopamine D1-like receptor agonists SKF81297 compared to controls. In contrast, we showed that the absence of GR in dopaminoceptive neurons dampened locomotor responses to MK801, a NMDA receptor antagonist. Given that c-Fos induction by amphetamine is specifically dampened in the NAc, this suggests that glutamate neurotransmission could potentially be impaired within this brain region. This is confirmed by our electrophysiological data. In GR<sup>D1Cre</sup> mice, about half of NAc medium spiny neurons were found to be less reactive to glutamate while the remaining neurons had normal responses. Medium spiny neurons in both CPu and NAc can be segregated in two neuronal populations expressing either D1 or D2 dopamine receptors with some overlap between these two populations (Bertran-Gonzalez et al., 2010). The bimodal distribution could therefore be the result of this segregation. However, GR<sup>D1Cre</sup> mice are likely to be recombined in both populations (Barik et al., 2013), potentially because of transient developmental expression of the D1 receptor. GR may differentially regulate glutamate reactivity in D1- or D2-expressing neurons and future studies will be needed to directly test this hypothesis. The alteration of glutamate neurotransmission within the NAc could explain the decreased responses to psychostimulants, which also largely relies on glutamate transmission (Kalivas, 2000). A body of evidence suggests that GR can modulate glutamate transmission in the brain (Popoli et al., 2012). In addition, in a previous study, microarray and RT-qPCR revealed changes in the expression of NMDA receptor subunits and in AMPA/kainate signaling pathways within the striatum of GR<sup>D1Cre</sup> mice (Barik et al., 2010). These changes could account for the alteration of glutamate neurotransmission in these mice.

This study, as well as our previous work, clearly demonstrate that the absence of GR in dopaminoceptive neurons diminish reinforcing, rewarding, and sensitizing effects of psychostimulants. As the same brain circuits are involved in behavioral responses to abused drugs and natural rewards, we also investigated the effect of inactivating GR within dopaminoceptive cells on the hedonic reactions to food reward (either regular chow or palatable food) in a CPP paradigm and on the willingness to expend effort to obtain a food reward in a progressive ratio task. Both meso-accumbens and nigro-striatal dopamine pathways have been involved in the modulation of motivation and decision-making processes essential to reach a goal (Schultz, 2006; Wise, 2008). Moreover, deficits in motivation are hallmark features of many psychiatric disorders including depression for which stress exposure is an important environmental risk factor. A recent study in rat showed that chronic stress exposure impairs the sensitivity to changes in outcome value and in response-outcome contingency suggesting that chronic stress exposure might induce deficits in reward expectation (Dias-Ferreira et al., 2009). Along with these deficits, structural changes in prefrontal areas and in the dorsal striatum have been observed suggesting that stress exposure may lead to goal-directed behavior impairments by altering cortico-striatal circuits (Dias-Ferreira et al., 2009). Interestingly, these results were mimicked by chronic GCs administration. Indeed, chronic GCs treatments have been shown to impair goal-directed response-outcome associations as well as motivation to obtain food reward in a PR schedule. On the other side, acute pharmacological blockade of GR only impaired response-outcome association sparing motivation (Gourley et al., 2012). Surprisingly, we did not find any difference between GR<sup>D1Cre</sup> mice and control littermates in both CPP and PR tasks suggesting that GR in this cell population may not be necessary to modulate rewarding and reinforcing properties of food as it is for psychostimulant drugs. In GR<sup>D1Cre</sup> mice, most of striatal and NAc neurons show an inactivation of GR. Indeed, GR ablation was observed in more than 85% of striatal neurons. However, within the cortex, only neurons located within deep layers (V/VI) exhibit a percentage of recombination comparable to that of striatal neurons, while most of neurons from upper layers still express GR (Barik et al., 2013). Thus, the effects of stress and GCs on goal-directed behavior and motivation might be rather mediated by an impact at the level of the PFC rather than the striatum or the NAc. Another possibility is that the inactivation of GR in dopaminoceptive neurons may protect from deleterious effects of chronic stress exposure rather than having an effect at basal stress levels. Further studies will be required to explore these hypotheses.

Our findings along with previous studies show that GR in dopaminoceptive neurons selectively modulates reinforcing, rewarding, and sensitizing properties of psychostimulant drugs such as cocaine and amphetamine. These effects seem mediated by alterations of integration of glutamate signaling within the striatum and NAc. On the other side, behavioral responses to food remained unchanged in the absence of GR within dopaminoceptive neurons. These results could be interesting in the context of the development of new medications able to decrease sensitivity to abused drugs while sparing general motivation for natural reinforcers.

**AUTHOR CONTRIBUTIONS**

Sébastien Parnaudeau, François Tronche, and Jacques Barik designed the study. Sébastien Parnaudeau, Frédéric Ambroggi, Marc Turiault, Marie-louise Dongelmans, Anne-Sophie Delbes, Céline Cansell, Serge Luquet, and Jacques Barik performed research. Sébastien Parnaudeau, Frédéric Ambroggi, Marc Turiault, Marie-louise Dongelmans, Pier-Vincenzo Piazza, Jacques Barik, and François Tronche analyzed the data. Sébastien Parnaudeau, Frédéric Ambroggi, Françoise Tronche, and Jacques Barik wrote the manuscript.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Research report

The differential effects of OX1R and OX2R selective antagonists on morphine conditioned place preference in naïve versus morphine-dependent mice

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HIGHLIGHTS

► We tested the effect of SB 334867 and TCS-OX2-29 on morphine CPP.
► Both antagonists in naïve mice inhibited morphine CPP acquisition and expression.
► SB 334867 did not suppress CPP acquisition and expression in dependent mice.
► TCS-OX2-29 inhibited CPP acquisition and expression in dependent mice.

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ABSTRACT

Conditioned place preference (CPP) has been associated with orexinergic (hypocretinergic) system activation in naïve mice; however, the distinct role of different receptors of orexin in this paradigm has not been characterized yet. Moreover, the relationship between orexins and morphine in dependent mice may not be equal to naïve mice and seems noteworthy to investigate. We investigated the effects of systemic administration of orexin-1–receptor antagonist, SB 334867, and orexin-2 receptor antagonist, TCS-OX2-29 on the acquisition and expression of morphine conditioned place preference (CPP) in both naïve and morphine-dependent mice. We tested SB 334867 in three doses (10, 20 and 30 mg/kg), TCS-OX2-29 in two doses (5 and 10 mg/kg) and morphine with highest effective dose based on our dose–response experiment (5 mg/kg). Our results revealed that while SB 334867 suppressed CPP acquisition and expression in naïve mice, it failed to block CPP acquisition and expression in morphine dependent animals. In contrast, TCS-OX2-29 suppressed CPP acquisition and expression in both naïve and dependent mice significantly. The rewarding effect of morphine has stronger correlation with orexin-2 receptors in morphine-dependent mice while it depends on both kinds of receptors in naïve mice. This finding, if confirmed in other studies, persuades us to further investigate the role of orexin-2 receptor antagonists as potent drugs in addiction treatment.

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1. Introduction

Orexins (hypocretins) are neuropeptides exclusively produced in hypothalamic neurons including dorso medial hypothalamus (DMH), perifornical area (PFA), and lateral hypothalamus (LH) [1–4]. They constitute a peptide family with two ligand subgroups, orexin A and orexin B [5] affecting G protein-coupled orexin-1 (OX1R) and orexin-2 receptors (OX2R). Orexin A is selective for orexin 1 receptor whereas both orexin A and B act upon orexin 2 receptor [4]. These receptors are differentially distributed throughout the brain [6].
Early studies suggest a role of orexin in sleep regulation as dysfunction of the orexin system is strongly associated with narcoleptic symptoms in animals [7,8]; however, further studies showed the important role of orexin neurons in reward-related behaviors [4–9]. Administration of SB 334867 (OXIR antagonist) reduced the acquisition and expression of morphine place preference conditioning [10,11], and orexin gene knockout mice lacked conditioned place preference (CPP) for morphine [11], with controversial result in another study which demonstrated while orexin antagonist, SB 334867 attenuated conditioned place preference, orexin gene knockout mice still acquired CPP [12]. The effects of independent OX2R antagonists like TCS-OX2-29 or dual receptor antagonists like almorexant have not been evaluated in morphine conditioned place preference yet while distinct role of these antagonists in other phenomenon like sleep–wakefulness cycle has been described, for example several lines of evidence support the pivotal role of OX2R in the regulation of sleep–wakefulness [5,8,13]. An investigation comparing the selective OX2R antagonist JNJ-10397049, selective OX1R antagonist SB-408124 and almorexant as the dual receptors antagonist in rats demonstrated that although both JNJ-10397049 and almorexant induced and prolonged sleep time, the selective OX2 receptor antagonist had a 10-fold higher potency than dual OX1/OX2 antagonist. On the contrary, SB-408124 had no effect on any tested sleep parameter independently and even attenuated the sleep-promoting effect of JNJ-10394079 administered simultaneously [14].

Current data suggest the VTA as an essential site, which the orexin acts on, for the acquisition and expression of morphine place preference in non-dependent mice [10,11].

In addition to effects of acutely administered drugs, chronic administration of drugs also influences the orexin neural activation and mRNA levels of orexin or its receptors. The effect of chronic administration of drugs – either repeated equivalent doses or escalating doses – on addiction may differ from acute administration of them [15–17].

The role of orexergic system in morphine-induced conditioning while the subjects are already dependent to morphine has not been investigated yet. Orexin might play different role in morphine dependent versus naive mice due to novel neuronal pathway development in dependent animals and it can potentially change behavioral responses, which is noteworthy to study. The major goal of the present study is to evaluate the effects of SB 334867 and TCS-OX2-29 as the independent antagonists of two known orexin receptors administered in both naive and morphine-dependent mice. This can reveal possible distinct role of two receptors in rewarding effects of morphine.

2. Materials and methods

2.1. Animals

We began the study with a total of 440 (including preliminary study) adult male NMRI mice (Pasteur Institute of Iran, Tehran, Iran), weighing 25–30 (g). Animals were housed 5–8 per cage in transplant Plexiglas cages in a temperature and humidity controlled colony room under a 12/12 light/dark cycle (lights on at 7:00 AM), with ad libitum access to the food and water except during experiments. Subjects were experimentally naive and each animal was used only once. They were assigned randomly to each treatment group consisting of 6–8 animals. Mice were allowed a week to acclimatize to the laboratory environment before testing started and during this period they were handled, weighed, and habituated to the drug administration procedure. All procedures were performed according to the institutional guidelines for animal care and use. The protocol was approved by the Committee of Ethics of the Faculty of Sciences, University of Tehran.

2.2. Drugs

The following drugs were used: morphine sulfate (Tmenad, Karaj, Iran), SB 334867 (Tocris Bioscience, Ellisville, MO, USA) and TCS-OX2-29 (Tocris). Morphine was prepared freshly in sterile 0.9% NaCl solution and injected subcutaneously (s.c) with maximized effective dose of 5 mg/kg derived from our preliminary dose–response study. SB 334867 (OXIR antagonist) was dissolved in 10% (w/v) (2-hydroxypropyl)-B-cyclodextrin (10% dimethyl sulfoxide (DMSO) and was administered intraperitoneally (i.p.) at different doses of 10, 20 and 30 mg/kg. SB 334867 was injected 30 min prior to each conditioning session in experiments 1 and 3, and 30 min prior to post-conditioning test session in experiments 2 and 4; this time and doses have been reported to be effective in several different behavioral procedures both in mice and rats [10,18–23], in fact in rats the highest plasma and brain levels of the drug have been achieved 30 min after injection [23] but this finding cannot be necessarily attributed to the mice because of probable different pharmacokinetics of drugs in different species. The OX2R antagonist, TCS-OX2-29 was dissolved in physiological saline (0.9% NaCl) and administered intraperitoneally at doses of 5 and 10 mg/kg based on a previous study [24].

Dependence to morphine was achieved by consecutive escalating doses of morphine (30, 45, 60, 90, 120 and 120 mg/kg) injected every 12 h on 3 consecutive days followed by CPP procedure [25]. The dependent mice also received a daily dose of morphine (10 mg/kg) to avoid withdrawal syndrome during conditioning and test sessions [26]. It was injected late in the evening (10 PM), while animals underwent conditioning sessions in the next day (10:00 AM–2:00 PM) to make the interference of morphine maintenance dose with morphine conditioning (made by administration of 5 mg/kg morphine, just before the conditioning) less likely and to avoid the state-dependent retrieval on postconditioning day.

2.3. General behavioral procedure

The place preference apparatus was made of wood and consisted of two squared-base compartments (15 cm × 15 cm × 30 cm H each). To distinguish two compartments, visual and sensory texture cues were used: the inner surface of one compartment was painted in black with a smooth floor; the other side was white with a textured floor to create equally preferred compartments. Place conditioning was conducted using an unbiased procedure. In this design, animals did not show a significant preference toward any compartment in the pre-conditioning test and drug administration was randomly paired with either of the compartments [27]. The CPP paradigm took place in nine consecutive days, which consisted of three phases: familiarization and pre-conditioning, conditioning and post-conditioning. All trials were done between 10:00 AM and 2:00 PM. On the first (i.e. familiarization) and second (i.e. pre-conditioning) days, each mouse was placed separately into the apparatus for 10 min with free access to both compartments. The time spent in each compartment was recorded on the pre-conditioning day to determine any individual innate preference for either of the two compartments. Placement in each compartment was assigned as placement of the front paws and head. Animals showing strong unconditioned aversion for any of the compartments (time spent in either of the two compartments > mean ± 2SD) were excluded from the experiment (totally 31 animals). This familiarization phase was performed in all experiments including experiments 3 and 4 with dependent mice.

Conditioning phase consisted of six 40-min conditioning sessions held on six consecutive days. Every other day, each animal was confined to one of the differently textured compartments by isolating removable sheet. The mice received the drugs on days 1, 3 and 5 and the vehicle on days 2, 4 and 6 of the conditioning phase according to the experimental design. Treatment compartments were counterbalanced for all groups. The locomotor activity of each animal was measured after receiving the drugs or vehicles on conditioning sessions or on the post-conditioning session by dividing the ground of each compartment into 100 cm² small squares (7.5 cm × 7.5 cm), and recording the number of mice entrances to every one of them by an unbiased observer as the locomotor activity index [27]. This index was used as the physical indicator of morphine sensitization in our study based on previous studies [28–30]. For each animal, conditioning locomotor activity was recorded during each conditioning session and the mean of all amounts was calculated and applied for comparison tests.

Post-conditioning phase was carried out on the ninth day of the trial (24 h after the last conditioning session) in a drug-free state except for the daily treatment of morphine in dependent mice, which they received 12 h before the post-conditioning test, on the afternoon of the previous day. As in the pre-conditioning phase the partitions were raised and the animals were placed in the apparatus for 10 min with free access to both compartments. An observer who was unaware of mice and treatments recorded the time spent in the compartments. Change in preference (CPP) was calculated as the time spent in the drug-paired compartment on the post-conditioning day minus the time spent in the same compartment in the pre-conditioning day.

2.4. Experimental design

2.4.1. Preliminary dose–response study

We tested different doses of morphine (0, 2.5, 5, 10 and 20 mg/kg) to achieve the maximum conditioned place preference produced by this drug in groups of animals either in naive or dependent states. They were also NMRI mice from the same institute with each group contains 6–8 mice.
2.4.2. Experiment 1: effects of SB 334867 and TCS-OX2-29 on acquisition of place preference perse and induced by morphine in naive mice

In this experiment 3 groups of animals received SB 334867 in 3 different doses including 10, 20 and 30 mg/kg, 2 groups received TCS-OX2-29 and one group received saline, all of them 30 min prior to the administration of morphine. On the other hand, equal control groups of animals received saline in turn of morphine to investigate the pure effect of SB 334867 or TCS-OX2-29 on place preference.

2.4.3. Experiment 2: effects of SB 334867 and TCS-OX2-29 on expression of place preference perse and induced by morphine in naive mice

This experiment was carried out to see the effect of orexin antagonists on the expression of morphine-conditioned preference acquired formerly by mice. The animals underwent CPP paradigm as described above, except that they received SB 334867, TCS-OX2-29 or vehicle 30 min prior to the post-conditioning test.

2.4.4. Experiment 3: effects of SB 334867 and TCS-OX2-29 on acquisition of place preference perse and induced by morphine in dependent mice

In this experiment, animals first underwent a protocol to become dependent to morphine (as described above) before the familiarization phase of the CPP. Dependent mice were also treated with a daily dose of 10 mg/kg on familiarization, conditioning and test days to avoid withdrawal [26]. The signs of withdrawal including jumping, wet dog shakes, diarrhea, ptosis, body tremor, piloerection and weight loss were recorded for each animal every day, together with recording locomotor activation during both conditioning and test sessions and the animals with withdrawal signs were excluded from the study in a total count of 34 animals. All other steps were similar to experiment 1.

2.4.5. Experiment 4: effects of SB 334867 and TCS-OX2-29 on expression of place preferenceperse and induced by morphine in dependent mice

In this experiment, animals first underwent a protocol to become dependent to morphine before the familiarization phase of the CPP and then were treated daily with morphine (as described above). All other steps were similar to experiment 2.

2.5. Data analysis

All results are presented as mean ± SEM. The effect of each OXR antagonists on the change in preference (CIP) of morphine treated animals or vehicle treated animals were assessed by two-way analysis of variance (ANOVA); separately. One-way ANOVA was also applied to assess the independent effects of morphine or antagonists on CIP and locomotor activity in each experiment. Post hoc analysis (Tukey–Kramer) was performed to determine the effects of various doses. P values less than 0.05 were considered as significant.

3. Results

3.1. Preliminary dose–response study

A significant dose-dependent effect of morphine on conditioning was found (one-way ANOVA, F4,42 = 5.953, P < 0.001). Post hoc analyses revealed that the doses of 2, 5 and 10 mg/kg induced significant place preference (P < 0.01, P < 0.001, P < 0.01 respectively) compared to saline treatment. The maximum response was observed with 5 mg/kg of morphine. The larger dose, 20 mg/kg of morphine failed to produce CPP (P > 0.05) (Fig. 1).

3.2. Effect of SB 334867 or TCS-OX2-29 on acquisition of morphine CPP in naive mice

A significant interaction between morphine treatment and OX1R antagonist pretreatment on the acquisition of morphine CPP was indicated applying two-way ANOVA (factor morphine F1,16 = 27.340, P < 0.001; factor SB 334867 F1,25 = 0.843, P > 0.05; factor morphine × SB 334867 F1,50 = 3.349, P < 0.05) in naive mice. Further analysis with Tukey–Kramer’s multiple comparison tests in the first experimental group indicated that morphine conditioned animals which were not pretreated with SB 334867 showed a significant preference to the drug-paired chamber in comparison to all SB 334867 pretreated groups (P < 0.05, P < 0.001, P < 0.05 for 10 mg/kg, 20 mg/kg and 30 mg/kg respectively) and saline conditioned animals (P > 0.01). Post hoc analysis also revealed that animals receiving SB 334867, which was not followed by morphine, did not show any significant preference toward each of the compartments in comparison to saline treated mice (P > 0.05 for 10 mg/kg, 20 mg/kg and 30 mg/kg of OX1R antagonist) (Fig. 2.1).

Animals, which received morphine before conditioning sessions including those which were pretreated by SB 334867 showed significant, enhanced locomotor activity compared to saline treated mice (one-way ANOVA; F4,33 = 10.659, P < 0.001); however, none of doses of SB 334867 suppressed this enhanced locomotion significantly when they were administered before morphine (post hoc analysis indicated P > 0.05 for 10, 20 and 30 mg/kg).

A significant interaction between morphine treatment and OX2R antagonist pretreatment on the acquisition of morphine CPP was indicated applying two-way ANOVA (factor morphine F1,16 = 27.340, P < 0.001; factor TCS-OX2-29 F2,24 = 0.895, P > 0.05; factor morphine × SB 334867 F2,48 = 8.312, P < 0.001) in naive mice. Further analysis with Tukey–Kramer’s multiple comparison tests in the first experimental group indicated that morphine conditioned animals which were not pretreated with TCS-OX2-29 showed a significant preference to the drug-paired chamber in comparison to all TCS-OX2-29 pretreated groups (P < 0.001 for both 5 and 10 mg/kg) and saline conditioned animals (P > 0.01). Post hoc analysis also revealed that animals receiving TCS-OX2-29, which was not followed by morphine, did not show any significant preference toward each of the compartments in comparison to saline conditioned mice (P > 0.05 for 5 mg/kg and 10 mg/kg of OX2R antagonist) (Fig. 2.2).

Animals, which received morphine before conditioning sessions including those pretreated by TCS-OX2-29 showed significant, enhanced locomotor activity compared to saline treated mice (one-way ANOVA; F3,32 = 25.360, P < 0.001); however, none of doses of TCS-OX2-29 suppressed this enhanced locomotion significantly when they were administered before morphine, compared to animals which were treated only with morphine (post hoc analysis indicated P > 0.05 for 5 and 10 mg/kg).

Morphine-conditioned animals showed enhanced locomotion during the post-conditioning test compared to SB 334867 pretreated group (one-way ANOVA F2,24 = 5.641, P < 0.01, post hoc analysis P < 0.05 for 10 mg/kg, P < 0.05 for 20 mg/kg and P < 0.032 for 30 mg/kg), TCS-OX2-29 pretreated group (one-way ANOVA F2,24 = 7.041, P < 0.01, post hoc analysis P < 0.05, P < 0.01 respectively for 5 mg/kg and 10 mg/kg) and saline control group (one-way ANOVA, F1,16 = 11.029, P < 0.01).

3.3. Effect of SB 334867 or TCS-OX2-29 on the expression of morphine CPP in naïve mice

A significant interaction between morphine treatment and OX1R antagonist administration, received on the post-conditioning test date, on the expression of morphine CPP was indicated by two-way ANOVA (factor morphine, F1,16 = 17.745, P < 0.001; factor SB 334867, F1,25 = 0.497, P > 0.05; factor morphine × SB 334867, F2,52 = 2.910, P > 0.05) in naïve mice. Further analysis with Tukey–Kramer’s multiple comparison tests in the second experimental group indicated that morphine conditioned animals which did not receive SB 334867 on the post-conditioning test day showed a significant preference to the drug-paired chamber compared to all SB 334867 treated groups (P < 0.05, P < 0.001, P < 0.05 for 10 mg/kg, 20 mg/kg and 30 mg/kg, respectively) and saline treated animals (P < 0.01) (Fig. 3.1).

A significant interaction between morphine treatment and OX2R antagonist administration, received on the post-conditioning test date, on the expression of morphine CPP was indicated by two-way ANOVA (factor morphine, F1,16 = 17.745, P < 0.001; factor TCS-OX2-29, F2,23 = 0.174, P > 0.05; factor morphine × TCS-OX2-29, F2,48 = 7.596, P < 0.001) in naïve mice. Further analysis
with Tukey–Kramer’s multiple comparison tests in the second experimental group indicated that morphine conditioned animals which did not receive TCS-OX2-29 on the post-conditioning test day showed a significant preference to the drug-paired chamber compared to all TCS-OX2-29 treated groups (P<0.001, P<0.01 for 5 and 10 mg/kg respectively) and saline treated animals (P<0.001) (Fig. 3.2).

Considering all SB 334867 treated groups and their controls, animals which received morphine before conditioning, showed enhanced locomotor activity during conditioning sessions (one-way ANOVA, \( F_{1,52} = 34.156, P<0.001 \)). Morphine-conditioned animals showed enhanced locomotion in the post-conditioning test compared to SB 334867 treated group (one-way ANOVA \( F_{3,26} = 4.746, P<0.010 \), post hoc analysis \( P<0.041 \).

![Fig. 1](image1.png)

**Fig. 1.** The dose–response experiment to find the highest effective dose of morphine in producing CPP. Different doses (0, 2, 5, 10 and 20 mg/kg) were tested. All data are shown as mean ± SEM of change in preference (CIP). Significant differences are illustrated as *P<0.05 and **P<0.001.

![Fig. 2](image2.png)

**Fig. 2.** The interaction of morphine and orexin antagonists on acquisition of CPP in naïve mice. (1) SB 334867 (OX1R antagonist) different doses (10, 20 and 30 mg/kg) or vehicle (saline) were administered 30 min prior to morphine (5 mg/kg) or vehicle (saline) before each session of conditioning. SB 334867 pretreated morphine-conditioned animals are compared to vehicle pretreated morphine-conditioned animals, whereas SB 334867 pretreated animals not conditioned with morphine are compared to saline-treated group. (2) TCS-OX2-29 (OX2R antagonist) different doses (5 and 10 mg/kg) or vehicle (saline) were administered 30 min prior to morphine (5 mg/kg) or vehicle (saline) before each session of conditioning. TCS-OX2-29 pretreated morphine-conditioned animals are compared to vehicle pretreated morphine-conditioned animals, whereas TCS-OX2-29 pretreated animals not conditioned with morphine are compared to saline-treated group. All data are shown as mean ± SEM of change in preference (CIP). Significant differences are illustrated as *P<0.05 and **P<0.001.

![Fig. 3](image3.png)

**Fig. 3.** The interaction of morphine and orexin antagonists on expression of CPP in naïve mice. (1) SB 334867 (OX1R antagonist) different doses (10, 20 and 30 mg/kg) or vehicle (saline) were administered 30 min prior to post-conditioning test. The data are shown as mean ± SEM of change in preference (CIP). SB 334867 treated morphine-conditioned animals are compared to morphine-conditioned vehicle treated animals, whereas SB 334867 treated animals not conditioned with morphine are compared to saline-treated group. (2) TCS-OX2-29 (OX2R antagonist) different doses (5 and 10 mg/kg) or vehicles (saline) were administered 30 min prior to post-conditioning test. TCS-OX2-29 treated morphine-conditioned animals are compared to vehicle treated morphine-conditioned animals; whereas TCS-OX2-29 treated animals not conditioned with morphine are compared to saline-treated group. All data are shown as mean ± SEM of change in preference (CIP). Significant differences are illustrated as *P<0.05 and **P<0.001.
for 10 and 30 mg/kg and P < 0.05 for 20 mg/kg), TCS-OX2-29 treated group (one-way ANOVA, F2,24 = 6.108, P < 0.01, post hoc analysis P < 0.05 for 5 mg/kg and P < 0.05 for 10 mg/kg) and saline control group (one-way ANOVA, F1,16 = 7.039, P < 0.05).

Considering all TCS-OX2-29 treated groups and their controls, Animals, which received morphine before conditioning, showed enhanced locomotor activity during conditioning sessions (one-way ANOVA, F1,48 = 77.209, P < 0.001).

3.4. Effect of SB 334867 on the acquisition of morphine CPP in dependent mice

There was no significant interaction between morphine treatment and OX1R antagonist pretreatment on the acquisition of morphine CPP, indicated by two-way ANOVA (factor morphine, F1,11 = 30.459, P < 0.001; factor SB 334867, F2,24 = 0.332, P > 0.05, factor morphine × SB 334867, F3,49 = 0.136, P > 0.05) in morphine dependent mice. Further analysis with Tukey–Kramer’s multiple comparison tests in the third experimental group indicated that morphine conditioned animals which were not pretreated with SB 334867 did not show a significant difference in their preference to the drug-paired chamber in comparison to all SB 334867 pretreated groups (P > 0.05 in 10 mg/kg, 20 mg/kg and 30 mg/kg), however all of morphine-conditioned animals including those pretreated with SB 334867 displayed significant preference to the drug-paired compartment compared to saline conditioned animals (P < 0.01). Post hoc analysis also revealed that animals receiving SB 334867, which was not followed by morphine, did not show any significant preference toward each of the compartments in comparison to saline-treated mice (P > 0.05 for 10 mg/kg, 20 mg/kg and 30 mg/kg of SB 334867) (Fig. 4.1).

Locomotor activity was enhanced during conditioning sessions in groups of animals, which received morphine compared to saline treated group (one-way ANOVA, F4,30 = 14.114, P < 0.001) but SB 334867 did not suppress this enhancement when administered before morphine. Post hoc analysis with Tukey test indicated P > 0.05 for 10, 20 and 30 mg/kg of SB 334867.

A significant interaction between morphine treatment and OX2R antagonist pretreatment on the acquisition of morphine CPP was indicated applying two-way ANOVA (factor morphine, F1,11 = 30.459, P < 0.001; factor SB 334867, F2,24 = 0.040, P > 0.05; factor morphine × SB 334867, F2,43 = 17.008, P < 0.001) in dependent mice. Further analysis with Tukey–Kramer’s multiple comparison tests in the third experimental group indicated that morphine conditioned animals which were not pretreated with TCS-OX2-29 showed a significant preference to the drug-paired chamber in comparison to all SB 334867 pretreated groups (P < 0.001 for both 5 and 10 mg/kg) and saline treated control animals (P < 0.001). Post hoc analysis also revealed that animals receiving TCS-OX2-29, which was not followed by morphine, did not show any significant preference toward each of the compartments in comparison to saline treated control mice (P > 0.05 for 5 mg/kg and 10 mg/kg of OX2R antagonist) (Fig. 4.2).

Animals, which received morphine, showed enhanced locomotor activity in comparison to saline treated animals (one-way ANOVA, F2,27 = 25.660, P < 0.001), however TCS-OX2-29 did not suppress this enhancement when administered prior to morphine (post hoc analysis indicated P > 0.05 for 5 mg/kg and 10 mg/kg of TCS-OX2-29).

Morphine-conditioned animals did not show significant different locomotor activity in the post-conditioning test compared to SB 334867 pretreated group (one-way ANOVA, F1,24 = 0.625, P > 0.05, post hoc analysis P > 0.05 for 10 mg/kg, 20 mg/kg and 30 mg/kg), however they displayed enhanced locomotion compared to TCS-OX2-29 pretreated group (one-way ANOVA, F2,21 = 5.563, P < 0.05, post hoc analysis P < 0.05 for 5 mg/kg and P < 0.019 for 10 mg/kg) and saline control group (one-way ANOVA, F1,11 = 10.938, P < 0.01).

3.5. Effect of SB 334867 on the expression of morphine CPP in dependent mice

There was no significant interaction between morphine treatment and OX1R antagonist treatment, received on the post-conditioning test date, on the expression of morphine CPP, indicated by two-way ANOVA (factor morphine, F1,12 = 48.255, P < 0.001; factor SB 334867, F2,24 = 0.377, P > 0.05; factor morphine × SB 334867, F3,49 = 0.224, P > 0.05) in dependent mice. Further analysis with Tukey–Kramer’s multiple comparison tests in the fourth experimental group indicated that morphine conditioned animals which did not receive SB 334867 on the post-conditioning test day did not show a significant preference to the drug-paired chamber compared to all SB 334867 treated groups (P > 0.05 for 10 mg/kg, 20 mg/kg and 30 mg/kg), however all morphine-conditioned animals including those receiving SB 334867 at post conditioning test day showed significant preference toward the drug-paired chamber compared to saline conditioned animals (P < 0.001) (Fig. 5.1).

A significant interaction between morphine treatment and OX2R antagonist administration, received on the post-conditioning test date, on the expression of morphine CPP was indicated by two-way ANOVA (factor morphine, F1,12 = 48.255, P < 0.001; factor TCS-OX2-29, F2,21 = 1.725, P > 0.05; factor morphine × TCS-OX2-29, F3,52 = 15.299, P < 0.001) in dependent mice. Further analysis with Tukey–Kramer’s multiple comparison tests in the fourth experimental group indicated that morphine conditioned animals which did not receive TCS-OX2-29 on the post-conditioning test day showed a significant preference to the drug-paired chamber compared to all TCS-OX2-29 treated groups (P > 0.05 for both 5 and 10 mg/kg) and saline treated animals (P > 0.001) (Fig. 5.2).

Considering all SB 334867 treated groups and their controls, animals, which received morphine before conditioning, showed enhanced locomotor activity during conditioning sessions (one-way ANOVA, F4,48 = 95.935, P < 0.001).

Morphine-conditioned animals did not show significant different locomotor activity in the post-conditioning test compared to SB 334867 pretreated group (one-way ANOVA, F2,24 = 0.511, P > 0.05, post hoc analysis P > 0.05 for 10 mg/kg, 20 mg/kg and 30 mg/kg), however they displayed enhanced locomotion compared to TCS-OX2-29 pretreated group (one-way ANOVA, F2,21 = 5.568, P < 0.05, post hoc analysis P < 0.05 for 5 mg/kg and P < 0.05 for 10 mg/kg) and saline control group (one-way ANOVA, F1,11 = 12.991, P < 0.01).

Considering all TCS-OX2-29 treated groups and their controls, Animals, which received morphine before conditioning, showed enhanced locomotor activity during conditioning sessions (one-way ANOVA, F4,43 = 56.836, P < 0.001).

4. Discussion

Orexin’s contribution to drug-related behaviors appears to be drug specific and model-specific too [9,16,17,31–36]. These responses may be due to different anatomical targets which orexin selects or uneven expression of orexin receptor types throughout these sites [10–12,16,19,35], as well as interaction with other neurotransmitters from distinct circuits and pathways [10,12,15–17,37]. Moreover, it is possible that, chronic administration of drugs would act differently [16,17].

We designed our study to investigate the effect of systemic administration of selective orexin receptor (both OX1R and OX2R)
antagonists on morphine's CPP acquisition and expression in naïve and morphine-dependent mice while selective OX1R antagonist, SB 334867, had been demonstrated to block both acquisition and expression in naïve mice in previous studies [10,11], the finding, which also was confirmed in our study. It is reasonable to evaluate acquisition and expression of CPP separately because it has been shown previously that orexin may act on any of these stages differently, for example Aston-Jones et al. reported that administration of 30 mg/kg SB 334867 prior to the Pavlovian conditioning session had no effect on subsequent cue-elicited reinstatement; nevertheless, it reduced the expression of conditioned-cue-elicited drug-seeking when administered just prior to a subsequent reinstatement session [38].

We found that in contrary to naïve mice, the suppressing effect of OX1R antagonist, SB 334867, on acquisition and expression of morphine CPP, was not seen in morphine dependent mice, however, OX2R antagonist, TCS-OX2-29, appeared to inhibit acquisition and expression of morphine CPP not only in naïve mice, but also in morphine-dependent mice. Previously, another study demonstrated that selective blockade of OX2Rs by JNJ-10397049 attenuated the acquisition, expression and reinstatement of ethanol CPP while SB-408124, selective OX1R antagonist, did not have any of these effects [39].

It is possible that the effect of orexin on reward seeking and learning the association between reward and cue is mainly mediated through OX2R in dependent mice whereas OX1R has been implicated to have the essential role in naïve mice. In fact, OX1R and OX2R have been reported to be expressed in both VTA and NA but orexin's actions in the NA have been attributed to OX2R binding because of the very low levels of OX1R in this area [6,37]; therefore a suggestion might be that direct action of Orexin at the NA via projections from LH is the dominant circuit in turn of indirect pathway modulated by VTA. Previous studies indicated that chronic cocaine administration increased OX2R levels in NA in a long-lasting manner and naloxone-precipitated morphine withdrawal is accompanied by an increase in c-Fos expression in the NA shell [16–19]. In addition, although we did not find the same evidence about morphine, it has been reported that chronic administration of cocaine up-regulates OX2R – but not OX1R – levels in NA. The up-regulation has not occurred in frontal cortex, VTA and the dorsal striatum. This effect in the NA persisted even months after the cessation of the cocaine treatment [16]. A limitation of our study is that we could not take this hypothesis as a direct conclusion of the results and it may require to be examined by local injection of both orexin receptors specific antagonists, into different sites of the circuit including NA. It should also be considered that the role of OX2R is not necessarily limited to NA; one study demonstrated that dual receptor antagonist, almorexant, in concentrations, which are exclusively efficient on OX2R can slow the firing rate of VTA dopaminergic neurons effective in reward-related behaviors, and suggested this effect, which is more potent than the influence of OX1R antagonists, as an evidence of greater role of OX2R in response to orexin A in this area. The same effect was also observed by the administration of EMPA, a selective OX2R antagonist [40].

CPP in morphine dependent mice may include a stress component which increases Fos activation in DMH and PFA rather than LH and VTA [15] and is therefore less sensitive to antagonistic effect.
may inhibit this orexin release. This is consistent with the finding of Malherbe et al., which demonstrated the more potent effect of OX2R antagonists on slowing the firing rate of dopaminergic neurons [40]. The current doses which we used in our study (10, 20 and 30 mg/kg of SB 334867) all failed to block the development or expression of CPP in dependent mice. Further studies associated with local injection of different doses of both antagonists and measuring the level of drugs and orexin in the particular site can be useful. Lacking molecular survey was a limitation of our study.

Altogether, our results show that systemic administration of OX1R antagonist attenuates acquisition and expression of morphine CPP only in naïve animals but OX2R antagonists has a preventive effect not only in naïve mice, but also in morphine-dependent mice. It would be important because any manipulation of the orexin (hypocretin) system intending to treat or control the addiction, is more applicable in substance-dependent subjects and this finding, if confirmed in other experiments, proposes clinical and practical benefits of OX2R antagonists and encourages us to evaluate their effects on addicted individuals. This is specifically reasonable because sleep-promoting effects of these drugs can potentiate their activity against rewarding effects of opioids, making them more favorable medications to treat addicted persons.

5. Conclusions

In this study, we have shown that systemic administration of an OX1R antagonist, SB 334867, suppressed CPP acquisition and expression in naïve mice; however, it failed to block CPP acquisition and expression in morphine dependent animals. In contrast, selective OX2R antagonist, TCS-OX2-29 inhibited CPP acquisition and expression not only in naïve mice but also in morphine dependent mice. It is suggested that the effect of orexin on reward seeking and learning the association between reward and cue, might be mainly mediated through OX2R in dependent mice; whereas both receptors could be implicated to manifest the essential role in naïve mice.

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Early adolescent nicotine exposure affects later-life cocaine reward in mice

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Abstract

Adolescence represents a unique developmental period associated with increased risk-taking behavior and experimentation with drugs of abuse, in particular nicotine. We hypothesized that exposure to nicotine during early adolescence might increase the risk for drug reward in adulthood. To test this hypothesis, male ICR mice were treated with a subchronic regimen of nicotine or saline during adolescence, and their preference for cocaine, morphine and amphetamine was examined using the conditioned place preference (CPP) test in adulthood. Long-term behavioral changes induced by nicotine suggested a possible role of altered gene transcription. Thus, immunoblot for ΔFosB, a member of the Fos family of transcription factors, was conducted in the nucleus accumbens of these mice. Mice treated with nicotine during early but not late adolescence showed an increase in CPP for cocaine, morphine and amphetamine later in adulthood. This effect was not seen in mice pretreated with a subchronic regimen of nicotine as adults, suggesting that exposure to nicotine specifically during early adolescence increases the rewarding effects of other drugs in adulthood. However, adolescent nicotine exposure did not alter highly palatable food conditioning in mice. The enhancement of cocaine CPP by nicotine was strain-dependent and was blocked by pretreatment with nicotinic antagonists. In addition, nicotine exposure during early adolescence induced ΔFosB expression to a greater extent than identical nicotine exposure in adulthood, and enhanced cocaine-induced locomotor sensitization later in adulthood. These results suggest that nicotine exposure during early adolescence increases drug-induced reward in adulthood through mechanisms that may involve the induction of ΔFosB.

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1. Introduction

Adolescent drug use is highly predictive of susceptibility to drug abuse and addiction later in life. Adults with substance abuse disorders typically initiate drug use as adolescents, and the earlier the age drug use begins, the greater the likelihood of developing addition and with worse outcomes. For example, initiation of nicotine and tobacco product use typically begins during adolescence, at the average age of 13, and an estimated nine out of 10 adult smokers began smoking before age 18 (Kota et al., 2009; Dickson et al., 2011). In addition, tobacco and nicotine use during adolescence has been linked to use of tobacco, alcohol, cocaine and other illicit drugs in adulthood. For example, individuals who smoke cigarettes before the age of 15 are estimated to be 80 times more likely to use illegal drugs such as cocaine (Lai et al., 2000). Given the widespread use of tobacco and nicotine products among adolescents, there is a critical need to understand the relationship between adolescent use of nicotine and the future risk of addiction. While both human and animal studies are necessary to address this important issue, controlled animal studies are needed to fully understand the possible mechanisms for this age vulnerability.

Studies in rodents have also shown that adolescent nicotine exposure affects nicotine and cocaine reward and sensitivity later in adolescence (McQuown et al., 2007; Dao et al., 2011). We showed that mice exposed to nicotine for one week during early
adolescence exhibited increased rewarding effects of nicotine in the conditioned place preference (CPP) test in adulthood (Kota et al., 2009). Nicotine administration during adolescence also alters the effects of psychomotor stimulants in adulthood. Nicotine exposure during adolescence has been reported to increase cocaine reward in adult rodents (Dickson et al., 2011, 2014; McMullen et al., 2005). Nicotine treatment during adolescence also enhanced the locomotor effects of psychomotor stimulants in adult animals (Collins and Izenwasser, 2004). However, neither the behavioral and age specificity of this increase in psychostimulant reward by nicotine nor the role of nicotinic receptor subtypes involved have been clearly identified.

In addition, the molecular and genetic mechanism(s) underlying these observations are not well understood. Although drugs of abuse have different initial pharmacological targets, most enhance activity in the mesolimbic system, which projects from the ventral tegmental area to nucleus accumbens (Wise, 1996). The persistence of nicotine-mediated effects into adulthood, long after the termination of adolescent nicotine exposure, suggests that long-lasting adaptations in the mesolimbic system might contribute to enhancement of reward in adulthood. One mechanism that could underlie these adaptations is induction of ΔFosB, a stable transcription factor that accumulates with repeated drug exposure (Chen et al., 1997; Nestler et al., 2001). ΔFosB is induced in the NAc of adult animals following repeated treatment with drugs of abuse that include nicotine (Martìlia et al., 2006; Pich et al., 1997; McClung et al., 2004; Xiong et al., 2011). The effect of nicotine treatment on ΔFosB expression in adolescent rodents has not been clearly defined. FosB, as measured by immunohistochemistry that detects both ΔFosB and full-length FosB proteins, was increased in the NAc and hippocampus of both peri- and post-adolescent rats after nicotine exposure (Soderstrom et al., 2007). Increases in FosB immunoreactivity persisted at least until postnatal day 80 in both groups that received nicotine, suggesting that FosB and/or its splice variant ΔFosB, could contribute to long-lasting adaptations. Exposure during the adolescent period to nicotine may therefore regulate behavioral and molecular responses later in life. The current study was designed to determine the effect of adolescent nicotine treatment on drug reward in adulthood and to explore possible mechanisms underlying enhancement of reward.

2. Materials and methods

2.1. Subjects

Male ICR mice were obtained from Harlan Laboratories (Indianapolis, IN) and male C57BL/6] (B6) and DBA/2J (D2) mice were obtained from Jackson Laboratory (Bar Harbor, ME). Mice were treated during early (PND 28-34) or late (PND 47-59) adolescence, as defined in the literature (Spear, 2000). Possible litter effects were controlled by including mice from different litters in each test group. Mice were housed 4 per cage in a humidity and temperature controlled vivarium on a 12-h light/dark cycle with food and water ad libitum and were acclimated for 7 days prior to experiments. Animals were maintained in an AAALAC approved facility, and procedures were approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University and in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

2.2. Drugs

(-)-Nicotine tartrate salt [(−)-1-methyl-2-(3-pyridyl)pyrrolidine (+)-bitartrate salt, methyllycaconitine citrate salt (MLA), mecamylamine hydrochloride [2-(methylamino)isocamphane hydrochloride] and dihydro-β-erythroidine (DHβE) were purchased from Sigma–Aldrich Inc. (St. Louis, MO, USA). The α7 nicotinic agonist PHA-543613 (N-(3R)-1-Azabicyclo[2.2.2]oct-3-yl-furo[2,3-c]pyridine-5-carboxamide hydrochloride), morphine sulfate, 6-amphetamine and cocaine HCl were provided by the Drug Supply Program of the National Institute on Drug Abuse (Rockville, MD). All compounds were injected subcutaneously (s.c.) except for cocaine, which was injected intraperitoneally (i.p.) at a volume of 10 ml/kg body weight. Control groups received saline injections at the same volume and by the same route of administration. All drugs were dissolved in sterile saline (0.9% sodium chloride) and prepared fresh before each experiment. All doses are expressed as the free base of the drug.

2.3. Drug exposure protocol

Nicotine (0.1, 0.5 and 1 mg/kg) or saline was administered to early adolescent (PND 28) mice s.c. twice daily (09:00 and 16:00) for either 1 (acute) or 7 (repeated) days. Mice were then housed in their home cages and allowed to reach adulthood (>PND 70), at which point they were evaluated as described below. For the studies with the α7 nicotinic agonist, PHA-543613 (8 mg/kg, s.c.) or saline was given to early adolescent (PND 28) mice s.c. twice daily (09:00 and 16:00) for 7 days. Mice were then housed in their home cages and allowed to reach adulthood (>PND 70), at which point they were tested.

Control studies to examine nicotine treatment in late adolescent and adults were performed using a separate group of mice. Nicotine (0.5 mg/kg) or saline was administered to late adolescent (PND50) and adult (PND 70) mice s.c. twice daily (09:00 and 16:00) for 7 days. Mice were then housed in their home cages for 35 days and then tested in behavioral and molecular experiments.

For the strain differences study, nicotine (0.5 mg/kg) or saline was administered to early adolescent (PND 28) C57BL/6J and DBA/2J mice s.c. twice daily (09:00 and 16:00) for 7 days. Mice were then housed in their home cages and allowed to reach adulthood (>PND 70), at which point they were tested.

2.4. Conditioned place preference test

Mice were tested for morphine, amphetamine- and cocaine-induced preference using the CPP paradigm. The place conditioning chambers and software were purchased from Med Associates (St. Albans, VT). Place conditioning boxes consisted of two distinct sides (20 cm × 20 cm × 20 cm) separated by a smaller center gray compartment. Openings from the center compartment allowed access to either side of the chamber. An unbiased CPP paradigm was utilized in this study as described in Kota et al. (2007). On day 1, animals were placed in the boxes and allowed to move freely from side to side for 15 min, and time spent in each side was recorded. On days 2–4 (conditioning days), twice per day, mice were injected with saline or drug [morphine (5 mg/kg, s.c.), amphetamine (0.2 mg/kg s.c.) or cocaine (1, 5 or 10 mg/kg i.p.)] and subsequently paired with either the white or black chamber, where they were allowed to roam for 15 min. Vehicle-treated animals were paired with saline in both chambers and drug-treated animals received saline in one chamber and drug in the opposite chamber. Pairing of the drug with either the black or white chamber was randomized within the drug-treated group of mice. Animals in the drug group received drug each day. Injections were counterbalanced so that some mice received drug in the morning, others in the late afternoon. On day 5 (test day), mice did not receive an injection. They were placed into the center chamber for 5 min. The partitions were lifted, and they were allowed to roam freely for 15 min. Data are expressed as preference score (time spent on drug-paired side...
To determine if adolescent nicotine exposure broadly affects conditioning, food place conditioning was measured using highly palatable food (Kraft Classic Philadelphia Cheesecake, Deerfield, IL) or standard laboratory chow (Harlan, Laboratories; Indianapolis, IN), as we previously described in Sanjakdar et al. (2015). Briefly, mice that were exposed to nicotine or saline during early adolescence were conditioned as adults (PND70) as described with drug CPP with the following exceptions. Immediately after establishing baseline preference (Day 1), mice were allowed to consume the highly palatable food (or standard chow) for the next 4–6 h in the home cage. Next, the highly palatable food (or standard chow) was paired with one large chamber during which the standard chow was paired with the other large chamber during daily 40 min sessions that occurred for the next 6 days.

2.5. Cocaine locomotor sensitization

For this study, only early adolescent mice (PND 24–30) were pretreated with saline or nicotine (0.5 mg/kg) injections for 7 days twice daily as described above. Briefly, once the mice had reached PND 70, a 13-day cocaine sensitization protocol began. All of the behavioral procedures were performed during the light phase of the light/dark cycle between 10:00 am and 2:00 pm. Three days before starting the experiments, mice received a daily i.p. saline injection and were subjected to the locomotor activity chambers (30 min) to minimize stress induced by experimenter handling, the injection procedures, and exposure to the novel environment. The activity count of the last day was considered as the baseline activity of mice. Mice were randomly divided into three groups: saline–saline, saline–cocaine, and cocaine–cocaine (groups represent the acquisition day drug followed by the challenge day drug). Mice were then given another injection of either saline or cocaine 20 mg/kg (i.p.), depending on the assigned group, and placed in the chambers again for a 30 min acquisition period. This procedure was repeated on days 2–5. Days 6–12 were considered a drug free week in which the animals were not given injections or exposure to the chambers. On day 13, mice were tested again in the same manner as described for days 1–5, but mice in the cocaine group received a challenge dose of cocaine of 5 mg/kg (i.p.). Counts were recorded as number of photocell interrupts after a 30 min test period.

2.6. Plasma nicotine and cotinine levels

PND 28 male B6 and DBA mice were exposed to nicotine for 7 days as detailed above. Fifteen minutes after the last injection, mice were decapitated and trunk blood was collected and immediately centrifuged for 10 min. Blood plasma was stored for 7 days at −80°C. Plasma nicotine and cotinine levels were measured using high-performance liquid chromatography/tandem mass spectrometry (HPLC/MS/MS) as previously described (AlSharari et al., 2015). At least five animals were used per group.

2.7. Immunoblotting

Adolescent and adult mice received nicotine or saline twice daily for 7 days as described above and were then sacrificed for immunoblot studies (Zachariou et al., 2006; Lazenka et al., 2014). Tissue from the nucleus accumbens (NAc) was homogenized in 20 mM HEPES buffer (pH 7.8) with 0.4 M NaCl, 20.0% glycerol, 5.0 mM MgCl₂, 0.5 mM EDTA, 0.1 mM EGTA, and 1% NP-40 (EMSA buffer) containing 500 μM dithiothreitol and Halt™ protease inhibitor cocktail. Samples were loaded in 10% Tris–HCl gels and separated by electrophoresis. Gels were transferred onto nitrocellulose paper, blocked and incubated in FosB (1:1000; Cell Signaling Technology, Beverly, MA, USA) and α-tubulin loading control (1:5000; Upstate, Temecula, CA, USA) antibodies. Blots were washed and incubated with Alexa 680 goat anti-rabbit IgG (1:12,000) and Alexa 800 goat anti-mouse IgG (1:12,000) for 45 min at room temperature. Fluorescent intensity was visualized using the Odyssey LI-COR infrared scanner. LI-COR software version 2.1 was used to measure integrated intensity between treatments for the band of interest, with subtraction of the background (average of intensities 3 border widths above and below the band).

2.8. Data analysis

For all data, graphs and statistical analyses were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software; San Diego, CA). All CPP results were expressed as mean preference scores ± standard error of the mean. Preference scores were measured in seconds, and indicate time spent in the drug-paired side during post-conditioning – time spent in the drug-paired side pre-conditioning (baseline). Statistical analyses of all behavioral and ΔFosB studies were performed with mixed-factor (one or two-way) ANOVA and Bonferroni post hoc analyses were used to determine significant differences between groups (p < 0.05).

3. Results

3.1. Early-adolescent nicotine exposure enhances cocaine-induced CPP in adulthood

To assess the effect of early-adolescent nicotine exposure on cocaine-mediated reward-like effects in adulthood, ICR mice were pretreated with nicotine (s.c.) during early adolescence (PND 28), and then allowed to mature to adulthood (PND 70) before CPP testing. In the first study, the effect of a one-day exposure to either saline or nicotine (0.1 or 0.5 mg/kg, two injections at 8 am and 5 pm) in PND 28 mice was investigated. At PND 70, these mice were then conditioned with cocaine or saline in the CPP test. As shown in Fig. 1A, mice developed a significant preference [F(1, 36) = 104, p < 0.0001] for the chamber paired with cocaine (10 mg/kg) as compared to saline-conditioned mice. However, two-way ANOVA analysis revealed no significant effect of one-day adolescent nicotine pretreatment [adolescent pretreatment × adult CPP treatment; F(2, 36) = 0.12, p = 0.8906] on cocaine preference. Since one-day exposure to nicotine did not affect cocaine CPP, ICR mice were treated with three different doses of nicotine (0.1, 0.5, or 1.0 mg/kg) for 7–days during adolescence (PND28–35) and tested for cocaine CPP at PND 70. As shown in Fig. 1B, one-week exposure to nicotine during early adolescence significantly increased the preference for cocaine in adulthood [two-way ANOVA adolescent pretreatment × adult CPP treatment; F(7, 42) = 30.49, p < 0.0001] in a nicotine treatment dose-related manner as compared to mice that received saline during adolescence. In addition to the first group that was treated with nicotine and then cocaine 10 mg/kg, another group of early-adolescent mice (PND 28) received 0.5 mg/kg nicotine (2×/day for 7 days) and animals were tested at PND70 with various doses of cocaine (1, 5, and 10 mg/kg) in the CPP procedure. Pretreatment with 0.5 mg/kg nicotine during adolescence produced a leftward shift in the cocaine dose–response curve (Fig. 1C), and subsequent doses of 5 and 10 mg/kg cocaine evoked a significant CPP response in adulthood as compared to saline control mice [F(2, 40) = 14.33, p < 0.0001]. The role of nicotinic receptor subtypes in enhancing the effect of cocaine was investigated using three nicotinic antagonists and a selective agonist. Studies were first conducted by pretreating adolescent ICR mice with the nonselective nicotinic antagonist...
mecamylamine followed 10 min later by nicotine administration to determine whether blocking nicotinic receptors prevents nicotine-induced enhancement of cocaine preference in adulthood. Fig. 2A shows that pretreatment of early adolescent mice with mecamylamine (2 mg/kg s.c.) and nicotine (0.5 mg/kg, s.c. 2×/day for 7 days) prevented the nicotine-induced enhancement of cocaine CPP in adulthood [F(4,28) = 4.169, p = 0.009]. These results suggest that the activation of nicotine receptors (nAChRs) is required for nicotine-induced enhancement of cocaine in the CPP test. Furthermore, administration of DHßE (2 mg/kg), a selective antagonist for β2* nAChR subtypes, before daily nicotine pretreatment in adolescence, also blocked nicotine-induced enhancement of cocaine preference in adulthood (Fig. 2A). In contrast, the α7 nicotinic antagonist MLA (10 mg/kg, s.c.) failed to block nicotine-induced enhancement of cocaine in the CPP test (Fig. 2A). Pretreatment with nicotinic antagonists alone during early adolescence did not affect cocaine preference in adulthood (Table 1).

To furthermore probe the role of the α7 nAChR subtype, a group of early-adolescent ICR mice (PND 28) received 8 mg/kg of the selective α7 nAChR agonist PHA-543613 (2×/day for 7 days, s.c.) and animals were tested at PND70 with 10 mg/kg of cocaine (10 mg/kg) in the CPP procedure. As seen in Fig. 2B, both PHA-543613- and saline-treated mice during adolescence developed a significant preference [F(1, 24) = 57.91, p < 0.0001] for the chamber paired with cocaine (10 mg/kg) as compared to saline-conditioned mice tested later in adulthood. However, two-way ANOVA analysis revealed no significant effect of seven-day adolescent PHA-543613 pretreatment [adolescent pretreatment × adult CPP treatment; F(1, 24) = 0.272, p = 0.606].

The early adolescent period plays a critical role in the effects of nicotine on cocaine preference in adulthood.

To determine whether enhancement of cocaine preference is specific to nicotine treatment during early adolescence, late adolescent (PND 50) and adult (PND 70) ICR mice were administered nicotine (0.1 or 0.5 mg/kg) for 7 days, and then tested for cocaine-induced CPP after the same drug-free period (36 days) as early adolescents (Fig. 3A and B). All mice conditioned with cocaine in the CPP test exhibited a significant preference for the cocaine-paired side, as shown by one-way ANOVA [F(2, 27) = 124.9; p < 0.0001] (Fig. 3A). In contrast to data from mice that received nicotine during early adolescence (Fig. 1B), mice treated with nicotine during late adolescence did not demonstrate any

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**Fig. 1.** The effect of early-adolescent nicotine exposure on cocaine-induced CPP in adult mice. Early adolescent (PND 28) male ICR mice were injected s.c. with either saline or nicotine (0.1 or 0.5 mg/kg) twice for (A) one day or (B) seven days and were assessed for cocaine preference in the CPP test on PND 70. (C) Dose–response relationship for cocaine-induced CPP in mice that were exposed to 0.5 mg/kg nicotine in adolescent (7-day protocol). Results are expressed as mean ± SEM of n = 8/group. *p < 0.05 from respective saline control. †p < 0.05 from respective saline—cocaine.
significantly different from mice pretreated with saline when assessed for cocaine CPP in adulthood \[F(5,27) = 0.57; p > 0.05\] (Fig. 3A). Similarly, mice that received either nicotine or saline during adulthood displayed similar preference for cocaine \[F(5,27) = 34; p > 0.05\] (Fig. 3B).

### 3.2. Early-adolescent nicotine exposure enhances amphetamine- and morphine-induced CPP in adult mice

Studies were then conducted to determine whether nicotine treatment during early adolescence affects preference for other abused drugs in adulthood. Early-adolescent ICR mice (PND 28) were pretreated with nicotine (0.5 mg/kg, s.c.) or saline twice daily with injections approximately 6 h apart (9:00 am and 3:00 pm) for 7 days. Mice were then housed in their home cages and allowed to mature until they reached adulthood (>PND 70), at which point they were evaluated in the CPP test as described in Materials and Methods. Data are expressed as mean ± S.E.M. of n = 6 mice/group.*p < 0.05 vs saline control group.

### Table 1

Effect of nicotinic antagonist pretreatment during early adolescence on cocaine CPP in adulthood. Male adolescent (PND 28) ICR mice received mecamylamine (2 mg/kg, s.c.), dihydro-beta-erythroidine (2 mg/kg, s.c.) or saline twice daily with injections approximately 6 h apart (9:00 am and 3:00 pm) for 7 days. Mice were then housed in their home cages and allowed to mature until they reached adulthood (>PND 70), at which point they were evaluated in the CPP test as described in Materials and Methods. Data are expressed as mean ± S.E.M. of n = 6 mice/group. *p < 0.05 vs saline control group.

<table>
<thead>
<tr>
<th>Adolescent treatment</th>
<th>CPP treatment</th>
<th>Preference score (mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline/Saline</td>
<td>Saline</td>
<td>13 ± 9</td>
</tr>
<tr>
<td>Saline/Saline</td>
<td>Cocaine</td>
<td>154 ± 35*</td>
</tr>
<tr>
<td>DH(\beta)E/Saline</td>
<td>Saline</td>
<td>15 ± 17</td>
</tr>
<tr>
<td>DH(\beta)E/Saline</td>
<td>Cocaine</td>
<td>156 ± 43*</td>
</tr>
<tr>
<td>MLA/Saline</td>
<td>Saline</td>
<td>11 ± 8</td>
</tr>
<tr>
<td>MLA/Saline</td>
<td>Cocaine</td>
<td>167 ± 21*</td>
</tr>
<tr>
<td>MLA/Saline/Mecamylamine</td>
<td>Saline</td>
<td>15 ± 31</td>
</tr>
<tr>
<td>MLA/Saline/Mecamylamine</td>
<td>Cocaine</td>
<td>162 ± 25*</td>
</tr>
</tbody>
</table>

Fig. 2. Nicotinic receptors mediating the effect of early-adolescent nicotine exposure on cocaine-induced CPP. (A) Early-adolescent mice (PND 28) were pretreated s.c. with mecamylamine \(\text{MEC}\) or dihydro-beta-erythroidine \(\text{DH\(\beta\)E}\) before daily nicotine treatment twice per day for 7 days. (B) Early-adolescent mice (PND 28) were pretreated s.c. with PHA-543613 (8 mg/kg, s.c.) twice per day for 7 days. The x-axis shows conditioning treatment in the CPP test. The legend represents the treatment group during early adolescence. Results are expressed as mean ± SEM of n = 8/group. *p < 0.05 from respective saline control.

Fig. 3. The influence of adolescent period of nicotine exposure on cocaine condition preference in adulthood. (A) Late adolescent (PND 50) mice were injected s.c. with either saline or nicotine (0.1 or 0.5 mg/kg) twice a day for 7 days, and were assessed for cocaine CPP on PND 92. (B) Adult (PND 70) mice were injected s.c. with either saline or nicotine (0.1 or 0.5 mg/kg) two times per day for 7 days, and were assessed for the cocaine CPP test on PND 112. The legend represents the pretreatment group during early adolescence. Results are expressed as mean ± SEM of n = 8/group. *p < 0.05 from respective saline control.
significant preference for the drug-paired side as compared to their respective saline controls. Interestingly, mice that received nicotine during adolescence displayed a significantly enhanced preference to amphetamine compared to mice that received saline during adolescence \( F_{(3, 26)} = 17.18, \ p < 0.05 \). A similar increase in morphine-induced CPP was also observed (Fig. 4B). Indeed, mice that received nicotine during adolescence displayed a significantly enhanced preference to morphine compared to mice that received saline during adolescence \( F_{(3, 23)} = 17.44, \ p < 0.05 \).

### 3.3. Enhancement of cocaine CPP by adolescent nicotine exposure is strain-dependent

The effect of genotype on nicotine enhancement of cocaine CPP was investigated by treating B6 and D2 mice, well known and used inbred strains in drug abuse studies, with nicotine during early adolescence (0.5 mg/kg twice per day for 7 days). Two-way ANOVA \( F_{\text{strain}(1, 47)} = 208.7, \ p < 0.0001 \) showed that cocaine CPP tested in adulthood was significantly enhanced in B6 mice treated with nicotine during adolescence as compared to mice that had received saline (Fig. 5). In contrast, cocaine preference in D2 mice that received nicotine during adolescence did not significantly differ from their saline pretreated controls (Fig. 5). In addition, similar plasma levels of nicotine were found in the blood of B6 and D2 mice treated with nicotine for 7 days (Table 2). A slightly but significant higher plasma levels of cotinine, a main and inactive metabolite of nicotine, was observed in D2 mice compared to B6 animals.

Taken together, the results of behavioral studies demonstrate that repeated nicotine administration in early, but not late, adolescence enhances the potency of cocaine to induce CPP in adulthood, and that this effect is both \( \beta^2 \) nAChR-dependent and strain-specific.

### 3.4. Adolescent nicotine exposure did not alter highly palatable food conditioning

To assess whether the effect of adolescent nicotine treatment is specific for drug place conditioning, place conditioning for highly palatable food was examined. Fig. 6 shows that a significant preference was observed for highly palatable food compared to standard chow \( F_{(1,24)} = 31.85; \ p < 0.0001 \) in ICR mice. However, no effect of adolescent nicotine pretreatment was observed on palatable food conditioning (p > 0.05). These data suggest that adolescent nicotine does not generally alter reward-related behaviors using these food treatment conditions.

**Table 2**

<table>
<thead>
<tr>
<th>Mouse strain</th>
<th>Nicotine levels (ng/ml)</th>
<th>Cotinine levels (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6J</td>
<td>45.7 ± 3.6</td>
<td>30.5 ± 6.2</td>
</tr>
<tr>
<td>DBA/2J</td>
<td>41.7 ± 6.1</td>
<td>43.4 ± 2.8*</td>
</tr>
</tbody>
</table>

\( ^* p < 0.05 \) vs C57 mice.
induction of locomotor sensitization to cocaine. Demonstrate that early adolescent nicotine exposure enhances the saline pretreated animals (p < 0.05) (Fig. 7C). These results demonstrate that early adolescent nicotine exposure enhances the induction of locomotor sensitization to cocaine.

ΔFosB induction is greater in mice treated repeatedly with nicotine during early-adolescence than mice that received nicotine as adults. The longevity of the enhanced behavioral responses to cocaine after adolescent treatment with nicotine suggests that persistent molecular changes occur. Studies were therefore conducted to measure expression of ΔFosB, a stable transcription factor that has been implicated in the rewarding effect of drugs of abuse.

Adult ICR mice (PND 70) were treated with nicotine (0.5 mg/kg, 2 injections each day) for 7 days, and the NAc was collected 24 h after the last nicotine injection (PND77). Immunoblot showed that ΔFosB levels in the NAc increased by approximately 1.4-fold [p < 0.005] compared to levels in saline-treated control mice (Fig. 8). Early-adolescent mice (PND 28) were similarly treated with saline or nicotine for 7 days, and the NAc was collected 24 h later (PND 35). Results showed that repeated nicotine exposure during early adolescence significantly increased ΔFosB by approximately 4-fold [p < 0.005] compared to saline-treated controls (Fig. 8). Therefore, while repeated administration of nicotine induced ΔFosB expression in both adolescent and adult mice, a greater induction was measured in mice treated during early adolescence.

4. Discussion

In the present study, we have shown that repeated, but not short-term administration of nicotine during early adolescence enhanced cocaine CPP in adult mice. These behavioral changes are long-lasting and dependent upon the age at which nicotine is administered. Furthermore, the effects of nicotine are mediated by β2* nAChR subtypes and are strain-dependent. In addition, nicotine’s effects in enhancing CPP extended to other drugs of abuse (amphetamine and morphine) but not to CPP induced by highly palatable food. Furthermore, exposure to nicotine during early adolescence enhanced locomotor sensitization to cocaine during adulthood. Finally, our initial molecular studies showed that ΔFosB accumulation in the NAc might be an important player in these maladaptive events after exposure to nicotine in adolescence because ΔFosB is induced to a greater extent by nicotine in early adolescence than in adulthood.

Repeated but not acute pretreatment with nicotine in early adolescence increased cocaine-induced CPP in a dose-related manner during adulthood, suggesting a sustained effect of nicotine exposure. Thus, enhanced cocaine conditioned reward as a result of adolescent exposure to nicotine may lead to neuro-adaptations that predispose individuals to consume more cocaine. Importantly, the enhancement of cocaine CPP by nicotine depends on the age of exposure to the drug. Indeed, an increased behavioral response to cocaine was observed when nicotine exposure occurred during early adolescence (PND 28–34), but not during late adolescence or adulthood. These findings indicate that early adolescence is a critical period of vulnerability for nicotine exposure. Relevant to our findings, Belluzzi et al. (2004) demonstrated that early-adolescent rats displayed a preference for an environment paired with a single injection of nicotine, whereas late-adolescent and adult animals did not. Similarly, Dao et al. (2011) showed that early but not late adolescent pretreatment with nicotine enhanced subsequent cocaine self-administration in rats. These similarities in findings across species and behavioral tests provide strong support for early adolescence as a critical period mediating the increase in sensitivity to nicotine.

Our results are consistent with previous behavioral studies that have shown differences in responses to cocaine in rodents treated with nicotine during adolescence compared with animals that receive nicotine as adults. In adolescent mice and rats, pre-exposure to nicotine during adolescence enhanced cocaine-mediated locomotor responses, CPP and self-administration of cocaine (Collins and Izenwasser, 2004; McMillen et al., 2005; McQuown et al., 2007; Dao et al., 2011; Dickson et al., 2014). In contrast to these findings, (Kelley and Rowan, 2004) found that mice demonstrated a decrease in cocaine-induced CPP after adolescent nicotine exposure. This difference could be due to the nicotine dose administered (a high dose of 3 mg/kg vs. our low dose of 0.5 mg/kg), the length of nicotine exposure (25 days vs. our 7 days), or the age of animals during CPP testing (PND 142 vs. our PND 72). Regardless, the preponderance of evidence in the literature indicates enhancement rather than suppression of cocaine abuse-related effects by adolescent nicotine exposure.

We have also shown that the enhancement of the drug reward in the CPP test by prior adolescent nicotine exposure was also observed with amphetamine and morphine. These results are in agreement with previous reports showing that prior treatment with nicotine during early adolescence in rodents sensitized the behavioral effects of amphetamine and nicotine in adulthood (Collins et al., 2004; Adriana et al., 2003; Kota et al., 2009; Dao et al., 2011). While various mechanisms could mediate this cross-
sensitization between nicotine and other drugs of abuse, it is likely that changes in the dopaminergic system affected by nicotine exposure during the early developmental period, play an critical role. Importantly, since we found no effect on conditioning for highly palatable food, this suggests that adolescent nicotine exposure exhibits some behavioral specificity toward drug-induced reward.

The protracted behavioral response suggests the involvement of persistent neuroadaptations underlying the enhancement of cocaine-rewarding effects. This idea is consistent with the recent study by Doura et al. (2010) showing that adolescent rats subjected to chronic nicotine exhibited age-specific persistent gene expression changes in the ventral tegmental area. Indeed, over 500 adolescent-specific genes showed no initial response to chronic nicotine at the end of the 2-week treatment period but showed significant up- or down-regulation 30 days after the cessation of the drug.

We also showed that nicotine-pretreated adolescent C57BL/6J mice displayed a significantly higher level of cocaine-induced preference as adults compared with saline-pretreated mice. In contrast, nicotine failed to enhance the CPP response to cocaine in DBA/2J mice. This is unlikely to be attributable to a pharmacokinetic difference because nicotine blood levels were similar in the two strains of mice after chronic exposure to the drug during adolescence. However, differences in the expression and function of different targets and pathways of nicotine between the two strains could play an important role. Interestingly, these two strains also differ in their responses to nicotine in the CPP test, with DBA/2J mice much less sensitive to the rewarding effects of nicotine compared to C57BL/6J (Jackson et al., 2009).

The enhancement of cocaine-induced behaviors by adolescent nicotine treatment was mediated by nAChRs because it was blocked by co-administration of mecamylamine, a nonselective nicotinic receptor antagonist. In addition, co-administration of the selective α4β2* nAChR antagonist but not the α7 antagonist MLA during adolescence blocked nicotine-induced enhancement of

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**Fig. 7.** The effect of early-adolescent nicotine exposure on cocaine-induced locomotor sensitization in adult mice. (A) Experimental protocol of cocaine-sensitization in saline- and nicotine-treated mice. Early adolescent (PND 28) mice were injected s.c. with saline or nicotine (0.5 mg/kg) twice daily for seven days. At PND70 mice were they were started in the locomotor sensitization protocol. (B) Results of baseline activity before the start of the acquisition phase. (C) Effect of adolescent nicotine treatment on the level of cocaine-induced behavioral sensitization in mice. Mice received an i.p. injection of saline or cocaine (5 mg/kg) and were measured locomotor activity for 30 min. Results are expressed as mean ± SEM of n = 8/group. Statistical analysis was performed with mixed-factor (two-way) ANOVA and Bonferroni post hoc analyses were used to determine significant differences between groups (*p < 0.05) as indicated by the lines in the figure.
adolescent rats increased mRNA expression of nAChR subtypes in the ventral midbrain of rats in adulthood (Kota et al., 2007).

Our finding that nicotine treatment during adolescence increases cocaine sensitization in adulthood agrees with the results of our CPP studies. We found that a 7-day nicotine pretreatment in early adolescence enhanced locomotor sensitization to cocaine on challenge day as compared to saline pretreated-mice. In rodents, sensitization has been shown to correlate with enhanced predisposition to self-administer psychostimulants (Schenk and Partridge, 2000; Vezina et al., 2002) and reinstatement of extinguished self-administration (De Vries et al., 1998; Suto et al., 2004).

In human studies, adolescents who had previously smoked cigarettes were found to have higher initial “wanting” scores and were more likely to become cocaine-dependent than non-smokers (Lambert et al., 2006). Our findings suggest that nicotine exposure during adolescence enhances long-lasting neuronal alterations in neurochemical systems mediating locomotor sensitization in animals.

Several mechanisms could underlie the protracted behavioral sensitization induced by nicotine during adolescence. Substantial evidence suggests that ΔFosB produces long-term neural adaptations that contribute to drug addiction. Indeed, the induction of ΔFosB in the striatum after chronic exposure to various drugs of abuse persists for at least several weeks (Nestler, 2008). Furthermore, mice overexpressing ΔFosB in NAc exhibited enhanced sensitivity to both acute locomotor activity and rewarding effects of cocaine (Kelz et al., 1999). In addition, ΔFosB overexpression in the NAc increased morphine CPP (Zachariou et al., 2005). These findings suggest that ΔFosB accumulation in the NAc might play a role in nicotine-mediated enhancement of cocaine sensitization and reward. As a first step to investigate this possibility, ΔFosB was measured in the NAc of adolescent and adult mice that received nicotine. Nicotine treatment in adult mice produced a 1.4 fold increase in ΔFosB. The ability of nicotine to induce ΔFosB in the NAc is similar to other drugs of abuse (Perrotti et al., 2008). These results are also consistent with recent behavioral studies showing that nicotine exposure (7 days) in adult mice enhanced cocaine CPP as compared to mice pretreated with saline (Levine et al., 2011; Li H et al., 2014). Interestingly, the 7 days of nicotine pretreatment also enhanced increases in ΔFosB expression produced by subsequent cocaine administration (Li H et al., 2014). Our results also showed an even greater (4-fold) increase in ΔFosB expression after nicotine treatment in adolescents as compared to adults. Considering the longevity of ΔFosB, this mechanism could contribute to the long-lived effect of adolescent nicotine treatment on drug reward in adulthood.

The induction of ΔFosB is long-lived but not permanent because it is degraded and returns to pre-drug levels after 1–2 months (Nestler, 2008). This suggests that the ΔFosB protein itself does not maintain drug dependence, but rather alters the expression of target genes expected to play a role in nicotine and other drug addictions. One possible target is the GluR2 subunit of the AMPA receptor (Kelz et al., 1999). Another putative target gene of ΔFosB in NAc is dynorphin, which is thought to activate kappa-opioid receptors on VTA dopaminergic neurons and inhibit dopaminergic transmission, thereby decreasing reward. Nestler (2008) and Zachariou et al. (2006) have shown that the induction of ΔFosB represses dynorphin gene expression in the NAc, which could contribute to the enhancement of reward mechanisms mediated by ΔFosB.

Overall, our results suggest that nicotine use during early adolescence may convey a greater risk than nicotine use during adulthood. Thus, adolescent smokers may be particularly vulnerable to the risks of drugs of abuse.

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All authors declare no conflict of interest.

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References


Abstract  Rationale: Recent advances in neurobehavioral genetics have increased the importance of research on the behavioral patterns of different mouse strains. A comprehensive comparison of inbred and outbred mouse strains was conducted to provide information on the range of performance and pharmacological effects in the forced swimming test, a behavioral test commonly used to measure the effects of antidepressant drugs.

Objectives: Baseline performance and pharmacological responses to desipramine, a selective norepinephrine re-uptake inhibitor, and fluoxetine, a selective serotonin re-uptake inhibitor, were compared in seven inbred and four outbred mouse strains in the forced swimming test.

Methods: Swim sessions were conducted by placing mice in individual glass cylinders filled with water for 6 min. The duration of behavioral immobility during the last 4 min of the test was scored from videotapes.

Results: A 10-fold range of immobility values and coefficient of variation supported the existence of substantial behavioral differences between mouse strains in baseline performance in the FST. In general, inbred strains demonstrated lower variability than outbred strains. Desipramine dose-dependently reduced immobility in seven of the 11 strains tested, with DBA/2J and the C57BL/6J mice showing greater sensitivity than the other strains. In contrast, fluoxetine reduced immobility in only three out of the 11 strains tested, DBA/2J, BALB/cJ and NIH Swiss mice.

Conclusions: Background strain is a critical variable in determining baseline performance and the sensitivity to different types of antidepressant drugs in the mouse FST. The use of such mouse strains may provide information on the genetic basis for strain differences in depressive behavior and differential sensitivity to diverse classes of antidepressants.

Keywords  Forced swimming test · Depression · Fluoxetine · Desipramine · Genetics · Pharmacogenetics · Mouse

Introduction

A strong knowledge base on behavioral differences between mouse strains has become increasingly important for researchers in the field of behavioral genetics and behavioral pharmacology. Such information can be used by molecular geneticists to map the genes that mediate specific phenotypes. The interpretation of behavioral effects of targeted gene deletions or transgenic manipulations in mice depend on understanding normal variations in behavior from the background genotype (Gerlai 1996; Crawley et al. 1997; Rady et al. 1999). In addition, strain differences in sensitivity to psychoactive compounds may provide important information on the neurobiological substrates associated with behavioral responses to drugs. Differences between mouse strains in the behavioral effects and/or mechanisms of action of a number of drugs have been described, including ethanol (Crabbe et al. 1982, 1996), opiates (Belknap and O’Toole 1991; Mogil et al. 1996; Rady et al. 1999), cocaine (Ruth et al. 1988; Heyser et al. 1997), nicotine (Marks et al. 1989; Miner and Collins 1989; Stolerman et al. 1999) antipsychotics (Kanes et al. 1996) and anxiolytics (Crawley and Davis 1982; Mathis et al. 1994; Belzung and Ågmo 1997; Garrett et al. 1998; Griebel et al. 2000). However, there have been few comparisons of strain differences on behaviors sensitive to antidepressant drugs (Dalvi and Lucki 1999).

The forced swimming test (FST) evokes a characteristic behavioral immobility by exposing rodents to a swimming session within a confined cylinder. Because antidepressant drugs reduce the duration of immobility in the FST, the FST has become the most extensively used test used for measuring the behavioral effects of antidepressant drugs in versions of the test used with rats (Porsolt et al. 1977a, 1977b; Armario et al. 1988; Borsini 1995;
Lucki 1997) or with mice (Porsolt et al. 1977a; Redrobe et al. 1996). The FST is sensitive to all major classes of antidepressants, including tricyclics, selective norepinephrine and serotonin reuptake inhibitors, monoamine oxidase inhibitors and atypical antidepressants (Koe et al. 1983; Kulkarni and Mehta 1985; Borsini and Meli 1988; Cesana et al. 1993; Nixon et al. 1994; Bourin et al. 1996; Redrobe et al. 1996; Da-Rocha et al. 1997; Sánchez and Meier 1997). The immobility in the FST was originally considered a model of depression (Porsolt et al. 1977a, 1977b) and thought to represent the psychomotor retardation shown by many depressed patients. More recent theoretical formulations have suggested other behavioral accounts. Behavioral immobility in the FST provides adaptive disengagement from the persistent stress of forced swimming that alternates with active escape as part of the search-waiting coping strategy (Thierry et al. 1984), or immobility reflects a specific state of the mammalian defense repertoire known as “arrested flight” that is correlated with the psychological construct of “entrapment” in clinical depression (Dixon 1998; Gilbert and Allan 1998). Thus, the genetic basis for behavioral immobility in the FST may be related to variations in stress-induced behavioral depression.

The present study compared behavioral baselines of 11 mouse strains, seven inbred strains and four outbred strains, to examine the range of possible performance and variability in the FST. In addition, the responses to the two different types of antidepressant drugs were examined in each mouse strain. The effects of desipramine, a selective norepinephrine reuptake inhibitor, were compared with those of fluoxetine, a selective serotonin reuptake inhibitor (SSRI). In addition to examining the relationship between baseline performance and response to antidepressants across strains, behavioral responses to antidepressant drugs with distinct pharmacological mechanisms of action were compared within strains.

Materials and methods

Animals

Subjects were 10- to 12-week-old adult male mice housed in groups of four per cage (cage size: 28.5×17.5×13.0 cm) for at least 4 weeks prior to testing. Animals were maintained in a temperature-controlled environment (22±1°C) under a 12-h light-dark cycle, with lights turned on at 0700 hours. Food and water were freely available. All subjects were experimentally naive and used only once.

Mice from four commercially available outbred strains were obtained for testing: Swiss-Webster, CD-1 (Charles River, Wilmington, Mass., USA) and NIH Swiss (Harlan Sprague-Dawley Inc., Indianapolis, Ind., USA) and NIH Swiss, FVB/NJ; CD-1>Swiss-Webster, NIH Swiss, FVB/NJ; CD-1>C3H/HeJ, NIH Swiss, FVB/NJ; DBA/2J and BALB/cJ>NIH Swiss, FVB/NJ; A/J, 129/SvEmJ, C3H/HeJ, Swiss-Webster, NIH Swiss, FVB/NJ; CD-1>C3H/HeJ, NIH Swiss, FVB/NJ; DBA/2J and BALB/cJ>NIH Swiss, FVB/NJ; A/J, 129/SvEmJ, C3H/HeJ Swiss Webster, NIH Swiss>FVB/NJ.

Procedure

To facilitate adaptation to novel surroundings, mice were transported to the testing area from the animal colony at least 1 h prior to testing. All experimental sessions were conducted between 1200 and 1800 hours. Animals were randomly assigned to treatment conditions and tested in counterbalanced order. Briefly, swim sessions were conducted by placing mice in individual glass cylinders (46 cm tall×21 cm in diameter) filled with water (23–25°C water) to a depth of 15 cm. The depth was deep enough so that mice could not support themselves by placing their paws on the base of the cylinder. While the procedure was essentially similar to that described by Porsolt et al. (1977a), a cylinder of larger diameter was used because the larger swimming area has been reported to increase the predictive validity of the mouse FST (Sunal et al. 1994).

Behavioral scoring

A standard 6-min test duration was employed (cf. Borsini and Meli 1988). The water was changed between subjects. All test sessions were recorded by a video camera positioned directly above the cylinders. Videotapes were subsequently scored blindly by a highly trained observer. The behavioral measure scored from videotape was the duration of immobility during the last 4 min of the test period. Mice were judged to be immobile when making only those movements necessary to keep its head above water.

Drugs

Desipramine hydrochloride (Sigma, St Louis, Mo., USA) and fluoxetine hydrochloride (Eli Lilly, Indianapolis, Ind., USA) were prepared freshly on test days and administered IP 30 min prior to testing in a volume of 10 ml/kg. All drug doses were calculated as mg/kg base. Both drugs were dissolved in deionized water and fluoxetine required sonication to dissolve completely. Control animals received injections of 0.9% saline in a volume of 10 ml/kg.

Statistical analysis

Baseline performance and the coefficient of variation for each mouse strain (SD/mean) were calculated by combining scores from the saline treatment groups. Baseline immobility scores were analyzed by a single factor ANOVA, and Newman-Keul’s follow-up tests. Drug effects on immobility scores in each strain were analyzed by single factor analysis of variance (ANOVA). Dunnett’s t-test was used to compare values obtained at different doses of drug with corresponding saline-treatment values.

Results

The effects of strain on baseline and variability are shown in Table 1. Baseline immobility ranged from 0.16 to 1.62, a 10.1-fold range. ANOVA revealed significant inter-strain differences in baseline immobility [F(10,216)=9.52, P<0.0001]. Newman-Keuls post hoc analysis indicated that the following differences in baseline immobility were significant (P<0.05): C57BL10s-DBA/2, A/J, 129/SvEmJ, C3H/HeJ, Swiss-Webster, NIH Swiss, FVB/NJ; CD-1>C3H/HeJ, NIH Swiss, FVB/NJ; DBA/2J and BALB/cJ>NIH Swiss, FVB/NJ; A/J, 129/SvEmJ, C3H/HeJ Swiss Webster, NIH Swiss>FVB/NJ.

The effects of desipramine on immobility in the FST are shown in Fig. 1 for six inbred strains and in Fig. 2 for...
four outbred strains. The inbred strains that demonstrated significant changes with desipramine were: DBA/2J \([F(5,60)=6.10; \ P<0.005]\), C57BL/6J \([F(3,31)=5.50; \ P<0.01]\), BALB/cJ \([F(3,31)=13.72; \ P<0.005]\), 129/SvemJ \([F(3,36)=7.27; \ P<0.005]\), and A/J \([F(3,36)=4.90; \ P<0.01]\). Two outbred strains were also sensitive to the behavioral effects of desipramine: NIH Swiss \([F(4,62)=5.04; \ P<0.025]\) and CD-1 \([F(3,31)=4.72; \ P<0.01]\). Analyses of the individual strains indicated that desipramine was most sensitive in DBA/2J, C57BL/6J, and CD-1 mice, moderately sensitive in 129/SvemJ mice, and active only at 20 mg/kg in the A/J and NIH Swiss strains. In contrast, there were no significant effects of desipramine in C3H/HeJ \([F(3,36)=1.32; \ \text{NS}]\), Swiss Webster \([F(3,36)=0.82; \ \text{NS}]\), CF-1 \([F(3,47)=0.75; \ \text{NS}]\) or FVB/NJ mice (see below).

Figure 3 shows the effects of fluoxetine on immobility in the FST for seven inbred mouse strains. ANOVA revealed significant effects of fluoxetine on immobility in DBA/2J \([F(5,59)=6.41; \ P<0.005]\), BALB/cJ \([F(3,35)=3.22; \ P<0.022]\), and 129/SvemJ \([F(3,36)=3.92; \ P<0.05]\) mice. Analyses of the individual strains indicated that fluoxetine significant reduced immobility in DBA/2J (5.0–10.0 mg/kg) and BALB/cJ (10.0 mg/kg) mice. Fluoxetine increased immobility in 129/SvemJ mice (20.0 mg/kg) by interfering with the movement of their hindlimbs, perhaps a sign of the serotonin syndrome. Of the four outbred strains, fluoxetine was active only in NIH Swiss \([F(4,45)=2.91; \ P<0.05]\) mice, reducing immobility values significantly at 20.0 mg/kg. Fluoxetine was without significant effects on the behavior of the other strains tested: C57BL/6J
FVB/NJ mice failed to develop behavioral immobility during the test period. The response of FVB/NJ mice given desipramine (2.4±0.9 s) and fluoxetine (16.1±6.7 s), at 20 mg/kg, did not differ significantly from saline (13.4±6.7 s), according to ANOVA [F(2,29)=1.59, NS]. Because of the variable response of the FVB/NJ mice, only three out of ten mice given saline demonstrated immobility values that were greater than 5 s, complete dose-response curves for desipramine and fluoxetine were not determined.

Discussion

The existence of marked differences between 11 mouse strains in immobility baselines and pharmacological responses to antidepressants in the FST suggest that significant genetic factors contribute to the behavioral performance of rodents in this test. The mouse strains showed a 10.7-fold range of baseline immobility values in the FST. Baseline variability was calculated as a proportion of mean (i.e., coefficient of variation) as an estimate of the effect size required in pharmacological experiments that would be conducted with different mouse strains. In general, the outbred strains (CD-1, CF-1, Swiss-Webster and NIH Swiss) showed more variable baseline performances than inbred strains. Outbred strains ranked three of the four highest strains for absolute variability (standard deviation) and four of the six highest strains for variability relative to mean performance (coefficient of variation). Because genetic contributions are less variable for inbred than outbred strains, the comparison of performance variability between inbred and outbred strains emphasizes the relative roles of genetic background and environmental factors in determining the immobility values of individual subjects in the FST.

The relative variability of performance of certain mouse strains in the FST could also influence their selection for measuring changes with antidepressant drugs. The least variable strain at baseline, C57BL/6J, would be expected to provide the greatest precision of measuring changes in drug effects. Four of the six most variable strains were those that did not demonstrate significant effects to desipramine. It is also possible that mouse strains with the highest immobility values (e.g., C57BL/6J) may be more vulnerable to stress-evoked depressive behavior, as has been suggested for the WKY rat strain in the rat FST (Pare 1994). A consistent pattern of greater sensitivity on other behavioral tests sensitive to antidepressants, such as learned helplessness, tail suspension, and avoidance behavior, would help to support this hypothesis (Lucki 2001). In general, the survey failed to indicate that sensitivity to the effects of desipramine or fluoxetine across strains was determined by baseline levels of immobility. In comparison with previous reports on the mouse FST, C3H/He mice were
reported to display less immobility than C57BL/6 (Dubocovich et al. 1990; Nikulina et al. 1991), BALB/c, and DBA/2 (Nikulina et al. 1991), but the relative baselines of the BALB/c, C57BL/6 and DBA/2 mice in the current report did not agree with data obtained by Nikulina et al. (1991). These differences may derive from significant methodological differences. For example, Nikulina and associates used a 3-min test session, scored all 3 min, used a cylinder of smaller diameter and measured mobility by placing a wheel in the water. In contrast, the present research employed a 6-min test session, scored only the last 4 min, used a tank of larger diameter and measured immobility more directly. Differences in suppliers may also contribute to disparate behavioral results between studies.

The strain differences in baseline immobility did not appear to be explained by differences in locomotor activity. In contrast to their rank as the most immobile of strains tested in the FST, C57BL/6 mice were found to be the most active in novel environments of various descriptions (Trullas and Skolnick 1993; Logue et al. 1997). Furthermore, it is unlikely that the behavioral results could be explained in terms of a simple deficit or enhancement of learning across strains. C57BL/6 mice were better performers in a number of learning tasks, such as the Morris water maze compared with DBA/2, A/J and FVB/NJ mice (Upchurch and Wehner 1989; Owen et al. 1997), in spontaneous alternation in a T maze relative to DBA/2 mice (Paylor et al. 1993), in the radial arm maze compared with DBA/2, BALB/c and C3H/He strains (Ammassari-Tuele et al. 1993) and contextual fear conditioning relative to DBA/2 and FVB/N mice (Owen et al. 1997). In contrast, DBA/2 and BALB/c mice were superior in avoidance learning compared with C57BL/6 (Bovet et al. 1969; Buselmaier et al. 1981; Henderson 1989; Lipp et al. 1989; Weinberger 1992) and C3H/He mice (Bovet et al. 1969) and DBA/2 mice habituated to a novel environment faster than C57BL/6 mice (Ammassari-Tuele et al. 1995). It is not clear whether any other behavioral trait may be associated with the range of behavioral immobility values presented by the mouse strains in the present study.

The selective norepinephrine reuptake inhibitor desipramine was active in seven of 11 mouse strains tested. Three strains that were insensitive to desipramine (Swiss-Webster, C3H/HeJ and CF-1) were also among the most variable at baseline performance, a factor that may have made it more difficult to detect antidepressant effects. The fourth insensitive strain, FVB/NJ, did not demonstrate the development of sufficient immobile behavior for drug testing. Furthermore, there were inter-strain differences in sensitivity between the desipramine responders, with C57BL/6f, DBA/2J and CD-1 mice showing sensitivity at 5.0–20.0 mg/kg; BALBcJ, 129SvImJ at 10.0–20.0 mg/kg; and A/J at 20.0 mg/kg. Different mouse strains have been shown to differ in altering norepinephrine transmission after stress (Shanks et al. 1991). It is possible that similar strain differences in the response to stress on noradrenergic transmission could underlie the behavioral differences in the response to desipramine, a norepinephrine reuptake inhibitor.

Fewer mouse strains, only three of 11 strains tested, responded to the SSRI fluoxetine, suggesting the existence of greater genetic variability in the behavioral response to SSRIs in the mouse FST (Borsini 1995). Previously published reports on the effects of fluoxetine in the mouse FST have tested only outbred strains. The sensitivity of fluoxetine in NIH Swiss mice in the present study confirms the substantial work of M. Bourin and colleagues that have used NIH Swiss mice to study the neural basis for the effects of SSRIs in mice (Redrobe et al. 1996; Redrobe and Bourin 1998). Although several researchers have reported anti-immobility effects of fluoxetine at high doses in Swiss-Webster and CD-1 mice [Koe et al. 1983 (56 mg/kg); Cesana et al. 1993 (30 mg/kg); Redrobe et al. 1996 (32 mg/kg); Da-Rocha et al. 1997 (16 mg/kg); Sánchez and Meier 1997], there have also been negative reports at lower doses (<20 mg/kg; Kulkarni and Mehta 1985; Nixon et al. 1994; Bourin et al. 1996). As the dose of fluoxetine required to produce behavioral effects increases, however, it becomes less likely that the responses would be mediated by serotoninergic mechanisms, as are the effects of fluoxetine in the rat FST (Page et al. 1999). Fluoxetine at 20 mg/kg is sufficient to produce a 4-fold increase of striatal extracellular 5-HT levels in 129Sv mice (Knobelman and Lucki 2000). Some of the variance between studies may also result from testing procedures, such as differences in cylinder dimensions and water depth (Sunal et al. 1994) or mice obtained from different suppliers. The response to fluoxetine in the mouse FST has not previously been reported for inbred strains. Of the inbred strains tested in the present study, fluoxetine reduced immobility only in the DBA/2J and BALB/cJ strains, but not in five other strains. Fluoxetine actually increased immobility in one strain, 129/SvImJ, because of the production of hindlimb abduction when the mice were placed in the water.

Endogenous differences in the responses of 5-HT transmission to stressors and to 5-HT receptor activation between strains could provide a basis for understanding physiological differences that may account for divergence between two strains (DBA/2 and C57BL/6) in their behavioral sensitivity to fluoxetine. Thus, compared with DBA/2 mice, C57BL/6 mice had significantly higher baseline tissue concentrations of 5-HT or 5-HIAA in the hippocampus, caudate-putamen and ventral midbrain (Wimer et al. 1973; Shanks et al. 1991; Jones et al. 1996). Exposure of DBA/2 mice to a range of stressors reduced 5-HT levels in the amygda1a, hippocampus and mesocortex and increased 5-HT in the frontal cortex (Wimer et al. 1973; Shanks et al. 1991; Jones et al. 1996), whereas exposure of C57BL/6 mice to stress increased 5-HT levels in the hippocampus and 5-HIAA levels in the hypothalamos and mesocortex (Jones et al. 1996). The 5-HT2 receptor agonist, DOI, produced anxiogenic behaviors in DBA/2 mice but anxiolytic behaviors in C57BL/6 mice in the elevated plus maze (Onaivi
et al. 1995). Strain variations in stress neurobiology may also underlie differences in the behavioral response to fluoxetine. For example, exposure to stress produced opioid-mediated analgesia in DBA/2 mice but non-opioid analgesia, a more severe stress response, in Swiss-Webster or C57BL/6 mice (Przewlocka et al. 1988; Mogil and Belknap 1997). Fluoxetine may be sensitive to reducing the effects of a mild stressor but may be ineffective in more severely stressed mice.

The diameter of the cylinder used in the present study was larger than that used in the traditional forced swimming apparatus (Porsolt 1977a, 1977b). This modification was adopted because of the report that larger diameter cylinders increased the predictive validity of the mouse FST by reducing the number of false positive responses (Sunal et al. 1994). The present study suggests that this modification may produce additional behavioral differences. First, the doses of fluoxetine required to produce antidepressant-like effects in DBA/2J and BALB/c mice in the current series appeared lower than those previously reported for outbred strains. Secondly, the range of variability between inbred and outbred mouse strains in the present study appeared greater than shown previously using the traditional FST procedure. This observation would agree with the idea that larger cylinder dimensions and deeper water eliminate the development of adaptive positional responses using the cylinder bottom and walls that do not reflect immobility induced by prolonged swimming. This would increase variability between individuals and the sensitivity to antidepressants, as shown previously in the rat FST when the water depth was increased (Detke and Lucki 1995). To determine the generality of the current findings, similar strain comparison studies should be conducted in other mouse behavioral tests for antidepressants and behavioral responses to a chronic treatment regimen should be examined.

The overall picture provided by the survey of strain effects indicated that a greater number of mouse strains responded to desipramine (seven) than to fluoxetine (three). Desipramine, as a selective norepinephrine reuptake inhibitor, and fluoxetine, as a selective serotonin reuptake inhibitor, produce their pharmacological effects through different mechanisms (Frazer 1997). Previous studies in rodents have shown that both norepinephrine and serotonin mediate antidepressant behavioral responses, although desipramine and fluoxetine produce different specific behaviors through independent mechanisms (Lucki 1997; Page et al. 1999). Clinical studies have also shown that both norepinephrine and serotonin mediate antidepressant responses, because depletion of norepinephrine or serotonin in patients treated with antidepressants induced a rapid relapse of symptoms. The effects of norepinephrine and serotonin are independent, however, because subjects who relapsed after serotonin depletion were treated with serotonergic antidepressants and those who relapsed after norepinephrine depletion were treated with noradrenergic antidepressants (Heninger et al. 1996). The overall rates of treatment response for serotonergic and noradrenergic antidepressants appear to be relatively similar (Nelson 1999). However, their relatively similar rates of efficacy studies do not address whether the drugs treat the same patients or produce similar behavioral effects aside from treatment outcome, especially in more specific dimensions involving anxiety, psychomotor activation or psychosocial interactions. The differences in the effects of desipramine and fluoxetine between different mouse strains may represent a model for examining the genetic contribution to behavioral and neurochemical differences between pharmacological classes of antidepressant drugs. Given that noradrenergic and serotonergic antidepressants still produce treatment responses in only 60% of patients (Nelson 1999), the identification of pharmacogenetic markers that could predict greater efficacy for individual patients would improve the therapeutic outcome substantially for both drugs.

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Abstract

Immune challenge by bacterial lipopolysaccharide (LPS) causes short-term behavioral changes indicative of depression. The present study sought to explore whether LPS is able to induce long-term changes in depression-related behavior and whether such an effect depends on mouse strain and social context. LPS (0.83 mg/kg) or vehicle was administered intraperitoneally to female CD1 and C57BL/6 mice that were housed singly or in groups of 4. Depression-like behavior was assessed with the forced swim test (FST) 1 and 28 days post-treatment. Group-housed CD1 mice exhibited depression-like behavior 1 day post-LPS, an effect that leveled off during the subsequent 28 days, while the behavior of singly housed CD1 mice was little affected. In contrast, singly housed C57BL/6 mice responded to LPS with an increase in depression-like behavior that was maintained for 4 weeks post-treatment and confirmed by the sucrose preference test. Group-housed C57BL/6 mice likewise displayed an increased depression-like behavior 4 weeks post-treatment. The behavioral changes induced by LPS in C57BL/6 mice were associated with a particularly pronounced rise of interleukin-6 in blood plasma within 1 day post-treatment and with changes in the dynamics of the corticosterone response to the FST. The current data demonstrate that immune challenge with LPS is able to induce prolonged depression-like behavior, an effect that depends on genetic background (strain). The discovery of an experimental model of long-term depression-like behavior after acute immune challenge is of relevance to the analysis of the epigenetic and pathophysiologic mechanisms of immune system-related affective disorders.

Introduction

Experimental and clinical evidence indicates that activation of the immune system contributes to the pathogenesis of mood disorders [1–6]. Patients with major depression have frequently been observed to present with elevated levels of proinflammatory cytokines in blood plasma and cerebrospinal fluid [4]. There is extensive comorbidity of major depression with medical conditions involving inflammation and an increased expression of cytokines, and the therapeutic use of cytokines such as interferons is known to induce a depression-like syndrome in a sizeable proportion of patients [5]. These lines of clinical evidence are complemented by a plethora of animal studies. Both peripheral induction of cytokines by infection [6–9] or cancer [10] and intracerebral administration of cytokines to rodents evoke depression-like symptoms which are abrogated by cytokine antagonists or cytokine synthesis blockers.

Peripheral induction of cytokines by systemic administration of bacterial lipopolysaccharide (LPS) at doses that are too low to evoke a shock-like condition is known to induce a behavioral syndrome that includes traits of depression and follows a distinct time course [3,6,11]. Initially, a response termed “sickness behavior” is prevailing, which includes fever, anorexia, reduction of locomotion and a decrease in social interaction. Once the sickness behavior in terms of anorexia and sedation is over, behavioral symptoms indicative of depression such as anhedonia and passive stress coping are observed 24–48 h post-treatment [12,13]. When neuropeptide Y receptors of subtype Y2 or Y4 have been knocked out, depression-like behavior is seen even 4 weeks after a single injection of low-dose LPS [14]. This observation led us to hypothesize that, depending on genetic background (mouse strain) and social context (single versus group housing), intraperitoneal (IP) injection of LPS is able to induce long-term depression-like behavior. We addressed this hypothesis by studying the behavioral effect of LPS with the forced swim test (FST), a measure of behavioral despair [15], and the sucrose preference test, a measure of depression-related anhedonia [12]. These tests were carried out 1 day and 4 weeks post-LPS treatment.

Two strains of mice, outbred CD1 mice and inbred C57BL/6 mice, were compared with each other. CD1 mice were selected because short-term behavioral changes indicative of a state of depression following LPS treatment have extensively been studied in this mouse strain [12]. C57BL/6 mice were chosen because the Y2 and Y4 receptor knockout mice that exhibit long-term depression-like behavior following LPS treatment have a 50% C57BL/6 background [14]. In addition, C57BL/6 mice harbor a
serotonin transporter haplotype defined by two non-synonymous coding variants, which have implications on serotonin transporter function [16]. The experiments were carried out with female mice, given that affective disorders are more prevalent in women than in men [17] and there is a need to overcome the sex bias that is present in the neurosciences [18].

Apart from genetic background, psychosocial context may be another factor relevant to the manifestation of mood disturbances due to immune challenge [19–22]. Psychosocial stress is able to evoke cerebral expression of cytokines [5,23,24], and prolonged separation of mice enhances the LPS-evoked sickness behavior [22]. Since social isolation of mice is also able to enhance depression-like behavior [25], we used this experimental paradigm to address the question whether social context modifies the effect of LPS to induce long-term changes in affective behavior. Thus, female CD1 and C57BL/6 mice were either kept in groups of 4 or housed singly throughout the course of the experiments. LPS-evoked peripheral immune challenge alters brain functions by a neural and an endocrine route [6,11]. In addition, LPS and proinflammatory cytokines are able to stimulate the hypothalamic-pituitary-adrenal (HPA) axis as revealed by a rise of circulating glucocorticoid levels [4–6,22]. For this reason, plasma levels of interleukin-6 and corticosterone were monitored to examine whether any behavioral changes are correlated with these factors.

Materials and Methods

Experimental animals

The study was carried out with age-matched, adult, 4–6 month old, female mice of the outbred strain CD1 and the inbred strain C57BL/6, which were obtained from Charles River Laboratories (Sulzfeld, Germany). The animals were kept in groups of 4 in cages of size Ill. (length × width × height = 26 cm × 20.5 cm × 14 cm) in the institutional animal house in which the temperature (set point 22°C), relative air humidity (set point 50%) and light conditions (lights on at 6:00 h, lights off at 18:00 h, maximal intensity 150 lux) were tightly controlled. Tap water and standard laboratory chow were provided ad libitum throughout the study.

Ethics statement

The experimental procedures and number of animals used in this study were approved by an ethical committee at the Federal Ministry of Science and Research of the Republic of Austria and conducted according to the Directive of the European Communities Council of 24 November 1986 (86/609/EEC). The experiments were designed in such a way that the number of animals used and their suffering was minimized.

Administration of lipopolysaccharide (LPS)

LPS extracted from E. coli 0127:B8 (purified by gel-filtration chromatography, catalogue number L3137, Sigma-Aldrich, Vienna, Austria) was dissolved in pyrogen-free sterile saline (0.9% NaCl) at a concentration of 1 mg/ml. This stock solution was diluted with pyrogen-free sterile saline to yield an injection solution of 0.083 mg/ml LPS, which was injected IP at a volume of 0.01 ml/g, equivalent to a dose of 0.83 mg/kg LPS [12,14]. Pyrogen-free sterile saline injected at the same volume was used as vehicle control.

Experimental groups and time lines

The experiments were started after the animals had become familiar with the institutional animal house over the course of at least 3 weeks. Prior to the behavioral tests, the mice were allowed to adapt to the test room (set points 22°C and 50% relative air humidity, lights on at 6:00 h, lights off at 18:00 h, maximal light intensity 100 lux) for at least two days.

Two different protocols were used (Figure 1). In protocol 1, the effect of IP injected vehicle and LPS on depression-like behavior in the FST was assessed 1 and 28 days post-injection. For this purpose a total of 64 CD1 and 64 C57BL/6 mice was employed. The animals of each strain were allocated to 8 experimental groups (Figure 1), each group comprising 8 mice. The animals were either kept in sets of 4 animals per cage (group housing) or housed individually (single housing). The single housing protocol was started 7 days before IP injection of vehicle or LPS (Figure 1). After the FST, the mice were instantly returned to their home cage, and care was taken not to change cage mates during the course of the experiment. Thirty minutes after the start of the FST, trunk blood was collected for determination of the circulating levels of corticosterone and interleukin-6.

In protocol 2, the effect of vehicle and LPS on the sucrose preference of 14 singly housed C57BL/6 mice was assessed during days –1–4 and days 26–27 post-treatment (Figure 1). The single housing protocol was started 7 days before IP injection of vehicle or LPS. The animals were offered the choice to drink from two bottles, one filled with tap water and the other filled with 1% sucrose in tap water. The daily intake of tap water and sucrose solution was estimated by weighing the bottles every day.

Forced swim test (FST)

Each mouse was placed individually in a glass cylinder (diameter: 16 cm, height: 23 cm) containing tap water at 25°C. The water was 16 cm deep, which prevented the mice from touching the bottom of the beaker with their paws or the tail. Three categories of behavioral activity (climbing, swimming, and immobility) during the 6 min test session [14,26] were scored by a trained observer. The time each mouse spent climbing, swimming and floating (immobile) during the FST was recorded and expressed as a percentage of the test duration. Mice were considered immobile when they floated passively in the water, performing only movements that enabled them to keep their heads above the water level [15]. After the FST, the mice were placed for 6 min under a warming lamp and then returned to their home cage.

Sucrose preference

The animals were offered the choice to drink from two bottles, one filled with tap water and the other filled with 1% sucrose in tap water. The daily intake of tap water and sucrose solution was estimated by weighing the bottles every day between 8:30 h and 9:00 h. After they had been weighed, the bottles were cleaned and refilled every day. In addition, the relative position of the two bottles in the cage lid was changed every day.

Circulating corticosterone

Thirty minutes after the FST, between 11:00 h and 13:00 h, mice were deeply anesthetized with pentobarbital (150 mg/kg IP) before they were decapitated. Within 2 min after the injection of anesthetic, trunk blood was collected into vials coated with ethylenediamine tetraacetate (Greiner, Kremsmünster, Austria) kept on ice. Following centrifugation for 10 min at 4°C and 1200 x g, blood plasma was collected and stored at −70°C until assay. The plasma levels of corticosterone were determined with an enzyme immunoassay kit (Assay Designs, Ann Arbor, Michigan, USA). According to the manufacturer’s specifications, the sensitivity of the assay is 27 pg/ml and the intra- and inter-assay coefficient of variation amounts to 7.7 and 9.7%, respectively.
Protocol 1

Group 1  
VEH  FST  
group housing

Group 2  
LPS  FST  
group housing

Group 3  
VEH  FST  
single housing

Group 4  
LPS  FST  
single housing

Group 5  
VEH  FST  
group housing

Group 6  
LPS  FST  
group housing

Group 7  
VEH  FST  
single housing

Group 8  
LPS  FST  
single housing

Time (day)

Protocol 2

Group 1  
single housing  VEH  Sucrose preference (SP)  

Group 2  
single housing  LPS  Sucrose preference (SP)  

Time (day)
Circulating interleukin-6

A part of the blood plasma collected for determination of corticosterone was used for the assay of circulating interleukin-6. The plasma levels of interleukin-6 were determined with an enzyme immunoassay kit (Fluorokine MAP Mouse IL-6 Kit, R&D Systems, Minneapolis, Minnesota, USA). According to the manufacturer’s specifications, the sensitivity of the assay is 1.1 pg/ml, and the intra- and inter-assay coefficient of variation amounts to 4.0 and 7.4%, respectively.

Statistics

Statistical evaluation of the results was made with SPSS 16.0 (SPSS Inc., Chicago, Illinois, USA). In general, the data were analyzed by two-way analysis of variance (ANOVA), in some cases for repeated measurements. The homogeneity of variances was assessed with the Levene test. In case of sphericity violations the Greenhouse-Geisser correction was applied. Post-ANOVA analysis of group differences was performed with the Tukey HSD (honestly significant difference) test, when the variances were homogeneous, and with the Games-Howell test, when the variances were unequal. Student’s $t$ test was used when only two data groups were compared with each other. Some experiments were analyzed by planned comparisons with the Mann-Whitney test [27]. In view of the exploratory nature of the study, probability values $P \leq 0.1$ [27–29] were regarded as statistically significant. All data are presented as means±SEM, $n$ referring to the number of mice in each group.

Results

LPS reduced body weight 1 day post-treatment

The body weight of the animals was recorded immediately before (day 0) and 1 day after IP injection of vehicle or LPS. The body weight before treatment did not differ between the experimental groups of CD1 and C57BL/6 mice under study. Specifically, the baseline weight of the CD1 mice was 31.6±0.34 g ($n = 64$) and that of C57BL/6 mice 19.1±0.10 g ($n = 64$), which is typical of these strains. Treatment with LPS reduced the body weight of CD1 (Figure 2A) and C57BL/6 (Figure 3A) mice to a significant extent, while vehicle treatment had no effect. The LPS-induced decrease in body weight was on average 2.1–2.6 g in CD1 mice and 2.4–2.5 g in C57BL/6 mice (Figures 2A and 3A). In both strains of mice this weight loss did not significantly differ between singly housed and group-housed animals (Figures 2A and 3A). The detailed results of the statistical analysis of the data are reported in Text S1.

LPS had no effect on body weight 28 days post-treatment

When CD1 and C57BL/6 mice were weighed 28 days post-treatment, their body weight was higher than immediately before IP injection of LPS or its vehicle (Figures 2B and 3B). The increase in body weight amounted on average to 2.1–2.6 g in CD1 mice and 2.4–2.5 g in C57BL/6 mice (Figures 2B and 3B) and was statistically significant in either strain of mice (see Text S1). However, the weight gain did not significantly differ between singly housed and group-housed animals (Figures 2A and 3A). The detailed results of the statistical analysis of the data are reported in Text S1.

LPS induced short-term depression-like behavior in group-housed CD1 mice

The behavior in the FST was assessed 1 and 28 days after IP injection of LPS or its vehicle and analyzed with respect to the factors time (1 day versus 28 days post-treatment) and treatment (LPS versus vehicle). Relative to vehicle, LPS failed to alter the duration of immobility and climbing in singly housed CD1 mice both 1 day and 4 weeks after treatment (Figure 4A,C). In contrast,
the duration of time spent swimming by singly housed CD1 mice was significantly reduced by LPS (see Text S1), but since there was no interaction between the factors time and treatment, this effect of LPS could not be further analyzed (Figure 4B).

The effect of LPS on the FST behavior in group-housed CD1 mice was much more pronounced than in singly housed CD1 mice (Figure 4). Relative to vehicle, LPS significantly prolonged the duration of immobility in group-housed CD1 mice 1 day, but not 28 days, post-treatment (Figure 4A). This LPS-induced extension of immobility was complemented by a highly significant shortening of the duration of swimming 1 day post-treatment, whereas 28 days post-treatment the effect of LPS to reduce the duration of swimming in group-housed CD1 mice was clearly less pronounced but still statistically significant (Figure 4B). In contrast, the duration of climbing spent by group-housed CD1 mice was not significantly altered by LPS 1 and 28 days post-treatment (Figure 4C).

LPS induced short- and long-term depression-like behavior in C57BL/6 mice

The experiments conducted with C57BL/6 mice were analogous to those carried out with CD1 mice, yet the outcome of the FST differed substantially between CD1 mice (Figure 4) and C57BL/6 mice (Figure 5). Two-way ANOVA disclosed an effect of treatment (LPS versus vehicle) on the duration of immobility, swimming and climbing (see Text S1). However, since there was no significant interaction between the factors treatment and time, the effect of LPS could not be subjected to post-hoc analysis of group differences. Notwithstanding this fact, LPS prolonged the duration of immobility in singly housed C57BL/6 mice (Figure 5A), while at the same time the duration of climbing was shortened (Figure 5C). In addition, the duration of swimming was reduced by LPS in a time-dependent manner (Figure 5B). A synopsis of the data obtained in singly housed C57BL/6 mice indicates that LPS prolonged the duration of immobility along with a shortening of the duration of both swimming and climbing, these changes being numerically more pronounced 1 day than 28 days post-treatment (Figure 5A,B,C).

The effect of LPS on the behavior of group-housed C57BL/6 mice in the FST showed a distinct time course over the 28-day period post-treatment. Thus, the duration of immobility was significantly shortened by LPS 1 day, but significantly prolonged 28 days post-treatment (Figure 5A). In addition, the duration of immobility recorded in vehicle-treated animals 1 day post-treatment was significantly longer than that recorded 28 days post-treatment (Figure 5A). The changes in immobility duration which LPS evoked in group-housed C57BL/6 mice (Figure 5A) were complemented by alterations in the duration of swimming (Figure 5B) and climbing (Figure 5C). Specifically, LPS shortened the duration of swimming in group-housed C57BL/6 mice 28 days post-treatment, but not 1 day post-treatment (Figure 5B). In addition, the duration of swimming recorded in vehicle-treated animals 1 day post-treatment was shorter than that recorded 28 days post-treatment (Figure 5B). The duration of climbing observed in group-housed C57BL/6 mice was significantly prolonged by LPS 1 day, but not 28 days, after treatment (Figure 5C). Taken all aspects together, it is evident that in group-housed C57BL/6 mice LPS shortened immobility 1 day post-treatment due to an increase in climbing, but prolonged immobility 28 days post-treatment at the expense of swimming (Figure 5A,B,C).

LPS caused short-term, but not long-term, increases in circulating corticosterone levels

The plasma concentrations of corticosterone were measured 30 min after the FST had begun. Two-way ANOVA of the corticosterone levels measured in singly housed CD 1 mice (Figure 6A) revealed an effect of treatment (LPS versus vehicle) but, since there was no significant interaction between the factors treatment and time (see Text S1), the effect of LPS could not be subjected to post-hoc analysis of group differences. Despite this fact it is evident from Figure 6A that LPS enhanced the post-FST levels of corticosterone in singly housed CD 1 mice and that this LPS-induced increase in circulating corticosterone was nominally more pronounced 1 day post-treatment than 28 days post-treatment (Figure 6A). A similar result was obtained in group-housed CD1 mice (see Text S1) in which the effect of LPS to enhance the post-FST plasma corticosterone levels was seen 1 day, but not 28 days, post-treatment (Figure 6A).

The effect of LPS on the post-FST plasma concentrations of corticosterone in C57BL/6 mice was analogous to that seen in
CD1 mice. One day post-treatment, LPS evoked a comparable rise of circulating corticosterone in both singly housed and group-housed C57BL/6 mice, an effect that was no longer observed 28 days post-treatment (Figure 6B). In addition, the post-FST plasma levels of corticosterone measured 28 days after vehicle treatment of singly housed and group-housed C57BL/6 mice were significantly lower than 1 day after vehicle treatment (Figure 6B).

Additional analysis revealed that the post-FST plasma concentrations of corticosterone depended on the housing conditions. Thus, the circulating levels of the glucocorticoid measured 28 days after vehicle/LPS treatment of singly housed CD1 and C67BL/6 mice were lower than those in the respective group-housed animals (Figure 6A,B).

The effect of LPS to transiently increase circulating interleukin-6 levels was more pronounced in C57BL/6 mice than in CD1 mice.

The plasma levels of interleukin-6 were measured 30 min after the FST had begun. Relative to vehicle, LPS increased the circulating concentrations of interleukin-6 1 day, but not 28 days, after treatment of singly housed and group-housed CD1 mice (Figure 7A). C57BL/6 mice turned out to be particularly sensitive...
Much as in CD1 mice, however, the effect of LPS had waned 28 days after treatment of singly housed and group-housed C57BL/6 mice (Figure 7B). In neither strain of mice did the interleukin-6 concentrations significantly differ between singly housed and group-housed animals.

LPS caused a long-term inhibition of sucrose preference in C57BL/6 mice

The effect of LPS on sucrose preference and total water intake was investigated in singly housed C57BL/6 mice for a period of 27 days. As expected, the animals strongly preferred sucrose (1%) solution over normal tap water (Figure 8A,B). Following injection of vehicle or LPS, there was a change in their drinking behavior. Vehicle treatment transiently increased the consumption of water and slightly decreased the intake of sucrose solution during the 24-h period post-treatment (Figure 8A). Planned comparison of the relative contribution of sucrose solution and water intake to the total consumption of fluid revealed that on day 26 after vehicle treatment the relative intake of sucrose solution and water was indistinguishable from that measured during the day before vehicle treatment (Figure 8A, insert). Both on day 1 before (day -1) and on day 26 after vehicle treatment, the intake of sucrose solution covered more than 80% of the total consumption of fluid (Figure 8A, insert).

In contrast to vehicle, LPS led to a short-term reversal of sucrose preference to water preference. As is shown in Figure 8B, the intake of sucrose solution was reduced by more than 70% during the 24-h period after LPS treatment, while the consumption of water was enhanced. The relative consumption of sucrose solution versus water normalized over the following days, but on days 26 and 27 post-treatment the relative intake of sucrose solution was nominally lower, and that of water nominally higher, than before LPS treatment (Figure 8B). This instance was also revealed by planned comparison of the relative contribution of sucrose solution and water intake to the total consumption of fluid (Figure 8B, insert). While on day 1 before LPS treatment (day -1) the intake of sucrose solution covered some 80% of the total consumption of fluid, the relative intake of sucrose solution and water was no longer significantly different from each other on day 26 after LPS treatment (Figure 8B, insert).

Further analysis demonstrated that the total intake of fluid significantly increased during day 1 after vehicle treatment but otherwise stayed constant during the 26-day observation period post-treatment (Figure 9). In contrast, the total consumption of fluid did not significantly change during the 26-day observation period after LPS treatment, but during days 1 and 2 post-treatment was significantly lower than after vehicle treatment (Figure 9).

Discussion

Behavioral analysis of female mice

The present data show that a single IP injection of LPS is able to induce long-term depression-like behavior in mice, an effect that depends on genetic background (strain) and, in part, on social environment. The dose of LPS used here (0.83 mg/kg) was identical to that used previously in the examination of its impact on depression-like behavior [12,14]. The current study was carried out with female mice because affective disorders are more prevalent in women than in men [17], a situation that need be reflected in the design of translational animal experiments [18]. We did not monitor the estrous cycle in order to avoid additional stress which may influence the behavior of the animals. On the one hand, stress can prolong the estrous cycle [30] while, on the

Figure 6. Effect of LPS (0.83 mg/kg injected IP), relative to vehicle (VEH), on plasma concentrations of corticosterone as recorded 1 and 28 days after treatment under single and group housing conditions. Trunk blood of CD1 (A) and C57BL/6 (B) mice for the corticosterone assay was taken 30 min after the FST had been started. The values are means±SEM, n = 7–8. *** P<0.01 versus vehicle-treated mice at the same time point post-treatment, + P≤0.1, +++ P<0.01 versus vehicle-treated mice tested 1 day post-treatment. In panel A it was not possible to apply a post-hoc test because two-way ANOVA failed to disclose any interaction between the factors time and treatment.

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other hand, the estrous cycle can modify neural responses to endotoxin [31], depression-like behavior [32,33] and circulating corticosterone [34]. Although being aware of these limitations, we consider it unlikely for a number of reasons that our data were significantly confounded by these factors. First, the experiments were carried out in the strict absence of male mice. Second, we have previously found that the estrous cycle is largely synchronized within the same and across different cages [35]. Third, the effects of LPS and its vehicle were examined in parallel under identical housing conditions. Fourth, the coefficient of variation of the data obtained in female mice did not appear anomalous in comparison to data obtained in male mice [36].

Short-term behavioral effects of LPS

The short-term effect of LPS on behavior in the FST was evaluated because it may be of relevance to its long-term effect. It has previously been shown that singly housed male CD1 mice exhibit enhanced depression-like behavior in the FST 24 h post-LPS, as revealed by an increase in the immobility time at the expense of swimming time [12]. In the current study with female CD1 mice, an increase in depression-related behavior 1 day post-LPS was seen only under group housing conditions. In C57BL/6 mice, to the contrary, depression-like behavior was markedly enhanced by LPS only under single housing conditions. In addition, the behavioral profile of the depressogenic effect of LPS in C57BL/6 mice (prolonged immobility time, shortened swimming and climbing time) differed from that of CD1 mice (prolonged immobility time, shortened swimming time). The finding that, in group-housed C57BL/6 mice, LPS reduced immobility and enhanced climbing could be related to the apparent ability of vehicle to increase immobility and decrease climbing.

In interpreting these diverse observations made 1 day post-treatment several factors need be considered. First, the acute effect of LPS on behavior in the FST may rather be related to the transient sickness response, which can last for up to 2 days [37], than reflect a truly depressogenic effect [37]. Second, affective behavior in group-housed animals may be influenced by strain-related empathy among the cage mates [38–40] under the distress caused by LPS. Third, social isolation of mice can, on the one hand, increase the LPS-evoked sickness behavior [22] and, on the other hand, enhance depression-like behavior [25,41–43]. Thus, the acute effect of LPS on the behavior in the FST under different housing conditions is a function of multiple interactive factors.

Long-term depression induced by LPS

The major advance put forward by the present study is the discovery that LPS is able to induce long-term depression-like behavior in a strain-related manner. While the acutely depressogenic effect of LPS in group-housed CD1 mice had largely waned 4 weeks post-treatment, long-term depression in C57BL/6 mice was induced whether or not there was an acutely depressogenic effect. Importantly, the behavioral profile of the LPS-evoked long-term depression in group-housed C57BL/6 mice (prolonged immobility time, shortened swimming time) differed from that in singly housed C57BL/6 mice (prolonged immobility time, shortened climbing time).

The profile of the LPS-induced behavioral changes in the FST is worth noting because antidepressants that enforce serotonergic transmission increase primarily swimming behavior, while antidepressants that enhance noradrenergic transmission increase predominantly climbing behavior [15,43]. In analogy, there is reason to speculate that the long-term depression-like behavior caused by LPS in group-housed C57BL/6 mice involves serotonergic but not noradrenergic pathways, while the depression-like behavior induced by LPS in singly housed C57BL/6 mice depends on noradrenergic but not serotonergic circuits. It would hence appear that the neurochemical substrates whereby LPS alters depression-like behavior in the FST differ with housing conditions. This instance provides a lead for targeted studies into

Figure 7. Effect of LPS (0.83 mg/kg injected IP), relative to vehicle (VEH), on plasma concentrations of interleukin-6 as recorded 1 and 28 days after treatment under single and group housing conditions. Trunk blood of CD1 (A) and C57BL/6 (B) mice for the interleukin-6 assay was taken 30 min after the FST had been started. The values are means±SEM, n = 7–8. ** P<0.05, *** P<0.01 versus vehicle-treated mice at the same time point post-treatment.

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A: VEH in C57BL/6 mice

B: LPS in C57BL/6 mice
The main graphs show the absolute daily intake of sucrose solution and water (ml). In the inserts, the daily intake of sucrose solution and water on the day before treatment (day -1) and on day 26 post-treatment. The values are means±SEM, n = 7. **P<0.01 versus water intake.

The prolonged depression-like behavior in LPS-treated C57BL/6 mice as observed in the FST was confirmed with the sucrose preference test in an independent cohort of animals. The two tests address different aspects of depression. While the FST measures behavioral despair [15], the sucrose preference test assesses anhedonia, a decrease in the ability to experience pleasure [12,45,46]. LPS exerted two distinct time-related effects on sucrose preference in singly housed C57BL/6 mice. One day post-treatment, there was a pronounced reduction of sucrose preference, an effect that waned during the subsequent days and had previously been noted in CD1 mice [12]. Twenty-six days post-treatment, however, the sucrose preference was found to be attenuated in LPS-treated C57BL/6 mice, which attests to a long-term effect of LPS to induce anhedonia-like behavior.

**Short-term and long-term effects of LPS on circulating interleukin-6 and corticosterone**

The LPS-induced changes in body weight did not provide any explanation for its strain-dependent impact on affective behavior in the short and long term. The initial decrease in body weight is part of the transient “sickness response” to LPS, which includes anorexia and a reduction of locomotion and exploration [3,6,11,47]. The sickness response arises from LPS-evoked induction of proinflammatory cytokines, including interleukin-6, that signal to the brain via a neural and endocrine route [6,11,22]. Our finding that LPS increased the post-FST plasma levels of interleukin-6 1 day but not 4 weeks post-treatment indicates that LPS, but not the FST, was responsible for the rise of this cytokine. The observation that circulating interleukin-6 in C57BL/6 mice increased to a much larger extent than in CD1 mice suggests that C57BL/6 mice are more sensitive to immune stress than CD1 mice. It remains to be shown whether the pronounced induction of interleukin-6 in C57BL/6 mice has any bearing on the long-term depression-like behavior induced by LPS in this mouse strain.

LPS and proinflammatory cytokines are able to stimulate the HPA axis as shown by a rise of circulating glucocorticoid levels [4–6,22]. In the present study, the plasma levels of corticosterone measured 30 min post-FST do not represent basal levels of the glucocorticoid but reflect levels that are elevated by the stress of the FST procedure [36]. Single housing is stressful for female mice [48] and results in a reduction of the basal levels of circulating corticosterone [25]. Likewise, the post-FST levels of corticosterone measured 28 days after vehicle/LPS treatment were lower under single housing than under group housing conditions. We do not think, however, that the LPS-evoked long-term depression-like behavior in C57BL/6 mice arises primarily from a change in the function of the HPA axis, although two distinct alterations in the dynamics of the HPA axis were observed. First, LPS enhanced the FST-evoked increase in circulating corticosterone 1 day post-treatment independently of strain and housing conditions, whereas 4 weeks post-treatment this effect of LPS was largely gone. This observation is in keeping with the contention that the HPA axis undergoes prolonged desensitization after immune challenge [49]. Second, the post-FST plasma levels of corticosterone measured in C57BL/6 mice 28 days post-treatment were in general significantly lower than 1 day post-treatment. This observation may reflect a particular trait of C57BL/6 mice to respond to experimental interventions by a long-term change in HPA axis dynamics. Elucidation of the full impact of the HPA axis on LPS-evoked long-term changes in affective behavior will involve analysis of basal and stress-related levels of corticosterone and of the time course of the HPA axis response to stress.

**Conclusion**

The current study demonstrates that immune challenge with LPS is able to induce prolonged depression-like behavior. Importantly, this effect depends on genetic background (strain). It has been noted before that the impact of immune challenge on affective behavior need be analyzed with respect to background conditions [22]. The availability of an experimental model of long-term depression-like behavior after acute immune challenge will aid the elucidation of the pathophysiology of immune system-related affective disorders and of the epigenome that is controlled by social environment and immune system activity [50].

**Supporting Information**

Text S1 Detailed Results of the Statistical Analysis of the Data.

(DOC)
Acknowledgments

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References


Author Contributions

Conceived and designed the experiments: EP MJK. Analyzed the data: EP MJK. Contributed reagents/materials/analysis tools: EP FS PH. Wrote the paper: EP MJK FS Frazier.
Research report

Behavioural analysis of four mouse strains in an anxiety test battery

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Abstract

Differences in locomotor activity, exploratory activity and anxiety-like behaviour of C57BL/6ChR, C57BL/6J, Swiss Webster/J and A/J strain were investigated in an anxiety battery. The battery consisted of paradigms studying spontaneous behaviour after a mild stressor, tasks of innate anxiety (light–dark box, elevated plus maze, novel object exploration), response to a conflict situation (Vogel conflict), conditioned fear and response to inescapable swim stress. Locomotor activity was studied in an open field and compared with locomotion in the other tests. Exploratory behaviour was studied in a 16-hole board task. The data confirm previous studies suggesting that A/J mice are a relatively anxious strain. Also, the data indicated that locomotor activity was independent of the paradigm employed, while the rank order of strain-dependent effects on anxiety-related behaviour changed as a function of the task under study. Our data provide further support for the notion that choice of strain is essential in studies of anxiety-related behaviour. Influence of strain should be considered in pharmacological and lesion studies, as well as in studies with mutant mice. In addition, the data indicate that different anxiety paradigms tax different aspects of anxiety, suggesting that a battery of different tests should be used in studies of anxiety-related behaviour. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Mice; Anxiety; Tests

1. Introduction

Recent advances in molecular biology have led to the generation of an increasing number of genetically engineered mice with specific mutations in single genes. However, such mutations can have very different phenotypes, for example with respect to anxiety-related behaviour, depending on genetic background [1,17]. Indeed, strain differences in murine anxiety paradigms are well established [10,18,21,32,36], differences which do not only affect the outcome of experiments with mouse mutants, but which are of course of equal relevance for pharmacological [6,11] or lesion studies.

Complexity increases by the fact that different types of anxiety-related behaviour have been distinguished in rodents, such as state and trait anxiety [20,23], and that different behavioural principles seem to underlie the design of the various anxiety tasks, with some paradigms being based on spontaneous exploration, some on a learned response, and yet others measure conflict behaviour. This diversity is reflected by the fact that different paradigms show different sensitivities for the anxiolytic- or anxiogenic-like actions of drugs, and task-dependent effects have been demonstrated following manipulations of several brain regions [19,22,26,28,35]. This in turn would suggest that testing of mouse mutants using a wide range of different anxiety paradigms would be advantageous. However, the majority of studies with mouse mutants employ only a very limited number of anxiety tasks, which poses the risk of detecting false positive or false negative results.

Therefore, the present study compared a wide range of anxiety tests, such as the elevated plus maze, the light–dark box, novel object exploration, the hole board, Vogel conflict and conditioned fear. Three in-
bred mouse strains were compared in these tests: the A/J strain, which has been suggested to show a high degree of anxiety-related behaviour [10,11], the C57BL/6J strain, which has been suggested to display intermediate levels of anxiety [11] and which is commonly used for the generation of mouse mutants, and the C57BL/6ChR strain. The C57BL/6ChR subline originated from the C57BL/6J line, but both sublines have been bred independently for several generations since 1974 and are provided by different suppliers. Earlier studies have shown genetic divergence in a number of inbred strains, and certain C57BL/6 sublines show marked differences in behaviour, for example in exploratory activity [13]. Finally, the Swiss Webster outbred strain was tested. This strain was chosen, as a literature search revealed the Swiss Webster mouse to be a popular mouse strain for pharmacological tests of anxiety-related behaviour.

2. Material and methods

2.1. Animals

Male A/J mice (n = 11), C57BL/6 mice (B6/J; n = 10) and Swiss Webster mice (n = 11) were supplied by Harlan Winkelmann (Borchcn, Germany), while C57BSW/6CrlBR (B6/ChR; n = 10) were supplied by Charles River (Schulzfeld, Germany). Animals were singly housed in type II cages and kept under 12:12 h light–dark cycle, with food and water ad libitum. All the testing took place during the light phase of the light–dark cycle of animals maintained in type II cages and kept under 12:12 h light–dark cycle, with food and water ad libitum. All testing was performed independently for several generations since 1974 and is provided by different suppliers. Earlier studies have shown genetic divergence in a number of inbred strains, and certain C57BL/6 sublines show marked differences in behaviour, for example in exploratory activity [13]. Finally, the Swiss Webster outbred strain was tested. This strain was chosen, as a literature search revealed the Swiss Webster mouse to be a popular mouse strain for pharmacological tests of anxiety-related behaviour.

2.2. Examination of spontaneous behaviour

Spontaneous behaviour was analysed in the home cage. Shortly before testing, the animal was aroused by placing it in a confinement for 1 min in order to ensure a certain level of behavioural activation. During this time, the bedding in the home cage was shuffled around and evenly redistributed. A clear plastic lid was placed on top of the cage, which prevented escape of the animal but allowed video recording, which started as soon as the mouse was returned to its home cage and lasted for 10 min. For behavioural analysis, the bottom of the cage was arbitrarily divided in 3 × 2 equally sized fields. The number of line crossings was scored. The time spent rearing, grooming and burying behaviour were scored, and were calculated as percentage of the total session time.

2.3. Elevated plus maze

Next, the animals were tested on the elevated plus maze. The maze was made of dark grey plastic and was elevated 72 cm above the floor. Four maze arms (28 × 5 cm) originated from a central platform (5 cm square), effectively forming a cross. Two of these arms (closed arms), located opposite each other, were enclosed by clear Perspex walls (15 cm high), while the other two arms (open arms) were equipped with a small threshold (0.5 cm high), which was fixed at the edges of the arms. Testing took place during the early dark phase, starting 2 h after the lights had been turned off. Dim illumination was provided by a red light. A test session started by placing the animal on the central platform, facing the enclosed arm. Over a period of 5 min, exploration was video recorded. Between sessions, the maze was thoroughly rinsed with water and dried with paper towel. Subsequently, the number of open and closed arm entries, and the time spent in the different compartments of the maze (central platform, open and closed arms) were scored.

2.4. Light–dark exploration

Testing took place in four boxes (Coulbourn Instruments, Allentown, USA), which allowed the testing of four animals in parallel. Each box was divided into two equally sized compartments (26 × 13 × 38 cm high each). One compartment was made of clear plastic walls and was illuminated by bright light (500 lux), while the other compartment was made of black plastic, not illuminated and covered by a black roof. An opening, 7.5 × 7.5 cm wide, connected the two parts of the box. Two infrared sensor rings (sensor spacings 1.52 cm) allowed the measurement of vertical and horizontal activity (sampling rate 250 ms), and were connected to a computer equipped with the Tru Scan Software Vers 1.1A (Coulbourn Instruments). Each box, including its sensor rings, was surrounded by an additional box (47 × 47 × 38 cm, white plastic) which prevented the animals from seeing each other.

A session started by placing the animal in the centre of the illuminated compartment, facing the opening, and lasted 5 min. From the raw data, the distance travelled and the number of rearsings made over the 5-min period were calculated. Furthermore, moving time and entries into the illuminated compartment, the relative distance travelled and the relative number of rairsings made in the illuminated compartment were calculated.

2.5. Novel object exploration

Avoidance of a novel object placed into a familiar environment was investigated. Animals were first habituated to an open field (26 × 26 × 38 cm high, 500-lux illumination), made of a white floor and clear plastic walls, and equipped with infrared photocell sensors. Habituation lasted for 30 min. Subsequently, animals
were briefly returned to their home cage and an object (a bolt, M 12 × 60 mm high) was placed into the centre of one of the quadrants of the field. Afterwards, each mouse was returned to the open field for an additional 15 min. During the habituation stage, the distance travelled was measured in 3 min time-bins. Furthermore, the number of entries and the time spent in the quadrant, which subsequently would contain the object, was measured during the last 15 min of the habituation stage and taken as baseline. The same measures were taken once the object had been introduced, and the number of entries and time spent in the object quadrant relative to baseline were calculated in order to correct for possible preferences for a particular quadrant in the testing box. In addition, the total time spent in the object quadrant was measured during this stage.

2.6. Vogel conflict task

Testing took place in a grey plastic chamber (20.5 × 20.5 × 30 cm, <1 lux illumination) equipped with a metal grid and a water spout, which was located in the centre of one of the walls, 1 cm above the grid. The spout and the grid were connected to a drinkometer and a shock generator (Coulbourn Instruments), which allowed to deliver a 0.15 mA shock over 10 ms. Prior to testing, animals were water deprived for 24 h. A session started once the animal had made 20 licks on the spout to ensure knowledge about the position of the spout prior to start, and terminated after 20 min. Every 20th lick, the mouse received a mild shock. Animals failing to make at least 20 responses necessary to start the session within 20 min were excluded. The latency to start the session and number of responses made during the session were registered by a computer.

2.7. Hole board

Mice were tested in the same square boxes as used for the novel object exploration (500 lux illumination), except that now a metal floor was used, containing 16 holes (2.2 cm in diameter), evenly distributed over the floor (4 × 4 holes). Animals were placed at the centre of the field and allowed to freely explore for 30 min. The distance travelled, number of rearing and number of holes visited were monitored by three infrared sensor rings, connected to a computer equipped with the Tru Scan Software Vers 1.1A (Coulbourn Instruments).

2.8. Conditioned fear

Two chambers were used. One chamber (the conditioning chamber) was identical to the box used for the assessment of the novel object exploration and hole board exploration, except that now a shock grid was placed on the floor, and that the box was dimly illuminated (1 lux). The shock grid was connected to a shock generator (Coulbourn Instruments) which allowed the delivery of a 0.35 mA shock over 2 s. The other chamber (the test chamber) was of similar dimensions, except that instead of a shock floor the box was equipped with a white plastic floor. The shock grid was cleaned with 70% ethanol while the white plastic floor was cleaned with a lemon-flavoured detergent after each animal, in order to change the olfactory properties of the two chambers. A light placed on top of the chambers allowed presentation of a flashing light stimulus for 15 s (26 lux at floor level, flash rate 5 Hz).

On the first day, a mouse was placed in the centre of the conditioning chamber. After 5 min of free exploration, a series of three light shock pairings was delivered, once every 100 s, with the shock always presented during the last 2 s of light presentation. Twenty-four hours later, the mice were exposed to the test box. After 5 min of exploration, the flashing light was presented for a period of 5 min, but no shock was delivered. The distance travelled and the resting time were measured, the latter being defined as the cumulative time during which an animal did not change its position within the sample time of 250 ms. Minute-by-minute analysis of the first 5 min of exploration on day 2 revealed that the animals reached stable baseline in terms of the distance travelled and the time not moving from the third minute onwards. Conditioned suppression of activity, i.e. the distance travelled and the time not moving, were taken as indicators for fear conditioning. The mean activities during the last 3 min prior to cue re-exposure were taken as baselines to calculate relative activities. Automated measurement of the conditioned suppression of ongoing spontaneous behaviour has been shown to be a reliable measure of fear conditioning [12,25], and earlier studies have shown a high correlation between freezing scored manually and automated measurement of locomotor activity in mice ([31], Steckler et al., unpublished results) and rats [4].

2.9. Hot plate

Pain threshold was investigated in the hot plate test. Here, an animal was placed on a square plate (27.5 × 22.5 cm), heated to 55°C. The latency to feet licking or jumping, whatever happened first, was scored.

2.10. Water intake

Twenty-four hour water intake was measured in the home cage.

2.11. Inescapable swim stress

A clear plastic cylinder (11 cm in diameter, 25 cm high) was filled to a depth of 7.5 cm with water (25°C).
Animals were placed in the water for 6 min. Behaviour was video recorded and immobility time during the last 3 min of testing was scored.

At least 5 days lapsed between tests.

2.12. Statistical analysis

Data were analysed by analysis of variance (ANOVA), followed by post hoc Tukey test, if appropriate.

3. Results

3.1. Examination of spontaneous behaviour

Locomotor activity in the home cage, as measured by the number of line crossings, was significantly different between strains ($F_{1,38} = 67.05, P < 0.001$; Fig. 1A). Post hoc analysis showed the following rank order, SW/J > B6/J = B6/ChR > A/J. Furthermore, locomotor activity decreased over time in all the strains ($F_{1,38} = 334.65, P < 0.001$). Analysis of the rearing activity also revealed an effect of strain ($F_{3,38} = 86.66, P < 0.001$; Fig. 1B), with the SW/J mice spending more time rearing than the other three groups, and the B6/ChR mice rearing more than mice of the A/J strain. Strains also differed in the time spent grooming ($F_{3,38} = 14.80, P < 0.001$; Fig. 1C), whereby A/J mice groomed significantly more than animals from the other strains. Finally, there was an effect of strain on the time spent digging in the sawdust ($F_{3,38} = 22.01, P < 0.001$; Fig. 1D), with the following rank order, B6/J > B6/ChR > SW/J = A/J.

3.2. Elevated plus maze

The time spent on the central platform was significantly different between strains ($F_{3,36} = 11.62, P < 0.001$), with the A/J strain spending more time on the central platform compared with all the other groups (Fig. 2A). A strain difference was also seen in terms of the total arm entries ($F_{3,36} = 45.07, P < 0.001$), and post hoc comparison indicating that the SW/J mice were more active than all the other strains (Fig. 2B). Latency to enter open arm also differed between strains ($F_{3,36} = 3.46, P = 0.026$). Post hoc analysis showed that A/J mice had longer latencies than B6/J animals (Fig. 2C). Moreover, strains differed in the total time spent on the open arms ($F_{3,36} = 8.74, P < 0.001$), and post hoc comparison indicating that the SW/J mice spent significantly more time there than the SW/J or the A/J strains (Fig. 2D). When the relative number of entries into the open arms was analysed, ANOVA revealed a further effect of strain ($F_{3,35} = 3.49, P = 0.026$; Fig. 2E), but post hoc testing failed to show significant group differences.

3.3. Light–dark exploration

There was a strain effect in terms of the total distance travelled ($F_{3,38} = 22.81, P < 0.001$). A/J animals were less active than all the other strains (Fig. 3A). The total number of rearings was also significantly different
**Elevated Plus Maze**

![Graphs showing exploration data](image)

Fig. 2. Elevated plus maze exploration: the time spent on the central platform (A), total arm entries (B), the latency to enter an open arm (C), the time spent on the open arms (D) and the relative number of open arm entries (E) are shown. Data are presented as means, with error bars denoting S.E.M. *P* < 0.05; **P** < 0.01; ***P** < 0.001, significant differences between strains.

between strains (*F*<sub>3,38</sub> = 4.61, *P* = 0.008), and the A/J mice reared less frequently than the two B6 strains (Fig. 3B). Furthermore, strains differed in the number of entries into the light (*F*<sub>3,38</sub> = 2.89, *P* < 0.048), which were increased in the B6/J animals in comparison with the B6/ChR mice (Fig. 3C), in terms of moving time in the light (*F*<sub>3,38</sub> = 3.83, *P* = 0.017; Fig. 3D), in the relative distance travelled (*F*<sub>3,38</sub> = 5.44, *P* = 0.003; Fig. 3E), and in the relative number of rearings made in the illuminated part of the box (*F*<sub>3,38</sub> = 6.35, *P* < 0.001; Fig. 3F). Post hoc analysis revealed that A/J animals made relatively more rearings and travelled a relatively greater distance in the illuminated half of the box than both the B6/ChR and SW/J mice, and spent more time moving in the illuminated part than B6/ChR animals.

### 3.4. Novel object exploration

A significant effect of time-bin was found on the distance travelled measure during the 30 min habituation stage (*F*<sub>9,342</sub> = 4.22, *P* < 0.001; Fig. 4). Furthermore, an overall significant strain difference (*F*<sub>3,38</sub> = 26.85, *P* < 0.001), but no strain × bin interaction (*F*<sub>27,342</sub> = 1.29, *P* > 0.05) was seen for this measure. Post hoc analysis indicated that the A/J mice were hypoactive compared with all the other strains, and that the SW/J animals showed significant higher locomotor activity than the B6/ChR mice. No preference for a particular quadrant of the field was observed during the habituation stage according to the time spent in the different quadrants (*F*<sub>3,152</sub> = 2.60, *P* > 0.05).
However, after introduction of the novel object, strains differed in the total time spent in the object quadrant \((F_{3,38} = 6.48, P = 0.001)\), and a significant genotype × bin interaction was observed \((F_{12,152} = 2.56, P = 0.010)\). This interaction was due to the fact that the SW/J mice spent more time in the object quadrant as compared with all other groups during the first 3 min of exposure to the object (Fig. 5A), and continued to differ significantly during the second 3-min bin from both the B6/J and A/J strains (data not shown). A strain effect was also seen if the time spent in the object quadrant relative to baseline was calculated \((F_{3,38} = 3.21, P = 0.034)\), and a significant strain × bin interaction was found for this measure \((F_{12,152} = 2.25, P = 0.012)\). As for the quadrant preference measure, SW/J mice spent more time in the object quadrant than all the other groups, if the relative change from the baseline was considered (Fig. 5B). In addition, analysis of the change from baseline also revealed that B6/ChR animals spent more time in the object quadrant than A/J mice during the first 3 min of object exposure. Strains did not differ during subsequent bins on this measure. Visual inspection of the data indicated that the strains reacted very differently to the introduction of a novel object, varying from avoidance, most pronounced in the A/J strain, to approach of the object quadrant, which was strongest in the SW/J strain, while the two B6 strains seemed to react rather indifferently (Fig. 5B).

Analysis of the number of entries into the object quadrant relative to baseline also showed an effect of strain \((F_{3,38} = 4.17, P = 0.012)\), and a strain × bin interaction was observed \((F_{12,152} = 2.24, P = 0.022)\). As before, A/J mice showed avoidance of the object during the initial 3 min, while a relative increase of entries into the object quadrant was seen in the B6/ChR strain, and both the B6/ChR and the SW/J strains differed from A/J mice during the first two time bins (Fig. 5C).

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**Light-Dark Box**

Fig. 3. Light–dark box exploration: the total distance travelled over 5 min (A), the total number of rearings; (B), the number of entries into the illuminated part of the box; (C), the time the animal spent moving in the illuminated part; (D), the relative distance travelled in the light; (E) and the relative number of rearings made in the illuminated area (F) are shown. Data are presented as means, with error bars denoting S.E.M. *\(P < 0.05\); **\(P < 0.001\), significant differences between strains.
3.5. Vogel conflict task

There was an effect of strain on the number of shocks delivered during the conflict session ($F_{1,28} = 3.50, P = 0.028$; Fig. 6A). SW/J mice received significantly more shocks than A/J mice. No significant difference was found for the latency to start the session measure ($F_{1,28} = 0.95, P = 0.432$; Fig. 6B).

3.6. Hole board

Strains differed in the distance travelled on the 16-hole board ($F_{3,41} = 31.90, P < 0.001$). Again, the A/J animals were hypoactive compared with the other three strains (Fig. 7A). Likewise, an effect of strain was found in the number of rearings ($F_{3,41} = 10.15, P < 0.001$), with the A/J strain being the least active (Fig. 7B). Analysis of the total number of nose pokes also revealed an effect of strain ($F_{3,41} = 16.26, P < 0.001$), with SW/J mice visiting more holes than the other strains. A/J mice did not differ from the two B6 strains on this measure (Fig. 7C).

3.7. Conditioned fear

When the relative time not moving during re-exposure to the light stimulus (conditioned stimulus; CS) was analysed, ANOVA showed a strain x minute interaction ($F_{12,132} = 4.89, P < 0.001$). Further analysis revealed that all the strains displayed a comparable rise in the time not moving following presentation to the CS (approximately 25% increase from baseline; Fig. 8). However, the strains showed different degrees of extinction to the stimulus. Thus, activity in the two B6 strains started to return to baseline after the first minute, while extinction was retarded in the SW/J strain compared with both the B6 strains. Although SW/J mice again reached baseline at the end of the session, they spent significantly less time moving than the B6/J mice during the 2nd, 3rd and 4th min, and also differed from the B6/ChrR mice during the 3rd min. In contrast, A/J mice did not return to baseline and differed from the two B6 strains from the 3rd min onwards. No significant differ
Vogel Conflict Task

Fig. 6. Vogel conflict: number of shocks received (A) and latency to start the session (B). Data are presented as means, with error bars denoting S.E.M. *P < 0.05, significant difference between strainings.

ences were found between the two B6 strains or between A/J and SW/J strains.

Analysis of the change in the distance travelled relative to baseline also revealed a strain × time interaction (F_{12,132} = 6.35, P < 0.001). Post hoc testing showed a pattern similar to the one described for the time not moving measure (data not shown).

3.8. Hot plate

During the hot plate test, no jumping was observed. Latencies to lick the feet were significantly different between the strains (F_{3,38} = 10.61, P < 0.001; Table 1), and a lower pain threshold was seen in SW/J mice in comparison with the other strains.

3.9. Water intake

Twenty-four hour water intake was also strain-dependent (F_{3,38} = 8.95, P < 0.001). Post hoc analysis indicated that SW/J animals ingested more water compared with the mice from the other strains (Table 1).

3.10. Inescapable swim stress

An effect of strain was also seen for immobility time (F_{3,37} = 7.90, P < 0.001), and SW/J mice spent more time immobile than B6/Chr and A/J animals. Furthermore, the immobility time was increased in B6/J mice relative to immobility in the A/J strain (Fig. 9).

Fig. 7. Hole board exploration: distance travelled (A), number of rearings (B) and number of nose pokes (C) made during a 30 min session are shown. Data are presented as means, with error bars denoting S.E.M. ***P < 0.001, significant difference compared with SW/J; # P < 0.05; ## # P < 0.001, significant difference compared with A/J.
4. Discussion

In this study, strains differed in measures of anxiety-related behaviour, exploratory behaviour, and locomotor activity. In general, SW/J mice were the most active strain in terms of locomotor activity (distance travelled), while locomotor activity was intermediate in the two B6 strains. A/J mice were the least active. A comparable rank order was found when the numbers of rearings were compared in the different paradigms. Although clear strain-dependent effects were also seen in measures of anxiety-related behaviour, the rank order of strain effects was more variable and task-dependent, which suggests that the different tasks may measure different aspects of anxiety-related behaviour independent of differences on locomotor activity.

4.1. Behavioural considerations

Conceptually, the paradigms employed in the present study can be grouped into different categories, such as tasks primarily based on free exploration of novel environments (elevated plus maze, light–dark box, hole board), free exploration of novelty, but based within a familiar surrounding (novel object exploration), conflict paradigms (Vogel conflict) and tasks involving a strong mnemonic component (fear conditioning).

The elevated plus maze has been suggested to be based on the natural aversion of rodents for heights and open spaces [28], and can be considered a standard paradigm for testing anxiogenic- and anxiolytic-like responses in mice [24]. The main anxiogenic factor suggested to underlie explorative behaviour in the light–dark box is the aversive nature of an open, brightly illuminated area [7–9]. Explorative anxiety paradigms like these have been suggested to measure state anxiety [2,3,23]. In contrast, in the novel object exploration task, mice are first habituated to an environment and subsequently exposed to novelty [27]. Exploration of novelty from an area known to be safe has been suggested to fundamentally differ from the exploration of a totally new environment and has been claimed to reflect trait rather than state anxiety [20].

The hole board, on the other hand, has been suggested to allow investigation of exploratory behaviour independent of locomotor activity in rats [15] and mice [32]. In mice, it has been shown that the number of hole visits decreases following restrained stress and is inverted by treatment with benzodiazepine and non-benzodiazepine anxiolitics, independent of locomotor activity [34]. A similar dissociation between locomotor activity and the number of hole visits was observed in the present study: SW/J mice visited more holes than mice from the other strains, but did not differ from the B6 strains in terms of locomotor or rearing activities. A/J mice were hypactive in terms of the distance
travelled and number of rearings measured, but did not differ from the B6 animals in terms of the number of hole visits. Thus, the distance travelled and rearings may give an indication of the general activity, while hole visits may be more indicative of exploratory behaviour.

When behavioural responses in the home cage were analysed, grooming was most extensive in the A/J strain. Grooming behaviour has been shown to increase after stress exposure in a range of paradigms [14,38]. Clearly, removing the animal from its home cage and manipulation of the bedding already reflects a mild stressor and it can be argued that the increased grooming activity in A/J mice reflects a form of displacement behaviour. However, the same arguments may account for the increase in sawdust digging, seen in the B6 strains, as this behavioural response increased in B6/ChR mice following social defeat (Van Gaalen and Steckler, unpublished results). Whether these types of behaviour really reflect anxiety-like behaviour in terms of displacement reactions needs further investigation.

4.2. Strain effects on anxiety-related behaviour

Differences between the two B6 sublines were absent with two exceptions: B6/J mice spent more time burying in the home cage and made more entries into the illuminated part of the light–dark box than the B6/ChR mice, but did not differ significantly from each other in any other measure. This suggests that both sublines display comparable levels of anxiety-related behaviour.

B6 mice of both lines were the least anxious on the elevated plus maze. No differences were found between the SW/J and the A/J mice in terms of open arm exploration. The A/J mice, however, spent more time on the central platform than the other three strains. These results are comparable with those reported by others comparing B6/J and A/J mice during this task [36]. The two B6 strains also displayed relatively low levels of anxiety-related behaviour in the fear-conditioning paradigm, while A/J mice showed strongest responses. Likewise, A/J mice spent more time grooming in the home cage, spent less time in the object quadrant than the other strains during the exploration of a novel object — in fact seemed to avoid this quadrant — and had the lowest number of licks in the Vogel conflict paradigm. These results support earlier findings, wherein A/J mice display higher level of anxiety-related behaviour when compared with other mouse strains [5,10,11]. Although it could be argued that A/J mice showed reduced anxiety-related behaviour in the light–dark box in terms of the relative activity in the illuminated compartment, which would contrast earlier reports of enhanced anxiety-related behaviour of A/J mice on this task [11], it should be noted that locomotor activity of A/J mice was very low in the present study. Thus, starting the mice in the illuminated part of the box may have skewed the data towards a ‘preference’ for the illuminated area.

According to the time spent in the object quadrant during novel object exploration, a rank order of SW/J < B6/ChR = B6/J < A/J was found. A similar rank order was seen for the number of licks in the Vogel paradigm. Since responses in the Vogel conflict and conditioned fear paradigms are potentially confounded by differences in the pain threshold, nociception was tested on the hot plate. These results suggest that the SW/J mice have a reduced pain threshold compared with the other strains. This in turn indicates that the increase in the time not moving in the conditioned fear paradigm seen in SW/J mice could be confounded by altered nociception. However, this explanation could not account for the performance in the Vogel conflict task, where the SW/J mice received the highest number of shocks. Motivational factors, however, could have affected the number of licks in SW/J mice in this task, as enhanced water intake was seen in this strain. Thus, the two tasks based on punishment would not allow firm conclusions about anxiety levels of the SW/J strain. However, SW/J showed higher explorative activity on the hole board, while general activity did not differ from that of B6 mice, which would again suggest that SW/J mice are less anxious than B6 animals.

Interestingly, the analysis of immobility time in the swimming paradigm also showed a rank order of SW/J > B6 > A/J. Immobility is unlikely to simply reflect locomotor activity, as a reversed rank order would have been expected. Immobility time has been suggested to be a useful predictor for antidepressant activity [30]. However, many antidepressant drugs are also potent anxiolytic substances [38], and immobility has also been reported to increase after treatment with benzodiazepines such as diazepam or flurazepam at doses which were not muscle relaxant [29]. Moreover, struggling in the swimming paradigm has been reported to be negatively correlated with anxiety-related behaviour [16]. Thus, it is possible that immobility time in this task also depends on the degree of anxiety-related behaviour.

One of the important findings of the present study was that the rank-orders in anxiety-related behaviour were task dependent, which might indicate that different aspects of anxiety can be taxed by different anxiety paradigms. A differentiation in trait and state anxiety has been suggested [20,23]. However, we found a different rank-order in the elevated plus maze compared with the light–dark box, and a lack of correlation between anxiety-related behaviour investigated in the open field and elevated plus maze has been reported by others [21]. Thus, a dissociation into trait and state anxiety cannot easily accommodate the present data.
However, this classification is not suitable to categorise paradigms such as conditioned fear and conflict tasks. Therefore, it might be necessary to extend this concept.

4.3. Neural considerations

Our results agree with a recent study by Brodkin et al. [5], showing that the latency to transverse from the familiar to the novel site of a place preference box was increased in A/J mice relative to the latency of B6/J animals. Moreover, these authors showed that immunoreactivities for the dopamine transporter in the nucleus accumbens, and for the transcription factor ΔFosB in dorsal striatum were lower in A/J mice than in the animals from the B6/J strain. Late afternoon plasma corticosterone levels, on the other hand, were higher in A/J than in B6/J mice, suggesting higher activity of the hypothalamic-pituitary-adrenal (HPA) axis in A/J mice. However, shock induced plasma corticosterone concentrations in B6/J mice have been reported to exceed those of A/J animals [33]. Although no correlations were found between these biochemical measures and levels of exploratory activity in the study by Brodkin and colleagues [5], it is tempting to speculate that these differences could form part of the neurochemical basis for the behavioural differences observed in the present study. In this respect, it is interesting to note that grooming, which was most pronounced by the A/J mice, has not only been shown to increase after stressors, but can be induced by stimulation of the paraventricular nucleus of the hypothalamus, which subsequently leads to HPA axis activation [14,39].

In summary, our data confirm earlier studies suggesting that A/J mice are a relatively anxious strain. Here, we extend these findings by showing these effects in several tasks that are based on different theoretical principles. Although our results also support the notion that different anxiety task may measure different aspects of anxiety, the data do not support current classifications of anxiety-related behaviour. It is also pertinent to point out that a battery of tests should be used for the measurement of anxiety rather than a limited number of tests, as frequently done in studies with mouse mutants.

References


Deficit in Attachment Behavior in Mice Lacking the \( \mu \)-Opioid Receptor Gene

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Endogenous opioid binding to \( \mu \) receptors is hypothesized to mediate natural rewards and has been proposed to be the basis of infant attachment behavior. Here, we report that \( \mu \)-opioid receptor knockout mouse pups emit fewer ultrasonic vocalizations when removed from their mothers but not when exposed to cold or male mice odors. Moreover, these knockout pups do not show a preference toward their mothers’ cues and do not show ultrasonic calls potentiation after brief maternal exposure. Results from this study may indicate a molecular mechanism for diseases characterized by deficits in attachment behavior, such as autism or reactive attachment disorder.

The opioid system controls nociceptive and addictive behaviors, with a prominent role of \( \mu \)-opioid receptors (\( Orpm^{\text{−/−}} \)) in these responses. Mice lacking the \( \mu \) receptor gene (\( Orpm^{\text{−/−}} \)) show a loss of morphine-induced analgesia, reward, and dependence (1); increased sensitivity to painful stimuli (2, 3); reduced reward to nonopioid drugs of abuse (4); and altered emotional responses (3). Endogenous opioid binding to \( \mu \) receptors has been considered one of the neural mediators of infant attachment behavior (6–10), although the role of \( \mu \) receptors in mediating the rewarding properties of mother-related stimuli has not been determined (11, 12).

Attachment behavior entails the display of affiliative behaviors and the establishment of a special bond with the animal’s

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caregivers (13). It is characterized by the selective approach to, and interaction with, specific individuals and by the display of emotional distress during acute periods of separation from these individuals. This behavioral system has been suggested to represent the developmental roots of the affiliative system emerging during the adult life and to modulate behaviors such as social play and sexual and parental behavior (12).

Scott (14) was the first to use the distress vocalizations as a model to study attachment behavior in puppies. In mouse pups, the response to separation from the mother consists of a protest phase characterized by a high rate of ultrasonic vocalization (UV) and hyperactivity, which facilitates reunion with her. UVs have been used as an index of distress in several experimental paradigms (15) and are strongly reduced in pups given μ-opioid agonist agents (16–18). However, the inconsistency in the results obtained with antagonist administration (19, 20) has questioned the role of μ-opioid receptors in the pup’s vocal response to social separation.

Orpm<sup>−/−</sup> pups emitted fewer calls than their wild-type controls (Orpm<sup>+/+</sup>) in response to isolation at various developmental time points (21) (Fig. 1). Moreover, morphine administration significantly reduced UVs in 8-day-old wild-type pups, whereas this drug had no effect on Orpm<sup>−/−</sup> calls (21). The lower UV performance of Orpm<sup>−/−</sup> pups was not associated with evident differences in maternal responsiveness toward the offspring. In fact, mothers of the two genotypes did not differ in their behavior toward pups in response to either nest relocation (Table 1) or to a test already validated for maternal emotionality and motivation (Table 2) (21). The absence of detectable differences in maternal responsiveness toward pups suggests that the fewer distress calls emitted by Orpm<sup>−/−</sup> pups are the result of a reduced sensitivity to isolation. We thus performed a second experiment to measure the UV response of isolated pups to stressful physical (cold) and social (clean and novel male bedding compared to nest odor bedding) stimuli (Fig. 2). Wild-type pups responded to isolation and exposure to clean bedding with a high frequency of ultrasonic calls; this effect was significantly reduced in the presence of familiar bedding. In contrast, isolated Orpm<sup>−/−</sup> pups showed very low amounts of UVs when isolated and exposed to clean bedding and no further reduction in the amount of calls emitted in the presence of familiar bedding. In fact the clean bedding condition was not sufficient to elicit the distress behavioral response in Orpm<sup>−/−</sup> pups. However, pups of both genotypes showed a similar UV response when exposed to stressful stimuli such as cold or strange male odors, ruling out the possibility that the mutant animals have a general impairment in their capacity to emit high levels of ultrasonic calls or in their sensitivity to olfactory and thermal stimuli.

These results suggest that the absence of mother and nest odor does not elicit a distress vocal response in Orpm<sup>−/−</sup> pups. Opioids have a facilitatory role in odor preference acquisition and nipple-milk conditioning (22). Orpm<sup>−/−</sup> pups may thus lack the normal activation of the endogenous opioid system, mediating the rewarding values of maternal stimuli that facilitate infant behavior toward the mother. This hypothesis was further investigated by assessing the maternal potentiation of UV (23). Maternal potentiation of UVs is suggested to have its basis in a learning process whereby pups associate their calling with positive maternal-related reinforcements. The removal of the mother, after a brief exposure, causes a violation of reward expectancy and an increase in ultrasonic calls. Wild-type pups showed the expected increase in UV after a short exposure to the mother, whereas mutant pups did not show a maternal potentiation effect (Fig. 3). The absence of maternal potentiation of UV in Orpm<sup>−/−</sup> pups supports the role of μ receptors in mediation of the positive affective state evoked by maternal cues.

Pup attachment to the mother, providing warmth, milk, and protection, has its basis primarily in olfactory cues. Therefore, we performed a second experiment to measure the number of ultrasonic calls of Orpm<sup>−/−</sup> (<i>n</i> = 17) and Orpm<sup>+/+</sup> (<i>n</i> = 16) isolated pups tested during a 5-min session in an empty beaker at room temperature. Pups’ ultrasound sounds were measured on postnatal days (PNDs) 4, 8, and 12. Four liters per genotype were tested, and litter size ranged between three and six in both lines. The two-way ANOVA for repeated measures indicated significant main effects [for genotype, <i>F</i>(1/62) = 5.08 and <i>P</i> < 0.05; for day, <i>F</i>(2/62) = 8.34 and <i>P</i> < 0.001] and no significant interaction effect.
evaluated the olfactory acuity and preference of Orpm<sup>+/+</sup> and Orpm<sup>−/−</sup> pups. We found no difference between the abilities of 8-day-old pups of the two genotypes to discriminate between their mother and nest and clean bedding. In contrast, when the pups had to choose between their own and an unfamiliar mother and nest bedding, only 36% of Orpm<sup>+/+</sup> compared with 100% of Orpm<sup>−/−</sup> pups chose the familiar bedding. In addition, the percentage of time spent in the familiar bedding was lower in the mutant pups (Fig. 4).

These results indicate that mice lacking μ-opioid receptors show deficits in two independent measures of attachment behaviors. Orpm<sup>+/+</sup> pups were not able to selectively approach their mothers, and maternal deprivation was not sufficient to elicit distress calls. Infant vocal behavior has been suggested to reflect anxiety in pups (24). However, lower fearfulness during maternal separation alone cannot account for the deficit in attachment behavior displayed by these knockout pups. Pups of both genotypes showed the same amount of UV when exposed to cold or male odor cues. Most interestingly, Orpm<sup>−/−</sup> pups seemed to be specifically less sensitive to the absence of maternal cues. This could be because of the absence of association between maternal stimuli and pleasurable states mediated by the μ-opioid system.

The mesocorticolimbic dopamine reward system has been implicated in social attachment whereby oxytocin and vasopressin peptides play a critical role in the association between the social stimuli and the brain reward circuits (25). Our data support the hypothesis that the μ-opioid system is a crucial component of the social reinforcement pathways modulating the positive affective states associated with mother stimuli. Panksepp and colleagues (7, 16, 26) elaborated a model in which the social contact provides comforting effects, in part opioid-mediated, and proposed that social separation determines a distress analogous to opiate withdrawal. Orpm<sup>−/−</sup> pups would thus not experience either state because of the absence of the receptors mediating the response to social isolation or social comfort. Pharmacological activation of μ-opioid receptors also decreased UVs (16–18), suggesting that exogenous opioids mimic the positive state associated with mother’s presence, thus reducing the UV response to isolation. We hypothesize that the deficit in the μ-opioid systems of our knockout mice annihilate the natural association between reward and maternal stimuli, making these animals less sensitive to maternal separation.

Alterations of the attachment system have been considered as etiological factors for several psychiatric syndromes. Our data show that μ receptors are critical players in attachment disorders, supporting the classical hypothesis of Panksepp and co-workers (27, 28) that a malfunctioning of the endogenous opioids system may be implicated in the social indifference displayed by autistic infants. The present data also highlight mice lacking μ-opioid receptors as a useful animal model to evaluate the consequences of deficits in the affiliative system during development and adulthood.

**References and Notes**

21. Information on materials and methods is available on Science Online.
26. Because of restricted space it was not possible to...
Robust Temporal Coding in the Trigeminal System

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The ability of rats to use their whiskers for fine tactile discrimination rivals that of humans using their fingertips. Rats perform discriminations rapidly and accurately while palpating the environment with their whiskers. This suggests that whisker deflections produce a robust and reliable neural code. Whisker primary afferents respond with highly reproducible temporal spike patterns to transient stimuli. Here we show that, with the use of a linear kernel, any of these reproducible response trains recorded from an individual neuron can reliably predict complex whisker deflections. These predictions are significantly improved by integrating responses from neurons with opposite angular preferences.

In many sensory systems (1–3), including the rodent whisker-trigeminal system (4, 5), complex stimuli elicit sparse spike trains in individual neurons. Surprisingly, a small number of spikes may be sufficient for rapid sensory discrimination (6). This suggests that the nervous system extracts sufficient information from sparse spike trains to accurately encode complex stimuli (7, 8). To test this hypothesis, it is essential to reveal coding strategies used by first-order neurons in the sensory system, because these will constrain all subsequent processing and coding strategies. In the rodent whisker pathway, the first-order neurons are in the trigeminal ganglion.

To mimic whisker contacts during tactile discrimination, we deflected individual whiskers with a 2-s-long white noise waveform with frequencies from 10 to 125 Hz (Fig. 1A, middle column). We recorded well-isolated extracellular spikes from individual trigeminal ganglion neurons in response to 50 presentations of this stimulus. Data were obtained from four adult female rats, anesthetized with Nembutal and prepared for recordings as previously described (9). Because trigeminal neurons display a strong angular preference (9, 10), we applied the stimuli in each neuron’s preferred direction. Figure 1B shows responses of an individual neuron. Most spikes occur at precisely the same time in every trial (11). These highly reproducible firing patterns suggest that a single spike train may contain sufficient information to encode the stimulus. We thus attempted to predict the stimulus from the recorded responses. We computed a linear kernel for stimulus-response pairs recorded during the first half of the stimulus. The kernel (K) identifies which features of the stimulus are present before each spike:

$$K = \frac{CSD_{\text{spikes, stimulus}}}{PSD_{\text{stimulus}}}$$

where CSD spk,stimulus is the cross-spectral density between the spike trains and the stimulus and PSD stim is the power spectral density of the stimulus. We then convolved this kernel with each spike train recorded during the second half of the stimulus to derive the prediction. We estimated the accuracy of this prediction by computing the cross-correlation coefficient between the actual and the predicted stimuli (R predict).

We illustrated this process for one neuron in Fig. 1C (middle column). The original stimulus features (black trace) are well captured by the predicted stimulus (red). The prediction does not fully capture the peak amplitude of stimuli applied in the downward direction, because these deflections are in the cell’s nonpreferred direction and produce no consistent spikes. Nevertheless, the predicted stimulus was highly correlated with the original stimulus. For the cell in Fig. 1, the prediction obtained by applying the kernel to a single spike train had a correlation coefficient, R predict, of 0.77 (125-Hz position). Predictions computed from each of the other spike trains recorded from this cell were also highly correlated with the original stimulus (mean ± SD = 0.75 ± 0.01 and range of 0.73 to 0.77). We performed similar predictions from 15 additional neurons. For each of these cells, stimulus predictions were significantly correlated with the original stimulus (group mean R predict = 0.66 ± 0.10 and an individual cell range of 0.45 ± 0.03 to 0.79 ± 0.02). We also computed, for each neuron, the coefficient of variation (CV) of R predict for each of the 50 trials: The CV was <10%, indicating that a spike train from any individual trial provides an equally accurate prediction of the stimulus.

Trigeminal ganglion neurons respond more robustly to whisker deflections at high velocity or acceleration (10, 12). We therefore asked whether encoding of velocity or acceleration is more accurate than that of whisker position. Fig. 1C and E, shows predictions for stimulus velocity and acceleration computed from the same spike train depicted in Fig. 1D. Both velocity and acceleration provided significantly better predictions than position [position R predict = 0.75 ± 0.01, velocity R predict = 0.85 ± 0.01, and acceleration R predict = 0.86 ± 0.01; analysis of variation (ANOVA) with Tukey’s honestly significant difference (HSD), P < 10^-5]. In 15 of 16 neurons, velocity and acceleration produced significantly better predictions than did position (P values < 0.01). Similarly, as a group, predictions of stimulus velocity (R predict = 0.76 ± 0.12) and acceleration (0.77 ± 0.13) were significantly more accurate than position (0.66 ± 0.10, Kruskal-Wallis, P = 0.004). This indicates that the neural code best captures abrupt changes in whisker trajectories. Such changes occur when a whisker contacts an object or when it encounters changes in an object’s shape or texture. Neurons upstream in the whisker-to-barrel pathway also respond more robustly to high-velocity whisker deflections (13–15). Thus, whisker-based tactile discrimination appears to use a coding strategy similar to that of the visual system, in which neurons are sensitive to changes in contrast (16).

We therefore compared predictions of stimuli with different velocity content: white noise bandpass filtered at 10 to 25 Hz (25-Hz stimulus, velocity ≤ 1 μm/s, and acceleration ≤ 0.01 μm/s^2), 10 to 625 Hz (625-Hz stimulus, velocity ≤ 20 μm/s, and acceleration ≤ 3 μm/s^2), and 10 to 125 Hz (125-Hz stimulus, velocity ≤ 5 μm/s, and acceleration ≤ 0.2 μm/s^2). We obtained...
Hair barbering in mice: Implications for neurobehavioural research

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Abstract

Barbering (fur/whisker trimming, the Dalila effect) is a behaviour-associated hair and whisker loss frequently seen in laboratory rodents, including mice. Here we analyse barbering behaviour in 129S1, NMRI, C57BL/6 and BALB/c mouse strains and some of their F1 hybrids. Our study shows that barbering in mice, depending on their genotype, is a complex behaviour with several distinct contexts or domains. We observed social (dominant) barbering in NMRI and C57BL/6 mice, sexual over-grooming in 129S1 and C57BL/6 mice, maternal barbering in lactating 129S1 and C57BL/6 mice, and stress-evoked barbering in F1 (NMRI × 129S1) hybrids. In contrast, aggressive BALB/c mice and their F1 progeny do not use barbering in their behaviour. We suggest that barbering may be an important complex multi-domain behaviour sensitive to various manipulations, and represent a useful index in neurobehavioural research.

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1. Introduction

Behaviour-associated hair loss has been observed in many species including dogs, cats, horses, cattle and non-human primates (Ehrenlechner and Unshelm, 1997; McElwee et al., 1999). Described in the literature as barbering, overgrooming, whisker/hair or fur trimming, nibbling, eating (trichophagia), plucking, pulling, de-whiskering or the Dalila effect (Jackson Laboratory, 1987; Carruthers et al., 1998; Sarna et al., 2000), it has long been observed in laboratory rodents. Barbering includes plucking of fur or whiskers from cage-mates (hetero-barbering) or oneself (self-barbering), and is common in mice (Long, 1972; Strozik and Festing, 1981; Sarna et al., 2000), rats (Beare-Rogers and McGowan, 1973; Sarna et al., 2000), and guinea pigs (Gerold et al., 1997).

Mounting data indicate that rodent barbering may be a form of dominant behaviour (Reinhardt and Militzer, 1979) and a strong indicator of social hierarchy (Long, 1972). In groups of mice and rats, there was usually one with unbarbered whiskers, playing a dominant role in the cage (Strozik and Festing, 1981; Bresnahan et al., 1983; Sarna et al., 2000). Whisker removal itself did not alter social dominance in male mice (Van de Weerd et al., 1992), suggesting that dominance determines barbering and not the otherwise. In addition, barbering occurred even if the mice were separated by wire mesh, indicating that both animals, either actively or passively, co-operate in this behaviour (Van den Broek et al., 1993).

In mice, barbering is particularly common in some strains, especially C57BL/6 (B6) and A2G (Long, 1972; Strozik and Festing, 1981; Jackson Laboratory, 1987; Sarna et al., 2000), suggesting a strong genetic component (Hauchka, 1952; Militzer and Wecker, 1986; Van den Broek et al., 1993; McElwee et al., 1999). It may also be socially transmitted, e.g., induced in a non-barbering group after introducing a barber (Reinhardt and Militzer, 1979). Several husbandry factors have also been reported to affect barbering (e.g., diet, weanling age and enrichment; Myers, 1997; De Luca, 1997), suggesting that barbering may represent stress-evoked behavioural response (e.g., coping with inappropriate housing; Van den Broek et al., 1993), or a pathological behaviour similar to human compulsive hair pulling (Garner et al., 2004a,b).

Why the Dalila effect is so important for neurobehavioural research? First, barbering is an interesting behaviour per se, representing as essential part of rodent waking activity (Sarna et al., 2000; Whishaw et al., 2001). Second, in most cases it affects whiskers, regarded as a crucial source of sensory input.
in rodents (Ahl, 1986; Staiger et al., 2000). Whisking represents an essential part of rodent behavioural repertoire (Prigg et al., 2002), as rodents actively use whiskers to locate and distinguish objects in their immediate environments, for texture discrimination, balance control, orienting and exploration (Vincent, 1912; Meyer and Meyer, 1992; Belzung, 1999; Prigg et al., 2002; Prchal et al., 2004). Therefore, altered whisker status in mice due to barbering may disorganize animal behaviours, also impairing their performance in various behavioural tests (Crawley, 1999).

Given the importance of behavioural phenotyping of various mice (Crawley et al., 1997; Greer and Capecchi, 2003), including mutants with abnormal barbering (Wang et al., 2001; Holmes et al., 2002a; Long et al., 2004), further studies are necessary to understand in detail the nature and etiology of barbering. The present study sought to extend the available literature on the Dalila effect by presenting a detailed systematic ethological analysis of barbering in several mouse strains widely used in behavioural neuroscience (Crawley and Paylor, 1997; MGI, 2001; MPD, 2001).

2. Materials and methods

The mouse colony consisted of approximately 560 male and female mice (2.5–4 months old) of different strains, including 129S1 (S1), NMRI, BALB/c (BC), B6 and their F1 inter-crosses (Table 3). All mice were bred in the University of Tampere (Finland) and maintained in a standard virus/parasite-free facility, exposed to a 12-h light:12-h dark cycle. Lights were turned off at 18.00 h and on at 6.00 h. Animals were experimentally naïve and housed in the groups of 2–9 (depending on the strain). All mice were weaned at 21 days of age, and housed in clear plastic cages (425 mm × 185 mm, Scanbur, Sweden) on aspen chips bedding (4 mm × 4 mm × 1 mm, Tapani Oy, Finland), with food and water freely available.

Hair loss was recorded by a highly experienced observer (intra-rater reliability ≥0.9) using a custom-made register. Each mouse was visually inspected on both the dorsal and ventral surfaces (Garner et al., 2004a) for at least 2 min. The following 5-point scale was used in the present study: 0, no barbering; 1, whisker removal or shortening (Figs. 1B right and 2A); 2, snout/face demudling (Fig. 1B middle); 3, individual bald patches on head and body (Fig. 1A middle); 4, multiple alopecic areas on head and/or body (Fig. 1A right); 5, severe alopecia including complete snout demudling and large pronounced alopecic areas on head and body (Fig. 2B). The observer was unaware of the genotype (except in cases when the strain could be easily recognized by coat color or body size, i.e. B6, NMRI). Hair loss was scored as barbering if the hair lesion was non-puritic, there was no scarring or scabbing around the lesion, and the animal was otherwise in good health and the fur (where present) was in good conditions (Garner et al., 2004a). For each individual strain, we analysed the number (%) of cages in which the barbering occurred and the average severity of barbering in each cage. In addition, for same-sex barbering (Experiments 1 and 3, see further) we analysed the percentages of barbers and barbered animals (of total animals of each strain). Barber animals were identified as the single intact mouse in the cage, according to (Sarna et al., 2000; Garner et al., 2004a).

In Experiment 1, we analysed social barbering in same-sex cages in BC, NMRI, B6 and S1 mice (average number of animals per cage: 2.8–4.6) housed socially for approximately 2.5 months since weaning. In Experiment 2, we examined the link between barbering and social rank, observing five cages with highly barbering NMRI strain. Twelve adult male mice (3–4 months old, previously housed individually for 1 month to stimulate intermale aggression) were put together (two to three animals per cage). Five days later (necessary to establish social hierarchy), robust hair loss due to barbering was observed in 100% cages. These cages were observed for 1 h, recording barbering activity and aggressive encounters of each individual mouse (animals were identified by marking their tails with colors). Inter-male aggression was also assessed by analysing scarring on the hind limbs, base of the tail and rear flanks (Garner et al., 2004a). Each mouse was assessed individually for 2–4 min by the same experienced observer, and a score of 0 (intact skin) or a score of 1 (scared/scrubbed skin) were given for each of these areas. Total score was obtained from the sum of the score of each areas. Two weeks later, these mice were re-examined in order to examine altered severity of barbering and scarring in socially stabilized groups.

Experiment 3 studied sexual barbering in breeding groups (Table 2) consisting of one male and one to three females (1–4 for S1 + B6) of B6, S1, BC and NMRI strains (average female/male ratio: 2:2). Preliminary assessment of barbering in these mice was performed during the first 5 days; final observations were made 10 days after mating, as described above. Experiment 4 analysed barbering in same-sex cages (with approximately the same average animal density) of selected F1 hybrid mice (Table 3), housed socially since weaning for approximately 3 months.

In Experiment 5, using an additional mouse colony of approximately 20 adult male mice (University of Tampere, Finland), we assessed same-sex barbering in several other mouse strains, including A/J and F1 B6129SvJ (two to three animals per cage), showing interesting strain-specific patterns of barbering. Essentially the same animal housing and barbering assessment procedures were used for these animals.

In Experiment 6, based on earlier data showing barbering in lactating rats (Harkness, 2001), we assessed possible hair loss in lactating mice. Adult 3–3.5-month-old females of S1 and B6 strains (n=10; five to seven pups per dam) were examined immediately after weaning, as described earlier. Experiment 7 examined the role of self-barbering in the Dalila effect, analysing the occurrence of fur barbering in S1, BC, B6 and NMRI males (3 months old, n=8 in each group) housed individually for 3–4 weeks in small clear plastic cages (267 mm × 207 mm × 140 mm, Scanbur, Sweden) in the same facility.

All animal housing and experimental procedures used in this study were in full compliance with the European legislation on animal experimentation (86/609/EEC) and approved by the Ethical Committee of the University of Tampere (Finland).
Fig. 1. Patterns of hair loss due to barbering in different mouse strains. (A) Sexual barbering by female barbers, left to right: 129S1, NMRI, C57Bl/6 males. (B) Social (dominant) barbering in same-sex cages, left to right: C57BL/6 females (whisker removal and bald patches on head and neck), NMRI females (nasal alopecia and snout denuding; see Fig. 2 for similar pattern in males), A/J males (shortened whiskers and intact fur). (C) Barbering in male mice of F1 hybrid strains, left to right: social stress-evoked barbering in NMRI-129S1 (no single barber, all mice equally affected), dominant barbering in C57BL/6-129S1 (seems to be a mixture of both parental styles, Fig. 2B and C), C57BL/6-129SvJ mice (note severe complete face denuding specific for this strain, as well as multiple alopecic areas on the body).

3. Statistics

Data were analysed using two-way ANOVA (factors: strain, sex, Table 1) for social barbering and one-way ANOVA (factor: groups) for sexual and F1 barbering, followed by a post-hoc Mann–Whitney U-test. Scarring scores for NMRI mice (Experiment 2) and barbering occurrence in F1 NMRI/S1 mice (Experiment 4) were analysed by Mann–Whitney U-test. In all tests, \( P < 0.05 \) was considered statistically significant.

4. Results

Overall, we found strong “social dominance” context of barbering in Experiment 1 (% barbering cages: \( F(2, 35) = 6.04, P < 0.05 \) between genotypes, \( F(1, 35) = 40.2, P < 0.001 \) between sexes, \( F(2, 35) = 0.1, P < 0.001 \) genotype \( \times \) sex (Table 1). Robust dominant barbering was seen in NMRI (80–100% of cages, \( P < 0.05 \), U-test) but not BC (0%) or S1 (0–33%) adult mice socially housed since weaning. Mice of a highly aggressive BC strain (Wood, 2000; Van Loo et al., 2004; Kalueff and Tuohimaa, 2006).
Fig. 2. Social barbering in adult male mice of different strains (dominant barbers indicated by asterisks). (A) NMRI mice. Note intact whiskers and fur in the dominant barber, and denuded snout, lacking whiskers and a bald patch between ears (indicated by arrows) in the recipient mouse. (B) C57Bl/6 mice. Note severe barbering of whiskers, head, neck and body (indicated by arrows) in two recipient animals. Barber is unaffected dominant male in the center. (C) Barbering in two F1 C57Bl/6–129S1 mice. Mouse tails are marked with colors; recipient is in subordinant prone position. Note that in some mice several biting scars (D, enlarged, indicated by arrows) could be seen on the base of their tails, indicating lower social rank. Barber is a bigger dominant male with intact fur and skin.

2005), never displayed barbering in the present study, despite active fighting and numerous scars, especially in male cages. In most cases, barbering targeted whiskers, face, head and body, and in all cages there was a single dominant animal whose fur and whiskers remained fully intact. In addition, we found interesting patterns of social barbering in several other strains, including whisker shortening in socially housed A/J and peculiar snout denuding in F1 B6129SvJ mice (Fig. 1B and C), also performed by a single intact barber (Experiment 5).

In adult NMRI mice housed socially for 5 days (Experiment 2, trial 1), barbering was observed in 100% of cages (Fig. 2A), developing within 2–5 days and including whiskers removal and snout/head denuding (average severity: 2.2 ± 0.3). During this period, active fighting occurred, and social hierarchy was established. Overall, barbers demonstrated significantly lower scarring than their barbered counterparts (average score: 0.2 ± 0.2 versus 1.8 ± 0.3, respectively; P < 0.01, U-test), showing clear correlation of barbering with social rank. Our 1-h homecage observations in these mice also confirmed that barbers were always the dominant animals more frequently engaged in attacking other males (barbers: 12 ± 2, non-barbers: 3 ± 1, P < 0.05, U-test) during the establishment of social hierarchy. Two weeks later (trial 2), pronounced barbering patterns (slightly more severe, average score: 3.1 ± 0.7) was observed in all cages, whereas no biting scars could be found in these mice, significantly differing from trial 1.

In Experiment 3, robust sexual barbering in breeding mice was seen in our experiments in S1 and B6 mice (genotype effect: F(5, 44) = 3.6; P < 0.001 (average barbering score); F(5, 44) = 3.3, P < 0.05 (% of cages with barbering); Table 2. Head, shoulders and flanks were the most barbered areas, and the overall severity was much higher compared to social barbering. In all

<table>
<thead>
<tr>
<th>Parameters</th>
<th>S1</th>
<th>BC</th>
<th>NMRI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Males</td>
<td>Females</td>
<td>Males</td>
</tr>
<tr>
<td>Number of animals</td>
<td>37</td>
<td>23</td>
<td>21</td>
</tr>
<tr>
<td>Number of cages used</td>
<td>8</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>Average animals per cage</td>
<td>4.6</td>
<td>3.8</td>
<td>4.2</td>
</tr>
<tr>
<td>% Cages with barbering</td>
<td>0 ± 0a</td>
<td>33 ± 2.2a</td>
<td>0 ± 0a</td>
</tr>
<tr>
<td>Average barbering score</td>
<td>0 ± 0a</td>
<td>3.8 ± 0.3b</td>
<td>0 ± 0a</td>
</tr>
<tr>
<td>% Barbers</td>
<td>0 ± 0a</td>
<td>9 ± 5a</td>
<td>0 ± 0a</td>
</tr>
<tr>
<td>% Barbered mice</td>
<td>0 ± 0a</td>
<td>30 ± 6b</td>
<td>0 ± 0a</td>
</tr>
</tbody>
</table>

Table 1

Dominant (social) barbering in same-sex cages in 129S1 (S1), BALB/c (BC) and NMRI mice

Groups sharing common letters (a and b) are not statistically different (P > 0.05, U-test). ANOVA data (factor: strain) for male mice: average barbering score F(2, 15) = 195.0, P = 0; % barbers F(2, 15) = 61.1, P = 0; % barbered mice F(2, 15) = 1460.0, P = 0 and female mice: average barbering score F(2, 15) = 80.0, P = 0; % barbers F(2, 17) = 4.2, P < 0.05; % barbered mice F(2, 17) = 15.3, P < 0.001.
Table 2. Sexual barbering in C57BL/6 (B6), 129S1 (S1), BALB/c (BC) and NMRI male and female mice.

<table>
<thead>
<tr>
<th>Breeding groups</th>
<th>n</th>
<th>M</th>
<th>F</th>
<th>Barbering score</th>
<th>% Cages with barbering</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1 + S1</td>
<td>16</td>
<td>28</td>
<td>16</td>
<td>4 ± 1a</td>
<td>38 ± 13ac</td>
</tr>
<tr>
<td>S1 + B6</td>
<td>11</td>
<td>13</td>
<td>11</td>
<td>3 ± 0a</td>
<td>45 ± 16ac</td>
</tr>
<tr>
<td>BC + S1</td>
<td>5</td>
<td>19</td>
<td>5</td>
<td>0 ± 0b</td>
<td>0 ± 0b</td>
</tr>
<tr>
<td>S1 + BC</td>
<td>6</td>
<td>21</td>
<td>6</td>
<td>3 ± 0b</td>
<td>0 ± 0b</td>
</tr>
<tr>
<td>NMRI + S1</td>
<td>4</td>
<td>6</td>
<td>4</td>
<td>4 ± 1a</td>
<td>100 ± 0a</td>
</tr>
<tr>
<td>S1 + NMRI</td>
<td>3</td>
<td>5</td>
<td>3</td>
<td>1 ± 0a</td>
<td>33 ± 33abc</td>
</tr>
</tbody>
</table>

N, number of cages studied; n, number of animals. F:M, average female/male ratio. Groups sharing common letters (a–c) are not statistically different (P > 0.05, U-test). Note that female mice were barbers in all these experiments.

Examining several hybrid mouse strains in Experiment 4, we found significant strain effect in these mice (% barbering: F(9, 71) = 69.8, P < 0.001; average barbering score: F(9, 71) = 109.5, P < 0.0005; % barbered animals: F(9, 71) = 92.3, P < 0.0005), with several barbering (e.g., BC × S1) and non-barbering (S1 × B6, S1 × NMRI) F1 strains (Table 3).

While S1 × B6 hybrids displayed moderate 33–43% barbering (Fig. 2D), F1 derived from NMRI and S1 strains generally did not barber, if kept <5 animals per cage, showing 80–100% barbering in “overcrowded” cages containing five to nine mice (P < 0.01, U-test). In these cages, all animals were equally barbered, showing no apparent dominant barbers (Fig. 1C). Overall, severity of this barbering was lower than in two previous contexts, predominantly showing snout denuding and whisker removal (scale 1–2), Table 3.

Examining “maternal” barbering in lactating mice performed by suckling pups (Experiment 6), we found that ≈25–30% of S1 and B6 mothers may display pronounced hair loss on their ventral surfaces, ranging from balding areas (B6) to complete fur removal (S1; Fig. 3). No hair loss was observed on head or dorsal surfaces of these female mice. Finally, in Experiment 7 we did not see any hair loss due to self-barbering (data not shown) in isolated mice of S1, BC, B6 and NMRI strains.

Table 3. Barbering in same-sex cages in mice of different F1 hybrid strains.

<table>
<thead>
<tr>
<th>Strain and sex</th>
<th>n</th>
<th>M</th>
<th>F</th>
<th>Barbering score</th>
<th>% Cages with barbering</th>
</tr>
</thead>
<tbody>
<tr>
<td>B6–S1 (M)</td>
<td>36</td>
<td>7</td>
<td>5</td>
<td>43 ± 20a</td>
<td>1.9 ± 0.3a</td>
</tr>
<tr>
<td>B6–S1 (F)</td>
<td>33</td>
<td>9</td>
<td>3</td>
<td>33 ± 17a</td>
<td>2.2 ± 0.4a</td>
</tr>
<tr>
<td>NMRI–S1 (M)</td>
<td>61</td>
<td>13</td>
<td>4</td>
<td>31 ± 13a</td>
<td>1.9 ± 0.3a</td>
</tr>
<tr>
<td>NMRI–S1 (F)</td>
<td>45</td>
<td>9</td>
<td>5</td>
<td>50 ± 33</td>
<td>2 ± 0a</td>
</tr>
<tr>
<td>S1–NMRI (M)</td>
<td>23</td>
<td>7</td>
<td>3</td>
<td>3 ± 0b</td>
<td>0 ± 0b</td>
</tr>
<tr>
<td>S1–NMRI (F)</td>
<td>25</td>
<td>7</td>
<td>3</td>
<td>3 ± 0b</td>
<td>0 ± 0b</td>
</tr>
<tr>
<td>S1–BC (M)</td>
<td>13</td>
<td>3</td>
<td>2</td>
<td>6 ± 0b</td>
<td>0 ± 0b</td>
</tr>
<tr>
<td>S1–BC (F)</td>
<td>19</td>
<td>5</td>
<td>3</td>
<td>8 ± 0b</td>
<td>0 ± 0b</td>
</tr>
<tr>
<td>BC–S1 (M)</td>
<td>15</td>
<td>4</td>
<td>3</td>
<td>8 ± 0b</td>
<td>0 ± 0b</td>
</tr>
<tr>
<td>BC–S1 (F)</td>
<td>21</td>
<td>6</td>
<td>3</td>
<td>8 ± 0b</td>
<td>0 ± 0b</td>
</tr>
</tbody>
</table>

Legend as in Table 2: M, males; F, females; Av, average number of animals per cage (n/N). N/A, data not available (in these mice in all cages where barbering was observed, all animals were equally barbered, and it was impossible to identify the barbers). Groups sharing common letters are not statistically different (P > 0.05, U-test between strains).

Fig. 3. “Maternal” barbering (produced by suckling pups) in lactating mice of C57BL/6 (A) and 129S1 (B) strains, photographed immediately after weaning. All dams have enlarged nipples, intact whiskers and unaffected fur on their dorsal surfaces. Note that ventral surfaces of some dams (A and B, left) may also remain unaffected, whereas pronounced alopecic areas can be seen in other females of the same strains (A and B, right, indicated by arrows): C57BL/6—balding patch with hair cut close to the skin. 129S1—large barbered area from tail to chin, including complete denuding of ano-genital region.
5. Discussion

In general, the most frequent location of hair loss observed here (snout, neck, head denuding) cannot be explained by self-barbering, clearly implying that this type of barbering did not occur in this study. Together with the lack of hair loss in single housed animals (Experiment 7), this allows to dissociate self- and hetero-barbering, clearly representing two different behavioral domains, and not a unitary phenomenon (but see Garner et al., 2004a,b). Although self-barbering may occur in some circumstances, our data suggest that it did not contribute to pronounced behaviour-associated hair loss in any of the strains examined in the present study.

Is barbering observed here a dominant behaviour? Although some researchers question this possibility (Garner et al., 2004a,b), our present findings in mice (Experiments 1 and 2; Table 1; Fig. 2) strongly support this notion, and coincide with numerous previously published studies (review in Sarna et al., 2000), showing single unaffected dominant barbers in all cages where social barbering occurred. In line with Long (1972), reporting barbering only after the social hierarchy has been established within the cage through aggression, our NMRI male mice (Experiment 2) displayed robust barbering after 2–5 days of active fighting in all cages. This barbering was always performed by a dominant mouse, the bigger animal with intact hair and skin, to subordinant losers (usually displaying numerous scars). Similar phenomenon was observed in F1 B6S1 males (Fig. 2), confirming clear correlation between barbering and social rank. Notably, mice are known to co-operate in barbering (Van den Broek et al., 1993), while the barber is as likely to approach as to be approached by a recipient (usually adopting a subordinate immobile posture, Sarna et al., 2000). In our study, all NMRI recipients did not try to escape, and were immobile in a prone posture, with eyes closed and ears pulled back. In contrast, NMRI male barbers performed barbering and allo-grooming by holding the recipient’s head or restrained the recipient by laying on top of it (also see Fig. 2C for similar behaviour in F1 B6S1 mice). In NMRI females, social barbering was also robust (Table 1, Fig. 1B), and although similar dominant/subordinant postures were observed, their barbering was not accompanied by fighting; see similar data in (Sarna et al., 2000) for B6 mice.

Our homecare observations in NMRI mice also showed strong association between barbing and allo-grooming, since all barbering episodes observed here ended with an intense face/head allo-grooming performed by a barber. This confirms that barbering may arise as a product of grooming activity (Militzner and Wecker, 1986; Sarna et al., 2000), whose biological role evolved from mutual body care to maintaining social hierarchy in colonies. Since B6 and NMRI mice are known as relatively non-aggressive strains (MPD, 2001), we suggest that their intensive use of barbering in established groups (e.g., during long-term social housing; Table 1; Sarna et al., 2000; Garner et al., 2004a) serves to substitute aggression. Indeed, while both barbering and scarring were observed in newly established NMRI groups (Experiment 2), these mice showed pronounced barbering and no scarring 2 weeks later, when stable social organization was established. This suggests that barbering, and not aggression, may be an essential tool in some strains to maintain social hierarchy once it was established.

In line with this, S1 mice displayed mild barbering and moderate aggression (own homecage observations), whereas aggressive BC mice did not barber (Table 1). Moreover, no barbering was observed during mating of BC and S1 mice, and in their F1 progeny (also characterized by poor barbering (Table 3) and high aggressiveness; own homecage observations). Not surprisingly, ByJ sub-strain of BC mice shows both low aggressiveness (Wood, 2000) and robust barbering (Jackson Laboratory, 1987). Taken together, all these observations support the idea that strain aggressiveness may negatively correlate with barbering activity (aggression: BC > F1 BCS1 > S1 > F1 S1NMRI > NMRI; barbering: BC = F1 BCS1 = S1 < F1 S1NMRI ≤ NMRI, such as reported here).

In addition to social (dominant) barbering observed in same-sex cages in both males and female mice, we found robust sexual barbering in some mouse strains (Table 2). This context differed markedly from social barbering seen in same-sex cages because it occurred without fighting and did not reflect dominance (i.e. males were always barbered by females, apparently subordinant members of breeding groups). In addition, here we report “maternal” barbering produced in lactating S1 and B6 females by sucklings (Fig. 3), which was unique in targeting ventral body surfaces, usually unaffected by other barbering contexts described here. Previously reported in lactating rats (Harkness, 2001), this phenomenon is now confirmed in mice, further contributing to “ethological richness” and complexity of rodent barbering.

Several interesting observations can be made on behavioural genetics of barbering. For example, non-barbering BC genotype was generally preserved in F1 (Table 3), while crossing low-barbering S1 mice with high-barbering phenotype (NMRI) led to altered barbering patterns in F1 hybrids (lower social context, higher environmental context, Table 3). In contrast, B6 × S1 hybrids displayed moderate-barbering (Table 3; Fig. 1C) and mixed cutting styles of both parental strains (Fig. 2B and C). Together, these data suggest that BC genes are stronger than S1 genotype in influencing the mouse barbering, whereas S1 background may interact with B6 in an additive manner. Finally, NMRI and S1 backgrounds seem to interplay (having equally strong effects) in F1 progeny, whose barbering contexts differed markedly from both parental strains. Importantly, distinct types of hair removal by barbers have been described in the literature (Sarna et al., 2000). While hair trimming is painless and does not affect follicle and skin receptors, plucking the whiskers out induces pain, affects follicular integrity and disrupts signaling between the receptors and nerve terminals (Prchal et al., 2004). In our study, mice did not show hair plucking, demonstrating “painless” whisker-cutting (accompanied by snout denuding). This is particularly evident in A/J mice with shortened whiskers, and B6129SvJ hybrids, whose extremely sophisticated barbering style may not be attributed to hair plucking (Fig. 1B and C). Collectively, this underlines the heterogeneity of both hair-loss styles.
and hair-removing behaviours in different mouse strains (Sarna et al., 2000; see Figs. 1–3 for the diversity of barbering patterns observed in the present study).

Can barbering be a form of stress response? Indeed, snout denuding was almost invariably seen in “overcrowded” cages with F1 NMRIS1 mice (Table 3), but was not seen in these mice kept two to four per cage. Clearly, this pattern of barbering was highly sensitive to environmental stress, and differed from all barbering contexts reported previously, confirming the mouse barbering sensitivity to various external factors (also see: De Luca, 1997; McElwee et al., 1999 for discussion).

Interestingly, the percentage of hair-barbering varies widely from strain to strain. For example, the BC mice showed no barbering (Table 1), in contrast to >20% of B6 and A/J (Long, 1972; Landau et al., 2001), >75% of A2G (Stirozik and Festing, 1981), and >80–100% of NMR1 mice (Table 1). Sarna et al. (2000) have recently reported that B6 mice may demonstrate individual “cutting styles”. In our study, mice also displayed strain- and context-specific cutting (Figs. 1 and 2), further supporting the idea of complexity and multi-factorial nature of barbering. For example, given the regional specificity of pheromonal release, and their role in social, sexual, maternal and stress-evoked rodent behaviours (Makarchuk and Kalueff, 2000; Iyikawa et al., 2004), different pheromones may control barbering, underlying its complex patterning in various strains in different contexts (Figs. 1–3).

Several neurobehavioural consequences of barbering, especially devirgulation, may include altered cortex plasticity (Maier et al., 2003), “tonic” modulation of neuronal excitability (Pechal et al., 2004), social and motor activity (Ehrenlechner and Unshelm, 1997) and emotional reactivity (Kozlovski and Prakh’e, 1995; Kozlovski et al., 1997). In addition, barbering (an important part of mouse agonistic interactions; Jackson Laboratory, 1987) may be impossible in hairless mice with mutation-induced hair/skin anomalies (e.g., Kaljis, 1992; Kalueff et al., 2004a,b). Unable to establish social hierarchy through barbering, such mice may develop non-specific pathological behaviours, including impaired cognitions, exploration and social interaction. Moreover, several known mouse mutations display aberrant barbering phenotypes (MGL, 2001; Sirito et al., 1998; Glynn et al., 2003). For example, poor-barbering mutants show impaired social interaction, representing genetic models of human abnormal social behaviours (Lijam et al., 1997; Long et al., 2004; Kassed and Herkenham, 2004). In contrast, mutants with increased barbering (e.g., Holmes et al., 2002a,b) display predictably lower aggression. Taken together, these findings indicate the potentially important behaviour-modulating role of barbering in rodents.

In conclusion, we view barbering as an essential part of mouse social, sexual and maternal behaviour, in some strains representing an important behavioural phenotype (also see: Jackson Laboratory, 1987). We suggest that the Dalda effect is a complex behavioural phenomenon with multiple mechanisms, underlying neural substrates, forms and contexts, collectively underlying the utility of rodent barbering analysis as a rich source of information about normal and abnormal brain functions.

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Sociability and preference for social novelty in five inbred strains: an approach to assess autistic-like behavior in mice


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Deficits in social interaction are important early markers for autism and related neurodevelopmental disorders with strong genetic components. Standardized behavioral assays that measure the preference of mice for initiating social interactions with novel conspecifics would be of great value for mutant mouse models of autism. We developed a new procedure to assess sociability and the preference for social novelty in mice. To quantitate sociability, each mouse was scored on measures of exploration in a central habituated area, a side chamber containing an unfamiliar conspecific (stranger 1) in a wire cage, or an empty side chamber. In a secondary test, preference for social novelty was quantitated by presenting the test mouse with a choice between the first, now-familiar, conspecific (stranger 1) in one side chamber, and a second unfamiliar mouse (stranger 2) in the other side chamber. Parameters scored included time spent in each chamber and number of entries into the chambers. Five inbred strains of mice were tested, C57BL/6J, DBA/2J, FVB/NJ, A/J and B6129PF2/J hybrids. Four strains showed significant levels of sociability (spending more time in the chamber containing stranger 1 than in the empty chamber) and a preference for social novelty (spending more time in the chamber containing stranger 2 than in the chamber containing the now-familiar stranger 1). These social preferences were observed in both male and female mice, and in juveniles and adults. The exception was A/J, a strain that demonstrated a preference for the central chamber. Results are discussed in terms of potential applications of the new methods, and the proper controls for the interpretation of social behavior data, including assays for health, relevant sensory abilities and motor functions. This new standardized procedure to quantitate sociability and preference for social novelty in mice provides a method to assess tendencies for social avoidance in mouse models of autism.

Keywords: Autism, exploration, inbred strains, locomotion, mice, olfaction, rotarod, sociability, social interaction, social preference

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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 (American Psychiatric Association 1994; see also Folstein & Rosen-Sheidley 2001; Kanner 1973; Lord et al. 2000a; Paul 2003; Piven et al. 1997; Piven 2001; Schloper & Mesibov 1987). The prevalence of autism is estimated at 1/1000 (with rates of 2.5/1000 when individuals with Asperger’s syndrome and pervasive developmental disorder are included) (Fombonne 2003). A large body of evidence from twin and family studies supports the important role of genetic factors in the etiology of autism (Bailey et al. 1995; Ritvo et al. 1989; Szatmari et al. 1998). Linkage and candidate gene studies, aimed at identifying autism susceptibility genes, are currently the focus of research in numerous laboratories (Barrett et al. 1999; Folstein & Rosen-Sheidley 2001; Philippe et al. 2002; Shao et al. 2002). Neuroimaging and post-mortem studies have implicated a number of neural structures and circuits in autism (Bauman & Kemper 1985; Happé et al. 1996; Schultz et al. 2000). A non-human primate model, resulting from neonatal lesions to the medial temporal lobe, has been described (Bachevalier 1991). Given the complexity of this syndrome, multiple, complementary approaches will be necessary to identify the underlying neural and etiologic mechanisms, and gene-brain-behavior relationships, in this disorder. The study of genes, brain and behavior relationships in mice potentially provides an important addition to current approaches to studying the pathogenesis of autism. However, to date, behavioral models of autistic-like responses in mice have received little attention.
Social behavior is a complex construct. The particular set of behaviors that define the syndrome of autism are wide ranging in severity and type, and change in form with chronological development. So, for example, in the original descriptions of autism by Kanner (1943), autistic children were characterized by their dramatic lack of interest in others, whereas current DSM-IV criteria identify substantially more subtle deficits in reciprocal social interaction as sufficient for meeting criteria for this disorder (American Psychiatric Association 1994). Behavioral abnormalities in this domain in autism are varied and include such characteristics as deficits in non-verbal expression (e.g. deficits in eye-to-eye gaze and diminished expression of emotion as measured by lack of gesturing or facial expression), abnormalities in the social use or understanding of language, deficits in social approach (such as the absence of coming for comfort upon injury), or markedly diminished peer relationships.

Mouse models may provide a useful research tool to advance the investigation of genes associated with autism (Insel 2001). In the present study, we sought to develop methods for characterizing some of the social behaviors in mice that could be viewed as qualitatively similar to social deficits that define autism. Mice are a highly social species (Blanchard et al. 2003; Grant & Macintosh 1963; Laviola & Terranova 1998; Scott & Fredericson 1951; Valzelli 1973; Vandenberghe 1989; Winslow & Insel 2002). Social behaviors have been explored in mice with mutations in candidate genes such as oxytocin (Winslow & Insel 2002; Young et al. 2002), monoamine oxidase-A (Shih & Chen 1999), fragile X (Churchill et al. 2002; Mineur et al. 2002) and dishevelled-1 (Li & et al. 1997). Good mouse models will require tasks specific for the types of social deficits that are considered the core symptoms of autism, including very low levels of social approach behaviors (Folstein & Rosen-Sheidley 2001; Lord et al. 2000a; Piven et al. 1997).

To quantify sociability tendencies in mice, we developed a set of tasks that measure (a) time spent with a novel conspecific and (b) preference for a novel vs. a familiar conspecific. Conceptually similar to previous social interaction tests (Bales & Carter 2003; File & Seth 2003; Gheusi et al. 1994; Insel & Young 2001; Tang et al. 2003; Winslow 2003), the present behaviors focus on a more narrowly defined set of parameters. We reasoned that most autistic individuals demonstrate reduced or unusual social approach. Therefore, subject mice were given the choice between exploring an empty, novel chamber vs. exploring an identical novel chamber containing a mouse with which the subject had no previous interaction (stranger 1). Autistic individuals may further avoid unfamiliar social partners and display diminished interest in novelty (American Psychiatric Association 1994). Subject mice were given another choice between exploring the chamber containing the now-familiar stranger 1, vs. exploring an identical chamber containing a new mouse (stranger 2). Time spent in each chamber and number of entries into each chamber were scored by observation. These measures were subsequently automated, as described in a companion paper (Nadler et al. 2004).

Previous methods for testing social behavior in mice employed tethered novel conspecifics and other approaches to maximize the contribution of the subject mouse to the social interactions, and minimize the contributions of the target stranger mouse (Brodkin et al. 2004; Carter et al. 1995; Winslow 2003). The present method contains the target stranger within an inverted round wire cage that permits visual, tactile, auditory and olfactory communication. This modification focuses the task on social approach tendencies, relevant to the social approach deficit in autism, while avoiding potential confounds resulting from aggressive or sexual interactions.

To ensure that differences in social behaviors were not artifacts of other aspects of mouse behaviors, a series of independent control experiments was conducted to evaluate potential confounding factors. Measures of general health, home cage behavior and neurological reflexes were conducted to rule out gross physical abnormalities (Crawley 2000). Olfaction abilities are considered essential for mouse social interactions (Cheal & Sprott 1971; Liebenau & Slotnick 1996; Wrenn et al. 2003). Retrieval of food buried in cage bedding was quantitated in a 15-minute olfactory test, to ensure that the subject mice were not grossly impaired in olfactory abilities. Further controls were designed to detect additional symptoms relevant to autism, such as abnormalities in locomotion or motor coordination (Hallett et al. 1993; Piven et al. 1997), that could directly affect performance of the social task.

Parameters scored within the test sessions in the social apparatus provided additional information on procedural abilities necessary for social behaviors. Before the start of the choice tests, subject mice were given a habituation session in a neutral central chamber. The number of times that the mouse exited from the habituated empty central chamber into the novel empty chamber provided a general measure of locomotor function. In addition, time spent in the habituated center chamber provided a preliminary measure of anxiety-like behaviors, analogous to the open field emergence test (Holmes et al. 2003; Smith et al. 1998).

Five inbred strains of mouse were chosen for the present evaluation of autistic-like social behaviors in mice: C57BL/6J, DBA/2J, FVB/NJ, A/J and the hybrid B6129PF2/J. Many approaches to studying mouse models of human disease have focused on mice with specific targeted mutations in single genes. Available evidence from family and twin studies, and the results to date from linkage studies, suggest that, for most cases, the simplest model of autism involves the interaction of multiple genes (Risch et al. 1999). Given data from family genetic studies showing a broad range of the behavioral expression of the underlying genetic liability for autism in nonautistic relatives (Piven et al. 1997), the possibility must also be considered that variation in the social and cognitive behaviors manifested by autistic individuals...
represents one end of the extreme of normal genetically-controlled variation. As an initial effort to characterize social behaviors in mice that might be relevant to the study of autism, we therefore focused on comparing and contrasting the continuum of social behaviors that could be observed across several ‘normal’ strains of inbred mice.

The present study presents the first results using the three-chambered apparatus to score social approach behaviors relevant to autism. For the purposes of this study, ‘sociability’ is defined as a significant propensity to spend time with another mouse, as compared to time spent in an identical but empty chamber. ‘Preference for social novelty’ is defined as a significant propensity to spend more time with a new mouse than with a familiar mouse. Most experimental groups consisted of male mice, for consistency with the 4:1 male:female difference in the diagnosis of autism (Folstein & Rosen-Sheidley 2001). Mice were tested at both juvenile and adult ages, consistent with the neurodevelopmental aspects of autism. The choice of inbred strains was designed to represent those commonly used in behavioral genetics, and to provide baseline scores that could later be compared to lines of targeted gene mutations bred into these background strains.

Materials and methods

Animals
Male mice from four inbred strains, C57BL/6J, DBA/2J, FVB/NJ, AVJ and the hybrid B6129PF2/J, were purchased from The Jackson Laboratory (Bar Harbor, ME). Male DBA/2 mice were purchased from Harlan (Indianapolis, IN). Mice ranged in age from 3 to 10 weeks upon arrival at the University of North Carolina animal facility in Chapel Hill, NC. An additional group of male and female C57BL/6J mice, used in only one experiment (data shown in Figs 5 and 6), were the first generation derived from stock purchased from The Jackson Laboratory. All animals were housed in group cages by strain and gender, three to four per plastic tub cage, and provided with food and water ad libitum. The housing room was maintained at 23 °C on a 12-h light/dark cycle (lights off at 19:00). All procedures were conducted in strict compliance with the policies on animal welfare of the National Institutes of Health and the University of North Carolina (stated in the Guide for the Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources, National Research Council, 1996 edition), and approved by the University of North Carolina Institutional Animal Care and Use Committee.

Test procedures
The first series of behavioral assays was conducted with 20 male mice from each of three strains, C57BL/6J, DBA/2J and FVB/NJ, aged 3–4 weeks at the time of arrival into the animal facility. Mice were evaluated on measures of home cage behavior, neurological reflexes, locomotor activity, motor coordination and balance, olfaction and social behavior.

Home cage behavior
During the first week in the animal facility, assessments of home cage behavior were conducted by investigator observations at three different time points: 8:00, 12:00 and 18:50. Observations were taken across two to three days, for 20 min at each time point, for a total of 60 min of home cage observation. Two hours before the noon observation, one white cotton nestlet square (Ancare Corp., Bellmore, NY) was added to each cage, in order to assess nest-building behavior. The evening observation was conducted 10 min before lights off, and then for another 10 min after the lights had gone off, using red light illumination. Records were taken for nestlet shredding, nest building, sleeping in huddles, activity, fighting and any aberrant behaviors, such as tremor or seizures.

General health and neurological reflexes
Behavioal testing began one week after arrival into the animal facility. The mice were first evaluated for general health, using several measures of overall appearance and behavior, as previously described (Crawley 2000; Holmes et al. 2003). The measures included general observations on the appearance of the fur and whiskers, body posture and normality of gait. Normal reflexive reactions to a gentle touch from a cotton swab to the whiskers on each side of the face, the approach of the cotton swab to the eyes, and the sound from a metal clicker (Preyer reflex) were assessed. Each animal was placed in a small, empty plastic cage, and its ability to remain upright when the cage was moved from side-to-side or up-and-down was noted. Animals were observed for the visual placing reflex (forepaw extension when lowered toward a visible surface), and for ability to grasp a metal grid with forepaws and hindpaws.

Locomotion
One day after the neurobehavioral screen, exploratory activity in a novel environment was assessed by a five-minute session in an open field chamber (40 cm L × 30 cm W × 40 cm H), constructed of clear Plexiglas. A grid of squares (10 × 6) was drawn on the floor of the chamber, and counts were taken of number of squares crossed and rears during the session.

Olfactory test
A simple test for olfaction was conducted two days following the activity test. One or two days before the olfactory test, an unfamiliar food high in carbohydrates (Froot Loops, Kellogg Co., Battle Creek, MI) was placed overnight in the home cages of the subject mice. Observations of consumption were taken to ensure that the novel food was palatable to the mice. On the day of the test, each mouse was placed in a large, clean tub cage (46 cm L × 23.5 W cm × 20 H cm), containing 3 cm deep paper chip bedding (Canbrands Product, Moncton NB, Canada), and allowed to explore for five minutes. The animal was removed from the cage, and one Froot Loop was buried in the cage bedding. The animal was
then returned to the cage and given 15 minutes to locate the buried food. Measures were taken of latency to find the Froot Loop and whether it was consumed. In addition, observations were taken of behavioral responses (sniffing, digging, locomotion) during the test session.

**Rotarod performance**

One day following the olfactory test, the mice were assessed for balance and motor coordination on an accelerating rotarod (IITC Inc., Woodland Hills, CA). Mice were placed on a cylinder which slowly accelerated to a constant rotating speed. The task required the mouse to walk steadily forward in order to remain on top of the barrel. Revolutions per minute (r.p.m.) were set at an initial value of 3, with a progressive increase to a maximum of 30 r.p.m. across five minutes, the maximum trial length. Each animal was given a single test session consisting of two trials, a practice trial and a test trial, with 45 seconds between each trial. Latency to fall from the top of the rotating barrel was recorded by the rotarod timer.

**Tests for sociability and social novelty preference**

The initial set of 60 mice was tested in the social task at 5–6 weeks of age, after completion of the control tasks described above. Methods were adapted from established procedures for testing pair-bonding in voles (Dean, S.M. & Vandenbergh, J.G. (2001) Prenatal exposure to antiandrogenic or estrogenic compounds alters monogamous behavior in pine voles (Microtus pinetorum). Presented at the Society for Behavioral Neuroendocrinology Annual Meeting, Tempe, Arizona; Winslow 2003). The social testing apparatus, illustrated in Fig. 1, was a rectangular, three-chambered box fabricated by Philip L. Thompson and Steve Medlin, University of North Carolina Physics Instrument Shop. Each chamber was 20 cm L \times 40.5\,cm W \times 22\,cm H. Dividing walls were made from clear Plexiglas, with small circular openings (3.5 cm in diameter) allowing access into each chamber. The chambers of the social apparatus were cleaned and fresh paper chip bedding was added between trials.

The test mouse was first placed in the middle chamber and allowed to explore for five minutes. The doorways into the two side chambers were obstructed by plastic boxes during this habituation phase. After the habituation period, an unfamiliar C57BL/6J male (stranger 1), that had no prior contact with the subject mice, was placed in one of the side chambers. The location of stranger 1 in the left vs. right side chamber was systematically alternated between trials. The stranger mouse was enclosed in a small, round wire cage (Galaxy Cup, Spectrum Diversified Designs, Inc., Streetsboro, OH), which allowed nose contact between the bars, but prevented fighting. The cage was 11 cm in height, with a bottom diameter of 10.5 cm and bars spaced 1 cm apart. A weighted cup was placed on the top of the cage to prevent climbing by the test mice. The animals serving as strangers were male C57BL/6J mice that had previously been habituated to placement in the small cage. Both doors to the side chambers were then unblocked and the subject was allowed to explore the entire social test box for a 10-minute session. Measures were taken of the amount of time spent in each chamber and the number of entries into each chamber, by a human observer seated five feet from the apparatus. An entry was defined as all four paws in one chamber. All data were entered via an event keyboard connected to data collection software on a PC. Interrater reliability was greater than 95% when results were compared between two trained observers scoring the same subject mice simultaneously.

At the end of the first 10 minutes, each mouse was tested in a second 10-minute session to quantitate social preference for a new stranger. A second, unfamiliar mouse was placed in the chamber that had been empty during the first 10-minute session. This second stranger was also enclosed in an identical small wire cage. The test mouse had a choice between the first, already-investigated unfamiliar mouse (stranger 1), and the novel unfamiliar mouse (stranger 2). As described above, measures were taken of the amount of time spent in each chamber and the number of transitions between chambers of the apparatus during the second 10-minute session.

**Critical elements of the sociability and social novelty preference tests**

Results from the initial group of mice indicated significant sociability and a preference for social novelty in young male...
mice from three different inbred strains. Potential modifier variables concerning sociability were then evaluated, including assessment of two additional strains, a comparison between male and female mice, replicability with repeated testing of the same subjects and a comparison between juvenile and adult mice.

**Social tests in hybrid male mice**
A group of 10 male B6129PF2/J mice, age 6 weeks at the time of testing, were assessed with the same social test procedures as used with the initial group of mice. This hybrid mouse strain was of particular interest, because it has been used as the genetic background control for transgenic lines maintained on a mixed C57BL/6J x 129P3/J background (e.g. Waite et al. 2002; Zhu et al. 2003), including mutant lines phenotyped by our laboratory (unpublished results).

**Social tests in male and female mice**
Sex differences were investigated using a group of 12 male and 8 female C57BL/6J mice, age 6 weeks at the time of testing. These animals were assessed with the same procedures as used with the initial group of mice. In this case, the males and females were tested in separate social test boxes. For both sexes, C57BL/6J males served as the unfamiliar mice (stranger 1 and stranger 2). There were no confounding sexual or aggressive behaviors, because the strangers were contained within the wire cages.

**Test for sociability in adult male mice**
In comparison to the juvenile male mice tested in the initial experiment, adult male mice were tested at three months of age. Three inbred strains were analyzed for social behavior in adult males: C57BL/6J, DBA/2 and A/J, 10 mice per inbred strain. In this case, the mice were each given a five-minute habituation period in the social test box with the doors open, and then a 10-minute choice task between the chamber containing an unfamiliar mouse and the empty chamber (the social novelty test was not conducted).

Because the A/J mice showed markedly low levels of entry into the different chambers, concern was raised that the small openings between chambers presented a physical barrier to exploratory locomotion in this strain. Low levels of exploration throughout the three chambers represent a potential artifact in the interpretation of social behavior deficits. Therefore, the A/J mice were given a second test one week later, with the dividing walls between the chamber sides partially retracted to facilitate exploration.

**Replicability within subjects**
A subset of the adult C57BL/6J mice (n = 5) and the DBA/2 mice (n = 6) was retested with the sociability procedure 11–12 days following the first test, to determine similarity of results across repeated testing of the same mice.

**Statistical analysis**
One-way Analysis of Variance (ANOVA) was used to compare data for the inbred strains on measures of body weight, activity, rotarod performance and latency to find buried food. Social data were first analyzed using repeated measures ANOVAS, with the factors of strain (or sex), test condition (sociability or social novelty) and chamber side (e.g. stranger 1 side or the opposite side). Within-group repeated measures ANOVAS were used to determine side preferences, and to compare results from retesting in the sociability procedure. Fisher’s protected least-significant difference (PLSD) tests were used to compare group means only when a significant F-value was determined. For all comparisons, significance was set at P < 0.05.

**Results**

**General health, home cage behavior, neurophysiological reflexes and sensory and motor function**
As shown in Table 1, preliminary observations of the inbred strains provided evidence that the mice were in good general health, without overt signs of impairment or aberrant behaviors. In the home cages, all groups of mice made nests and, in almost all cases, were observed sleeping together in huddles. There were no significant differences between the strains in body weight or rotarod performance (see Table 1 for mean latency to fall on the second trial). Overall, neurophysiological reflexes in response to visual, tactile or auditory stimuli were normal. For the olfactory test, the majority of the subjects successfully located and retrieved the buried food. The FVB/NJ mice were particularly adept at this task, with significantly lower latency scores to find the buried food, in comparison to the other two groups (post hoc tests following significant effect of strain; F2,56 = 4.399, P = 0.0168). Behavioral observations recorded during the olfactory task confirmed that all of the subjects showed exploration of the novel test cage. On measures of activity, the FVB/NJ mice had higher levels of locomotion in an open field than the other two groups, and the DBA/2J mice showed less locomotion (post hoc tests following significant effect of strain; F2,57 = 44.664, P < 0.0001). The FVB/NJ mice also showed more rearing responses in the open-field, in comparison to the other two strains (post hoc tests following significant effect of strain; F2,57 = 86.234, P < 0.0001).

**Social behavior**
Test for sociability and social novelty in three inbred mouse strains
C57BL/6J, DBA/2J and FVB/NJ male juvenile mice demonstrated a significant preference for spending time in the chamber containing stranger 1, vs. time spent exploring the empty chamber (Fig. 2a; post hoc tests following significant main effect of side; F1,59 = 39.29, P < 0.0001). When the choice was between the first stranger mouse and a second
stranger mouse (Fig. 2b), all three inbred strains demonstrated a preference for social novelty (post hoc tests following significant main effect of side; \( F_{1,59} = 42.867, P < 0.0001 \)). An overall repeated measures ANOVA was performed, looking at the effects of strain, side of chamber (stranger 1 side or opposite side) and test condition (sociability or social novelty). A significant interaction was found between side of chamber and test condition (\( F_{1,57} = 67.634, P < 0.0001 \)), reflecting the finding that preference was for the stranger 1 side during the first half of the session (the sociability condition), but switched to the stranger 2 side during the last part of the session (the social novelty condition). There were no significant differences between the three inbred strains (C57BL/6J, DBA/2J and FVB/NJ) on measures of duration (no significant effect of strain, \( F_{2,57} = 2.369, P = 0.1027 \)).

As shown in Fig. 3, the measure of entries into each side of the social test box did not follow the pattern seen for the measure of time spent in each chamber. Significant preferences for sociability (Fig. 3a) or preference for social novelty (Fig. 3b) were not observed for number of chamber entries in any of the inbred strains. As seen in the previous test for open-field locomotion, the DBA/2J mice appeared less active, as evidenced by significantly lower numbers of entries into each side, in comparison to the other two groups (post hoc tests following significant main effect of strain, \( F_{2,57} = 44.322, P < 0.0001 \)).

### Test for sociability and preference for social novelty in hybrid mice

As observed with the three inbred strains, juvenile male mice from a mixed B6/129 background had a clear preference for spending time with an unfamiliar conspecific, vs. exploring an empty chamber (Fig. 4a, left) and a significant preference for social novelty, measured by comparing time spent with the new stranger 2 as compared to time spent with the now-familiar stranger 1 (Fig. 4b, left; significant main effect of side, \( F_{1,9} = 9.645, P = 0.0126 \), and significant interaction between side and test condition, \( F_{1,9} = 64.427, P < 0.0001 \)). Interestingly, in the hybrid strain, this same pattern for preference was reflected in the measure of entries, both for the sociability test (Fig. 4a, right) and the test for social novelty preference (Fig. 4b, right; significant main effect of test condition, \( F_{1,9} = 7.978, P = 0.0199 \), and significant

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### Table 1: General health, home cage behaviors, neurological reflexes and motor function

<table>
<thead>
<tr>
<th></th>
<th>C57BL/6J</th>
<th>DBA/2J</th>
<th>FVB/NJ</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Physical characteristics</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>19.9 ± 0.3</td>
<td>18.8 ± 0.5</td>
<td>19.6 ± 0.3</td>
</tr>
<tr>
<td>Poor coat condition</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>Piloerection</td>
<td>0%</td>
<td>0%</td>
<td>35%</td>
</tr>
<tr>
<td><strong>Home cage behaviors</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type of nest</td>
<td>spherical</td>
<td>partial, flat</td>
<td>spherical, flat</td>
</tr>
<tr>
<td>Huddling together (% cages)</td>
<td>100%</td>
<td>100%</td>
<td>80%</td>
</tr>
<tr>
<td>Sleep during day (% subjects)</td>
<td>95%</td>
<td>100%</td>
<td>80%</td>
</tr>
<tr>
<td>Lights-off locomotion (% subjects)</td>
<td>25%</td>
<td>100%</td>
<td>20%</td>
</tr>
<tr>
<td>Aberrant responses (% subjects)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Neurological reflexes (% normal)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corneal</td>
<td>90%</td>
<td>80%</td>
<td>100%</td>
</tr>
<tr>
<td>Visual placing</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>Vibrissae orientating</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>Preyer reflex</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td><strong>Olfaction</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uncovered buried food (% subjects)</td>
<td>75%</td>
<td>68%</td>
<td>100%</td>
</tr>
<tr>
<td>Latency to uncover buried food (seconds)</td>
<td>422.5 ± 74.8</td>
<td>459.5 ± 78</td>
<td>198.4 ± 46.2#</td>
</tr>
<tr>
<td><strong>Motor/muscular abilities</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Locomotor score</td>
<td>297.0 ± 19.2</td>
<td>198.2 ± 9.3#</td>
<td>418.1 ± 19.0#</td>
</tr>
<tr>
<td>Rears</td>
<td>29.0 ± 1.9</td>
<td>28.3 ± 1.9</td>
<td>64.0 ± 2.7#</td>
</tr>
<tr>
<td>Rotarod latency (seconds)</td>
<td>157.5 ± 21.4</td>
<td>130.0 ± 16.1</td>
<td>128.7 ± 15.6</td>
</tr>
</tbody>
</table>

Results for home cage behavior were compiled across three different observation periods, in most cases. Observation of lights-off locomotion was taken for 10 min following the initiation of the dark cycle in the animal housing room, under red light illumination. Neurological reflexes included reaction to a sterile cotton swab approaching the eye (corneal) or touching whiskers (vibrissae orienting). For visual placing, mice were lowered towards a mesh grid and observed for forepaw-reaching. Preyer reflex was elicited by a loud metal clicker. Rotarod data are the group means for the second of two trials. Data are expressed as percentage of the total number of mice tested, or as mean ± standard error of the mean (SEM). \( n = 20 \) for each strain. Data were lost from one DBA/2J mouse for olfaction test. \( \# P < 0.05 \), strain different from other two strains on comparable measures.
mouse sociability tasks

Figure 2: Social behavior in juvenile C57BL/6J, DBA/2J and FVB/NJ mice. Duration measures were taken for (a) sociability, i.e. preference for a stranger mouse vs. an empty chamber, and (b) preference for a novel stranger (stranger 2) vs. the first unfamiliar mouse (stranger 1). See text for further description. Data shown are mean ± SEM for each group. n = 20 for each strain. *P < 0.05, within-group comparison between stranger 1 side and opposite side. All three strains demonstrated significant preference for spending time in the chamber containing the strange mouse.

Interaction between side and test condition, F_{1,9} = 22.821, P = 0.001.

Comparison of male and female mice on sociability and preference for social novelty

Figure 5 presents the results of a comparison between duration measures in juvenile male and female C57BL/6J mice. An overall repeated measures ANOVA indicated significant main effects of sex (F_{1,18} = 9.374, P = 0.0067) and a significant interaction between side and test condition (F_{1,18} = 108.127, P < 0.0001). Male and female mice demonstrated similar sociability, in terms of time spent in the chamber containing stranger 1 (Fig. 5a; significant main effect of side, F_{1,18} = 80.003, P < 0.0001), and similar preference for social novelty, in terms of time spent in the chamber
containing stranger 2 (Fig. 5b; significant main effect of side, $F_{1,18} = 63.271, P < 0.0001$). Further analysis revealed that the significant effect for sex stemmed from slightly less time spent in the side compartments in the females, in comparison to the males, during the test for social novelty (Fig. 5b; significant main effect of sex, $F_{1,18} = 9.669, P = 0.0061$).

There were no significant differences between the male and female mice for number of entries (Fig. 6), although a significant main effect of test condition ($F_{1,18} = 9.722, P < 0.0059$) and a significant interaction between side and test condition ($F_{1,18} = 21.917, P < 0.0001$) were found. Differences in number of entries into the two sides were not observed during the test for sociability (Fig. 6a). In contrast, both the male and female mice were found to have more entries into the chamber containing stranger 2, in comparison to the side containing stranger 1 (Fig. 6b, $F_{1,18} = 28.972, P < 0.0001$).

**Test for sociability in adult mice from three inbred mouse strains**

Measures of duration in each side of the social test box and number of entries were taken during the five-minute habituation period, in order to demonstrate that mice did not have a bias for spending time in one particular side, and to establish baseline levels of activity when no stranger mouse was present. Overall, the mice did not show a preference for either the right or left sides of the test box during habituation (Fig. 7a; $F_{1,27} = 0.158, P = 0.6945$). The adult male mice from the A/J inbred strain spent significantly less time in either of the side chambers, in comparison to the C57BL/6J and DBA/2 animals (post hoc tests following significant main effect of strain, $F_{2,27} = 32.234, P < 0.0001$). The A/J group also made fewer entries into either side during habituation, in comparison with the other two strains, while the C57BL/6J mice had higher levels of entries than the other groups (Fig. 7b; post hoc tests following significant main effect of strain, $F_{2,27} = 56.14, P < 0.0001$).

As depicted in Fig. 8a, adult male mice from the C57BL/6J and DBA/2 strains showed a preference for spending more time investigating an unfamiliar mouse, vs. time in the empty side, in the sociability test (post hoc tests following repeated measures ANOVA, significant main effects of strain, $F_{2,27} = 30.648, P < 0.0001$; side, $F_{1,27} = 76.646, P < 0.0001$; and significant interaction between strain and side, $F_{2,27} = 22.233, P < 0.0001$). These results were analogous to the data obtained with juvenile male mice of these two inbred strains. In this set of experiments with adult males, a comparable pattern emerged for entries (Fig. 8b), where higher numbers of entries were associated with the side containing the stranger mouse in the C57BL/6J and DBA/2 strains (post hoc tests following repeated measures ANOVA,
significant main effects of strain, $F_{2,27} = 32.245$, $P < 0.0001$; side, $F_{1,27} = 9.353$, $P = 0.005$; and significant interaction between strain and side, $F_{2,27} = 3.96$, $P = 0.031$.

In contrast, adult males of the A/J strain did not spend more time in the chamber containing stranger 1 than in the empty chamber (within-group repeated measures ANOVA, $F_{1,9} = 0.096$, $P = 0.7639$). As clearly indicated in Fig. 8a, the A/J mice stayed in the center region for most of the session. The low number of entries observed in the A/J mice was significantly different from both of the other inbred strains (post hoc tests following repeated measures ANOVA, $F$-values above).

Sociability procedure with modified social test box

Observations of the A/J mice during the social test indicated that the animals explored the center region of the social test box, but did not readily enter the openings in the dividing walls. Therefore, these A/J mice were retested one week following the initial test, to explore the possibility that partial removal of the dividing walls would facilitate entry into the chambers adjacent to the center region. As shown in Fig. 9, the A/J group did show significant increases in time spent in the side regions (Fig. 9a, significant main effect of retest, $F_{1,9} = 11.343$, $P = 0.0083$) and in number of entries (Fig. 9b, significant main effect of retest, $F_{1,9} = 13.302$, $P = 0.0053$) when the openings between chambers were more accessible. However, A/J mice still did not show a preference for the side containing the unfamiliar mouse, in comparison to the empty side ($F_{1,9} = 0.531$, $P = 0.4849$).

Repeated testing for sociability

To assess replicability of the procedure when the same mouse was tested twice, a subset of the C57BL/6J and DBA/2 mice was given a retest, using procedures identical to their first test. As shown in Fig. 10, a preference for spending more time with the unfamiliar mouse, in comparison to exploring an empty chamber, was again evident in both strains when retested 11–12 days following the first test (Fig. 10a; significant main effect of side, $F_{1,9} = 285.705$, $P < 0.001$; and significant interaction between side and retest, $F_{1,9} = 7.16$, $P = 0.0254$). Further analysis revealed that the DBA/2 mice did not show any differences in duration between the first and second tests, while the C57BL/6J
mice had lower times in the stranger side during the retest (post hoc test following within-group comparison, significant main effect of side, $F_{1,4} = 72.372, P = 0.001$; and significant interaction between side and retest, $F_{1,4} = 35.876, P = 0.0039$).

Differences between the two strains emerged for the measure of entries. DBA/2 mice tended to have lower numbers of entries than the C57BL/6J mice (Fig. 10b; significant main effect of strain, $F_{1,9} = 16.156, P = 0.003$; retest, $F_{1,9} = 36.215, P = 0.0002$; and side, $F_{1,9} = 8.723, P = 0.0161$). DBA/2 mice did not show significant differences between number of entries recorded for the first test and the second test. C57BL/6J mice showed higher numbers of entries into the empty side of the social test box during the retest, in comparison to the first test (post hoc tests following within-group comparison, significant main effect of retest, $F_{1,4} = 103.143, P = 0.0005$; and significant interaction between side and retest, $F_{1,4} = 8.963, P = 0.0402$).

**Discussion**

Deficits in social interaction and communication are primary diagnostic indicators of autism (e.g. American Psychiatric Association 1994; Lord et al. 2000b). From an early age, autistic children show low levels of eye contact and affective responses, less orientation toward others and language deficits, with a concomitant paucity of speech and social communication (for review, see Tager-Flusberg et al. 2001). Given the primacy of social impairment in autism, we considered this symptom to be fundamental to a mouse model of autism (Insel 2001). Therefore, the present methods were designed to quantitate levels of sociability, i.e. tendency to initiate social contact, and preference for social novelty, i.e. tendency to initiate social contacts with a new individual as compared to someone familiar from past experience, using a simple scoring approach for laboratory mice.

In the present studies, sociability was defined as the tendency to approach and remain proximal to an unfamiliar conspecific, vs. avoidance of the stranger mouse by remaining in the center chamber or exploring an equally novel chamber devoid of another mouse. Overall, four of the five inbred and hybrid strains tested spent more time in the side of the social test box containing the unfamiliar stranger, vs. time in the empty side. Juvenile male mice from three different inbred strains (C57BL/6J, DBA/2J and FVB/NJ) and one hybrid cross...
(B6129PF2/J), as well as older adult male mice (C57BL/6J and DBA/2), all showed significant levels of sociability, as evidenced by longer durations for time in the stranger side. Similar levels of sociability were clearly evident in a group of young female C57BL/6J mice.

When a novel unfamiliar conspecific was placed in the formerly empty side, a reversal of chamber preference was noted in all groups of juvenile mice from the three inbred strains and the hybrid cross. The higher duration of time spent with the novel stranger (stranger 2) was interpreted as a demonstration that the mice preferred to approach a new stranger, rather than restrict contact to a more familiar individual. It was also an indication of the ability of the mice to recognize a peer and seek social interaction.

**Figure 7: Exploration in social test box during 5-min habituation period for adult male mice from three inbred strains.** Measures were taken of (a) duration in and (b) entries into the right and left sides of the social test box. All three chambers were empty during this habituation period. Data shown are mean ± SEM for each group. n = 10 for each strain. # P < 0.05, strain different from other two strains on comparable measure.

**Figure 8: Sociability in adult male mice from three inbred strains.** Measures were taken of (a) duration in and (b) entries into the side of the social test box containing the unfamiliar mouse (stranger side) vs. the empty side. Data shown are mean ± SEM for each group. n = 10 for each strain. * P < 0.05, within-group comparison, different from stranger side. # P < 0.05, strain different from other two strains on comparable measure. A/J was the only strain tested that failed to spend more time with a conspecific, and spent most of its time in the central chamber after the initial 5-minute habituation session.
to discriminate between the two strangers, and to recognize the one that had not been encountered before. Previous work in social recognition has shown that mice show both habituation of olfactory responses directed toward an unfamiliar conspecific, and dishabituation, with a reinstatement of social sniffing, when a novel stimulus animal is presented (Bluthe et al. 1993; Dluzen & Kreutzberg 1993; Ferguson et al. 2000; Kogan et al. 2000; Winslow & Camacho 1995). The choice procedure used in the present study allows the investigator to determine whether the test mice
prefer proximity to a novel stranger mouse, vs. proximity to a previously encountered mouse, which may be more relevant to the core symptoms of autism than simple recognition tests.

The social preference pattern observed for the duration measures was not evident for the number of entries into each side for the initial set of juvenile male mice (C57BL/6J, DBA/2J and FVB/NJ). In fact, within each strain, the number of entries into either side of the social test box was almost equal for both the sociability and social novelty tests. However, differences between the three inbred strains were found, with the DBA/2J mice having significantly lower entries into each side, in comparison to the C57BL/6J and FVB/NJ mice. While the DBA/2J group also had significantly lower levels of locomotor activity in the open field test, the significantly higher locomotor scores observed in the FVB/NJ strain, in comparison to the other two groups, were not reflected in an overall higher number of entries. Therefore, for the juvenile male mice, number of entries did not reflect the trends for social preference seen in the duration measures, and only partially correlated with their locomotor activity independently quantitated in the open field.

In contrast, the hybrid B6129PF/2J mice, when tested for sociability, had more entries into the side of the social test box containing the unfamiliar stranger, in comparison to the empty side. There was a reversal of this pattern during the social novelty test, when a higher number of entries were made into the side with the second unfamiliar stranger (formerly the empty side). The pattern for the entries measured in the hybrid mice reflected the social preference pattern observed for the duration measure. In the second group of juvenile C57BL/6J mice, both males and females made equal numbers of entries into each side of the social test box during the assay for sociability, but showed an increased number of entries into the side of the social box containing the second, ‘novel’ unfamiliar stranger during the test for social novelty. Finally, the adult C57BL/6J and DBA/2 mice had higher numbers of entries into the side of the chamber containing the unfamiliar conspecific in the sociability test. Therefore, number of entries appears to vary in terms of its correlation to other parameters. Future studies with these and other inbred strains will be designed to understand the usefulness of the number of entries parameter as a measure of sociability and preference for social novelty.

Based on the present results, time spent in each chamber appears to be the most useful parameter. However, it will be important to differentiate time spent in the chamber from true social investigation of the stranger target mouse in its wire cage. One approach to confirming the social nature of the chamber exploration is to score sniffs directed towards the stranger. Number of sniffs of the wire cage containing the stranger vs. number of sniffs of an empty wire cage were compared in a companion study with an automated version of the present social test chambers (Nadler et al. 2004). Higher numbers of sniffs were directed toward stranger 1 than towards the empty wire cage, and towards the novel stranger 2 vs. the now-familiar stranger 1, in C57BL/6J, DBA/2J and FVB/NJ male mice (Nadler et al. 2004).

A particularly interesting finding from the present strain distribution analysis is the low level of sociability in the A/J inbred strain. Adult A/J males failed to show a significant preference for the side containing stranger 1, as compared to the empty side. The A/J mice spent significantly more time in the center chamber, indicating a strong preference for the start box that was not seen in the other strains. These findings may indicate a specific social deficit in the A/J strain. Alternatively, these data may reflect the low levels of open field exploratory behaviors and high levels of anxiety-like behaviors in the light/dark transitions test reported for A/J mice in other studies (Bouwknecht & Paylor 2002; Mathis et al. 1994, 1995; Thifault et al. 2002; Wahlsten et al. 2003) and reflected in the low number of entries in the present study. Arguing against an artifactual interpretation that hypolocomotion caused the social deficit in the A/J group are the data of Fig. 8. Repeated testing of A/J in the modified apparatus increased the number of entries, but did not yield significant sociability scores. In addition, low locomotor scores in DBA/2J and high locomotor scores in FVB/NJ appeared to be unrelated to amount of time spent in the chamber with stranger 1 and stranger 2. It will be important to address the potential confounding variable of locomotor activity in future investigations.

The present procedures for the sociability and social novelty tests are designed to be simple, quantitative and easily mastered by investigators. Methods are based on procedures examining pair-bonding in voles (Dean, S.M. & Vandenbergh, J.G. as above), as well as many other elegant tests for social behavior in rodents (Bluthe et al. 1993; Carter et al. 1995; Dluzen & Kreutzberg 1993; Gheusi et al. 1994; Insel and Young 2001; Kogan et al. 2000; Winslow & Camacho 1995; Winslow 2003). In most cases, these procedures have involved placing two animals together and recording behavioral responses, or using a stimulus animal that is tethered or otherwise confined to one side of a multichambered testing apparatus. Observations of mice in these settings have shown that exposure to an unfamiliar stranger typically evokes a period of intense social sniffing, which may be followed by escalating aggressive responses or sexual behavior, dependent upon such factors as age and sex of the unfamiliar conspecific (Bluthe et al. 1993; Kogan et al. 2000; Winslow & Camacho 1995). Use of the three-chambered apparatus for demonstrating social preference (Insel 2001) was adapted by Brodkin et al. (2004) to investigate social approach and avoidance in female mice. The center chamber was smaller than the side chambers, and Plexiglas cylinders with small air holes were used to contain the stranger. Juvenile Balb/cJ females demonstrated social avoidance, rather than social approach, in this study (Brodkin et al. 2004). The present protocol used a small wire cage to enclose the...
stimulus mouse, which allowed visual, auditory and olfactory contact between the mice, but prevented the occurrence of aggressive and other responses. This method also simplified the quantification of data. As noted by Winslow (2003), the analysis of the full behavioral series that can occur in social tests demands familiarity with the mouse ethogram, extensive training and the ability to discern the sometimes rapid and fleeting responses that mice can emit. In our experience to date with the present procedures, human observers attain a high level of proficiency after only one or two practice sessions, with high rates (greater than 95%) of interrater reliability between trained observers.

Several additional behavioral assays were performed in the initial set of juvenile male mice (C57BL/6J, DBA/2J and FVB/NJ). These assays were designed as controls for sensory and motor abilities, etc., to ensure that the test mice were physically able to perform the procedures necessary for the social tasks described above. Further, we included home cage observational periods to identify other behavioral parameters that could be useful in a mouse model of autism. Nesting and sleep patterns, and any incidents of stereotypy, hyperactivity and seizures were noted, relevant to common symptoms of autism. Observational findings in some of these behavioral domains have been reported in some targeted gene mutations, e.g. deficits in social interaction within the home cage have been reported for mice deficient in the dishevelled-1 gene (Lijam et al. 1997) or the NMDA NR1 subunit (Mohn et al. 1999). In the present study, the juvenile male mice made nests and huddled together while sleeping. General health, neurological reflexes, activity levels, motor functions and sensory abilities were normal in most regards for the strains tested. All of the inbred strains were able to locate buried food, supporting an interpretation that none were anosmic, which could have severely impacted social behaviors by blocking detection of olfactory cues emitted by the stranger mice. The FVB/NJ mice demonstrated a significantly shorter latency for locating the buried food, but as they did not have markedly higher levels of sociability or preference for social novelty, it seems likely that the shorter latency reflected higher levels of exploratory activity in these mice, as shown in Table 1.

These control parameters are not only important for the interpretation of the results from the social tests, but suggest additional components for putative mouse models of autism. For example, some autistic patients demonstrate aberrant motor responses, including repeated hand flapping, jumping and other repetitive and stereotyped behaviors (Bodfish et al. 2000; Tager-Flusberg et al. 2001). Altered sensory responses have been reported, including both hypo- and hyper-reactivity to environmental stimuli (Baranek 2002). Children with fragile X syndrome often show autistic behavior (Hagerman et al. 1986), and the mouse model of fragile X syndrome has been characterized by changes in activity (Bakker et al. 1994; Peier et al. 2000) and reactivity to acoustic stimuli (Chen & Toth 2001; Nielson et al. 2002).

Further work has suggested that the changes found in the mouse models of fragile X are dependent upon the particular background strain for the mutation (Dobkin et al. 2000; unpublished observations from our laboratory).

Development of a new animal model of a human disease is a long and iterative process. The present mouse tasks were designed to maximize face validity to some of the core symptoms of autism. Issues of construct validity and predictive value remain to be investigated. For example, the low sociability scores of the A/J strain require further consideration in terms of sex differences, age of onset and lifetime expression, previous life experiences and potentially confounding artifacts. Following detection of a social deficit in a line of mice, the model can be expanded to encompass a wider set of parameters, including other complex and sensitive measures of social behaviors, quantitated by video-tracking software. More in-depth analyses of other behaviors potentially relevant to autism will be useful, including measures of motor stereotypies, sleep dysfunction, sensory hypersensitivity and anxiety-like behavior. Potential applications of this social task include the testing of hypotheses about the etiology of autism, investigating transgenic and knockout mice with mutations in candidate genes for autism, screening large numbers of offspring obtained from chemical mutagenesis for mutations relevant to autism and comparing strains of mice with varying levels of social skills using DNA microarray and proteonomics analyses. Most importantly, the utility of these social behavior tasks in mice will require comprehensive testing to determine their predictive value for evaluating potential therapeutic approaches to treat or prevent the social deficits in autistic patients.

References


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