Genetic architecture and behavioral analysis of attention and impulsivity

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Chapter 4

Activity and impulsive action are controlled by different genetic and environmental factors

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Abstract

Both impulsivity in operant tasks and locomotor activity in a novel open field are known to predict the development of addiction-related behavior in rodents. Here, we investigated to what extent impulsivity in the 5-choice serial reaction time task and various measures of novelty exploration are controlled by shared genetic and environmental factors in twelve different inbred mouse strains. No genetic correlation was observed between the level of impulsivity and levels of activity, a low correlation was observed with traditional measures of anxiety-like behavior (impulsive strains tend to be less anxious) and a highly significant correlation was found between impulsivity and specific aspects of movement. Furthermore, we found that impulsivity and all measures of novelty exploration were under control of different environmental factors. Interestingly, in the dorsal medial prefrontal cortex, a brain region involved in impulsivity and activity in novelty exploration tests, these behavioral measures correlated with the expression of different genes (respectively *Frzb*, *Snx5, BC056474* and the previously identified *Glo1*). Taken together our study shows that impulsivity and activity in novelty exploration tests are genetically and environmentally distinct, suggesting that mouse models of these behaviors provide complementary insights into the development of substance abuse disorder.

Introduction

Impulsivity and novelty seeking are core aspects of human personality (Bouchard & McGue, 2003) that received a great deal of attention, not the least because they have been associated with substance abuse disorder (Kreek *et al.*, 2005; Wills *et al.*, 1994). In rats, individual differences in impulsivity and the locomotor response to a novel open field, a putative measure of novelty seeking are predictive of aspects of drug self-administration behavior (Belin *et al.*, 2008; Diergaarde *et al.*, 2008; Ellenbroek & Cools, 2002; Piazza *et al.*, 1989). These individual differences can arise from genetic differences among individual rats and/or from environmental factors. Given the relevance of animal models of predictive factors of addiction-related behavior, we examined whether shared, or alternatively, distinct genetic and/or environmental factors control impulsivity and various measures of novelty exploration.

In particular inbred strains of mice provide the opportunity to separate genetic and environmental factors. When kept under highly controlled conditions, differences between inbred mouse strains result from additive genetic effects and gene-by-environment interactions (Crabbe *et al.*, 1999). Thus, high correlation between measures of impulsivity and novelty exploration across strain means would indicate that these measures are controlled by common genetic or gene-by-environment interaction effects (*genetic correlation*), albeit such correlations contain some residual environmental influence due to estimation of strain means (Crusio, 2006). In contrast, behavioral differences between isogenic mice of the

same strain result from environmental effects idiosyncratic to each individual, and hence, high within-strain correlations of impulsivity and measures of novelty exploration would indicate that these are controlled by common environmental factors (*environmental correlation*).
Several operant tasks of impulsivity exist (Evenden, 1999), broadly categorized into those that measure impulsive action or impulsive choice (Winstanley *et al.*, 2006a). Here, the murine version of the 5-choice serial reaction time task (5-CSRTT; Pattij *et al.*, 2007; Robbins, 2002) was used, in which premature responses to a food predictive stimulus are a measure of impulsive action, previously shown to be predictive of increased motivation to self-administer addictive drugs in rats (Belin *et al.*, 2008; Diergaarde *et al.*, 2008).
The locomotor response in a novel open field, predictive of amphetamine self-administration (Piazza *et al.*, 1989), is the net result of several interacting behavioral constructs (most notably anxiety, novelty seeking and general activity). Therefore in this study novelty exploration was also measured in tests that differ in the anxiogenic nature of novel stimuli, the motivational salience of novel stimuli and the required activity to cover the novel stimulus. In addition to total activity, novelty exploration was also measured in terms of the locomotor strategy and location of activity.
Finally, because the dorsal part of the rodent medial prefrontal cortex (mPFC) is involved in both impulsivity in the 5-CSRTT (Muir *et al.*, 1996) and activity in a novel open field (Deacon *et al.*, 2003; Holmes & Wellman, 2008), we subsequently analyzed gene expression data of this brain region (Hovatta *et al.*, 2005) to identify genes whose expression was specifically correlating with either impulsivity or activity in a novel open field.

Materials and Methods

Subjects
Six-week-old male mice (n = 12 per strain) were obtained from Jackson Laboratory (A/J, C3H/HeJ, C57BL/6J, DBA/2J, FVB/NJ), from our own breeding colony (BXD2, BXD6, BXD11, BXD21, BXD32, and BXD40) or Taconic Farms (129S6/SvEvTac). Mice arrived in the facility in 6 different batches in a period spanning 18 weeks. Batches 1 through 5 consisted of BXD mice of different strains, batch 6 entirely consisted of the remaining 6 strains. Mice were housed individually in Macrolon cages on sawdust bedding, which were, for the purpose of animal welfare, enriched with cardboard nesting material and a curved PVC tube. A metal food cup was placed in the home cage (for the purpose of habituation; used in subsequent hypophagia test). Food (Harlan Teklad) and water was provided ad libitum, except food supply during the weeks of operant testing. All mice were habituated to the facility for 14 days before testing started. During habituation mice were familiarized 4 times, on separate

days, to a highly palatable snack (crumbles of cream cracker) placed in a metal food cup. Housing rooms were controlled for temperature, humidity and light-dark cycle (7 AM lights on, 7 PM lights off). All experimental procedures were approved by the local animal research committee and complied with the European Council Directive (86/609/EEC).

General procedure of behavioral testing

All mice were subjected to all behavioral tests, and the order of the tests was identical for all mice. Below, days are numbered with respect to the first testing day. All testing occurred exclusively between 8-12 AM. The housing and testing rooms (4 m apart) were separated by two sound attenuating doors. On testing days, mice were transferred one by one from the housing room to the testing room and immediately introduced into the test apparatus. The same experimenter performed all tests, except for daily training in the 5-CSRTT.

Home cage behavior

During the first 3 test days, the familiar snack was introduced into the familiar metal food cup in the home cage and the latency to start eating was scored (maximum duration 240 s). If the subject did not start eating within 240 s, the maximum time was assigned. The mean latency to eat was calculated over 3 days. On days 7 and 9, novel objects (metal spout and a blue plastic bottle cap respectively) were introduced into the home cage. Both latency until first touch of the object and cumulative time touching the object during 4 min were recorded manually. If a subject did not touch the object during the test, the latency was set to 240 s. The mean of both novel object sessions was used for analysis. On day 8, the familiar snack was introduced into the home cage in an unfamiliar glass cup. Latency until first touch of the unfamiliar glass cup, total duration of exploring the unfamiliar glass cup before eating, and latency to eat the snack were recorded for a maximum of 10 min. If a subject did not touch the object, the latency to explore the object was set to 600 s. If the subject did not eat the snack, the latency to eat was set to 600 s.

Novel cage

On days 4 and 11, mice were transferred to a novel clean cage with fresh bedding containing the metal cup with the familiar snack. Both the latency until the first touch of the cup and latency to start eating the snack were recorded manually. The mean of latencies of both days was used for analysis. If a subject did not eat within 720 s, the maximum time was assigned. After testing, both nesting material and tube were transferred to the novel home cage.

Open field (OF)

On day 14, mice were introduced into a corner of the white square open field (50 × 50 cm, walls 35 cm high) illuminated with a single white fluorescent light bulb

from above (130 lx), and exploration was tracked for 10 min (12.5 frames/s; EthoVision 3.0, Noldus Information Technology). Time spent in, and number of entries into the center square area (20 × 20 cm) was measured using EthoVision. The SEE software (Strategy for the Exploration of Exploration; Kafkafi *et al.*, 2005) was used to smoothen path shape to calculate the total distance moved. Furthermore, SEE uses the distribution of speed peaks to parse the locomotor data into slow local movements (lingering episodes) and progression segments, which together constitute all distance traveled. In addition to the traditional measures in the open field, describing the animal tendency to engage in exploratory behavior, SEE was used to calculate the number of progression segments and the median duration of a lingering episode. SEE also enables the calculation of measures that describe the strategy of movement once exploration has been initiated: the median distance traveled per progression segment, the median duration of a progression segment, the number of stops per distance and the median acceleration during a progression.

Dark-light box (DLB)

On day 15, mice were introduced into the dark compartment (<10 lx; 25 × 30 × 30 cm, length × width × height) of a dark-light box (TSE-Systems; Baarendse *et al.*, 2008), 15 s later the motorized black door to the identically-sized brightly lit compartment (320 lx) was opened and behavior was tracked for 5 min using infrared beams (1.4 cm apart). Complete transitions between dark and light compartment yielded the latency to enter the light, the number of entries into the light, and time spent in the light compartment.

Elevated plus maze (EPM)

On days 16 or 17, all mice were introduced into the same closed arm of an EPM (arms 30 × 6 cm, walls 35 cm high, elevated 50 cm above the ground), facing the closed end of the arm. The EPM was illuminated with a single white fluorescent light bulb from above (130 lx) and exploratory behavior was video tracked for 5 min (12.5 frames/s, EthoVision 3.0, Noldus Information Technology). The border between center and arm entries was defined at 2 cm into each arm, producing the number of entries into the open arms, into the closed arms, onto the center platform, and time spent on the open arms. In addition, latency to explore was defined by the time between introduction onto the maze and the first appearance in the maze center.

5-CSRTT

Between days 23 and 28, mice were food-restricted to gradually decrease their body weight to 90% of their free feeding weight. Operant chambers were equipped with 5 response holes, a food magazine at the opposite wall and a house light (MEDNPW-5M, Med Associates, St. Albans, VT, USA), and placed in sound attenuating ventilated cubicles. Both response holes and food magazine

contain yellow LED stimulus lights and infrared response detectors. On day 28, mice were placed into the operant chambers for a 20 min habituation session during which none of the stimulus lights was switched on. On days 29, 30 and 31, purified precision pellets (12 mg, Formula P, Research Diets, New Brunswick, NJ, USA) were distributed into the magazine at random fixed inter trial intervals (ITI; 4, 8, 16 and 32 s), which coincided with switching on the magazine stimulus light. An ITI was only initiated when the previous pellet had been collected as indicated by a magazine response, during which the magazine stimulus light was off. A session ended after 25 min or sooner when the criterion of 50 pellets was reached. During the 3rd session the mean number of responses into the empty non-illuminated magazine was recorded for further analyses. In the next sessions, a trial started by the illumination of all 5 stimulus holes. An instrumental response into any of these holes switched off the light in all 5 stimulus holes, switched on the stimulus light in the magazine and delivered a food pellet into the magazine. An ITI was only initiated when the previous pellet had been collected as indicated by a magazine response, after which the magazine stimulus light was off. Sessions lasted for 25 min or 60 earned food pellets. As soon as mice earned 50 or more pellets in two sessions, or after 17 sessions, they commenced to the next phase. In this phase, trials started by the illumination of only one stimulus hole. Responses into the non-illuminated holes were without consequence. As soon as mice earned 50 or more pellets in two sessions, or after 10 sessions, they commenced to the actual 5-CSRTT procedure. In a 5-CSRTT session, a trial started with a response of the subject into the illuminated magazine, which switched off the magazine light. After a delay of 5 s (ITI) a stimulus was switched on in one of the 5 stimulus holes for a limited duration (stimulus duration). A response in the correct stimulus hole, during stimulus presentation or within the limited hold of 4 s after termination of the stimulus, switched on the magazine light and delivered a food pellet. Both incorrect responses into non-illuminated stimulus holes and omissions of a response resulted in a 5 s time-out period, during which all stimulus lights and the house light were switched off. When the time-out period ended, both the house light and the magazine light were switched on and the subject could start the next trial. A premature response into a non-illuminated stimulus hole during the ITI also resulted in a time-out period, and a subsequent response into the illuminated magazine restarted the same trial. The percentage of omissions was defined as [100 × (omissions) / (omissions + number of correct and incorrect responses)]. The accuracy was defined as [100 × (number of correct responses) / (number of correct and incorrect responses)]. The percentage of premature responses (impulsivity) was defined as [100 × (number of premature responses) / (number of omissions + correct + incorrect responses)]. In the first 5-CSRTT session the stimulus duration was set to 16 s, which was gradually decreased in subsequent sessions to 8, 4, 2, 1.5 and 1 s if the subject reached the criterion (omissions < 30%, accuracy > 60%, started trials > 50) or after 10 sessions at the

same stimulus duration. Baseline 5-CSRTT performance was calculated from the 6th until 10th session at stimulus duration of one second. During these baseline sessions, the average percentage of premature responses was recorded and used for further analyses together with the average latency to collect the reward from the magazine after a correct response (calculated only if a subject responded correctly for 5 times during one or more baseline sessions). During the 14th session at stimulus duration of 1 second, the ITI was programmed to vary between three fixed intervals (5, 7.5 and 12.5 s), with each interval occurring an equal number of times for all mice.

Statistical analyses of behavioral measures

Before statistical analyses and graphical representation, all data were transformed to a normal distribution by normal scores transformation according to Blom's method (see Altman, 1991), except for **Figure 2** in which data is displayed in the unit of measurement Estimates of the genetic effect size (narrow sense heritability) were calculated according to Hegmann and Possidente (1981) using a custom function (Microsoft Excel 2003), which takes the differences in the number of animals per group into account when estimating the within and between-strain variance (Lynch & Walsh, 1998) as previously implemented by others (Heimel *et al.*, 2008). Environmental correlations between measures were calculated from the individual performance of all 144 individual mice after subtraction of their respective strain mean. Genetic correlations between measures were calculated using mean strain performances and using bootstrap analysis, 95% confidence intervals were calculated from the genetic correlations of 2000 resampled inbred strain means. Principal component analysis, discriminant analysis, and correlation analyses were performed with the Statistical Package for Social Sciences version 14.0 (SPSS, Chicago, IL, USA). For PCA, cases were excluded pair-wise in case of missing values. The PCA solution was promax rotated. For discriminant analysis, missing data were replaced by strain means. Discriminant analysis was performed by entering all 29 measures simultaneously into the analysis, followed by a varimax rotation of the structure matrix.

Gene expression

Raw gene expression data of 6 of the 12 strains in the study was downloaded (cingulate cortex samples of 2-5 male mice per array, 2-3 arrays per strain, Affymetrix U74Av2 arrays ((Hovatta *et al.*, 2005) NCBI GEO database, #GDS1406) and normalized using Robust Multi-chip Analysis (ArrayAssist version 3.2, Stratagene, La Jolla, CA, USA). The Excel plug-in of Significance Analysis of Microarrays (Tusher *et al.*, 2001) was used to generate a list of probe sets that were differentially expressed between these 6 strains (FDR < 0.01).

For real time quantitative PCR measurements (RT-qPCR), brains were rapidly frozen in –60 to –70ºC isopentane. In a cryostat, the brains were sliced into 150

μm thick coronal sections and the dorsal mPFC, encompassing the cingulate and prelimbic cortices, was dissected (according to Paxinos & Watson (Paxinos & Watson, 1998); Cg1, Cg2 and PrL from +2.68 to +1.34 mm anterior to Bregma). RNA was isolated, DNAse-treated and reverse transcribed to cDNA using random hexanucleotide primers (Spijker *et al.*, 2004). Gene expression was analyzed (ABI Prism® SDS 7900, Applied Biosystems) using a SYBR Green approach (300 nM gene specific primers and the cDNA equivalent of 40 ng RNA in a total volume of 10 μl) and normalized to the geometric mean of 2 housekeeping genes (GAPDH and HPRT). Normalized gene expression levels (GE*norm*) were calculated from detection cycle threshold values (Ct) as follows: GE*norm* = 2 ^ (- ($Ct_{gene\ x} - Ct_{HKgenes}$))

Results

Strain means of impulsivity in the 5-CSRTT and measures of novelty exploration in five different test settings, i.e. in the home cage, novel cage, open field, dark-light box and on the elevated plus maze are presented in **Supplementary Figure 1**. Together, the battery of impulsivity and novelty exploration tests yielded a set of 29 behavioral measures. The genetic effect size of these measures varied between 9% and 54% (**Fig. 1**), indicating that both environmental and genetic variation contribute to these measures. Data were obtained for 144 subjects, but for 9 subjects (of 6 different strains) magazine latencies in the 5-CSRTT were missing (too few correct responses) and for 1 subject open field data was missing. All 12 strains displayed both average and extreme performance (**Fig. 1**), indicating that all strains contributed to the complex pattern of strain differences and no strain constituted an outlier on all measures.

Stability of between-strain and within-strain differences in impulsivity

Reliability analysis revealed that the level of impulsivity was stable across 5 baseline sessions (Cronbach's $\alpha = 0.91$). Subsequent reliability analyses on the strain means (Cronbach's $\alpha = 0.99$) and on individual performance after subtraction of strain mean (Cronbach's $\alpha = 0.83$) indicated that both genetic and environmental factors contribute to the stability of impulsivity across baseline sessions. We observed a significant strain difference ($F(11,132) = 13.07$, $p < 0.001$) in the baseline level of impulsivity (**Fig. 2**) with an estimated genetic effect size of 34%. A within-session variable ITI, which makes the task temporally less predictable, significantly increased impulsivity (ITI; $F(1,128) = 378.60$, $p < 0.001$) in all strains (post hoc $p < 0.05$ for all strains) compared to baseline levels (**Fig. 2**). The known visual impairment of some strains (Wong & Brown, 2006) did not prevent any of these strains to perform significantly above chance level (20%, see **Supplementary Fig. 2**). Moreover, all 144 individual mice showed response accuracies above chance level (data not shown).

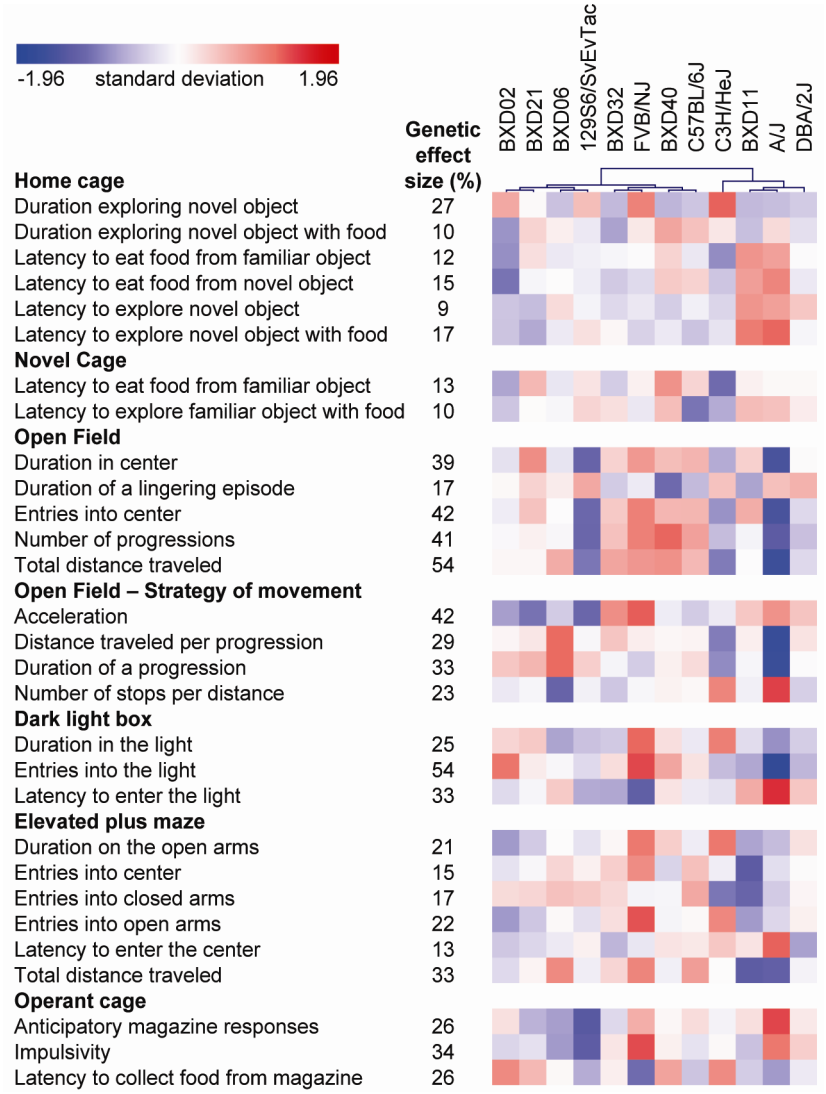

Figure 1 | Behavioral differences between twelve inbred strains of mice. In the left panel, tests are ordered chronologically and their measures are ordered alphabetically from top to bottom. The genetic effect size was estimated for each measure. The right panel is a graphical representation (heat map) of strain means after normal transformation of the data (the distributions of all measures have a mean of 0 and a standard deviation of 1). Intense colors represent a positive (red) or negative (blue) deviation from the mean (white) equal to 1.96 times the standard deviation as indicated in the legend (top right). The cluster tree represents the average distance (Pearson correlation) between the 12 inbred strains used (n = 12 per strain). Note that BXD strains segregate beyond the parental lines (C57BL/6J and DBA/2J) showing transgression for several phenotypes.

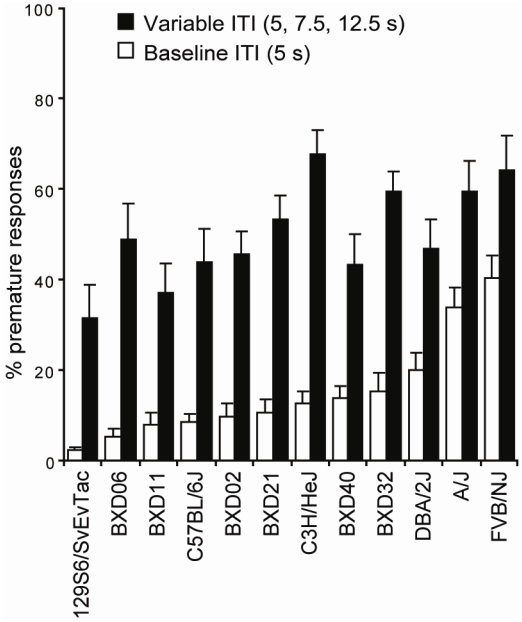

Figure 2 | Levels of impulsivity of 12 inbred strains during standard and variable inter-trial interval. The level of impulsivity, measured as percentage of premature responses of 12 inbred strains of mice ranked according to baseline performance (ITI of 5 s, white bars) and during a session with variable ITI (black bars).

Lack of genetic and environmental correlation between impulsivity and distance traveled in a novel open field

The inbred strains significantly differed in distance traveled in a novel open field ($F(11,132) = 21.60$, $p < 0.001$; estimated genetic effect size 54%). No significant correlation was observed between impulsivity and distance traveled in a novel open field when calculated across all 144 individual mice (i.e. no phenotypic correlation; **Fig. 3a**; Pearson $r = 0.01$, $p = 0.88$), nor when calculated across strain means (i.e. no genetic correlation; **Fig. 3b**; Pearson $r = 0.02$, $p = 0.96$). Impulsivity and distance traveled in a novel open field did also not correlate when calculated across individual mice after subtraction of strain means (i.e. no environmental correlation; **Fig. 3c**; Pearson $r = 0.01$, $p = 0.92$).

Lack of genetic correlation between impulsivity and novelty exploration in other tests

Inbred strain means were used to calculate genetic correlations between impulsivity and 28 measures of the test battery (**Table 1**). As inbred strain means calculated from the average of 12 individuals per strain are estimates of the real strain mean, they are not free of environmental variation. Therefore, 95% bootstrap confidence intervals were constructed for each genetic correlation based on resampling (2000 bootstrap draws) from the twelve subjects of each inbred strain (**Table 1**). No significant genetic correlations were observed between impulsivity and measures describing the activity in the novelty exploration tests. Some genetic correlations with traditional measures of anxiety-like behavior were not statistically significant even though the 95% bootstrap

confidence intervals did not include zero, illustrating that the sampling individuals in the current study may have contributed to the suppression of a possible genetic correlation. Strong significant genetic correlations were observed between impulsivity and measures describing the strategy of movement after initiation of exploration (median locomotor acceleration (Pearson r = 0.81, p < 0.01), the median duration of a progression segment in the open field (Pearson r = -0.70, p < 0.05), and the number of anticipatory magazine responses during the shaping phase of the 5-CSRTT (Pearson r = 0.81, p < 0.01; **Table 1**).

In contrast to impulsivity, total distance traveled in the open field significantly correlated with measures of activity from several other tests (**Table 1**). More specifically, genetic correlations were observed between total distance traveled in the open field and on the EPM and DLB (frequency of light compartment entries) and with all other measures obtained from the open field, except median locomotor acceleration.

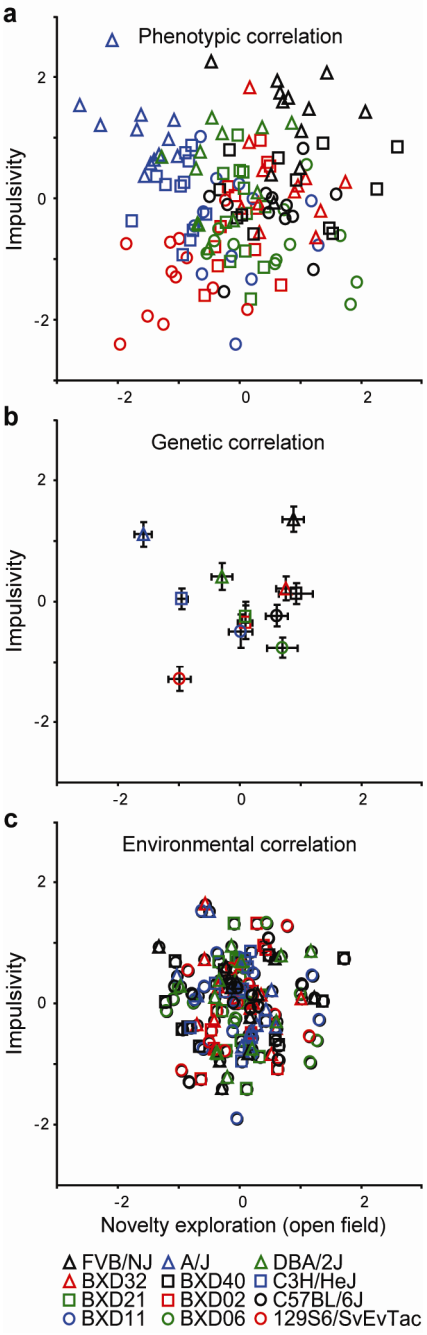

Figure 3 | Impulsivity does not correlate with distance traveled in a novel open field**.** (**a**) No significant phenotypic correlation (i.e. across all 144 mice in the experiment) was observed between percentage of premature responses and distance traveled in a novel open field. On both the x- and y-axis, scores of individual mice are displayed as the deviation from the mean of all 144 mice (fold difference). (**b**) No significant genetic correlation (i.e. across 12 strain means) was observed between percentage of premature responses and distance traveled in a novel open field. On both the x- and y-axis, strain mean scores are displayed as the deviation from the mean of all 12 strains (fold difference). (**c**) No significant environmental correlation (i.e. across all 144 mice in the experiment after subtraction of the respective strain mean) between percentage of premature responses and distance traveled in a novel open field was detected. On both the x- and y-axis, scores of individual mice are displayed as the deviation from its respective strain mean (fold difference).

Table 1 | Genetic and environmental correlation of all measures with impulsivity and the distance traveled in a novel open field.

	Impulsivity		**Distance traveled in open field**	
	r_G	r_E	r_G	r_E
Home cage				
Duration exploring novel object				
Duration exploring novel object with food				
Latency to eat food from familiar object				
Latency to eat food from novel object				
Latency to explore novel object			-.38#	
Latency to explore novel object with food			-.54#	
Novel cage				
Latency to eat food from familiar object				
Latency to explore familiar object with food				
Open field				
Duration in center			**.84****	**.43****
Duration of a lingering episode			**-.71****	**-.31****
Entries into center			**.90****	**.71****
Number of progressions			**.92****	**.54****
Total distance traveled			NA	NA
Open field – Strategy of movement				
Acceleration	**.81****			
Distance traveled per progression	-.43#		**.78****	**.51****
Duration of a progression	**-.70***		**.58***	**.34****
Number of stops per distance	.46#		**-.67***	**-.44****
Dark light box				
Duration in the light	.31#			
Entries into the light			**.77****	**.35****
Latency to enter the light				
Elevated plus maze				
Duration on the open arms	.43#			
Entries into center			.34#	**.34****
Entries into closed arms			.38#	
Entries into open arms	.51#			
Latency to enter the center			-.57#	
Total distance traveled			**.63***	**.37****
Operant cage				
Anticipatory magazine responses	**.81****			
Impulsivity	NA	NA		
Latency to collect food from magazine				

The Pearson correlation coefficients (r) of the genetic correlation (r_G) and the environmental correlation (r_E) with impulsivity and the exploration of a novel open field. Correlations with $|r| < 0.3$ are not displayed. Significant Pearson correlation coefficients are indicated in **bold** (* $p < 0.05$; ** $p < 0.01$). #For these non-significant genetic correlations bootstrap analyses showed that the Pearson correlation coefficients were greater than zero in more than 95% of the bootstrapped samples.

Discriminant analysis of impulsivity and novelty exploration

Multivariate analyses can be used to get insight in the structure of genetic correlations underlying impulsivity and novelty exploration. Although inbred strain means can be used in a PCA to extract genetic variation common to multiple measures, the limited availability of strain means (i.e. twelve) in this study made PCA inappropriate. Instead, a discriminant analysis was used. This analysis separates the complex pattern of strain differences into dimensions of behavior by using linear combinations (discriminant functions) of the original 29 behavioral measures. Those measures that show large differences between-strains, and hence are more influenced by genotype, receive larger weights in the discriminant functions than measures that show little between-strain differences. The use of twelve strains allowed for the extraction of a maximum of 11 discriminant functions. According to Wilks' lamba tests, the first 10 functions ($p < 0.001$) were required to explain the dimensions of the strain differences. Whereas the standardized coefficients for each variable in the discriminant function can be used to determine redundant measures in a test battery, correlation of the discriminant functions with the original measures can be used to interpret the discriminant functions (for details see Stevens, 2002). To interpret the discriminant functions in terms of genetic dimensions, we therefore calculated correlations between the strain means on the discriminant scores and the strain means on all 29 measures (**Table 2**). Impulsivity correlated strongly with discriminant function 1, together with two measures that describe the strategy of movement once exploration has been initiated and the number of anticipatory magazine responses during the shaping phase of the 5-CSRTT (see also **Table 1**). Measures that describe the tendency of an animal to engage in exploration of novelty correlated best with function 2 (exploration of OF and DLB), function 5 (exploration of novel object and DLB), function 7 (exploration of OF), function 8 (exploration of EPM), function 9 (exploration of novel object with food and EPM) and function 10 (exploration of novel object with food). Apart from its correlation with function 1, strategy of movement correlated well with function 4. Impulsivity significantly correlated with function 4 as well. The discriminant analysis was also performed after excluding subjects with missing values (i.e. excluding 10 subjects of 6 different strains) to investigate whether substituting missing data by strain means had any undue effects. The exclusion affected the discriminant analysis marginally; the most noteworthy change concerned the increased correlation of impulsivity with discriminant 3 ($|r| < 0.6$) which in turn correlated with measures describing the strategy of movement (like function 1) and the latency to explore a novel object with and without food. In summary, impulsivity and strategy of movement seem to constitute similar genetic dimensions, whereas there is less overlap between impulsivity and genetic dimensions describing other aspects of novelty exploration.

Table 2 | Impulsivity and novelty exploration correlate with different genetic dimensions.

	Discriminant functions									
	1	2	3	4	5	6	7	8	9	10
Home cage										
Duration exploration novel object					**.90**					
Duration exploration novel object with food										**.81**
Latency to eat food from familiar object										
Latency to eat food from novel object					-.59					
Latency to explore novel object			-.60		-.65	.65			-.67	
Latency to explore novel object with food			-.69			.60			**-.73**	
Novel home cage										
Latency to eat food from familiar object										
Latency to explore familiar object with food										
Open field										
Duration in center		**.72**					**.96**			
Duration of a lingering episode		**-.84**								
Entries into center		**.82**					**.91**			
Number of progressions		**.90**					**.76**			
Total distance traveled		**.93**		.67			.70			
Open field – Strategy of movement										
Acceleration	**.87**		-.63			.60				
Distance traveled per progression		.64		**.95**						
Duration of a progression	**-.73**		.70	**.97**						
Number of stops per distance				**-.95**						
Dark light box										
Duration in the light					**.89**					
Entries into the light		**.80**	.65		.61					
Latency to enter the light					-.67				-.61	
Elevated plus maze										
Duration on the open arms								**.92**		
Entries into center									**.89**	
Entries into closed arms									**.85**	
Entries into open arms								**.90**		
Latency to enter the center				-.69						
Total distance traveled									**.82**	
Operant cage										
Anticipatory magazine responses	**.89**			-.60						
Impulsivity	**.95**			-.59						
Latency to collect food from magazine						**-.96**				

Pearson correlation coefficient of the genetic correlation between the ten discriminant functions and 29 measures were calculated across strain means. Pearson correlation coefficients ($r \geq 0.576$, $p<0.05$ and $r \geq 0.708$, $p<0.01$ in **bold**) were used to interpret discriminant functions.

Lack of environmental correlation between impulsivity and novelty exploration in other tests

In addition to distance traveled in the open field, we analyzed the environmental correlations between impulsivity and 27 other obtained measures (**Table 1**). No substantial environmental correlations between impulsivity and other behavioral measures were observed ($|r| > 0.3$). In contrast, significant environmental correlations were observed between the total distance traveled in the open field and several measures of exploration in other tests (**Table 1**).

To better understand the structure of the environmental factors that underlie novelty exploration a PCA was performed on the matrix of environmental correlations among all 29 measures (**Table 3**). Ten principal components (PCs) were extracted (Eigenvalues > 1), together explaining 76% of the variance. Impulsivity and total distance traveled in the open field loaded onto different principal components (PC 10 vs. PC 1 & 2, respectively) clearly indicating that these two measures are influenced by different environmental factors.

Gene expression correlations with impulsivity and distance traveled in a novel open field

Previously acquired microarray data of gene expression in the dorsal mPFC were available for 6 (A/J, C3H/HeJ, C57BL/6J, DBA/2J, FVB/NJ and 129S6/SvEvTac) out of the 12 inbred strains in this study (Hovatta *et al.*, 2005). Out of ~12,000 probe sets on the arrays, 82 probe sets were differentially expressed (FDR < 1%) among these six strains, and correlation of their expression level with impulsivity and total distance traveled in the open field was evaluated. Fourteen probe sets with a Pearson correlation coefficient > 0.73 ($p < 0.1$) with either impulsivity (7 probe sets) or total distance traveled in the open field (7 probe sets) were selected (**Table 4**). Using RT-qPCR, the expression level of the respective genes was measured in the dorsal mPFC of individual mice from all 12 strains that were subjected to the test battery in this study. Correlations between expression level of these 14 genes and impulsivity or total distance traveled in the open field were analyzed. Significant correlations ($p < 0.05$) were observed between 3 genes and impulsivity (*Frzb*, $r = 0.71$; *Snx5*, $r = 0.64$; *BC056474*, $r = 0.59$) and 1 gene and total distance traveled in the open field (*Glo1*, $r = -0.73$; **Fig. 4**). Thus, alike the different genetic and environmental factors controlling impulsivity and the total distance traveled in a novel open field, also gene expression in the dorsal mPFC mapped differently onto these behaviors.

Table 3 | Impulsivity and novelty exploration load onto different environmental factors.

	Principal components									
	1	2	3	4	5	6	7	8	9	10
Home cage										
Duration exploration novel object			**-.78**							
Duration exploration novel object with food							**.98**			
Latency to eat food from familiar object			.64							
Latency to eat food from novel object			.45				.48			
Latency to explore novel object			**.75**							
Latency to explore novel object with food			.46						.65	
Novel home cage										
Latency to eat food from familiar object			.58							
Latency to explore familiar object with food			.53		-.41					
Open field										
Duration in center		**.84**								
Duration of a lingering episode	.53	-.47								
Entries into center		**.93**								
Number of progressions		.66								
Total distance traveled	.45	**.74**								
Open field – Strategy of movement										
Acceleration								**.80**		
Distance traveled per progression	**.99**									
Duration of a progression	**.91**									
Number of stops per distance	**-.99**									
Dark light box										
Duration in the light					**.82**					
Entries into the light					.74					
Latency to enter the light					**-.82**					
Elevated plus maze										
Duration on the open arms				**.96**						
Entries into center						**.78**				
Entries into closed arms						**.96**				
Entries into open arms				**.94**						
Latency to enter the center									.66	
Total distance traveled				.40		.58				
Operant cage										
Anticipatory magazine responses								-.69		
Impulsivity										**.86**
Latency to collect food from magazine										**.70**

The PCA solution was promax rotated and the loadings from the oblique rotated pattern matrix are shown. Loadings < 0.4 are omitted, loadings > 0.7 in **bold**.

Discussion

After separately analyzing genetic and environmental variation, we found no evidence for either a genetic or an environmental correlation between impulsivity and total distance traveled in a novel open field or activity in other novelty exploration tests. In addition, we showed that impulsivity and activity in a novel open field correlated with different genes in terms of gene expression in the dorsal mPFC.

Operant procedures, including the 5-CSRTT, allow measurement of the same behavior across multiple test sessions, unlike most novelty-related tests that by their nature can only be novel during the first session. By repeatedly measuring the individual level of impulsivity, we were able to show that impulsivity was stable across multiple test sessions. Reliability analyses on within-strain and between strain differences in impulsivity indicated that both environmental and genetic factors contribute to this stability of impulsivity in mice.

The 5-CSRTT employed in the current study requires animals to acquire a complex sequence of behaviors. We have two reasons to believe that strain differences in impulsivity are not the result of strain differences in task

Table 4 | Gene expression correlates of impulsivity and exploration of a novel open field.

Probe ID Affimetrix U74v2	Gene Symbol	Correlation with Array data	Correlation between array and RT-qPCR	Correlation reproduced using RT-qPCR
Impulsivity				
101579_at	Srp9	-0.96#	-0.72	No
99849_at	1200016E24Rik	0.89#	NA	NA[1]
101516_at	Cd59a	0.83#	NA	NA[2]
92621_at	Pcbp2	-0.79#	-0.99	No[3]
104225_at	Snx5	0.74#	0.86	0.65*
104672_at	Frzb	0.74#	0.91	0.71*
98516_at	BC056474	0.74#	0.56	0.61*
Distance traveled in the open field				
93269_at	Glo1	-0.92#	0.80	-0.73*
95430_f_at	Spg21	0.87#	-0.61	No
92995_at	Vsnl1	-0.84#	0.17	No
98531_g_at	Gas5	0.84#	0.09	No
93964_s_at	Ddx6	-0.76#	-0.43	No
97710_f_at	C530046L02Rik	0.75#	-0.79	No
96302_at	Sfrs7	-0.75#	-0.48	No

#Pearson correlation coefficient > 0.729 ($p < 0.1$) with impulsivity or the total distance traveled. *The correlation between gene expression level and impulsivity or the total distance traveled was reproduced at a more stringent level ($p < 0.05$) using RT-qPCR. [1]Probeset sequence corresponds to a repetitive sequence in the mouse genome of viral origin, no RT-qPCR was performed. [2]Primer sequences perfectly matched target, but no product was detected after 40 PCR cycles. [3]Correlation with impulsivity was significant ($p < 0.05$), but of opposite direction compared to micro array data.

acquisition or strategy to solve the task. First, general impairments in the acquisition of the task are unlikely, since under baseline task requirements all inbred strains made significantly more correct responses than could be expected only by chance. Secondly, manipulation of the temporal predicttability of the task by varying the ITI (Robbins, 2002) significantly increased impulsivity in all strains. This indicates that all strains displayed more difficulties in withholding responses particularly when the ITIs were long, suggesting similar inhibitory control mechanisms.

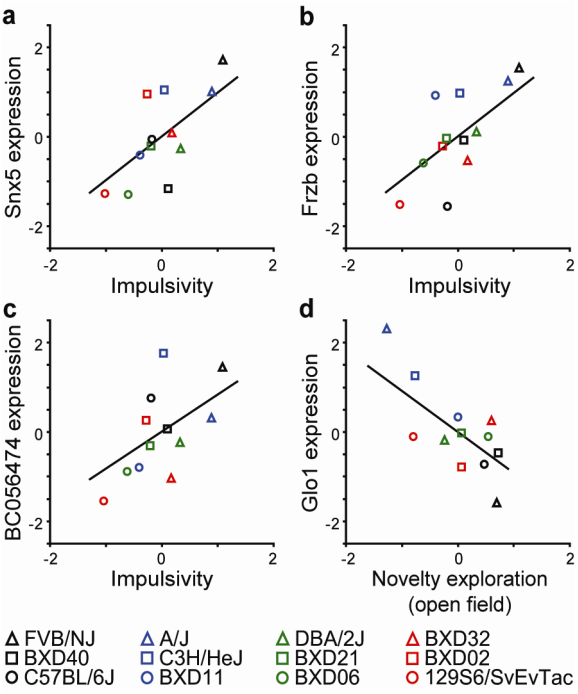

Figure 4 | Correlation of gene expression with impulsivity and distance traveled in a novel open field. Scatter plots of the significant correlations between normal transformed levels of (**a - c**) impulsivity (% premature responses) and (**d**) the distance traveled in a novel open field and the level of expression of four genes in the mPFC. Normalized levels of gene expression are displayed as deviation from the mean expression level (GE*norm*).

In particular the behavior of the A/J strain of mice exemplified the absence of a genetic correlation between impulsivity and measures of activity. The A/J strain of mice is well known for its docile, inactive appearance, while it turned out to be the second most impulsive strain in this study. Furthermore, we noticed the particular good performance of the 129S6/SvEvTac strain with respect to high accuracy and low impulsivity, in line with the performance of related 129S2/SvHsd strain published previously (Pattij *et al.*, 2007).

Because novelty exploration in tests such as the open field does not only depend on the animal's tendency to explore novelty but also on the induction of a state of anxiety and general levels of activity, we measured novelty exploration in several tests that varied in anxiogenic nature of the novel stimulus, motivation to move, and the required activity to cover the novel stimulus. Yet, no significant environmental or genetic correlations were found between impulsivity and measures of activity in these novelty exploration tests, indicating that impulsivity and activity in novelty exploration tests are influenced by different environmental and genetic factors.

In contrast to general measures of activity, specific measures of movement did genetically correlate with impulsivity, most notably locomotor acceleration. Acceleration was previously reported to be genetically different from other locomotion-related measures as well (Kafkafi *et al.*, 2005). Bootstrap analyses indicated that the presence of some environmental variation in the genetic correlations (see Introduction) may have prevented significant correlations between impulsivity and traditional measures of anxiety-like behavior (e.g. the duration on open arm and duration in the light compartment). This finding suggests that correlations between impulsivity and anxiety-like behavior may reach statistical significance in a different or larger sample of mice.

It is well known that prior experience affects behavior in subsequent tests (Henderson, 1969; McIlwain *et al.*, 2001). To minimize carry over effects in our battery, we administered the tests in the order of increasing putative anxiogenic content. However, in order to calculate environmental (within-strain) correlations between multiple measures in the test battery it was necessary to subject each individual mouse to all tests in the battery. With respect to the environmental correlations, we did not control the rearing environment of the subjects in this study. Hence, it could be possible that in the current study not all environmental factors were present that might have affected both impulsivity and novelty exploration.

In this study, genetic correlations were calculated from differences between inbred mouse strains. Between-strain differences result from additive genetic effects and gene-by-environment (e.g. laboratory) interactions (Crabbe *et al.*, 1999). Therefore, we note that the absence of genetic correlations between impulsivity and novelty exploration in our study does not entirely rule out the possibility of a correlation when these experiments are repeated with the same strains obtained from a different vendor, or when performed in a different laboratory. Furthermore, 6 out of 12 strains used in this study were recombinant inbred strains derived from a cross between C57BL/6J and DBA/2J (BXD strains). The behavioral phenotypes of these BXD strains transgressed beyond the phenotype of their parents on several measures, likely due to the novel combinations of parental loci in these strains, indicating that the BXD strains contributed to the genetic diversity in the current study. In addition, we calculated genetic correlations between impulsivity and all other measures while omitting the BXD strains, but no significant genetic correlations emerged other than those presented in **Table 1**. This suggests that the inclusion of recombinant inbred strain with common inbred strains did not specifically influence our results.

The dorsal part of the mPFC has been implicated in impulsivity as measured in the 5-CSRTT (Muir *et al.*, 1996) and activity in novelty exploration tests (Deacon *et al.*, 2003; Holmes & Wellman, 2008). Using previously acquired microarray data of the dorsal mPFC, we observed correlations between impulsivity and the expression level of the genes *Frzb*, *Snx5*, and *BC056474*.

These correlations were confirmed by RT-qPCR in the dorsal mPFC of mice that were subjected to the test battery in this study. The exploration of a novel open field showed a high correlation with the expression of *Glo1*, a gene involved in glutathione homeostasis. This correlation between expression of *Glo1* and behavior in an open field has been observed before (Ditzen *et al.*, 2006; Hovatta *et al.*, 2005). Knockdown and overexpression of *Glo1* in the dorsal mPFC, respectively decreased and increased time spent in the center of the open field, indicating that *Glo1* expression levels in the dorsal mPFC are crucial to behavior in the open field. The correlations between impulsivity and *Frzb*, *Snx5* and *BC056474* gene expression have not been described before. Frzb (Sfrp3) is a member of the secreted frizzled-related protein family that inhibits the Wnt signaling pathway. Among other functions, Wnt signaling is important in axon path finding (Bovolenta *et al.*, 2006), and synapse structure and function (Ataman *et al.*, 2008) Interestingly, *Frzb* was downregulated by acute ethanol treatment in the mPFC of mice (Kerns *et al.*, 2005), indicating that the expression of this gene is regulated by at least this type of drug of abuse. From the other two identified genes, Snx5 belongs to the sorting nexin family. Members of this family are involved in intracellular trafficking, and Snx5 may be involved in trafficking of cell surface receptors (Otsuki *et al.*, 1999). The gene BC056474 is an uncharacterized conserved protein (www.informatics.jax.org). The involvement of both Snx5 and BC056474 in behavioral functions is essentially unclear and needs to be established.

In conclusion, we provide evidence that two predictors of addiction-related behavior in rodents, namely impulsivity and activity in novelty exploration tests, which may be processed by similar underlying neuronal systems such as the mPFC, are under the control of different genetic and environmental factors. Rodent models of impulsivity and activity in novelty exploration tests will therefore act complementary in our understanding of the mechanisms underlying susceptibility for substance abuse (Belin *et al.*, 2008; Diergaarde *et al.*, 2008; Ellenbroek & Cools, 2002; Fattore *et al.*, 2009; Piazza *et al.*, 1989), and research defining the role of the identified genes may contribute to the understanding of the different molecular mechanisms involved.

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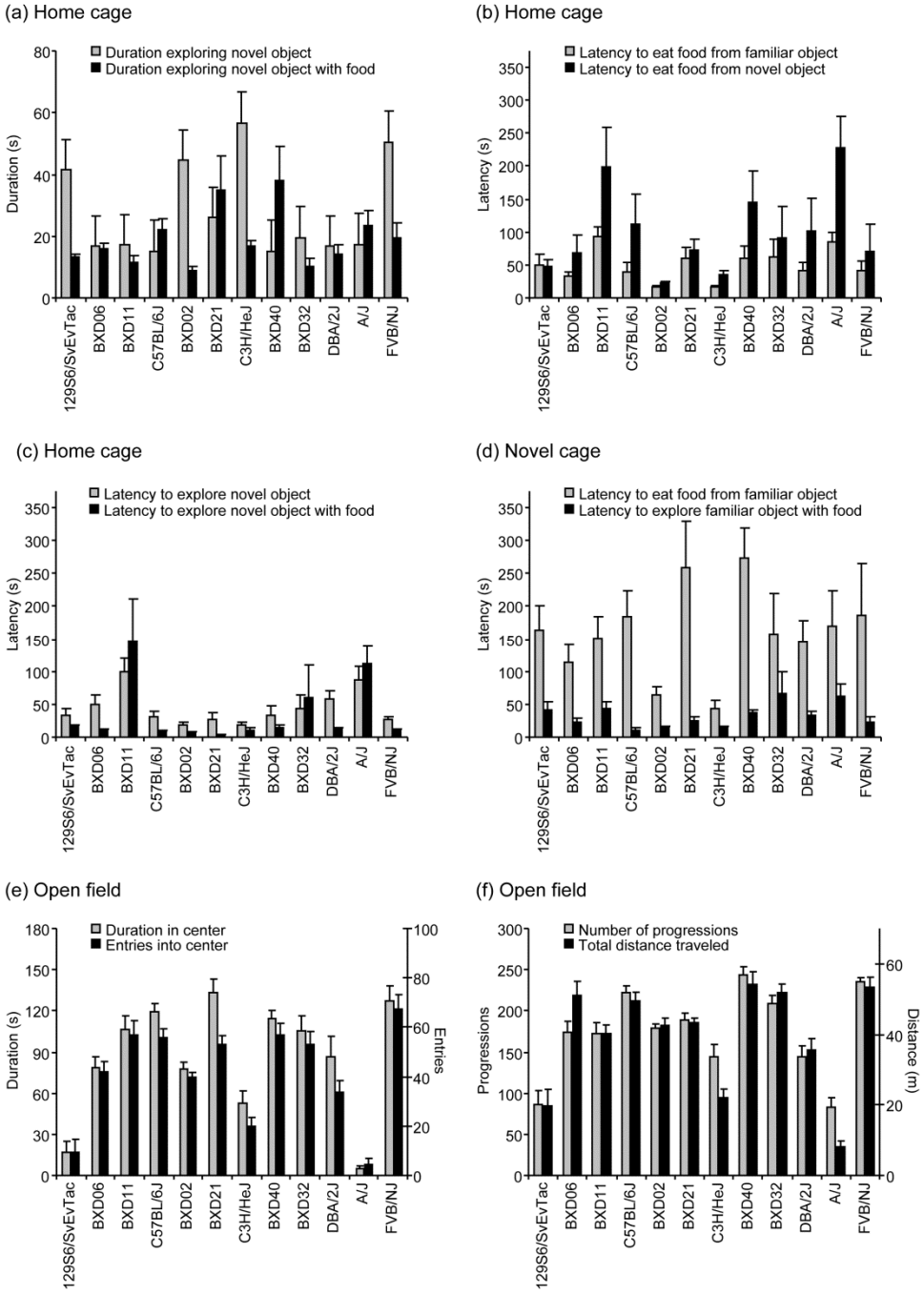
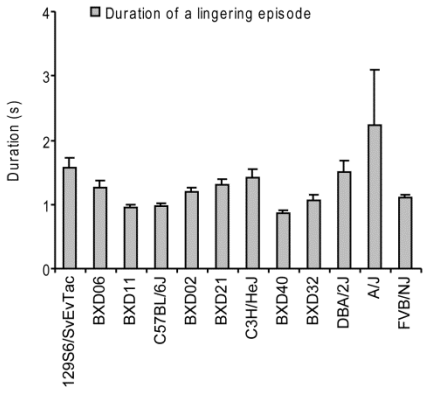
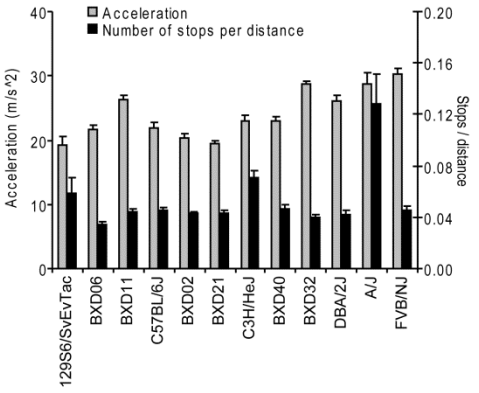
Supplementary data



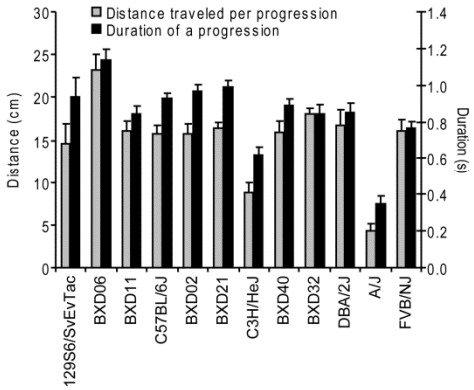
(g) Open field



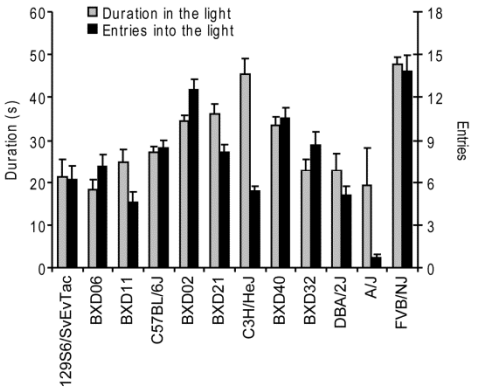
(h) Open field - Strategy of movement



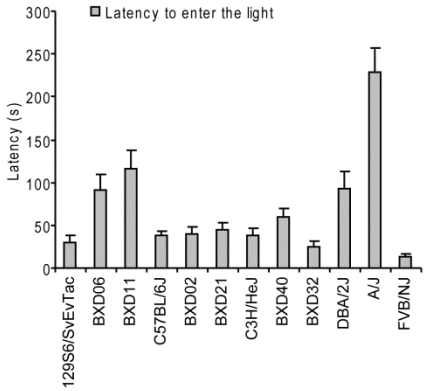
(i) Open field - Strategy of movement



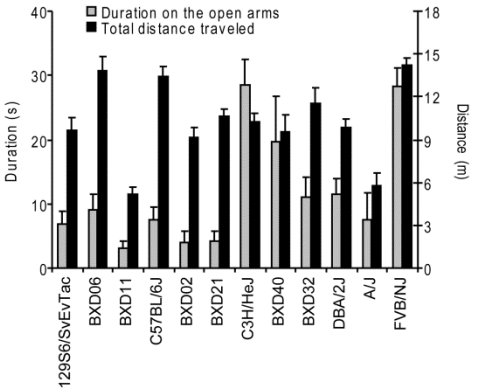
(j) Dark light box

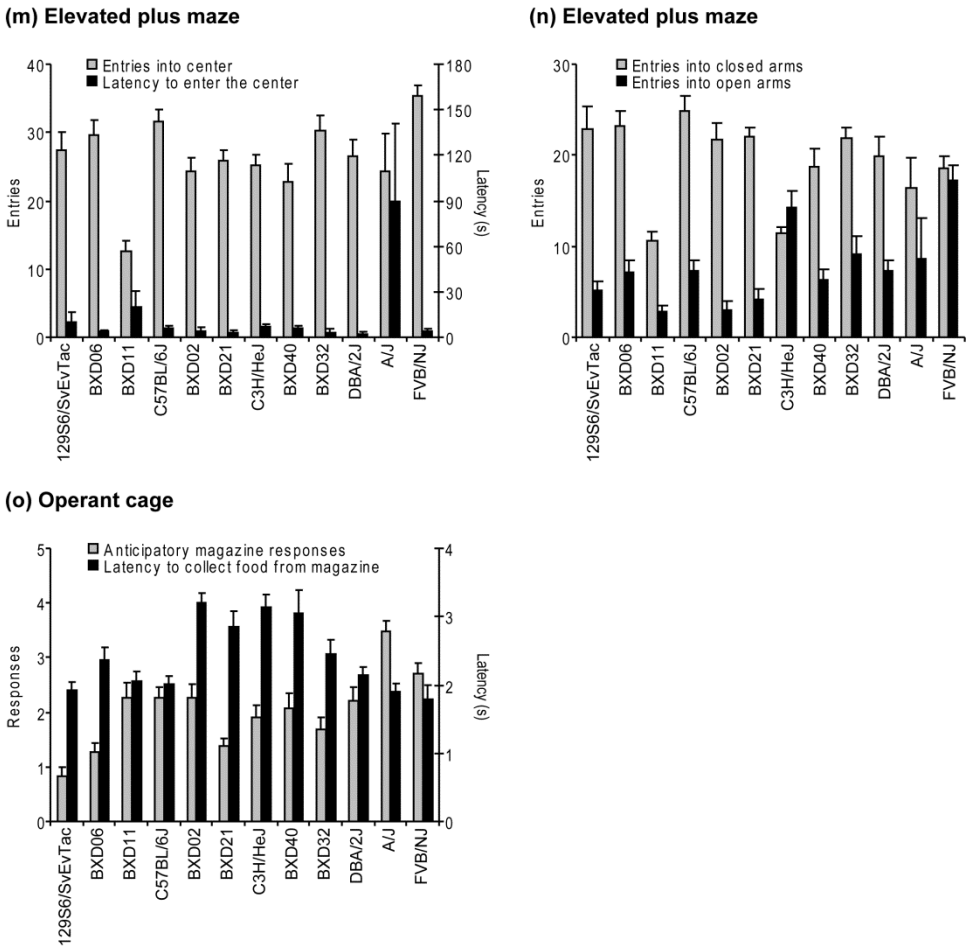


(k) Dark light box

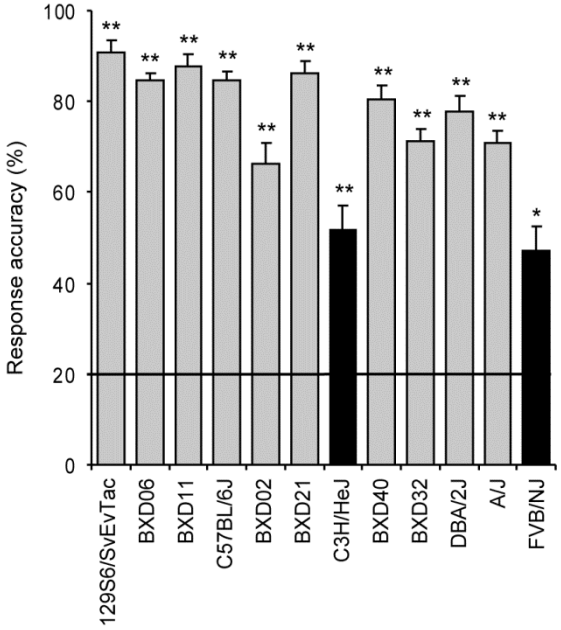


(l) Elevated plus maze





Supplementary Figure 1 | Strain means of measures in the home cage (**a – c**), novel cage (**d**), open field (**e – i**), dark light box (**j – k**), elevated plus maze (**l – n**) and operant cage (**o**).



Supplementary Figure 2 | The accuracy in the 5-CSRTT is defined by the number of correct responses divided by the sum of correct and incorrect responses. If a random nose-poke response is made in one of the 5 response holes, the chance level of a correct response is 20%. All strains, including the strains with early onset retinal degeneration (black bars), perform well above chance level (dashed line, one-sample t-test; *p<0.001, **p<0.0001).