Exploring the triad of behaviour, genes and neuronal networks: Heritability of instrumental conditioning and the Arc/Arg3.1 gene in hippocampal coding

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Chapter 2. Appetitive operant conditioning in mice: heritability and dissociability of training stages

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Abstract

To study the heritability of different training stages of appetitive operant conditioning, we carried out behavioural screening of 5 standard inbred mouse strains, 28 recombinant-inbred (BxD) mouse lines and their progenitor strains C57BL/6J and DBA/2J. We also computed correlations between successive training stages to study whether learning deficits at an advanced stage of operant conditioning may be dissociated from normal performance in preceding phases of training.

The training consisted of two phases: an operant nose poking phase, in which mice learned to collect a sucrose pellet from a food magazine by nose poking, and an operant lever press and nose poking phase, in which mice had to execute a sequence of these two actions to collect a food pellet. As a measure of magazine oriented exploration, we also studied the nose poke entries in the food magazine during the intertrial intervals at the beginning of the first session of the nose poke training phase.

We found significantly heritable components in initial magazine checking behaviour, operant nose poking and lever press–nose poking. Performance levels in these phases were positively correlated, but several individual strains were identified that showed poor lever press–nose poking while performing well in preceding training stages. Quantitative trait loci mapping revealed suggestive likelihood ratio statistic peaks for initial magazine checking behaviour and lever press–nose poking. These findings indicate that consecutive stages towards more complex operant behavior show significant heritable components, as well as dissociability between stages in specific mouse strains. These heritable components may reside in different chromosomal areas.
1. Introduction

Appetitive operant learning is often studied in a conditioning chamber in which animals learn to lever press and nose poke in order to receive a food or liquid reward delivered at a specific reward site. Operant learning usually results in complex behaviour that depends on a multitude of capacities such as forming place-reward and action-outcome associations, and chaining actions such as lever pressing and nose poking into a food magazine together. Little is known about the genetic basis of appetitive operant conditioning in general, or of the subsequent trainings stages that lead up to it. The first general goal of our study was to examine whether performance of a large group of mouse strains at consecutive training stages leading up to, and including operant conditioning, contain a heritable component. Secondly, for genetic mouse models of learning and memory, it would be an asset to identify strains that show a dissociation between poor performance at advanced training stages but good performance at preceding stages. Such dissociations may indicate which mouse strains present models specifically targeting more complex operant learning, or alternatively show deficits in more basic behaviors and learning processes.

In the past years, animal models with targeted mutations together with clinical findings in human populations have increased our understanding of the role of genes in cognitive processes such as memory and learning (The Dutch-Belgian Fragile X Consorthium et al., 1994; Khelfaoui et al., 2007; Morice et al., 2008). However, standard inbred mouse strains, such as the 129 strains, commonly used for transgenic studies differ in genetic background and behavioural phenotype, stressing the importance of characterization and selection of the background strain (Crusio, 1996; Gerlai, 1996).

Despite thorough genetic and behavioural characterization, the genetic components of the appetitive learning abilities of standard inbred mice (e.g. strains C57BL/6J and DBA/2J, A/J, Balb/c, C3H/HeJ, NOD/Ltj and 129S1/Sv) are largely unknown (but see Isles et al., 2004) for estimates of the genetic effect on choice bias for immediate rewards based on four standard inbred mouse lines and (Baron and Meltzer, 2001; McKerchar et al., 2005) for strain comparisons in an operant nose poke task and lever press task, respectively). Especially the behavioural characterization of learning in NOD/Ltj mice is far from complete. Overall, this
makes it difficult to select a background strain if one aims to develop a genetic mouse model for appetitive learning disabilities.

Susceptibility to cognitive dysfunctions is mostly affected by quantitative effects of groups of genes, rather than single genes (Valdar et al., 2006). Taking an approach opposite to studying targeted mutations, a behaviour-to-genes approach with genome-wide scanning for linkages between behavioural traits and chromosomal areas aims at elucidating the roles of genes in complex traits such as cognitive functions. Complementing the vast number of genetically engineered mouse lines, several sets of recombinant-inbred mouse lines have been created, perhaps most remarkably the BxD lines developed from the popular inbred laboratory mouse strains C57BL/6J and DBA/2J (Peirce et al., 2004). The high number of unique chromosomal recombinations in BxD lines, resulting in highly variable phenotypes, makes them very suitable for studying heritable components of cognition and behaviour.

However, many of the neuroscientific studies on recombinant inbred mouse lines published so far have focused on brain morphology (e.g. Martin et al., 2006; Badea et al., 2009) or behavioural traits associated with substance abuse, such as sensitization and tolerance to alcohol and cocaine (Tolliver et al., 1994; Gehle and Erwin, 1998; Jones et al., 1999; Kirstein et al., 2002). While some studies have managed to correlate morphology with behaviour (Yang et al., 2008), research on traits pertaining to learning behaviour motivated by naturalistic rewards has been scarce and, to our knowledge, heritability of operant tasks of different complexity, and motivated by appetitive rewards, has not been studied before.

In this study we characterized five commonly used inbred mouse lines and recombinant inbred (BxD) lines in their behavior across several consecutive training stages towards the acquisition of an appetitive, composite operant response, consisting of a lever press followed by a nose poke. The training stages preceding this final stage were magazine-checking behavior very early during training, in part reflecting exploratory behavior, and learning to nose poke for food reward. To lay a foundation for the development of novel mouse models for operant learning disabilities, we examined whether performance at these stages has a heritable background. We also investigated whether performance at more advanced stages of training can be dissociated from preceding stages, which may yield more specific mouse models deficient in operant learning. To expand the heritability analysis, we carried out a QTL mapping study based on the
data from 28 BxD lines and their progenitor lines to study whether these task stages are regulated by different chromosomal areas.

2. Methods

2.1. Animals

The BxD recombinant-inbred mouse lines used in this study were originally created in The Jackson Laboratory (http://www.jax.org). Both BxD and standard inbred mouse lines were bred locally at Harlan Netherlands (http://www.harlaneurope.com). The socially housed male mice (8-9 weeks of age at the beginning of experimental training) were kept in a reversed day–night cycle (7.00 lights off, 19.00 lights on). Each tested strain (standard mouse lines A/J, Balb/c/ByJ, C3H/HeJ, NOD/Ltj and 129S1/Sv, BxD-lines 1, 2, 8, 14, 16, 19, 21, 23, 27, 29, 31, 32, 33, 36, 39, 40, 42, 43, 51, 61, 62, 65, 68, 69, 73, 75, 87, 90 and their progenitor strains C57BL/6J and DBA/2J, N=5-19 mice per line, in total 343 animals, on average 9.8 animals per strain) consisted of several batches of mice with at least two litters from separate mothers. Prior to the beginning of the experiments, mice were habituated to the colony room for four weeks. Food and water were available ad libitum. In the week preceding the experiments, the mice were handled daily by the experimenter, habituated to the operant boxes for one hour per day and given samples of food pellets (14 mg dextrose-sucrose precision pellet produced by Bio-Serv, Frenchtown, NJ, http://www.bio-serv.com) in the home cage.

During the course of experiments, the mice were food-restricted by removing the food prior to the beginning of each training session to achieve about 5% weight loss. After the training session (once daily), food was available ad libitum until the beginning of the next restriction period on the following day. Water was provided in the home cages ad libitum at all times. All experimental procedures were approved by the institution’s Animal Welfare Committee and were in compliance with the European Council Directive (86/609/EEC) and Principles of laboratory animal care (NIH publication No. 86-23, revised 1985).
2.2. Behavioural apparatus

Mouse operant boxes (classical mouse modular test chamber, model ENV-307A, inside dimensions 15.9 x 14.0 x 12.7 cm, see Fig. 1) were equipped each with two stimulus lights above two retractable levers and a reward tray in the front panel. Each of the eight boxes was positioned inside a sound-attenuating cubicle (standard medium-density fiberboard cubicle, model ENV-022MD, inside dimensions 55.9 x 38.1 x 40.6 cm); the chambers were placed in parallel on two shelves, each holding four boxes. Control of the operant boxes and recording behavioural data was carried out by a MED-PC research control and acquisition system (version IV). Behavioural hardware and controlling software were provided by MED Associates, St. Albans, VT (http://www.med-associates.com).

![Behavioural testing chamber](image)

**Figure 1. Behavioural testing chamber.**
The behavioural box with stimulus lights, two operant levers (shown in the withdrawn state) and food magazine (in the middle) on the front panel.
2.3. Behavioural training and parameters

Every training session began with a habituation period during which the mice were placed in the operant box for one hour before training onset. To avoid decreased motivation by satiety, mice were able to collect a maximum of 30 pellets in one training session. Each training session was terminated when the mice reached this maximum or when the training session exceeded the maximum length of 60 minutes. The mice were trained for one session per day.

2.3.1. Operant nose poke task

Trial onset was marked by one of the green LED lights on the front panel of the operant box being lit up for 30 seconds or until the mouse collected a reward (one 14 mg sucrose pellet) with pseudorandom intertrial intervals of 5-25 seconds (15 s on average). While the light was on, the mice were able to collect a reward by the operant behavior of approaching and poking the food magazine in the front panel. Food magazine entries were detected by a photobeam detector. To prevent accumulation of sucrose pellets in the magazine tray, the pellet was only delivered at the moment the mouse put its nose into the tray. The mice were trained on this task for three sessions.

We will refer to this task as operant nose poking (NP) and not as (Pavlovian) cue conditioning (i.e., to the LED light) because no evidence was obtained that specific cue-reward associations were formed and expressed in behaviour, and furthermore the mice had to poke their nose in the feeder tray in order to obtain a pellet. We assessed the occurrence of cue conditioning by measuring the selectivity ratio, defined as the nose poke rate during stimulus light onset divided by nose poke rate during the intertrial interval. Cue conditioning should lead to a ratio clearly above one (Nordquist et al., 2003), but this was not the case in any of the mouse strains studied.

2.3.2. Operant lever press–nose poke task

In the lever press–nose poke task, the two levers (one on each side of the food magazine) protruded from the operant box wall. While the lever was in a protruded position, the mouse could obtain a sucrose pellet by first pressing the lever and subsequently poking its nose in the food magazine, thus expressing a chaining of two operant behaviors. Following a lever press or
timeout after 150 seconds without response, the levers were retracted to prevent possible extinction behaviour during the course of training, and a pseudorandom intertrial interval of 5-25 seconds followed. The mice were trained on this more complex operant task for five sessions in total. LED lights were not in use during this task.

2.4. Quantification and statistical analysis of behavioural parameters and heritability

2.4.1. Behavioural parameters

The following three parameters were analyzed in this study: (1) Initial magazine checking behaviour at the beginning of the first session of nose poke training; this behavior is taken to reflect primarily environmental exploration although early nose–poke learning may also contribute; (2) nose poke success at the end of nose poke training, and (3) lever press–nose poke performance at the end of training.

Initial magazine checking behaviour was defined as the number of nose poke entries in the food magazine per minute of inter-trial interval (ITI) during the first ten trials of the first session. Nose poke success was defined as the number of trials in the third, last session of training in which the mouse collected the reward during the trial by approaching and poking the food magazine, divided by the total number of trials in which pellet acquisition was possible. Lever press–nose poke performance was defined as the percentage of action sequences leading to reward deliveries relative to the total number of trials in the fifth, last session of training.

To quantify correlations between behavioural parameters, we computed standard Spearman's rank correlation coefficients and partial correlation coefficients on strain means. To assess the overall significance of behavioural differences between strains, we carried out one-way ANOVAs and post-hoc t-tests (Tukey's least significant difference procedure). All analysis was carried out in MATLAB (MathWorks, Natick, MA).

2.4.2. Heritability

Behavioural parameters from BxD lines, progenitor and standard inbred mouse strains were pooled to estimate narrow-sense heritabilities, which reflect the proportion of total phenotypic
variation that is due to the allelic effects of genes, thus excluding environmental factors, epistatic interactions, etc. ($h^2$; Hegmann and Possidente, 1981)) for behavioural patterns. To estimate the heritability, we used a procedure which controls for variable group sizes in different strains (Isles et al., 2004), where $N$ is the total number of animals, $S$ is the total number of tested strains, $n_s$ is the number of animals for a given strain $s$, $t_s$ is the trait average for a given strain, $v_s$ is the trait variance for a given strain and $T$ refers to the trait average across all strains:

\[ h^2 = \frac{(A - B)}{(A + 2kB - B)} \]

\[ k = \frac{(N - 1) / N \sum_s n_s^2}{(S - 1)} \]

\[ A = \left[ \sum_s n_s (t_s - T)^2 \right] / (S - 1) \]

\[ B = \left( \sum_s n_s v_s \right) / (N - S) \]

The p-levels of the heritability estimates were calculated by a permutation test with 1000 permutations (Moore and McCabe, 2000). Both the heritability estimates and their significance were calculated with a custom Matlab script (Heimel et al., 2008), available at http://www.nin.knaw.nl/~heimel/software/heritability.

2.4.3. Mapping of quantitative trait loci

Mapping of quantitative trait loci (QTLs) was performed by standard interval mapping scripts available at the WebQTL interface at http://www.genenetwork.org/ that link the observed behavioural traits with chromosomal areas with the help of established single nucleotide polymorphism (SNP) data. Likelihood ratio statistics (LRS) were calculated for each marker locus (Chesler et al., 2003, 2004; Wang et al., 2003). The whole-genome significance threshold for QTLs was defined by using a 1000x permutation test. We did not enable use of parent strains in order not to bias the permutation test.
4. Results

4.1. Initial magazine checking behaviour

The average nose poke rate during intertrial intervals of the first 10 trials of reward collection training varied from 0.53 pokes / min (129S1/Sv) to 10.28 pokes / min of ITI (NOD/Ltj), the mean ± SEM being 3.35 ± 0.34 pokes / min of ITI (Fig. 2A). While none of the BxD lines expressed more initial magazine checking behaviour than the progenitor line DBA/2J, the majority of the mouse lines showed less initial magazine checking than either of the progenitor lines, demonstrating transgressive segregation in the trait (see e.g. (Jones and Mormède, 2007), chapter 25). The heritability of this behavior was 10.4% (p < 0.001). A one-way ANOVA test revealed a significant strain effect (F(34,315) = 3.31, p = 0.15 * 10⁻⁷). Post-hoc testing indicated that out of 595 possible pair-wise comparisons between strains, 123 were significantly different from each other.

4.2. Nose poke task

During the third, last session of the nose poke task, the performance scores defined as the number of trials where the mouse collected the pellet divided by all trials ranged from 20.0% in BxD-73 to 93.2% in NOD/Ltj, the mean being 55.0 ± 3.5 % (Fig. 2B). As with initial magazine checking, the majority of the BxD mouse lines showed lower performance levels than either of the progenitor lines, but three BxD lines (2, 16 and 32) achieved higher levels than either progenitor line. The heritability for nose poke success in the last session of training was 19.6% (p<0.001). Also the nose poke task showed a significant strain effect in one-way ANOVA (F(34,315) = 5.85, p = 0), with 254 out of 595 possible pair-wise comparisons significantly differing from each other.

4.3. Lever press–nose poke task

Over the course of training on this task, one line (BxD-90) failed to complete any trials despite showing clear nose poke behaviour in the earlier training phase (Fig. 2C). Most strains showed improving performance over the five training sessions (Fig. 3). In the last, fifth session of training, performance in the operant task varied remarkably across strains: performance varied
Figure 2. Variability of behavioural parameters across the 35 strains tested. 129S1/Sv, NOD/Ltj and BxD-43 (discussed in the text) are indicated with arrows.

A | Initial magazine checking behaviour at the beginning of the first session of training. Initial magazine checking behaviour is presented by the number of nose pokes in the food magazine per minute of intertrial interval during the first ten trials of the first session of behavioural training. Values shown are strain means and SEMs.

B | Nose poke success per strain. Nose poke success in the third session of training is presented as the percentage of trials during which the mouse collected sucrose pellets during an interval of 30 s following trial onset, relative to the total number of trials.

C | Lever press–nose poke performance at the end of training. Performance in the last (fifth) session of training is presented as the percentage of trials during which the mouse presses the lever and nose pokes into the magazine to collect the sucrose pellet during the trial period (150 s following trial onset).

From 0.0% (BxD-90) to 99.6% (NOD/Ltj), the average being 46.4 ± 4.9% (Fig. 2C). A number of BxD lines (27, 8, 2, 33, 51 and 43) outweighed both of the progenitor strains in performance, but none of the above mentioned lines was topping the progenitor strain DBA in the initial magazine checking behaviour. Similarly, mouse lines 69, 31 and 16 that showed the highest initial magazine checking activity among the BxD lines, were performing worse in the lever press–nose poke task than either progenitor line. For many strains, a clear dissociation was found between initial magazine checking and lever press–nose poke performance, or between
nose poke success and lever press–nose poke performance. For instance, BxD-43, the top BxD line for lever press–nose poke learning, was amongst the lines expressing the least initial magazine checking behaviour and below the average in the nose poke task.

Figure 3. Acquisition of lever press–nose poke performance.
Lever press–nose poke performance percentages over the course of five training sessions for each strain. Strain means presented in colour-coding, see colour bar on the right; colour scale ranges from 0 to 100% correct performance. Strains are sorted based on their level of performance. A few of the mice did not complete the 5th session of training (which causes the drop in performance of lines 87 and 1), in which case we used the average of the last session of each mouse as the best available approximation for the final performance of the strain (Figure 2C).

Of most interest from the viewpoint of deficient operant learning were the lines showing poor lever press–nose poke learning but moderate or normal levels of nose poking behavior; these lines included BxD strains 23, 19, 21 and 32, and to a lesser extent C3H. For instance, BxD-32
had a low lever press–nose poke performance of about 20% despite it being among the top lines in during operant nose poke learning.

We found a significant (p < 0.001) heritable component (21.3%) in lever press–nose poke performance in the fifth session. Moreover, we found a significant strain effect in one-way ANOVA (F(34,309) = 3.22, p = 0.01), with 277 significantly different pair-wise comparisons out of 595.

4.4. Correlations between different training stages

There was a significant positive correlation between the strain means of initial magazine checking behaviour and the nose poke task (r = 0.63, p = 0.00006; Table 1), the nose poke task and lever press–nose poke performance (r = 0.52, p = 0.00143) and lever press–nose poking versus initial magazine checking behaviour (r = 0.53 p = 0.00116).

We also examined how performance levels at two of the three task stages were correlated when the influence of the third stage was taken into account (Table 1). The partial correlation for initial magazine checking and the nose poke stage per strain mean remained significant (r = 0.48, p = 0.00382; with lever press–nose poke learning partialed out). In contrast, the partial correlations between nose poke and lever press – nose poke performance (r = 0.29, p = 0.09828; with initial magazine checking partialed out) and initial magazine checking and lever press–nose poke performance (r = 0.31, p = 0.07664; with reward collection partialed out) showed a slight trend towards correlation but were insignificant. Especially the lack of a significant partial correlation between the nose poke and lever press–nose poke stages is notable, because these were contiguous in time and both represent a form of operant conditioning.

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<th>Table 1. Correlations and partial correlations between initial magazine checking (IMC), nose poke success (NP) and lever press–nose poke performance (LPNP).</th>
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4.5. QTL mapping results

4.5.1. Initial magazine checking behaviour

The QTL map for initial magazine checking behaviour showed suggestive peaks on chromosomes 4 and 6 (Fig 4A). When zoomed in further, the LRS is above the threshold for a suggestive QTL around 47-48 megabases on chromosome 4 (Fig. 4B) and around 93-95 MB on chromosome 6 (Fig. 4C). In both cases, the peaks were relatively flat and had several genes expressed in the central nervous system under them, meaning that it was not possible to point out a single candidate gene. Genes found under the peak are listed in Table 2A and 2B for chromosomes 4 and 6, respectively.

4.5.2. Nose poke task

Mapping the nose poke performance at the end of the training either as percentage of correct trials or percentage of correct trials normalized on the total number of nose pokes failed to reveal suggestive QTLs.

4.5.3. Lever press–nose poke task

QTL mapping of lever press–nose poke performance in the last session of training resulted in a suggestive peak on chromosome 9, (58 MB; Fig. 5). Normalizing lever press–nose poke performance on the total number of nose pokes in the preceding phase did not cause a notable change in the location or significance of the LRS. Due to the relative flatness of the peak combined with a high number of genes situated under the peak area being expressed in the mouse central nervous system, it was not feasible to point out a single candidate gene for this final stage of operant learning. Genes found under the peak are listed in Table 2C.

5. Discussion

The main results of this study can be summarized as follows: First, all three task stages studied here showed significant levels of heritability, ranging from 10.4% for initial magazine checking behavior to 21.3% for the final and most complex stage, lever press–nose poke learning. A significant strain effect due to multiple differences between mouse lines, not just a few outlier
Figure 4. QTL maps for initial magazine checking behaviour

A | Quantitative trait loci (QTL) map for initial magazine checking behaviour. QTL map for initial magazine checking behaviour presents likelihood ratio statistics for the trait over the whole genome. Numbers on the X axis represent chromosomes. Suggestive and conservative significance thresholds are marked by gray horizontal lines. The likelihood ratio statistic (LRS) score reaches the suggestive threshold on chromosomes 4 and 6.

B | QTL map for initial magazine checking behaviour, zoomed in on chromosome 4. LRS for the chromosome 4 reaches its peak around 47-48 megabases (MB). The abscissa runs from 0 to 155 megabases.

C | QTL map for the same trait, zoomed in on chromosome 6. LRS reaches the threshold for suggestive QTL around 93-95 MB. The abscissa runs from 0 to 145 megabases.

Figure 5. QTL maps for lever press–nose poke performance

A | QTL map for lever press–nose poke performance in the fifth session of training. LRS reaches suggestive threshold on chromosome 9.

B | QTL map for lever press–nose poke performance in the fifth session of training, zoomed in on chromosome 9. LRS peak is situated around 58 MB. The abscissa runs from 0 to 124 megabases.
strains, could be seen at all stages. In our QTL mapping analysis, suggestive LRS peaks were found for initial magazine checking (on chromosome 4 and 6) and for the lever press–nose poke task (chromosome 9), but not for nose poke learning. When analyzing correlations and dissociations between task stages, it should be emphasized that the analysis of heritability and quantitative trait loci hinges on high-throughput screening of many mouse lines, making it unfeasible to study different learning traits in separately trained subgroups of the same strain. In our paradigm where consecutive learning stages could be monitored in the same mouse, strong correlations were found between all three stages, but the correlations between initial magazine checking and lever press–nose poking, and between nose–poking and lever press–nose poking became insignificant when the third stage was factored out. The dissociability of nose–poking and lever press–nose poking was most poignantly illustrated by several BxD lines (especially BxD-32, but also e.g. 21, 19, 23 and 90) showing high performance on operant nose poking but low success on lever press–nose poking.

5.1. Operant nose poke learning and initial magazine checking behaviour

Although seemingly simple, the stage of operant nose poking for food reward may allow several associations to be formed. Apart from action–outcome (nose poke–food) learning, the animal may have formed cue-outcome (Ito et al., 2005; for a review see e.g. (Savage and Ramos, 2009) as well as place-outcome associations (McAlonan et al., 1993; Ito et al., 2008), but in the current study no evidence for conditioning to the cue light was found. Initial magazine checking behavior marks the very beginning of learning to approach the magazine and nose poking into it, and this stage is likely to be dominated by environmental exploration, as it was measured during the intertrial intervals of the first 10 trials.

We found that both magazine checking and nose poke performance had a significant heritable component. The positive correlation between nose poke performance and initial magazine checking behaviour (Table 1) remained significant when lever press–nose poke performance was partialed out. This can be explained, at least in part, by the notion that exploratory behaviour is an essential early step in operant nose poke behaviour: nose pokes in the feeder tray are required for the animal to discover a reward. Furthermore, during the course of
training the animals expressing more pronounced initial magazine checking behaviour are likely to visit the reward site at a higher rate, which directly facilitates performance success. Thus, our measures of magazine checking and final nose poke performance can be taken to reflect a continuum of learning, sampled at extreme time points, making the high correlation between these two stages a logical and expected result.

Of the standard inbred mouse lines, NOD/Ltj mice were expressing the most and 129S1/Sv the least initial magazine checking (Fig. 2A, indicated with arrows), which is in accordance with previous open field exploration and locomotor activity studies (for NOD/Ltj, see Bothe et al., 2005, for various 129 substrains, see Baron and Meltzer, 2001; Isles et al., 2004; Bothe et al., 2005).

5.2. Lever press–nose poke performance

To our knowledge, the heritability of appetitive lever press–nose poke learning has not been previously studied. Isles et al. (2004) studied several inbred mouse lines using an appetitive operant delayed-reinforcement paradigm in which mice were trained to respond to visual stimuli with a nose poke in order to get condensed milk as a reward, but this study did not focus directly on heritability of operant conditioning but on heritability of choice bias for immediate reward (15.8% and 16.5% depending on parameter definition; Isles et al., 2004). Studies using a non-appetitive, escape/avoidance lever press task (Brennan, 2004) and an appetitive task using condensed milk as reward (Baron and Meltzer, 2001; McKerchar et al., 2005) reported notable differences between inbred mouse lines but did not estimate the heritability.

Learning to perform an operant sequence of lever press–nose poking can be argued to be a complex process that depends on multiple components and is influenced, first, by several background factors such as basal exploratory activity, neophobia towards protruding levers, stress, motivational variables and incentive learning of pellet value (Luksys et al., 2009). These factors are not specific to our task per se, but affect learning indirectly, for instance by limiting the number of trials the animal will engage in. A second group of factors is discussed below and relates to learning that a previously effective operant response (nose poke) has to be preceded in this subsequent stage by a second, novel operant response (lever pressing). Given this complexity, it is not too surprising that acquisition of operant behavior varied highly across the
strains. Considering all contributing factors and associated behavioural variability, the significantly heritable component of 21.3% in lever press-nose poke performance may be considered rather high.

5.3. Correlation analysis and dissociations between subsequent operant tasks

While initial magazine checking behaviour, nose poke success and lever press–nose poke performance all appeared to have a positive correlation with each other, the individual positive correlations between lever press–nose poke performance and the two other stages disappeared when taking into account the effect of the third trait. This is less remarkable for initial magazine checking and the lever press-nose poke task because the variable performance on nose poking for food was temporally situated in between these two stages. Of more interest is the finding that the nose poke and lever press–nose poke stages lost their significant correlation when the influence of initial, exploratory magazine pokes was taken out, because these stages were temporally contiguous and both represent forms of operant conditioning.

In our experiments neither high expression of initial magazine checking behaviour nor nose poke success provided reliable predictive power for the outcome of the lever press–nose poke training – a finding which is also reflected by some individual mouse lines. While lines BxD-90 and NOD/Ltj were clearly on the lower or higher end of performance at each training stage, a dramatic dissociation was seen in mouse line BxD-43. When comparing initial magazine checking activity and lever press–nose poke performance, BxD-43 (Fig 2, indicated with an arrow) was among the lines having the lowest initial magazine checking activity, and its nose poke success was below average. Nevertheless, it had the highest lever press–nose poke performance of all the BxD-lines, second only to NOD/Ltj. Conversely, C3H and BxD-32 mice performed well above average on the nose poke task, but clearly below average on the subsequent lever press–nose poke task. A similar trend was observed in BxD lines 21, 19, 23 and 90, which were notably worse in lever press–nose poke task.

Several explanations may be noted for a poor performance on lever press–nose poking given moderate to high levels of nose poke learning. First, animals may be neophobic towards protruding levers and may have difficulty forming a trace between the act of lever pressing and
obtaining a reward later on in the trial. Second, the previously established nose poke–reward
association may impair or even block acquisition of a novel lever press–reward association.
Third, animals may lack the capacity to ‘chain’ lever press and nose poke actions in the correct
order (Balleine and Dickinson, 1998; Graybiel, 1998; Suri and Schultz, 1998; Corbit and Balleine,
2003) In all three scenarios, C3H and the BxD-lines 32, 21, 19, 23 and 90 may be regarded as
interesting models for further exploring deficiencies in more advanced forms of instrumental
learning and cognitive flexibility.

It is difficult at present to link the different stages of training to neuroanatomical substrates.
Nevertheless, both for the nose poke and lever press–nose poke tasks the learning of action-
outcome associations is important, depending on medial prefrontal-dorsomedial striatal
systems (Balleine and Dickinson, 1998; Dalley et al., 2004; Yin et al., 2005). Secondly, these
systems have also been implicated in the process of chaining or ‘chunking’ two or more actions
together (Ostlund et al., 2009; Pennartz et al., 2009). Thirdly, success on the nose poke task also
depends on learning place-reward associations, and in general appetitive contextual
conditioning is mediated at least in part by the hippocampal-ventral striatal system (Schacter et
al., 1989; Sutherland and Rodriguez, 1989; Ito et al., 2008). These dorsal and ventral striatal
systems are anatomically and physiologically linked in multiple ways, e.g. via the dopaminergic
mesencephalon and via connected cortico-basal ganglia loops (Haber, 2003; Voorn et al., 2004).

Although the dissociability of lever press–nose poke performance and initial magazine checking
may not be entirely surprising, it is of note that the QTL maps for initial magazine checking and
lever press–nose poke learning showed no overlapping loci (Fig. 4 and 5). This result, together
with the heritability and correlation analysis, indicates that the neural processes mediating
these two task stages have a heritable background and suggests that they are genetically
dissociable.

5.4. Comparisons to other studies of common inbred mouse
lines

Taken the importance of standard inbred mouse lines as disease models and background in
gene-targeting studies, it is interesting to note that the NOD mice, of which relatively little
behavioural data is available, were showing not only the highest initial magazine checking
activity (consistent with their reportedly high exploratory activity; (Bothe et al., 2005) and nose poke success amongst the strains, but also the highest lever press–nose poke performance, and that 129S1/Sv had little success on the lever press–nose poke task, with only one of the eight tested subjects making any lever press responses in this task. Although the large variety in 129 (sub)strains used in various studies makes interpretation of the behavioural data obtained in different laboratories challenging, our results on the 129S1/Sv strain are in agreement with previous reports of poor performance of this strain in an appetitive lever press task (McKerchar et al., 2005), and aversively motivated escape/avoidance lever press task (129S6/SvEvTac; Brennan et al 2004). McKerchar et al. (2005) also reported a positive correlation between locomotor activity and operant performance, which is in line with our data on magazine checking and operant learning.

In a study using a delayed-reinforcer task, the 129S2/SvHsd strain learned to respond to a light cue with a nose poke in order to receive a reward. Despite showing lower spontaneous locomotor activity, its start latency, choice latency and number of non-started trials in a delayed-reinforcer task were not significantly different from the highly active BALB/c and C57BL/6J mice (Isles et al., 2004). In a touchscreen-based appetitive operant task, 129S1/SvIMJ performed similarly to C57BL/6J (Hefner et al., 2008) and in a task where the mice had to make a nose poke into an illuminated hole, the latency of 129X1/SvJ and 129X1/SvJ mice to emit 50 operant responses was at the same level as C3H/Hej and DBA/2J mice were performing, while the difference to DBA/2J and Balb/cByJ was significant, but not as dramatic as was the case in operant lever press tasks (Baron and Meltzer, 2001). Together with our finding that 129 S1 mice performed moderately on the operant nose poke task (Fig. 2B), these findings suggest that the poor operant lever press–nose poke performance of 129 strains may be due to a specific learning deficit related to lever pressing rather than insensitivity to a reinforcer or low activity levels. Alternatively, 129 strains may be capable of acquiring high asymptotic levels of lever press performance after more prolonged autoshaping or training than was done in our study (5 sessions; cf. Isles et al. 2004).
5.5. Genetic loci potentially contributing to operant learning performance

To complement the behavioural and heritability analysis, we also conducted QTL mapping. For initial magazine checking behaviour, we found suggestive likelihood ratio statistic (LRS) peaks on chromosomes 4 and 6 (Fig. 4), indicating areas containing a number of genes expressed in the mouse central nervous system, but could not point out single candidate genes for this task stage. For nose poke performance, no LRS peaks were found.

For lever press–nose poke learning, we identified a suggestive LRS peak on chromosome 9 (Fig. 5). Also this peak was relatively flat due to a low number of local SNPs and/or an insufficient number of unique recombinations in the area of interest. Although it was not possible to point out a single candidate gene, it is interesting to note that the genes under the peak region included Bbs4, a locus known to be associated with human Bardet-Biedl syndrome type 4 which is characterized by deficits in sensory function and learning disabilities in addition to physiological symptoms such as obesity (Beales et al., 1997).

Despite careful standardization of the experiments, none of the observed LRS peaks exceeded the conservative threshold for significance. Even with low variability – high heritability traits such as morphometric data on brain area size, QTLs may not be detected due to a relatively small contributing effect of each individual QTL (Crusio, 2004) which often results in difficulties finding highly significant QTLs for complex traits. Despite these limitations, the present QTL results are useful for comparisons with further studies, which may help pinpointing the polygenic nature of learning behaviour to specific gene groups. Furthermore, the finding that QTL maps for initial magazine checking and lever press–nose poke learning showed no overlap supports our hypothesis that these processes are genetically dissociable.

According to the www.ensemble.org database, the progenitor lines C57BL/6J and DBA/2J show a small difference in the coding region of Bbs4 (human Bardet-Biedl syndrome type 4 gene, the mouse homolog of which is located on chromosome 9, under the QTL peak for operant performance), raising the possibility that some of the BxD lines may also differ in this locus, although this information is not available in the BXD Genotypes Database (see www.genenetwork.org), so we could not correlate the Bbs4 sequence in BxD lines with their
learning performance. No studies describing the cognitive-behavioural abilities of an existing Bbs4 knockout mouse line (Mykytyn et al., 2004) have been published, leaving open the question whether this locus may partially explain the observed variance in our lever press–nose poke task.

Even though the involvement of brain areas regulating operant conditioning has been studied extensively, its genetic background remains far from unraveled. One of the few identified genes known to regulate operant learning is Gpr6 on mouse chromosome 10, deletion of which facilitates acquisition of lever press behaviour in mice. It codes G protein-coupled receptor 6, which is known to be selectively expressed in striatal neurons projecting to the pallidum (Lobo et al., 2007). However, C57BL/6J and DBA/2J do not differ in this locus, so we could not assess its role in the operant performance variability by QTL mapping based on BxD mouse lines. Interestingly, 129S1/Sv, which had the worst lever press–nose poke performance of the standard mouse lines in our study, and also showed poor lever-press performance in previous studies, differs in amino acid sequence from C57BL/6J and DBA/2J in this locus. The QTLs found in previous contextual and auditory-cued fear conditioning studies (Owen et al., 1997a; Reijmers et al., 2006) did not appear in our study, which suggests that appetitive operant learning may be genetically dissociable from these aversively motivated types of learning – a subject awaiting further examination.

In conclusion, this study first showed that various task stages leading up to appetitive learning of sequential operant actions have a heritable component. Second, lever press–nose poke performance was only poorly predictable from the preceding task stage of operant nose poke learning, as illustrated by BxD line 43 showing poor nose poke performance but high lever press–nose poke learning, and by BxD lines 32, 21, 19, 23 and 90 as well as strain C3H, showing an opposite tendency. Especially the latter lines may provide mouse models to study deficiencies in learning more complex, chained operant responses. Together, the correlation analysis and novel QTL maps suggest that different task stages of appetitive operant learning are regulated by different sets of genes.
Acknowledgements

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References


Table 2. List of genes under suggestive LRS peaks. Genes indicated by underscores are known to be expressed in the CNS according to Mouse Genome Informatix gene expression database (www.informatics.jax.org) are underlined.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Full name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gabbr2</td>
<td>gamma-aminobutyric acid (GABA) B receptor, 2</td>
</tr>
<tr>
<td>Anks6</td>
<td>ankyrin repeat and sterile alpha motif domain containing 6</td>
</tr>
<tr>
<td>Gm568</td>
<td>predicted gene 568</td>
</tr>
<tr>
<td>Galnt12</td>
<td>UDP-N-acetyl-alpha-D-galactosamine: polypeptide N-acetylgalactosaminytransferase 12</td>
</tr>
<tr>
<td>Col15a1</td>
<td>collagen, type XV, alpha 1</td>
</tr>
<tr>
<td>Tgfrb1</td>
<td>transforming growth factor, beta receptor I</td>
</tr>
<tr>
<td>Alg2</td>
<td>asparagine-linked glycosylation 2 homolog (yeast, alpha-1,3-mannosyltransferase)</td>
</tr>
<tr>
<td>Sec61b</td>
<td>Sec61 beta subunit</td>
</tr>
<tr>
<td>Nr4a3</td>
<td>nuclear receptor subfamily 4, group A, member 3</td>
</tr>
<tr>
<td>Stx17</td>
<td>syntaxin 17</td>
</tr>
<tr>
<td>Erp44</td>
<td>endoplasmic reticulum protein 44</td>
</tr>
<tr>
<td>Invs</td>
<td>inversin</td>
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<tr>
<td>C030004N09Rik</td>
<td>RIKEN cDNA C030004N09 gene</td>
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<tr>
<td>Tex10</td>
<td>testis expressed gene 10</td>
</tr>
<tr>
<td>5730528L13Rik</td>
<td>RIKEN cDNA 5730528L13 gene</td>
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<tr>
<td>Tmeff1</td>
<td>transmembrane protein with EGF-like and two follistatin-like domains 1</td>
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<tr>
<td>Murc</td>
<td>muscle-related coiled-coil protein</td>
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<td>4933437F24Rik</td>
<td>RIKEN cDNA 4933437F24 gene</td>
</tr>
<tr>
<td>E130309F12Rik</td>
<td>RIKEN cDNA E130309F12 gene</td>
</tr>
<tr>
<td>Acnat2</td>
<td>acyl-coenzyme A amino acid N-acyltransferase 2</td>
</tr>
<tr>
<td>Acnat1</td>
<td>acyl-coenzyme A amino acid N-acyltransferase 1</td>
</tr>
<tr>
<td>9030417H13Rik</td>
<td>RIKEN cDNA 9030417H13 gene</td>
</tr>
<tr>
<td>Baat</td>
<td>bile acid-Coenzyme A: amino acid N-acyltransferase</td>
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<tr>
<td>Mrpl50</td>
<td>mitochondrial ribosomal protein L50</td>
</tr>
<tr>
<td>Zfp189</td>
<td>zinc finger protein 189</td>
</tr>
<tr>
<td>Aldob</td>
<td>aldolase B, fructose-bisphosphate</td>
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<tr>
<td>2810432L12Rik</td>
<td>RIKEN cDNA 2810432L12 gene</td>
</tr>
<tr>
<td>Rnf20</td>
<td>ring finger protein 20</td>
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<tr>
<td>Grin3a</td>
<td>glutamate receptor ionotropic, NMDA3A</td>
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<tr>
<td>Ppp3r2</td>
<td>protein phosphatase 3, regulatory subunit B, alpha isoform (calcineurin B, type II)</td>
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### B | Initial magazine checking, chromosome 6

<table>
<thead>
<tr>
<th>Gene</th>
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<tr>
<td>C130022K22Rik</td>
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<td>4930590J08Rik</td>
<td>RIKEN cDNA 4930590J08 gene</td>
</tr>
<tr>
<td>Fgd5</td>
<td>FYVE, RhoGEF and PH domain containing 5</td>
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<tr>
<td>4930466I24Rik</td>
<td>RIKEN cDNA 4930466I24 gene</td>
</tr>
<tr>
<td>Nr2c2</td>
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</tr>
<tr>
<td>9430019H13Rik</td>
<td>RIKEN cDNA 9430019H13 gene</td>
</tr>
<tr>
<td>Mrps25</td>
<td>mitochondrial ribosomal protein S25</td>
</tr>
<tr>
<td>Zfyve20</td>
<td>zinc finger, FYVE domain containing 20</td>
</tr>
<tr>
<td>Trh</td>
<td>thyrotropin releasing hormone</td>
</tr>
<tr>
<td>Prickle2</td>
<td>prickle homolog 2</td>
</tr>
<tr>
<td>Adamts9</td>
<td>a disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 9</td>
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<td>A730049H05Rik</td>
<td>RIKEN cDNA A730049H05 gene</td>
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<td>9530026P05Rik</td>
<td>RIKEN cDNA 9530026P05 gene</td>
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<tr>
<td>D630004L18Rik</td>
<td>RIKEN cDNA D630004L18 gene</td>
</tr>
<tr>
<td>Magi1</td>
<td>membrane associated guanylate kinase, WW and PDZ domain containing 1</td>
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<tr>
<td>B430316J06Rik</td>
<td>RIKEN cDNA B430316J06 gene</td>
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<tr>
<td>8030459D09Rik</td>
<td>RIKEN cDNA 8030459D09 gene</td>
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<tr>
<td>4930511A08Rik</td>
<td>RIKEN cDNA 4930511A08 gene</td>
</tr>
<tr>
<td>Slc25a26</td>
<td>solute carrier family 25 (mitochondrial carrier, phosphate carrier), member 26</td>
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<tr>
<td>Lrig1</td>
<td>leucine-rich repeats and immunoglobulin-like domains 1</td>
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<td>4930511E03Rik</td>
<td>RIKEN cDNA 4930511E03 gene</td>
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<td>2410024F20Rik</td>
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<tr>
<td>1700010K10Rik</td>
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<tr>
<td>Kbtbd8</td>
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### C | Lever press-nose poke performance, chromosome 9

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<tr>
<th>Gene</th>
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<tbody>
<tr>
<td>Scamp 5</td>
<td>secretory carrier-associated membrane protein 5</td>
</tr>
<tr>
<td>Rpp25</td>
<td>ribonuclease P protein subunit p25</td>
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<tr>
<td>Cox5a</td>
<td>cytochrome c oxidase, subunit Va</td>
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<tr>
<td>LOC735298</td>
<td>hypothetical locus LOC735298</td>
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<tr>
<td>2310046O06Rik</td>
<td>RIKEN cDNA 2310046O06 gene</td>
</tr>
<tr>
<td>Mpi</td>
<td>mannose phosphate isomerase</td>
</tr>
<tr>
<td>Scamp2</td>
<td>secretory carrier membrane protein 2</td>
</tr>
<tr>
<td>Ulk3</td>
<td>unc-51-like kinase 3</td>
</tr>
<tr>
<td>Cplx3</td>
<td>complexin 3</td>
</tr>
</tbody>
</table>
Lman1l  lectin, mannose-binding 1 like
Csk  c-src tyrosine kinase
Cyp1a2  cytochrome P450, family 1, subfamily a, polypeptide 2
Cyp1a1  cytochrome P450, family 1, subfamily a, polypeptide 1
Edc3  enhancer of mRNA decapping 3 homolog
Clk3  CDC-like kinase 3
Arid3b  AT rich interactive domain 3B (BRIGHT-like)
Ubi7  ubiquitin-like 7 (bone marrow stromal cell-derived)
Sema7a  sema domain, immunoglobulin domain (Ig), and GPI membrane anchor, (semaphorin) 7A
Cyp11a1  cytochrome P450, family 11, subfamily a, polypeptide 1
Ccdc33  coiled-coil domain containing 33
E330033L03  hypothetical protein E330033L03
1700120E02Rik  RIKEN cDNA 1700120E02 gene
Stra6  stimulated by retinoic acid gene 6
Isrl  immunoglobulin superfamily containing leucine-rich repeat
Islr2  immunoglobulin superfamily containing leucine-rich repeat 2
1600029O15Rik  RIKEN cDNA 1600029O15 gene
Pml  promyelocytic leukemia
Stoml1  stomatin-like 1
Loxl1  lysyl oxidase-like 1
Tbc1d21  TBC1 domain family, member 21
1700072B07Rik  RIKEN cDNA 1700072B07 gene
4930461G14Rik  RIKEN cDNA 4930461G14 gene
6030419C18Rik  RIKEN cDNA 6030419C18 gene
Cd276  CD276 antigen
Nptn  neuroplastin
2410076i21Rik  RIKEN cDNA 2410076i21 gene
A130026P03Rik  RIKEN cDNA A130026P03 gene
Hcn4  hyperpolarization-activated, cyclic nucleotide-gated K+4
Neo1  neogenin
Adpgk  ADP-dependent glucokinase
Bbs4  Bardet-Biedl syndrome 4
Arih1  ariadne ubiquitin-conjugating enzyme E2 binding protein homolog 1
Tmem202  transmembrane protein 202
Hexa  hexosaminidase A
Celf6  CUGBP, Elav-like family member
Parp6  poly (ADP-ribose) polymerase family, member 6