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Analysis of cognitive functions in recombinant inbred strains of rats produced by crossbreeding of SHR and BN-Lx/Cub lines

Analýza kognitivních funkcí u rekombinantních inbredních kmenů potkanů vzniklých křížením linií SHR a BN-Lx/Cub.

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Declaration

I declare that I have written this thesis by myself using literature listed in References section. I have acquired the raw data from the set of recombinant inbred strains and pursued the numerical analysis under supervision of thesis leader, consultant and other colleagues.

Hana Hatalová, Prague, Sep 28, 2011

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Abstrakt

Táto diplomová práca sa zaoberá prepojením medzi priestorovou pamäťou, genetikou a metabolickým syndrómom. Priestorová pamäť je veľmi komplexný znak, ovplyvnený množstvom faktorov. Pre tento experiment boli použité rekombinantné inbredné kmene HXB/BXH (n= 30) a ich parentálne línie (n=2) trénované v hipokampálne závislej úlohe nazývanej aktívne alotetické vyhýbanie sa miestu (AAPA). Úlohou potkana bola zapamätať si sektor v rotujúcej kruhovej aréne ktorému sa mali vyhýbať. Ako trest slúžil mierny elektrický šok po vstupu do zakázaného sektoru (4 dni tréningu so zakázaným sektorom smerujúcim na sever, jedno vybavovací (retrieval) sedenie, a tri sedenia zo šokovým sektorom obráteným na juh. Každé sedenie trvalo 20 minút, "retrieval" 10 minút). Kontrolné pokusy na vylúčenie vplyvu motorických a senzorických rozdielov prebehli vo forme testu otvoreného poľa a chodenia po latke. Korelácia z metabolickými fenotypmi boli prevedené pomocou internetovej databáze známych HXB/BXH fenotypov (GeneNetwork.org). Rozdiely v učení medzi skupinami boli signifikantné (jednosmerná ANOVA); avšak korelačná analýza neukázala žiadne zásadné prepojenie medzi metabolickými fenotypmi a pamäťou. Genetická analýza ukázala sugestívny lokus pre vybraný parameter učenia na chromozóme 20 a opakovane pre pohybovú aktivitu na chromozóme 4. Lineárna regresná analýza neukázala signifikantný vplyv anxiety ani motorických a senzorických rozdielov na učenie v tejto úlohe.

Kľúčové slová: HXB/BXH inbredné kmene - pamäť - genetika - metabolický syndróm - QTL

Abstract

This MSc. thesis deals with dissecting the link between memory, genetics, and metabolic syndrome. Memory is a very complex behavioral trait, probably influenced by innumerable factors. For this experiment HXB/BXH rat recombinant inbred lines (n= 30) and their parental strains (n=2) were used to be trained in the hippocampus dependant spatial learning task called Allothetic Active Place Avoidance. Rats were to memorize sector of a rotating circular arena, which they were to avoid, being motivated by receiving an electric shock upon entering the forbidden sector (4 training sessions; shock sector on the North, 1 retrieval session (no shock), and 3 reversal sessions, to-be-avoided sector facing South; each session 20-min long, retrieval 10-min). Control experiments to exclude impact of motor or sensory abnormalities were run in a form of open-field test and beam-walking test. Correlation with metabolic phenotypes was conducted in an online database of known HXB/BXH phenotypes (GeneNetwork.org). The results showed that differences in learning were significant between the groups ($p<0.05$); correlation analysis indicated no putative link between selected traits related to metabolic syndrome and memory in rats. The genetic analysis showed a suggestive locus on chromosome 20 for a learning parameter, and repeatedly a suggestive locus on chromosome 4 for a locomotor phenotype. Linear regression analysis showed no link of anxiety levels or possible sensory or motor impairments on spatial learning.

Keywords: HXB/BXH inbred lines - memory - genetics - metabolic syndrome - QTL

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1. List of abbreviations

AAPA - Active Allothetic Place Avoidance

ApoE - apolipoprotein E

BN-Lx/Cub - parental Brown Norway strain carrying Lx gene

DG - dentate gyrus

HDL - high density lipoprotein

HXB/BXH - a rat RI panel generated by crossing SHR and BN-LX/Cub

LOD - logarithm of odds

LRS - likelihood ratio statistics

PLS - polyluxate syndrome

QTL - quantitative trait locus

RI- recombinant inbred (strains)

SHR/Ola - spontaneously hypertensive rat

VA - additive genetic variance

VE - environmental variance

2 Introduction

My thesis is part of the larger project focused on identifying links between memory, metabolic syndrome and genetics. The cognition was shown to be heritable to a certain degree (Plomin et al., 1994). Additionally, it was observed before, that signs of metabolic syndrome often correlate with decreased cognitive abilities and dementia (Farmer et al., 1990). Metabolic syndrome is the collection of symptoms including high blood pressure, obesity, and insulin resistance (Scott et al., 2002). The connection between these two conditions may share a common molecular mechanism. This project utilizes inbred lines to elucidate the link between these two seemingly distant conditions.

The HXB/BXH inbred line platform was specially selected for this study because the two parental strains display some of the metabolic abnormalities seen in humans with a metabolic syndrome condition. One of the progenitor strains - spontaneously hypertensive rat (SHR), apart with naturally elevated blood pressure, shows progressively decreasing hippocampus volume paralleled by blood pressure increase (Sabbatini, 2000). The latter parental strain - Brown Norway (BN-Lx/Cub), was found to carry genes for hypertriglyceridemia (Vrana et al., 1993) and insulin resistance (Sedova et al., 2000). Their intercrosses, HXB/BXH recombinant inbred strains, carry the different combinations of these and other parental genotypes. Each strain indeed displays varied metabolic phenotypes. Later it was shown, that not only is metabolic variation present among HXB/BXH lines but also a behavioral variability (Conti et al., 2004; Bielavska et al., 2002). Thus, HXB/BXH platform is ideal for testing if memory and cognition correlates with metabolic parameters, and when they do, to explore a possibility of a genetic linkage.
The Allothetic Active Place Avoidance (AAPA) behavioral task was chosen because of its special utilization of hippocampus based spatial memory - an observable animal model of higher mental function. Although the task requires overall cognitive coordination as well, still the most putative to the tasks is the hippocampus function (Wesierska et al., 2005). Beside hippocampus, AAPA is known to require intact retrosplenial cortex (Wesierska et al., 2009).

The name of the task explains its nature, for the rat has to move **actively and allothetically** navigate in order to **avoid** punishment in a specific **place** it receives in a form of an electric shock. Allothetic orientation utilizes internal cognitive map, created to represent the arrays of external, mainly visual cues in the environment in which animal is

moving. This contrasts to idiothetic navigation where animal has to keep track of its own movement without external cues or with updating erroneous integration of path using local cues on the arena.. Spatial orientation or navigation to or away from directly imperceptible goals is often considered an animal analogue to the human declarative memory.

In this experiment the animal has to avoid part of the area that is designated for locomotion in the 20-min sessions. The arena is specially designed, rotating at the slow speed in the centre of the room (1 revolution per minute). Electric shocks are applied whenever rat enters to-be avoided section. Shock sector does not move with the arena, it is fixed in the room coordinates, stable in one cardinal compass directions (such as the North). The expected and the most efficient strategy of the experimental animal is to walk in the counter-rotational direction as to compensate for the drag of the arena into the shock sector. The necessity of the active movement forbids rat to use passive strategies such as immobility. It was demonstrated that beside allothetic navigation, this test involves ´cognitive coordination´, defined as ability to segregate spatial stimuli in the task into coherent representations of arena and room (which are dissociated by arena rotation) and to select the room frame for navigation while ignoring the substratal orienting cues (scant marks, etc) in the arena frame (Wesierska et al., 2005)

The control experiments are essential to exclude any influence of motor, sensory or motivational abnormalities and differences, as well as anxiety, on the result of behavioral testing. The open-field test and beam-walking test were run along with the Allothetic Active Place Avoidance test for this reason. Also, correlation with anxiety assays such as an open arm entries in plus maze run in other laboratory will be assessed to evaluate contribution of the anxiety on the test performance. The correlations will be made with numerous metabolic phenotypes as well.

Metabolic syndrome has been associated with cognitive decline in elder as well as middle age persons (Yaffe et al., 2007). Metabolic syndrome is a collection of the symptoms that increase the chances of developing cardiovascular disease or diabetes later in life. In humans, the metabolic syndrome comprises of five cardiovascular risk factors: abdominal obesity, hypertriglyceridemia, low high-density lipoprotein levels, hypertension, and hyperglycemia (Yaffe et al., 2007). Main factors contributing to the development of metabolic syndrome are genetics (Poulsen et al., 2001), aging, and lifestyle choices: sedentary lifestyle, low physical activity and excess caloric intake (Katzmaryk et al., 2003). The metabolic syndrome and the associated cognitive decline could be caused

by a common molecular mechanism or could have physiological causes such as increased inflammation (Oliveira et al., 2008).

The very useful tool for finding correlation within the inbred strains platform is the online GeneNetwork.org database, which stores all published phenotypic, genotypic and proteosome information concerning mouse, rat and other inbred lines - including HXB/BXH RI set. Uploaded data is simply added to the database and the correlation with the selected phenotypes or genotypes can be made. The online database is very sophisticated, its function ranging from correlations, through permutation tests to QTL analysis. The online GeneNetwork.org database will be the main aid in data analysis of HXB/BXH behavior in this experiment. It should be emphasized that all data in this database are cumulative and results of this study will be added to known phenotypes upon publishing.

One of the important applications of RI strains is a search for quantitative trait loci (QTL). The quantitative trait locus is a locus responsible for a trait that is quantitatively inherited, such as height, intelligence, etc. Many loci are responsible for the degree of the expression of a trait, each with a varying influence (polygenic traits). QTL analysis searches for the loci and assesses the degree of its influence. QTL analysis correlates the expression of the trait with the occurrence of the either of parental marker at each locus within each inbred strain.

This experiment will show, if there is a putative locus responsible for memory formation. However, the memory being a very complex behavioral trait many loci with small additive effect might be responsible for the final phenotypic expression instead of oligogenetical coding. However, this project will shed more light on genetic basis of the memory and on the possible genetic link between metabolic syndrome and cognition.

2.1 Recombinant inbred strains

HXB/BXH strain set is the largest rat recombinant inbred platform available. It allowed for genetic mapping of very complex and divergent traits (Printz, 2003). The RI inbred strains are created by gender reciprocal crossing of two divergent parental strains SHR/Ola and BN-Lx/Cub. Purpose of HXB/BXH strains was to study genetic basis of the metabolic phenotypes, and morphological abnormalities associated with polyluxate

syndrome (PLS). Development of the HXB/BXH strain set begun in 1982 by Vladimir Kren at the Institute of Biology of Charles University in Prague, Czech Republic, and by Michal Pravenec at the Institute of Physiology of the Czech Academy of Sciences (Printz, 2003).Parental strains of HXB/BXH inbred lines - SHR/Ola and BN-Lx/Cub - have a very different origin and are very varied in their phenotype at many levels.

The spontaneously hypertensive rat (SHR) is the most widely studied genetic animal model of human hypertension. The SHR strain originated from an outbred Wistar colony where the trait for spontaneous hypertension was selected by Okamoto and Aoki (1963), and subsequently inbred to form the SHR/Kyoto. Rats were later transferred to Hanover, Germany, and thereafter to Prague as the SHR/Ola. This rat line displays normal arterial pressure in early development but in the first months develop a chronic hypertension. Behaviorally, SHR rats differ form the normal Wistar rats by having higher locomotion and increase exploratory rearing behavior in the open-field (Van den Buuse and de Jong, 1988). They also display more intense humoral response to stressful stimuli (Tucker and Johnson, 1981).

The studies of the brain reveal reduced brain weight compared to normotensive age and weight matched Wistar-Kyoto (WKY) rats (Lehr et al., 1980). The consistent high pressure apparently causes ventricular enlargement which in turn decreases the brain mass (Ritter and Dinh, 1986). SHR display a very visible hippocampus volume decrease, and the loss of grey matter in both CA1 and dentate gyrus (DG) with age (Sabbatini, 2000), which are the brain regions responsible for learning and memory and essential for learning in the active allothetic place avoidance task (Cimadevilla et al., 2000; 2001).

Original Brown Norway was inbred from the wild rat in 1917, and in 1964 transferred to the Institute of Biology at Charles University (Cub). Congenic BN-Lx/Cub strain was created by transfer of Lx gene from outbred Wistar strain carrying a spontaneous mutation (*Lx*) predisposing to PLS (Kren, 1975). The original aim was to study origins of polyluxate syndrome, and the genetics responsible for the limb development. However along with *Lx* gene was unknowingly transferred the gene for hypertriglyceridemia (Vrana et al., 1993) and for the insulin resistance (Sedova et al., 2000). This increased even more the utility of HXB/BXH strains as a platform to study metabolic phenotypes. The *Lx* mutation was later mapped to a segment of chromosome 8 in close linkage with other important genes such as Drd2, Ncam, and apolipoprotein clusters: Apoa1, Apoa4, Apoc3, and ApoaV (Krenova et al., 1997).

HXB/BXH inbred strains and the two progenitor strains are very well genetically characterized. The marker-based map and strain distribution patterns (SDPs) of polymorphic markers allows studying genetic basis of many endophenotypes in a great depth (Printz, 2003).

As reviewed in (Silver, 1995), the recombinant inbred strains are created by mating two inbred progenitor strains, as genetically distant as possible, to produce F2 hybrids. Progenitor strains are homozygous at all their loci. Consequently, F1 offspring is a heterozygote at all loci that were different in its parental strains. Phenotypes of alleles at these loci now show an intermediate phenotype. During gametogenesis in F1 generation, due to crossing over, varied and irreproducible F2 generation is created. F2 progeny are randomly paired and brother-sister mated for many generations (Figure 1).

In each subsequent generation, genetic homogeneity is slowly established again (Figure 2). This process is referred to as *inbreeding*. Eventually, this process will lead to the production of inbred rat strains that are genetically homogeneous and homozygous at all loci, each with a unique combination of alleles of either BN-Lx or SHR/Ola origin. It was shown that inbred strain can be considered "inbred" after generation F_{20} (Green, 1981).

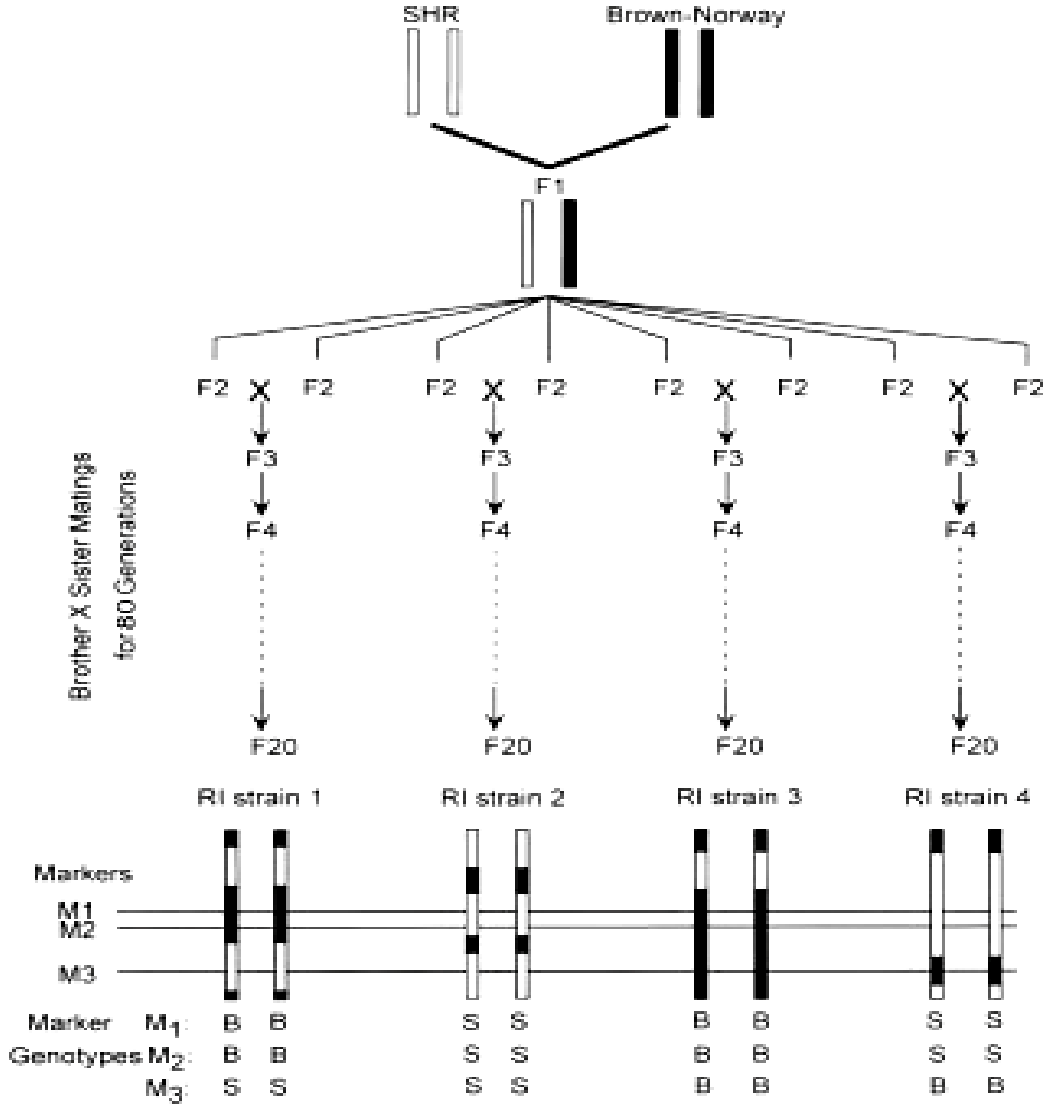


Figure 1: The standard method to produce RI strains is to cross Strain A and Strain B to give F1 progeny. The F1 rats are then crossed to produce an F2 generation. Randomly chosen F2 individuals are then continuously brother-sister mated for twenty or more generations when they are genetically fixed at more than 98% of the loci that differ between the two progenitor strains. Individual RI strains generated from the same initial cross each have a unique combination of loci derived by recombination of the alleles present in the original parental strains (adapted from Silver, 1995).

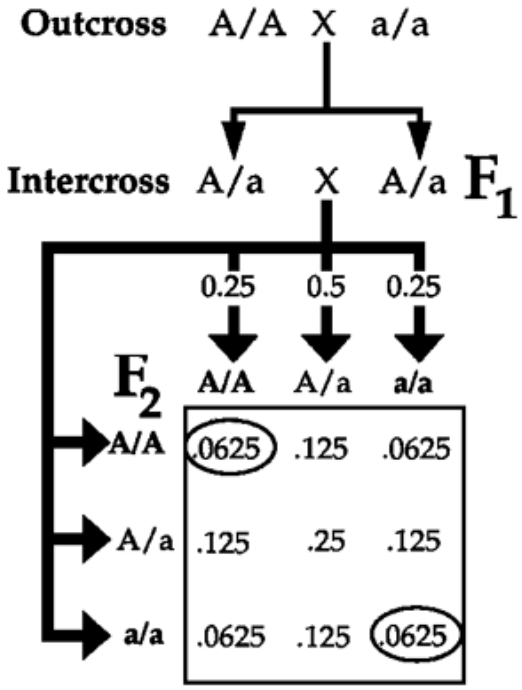


Figure 2: Due to inbreeding at the F2 generation. The first cross illustrated is an outcross between animals homozygous for alternative alleles at the A locus. The F1 offspring are all identically heterozygous A/a. An intercross between two F1 animals will produce an F2 generation with three possible genotypes having the probabilities shown. The final box indicates the different combinations of F2 by F2 matings that are possible and the probabilities associated with each one. The two mating combinations that cause fixation for one allele are circled (adapted from Silver, 1995).

In addition, development of the HXB/BXH strain set utilizes gender reciprocal crossing, which provides two sets of strains differing in the source of mitochondrial DNA and Y chromosome DNA. The HXB set where H represents SHR of female gender, and B represents male BN-Lx, was bred at the Czech Academy of Sciences. Thus all descendants carry mitochondrial DNA of SHR origin with the Y chromosome from the male BN-Lx strain. Originally, brother-sister inbreeding developed 26 HXB strains but five strains (HXB 9, 14, 16, 19, and 30) were lost after inbreeding due to poor breeding performance. The BXH set was derived at Charles University from female BN-Lx and male SHR, therefore the BXH set carries mitochondrial DNA of BN origin and males carry the Y chromosome from SHR. Altogether, 32 RI strains presently exist that are all beyond 50 generations of inbreeding. There are 21 HXB, and 11 BXH (Printz, 2003).

There are many advantages of recombinant inbred strains over single-generation intercross or backcross progeny:

1) They exhibit homozygosity at all loci.

2) All individuals of a particular RI strain are identical replicas so that studies may be replicated, permitting verification of any experimental result by independent testing of genetically identical animals.

3) Phenotyping and genotyping data are fully cumulative.

4) Studies may be conducted during pre and postnatal stages of development.

2.2 Place navigation and spatial memory

Memory is an organism's ability to store, retain, and recall information and experiences. It is essential for organism's survival. Some form of memory can be observed even in the invertebrate organisms such as *Drosophila melanogaster* (Ofstad et al., 2011). Memory is generally divided into declarative and non-declarative memory. Non-declarative (implicit) memory involves associative learning, priming, and procedural learning (motor skills), latter requiring mainly cerebellum and basal ganglia function. Declarative memory is subdivided into semantic and episodic memory. Semantic memory contains knowledge of facts independently of their context and allows the encoding of abstract knowledge about the world. Episodic memory, on the other hand, is used for more personal memories, such as the sensations, emotions, and associations of a particular place or time. Episodic memory usually inherently contains the components: "what, where and when" (it happened).

The spatial orientation is achieved by two complementary mechanisms: allothesis, the processing the information about spatial relationships between the animal and perceptible landmarks, and idiothesis, processing the substratal and sequential information produced by the animal’s active or passive movement through the environment. Both systems allow the animal to calculate its position with respect to the perceptive landmarks.

It is long known that rodents can construct the ‘‘cognitive maps’’ (Tolman, 1948), i.e., neural representations of spatial relationships between perceptible environmental landmarks. This "map" is thought to reside mainly in the hippocampus (O’Keefe and Nadel, 1978). Spatial memory is considered animal analogue of human declarative memory and therefore the highest form of memory that can be observed in the animal.

2.3 Hippocampus, memory, and navigation

The hippocampal formation is a part of a group of structures forming the limbic system, and is a component of the hippocampal formation, which includes the *Cornu ammonis* (CA) subfields, dentate gyrus (DG), subiculum, and entorhinal cortex. Different parts of the limbic system have been shown to play an essential role in all aspects of emotions, fear, learning and memory (Geinisman, 2000).

The first insights on the function of the hippocampus came from amnesia in human epilepsy patients following removal of the hippocampus and neighboring medial temporal structures (Scoville and Milner, 1956). Wide-ranging evidence suggests that the hippocampus and the related structures are important in the formation of episodic memories and consolidating information into long-term declarative memory in humans and in rodents (Reilly 2001; Mumby et al., 1999). Later, an influential book on the hippocampus (*Hippocampus as a cognitive map;* O´Keefe and Nadel, 1978) had first proposed a function of the hippocampus as a spatial memory module, allowing for a proper spatial decisions and movements in the environment. This view of the hippocampal function is vivid and very widespread until today.

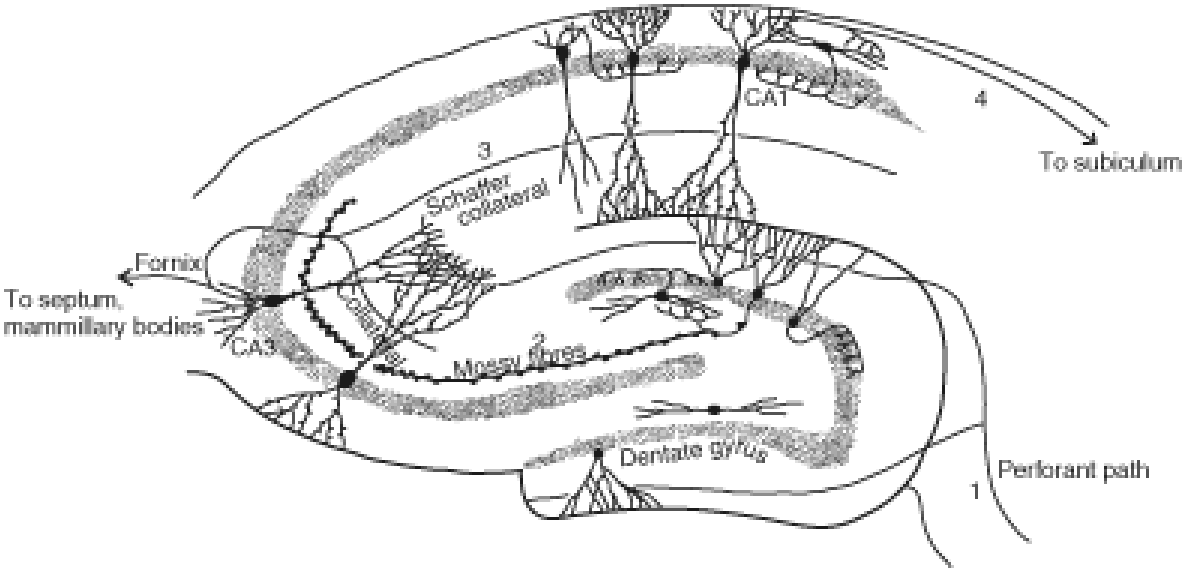


Figure 3: Visual representation of hippocampus. Depicted are Dentate gyrus (DG), CA1 and CA3 areas. The main input to the hippocampus comes from entorhinal cortex (adapted from Rolls, 2009).

Hippocampus is located in the medial temporal lobe of the brain and has a distinctive, curved shape resembling a seahorse. Although the hippocampus lies deep in the brain; beneath the cerebral neocortex, it is not a subcortical structure, but a cortical infolding. The structure is phylogenetically archicortical, i.e., much older and more primitive than the surrounding neocortex which has overgrown over it. Hippocampus contains three layers, compared to six layers present in neocortex. It is divided into four functionally different areas: dentate gyrus and CA1-CA3 areas (Figure 3). Dentate gyrus is one of the few places displaying adult neurogenesis (others are olfactory bulb and cerebellum). DG harbors predominantly granule cells and newly formed neurons, which are supplied to the CA1 area for maturation. CA1-CA3 areas contain primarily pyramidal cells. Pyramidal cells of CA1 area are thought to be “place cells”, by means of which cognitive map is formed (O’Keefe and Dostrovsky, 1971).

The place cells (Figure 4) are characterized by location-specific firing and derive their name from the fact that they fire only when an animal is in a part of its environment that the cell responds to (Kentros et al., 1998; O’Keefe and Dostrovsky, 1971). The place cells, which are found in hippocampus area CA1, receive positional information from two different areas: the intra-hippocampal network in area CA3, and directly from the entorhinal cortex containing grid cells (Brun et al, 2002).

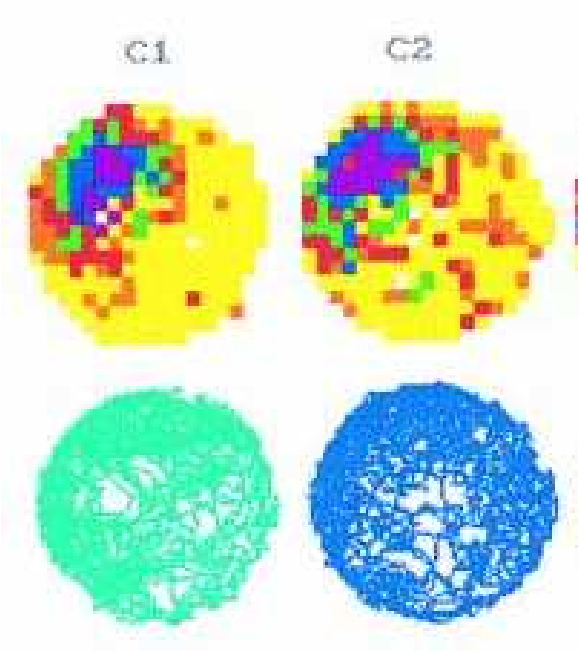


Figure 4: Firing of a hippocampal place cell in the circular arena. Upper images show the color-coded maps of firing rate in pixels of the arena, lower images show the tracks of animals in the arena (note that animals almost entirely covered the arena with locomotion) (Adapted from Bures et al., 1997)

The existence of place cells together with the lesion studies suggests that the hippocampus indeed plays a very important role in the processing of the spatial information (O'Keefe and Nadel, 1978). In literature, there are numerous evidences that removing or inactivating hippocampus indeed induces strong and permanent deficits in a wide variety of spatial memory tasks (O'Keefe and Nadel, 1978).

One of the tasks which are dependant upon hippocampal learning is Active Allothetic Place avoidance (AAPA) (Cimadevilla et al., 2000; 2001). In this task rat is placed into the rotating arena where a sector of the arena is allocated as a punished sector. The shock sector does not move with the arena movement but remains fixed in the room coordinates. Upon entering it, an animal receives a mild electric foot shock. Compared to some other spatial learning tasks AAPA require an overall cognitive coordination. Animal has to move during the whole time in order to avoid a drag into the punished sector. This demands much longer concentration and comprehension of the nature of the task. Also, animal must display only a limited anxiety as to avoid freezing reaction. Lastly, animal has to exhibit a learning flexibility as shock sector is changed to the different position in the middle of the protocol.

2.4 Genetic analysis

Before genotyping rat genome, only the genetic map could be relied on. Genetic map does not give a precise position of the allele; it just shows the relative position based on the distance to the other markers based on recombination frequency measured in cM, with one centimorgan being equivalent to a crossover rate of 1%. After completion of the rat map, the new and more precise genetic map was created. This was done in La Jolla and in Prague by Jirout et al. (2004). This endeavor resulted in the map of the 20 rat autosomes with 245 microsatellite markers. The total mapped length is 1,789 cM agreeing with the newly completed rat map. The coverage is throughout the whole genome with the inter-marker distance ~ 0.8 cM. The map is available at **http://www.ratmap.gen.gu.se**. Detailed map was a great opportunity to analyze Recombinant inbred strains for the advantage of QTL mapping (Jirout at al., 2004). Progenitors' polymorphic markers are distributed evenly over the strains except for the similarity between BXH8 and HXB8 because the strains diverged after F2 generation and HXB3 with HXB15 exhibit 78% similarity for unknown reasons (Jirout at al., 2004).

In addition, three areas with the same sequence were identified, as well as three sequences with a mirror image sequence. In both of these instances there is a chance of mistakenly assigning multiple QLT, or opposite allelic influence therefore they must be taken into the account during the QTL analysis (Jirout et al., 2004).

2.5 Quantitative trait loci (QTL)

A significant fraction of genes are polymorphic—that is, they exist in multiple forms or alleles. Differences in alleles generate variation in CNS structure that may be subtle or very prominent. Normal variation is a significant source of behavioral and other differences among humans and animals. These differences are caused not by mutations but by the cumulative action of many normally variable genes and by the action of numerous developmental and environmental factors. This is a very important source of variance: different forms of alleles (Williams et al., 1988).

QTL analysis provides information about these common gene variants. In contrast with knockout experiments and mutation studies which produce extreme phenotypes, QTL analysis shows fine differences between alleles and the effect they have upon the phenotypes in natural populations. QTL analysis was made much easier availability of Recombinant inbred strains, with all its lines very well genetically characterized.

The complex trait analysis was developed in the late 1990s as a result of the mergence of quantitative and molecular genetics (Lander and Schork, 1994). The techniques associated with this approach have greatly extended the amount of CNS phenotypes that can be subjected to the molecular analysis.

The QTL analysis links the behavioral and other phenotypic variance with a segment of the genome. The link exists if the variance in the given locus correlates with the variance in the phenotype. Although the QTL analysis only pinpoints the area in between the two markers instead of an exact gene locus it nevertheless remains an important step in finding candidate genes responsible for the differences important to these behavioral phenotypes.

The whole rat genome is covered by microsatellite markers with the inter-marker distance 0.8 cM. A microsatellite analysis begins with the identification of regions of non-coding DNA containing short repeats. Each repeat motif is usually two to four base pairs in size, and the number of these repeats is highly variable between the parental strains. Microsatellite polymorphisms can arise through the replication slippage, unequal crossing over, or mutations extending or shortening the series of repeats. Each RI strain contains a unique combination of parent specific microsatellite markers due to the segregation and recombination of alleles in F1 generation. It must be noted that the QTL analysis of the RI strains compares only two allelic variants, in comparison to many allelic variants possibly present in a natural population at each locus. The Recombinant inbred strains are small models of populations containing infinite copies of each individual. This ensures cumulativeness of data and gives a possibility of repeated experiments on naive animals. This is very important, for such convenience is not possible in experiments in a natural population. Each strain is sequenced, with the dense microsatellite map, and therefore observations about correlation between phenotype and allelic variants is made much less complicated.

A variation in hippocampus function is the biggest candidate for the spatial learning differences found among HXB/BXH strains. As mentioned before, HXB/BXH strain set displays different combination of alleles at their loci. The different combination of these alleles at loci influencing a hippocampus function will yield a different range of learning capability. Differences in alleles influencing hippocampus function will have either positive or negative impact on its function. The challenge is to identify the most consequential loci responsible for this variance, and their location on the chromosomes. As

memory is a very complex behavior, therefore it is expected that additive effect of many loci of small influence will be responsible for the resultant trait. Once the most prominent loci are indentified to a particular segment of DNA, the responsible genes can be indentified. The analysis is simplified by a recently completed transcriptosome of hippocampus mRNA for all HXB/BXH strains (Williams, unpublished results). The collection includes all mRNA transcripts and their amounts in hippocampus extracted from each strain (three animals per strain). The chromosomal location of each mRNA transcript is known.

A measurement of association of phenotype and genome sequence is given as a *logarithm of odds* (LOD) or *likelihood ratio statistics* (LRS) scores. Both of these measurements stand for a logarithm of a ratio of likelihood that correlation between observed phenotype and genotype indeed exists (hypothesis) to a likelihood that observed correlation is occurring by a chance (null hypothesis). Therefore, if the chance of a false positive correlation is for a certain locus and a phenotype 1: 1000 the LOD score for the correlation is log1000 that is 3. LRS score is simply LOD score multiplied by 4.61. Usually, LOD score above 1.9 is considered suggestive, and represents 65% probability of falsely rejecting null hypothesis. These are the loci worth paying special attention to, especially when receiving a recurrent result from repeated measurements. LOD score above 3.2 is usually considered significant meaning 5% chance of falsely rejecting null hypothesis (p=0.05)(Williams RW and Manly KF, Nov 15, 2004 - GeneNetwork). To estimate suggestive and a significant loci, permutation tests are used. Permutation tests are essential to exclude falsely significant QTLs.

Complex trait analysis is very complex and great care has to be taken to exclude a chance and other influences when detecting QTLs. Linear regression tests to rule out the QTLs that are linked to the other traits that significantly influence the trait in question.

2.6 Genetic variation and estimating heritability

The variation is simply the natural differences in the value of the trait. It is unwanted in most experiments, however in the search for the alleles and the loci responsible for natural variation it is an absolute must. The greater is the natural variance, the greater success of mapping a significant QTL. The variance is measured as the square of the standard deviation. The variation has many sources (Falconer and Mackay, 1996) and can be partitioned into three major components. They are technical and sampling

variance (VT), environmental variance (VE), and additive genetic variance (VA). There are several ways to separate them (Williams et al., 1996).

All the variance within the inbred stain is due to technical and environmental error, since all the individuals are genetically identical. A technical error can be estimated by repeated analysis. To reduce it, it is advantageous to have only one experimenter to produce the results. Thus, the technical error is same for each animal, and does not have to be regarded as much. Other means to reduce the technical error is to increase a number of sampled animals, 6-8 animals is thought to be reasonable to get reliable strain averages (Williams et al., 1996).

The variance within the strain after the subtraction of the technical error is purely environmental. It includes variation in age, body weight, litter size, age of mother and differences in litter size, exposure to pathogens, temperature and humidity variation, differences in the food chow, seasonal fluctuation in water quality, and a many other environmental factors that can be the source of variation (Williams et al., 1996). The ratio between variance within the strain and overall variance indicates the true level of genetic variability (Williams et al., 1996).

The heritability is the proportion of the total variation between individuals in a given population due to the genetic variation. This value can range from 0, meaning no genetic contribution to the variance, to 1, suggesting that all the variances in a trait is reflected by genetic variation. In human population, the heritability for the behavioral traits based on twin studies ranges from 0.3 to 0.6 (Plomin et al., 1994). In inbred lines heritability is counted as the fraction of the total variance throughout the strains divided by the variance within the strain. Homozygosity at each locus within the recombinant inbred strain must be accounted for; therefore variability values are divided by 2.

2.7 Metabolic syndrome

The metabolic syndrome is a collection of symptoms predisposing an individual to a much higher risk of cardiovascular disease and diabetes type 2. There are several metabolic risk factors constituting metabolic syndrome (Scott, et. al., 2002). These are:

- *Abdominal obesity*, the form of obesity most strongly associated with the metabolic syndrome. It presents itself as an increased waist circumference.

- *Atherogenic dyslipidemia,* manifested by raised triglycerides and low concentrations of HDL cholesterol. There could be present other lipoprotein abnormalities as well, e.g., increased lipoproteins, elevated apolipoprotein B, small LDL and HDL particles. All of these abnormalities have been implicated as being atherogenic (Kolovou et al., 2005; Ginsberget al., 2000).
- *Elevated blood pressure* strongly associates with obesity and commonly occurs in insulin-resistant persons.
- *Insulin resistance* is present in the majority of people with the metabolic syndrome. It strongly associates with other metabolic risk factors and correlates with CVD risk. The insulin resistance is not the cause of the metabolic syndrome; more likely it is a passive by product of fat deposition in the liver and muscle. It appears that the fat accumulation in the liver is associated with several features of insulin resistance even in normal-weight and moderately overweight individuals (Fodor, 2011).
- *glucose intolerance*, is a symptom much connected to the insulin resistance. When the glucose intolerance evolves into diabetes-level hyperglycemia, elevated glucose constitutes a major, independent risk factor for cardiovascular disease.
- *pro-inflammatory state*, recognized by elevations of C-reactive protein (CRP), is commonly present in persons with the metabolic syndrome. Multiple mechanisms seemingly underlie elevations of CRP. One cause is obesity, because an excess adipose tissue releases inflammatory cytokines that may elicit higher CRP levels (Oliveira et al., 2008).
- *prothrombotic state*, characterized by increased plasma plasminogen activator inhibitor (PAI)-1 and fibrinogen, also associates with the metabolic syndrome (Mauras et al., 2010).

Some studies show the symptoms of metabolic syndrome – the high blood pressure specifically - increases cognitive defects observed in elderly population over the 20 year study period (Farmer et al., 1990). Even middle age people display an impaired cognitive functioning compared to their non-metabolic age matched controls (Hassenstab, 2010). Many other studies directly show that subjects with hypertension, but with no clinical evidence of a vascular disease or diabetes, display impaired cognition in a broad range of tests of attention and short- and long-term memory (Harrington et al., 2000; Breteler et al., 1994). The suspected connecting link between symptoms of the metabolic syndrome and a cognitive decline observed is apolipoprotein E (ApoE), protein involved in the lipid

catabolism. ApoE allelic frequencies are connected to vascular disease risk (Vaukonen et. al, 1997). However, the effect of the metabolic syndrome and ApoE may contribute to the observed cognitive decline independently as some studies show (Knopman et al., 2009). An interactive effect between triglyceride, HDL levels and ApoE on cognition was shown to have a detrimental effect on cognition, although separately the effect of each component did not have as much effect (Lee et al., 2010). The link with cognition is also suggested with senile plaques (Sparks et. al, 1995), inflammation (Yaffe et al., 2004) and importantly Alzhemer's disease (Strittmatter et al., 1993). Men, far more than women, are affected by the cognitive decline as a side effect of metabolic syndrome (Cavalieri et al., 2010).

3 Aims of the study

1. Evaluation of hippocampus-dependent learning in the AAPA in the RI set.

2. Testing the hypothesis that there will be significant differences between strains.

3. Exclusion of the possibility that differences in AAPA learning will correlate with performance in control tests (open-field and beam walking tests)

4. Search for quantitative trait loci (QTLs) that appear to harbor alleles that are responsible for the observed differences in the learning behavior.

5. Search for correlations between memory and metabolic phenotypes that might support the relationship of learning and metabolic syndrome.

4 Methods

4.1 Animals

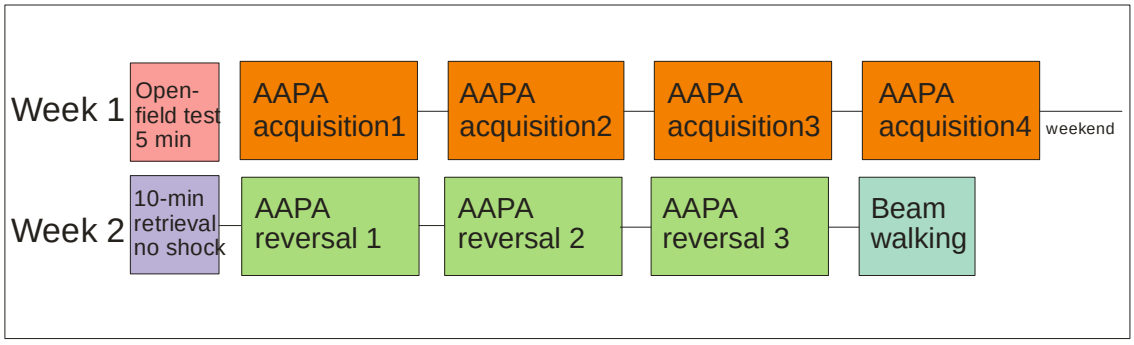
The behavioral part of the experiment included six to eight rats per one RI strain. Animals were obtained from Dpt. of Genetics of Model Diseases at the Institute of Physiology ASCR (Dr. M. Pravenec). All animals were males aged from 11 to 14 weeks. They were housed in an air-conditioned animal room with a constant temperature and regular 12/12 light/dark cycle. The rats received water and food *ad libitum*, with 12/12 light dark cycle, and all experiments run during the light period. All unhealthy or overly stressed animals were removed from the experiment.

Prior to the experiment the rats were gently handled 2 minutes daily for 3 consecutive days. Immediately before the experiment, surgical needle was applied to the skin on the neck of each animal and twisted to ensure that it will not fall out during the course of the experiment. This needle is to conduct electric current through the animal during the experiment.

The behavioral protocol consists of three distinct parts: open-field, active allothetic place avoidance (AAPA) and beam-walking - they will be described in this order.

4.2 Behavioral tasks

Beside prior habituation, the behavioral phenotyping took two weeks. The schematic diagram of study design is depicted in the following scheme (Scheme 1)



Scheme 1: Block diagram of behavioral phenotyping in the open-field test, AAPA and beam walking.

4.2.1 Open-field task

The open-field is a test of spontaneous activity in an enclosed space, usually a square. The rat is monitored by the overhead camera and observed by an experimenter within the 5 minute time window in 1x1 meter white plastic arena.

The arena does not contain any objects, or stimuli of interest. All the walls are painted white, except one wall made of glass used by an experimenter to observe the rat behavior. Before each use the arena is purged of any smells and waste with dishwashing liquid.

In the open-field the total distance, thigmotaxis, and complete ethographs is recorded. The total distance report is generated by a track analysis program. The distance walked indicates the motor abilities and presence of locomotion defects that may impede on the capacity of the animal to move in the arena in the later AAPA testing.

Thigmotaxis is the movement along the walls - the common measure of anxiety in laboratory animals. To measure thigmotaxis the arena is divided into 9 sectors. The level of thigmotaxis is given by the ratio of time spent in the sectors adjacent to the walls and in the central segments.

Ethograph is the quantitative description of an animal's normal behavior. It includes a record of sniffing, cleaning and rearing behaviors during the 5-minute window the rat is in the open-field arena. The sniffing behavior was subdivided into sniffing the surfaces of the open-field arena and sniffing air. The cleaning behavior was partitioned into cleaning head and cleaning rest of the body. The rearing was counted as standing upright on the hind legs without assistance of the arena walls.

Beside spontaneous activity and exploration, some measures (such as the thigmotaxis or time in the center of arena) can be used to estimate the anxiety levels.

4.2.2 Active Allothetic Place Avoidance task (AAPA)

Active allothetic place avoidance task (AAPA) is a hippocampus dependent learning paradigm. The apparatus is located in a rectangular room (4m x3m) illuminated by the shaded light in one corner. Walls of the arena are made of see-through plastic offering a view to abundant landmarks which is the animal to orient by such as windows, shelves, a door, a table and added colored papers with symbols pasted on the walls. The rat is placed into an elevated rotational circular arena. It has a light and current source attached to the

back, so its movement is detected and it can receive electric shocks, respectively (Figure 5).

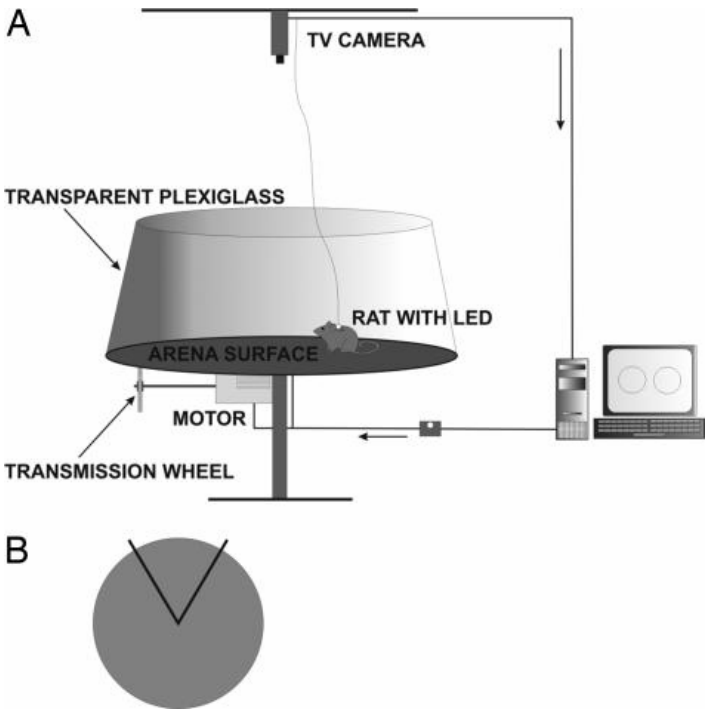


Figure 5: The illustration depicting sideway view of the experimental arena with the computational aperture; computer and the monitor present in different room. The bottom illustration shows the areal view of the experimental arena. The punished area always remains in the same location, although the floor of the arena rotates underneath and conducts electric current. (Adapted from Telensky et al., 2011)

Arena rotates at speed 1 rpm (1 revolution per minute), in the clockwise direction. Defined in the coordinates of the room, facing north, is a 60 degree section of the arena designated as a forbidden sector. In the experiment an animal is to learn to avoid this sector. The forbidden sector stretches from the centre to the outside edge of the arena. Upon entering it a rat is punished by an electric shock with intensity cca 0.6 mA. The shock is received every second during the whole time the rat is inside the forbidden sector. The rat is introduced to the arena in the same position every time, opposite to the shock sector.

The whole protocol lasts 10 days, including three days of rest following the fourth day. First four days rat is trained in the arena - acquisition sessions, next three days are the rest period, and the last three days are the testing period of the long term memory - retrieval together with learning to solve the task with the reversal of the shock sector to the opposite side of the arena - reversal.

On days 1, 2, 3 and 4 each rat is placed into the AAPA arena for the 20 minutes. This is called **acquisition session**, because the rat is learning, acquiring the skill of avoiding the shock sector and learning the principle of the task. By the day 4 rats should be able to avoid the shock sector with a success.

Next three days are the resting days, where rat is to consolidate what he learned into the long term memory. On experimental day 5 of the experiment the rat is placed into the arena for 10 minutes with the shock sector turned off. It is called **retrieval session** and it is to test for the confidence of the animal in the precise location of the shock sector. The result of the retrieval session is a very sensitive measure of the long-term memory.

1-2 hours later animals begin their **reversal sessions**. Shock sector is moved 180 degrees, now facing opposite side of the room as before (south). A reversal session lasts three days, for 20 minutes every day for each animal.

During the analysis the focus will be on selected parameters:

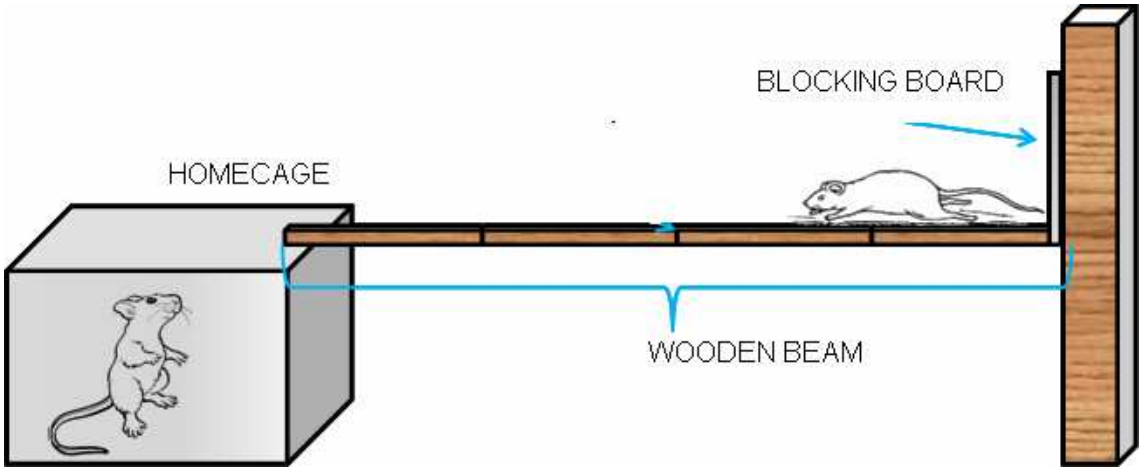
- time to the first entrance to the forbidden sector (variable name: T.FIRST), seconds
  - *showing a memory trace remembered from the previous session.*
- number of entrances to the shock sector (ENT), count
  - *showing the overall performance in the task*
- maximum time avoided (MAX.T), seconds
- *another measure of cumulative performance within a session*
- total distance (DIST), meters
  - *reflected total locomotor activity*

We have selected the following sessions as being most relevant for behavioral interpretation: the final day of acquisition (day 4), retrieval, first day of reversal (day 5) and last day of reversal (day 7) and a 10-min retrieval session (prior to reversal). Performance on the final day of acquisition reflects asymptotic performance in the test (Wesierska et al., 2009), whereas the performance on non-reinforced retrieval (shock off) served as a marker of memory retention. Performance in the first reversal session (obviously here with no usage of T.FIRST) shows the fast capture of changed rules in the task and might be specifically dependent on the dentate gyrus function and finally, the last session of reversal training (day 7) reflects final performance in the reversal condition, All these session will be included in the analysis.

4.2.3 Beam-walking test

The beam-walking is a task demanding sensorimotor coordination. In this experiment the beam-walking test serves as a control to exclude sensory or motor defects that could impede on the AAPA performance. The task for an animal is to walk on the thin wooden beam from one end to the other into the home cage placed at the other end of the beam as a positive motivation. The animal trains first on the 5 cm wide beam, later on a thin 2cm beam. On both thick and thin beams each animal has six runs - always the first three starting half way to the home cage and than the full distance. Immediately after the training, the rats were tested three times.

The time to cross the beam, number of footslips, and number of falls are recorded. An averages are computed for each animal and consequently for each strain. Animals with a severely developed luxation remain on a wide beam throughout the whole beam-walking experiment and could not be tested on the narrow beam.



Scheme 2: Schematic illustration of the beam-walking test (author: Anna Zemanová; reproduced with permission)

4.3 Data analysis and statistics

Statistical tests include two-way ANOVA followed by Student-Newman-Keuls method, regression analysis (linear regression), permutation tests, and Pearson's correlation.

To ensure that the desired traits are mapped, caution hat to be taken because a trait may be tightly correlated with other traits. It is not desirable to unintentionally map genes that control motility or anxiety. Therefore there is a possibility of mapping wrong QTL or no QTL at all. This has to be accounted for specially when mapping QTLs that modulate

rat behavior (Williams et al., 1998). For this reason linear regression analysis has to be preformed to explore and exclude these co-variances (Williams, 2000). The regression analysis is used to test significance of the effects of variance in spatial memory due to differences in motility, exploratory behavior, and anxiety levels. To analyze how these factors influence the result of the experiment, important parameters from the AAPA will be selected and compared to the control parameters by the regression analysis. For linear regression analysis SYSTAT 12 statistic program will be used.

Permutation test is a non-parametric method designed to reduce false positive results. Each QTL will be tested by the permutation tests in order to assess the probability of similarly significant results occurring by a pure chance. This is to make sure that the possibility of making a type I statistical error (declaring the presence of a QTL when there is none) is under 5% (Elston, 1998). Permutation test will be used with 2000 permutation, a method to determine the genome-wide 0.05 level (and 0.65 for suggestive loci) for each trait that is mapped (Churchill and Doerge, 1994). In the permutation test trait values are randomly reassigned to the genotypes. These permuted datasets are treated in the same way as the original data. Consequently, it is apparent how often similarly significant/suggestive QTLs are successfully mapped with the disordered data. If many of the permuted datasets produce LOD scores that are as good as or better than ones generated by the correctly ordered data, not much confidence can be placed in an observed QTL. In contrast, if fewer than 1 in 100 of the permuted datasets reaches the level of correctly ordered data, there is a confidence of the probability of having made a type I error under 0.01. In this study, 2000 permutations are run, using GeneNetwork.org application. Using the generated values a threshold for suggestive and significant LOD scores are estimated.

4.3.1 Heritability

Since animals are isogenic within each strain, the strain phenotypic means represent genetic, more than environmental, influences over the trait (Falconer, 1989). The genetic variation of a given trait is represented by the variance between strains. Thus, HXB/BXH RI strain data allow a direct estimate of heritability. Assuming no dominance, this genetic variation is equal to the additive genetic variance (VA). Inbreeding during the production of the RI strains doubles the genetic variance (Falconer, 1989). Therefore, to obtain an accurate estimate of VA, the between-strain component of variance was divided by two.

The pooled within-strain variance represents the environmental variability (VE). Thus, heritability is estimated by VA/VP, where VP = VA + VE:

h^2 = 0.5Va / (0.5Va+Ve) (Hegmann & Possidente, 1981)

Calculation of heritability will be made using Microsoft Office Excel 2007 application.

4.3.2 QTL analysis

Potential association between a trait and locus is measured using the logarithm of odds (LOD). The significance threshold for the genome wide scan was empirically determined by the GeneNetwork permutation test using 2000 permuted datasets. Significant linkage was defined in accordance with the guidelines (Lander and Kruglyak, 1995) as statistical evidence occurring by chance in the genome scan with a probability o less than 5%, and suggestive linkage as less than 65%. Based on these criteria and the results of the permutation test LOD score were established as the thresholds for significant and suggestive linkages in the HXB/BXH strain dataset. The Haplotype Analyst method of GeneNetwork was used to pinpoint the potential QTL between two markers.

5 Results

5.1 Distribution of the data

Parameters from the AAPA task were selected based on a previous experience of their relevance as a measure of learning. All behavioral values are expressed as means and standard error of the mean (S.E.M.) The chosen parameters were: number of entrances to the shock sector (ENT, measured in number of entrances into shock sector), maximum time of avoidance of the shock sector (MAX.T, measured in seconds), time to the first entrance to the shock sector (T.FIRST, measured in seconds) and lastly a locomotion (DIST, measured in meters). Distributions of mean values of these parameters and S.E.M. at selected days are included as their graphical representations in appendix. Illustratively, values of number of entrance (ENT) on day 4 are shown in Figure 6.

All but one parameter (T.FIRST) display normal distribution patterns in their values. A data with a skew value in range 1 to -1 were considered acceptable. A skew value is a measure of symmetry of probability distribution. Data for T.FIRST were

transformed by the powers of -0.71 to 0.21 to reach zero skew value. Microsoft Office Excel 2007 application was used.

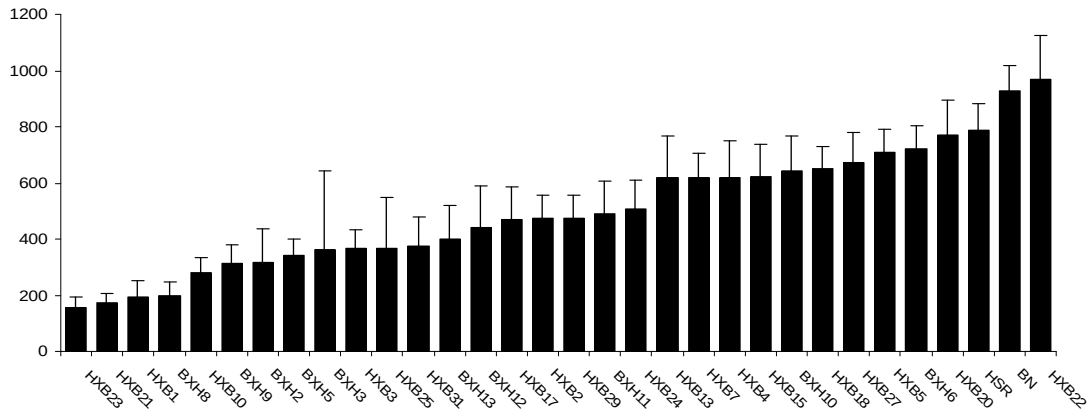


Figure 6: Distributions of mean values of these parameters and S.E.M. in the SHR/Ola, BN-Lx/Cub, and HXB/BXH recombinant inbred strains for day 4 (last day of acquisition) number of entrances to the forbidden sector.

5.2 Comparison of the strains by an analysis of variance (ANOVA)

Despite the fact that the main aim of the study was to map QTLs and correlations of learning parameters in the whole RI set, analysis of the between-strain differences was pursued as well. Following sessions were selected as being behaviorally relevant: performance in day 4 shows the asymptotic performance of the control animals in the AAPA; retrieval session reflects the memory retention from day 4 and is not reinforced (shock off); learning in day 5, first reversal session, reflected the fast capture of the spatially-reversed condition; and finally, the behavior on the final reversal session (day 7) showed the asymptotic performance in the reversal training. Strain effect on the different parameters of learning (T.FIRST, ENT, MAX.T) and locomotion phenotype (DIST) was evaluated in these putative sessions with one-way ANOVA followed by Student-Newman-Keuls post hoc test when appropriate and using a Bonferroni´s correction for multiple comparisons.

One-way ANOVA carried on a parameter 'maximum time avoided' (MAX.T) displayed a significant effect of Strains in day 4: ($F(31,181) = 3.90$; $p < 0.05$, day 7 ($F(31,182) = 3.37$; $p < 0.05$and retrieval session ($F(31,182) = 3.00$; $p < 0.05$.The *post-hoc* test showed that BN-Lx progenitor strain exhibited significantly higher avoidance times on days 4, 7, and retrieval session ($p<0.05$). HXB22 exhibited a significantly better learning compared to the other strains in day 4; and HXB27 on reversal and day 7 sessions ($p<0.05$).

One-way ANOVA carried on a parameter 'number of entrances' (ENT) showed a significant effect of Strains in day 4: ($F(31,152) = 2.28$; $p < 0.05$, day 5 ($F(31,182) = 2.56$; $p < 0.05$; day 7 ($F(31,181) = 2.95$; $p < 0.05$; and retrieval session ($F(31,180) = 2.49$; $p < 0.05$. The post-hoc test showed that BN-Lx progenitor strain significantly differed from the other strains with fewer entrances in all sessions except day 4 session ($p<0.05$). BXH13 and HXB21 strains entered the shock sector significantly more than other strains in day 7 ($p<0.05$).

One-way ANOVA carried on a parameter 'time to the first entrance' (T.FIRST) showed a significant effect of Strains in day 4: ($F(31,181) = 2.35$; $p < 0.05$; day 5 ($F(31,182) = 1.91$; $p < 0.05$; day 7 ($F(31,182) = 2.89$; $p < 0.05$; and retrieval session ($F(31,181) = 1.95$; $p < 0.05$.The post-hoc test showed that the significantly longer time spend in time to the first entrance was exhibited by strains HXB25 and HXB20 ($p<0.05$) compared to the other strains.

One-way ANOVA carried on a distance walked parameter (DIST) showed a significant effect of Strains in day 4: ($F(31,181) = 6.42$; $p < 0.05$, day 5 ($F(31,181) = 11.80$; $p < 0.05$; day 7 ($F(31,181) = 6.23$; $p < 0.05$; and retrieval session ($F(31,181) = 6.31$; $p < 0.05$. The *post-hoc* test showed that SHR progenitor strain exhibited a significantly higher locomotor activity compared to the other strains in day 5; HXB18 and BXH10 displayed significantly higher locomotor activity in all tested days; and HXB7 higher mobility only in day 7 ($p<0.05$). Significantly lower locomotion was displayed by HXB5 on day 7 and in retrieval session and BXH3 on day 4 ($p<0.05$).

Distribution was of learning parameter values among RI strains was continuous, suggesting a polygenic mode of inheritance (Figure 6, appendix.). The differences between RI strains were statistically significant in all selected learning parameters (ENT, T.FIRST, MAX.T) in all days except for day 5; and for a locomotion phenotype in all days at a significance level $p< 0.05$.

5.3 Heritability of the traits in the AAPA task

As seen from the graph (Figure 8), the most heritable component of the learning/behavior in the carousel maze is the locomotion (DIST), with average heritability 0.48; the least heritable component of learning is time to the first entrance (T.FIRST) with average heritability 0.34 (figure 7). The average heritability for all traits is 0.4.

	day 1	day 2	day 3	day 4	day 5	day 6	day 7	retrieval	average
ENT	0.42	0.42	0.44	0.41	0.37	0.37	0.37	0.37	0.4
MAX.T	0.41	0.41	0.37	0.42	0.36	0.39	0.41	0.35	0.39
DIST	0.4	0.49	0.46	0.48	0.58	0.45	0.48	0.48	0.48
T.FIRST	0.35	0.37	0.37	0.35	0.35	0.29	0.33	0.32	0.34

Figure 7: The table illustrating the heritability calculated for each day and measured parameters. The retrieval session is placed at the end, although chronologically belongs after day 4. The last column depicts averages for each measured parameter.

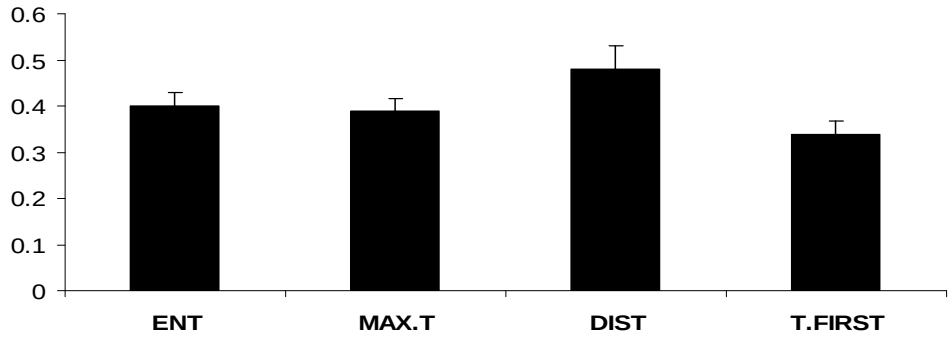


Figure 8: a graph exemplyfing average heretability values throughout the whole experiment for a selected learning parameters. As can ve seen the lowest heretability is fot the time to the first entrance to the arena - 0.34±0.03, the highers heretability is for locomotor activity (distance) - 0.48±0.05.

5.4 QTL analysis

All suggestive and significant LOD score thresholds were calculated with 2000 permuted datasets using GeneNetwork.org application. The suggestive and significant QTL's were calculated without the use of parental strains.

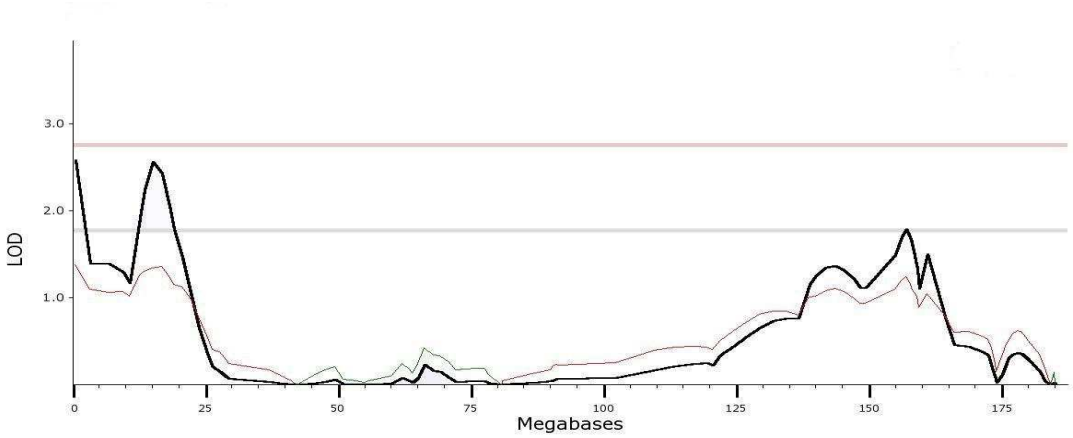


Figure 9a: figure shows suggestive locus for locomotion on day 4 on the chromosome 4 located between markers D4Utr21 and D4Ucsf1, max LOD= 2.6

The two horizontal lines represent the threshold LOD scores of suggestiveness (the lower line) and significance (the upper line), as estimated by the permutation tests. The thick line indicates LOD score for the trait - retrieval. Red line indicates that BN alleles increase trait values, while green line indicates that SHR alles increase the trait value

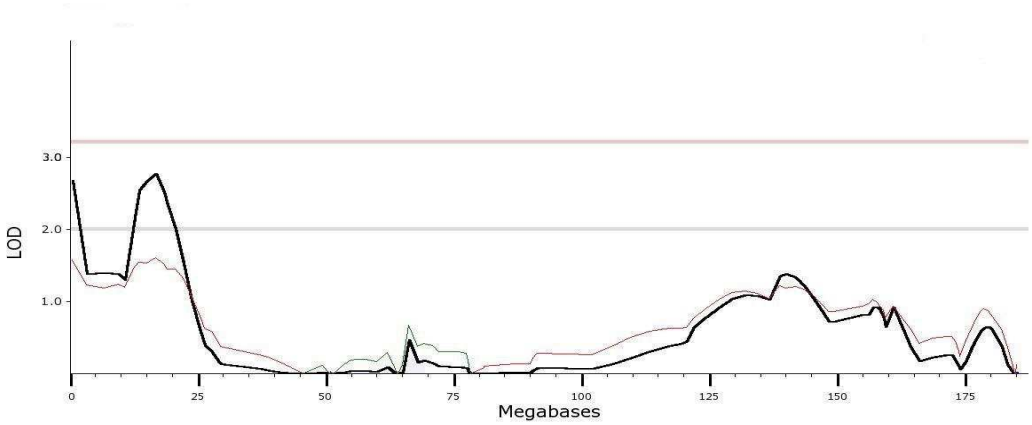


Figure 9b: figure shows suggestive locus for locomotion on day 5 (first day reversal) on the chromosome 4 located between markers D4Utr21 and D4Ucsf1, max LOD= 2.8

The two horizontal lines represent the threshold LOD scores of suggestiveness (the lower line) and significance (the upper line), as estimated by the permutation tests. The thick line indicates LOD score for the trait - retrieval. Red line indicates that BN alleles increase trait values, while green line indicates that SHR alles increase the trait value

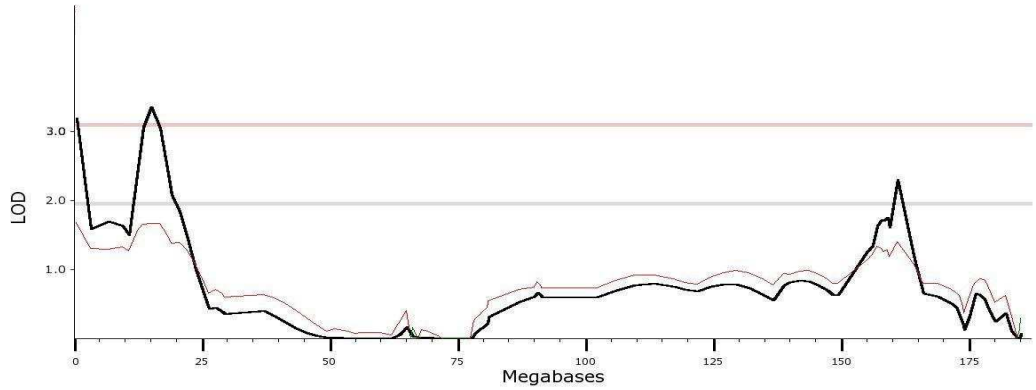


Figure 9c: figure shows a significant QTL for locomotion on day 7 (final day of reversal) on the chromosome 4 located between markers D4Utr21 and D4Ucsf1, max LOD=3.2

The two horizontal lines represent the threshold LOD scores of suggestiveness (the lower line) and significance (the upper line), as estimated by the permutation tests. The thick line indicates LOD score for the trait - retrieval. Red line indicates that BN alleles increase trait values, while green line indicates that SHR alles increase the trait value

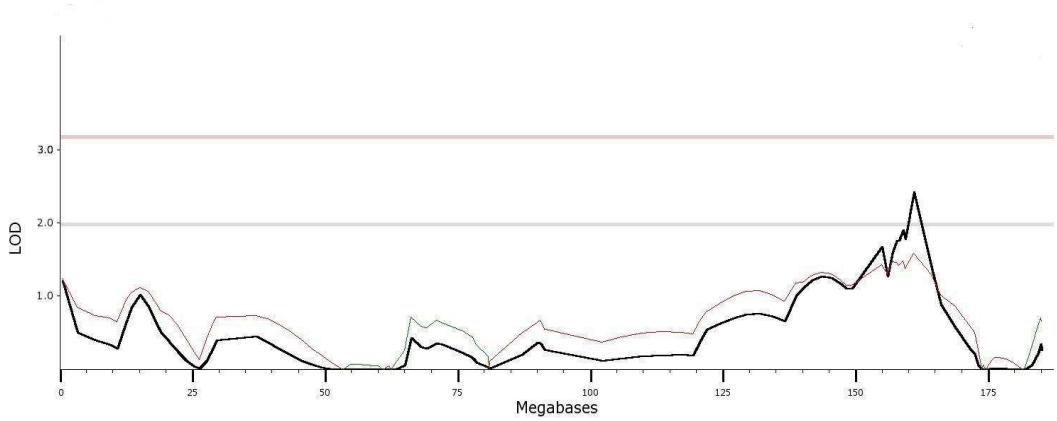


Figure 9d: figure shows a significant locus for locomotion on retrieval session on the chromosome 4 located between markers D4Rat202-D4Utr41, LOD=2.4.

The two horizontal lines represent the threshold LOD scores of suggestiveness (the lower line) and significance (the upper line), as estimated by the permutation tests. The thick line indicates LOD score for the trait - retrieval. Red line indicates that BN alleles increase trait values, while green line indicates that SHR alles increase the trait value

A suggestive locus for locomotion activity in days 4 chromosome 4 (D4Utr21 - D4Ucsf1, max LOD= 2.6). According to permutation tests (2000 permutations) a suggestive is a LOD > 1.77 (Figure 9a). In the same chromosomal location, there is a suggestive QTL for locomotion on day 5, the first day of reversal, with a max LOD= 2.8 (suggestive is LOD > 2.00) (Fig 9b).

A significant LOD score is located again in chromosome 4 for a locomotor activity on day 7 (last day of reversal) with a LOD = 3.2 (significant LOD> 3.10). In all cases, computer analysis indicates that BN allele increases the trait value (Figure 9c).

However, max LOD score location is different for a retrieval session, where a suggestive loci lies on different chromosome 4 location (D4Rat202-D4Utr41, LOD=2.4, suggestive LOD>1.98). Again, BN allele increases this trait value. In between these markers is found a gene for enolase 2, gamma (Figure 9d).

This suggests a possibly two important regulation loci of locomotor activity on chromosome 4.

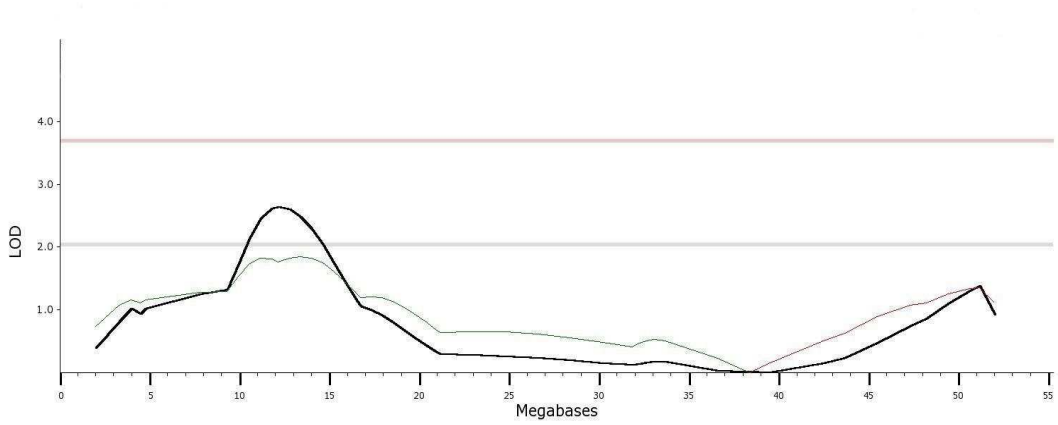


Figure 10: showing a segment of chromosome 20 for a 'time to the first entrance' on day 7 (LOD = 2.7).
The two horizontal lines represent the threshold LOD scores of suggestiveness (the lower line) and significance (the upper line), as estimated by the permutation tests. The thick line indicates LOD score for the trait - retrieval. Red line indicates that BN alleles increase trait values, while green line indicates that SHR alles increase the trait value

S suggestive locus (LOD = 2.7, suggestive LOD> 2.04) was discovered on chromosome 20 for a 'time to the first entrance' on day 7 in between markers D20Mqh5 and D20Rat75.

No other marker achieved a significant or a suggestive association with the any other learning parameter. The presence of a suggestive locus for locomotion deserves a further follow up study; also an attention should be paid to the chromosome 20 which potentially harbors a locus responsible for a memory formation.

5.5 Control test results

Control experiments were run in order to exclude an influence of behavioral or sensorimotor upon the preformance of animals in the arena. Avaliable measures were used and correlation matric was created. Open arm entries (Conti et al., 2002) was used as an anxiety assay - an behavioral experiment previously done on HXB/BXH platform. Sniffing and rearing are ethogram parameters from the open-field test. Beam-slips is the output from a beamwalking test. Number of slips best indicates sensorimotor capabilities.

	OPEN ARM	SNIFFING	REARING	BEAM-SLIPS
T.FIRST day 4	0.25	0.06	-0.54	-0.13
T.FIRST day 5	-0.16	-0.04	-0.24	-0.26
T.FIRST day 7	0.17	0.06	-0.25	-0.26
T.FIRST retr	-0.04	0.34	-0.6	-0.09
MAX.T day 4	0.31	0.46	0.26	-0.23
MAX.T day 5	0.28	0.07	0.44	-0.5
MAX.T day 7	0.32	0.03	0.34	-0.39
MAX.T retr	0.11	0.18	-0.54	-0.18
ENT day 4	-0.34	-0.41	-0.04	0.47
ENT day 5	-0.04	-0.22	-0.52	0.5
ENT day 7	-0.16	-0.07	-0.16	0.45
ENT retr	0.06	-0.24	0.62	0.17
DIST day 4	0.26	0	-0.08	-0.24
DIST day 5	0.23	0.05	-0.07	-0.31
DIST day 7	0.24	-0.17	-0.35	-0.25
DIST retr	0.37	-0.33	-0.2	-0.13

Figure 12: A correlation matrix illustrating Pearson's coefficients for selected learning variables compared to the behavioral control variables (open arm entries in a plus maze, sniffing and rearing in open-field, beam-walking slips).

Linear regression model was applied during the analysis of significance of linkage between learning parameters and selected behavioral parameters thought as possible

modifiers of the learning in AAPA task (figure 12). In analysis, learning parameters were used as a dependant variable, metabolic phenotypes as independent variables.

Neither open arm entries in a plus maze (OPENARM), number of slips in a beam-walking task (BEAM-SLIPS), nor sniffing activity in open-field (SNIFFING) had any significant effect on any of the selected learning parameters. However a significant correlation was found for time to the first entrance (T.FIRST), number of entrances (ENT) and maximum time of avoidance (MAX.T) on retrieval session with rearing activity ($p < 0.05$ in all three cases) Rearing activity did not correlate with any of the other learning parameters.

5.6 Metabolic correlations

The table (Figure 13) depicts all the correlation coefficients (Pearson's) for the selected metabolic parameters associated with the metabolic syndrome (serum triglycerides, body weight, fat proportion, fat cell size, LDL and HDL levels, blood pressure, insulin levels, glucose tolerance and C- reactive protein (CRP)) compared to the selected learning parameters from the AAPA task. The metabolic parameters were selected from the online database on GeneNetwork.org. Parameters were selected as well age matched to the animals used in behavioral experiment as possible. Correlations were made in SYSTAT statistics program.

The selected metabolic variables were:

- INSULIN - serum insulin (nmol/L), nonfasted, 10 wks, 10 wk males (Pravenec et al., unpublished results)
- (Bottger et al., 1996)
- HDL - serum HDL cholesterol concentrations in male rats, 10 weeks old, fed a diet with 60% fructose from 8 weeks to 10 weeks (Pravenec et al., 2002)
- LDL - serum LDL cholesterol concentrations in 11 week old males fed a high fat high cholesterol diet for 4 weeks (Bottger et al., 1996)
- GLUC. - glucose concentrations during intraperitoneal glucose tolerance test (60 min after glucose load) in male rats, 10 weeks old, fed a diet with 60% fructose (Pravenec et al., 2002)
- FAT C. -fat cell volume, otherwise unspecified (Aitman et al., 1997)

- PRESS. -mean arterial pressure determined by direct puncture of the carotid artery under light ether anesthesia in 12 week old males fed a normal lab chow at generations F16-F17 (Pravenec et al., 1989)
- WEIGHT - weight of rats, males 12 weeks old (Aitman et al., 1997)
- CRP - serum C reactive protein (mg/ml), 6 wk males (Jirout et al., unpublished results)
- TRIG. - serum triglyceride concentrations in male rats, 10 weeks old, fed a diet with 60% fructose from 8 weeks to 10 weeks (Pravenec et al., 2002)
- FAT R. - relative weight of the fat pad (Aitman et al., 1997)

	INSULIN	HDL	LDL	GLUC.	FAT C.	PRESS.	WEIGHT	CRP	FAT R.	TRIG.
T.FIRST day 4	-0.28	-0.05	-0.17	-0.16	0.55	-0.02	0.45	-0.04	-0.32	-0.17
T.FIRST day 5	-0.23	0.23	-0.05	-0.32	0.07	-0.4	-0.16	-0.25	-0.19	-0.41
T.FIRST day 7	-0.32	0.15	-0.12	-0.23	0.14	-0.49	0.15	-0.3	-0.19	-0.37
T.FIRST retr	-0.21	0.05	-0.23	0.02	0.5	0.28	0.25	0.2	-0.28	-0.1
MAX.T day 4	-0.03	-0.26	0.01	-0.06	-0.18	0.29	0.04	-0.08	-0.65	-0.19
MAX.T day 5	-0.22	-0.28	-0.07	0.24	-0.12	0.04	0.14	0.07	-0.4	0
MAX.T day 7	-0.1	-0.42	0.14	0.2	-0.07	-0.01	0.33	0.2	-0.25	-0.03
MAX.T retr	-0.11	-0.09	-0.05	0.11	0.13	0.18	0.28	0.07	-0.31	-0.25
ENT day 4	0.29	-0.07	0.18	-0.22	-0.04	-0.11	0.01	0	0.47	0.23
ENT day 5	0.45	0.28	0.29	-0.2	0.01	-0.01	-0.15	-0.06	0.5	0.21
ENT day 7	0.14	0.26	-0.08	-0.16	-0.18	-0.14	-0.22	-0.27	0.32	0
ENT retr	0.26	-0.32	0.3	-0.27	0.22	0.13	0.06	-0.1	0.18	0.54
DIST day 4	0.3	-0.16	0.33	-0.15	-0.05	0.47	-0.2	-0.37	-0.27	0.11
DIST day 5	0.3	-0.21	0.32	-0.09	0.12	0.54	-0.09	-0.28	-0.09	0.09
DIST day 7	0	0.17	0.2	-0.03	0.27	-0.18	0.07	-0.23	0.2	0.05
DIST retr	0.55	-0.22	0.49	0.05	0.31	0.19	0.3	-0.11	0.56	0.1

Figure 13: A correlation matrix illustrating Pearson's coefficients for selected learning variables compared to the metabolic phenotypes.

No significant effect of any of the metabolic phenotypes was demonstrated even in pairs with a high Pearson's correlation when significance was evaluated by the linear regression analyses. In the linear regression analysis learning parameters were used as dependant variable, metabolic phenotypes as independent variables, as to test for effect of metabolic parameters on learning capability.

6 Discussion

This experiment indicates that spatial cognition is genetically based. There is a significant natural variation in the spatial memory among HXB/BXH inbred rat strain ($p<0.005$). The data display normal distribution; therefore it can be claimed that many genes are influencing the trait expression - the trait is polygenic. This result is not surprising since memory formation is a very multi-componential process. Also, different strains handled different components of the task differently. There was a difference between relative learning between the strains in the original task, retrieval and the reversal learning. Several were unable to learn the latter reversal task although handling the acquisition learning perfectly (notably strains HXB29 and HXB17). This could be because of different mechanism underlying learning new information and re-modifying old information (reversal task).

Linear regression analyses proved that the task is not influenced to a significant degree by anxiety (open arm entries in plus maze), or sensorimotor capability (beam-walking). Although an significant effect ($p<0.05$) was found for rearing activity influencing all three parameters of learning on retrieval session, the effect is isolated to only one session and is not observable either in end of learning acquisition (day 4) or end of reversal session (day 7). Thus it can be claimed that rearing activity is not an important factor influencing the learning behavior in HXB/BXH inbred lines. The increase in rearing activity decreased the measure of learning is not explainable by current knowledge of rat behavior. By these results it can be claimed that the tested parameters in active allothetic place avoidance are a reflection of cognitive abilities of experimental animals.

Due to cumulativeness of the data varying phenotypes were discovered within the strains, which allows the traits to be compared with each other, and not only with the reference marker map. Since the HXB/BXH platform was originally created as a tool to study genetic basis of the metabolic phenotypes it is no wonder that these phenotypes are the most numerously recorded traits of the RI strains. Traits such as arterial pressure, insulin resistance and triglyceride counts were measured among others. Most of these traits are multigenic and thus measures are continuously distributed across the HXB/BXH RI strains (Printz, 2003). However, these measures, as most of the others, are environment dependant and therefore are not as straightforward to measure. Nonetheless, in the experimental setting these environmental factors can be to a greater degree eliminated.

One of the most exciting and unexpected findings was, that strains display wide spectrum of behavioral differences. Startling strain dependant difference in anxiety levels (Conti et al., 2004), response to startling stimuli (Jaworski et al., 2002), and conditioned taste aversion (Bielavska et al., 2002) prove that HXB/BXH platform is inarguably a viable tool to characterize behavioral alike metabolic phenotypes genetically. Surprisingly, memory, or other higher mental functions were never studied on these inbred strains. This project aim was to elucidate genetic basis of this very complex trait, and search for a connection with metabolic traits.

The AAPA testing is by far the most sophisticated of the behavioral tests that were studied on the HXB/BXH inbred strains. Therefore it does not come as a surprise that there is no pronounced locus responsible for the difference in learning between the strains. The task itself is complex having three cognitive subparts: memory, comprehension and flexibility. Fortunately, neither stress nor luxation mobility handicap seem to exert any significant effect on the AAPA performance. Although anxiety specifically did not seem to have an effect on learning capacity of animals, some animals displayed very much anxiety like behavior.

Animals select different strategies when solving the AAPA task. The most effective strategy for animal to avoid punishment is to walk in the opposite side of the arena to the shock sector. Using this strategy animal never gets an electric shock, which is taken as a proof of excellent memory. The other strategy is to get a shock and run away (no. shocks = no. entrances). This is considered as a deficit in spatial memory, or lack of motivation; however, the rat understands where the shock is and always runs away from the shock sector. There is often a continuum between these two strategies. The last "strategy" is the "sitter", where rat abandons all the movement and remains on one place receiving all the electric shocks (no. shocks is more than 10 times no. entrances). Although in this case it appears as if the animal is not learning, when listening to the sound emitted by the rat, its calls sound much more distressed when rat is to enter the shock sector. This was preliminarily experiment tested only on few animals and is not part of the study. It is possible that for the animals that failed to avoid the shock sector by using "sitter" strategy that they had prolonged freezing reaction as a anxiety coping strategy - an expression of stress in rodents and other animals is a freezing reaction; or the electric shock was not sufficient motivation, which could be caused by differential pain sensitivity, a parameter not tested on HXB/BXH strains. Due to the possibility that rats were aware of the exact

location of the shock sector, but failed to avoid for some other reason, but not a memory deficit (which is suggested by other experiments from our laboratory) it was decided to include all animals with a "sitter" strategy in the study.

The heritability values are expected values for a behavioral trait, and are in accordance with other behavioral studies done on HXB/BXH inbred lines (Conti et at., 2004; Bielavska et al., 2002). The highest heritability score was observed for the locomotion phenotype; it is not surprising that locomotion phenotype displayed most consistent suggestive loci as well on chromosome 4. The least heritable is the time to the first entrance to the arena (T.FIRST). This may be due to the coincidence, or the initial confusion of the animal connected to other factors.

A suggestive locus was discovered repeatedly on chromosome 4 for locomotion phenotype on different experimental days. A different suggestive locus was found on chromosome 4 for locomotor activity on retrieval session. This could be because of the difference in activity level at the beginning and at the latter phases of the animal's stay in the carousel arena. The suggestive locus for locomotion phenotype is different from one detected in elevated plus maze on chromosomes 3 and 18 (Conti et at., 2004).

A suggestive locus on chromosome 20 was discovered for one learning parameter on the last day of reversal task (day 7). It must be noted that reversal task is more difficult to handle for the animal than acquisition task (compare day 3 of acquisition to day 7; results not shown). Therefore the result may be to more discrete abilities of different strains to handle the task. Locus on chromosome 20 has to be further investigated as a potential candidate for harboring a locus responsible for differential spatial learning in rats and possibly humans.

The putative link between metabolic phenotypes and parameters expressing memory was not found in this experiment. The negative result is possibly due to non-detectable weak linkage for which the number of animals used is not big enough for the linkage to be detected. However, in large populations even a small linkage can be significant. Most studies focused on metabolism and cognition regarded elderly humans, and the animals used in this experiment barely reached adulthood. This is important because the connection between metabolic syndrome symptoms and cognition may be of physiologic nature and cognitive side effect is detectable only after a longer exposure to the metabolic disturbances (high cholesterol, blood pressure).

7 Conclusion

The aim of this project was to evaluate spatial learning in HXB/BXH recombinant inbred strains in hippocampus-dependent learning in Allothetic Active Place Avoidance task (AAPA). Next aim was to evaluate non-cognitive effects on performance (sensory, or motor impairment) and exclude the possibility that they bias learning by correlation of performance in control tests with learning. We have also searched for a quantitative trait loci that appear to harbor alleles responsible for learning in the active place avoidance. Another aim of the study was to inspect the possible connection of memory preformance and metabolic phenotypes to support link between memory and metabolic syndrome (as it is exemplified e.g. in dementias comorbid with metabolic syndrome) . HXB/BXH rat platform showed a strain-dependent differences in performance of this cognitive task as revealed by an ANOVA ($p<0.05$).. Average heritability of selected parameters was 0.40 ± 0.06, suggesting that these traits in the task are at least partly genetically-based. No significant correlation with a performance in control tests was found, suggesting that general impairments could not explain differences in the learning in the AAPA test (except for a correlation between rearing activity with a memory retention in the 10-min retrieval session, which might be related to exploration; but there is no straightforward explanation). QTL analysis with GeneNetwork.org showed a suggestive locus for learning (T.FIRST) in the final day of reversal testing on the chromosome 20. A significant QTL was detected on chromosome 4 for locomotor phenotype, despite the fact that AAPA task involves not spontaneous but forced locomotion (as being active avoidance task). Significance of these loci has to be investigated further. This project also failed to show a significant correlation of any learning larameter with selected metabolic phenotypes, suggesting that learning in the AAPA task is not directly related to metabolic dysfuctions.

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Presentation of this project in scientific meetings

Hatalova H, Grzyb A, Vales K, Overall R, Pravenec M, Kempermann G, Stuchlik A. Hippocampus-dependent spatial learning and memory in a place avoidance task in HXB/BXH recombinant inbred rat strains., *IBANGS meeting, Dresden, 2009*

Hatalova H., Grzyb A, Vales K, Lu L., Williams R. W., Overall R., Silhavy J., Zidek V., Pravenec M., Kempermann G. & Stuchlik A. Spatial learning and memory of HXB/BXH recombinant inbred rat strains in a hippocampus-dependent spatial learning task, active place avoidance. *FENS Forum, 2010, Amsterdam, 2010*

Ales Stuchlik, **Hana Hatalova**, Anna N. Grzyb, Tomas Petrasek, Iva Prokopova, Rupert Overall, Jan Silhavy, Vaclav Zidek, Gerd Kempermann, Michal Pravenec, Karel Vales Genetic and correlation analyses of hippocampus-dependent learning in Carousel arena and spontaneous behavior in the open-field. *IBRO meeting, Florence, July, 2011*

Hana Hatalova, Anna N. Grzyb, Tomas Petrasek, Iva Prokopova, Rupert Overall, Jan, Silhavy, Vaclav Zidek, Gerd Kempermann, Michal Pravenec, Karel Vales, Ales Stuchlik: Genetic and correlation analyses of hippocampus-dependent learning in Carousel arena. *1. Konference Neuropsychiatrického Fóra, Praha, 2011*

Supplementary Figures

Means for particular RI strains in selected sessions

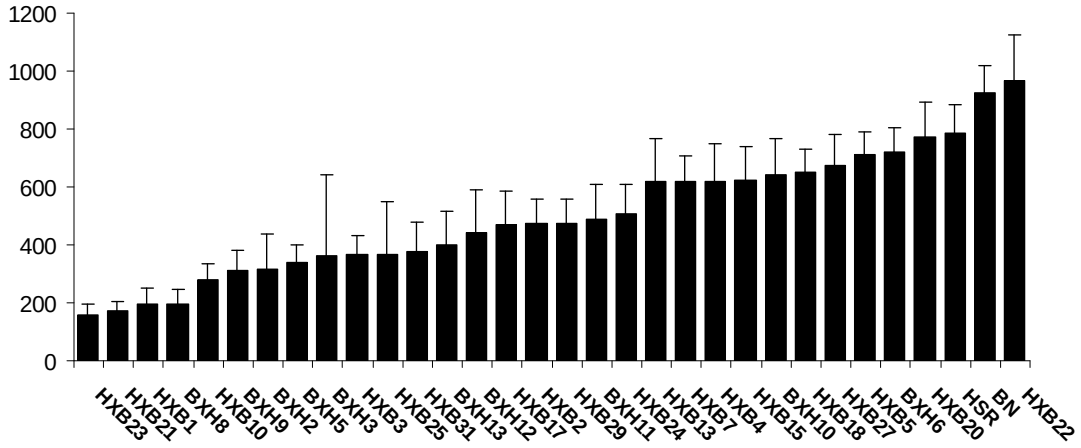

Fig. S1: A graph illustrating maximum time avoided (MAX.T day 4) averages for each strain ± S.E.M. Measure is described as a time in seconds (s).

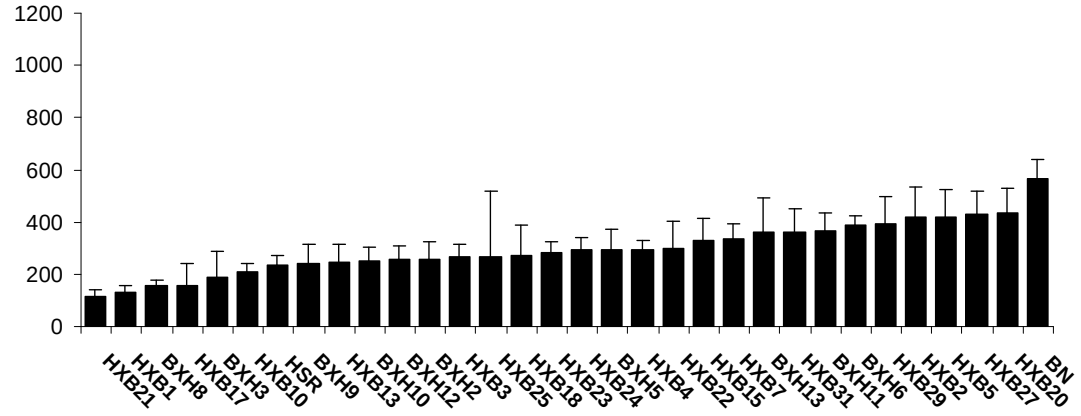

Fig. S2: A graph illustrating maximum time avoided (MAX.T day 5) averages for each strain ± S:.E.M. Measure is described as a time in seconds (s).

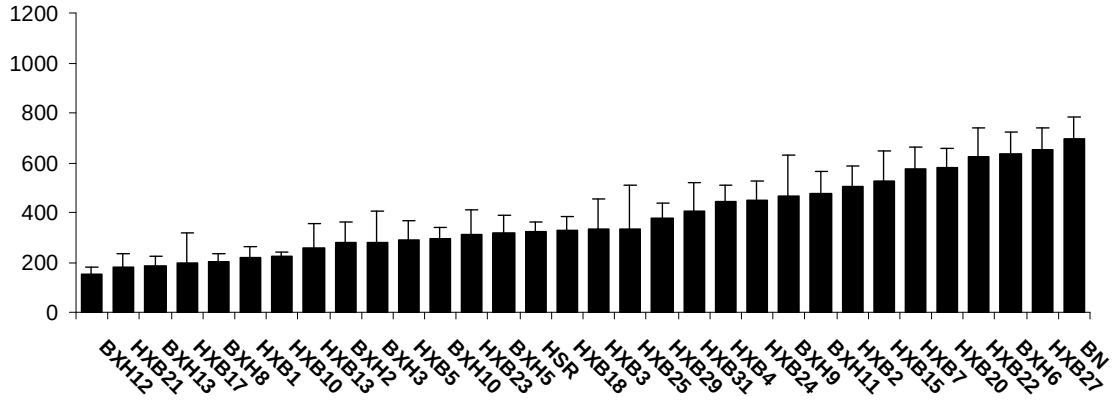

Fig. S3: A graph illustrating maximum time avoided (MAX.T day 7) averages for each strain ± S.E.M. Measure is described as a time in seconds (s).

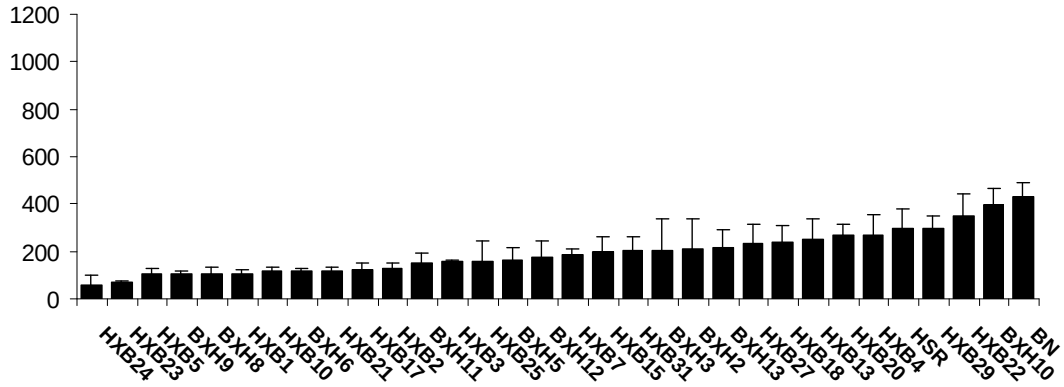

Fig. S4: A graph illustrating maximum time avoided (MAX.T retrieval) averages for each strain ± S.E.M. Measure is described as a time in seconds (s).

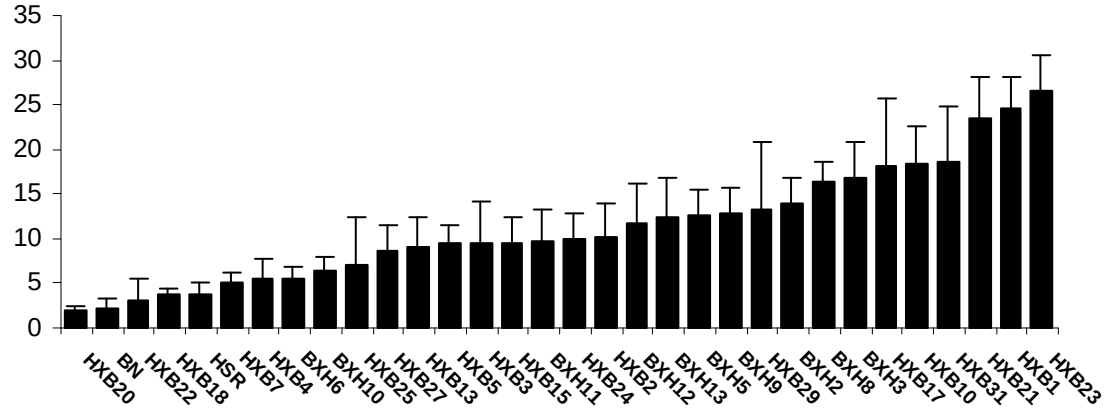

Fig. S5: A graph illustrating mumber of entrances (ENT, day 4) averages for each strain ± S.E.M.

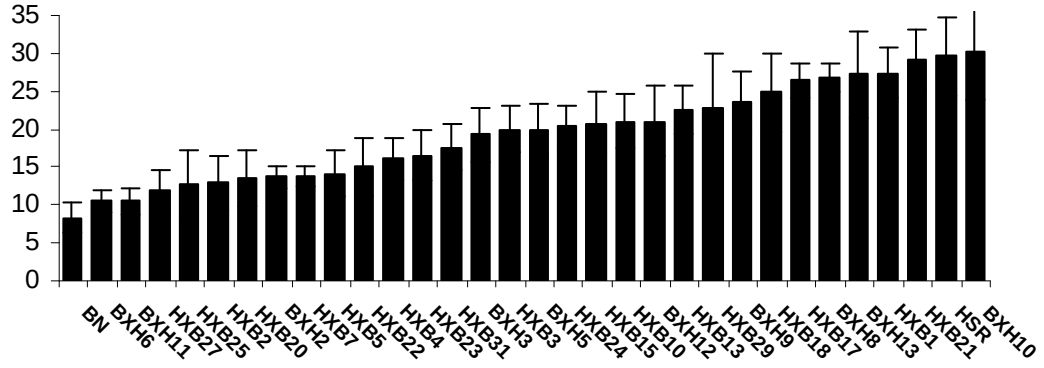

Fig. S6: A graph illustrating mumber of entrances (ENT, day 5) averages for each strain ± S.E.M.

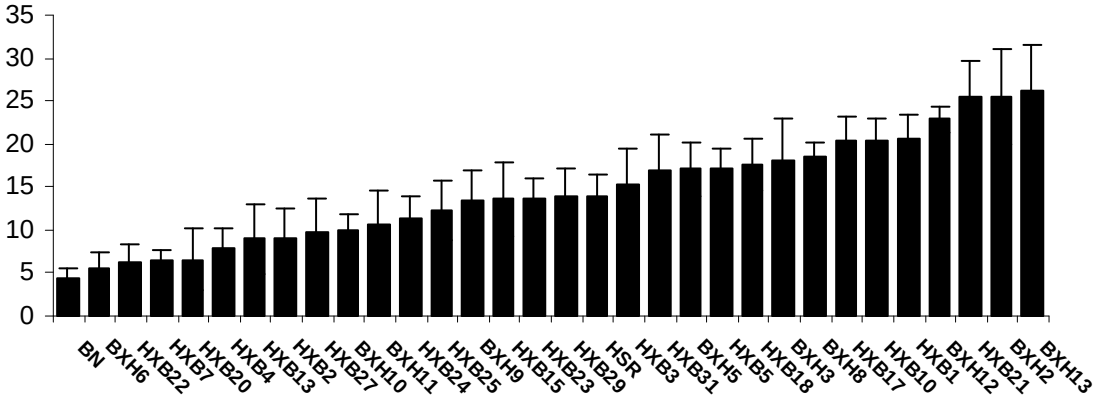

Fig. S7: A graph illustrating mumber of entrances (ENT, day 7) averages for each strain ± S.E.M.

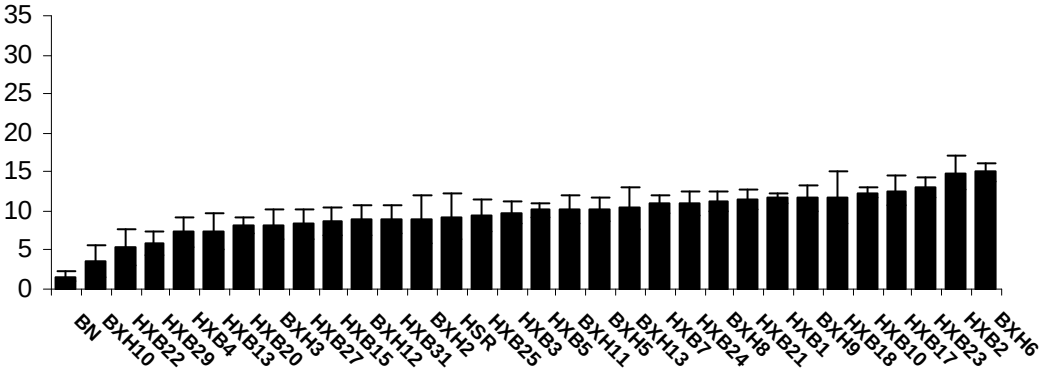

Fig. S8: A graph illustrating mumber of entrances (ENT, retrieval) averages for each strain ± S.E.M.

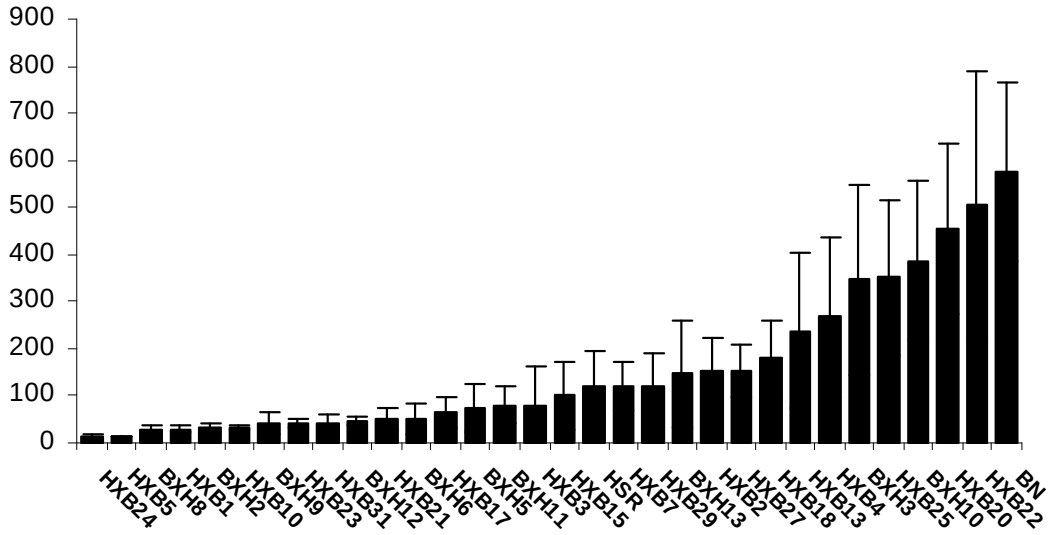

Fig. S9: A graph illustrating time to the first entrance (T.FIRST day 4) averages for each strain ± S.E.M. Measure is described as a time in seconds (s).

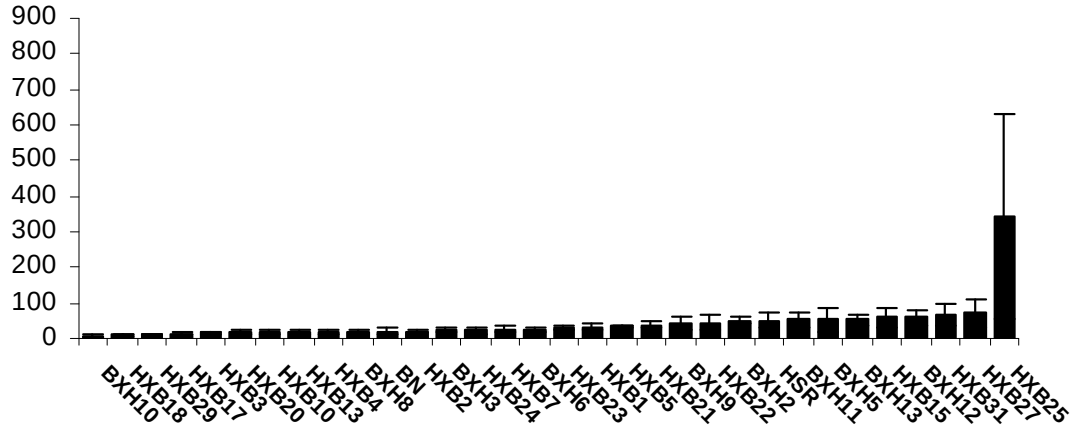

Fig. S10: A graph illustrating time to the first entrance (T.FIRST day 5) averages for each strain ± S.E.M. Measure is described as a time in seconds (s).

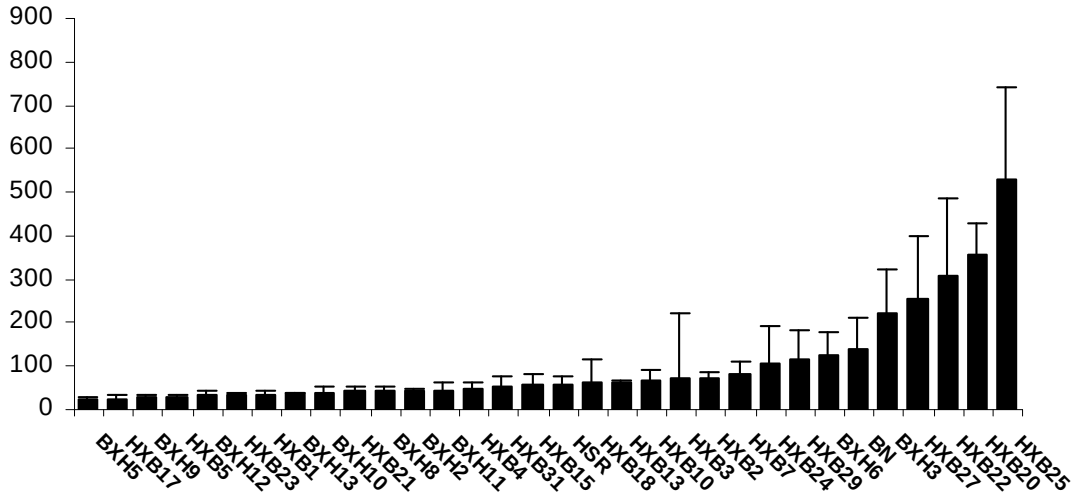

Fig. S11: A graph illustrating time to the first entrance (T.FIRST day 7) averages for each strain ± S.E.M. Measure is described as a time in seconds (s).

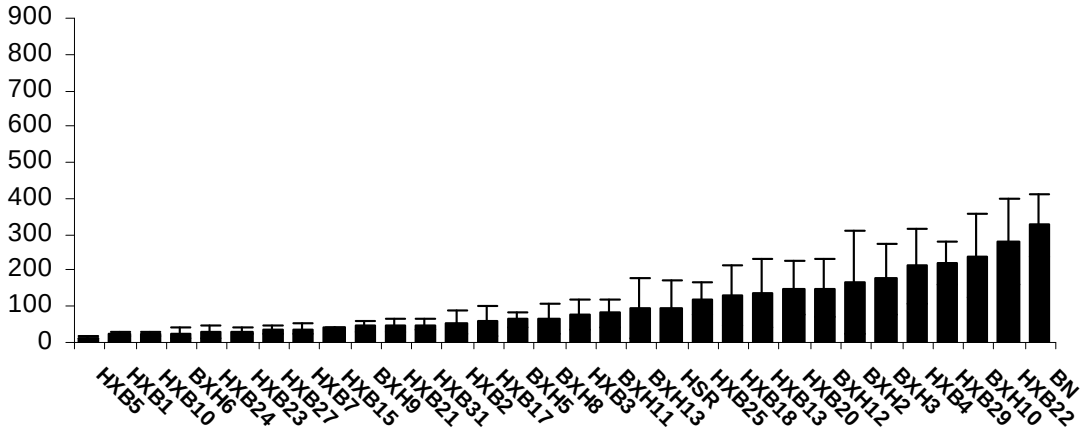

Fig. S12: A graph illustrating time to the first entrance (T.FIRST, retrieval) averages for each strain ± S.E.M. Measure is described as a time in seconds (s).

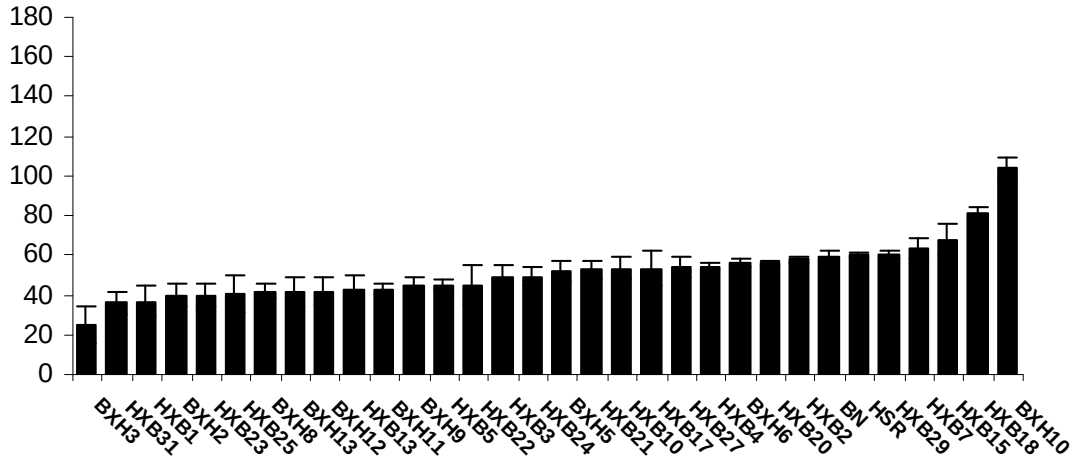

Fig. S13: A graph illustrating distance walked (DIST, day 4) averages for each strain ± S.E.M. Measure is described as a distance in meters (m).

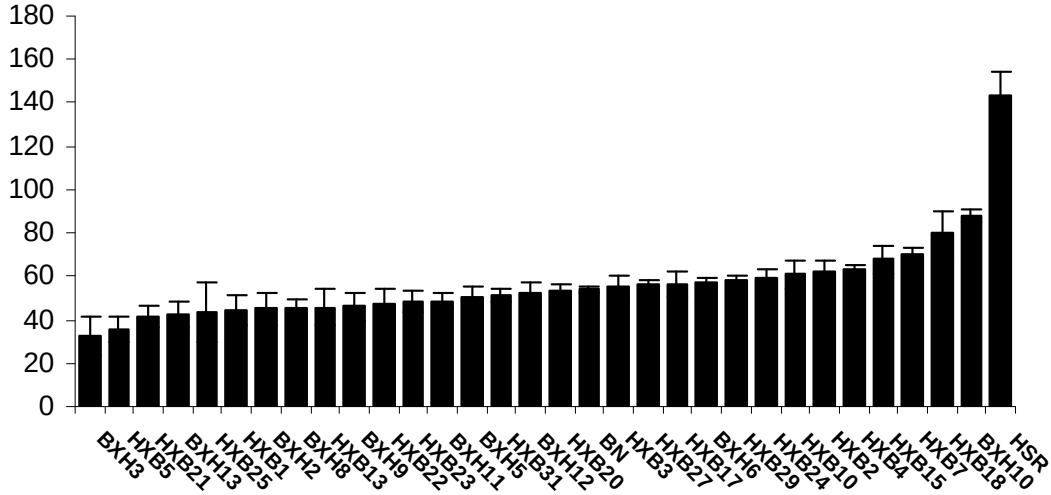

Fig. S14: A graph illustrating distance walked (DIST, day 5) averages for each strain ± S.E.M. Measure is described as a distance in meters (m).

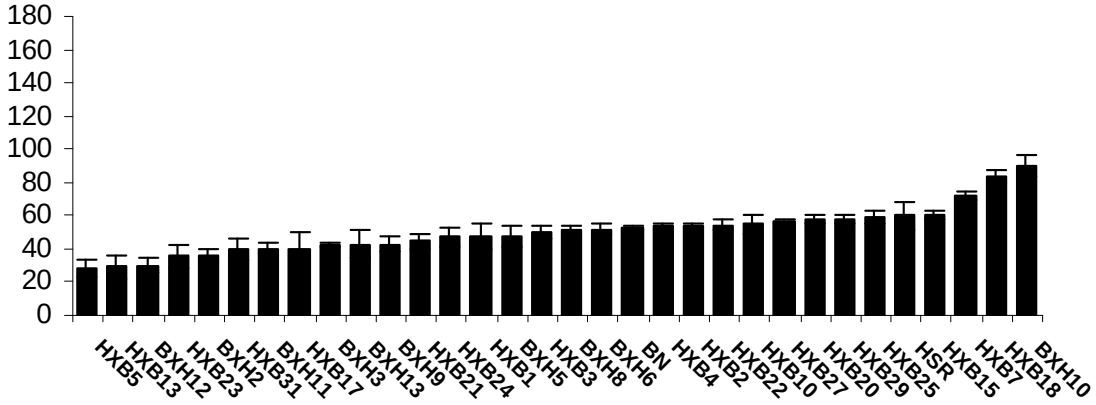

Fig. S15: A graph illustrating distance walked (DIST, day 7) averages for each strain ± S.E.M. Measure is described as a distance in meters (m).

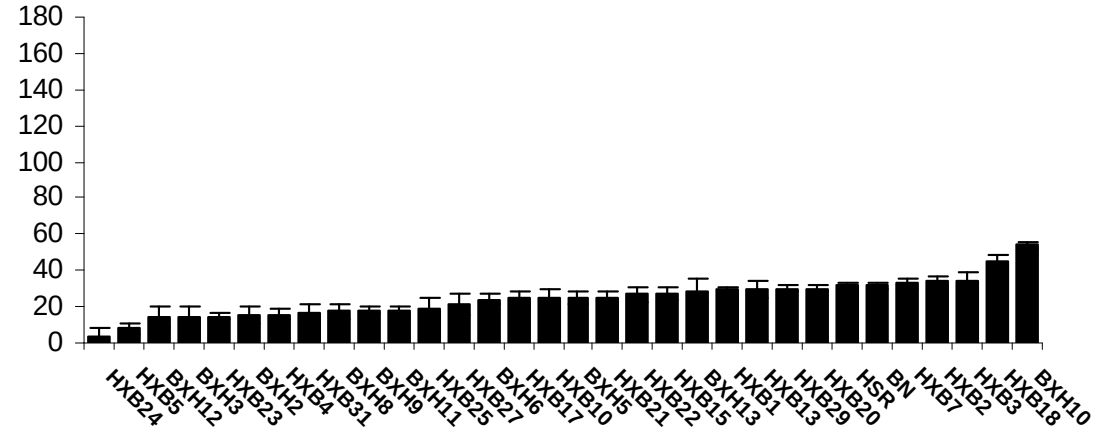

Fig. S16: A graph shows the means and S.E.M. for distance walked in the retrieval session