Network: Systematic Gene Identification and Functional Analyses in Common CNS Disorders

Project: A Phenotype-driven ENU Mutagenesis Screen for the Identification of Genes Involved in Alcohol Drinking and Relapse

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Introduction

One broad type of genetic screen is the phenotype-driven approach, in which mutations are generated at random across the genome, and offspring are scrutinized for phenotypes of interest. Mutant phenotypes that are heritable can then be mapped to the mutated region of the genome; next, the region is narrowed down, and the precise gene that is affected can be pinpointed. This kind of approach has driven genetics for years but has not so far been applied to the genetics of alcoholism. In mice, the chemical N-ethyl-N-nitrosourea (ENU) is the mutagen of choice for such screens and there are several large-scale ENU-based screens under way worldwide (Hrabé de Angelis et al., 2000; Nolan et al., 2000).

Phenotype-driven screens for dominant mutations as proposed here can be performed on a large scale in mice, because phenotypes can be identified in the first generation offspring of a mutated animal (a so-called ENU-mouse). Nevertheless, only about 1% of offspring reveal any sort of mutant phenotype, and good quantitative screening tools are needed in order to detect what can be a fairly subtle effect (Rossant, 2003). In this subproject, we propose a phenotype-driven approach in order to identify genes involved in alcohol drinking and relapse. In fact the screening test is the bottleneck of this procedure and only tests which measure accurately a specific behavior in a reliable and reproducible way are finally suitable for this procedure. Ideal for our purpose is the home cage, two- bottle free choice test to measure alcohol intake, preference and total fluid intake. This test can be performed on ethanol but not on other drugs owing to their pharmacokinetics, or to their aversive olfactory or taste characteristics (Spanagel and Sanchis-Segura, 2005). It should be noted that alcohol intake in mice is a very stable behavior even across several laboratories (Crabbe et al., 1999; Wahlsten et al., 2003). Relapse-drinking behavior can be measured by the alcohol deprivation effect (ADE). The ADE is one of the most robust phenomenon seen in rodents (Spanagel and Hölter, 1999) and can even be fitted with a mathematical equation demonstrating the robustness of this screen test.

Project Status

The ENU mice were generated at the GSF in Munich and the ENU mice were then transferred to the CIMH in Mannheim. Two months old F1 mice were used to investigate their “alcohol phenotype”. The number of offspring per mutagenized male is limited to 100 F1 mice animals, since clustered mutations can appear owing to the low number of remaining spermatogonial stem cells. 474 Male as well as 483 female F1 mice were analyzed in order to assess gender-specific differences in drinking behavior. However, before testing their “alcohol phenotype” general behavioral alterations were assessed by the use of a modified hole board test (Ohl et al., 2001). The modified hole board test can be performed within 5 min and can, therefore, be used as a high throughput test to investigate a variety of behavioral parameters such as anxiety, risk assessment, exploration, locomotion, food-intake inhibition, novelty-seeking, and arousal by using only one test. This test allows us (i) to eliminate mutated mice with strong behavioral abnormalities and (ii) to correlate an “alcohol phenotype” with a specific behavior. After this initial screen, F1 mice were single caged and underwent the large-scale screen for alcohol drinking behavior. In this test, all mice had free access to two bottles – one contained tap water, the other bottle was empty (dummy bottle) for one week. Positions of the bottles were changed each second day. This procedure abolishes initial side preferences. Then animals had the free choice between tap water and a 10% (v/v) ethanol solution for 8 weeks. After 8 weeks, a two week deprivation period was introduced and afterwards the ADE was determined.

Note: Spillage and evaporation were minimized by the use of self-made glass cannulaes in combination with a small plastic bottle. Under these conditions ethanol concentration in a given solution stays constant for at least 1 week, when measured with an alcoholometer. Bottles were weighed every three days and all drinking solutions were renewed every 3 days and the positions of the 2 bottles were changed to avoid side preferences.

Monitoring of alcohol intake and drinking patterns prior and following a deprivation phase was provided by a new developed device. Thus a fully automated “drinkometer/ lickometer e-motion” system combined with simultaneous home cage activity monitoring was used. This device was recently developed together with E-motion systems and allows us for the first time to study micro-drinking patterns over several months. Thus this device enables us to record even very small drinking amounts in the µl range. Along with the licking rates valuable information about drinking behaviour can be retrieved (e.g. bursts of licking and interburst intervals, clusters of bursts and intercluster intervals). Changes in parameters obtained by the lickometer system are very helpful to define the transistion from controlled drug-seeking and –taking behaviour to a compulsive one. Thus monitoring of micro-drinking patterns is essential for studying alterations in circadian drinking behavior and for monitoring compulsive drug-seeking components. In addition, the drinkometer e-motion system can monitor individual activity patterns in the home cage in parallel. Thus, we are able to closely correlate micro-drinking patterns with motor activity patterns of each individual rat. Those correlative measures can be considered as a major improvement for studies on ingestive behaviour.

Mutants with the most extreme (low or high) alcohol intake/preference were selected and their “alcohol phenotype” was confirmed in the next generation in the same two-bottle free choice paradigm (confirmation cross). We have already worked out the preconditions for selecting animals. All mice which show alcohol intake above or below 2 S.D. of the total mean are considered as a putative “alcohol phenotype”. Only confirmed “extreme alcohol phenotypes” will go into further behavioral analysis. Further phenotyping will involve detailed alcohol drinking patterns with our fully-automated drinkometer device. Operant self-administration procedures with progressive ratio and taste preference tests will also be performed. Furthermore, relapse behavior will be studied by yet another model – the reinstatement paradigm which has recently been established for mice in our laboratory (Spanagel and Sanchis-Segura, 2003).

The mutants which have been selected in the alcohol screen will be maintained at two different animal facilities and archived by embryo freezing. All confirmed mutants will undergo genetic analysis. The chromosomal localization of the mutation is achieved by outcross/backcross mapping strategies using another inbred mouse strain. For a dominant mutation to be mapped, 50 offspring from the second backcross are collected and phenotyped. Linkage of mutated allele with microsatellite markers is tested using the pooled DNA method. With the help of mouse linkage programs a

map with a resolution of 10 cM is obtained. From this stage on, the strategy is then to search for candidate genes or to establish a high resolution physical map for positional cloning.

Fig 1: *In a search for genes that regulate circadian rhythms in mammals, the progeny of mice treated with N-ethyl-N-nitrosourea (ENU) were screened for circadian clock mutations. A semidominant mutation, Clock, that lengthens circadian period and abolishes persistence of rhythmicity was identified. Clock segregated as a single gene that mapped to the midportion of mouse chromosome 5, a region syntenic to human chromosome 4. This approach shows that the power of ENU mutagenesis combined with the ability to clone murine genes by map position provides a generally applicable approach to study complex behavior in mammals. Interestingly, we have recently discovered that clock genes regulate alcohol consumption in mice as well as in humans.*

Outlook

Our preliminary experience shows that out of 957 screened mice 14 mice showed a putative “alcohol phenotype” which were then tested again in a confirmation cross. Confirmation of an “alcohol phenotype” requires 4 months (breeding + testing) but can be viewed as a parallel process. Only a confirmed “alcohol phenotype” will go into further detailed behavioral analysis. So far, we have confirmed two mice (Axel 67 and Anna 327). Currently, we try to build up stable lines for further in-depth behavioral analysis. These studies will require an additional 6 months. Only confirmed mouse lines which show a stable “alcohol phenotype” over several generations and which have been further validated by the in-depth behavioral analysis will finally undergo genetic analysis.

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