Novelty-Seeking Behaviors and the Escalation of Alcohol Drinking after Abstinence in Mice are Controlled by Metabotropic Glutamate Receptor 5 Receptors on Neurons Expressing Dopamine D1 Receptors

Supplementary Information

Y-maze

A single-trial version of the Y-maze test was performed as described previously (1). Briefly, each mouse was placed at the end of one arm and allowed to explore the apparatus freely for 5 min. The performance of each mouse was assessed visually by scoring the pattern of entries into each arm.

Morris Water Maze

The water maze spatial memory task (2) was performed as follows. The pool (1.2 m in diameter) was filled with water and made opaque by adding talcum. The escape platform (10 cm in diameter) was placed in the center of the designated quadrant with its top positioned 0.5 cm below the water surface. During testing, the room was dimly lit with diffuse white light (40 lux).

Mice were tested in three phases: acquisition, reversal and the probe trial. During the acquisition phase (4 trials/day for 5 days, with an inter-trial interval of ~5 min), mice were trained to find the hidden platform located in one of the quadrants. Each mouse was placed in a randomly assigned starting location (East, North, South, or West). If a mouse failed to find the platform within 90 s, it was guided to the platform and allowed to stay on it for 10 s. On day 5,
during the probe trial, the platform was removed, mice were allowed to swim in the pool for 90 s, and the time spent in each quadrant was recorded.

During reversal training (4 trials/day for 4 days), the platform was moved to the opposite quadrant, and the procedure was repeated. On the last day of reversal training, the platform was removed, and the time each mouse spent exploring the four quadrants was measured. The activity of each animal was measured using a video-based tracking system (AnyMaze).

**Immunostaining**

Animals were deeply anesthetized and transcardially perfused with saline followed by 4% paraformaldehyde. Dissected brains were post fixed for 12 hours in 4% paraformaldehyde and then cut into 40-μm sections with a vibratome (Leica). Coronal sections were incubated with antibodies against green fluorescent protein (1:500, rabbit-anti-GFP; Invitrogen A11122) and stained with diaminobenzidine (DAB) using the ABC kit (Vector Labs). Images of stained sections were acquired using a Nikon Eclipse 50i microscope equipped with a Nikon Digital Sight camera.

**Quantitative Polymerase Chain Reaction (PCR)**

Mice were sacrificed by cervical dislocation, and their brains were dissected, fixed overnight in RNAlater (Qiagen) and sectioned using a vibratome (Leica, Wetzlar, Germany). Then, the striatum and nucleus accumbens were microdissected under a binocular. Total RNA was prepared using the method of Chomczynski and Sacchi (3). RNA was reverse-transcribed
(Omniscript, Qiagen) and examined using real-time PCR with fluorescent probes for mGluR5 Mm0317986 and Hprt1 (Mm01545399_m1; Applied Biosystems, Foster City, CA).

**Tissue Levels of Neurotransmitters**

Neurotransmitter measurements were performed as described previously (1), using an Ultimate 3000 System (Dionex), a coulochemical detector Coulochem III (model 5300, ESA) with a 5020 guard cell, a 5014B microdialysis cell and a Hypersil Gold- C_{18} analytical column. The mobile phase was composed of 0.05 M potassium phosphate buffer adjusted to pH 3.9, 0.5 mM EDTA, 13 mg/l 1-octanesulfonic acid sodium salt, 3.1% methanol and 0.93% acetonitrile. The applied potential of the guard cell was +600 mV, whereas that of the microdialysis cell was $E_1 = -50$ mV, $E_2 = +300$ mV. The level of sensitivity was set to 50 nA/V.
Figure S1. Transgene expression in the brains of mGluR5<sup>KD-D1</sup> mice. Representative micrographs of coronal brain sections stained with an antibody against the green fluorescent protein (GFP), which is co-expressed with the synthetic miRs by the transgene. (A) The striatum of a wild-type control animal, (B) the striatum of a mGluR5<sup>KD-D1</sup> mouse, (C) the amygdala, (D) and (E) the hippocampus from a mutant animal are shown. GFP diffuses freely in neurons, hence both the cell body and its projections are stained. Arrows indicate regions with stained cell bodies in the (C) amygdala, (E) the subiculum and ventral CA1 of the hippocampus. Scale bars: 100 μm. (F) Abundance of metabotropic glutamate receptor 5 (mGluR5) mRNA in the striatum of mGluR5<sup>KD-D1</sup> animals. The bar graphs show the mean abundance of mGluR5 mRNA
measured by quantitative PCR in samples derived from the CPu or the NAc normalized to the average of control in the CPu. White bars correspond to wild-type mice, black bars to mGluR5\textsuperscript{KD-D1} animals and error bars indicate SEM (5 mice per group). (G) Monoamine levels and dopamine turnover in mGluR5\textsuperscript{KD-D1} mice. The graphs show the mean levels of dopamine (DA), noradrenaline (NA) and serotonin (5HT) as well as the ratios of DA to its metabolites, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), in the striatum of mGluR\textsuperscript{D1-KD} or wild-type mice (7 animals per group). Error bars represent the SEM, and no statistically significant differences between mutant and wild-type mice were found. Amy, amygdala; CA, cornus ammonis; CPu, caudate-putamen; NAc, nucleus accumbens; Sub, subiculum; SN, substantia nigra.
Figure S2. Memory and learning in the Morris water. Learning abilities were tested in the Morris water maze using a cohort of 12 mGluR5<sup>D1-KD</sup> and 17 wild-type mice. Each animal had to find the hidden platform in four consecutive trials, and the procedure was repeated over 5 days. The graph (A) shows the mean time required to reach the platform in the SW part of the maze on each trial. On the final day, the platform was removed, and the time each animal exploring the area that had previously contained the platform was measured (B). Then, animals were examined for their ability to learn that the platform had changed position (‘reversal learning’). The training procedure was repeated with the platform moved to the NE quarter of the maze (C), and the test in the absence of the platform was repeated (D). Error bars represent SEM; no significant differences in behavior in the water maze between mGluR5<sup>D1-KD</sup> and wild-type mice were observed.
Figure S3. Exploration of the Y-maze. Behavior in the Y-maze was tested with 7 mGluR^{D1-KD} and 8 wild-type mice. Each mouse was placed in a randomly selected arm of the maze and allowed to explore the apparatus for 5 minutes, while the behavior was recorded. Graphs show the mean percentage of spontaneous alternations (A), same arm returns (B) and the number of ambulations (C). The statistically significant difference ($p < 0.05$ by t-test) in the number of ambulations between control and mutant mice is marked with ‘*’.
**Figure S4. Object recognition.** During the pretest mice were placed in the open field apparatus containing a novel object (a metal ball or a blue plastic block) and their interaction with the object was measured for 10 minutes. Three hours later the procedure was repeated, but in addition to the familiar object (“old”), a second object (“new”) was introduced. The graph summarizes mean time spent interacting with the objects during the pretest and recognition test. A two-way analysis of variance of the data from the recognition test reveals a significant effect of *genotype* ($F_{1,26} = 14.36$, $p < 0.001$) and *object* ($F_{1,26} = 9.46$, $p < 0.01$) but no interaction between factors ($F_{1,26} = 1.086$, not significant). Thus although mGluR$_{5D1-KD}$ mice spent significantly less time exploring either familiar or novel object, they did spend more time interacting with the novel second object. This indicates normal object recognition despite the reduced exploratory behavior.
Supplemental References

