Supplementary Material

Supplementary Materials and Methods

Behavioural Animal Experiments

Generation of the Per1 mutant mice (1). A targeting vector was designed to replace a 4.3 kb *mPer1* genomic region encompassing 15 of the 23 exons of the *mPer1* gene with an *Hprt* minigene The vector was designed such that if splicing over the deleted region occurred in a mutant transcript (connecting exon 3 to exon 19), a shift in the reading frame would have precluded the translation of the C-terminal region of mPER1. The targeted allele, *mPer1*^{Brdm1}, was obtained in AB2.2 ES cells (derived from an XY 129S7 embryo) and used to generate chimeric mice. Intercrosses between heterozygous (C57BL/6*Tyr*$^{c\text{-}Brd}$ × 129S7) F_1 offspring gave rise to F_2 homozygous mutants at the expected Mendelian ratio. Homozygous mutants are viable, fertile, and morphologically indistinguishable from their wild-type littermates. Eight- to ten-week-old male *mPer1* mutant and wildtype mice were used in our 3 independent stress experiments. The animals were singly housed with food and water *ad libitum*. Artificial light was provided daily from 6 a.m. [Zeitgeber time (ZT)] to 6 p.m. (ZT12) (12 h of light/12 h of darkness =LD12:12 cycle) with room temperature and humidity kept constant (temperature: 22 ± 1°C; humidity: 55 ± 5%). The experiments were approved by the Committee on Animal Care and Use of the relevant local governmental body and carried out in accordance with German Law on the Protection of Animals.

Social defeat test: Aggressor CD-1 male mice were single-housed in a polycarbonate cage (24.0 cm × 46.0 cm × 15.5 cm) for 2 weeks. Control and Per1 mutant mice were subsequently placed into the resident CD-1 mouse's home cage into

which a 1/8-inch thick perforated transparent metal partition had been placed down the middle to separate the pair. The partition allowed olfactory, visual and auditory communication but prevented tactile contact. During the social defeat period, the aggressor attacked and defeated the intruder mice within 15 minutes of introduction into the cage. If fighting did not begin within the first 5 minutes after the intruder was introduced, the aggressor was removed and a new aggressor was introduced. At the end of the social defeat period, the intruder was returned to its cage and the residents were left undisturbed until the next day. This procedure was repeated for 3 consecutive days.

Diurnal pattern of home cage ethanol drinking. The home cage drinking behaviour was monitored by a so-called “drinkometer e-motion” system. This advice (INFRA-E-MOTION GmbH, Hagendeel, Germany) allowed us to study micro-drinking patterns and thus to record even very small drinking bouts in the µl range. Monitoring of micro-drinking patterns was essential for studying alterations in circadian drinking behavior as well as for monitoring stress-induced drug-taking components.

Adolescent sample (Suppl. Table 1A,B):

Infants were included consecutively into the study according to a two-factorial design intended to enrich and control the risk status of the sample (full details of the sampling procedure have been reported previously (2). As a result, approximately one third of the study sample had experienced moderate to severe obstetric complications, while about one third of the families suffered from moderate to severe psychosocial adversities. To control for confounding effects of family environment and infant medical status, only firstborn children with singleton births and German-speaking parents were enrolled in the study. Furthermore, children with severe physical handicaps, obvious

genetic defects, or metabolic diseases were excluded. Assessments were conducted at ages 3 months, and 2, 4, 8, 11, 15, and most recently at age 18 years. Of the original sample of 384 participants, 18 (4.7%) were excluded because of severe handicaps (neurological disorder or IQ < 70), 58 (15.1%) had incomplete data or were dropouts, and 35 (10.4%) refused to participate in blood sampling.

Adult patient and control samples (Suppl. Table 1C).

1006 adult alcohol dependent patients were recruited from the Department of Psychiatry at Regensburg University. All patients were admitted consecutively for inpatient treatment and met the criteria of alcohol dependence according to DSM-IV. Patients were also assessed for nicotine dependence using diagnostic criteria according to the DSM-IV and ICD-10. All participants were of German descent. Parents of the participants were living in Bavaria and participants themselves were born and raised in this area. Written informed consent was obtained for all participants before the investigation. Diagnosis was assessed after alcohol withdrawal by the Composite International Diagnostic Interview (3) performed by trained staff who rated participants independently. Patients with a lifetime history of schizophrenia or an addiction to drugs other than tobacco or alcohol were excluded from the study.

1178 control individuals from the region of Bonn, Germany were recruited from 2001-2003 within the German National Research Project to serve as controls for genetic studies in several neuropsychiatric phenotypes. Population-based recruitment was performed in collaboration with the local census bureau. Participants were screened for neurological and psychiatric disorders with self-report questionnaires adapted from the German version of the inventory to diagnose depression (4); While smoking and drinking were assessed with the Fagerström Tolerance Questionnaire (5) and the AUDIT (6) we

did not exclude any individual of this population-based control on the basis of their drinking behaviour. Any bias this might have introduced would be conservative and would thus not jeopardize our findings. More than 96% of the participants were of German or Western European origin as ascertained by place of birth of their grandparents. For all patients and controls, written informed consent was obtained before study participation.

Mutation screening and identification of SNPs

For each gene, primers were chosen in order to amplify the regulatory domains, the exon-containing DNA fragments, including exon-intron boundaries. PCRs were performed in a 15µl reaction mixture containing 25ng DNA. The list of the primers for each gene is available at www.cng.fr. Sequencing reactions were performed using an ABI PRISM 3700 DNA Analyser (Applied Biosystems, Foster City, CA). PCR reactions were performed in Biometra T1 thermocyclers, and fluorescence results were determined with the use of an ABI Prism 7900HT sequence-detector end-point read.

Cellular experiments

Epstein Barr Virus (EBV) transformed human lymphocytes cell lines (LCL) were established from alcoholic patients' blood lymphocytes, genotype specific for h*Per1* rs3027172. There were 7 cell lines carrying TT allele and 6 cell lines with CC allele. Cells were cultured in RPMI 1640 medium (Biochrom or Sigma) supplemented with 10% fetal bovine serum (FBS; Biochrom or Sigma), 2.4ug /ml Phytohemagglutinin L (PHA; Biochrom or Sigma), and 20 ug/ml gentamycin. Cells were maintained in a humidified 5% CO_2 atmosphere at 37°C.

Cell synchronization with 50% horse serum: Cells were subcultured 3-4 days before treatment and approximately 2-3×106 cells were plated in 25ml 10% FBS medium. At the point of 0 h, medium was changed to serum rich medium (PRMI 1640 + PHA +Gentamycin, supplemented with 50% horse serum (Gibco BRL)) and 2 h afterwards, the medium was replaced with serum-free PRMI 1640 supplemented with penicillin and PHA. Cells were plated in a 6-well plate and taken at the indicated time (0h, 1h, 4h, 8h, 12h, 20h and 24h). Collected cells were harvested by centrifuge (1000rpm, 5min) and washed once with PBS. These samples were frozen and stored at -80° C until the extraction of the whole cell RNA.

Cell treatment with cortisol: Cells were plated at the density of 1×10^6 cells per well in 12- well plates in 10%FBS PRMI1640 culture medium one day before the treatment. Cortisol (from Sigma) was dissolved in absolute ethanol, and at dilutions of <10, 000 an equal amount of ethanol was added to the control cultures. Cells were collected after treatment with 1μM cortisol continuously for 4 hours together with the control cultures.

Quantitative PCR: total RNA was extracted using RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Extracted RNA (500ng or 1000ng) were reverse-transcribed with Superscript II reverse transcriptase (Invitrogen) by using 1uM oligo d(T) primer at 42 °C for 1 h. The cDNA was then diluted 1:5 in RNase-free water for the polymerase chain reaction (PCR). Quantitative Real-time PCR (RT-PCR) was performed by using an ABI 7900(Applied Biosystem) with the SYBR Green Reagent as the fluorescent reporter molecule. For a 25-μl PCR reaction, 5-20 ng cDNA template was mixed with the forward and reverse primers to a final concentration of 600 nM each and

12.5 µl of 2x SYBR Green PCR Master Mix (Applied Biosystems). The reaction was first incubated at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. The house-keeping gene human ubiquitin C (UBC) was selected to normalize RT-PCR using the *geNorm*^{TM} program. (Primer sequences in supplementary materials and methods). Each gene-specific PCR was performed in triplicate. Relative quantitative was analyzed with ABI 7900 SDS software (Applied Biosystems).

Molecular experiments

Extract preparation. Tissue and cell homogenates were prepared as follows: 90 µl of buffer C (20 mM HEPES, pH 7.9, 0.42 M NaCl, 25% glycerol, 1.5 mM $MgCl_2$, 0.4 mM EDTA, 0.5 mM DTT, 0.2% NP-40), supplemented with 10µg / ml aprotonin, 10µg / ml leupeptin, 0.1 mM *p*-aminobenzamidine, 1µg / ml pepstatine, and 0.5 mM PMSF, was added to approx. 30 mg of powdered tissue / approx. 30 mg (wet weight) of pelleted cells. The tissue / cells was then homogenized using a plastic pestle (Sigma-Aldrich, USA), incubated for 10 min, rehomogenized, and centrifuged for 15 min at 20000 x g. All steps were carried out at 4°C. Bradford was used for protein determination.

qPCR Primers: PCR primers were designed with Primer Express software (Applied Biosystem), and were as follows (h*Per1*-F: CAGCACCACTAAGCGTAAATGTG, h*Per1*-R: TGCTGACGGCGGATCTTT) (hUBC-F: ATTTGGGTCGCGGTTCTTG, hUBC-R: TGCCTTGACATTCTCGATGGT).

Electro mobility shift assay. 10 µg of total protein was added to x µl of reaction mixture, so that the final volume of 20 µl contained: 10 mM Tris-HCl, pH 7.5, 20 mM KCl, 1 mM EDTA, 7.5% glycerol, 1.5 mM DTT, 20 µg BSA and ~80,000 cpm [^{32}P] – labeled

oligonucleotide. In competition experiments, 10 ng of competitor was added to the reaction mixture prior to adding protein. The samples were then incubated for approx. 30 minutes at RT and loaded onto 5% polyacrylamide gels in 0.5 x TGE buffer (25 mM Tris-HCl, 0.19 M glycine, 1 mM EDTA, pH 8.5). After electrophoresis, the gels were fixed in fixation solution (40% MetOH, 3% HAc) for 10 min, dried and exposed to films (Amersham, UK). The following oligonucleotides were SDS-PAGE or HPLC purified and [P^{32}] labeled with T4 kinase (plus strands are shown, DNA-binding sites including putative sites are in bold, mutated bases in italic, and the SNP in the human *PER1* promoter underlined): NF-κB, 5´- GGTGATCA**GGGACTTTCC**GCT**GGGGACT-TTCC**AGGAT-3´; c-Rel, 5´- GTCC**CGGAGTTTCC**TACCGGG -3´; C-*Per1*, 5´-CCGG**C**GGAGCTTCCACTCGGC-3´; T-*Per1*, 5´-CCGG**T**GGAGCTTCCAC-TCGGC-3´; E2-box, 5´- CGTGCGGCCTGA**CAGGTG**CTTTGA-3´; mE2-box (5), 5´-CGTGCG-GCCTGC***ACAC*TG**CTTTGA-3´.

In the depletion experiments, total extract of human prefrontal cortex (subject # 79) or nuclear extract from rat embryonic brain was incubated for 20 min at RT with labeled T-*Per1* oligonucleotide in the presence of anti-Snail1 (0.2 µg) - or GAP-34 (0.2 – 1.0 µg) monoclonal antibodies or 0.5-1 µl of Buffer solution (84 mM citrate, 163 mM KHPO4x3H20, 0.04% NaN3, 0.05% ProClin® 300, pH 7.0) used to dissolve anti-Snail1 antibodies, and analyzed by EMSA.

References

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Supplementary Tables:

Suppl. Table 1A: Characteristics of the MARS sample (adjusted for sex) by rs3027172 *hPER1* genotype

	TT (n = 159)	TC (n = 89)	CC (n = 25)
Sex: n (% females)	83 (52.2)	50 (56.2)	10 (40.0)
Age: mean (SE)	18.7 (.03)	18.8 (.04)	18.6 (.08)
IQ[1]: mean (SE)	103.4 (1.2)	102.4 (1.6)	103.3 (3.0)
Psychosocial adversity score: mean (SE)	2.16 (.17)	1.62 (.22)	2.12 (.42)
Frequency of monthly heavy drinking (SE) [2]	0.97 (.13)	1.30 (.18)	1.88 (.34)
Average amount of alcohol consumed/drinking day (SE) [3]	34.2 (2.2)	38.4 (2.9)	48.7 (5.5)

[1] nonverbal IQ (CFT 20) assessed at age 11 years

[2] defined as drinking more than five (females: four) standard drinks (each with 8-12 g alcohol) in a row

[3] g alcohol

Suppl. Table 1B: Definition of psychosocial adversity items

	Item	Definition
1	Low educational level of a parent	Parent without completed school education or without skilled job training
2	Overcrowding	More than 1.0 person a room or size of housing ≤ 50 m^2
3	Parental psychiatric disorder	Moderate to severe disorder according to DSM-III-R criteria (interviewer rating, kappa = .98)
4	History of parental broken home or delinquency	Institutional care of a parent / more than two changes of parental figures until the age of 18 or history of parental delinquency
5	Marital discord	Low quality of partnership in two out of three areas (harmony, communication, emotional warmth) (interviewer rating, kappa = 1.00)
6	Early parenthood	Age of a parent ≤ 18 years at child birth or relationship between parents lasting less than 6 months at time of conception
7	One-parent family	At child birth
8	Unwanted pregnancy	An abortion was seriously considered
9	Poor social integration and support of parents	Lack of friends and lack of help in child care (interviewer rating, kappa = .71)
10	Severe chronic difficulties	Affecting a parent lasting more than one year (interviewer rating, kappa = .93)
11	Poor coping skills of a parent	Inadequate coping with stressful events of the past year (interviewer rating, kappa = .67)

Suppl. Table 1C: Characteristics of the adult case-control sample

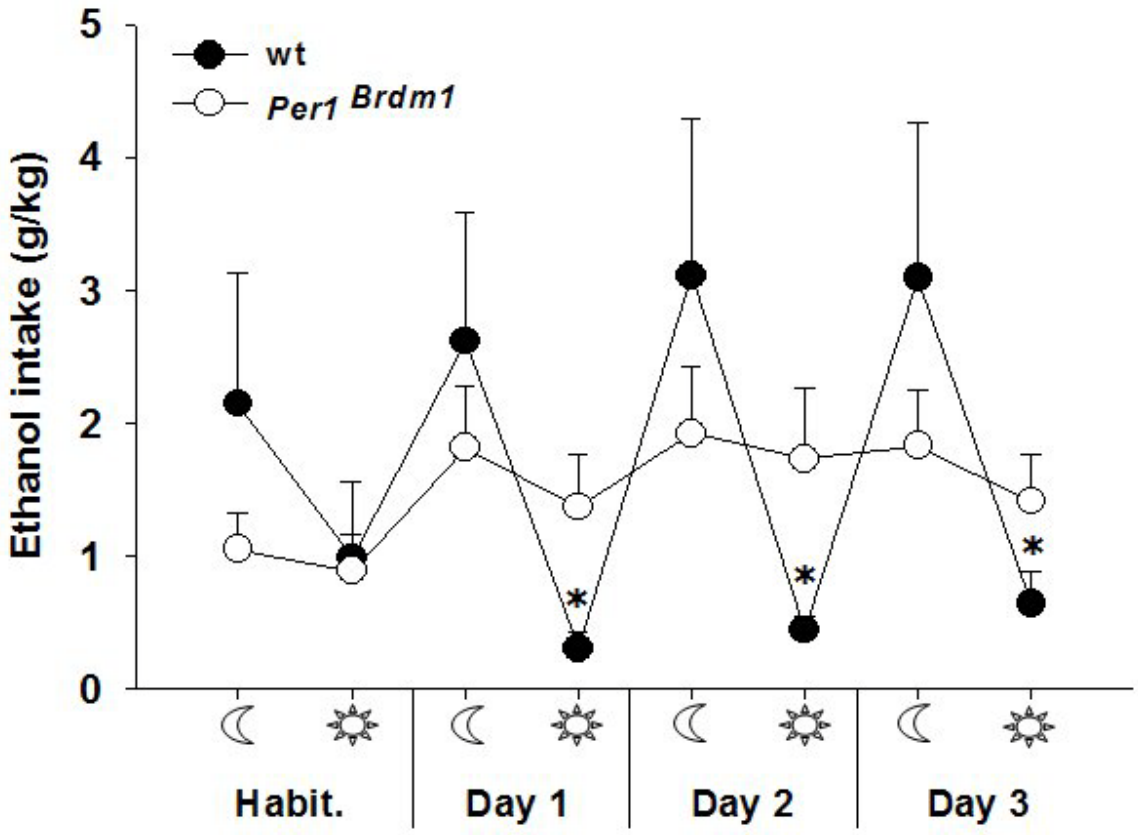
		TT (n = 1281)	TC (n = 799)	CC (n = 104)
Patients	Age: mean (SE)	43.2 (.40)	43.8 (.54)	43 (.98)
	Sex: n, % males	450 (35.1)	294 (36.8)	39 (37.5)
	n, % females	119 (9.3)	87 (10.9%)	17 (16.3)
Control	Age: mean (SE)	47.8 (.63)	47.5 (.83)	47.9 (2.6)
	Sex: n, % males	397 (31)	241 (30.2)	32 (30.8)
	n, % females	315 (24.6)	177 (22.1)	16 (15.4)

Suppl. Table 2: **Genetic variations identified in hPer1 gene:**

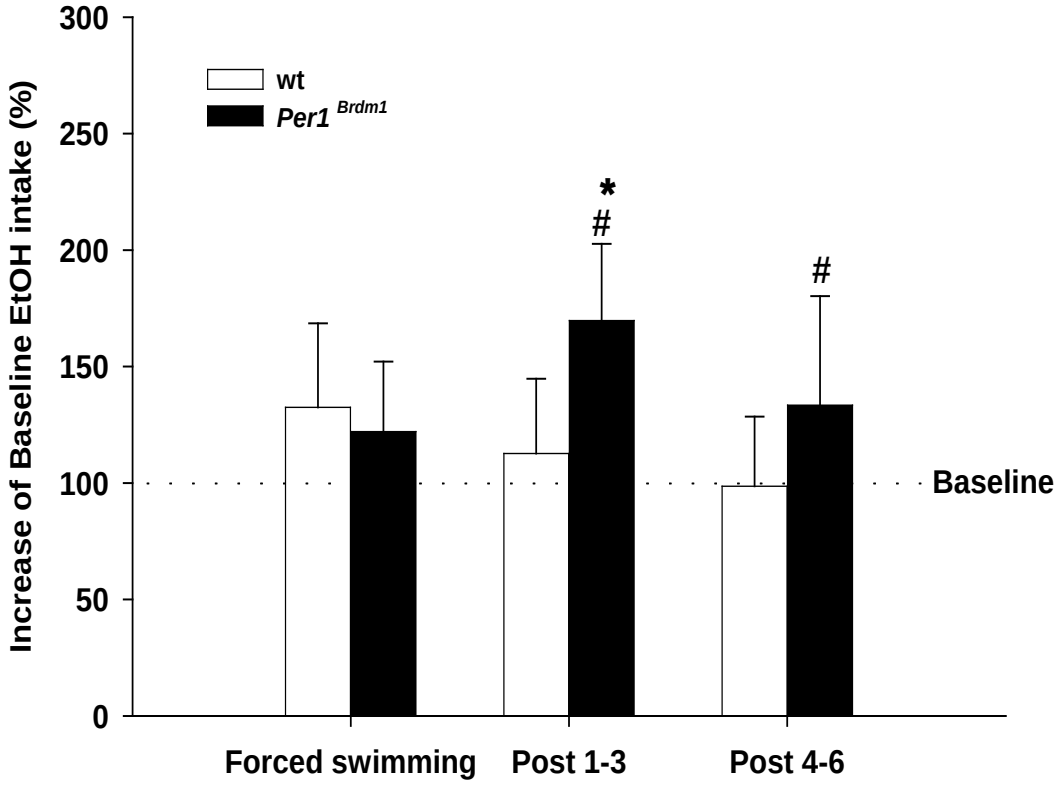
SNP		rs Number	Polymorphism flanking sequences	Base substitution		Allele frequency
SNP1	5´UTR		GCTCCGTCNGCAAGAG	G	C	0.016
SNP2	5´UTR	rs3027172	CCGCCCGGNGGAGCTT	T	C	0.25
SNP3	intron 1		CCACCCTCNCCCTGCC	-	T	0.063
SNP4	exon IV	rs35826160	AAGGGCCGNTCTGGGA	C	T	0.016
SNP5	exon V	rs3027178	GAGTACACNCTTCAGA	A	C	0.391
SNP6	intron 8		GCTGGGAGNAAGGACA	G	A	0.016
SNP7	intron 11	rs2304911	GTGGGCCANGCCCCCG	T	C	0.109
SNP8	intron 12	rs885747	TGGGAAGCNGGGTCAA	C	G	0.422
SNP9	intron 14		GATGATGTNTGGGTGA	G	A	0.016
SNP10	intron 17		GACCTTTCNCTATCTC	C	T	0.016
SNP11	exon XVIII	rs2735611	CTGCCTGGNCTAGCC	C	T	0.781
SNP12	exon XVIII	rs2253820	CTGCACACNCAGAAGG	A	G	0.781
SNP13	intron 18	rs2289591	GATTGTTGNGGGGTGG	G	T	0.266
SNP14	intron 18		CTCGCTAGNTTCTCCC	C	G	0.049
SNP15	intron 18		GCAGAGGTNCTGTCTC	G	A	0.016
SNP16	intron 18	rs3027191	CGCAGGACNCAGGACT	T	C	0.047
SNP17	intron 20		GAGTGGGCNTGCAGCC	A	G	0.016
SNP18	intron 22			A	-	1

Supplementary Figures:

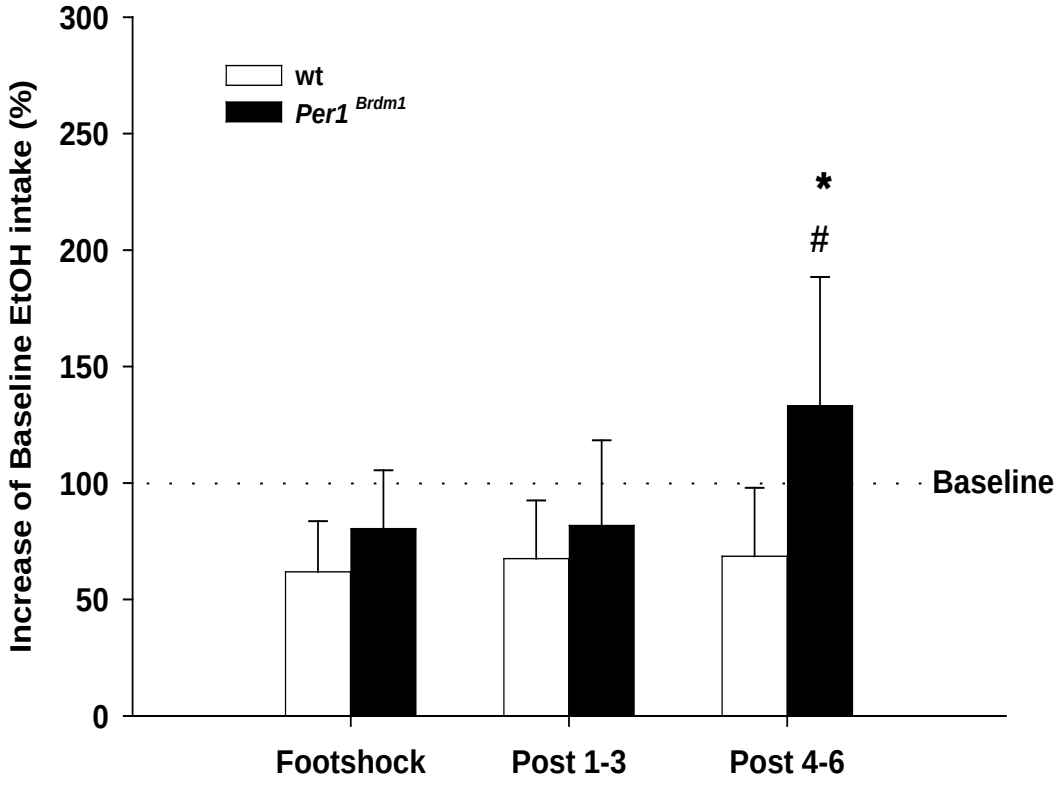
Suppl. Figure 1A: Lack of circadian rhythmicity in alcohol intake in *Per1Bdm1* mutant mice. 12 h cycle pattern of alcohol drinking behaviour (12%) in wildtype (wt) and *Per1Brdm1* mutant mice. *Per1Brdm1* mutant mice failed to show any rhythmicity as no differences were observed between the amount of ethanol consumed during the night (moon) and the light (sun) cycle (*p<0.01). The first day was used to habituate (Habit) the animals to the drinkometer system.



<u>Suppl. Figure 1B:</u> Effects of forced swimming on alcohol (EtOH) drinking behaviour (12%) in wildtype and *Per1*^{Brdm1} mutant mice. Both wildtype (wt) and *Per1*^{Brdm1} mutant mice showed a slightly higher alcohol intake relative to the baseline during forced swim stress administration which did not result in any statistical significance (Newman-Keuls test wild type p=0,11 ; *Per1*^{Brdm1} p=0,23). However, three (Post1-3) and 6 (Post4-6) days after stress application *Per1*^{Brdm1} mutant but not wildtype mice showed a significant and greater increase in alcohol consumption in respect to baseline consumption (vs. baseline *p<0.01) and compared to wildtype mice (wt vs. *Per1*^{Brdm1} in Post1-3 #p<0.01 and in Post 4-6 #p<0.05).



<u>Suppl. Figure 1C:</u> Effects of foot-shock on alcohol (EtOH) drinking behaviour (12%) in wildtype and *Per1*^{Brdm1} mutant mice. In this experiment foot-shock was administered, wild-type (wt) mice showed a reduction in alcohol consumption for the subsequent 6 days. *Per1*^{Brdm1} mutant mice showed significant higher alcohol intake when compared to. Post-hoc Newman-Keuls tests revealed significant decreases of alcohol intake during and after (Post1-3 and Post 4-6) footshock for the wildtype mice when compared to baseline (*p<0.01) and significant increase of alcohol intake 6 days after footshock (Post 4-6) for the *Per1*^{Brdm1} mutant mice (*p<0.01). In addition, both genotypes showed significant differences in alcohol intake during and more strongly after (Post 4-6) stress sessions (# p<0.01).



<u>Suppl.Fig. 1D:</u> Stress-induced blood corticosterone levels in mice. Before and following foot-shock stress we have measured blood corticosterone levels. At ZT4 we found 17 ± 4.4 ng/ ml in wild-type and 27 ± 3.7 ng/ml corticosterone in mutant mice - these differences, however, were not significant (two-way ANOVA, stress x genotype effect $F(1,42)=0.29$; $p=0.59$). Foot-shock stress led to an approx. 10-fold increase in corticosterone levels with 186 ± 23.7 ng/ml in wild-type mice and 209 ± 14.1 ng/ml corticosterone in *Per1*^{Brdm1} mutant mice (Newman Keuls test; $p<0.01$).