Introduction

Alcoholism is a devastating brain disorder that affects millions of people worldwide. The development of alcoholism is caused by alcohol-induced maladaptive changes in neural circuits involved in emotions, motivation, and decision-making. Because of its involvement in these processes, the amygdala is thought to be a key neural structure involved in alcohol addiction. However, the molecular mechanisms that govern the development of alcoholism are incompletely understood.

We have previously shown that in a limited access choice paradigm, C57BL/6J mice progressively escalate their alcohol intake and display important behavioral characteristics of alcohol addiction, in that they become insensitive to quinine-induced adulteration of alcohol. This study used the limited access choice paradigm to study gene expression changes in the amygdala during the escalation to high alcohol consumption in C57BL/6J mice. Microarray analysis revealed that changes in gene expression occurred predominantly after one week, i.e. during the initial escalation of alcohol intake. One gene that stood out from our analysis was the adapter protein 14-3-3ζ, which was up-regulated during the transition from low to high alcohol intake. Independent qPCR analysis confirmed the up-regulation of amygdala 14-3-3ζ during the escalation of alcohol intake. Subsequently, we found that local knockdown of 14-3-3ζ in the amygdala, using RNA interference, dramatically augmented alcohol intake. In addition, knockdown of amygdala 14-3-3ζ promoted the development of inflexible alcohol drinking, as apparent from insensitivity to quinine adulteration of alcohol. This study identifies amygdala 14-3-3ζ as a novel key modulator that is engaged during escalation of alcohol use.

Abstract

Alcoholism is a devastating brain disorder that affects millions of people worldwide. The development of alcoholism is caused by alcohol-induced maladaptive changes in neural circuits involved in emotions, motivation, and decision-making. Because of its involvement in these processes, the amygdala is thought to be a key neural structure involved in alcohol addiction. However, the molecular mechanisms that govern the development of alcoholism are incompletely understood.

We have previously shown that in a limited access choice paradigm, C57BL/6J mice progressively escalate their alcohol intake and display important behavioral characteristics of alcohol addiction, in that they become insensitive to quinine-induced adulteration of alcohol. This study used the limited access choice paradigm to study gene expression changes in the amygdala during the escalation to high alcohol consumption in C57BL/6J mice. Microarray analysis revealed that changes in gene expression occurred predominantly after one week, i.e. during the initial escalation of alcohol intake. One gene that stood out from our analysis was the adapter protein 14-3-3ζ, which was up-regulated during the transition from low to high alcohol intake. Independent qPCR analysis confirmed the up-regulation of amygdala 14-3-3ζ during the escalation of alcohol intake. Subsequently, we found that local knockdown of 14-3-3ζ in the amygdala, using RNA interference, dramatically augmented alcohol intake. In addition, knockdown of amygdala 14-3-3ζ promoted the development of inflexible alcohol drinking, as apparent from insensitivity to quinine adulteration of alcohol. This study identifies amygdala 14-3-3ζ as a novel key modulator that is engaged during escalation of alcohol use.
The aim of this study was to identify molecular mechanisms in the amygdala that contribute to the escalation of alcohol intake, which is an important stage in the development of drug addiction [31]. During the development of alcoholism, casual alcohol use escalates into excessive drinking, ultimately culminating in full-blown alcohol addiction, characterized by loss of control over alcohol intake. For this study we used a limited access choice paradigm, in which C57BL/6J mice show rapid escalation of alcohol consumption [32], which depends on processes within the CeA [30]. Moreover, using this paradigm C57BL/6J mice display alcohol use despite adverse consequences, an important behavioral characteristic of alcoholism, in that they fail to reduce their alcohol intake when an alcohol solution is adulterated with quinine and consume an aversive, quinine-containing alcohol solution despite the simultaneous availability of unadulterated alcohol [33]. Here, we studied gene expression patterns in the CeA during the escalation of alcohol intake in C57BL/6J mice, using microarray analysis followed by qPCR. We subsequently used RNA interference to pinpoint the involvement of 14-3-3ζ, a candidate gene that stood out from our analysis, in alcohol intake. Together, these data show that 14-3-3ζ signaling in the CeA controls the escalation of alcohol intake in mice.

Materials and Methods

Animals

8–10 Weeks old male C57BL/6J mice, derived from Jackson Labs (Bar Harbor, Maine, USA) and bred in our facility, were group-housed with food and water ad libitum under controlled conditions (20±2°C and 50–70% humidity) and acclimatized to a 12-h light/dark cycle (7:00 AM lights off) at least 2 weeks prior to testing. Experimental procedures were approved by the Animal Ethics Committee of Utrecht University and conducted in agreement with Dutch laws (Wet op dierproeven, 1996) and European regulations (Guideline 86/609/EEC).

Limited access alcohol consumption

Mice were trained to voluntarily consume alcohol using a limited access choice paradigm [30,34,35]: they had access to one drinking tube containing tap water and one containing alcohol (10–15% v/v) in daily 2 hour sessions, starting 3 hours into the dark cycle. Bottle positions were switched daily after 7 days to avoid side-preference. Fluid volumes were measured and alcohol intake, alcohol preference and total volume consumed were calculated.

For microarray analysis, mice were randomly assigned to 3 experimental groups that consumed alcohol for 1, 2 or 4 consecutive weeks (N= 14, 14 and 13), representing 3 stages of the development of alcoholism: initial escalation of alcohol intake (1 week), the stage where high alcohol intake is reached and insensitivity to quinine adulteration emerges [33] and the stage of stable high alcohol intake (4 weeks). A water control group (N=6) was included and a group of naive mice (N= 21) served as a reference sample. To ensure active engagement in the limited access choice paradigm and to exclude animals consuming extremely high or low amounts of alcohol, we applied the following inclusion criteria.

1) To ensure sufficient sampling of the fluids, total fluid intake should be ≥12 ml/kg on day 6 [30,32].

2) To ensure active engagement and motivation to consume alcohol, preference for alcohol over water should be >50% by day 6–8.

3) To ensure analysis of escalation, alcohol intake should be ≤2 g/kg by day 4 and ≥0.6 g/kg by day 6–8.

The final sample size after application of these criteria was N=6.

For qPCR validation, a separate batch of mice consumed alcohol for 1 week or 2 weeks (N=13); a total of 6 and 7 mice, respectively, met the inclusion criteria. Naïve mice (N=7) and a water group (N=6) were included as controls.

Tissue dissection

The mice were sacrificed by decapitation 10–11 hours after the final limited access choice session. Blood alcohol analyses using an NAD/ADH assay (Sigma, Germany) confirmed clearance of alcohol from blood at this time: blood alcohol levels were low (6.3–33 mg/dl) compared to levels of 97.7±24.9 mg/dl immediately after a drinking session [32]. Brains were dissected, snap frozen on dry ice and stored at −80°C. Amygdala samples were obtained using a 20G punch needle, aiming at the CeA [30,36] and were immersed instantly in RNAlater (Sigma, Germany). Total RNA was isolated from the amygdala using TRIzol (Invitrogen, NL), DNase treated (Ambion, TX, USA) and purified using the RNeasy MinElute Cleanup kit (Qiagen N.V., NL). RNA integrity was confirmed using the Bioanalyzer (Agilent Technologies Inc, CA, USA).

RNA isolation and Hybridization

Two-color oligonucleotide microarray analysis was performed as described [37]. RNA was amplified in a single round and complementary DNA (cDNA) was synthesized with Superscript III reverse transcriptase (Invitrogen) using a T7 oligo(dT)24VN primer [38]. Complementary DNA was transcribed in vitro using the T7Megascript kit (Ambion) in the presence of aminothiol-UTP, and copy ribonucleic acid (cRNA) quality was evaluated using the Bioanalyzer. Cy3 or Cy5 fluorophores (Amersham Biosciences, NL) were coupled to 1500 ng of cRNA, and label incorporation was monitored by spectrophotometry and hybridizations were set up with 1000 ng of Cy3-labeled and 1000 ng of Cy5-labeled cRNA. Each cRNA sample was labeled with Cy5 or Cy3 and was hybridized in dye swap against a common reference pool sample consisting of RNA from naïve mice; a total of 6 slides were hybridized for each experimental group. The mouse Array-Ready oligo set (version 3.0; Operon Biotechnologies GmbH, Germany) was printed on Corning UltraGAPS slides as previously described [37]. Slides were washed manually, scanned in the Agilent G2563AA DNA Microarray Scanner (100% laser power and 30% photomultiplier tube) and quantified and background corrected with IMAGENE (version 5.6.1; BioDiscovery, Inc., CA, USA) and Loess normalized per print-tip [39].

The microarray data were analyzed by ANOVA-modeling [40] to identify genes that show differential expression from naïve control mice. In a fixed effect analysis, sample, array and dye effects were modeled. Sample-specific differences between groups were then modeled and tested using permutations and family-wise error correction. MIAMEcompliant descriptions of protocols, experiment design, arrays, raw and normalized data have been deposited in the public microarray database ArrayExpress (http://www.ebi.ac.uk/arrayexpress/), all under the experiment accession number E-TABM-956. In addition, the microarray data was analyzed using the short time-series expression miner (STEM) with integrated gene ontology (GO) database [41].
qPCR Validation

Total RNA was isolated from the amygdala as described and cDNA was synthesized from the RNA samples using oligo-dT primers. qPCR analysis was performed using the LightCycler (Roche, NL), the Fast Start DNA Master PLUS SYBR Green I kit (Roche) and primers listed in Table 1. After initial normalization to the housekeeping gene beta-actin, gene expression was calculated as the ratio to levels of naive mice using the comparative Ct method [42].

RNA interference

Two shRNAs were designed for mouse 14-3-3ζ (YWHAZ, NCBI accession no. NM_011740): 1222 bp (GTGAAGAGTCGGCTACTCT), and 1854 bp (GAAGTTGTCTCTAGACAAG). A non-coding sequence was used as a control (GGCGTGAGGACCTATTGG). The sequences were cloned into a Lentivirus vector (pLL3.7, http://www.sciencegateway.org/protocols/lentivirus/plllmap.html).

Knockdown efficiencies were determined by transfection of Neuro2A cells with the respective lentiviral vectors and semi-lentivirus/

3.7 vector (pLL3.7, http://www.sciencegateway.org/protocols/lentivirus/plllmap.html).

To establish in vivo knockdown of 14-3-3ζ in the CeA and alcohol intake

To establish in vivo knockdown of 14-3-3ζ male C57BL/6J mice were anesthetized with ketamine (75 mg/kg i.p.) and medetomidine (1 mg/kg i.p.) and placed in a stereotaxic frame (David Kopf Instruments, CA, USA). The injectors (33G) were targeted at the CeA and alcohol (15% v/v) with graded quinine concentrations (100 μM, 250 μM, 350 μM, 500 μM and 750 μM) on 5 consecutive days [33]. Alcohol intake and preference were normalized to the group average over the last 3 days prior to quinine modulation. After completion of the alcohol consumption, intake of sweet (sucrose / saccharin) and bitter (quinine) solutions was also determined in two-bottle choice tests.

Post-mortem immunohistochemistry for GFP was performed using an anti-sheep antibody (1:5000, Biogenesis Ltd) to determine the infection site.

Data Analysis

Consumption data were analyzed by one-way repeated measures ANOVA with group as the between-subjects factor and time or quinine concentration as the repeated measures within-subjects factor. qPCR data were analyzed by one-way ANOVAs with group as the between-subjects factor, followed by Tukey HSD multiple-comparisons. Post-hoc analysis was performed by two-tailed t-tests where appropriate. Differences between pairs of means were considered significant at alpha < 0.05. SPSS 15.0 was used for data analysis.

Results

Gene expression during the escalation of alcohol intake

In order to trace molecular mechanisms engaged during the escalation of alcohol intake, gene expression in the CeA was studied by microarray analysis after 1 week, 2 weeks and 4 weeks of daily alcohol consumption (Fig. 1A–B). Alcohol preference was high from the first week of the experiment onwards (1 week F(time)5,65 = 1.7, N.S.; 2 weeks F(time)7,70 = 0.97, N.S.; 4 weeks F(time)10,50 = 1.1, N.S.) while alcohol intake stabilized only after two weeks of daily drinking. The experimental groups represent 3 stages of escalation to alcoholism-like behavior: the transition from low to high alcohol intake (1 week; intake: F(time)5,75 = 16.8, P<0.001), the stage when mice reach their highest levels of alcohol intake and start to show inflexible and indifferent alcohol intake (2 weeks; see Lesscher et al, 2010; intake: F(time)7,70 = 10.3, P<0.001) and finally also the stage where high alcohol intake has stabilized (4 weeks; intake: F(time)10,50 = 0.87, N.S.). Microarray analysis revealed marked changes in gene expression as a result of alcohol consumption, particularly during the early stages of alcohol intake (P<0.01 from control mice, Fig. 1D and Fig. 1E–F). After exclusion of those genes that showed differential expression after water consumption compared to naive mice, 267 genes in the CeA were found to be differentially regulated after 1 week of alcohol consumption. Of these 267 genes, 211 were up-regulated and 56 were down-regulated. By contrast, only 29 and 20 genes, respectively, were differentially regulated after 2 and

Table 1. Primer sequences for qPCR validation.

| Gene     | Primer Fwd | Primer Rev |
|----------|------------|------------|
| Gabrg2   | TGTGTGCTACCGAATGTTT | TACATTCTGTGCTACAAAGG |
| Gria3    | CTCGTGCTTCTAAACAGA | CCAAAACCGTCTGGGAGAAAT |
| YWHAZ    | AGCCAGCGAGGAGCTAATGAT | TTCTCTGGCTACATTCGGTCTT |
| Gprasp1  | CCATTAGATCCTCCGGTTG | CCTGTGATGGTCTTGTTGCTT |
| Ctnnd2   | CGCCAGCATCACTTGTCC | ACTGCTTCCTGGGACCAAT |
| Tmod2    | CCAGTTAATGTTGCCAGT | GGCCTGCTTCGGAGGTGAC |
| Gabrb3   | AGCAATCTACAACCAACTG | GGCCACCGAAAATACTGTA |
| Prkcb1   | TCTTTCTCTGCTCTACATGT | AGGGGAGCTGCTGTGAGA |
| Actb (beta-actin) | AGCCATGATCGTAGCTACCATC | CTCTCAGTCTGGTGCTGAA |

doi:10.1371/journal.pone.0037999.t001
4 weeks of alcohol consumption, i.e. when highest alcohol levels are reached and signs of alcoholism-like behavior emerge, and alcohol intake is stabilized. Regulated genes in all three experimental groups are presented in Table 2.

Gene Ontology for those genes that were regulated specifically during the first week of alcohol consumption identified significant enrichment of genes involved in transport, ligand-gated ion channel activity, synaptic transmission and cytoplasm ($P_{enrichment} < 0.01$, Table 3). Based on effect size, significance and evidence for involvement in processes that likely contribute to alcoholism, e.g. synaptic plasticity, memory processing or addiction-related signaling pathways, eight top candidate genes were selected for further investigation. The top candidate genes that we identified include genes which have previously been associated with alcoholism, such as Gabrg2, Gabrb3 and Gria3 [44,45], but also genes that have not been associated with alcoholism before. Those novel genes are the adapter protein 14-3-3ζ, the G-protein associated sorting protein Gprasp1, two genes involved in structural and functional plasticity (Tmod2 and Ctnnd2) and Prkacb, which has been postulated to affect cAMP-dependent gene expression. qPCR analysis for the 8 top candidate genes in an independent batch of mice confirmed significant up-regulation of Gria3, Gabrb3, 14-3-3ζ and Prkacb in the CeA after alcohol consumption (Fig. 2). Water consumption did not affect the expression of any of the genes tested (not shown).

**Amygdala 14-3-3ζ controls the development of alcoholism**

Of the genes that were consistently up-regulated during escalation of alcohol intake, we found 14-3-3ζ to be of particular interest. 14-3-3 Proteins are adapter proteins that have multiple...
Table 2. Significant effects of 1 week, 2 weeks and 4 weeks of daily alcohol consumption in the limited access choice paradigm on gene expression changes in the amygdala of C57BL/6J mice.

| Symbol | Gene description                                                                 | p value | EtOH/N |
|--------|----------------------------------------------------------------------------------|---------|--------|
|        | **1 week alcohol consumption***                                                   |         |        |
| Prkacb | cAMP-dependent protein kinase, beta-catalytic subunit                            | 0.0013  | 1.44   |
| Ywhaz  | 14-3-3 protein zeta/delta                                                        | 0.0001  | 1.43   |
| Mapk10 | Mitogen-activated protein kinase 10                                               | 0.0007  | 1.38   |
| Gprasp1| G-protein coupled receptor-associated sorting protein 1                          | 0.0034  | 1.36   |
| Rasgrp1| RAS guanyl releasing protein 1                                                    | 0.0037  | 1.35   |
| Rdm1   | RAD52 motif-containing protein                                                    | 0.0024  | 1.34   |
| Camk2a | Calcium/calmodulin-dependent protein kinase type II alpha chain                   | 0.0002  | 1.32   |
| Eif4a2 | Eukaryotic initiation factor 4A-II                                               | 0.0003  | 1.31   |
| Trim37 | Tripartite motif-containing protein 37                                            | 0.0098  | 1.30   |
| Pdia3  | Protein disulfide-isomerase A3 precursor                                         | 0.0005  | 1.30   |
| Slc2a17| solute carrier family 2 (organic cation transporter), member 17                  | 0.0055  | 1.30   |
| Atp6ap1| Vacuolar ATP synthase subunit 51 precursor                                       | 0.0002  | 1.29   |
| Zfp758 | zinc finger protein 758                                                           | 0.0001  | 1.28   |
| Arf1   | ADP-ribosylation factor 1                                                         | 0.0034  | 1.28   |
| Rtn4   | Reticulon-4 (Neurite outgrowth inhibitor)                                         | 0.0003  | 1.28   |
| Lgi1   | Leucine-rich glioma-inactivated protein 1 precursor                              | 0.0094  | 1.27   |
| Trim23 | GTP-binding protein ARD-1                                                        | 0.0018  | 1.26   |
| Cdksnap2| CDKS regulatory subunit-associated protein 2                                     | 0.0000  | 1.26   |
| Fbxl3  | F-box/LRR-repeat protein 3                                                        | 0.0079  | 1.26   |
| Cd47   | Leukocyte surface antigen CD47 precursor                                         | 0.0038  | 1.26   |
| Pcmtd1 | Protein-L-isosaspartate O-methyltransferase domain-cont protein1                 | 0.0036  | 1.26   |
| Arhgef9| Rho guanine nucleotide exchange factor 9                                          | 0.0016  | 1.26   |
| Actg2  | Actin, gamma-enteric smooth muscle                                               | 0.0000  | 1.26   |
| Ptk2b  | Protein tyrosine kinase 2 beta                                                   | 0.0022  | 1.25   |
| Homer1 | Homer protein homolog 1                                                           | 0.0003  | 1.25   |
| Lrp11  | Low-density lipoprotein receptor-related protein 11 precursor                   | 0.0008  | 1.25   |
| Dna1c5 | Dna1 homolog subfamily C member 5                                                | 0.0016  | 1.25   |
| Ybx1   | Nuclease sensitive element-binding protein 1                                     | 0.0024  | 1.24   |
| Gls    | glutaminase isoform 1                                                            | 0.0079  | 1.24   |
| Arf1   | ADP-ribosylation factor 1                                                         | 0.0038  | 1.24   |
| Eif4h  | Eukaryotic translation initiation factor 4H                                      | 0.0045  | 1.23   |
| Azin1  | Antizyme inhibitor 1 (AZI)                                                       | 0.0016  | 1.23   |
| Syn2   | Synapsin-2                                                                       | 0.0002  | 1.23   |
| Gapdh  | Glyceroldehyde-3-phosphate dehydrogenase                                         | 0.0037  | 1.23   |
| Lrcc58 | Leucine-rich repeat-containing protein 58                                         | 0.0011  | 1.23   |
| Tmod2  | Tropomodulin-2                                                                   | 0.0023  | 1.23   |
| Scl17a7| solute carrier family 17                                                          | 0.0001  | 1.23   |
| Wsb2   | WD repeat and SOCS box-containing protein 2                                       | 0.0033  | 1.22   |
| Gria3  | Glutamate receptor 3 precursor                                                   | 0.0001  | 1.22   |
| Hnrpk  | Heterogeneous nuclear ribonucleoprotein K                                        | 0.0007  | 1.22   |
| Ube2d3 | Ubiquitin-conjugating enzyme E2 D3                                               | 0.0001  | 1.22   |
| Calbp5 | Calcium-binding protein 5                                                        | 0.0000  | 1.22   |
| Sec23a | Protein transport protein                                                         | 0.0001  | 1.22   |
| Ddx3x  | ATP-dependent RNA helicase                                                        | 0.0020  | 1.22   |
| Gapdh  | Glyceroldehyde-3-phosphate dehydrogenase                                         | 0.0001  | 1.22   |
| Fbxl16 | F-box/LRR-repeat protein 16                                                       | 0.0010  | 1.22   |
| Usp31  | MKI/A1203 protein                                                                | 0.0026  | 1.22   |
| Symbol | Gene description | p value | EtOH/N |
|--------|------------------|---------|--------|
| Pja2   | E3 ubiquitin-protein ligase Prja2 | 0,0000  | 1,22   |
| Srp54a | Signal recognition particle 54 kDa protein | 0,0010  | 1,22   |
| Igf1r  | Insulin-like growth factor 1 receptor precursor | 0,0000  | 1,21   |
| Ube2i  | SUMO-conjugating enzyme UBC9 | 0,0026  | 1,21   |
| Arl8b  | ADP-ribosylation factor-like protein 8B | 0,0001  | 1,21   |
| Gabrb3 | Gamma-aminobutyric acid receptor subunit beta-3 precursor | 0,0002  | 1,21   |
| Lpgat1 | Acyl-CoA:lysophosphatidylglycerol acyltransferase 1 | 0,0002  | 1,21   |
| Mrfap1 | MORF4 family-associated protein 1 | 0,0073  | 1,21   |
| Gabrg2 | Gamma-aminobutyric acid receptor subunit gamma-2 precursor | 0,0000  | 1,21   |
| Tsnk13 | Thioredoxin domain-containing protein 13 precursor | 0,0003  | 1,21   |
| Rab18  | Ras-related protein Rab-18 | 0,0024  | 1,21   |
| Kcna1  | Calcium-activated potassium channel subunit alpha-1 | 0,0001  | 1,21   |
| Spag9  | C-jun-amino-terminal kinase-interacting protein 4 | 0,0010  | 1,21   |
| Peg3   | Paternally-expressed gene 3 protein (ASF-1) | 0,0002  | 1,21   |
| Pten   | Phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase | 0,0019  | 1,21   |
| Synj1  | Synaptojanin-1 | 0,0055  | 1,21   |
| Slc4a10 | Sodium-driven chloride bicarbonate exchanger | 0,0016  | 1,21   |
| Knap3  | Importin subunit alpha-3 | 0,0015  | 1,20    |
| Abr    | active BCR-related isoform 2 | 0,0052  | 1,20    |
| Ccnd132 | Coiled-coil domain-containing protein 132 | 0,0099  | 1,20    |
| Ap2a2  | AP-2 complex subunit alpha-2 | 0,0002  | 1,20    |
| Ppp2ca | Serine/threonine-protein phosphatase 2A catalytic subunit alpha | 0,0014  | 1,20    |
| Cap2   | Adenylyl cyclase-associated protein 2 | 0,0002  | 1,20    |
| Psap   | Sulfated glycoprotein 1 precursor (SGP-1) | 0,0021  | 1,20    |
| Elmo2  | Engulfment and cell motility protein 2 | 0,0080  | 1,20    |
| Kcnip2 | Kv channel-interacting protein 2 | 0,0043  | 1,20    |
| Atp5k  | ATP synthase subunit e, mitochondrial | 0,0001  | 0,81    |
| Mt2    | Metallothionein-2 | 0,0034  | 0,81    |
| Tsx    | Testis-specific protein | 0,0016  | 0,80    |
| Olfr779 | Olfactory receptor Olfr779 | 0,0002  | 0,79    |
| Olfr806 | Olfactory receptor 806 | 0,0085  | 0,77    |

2 weeks alcohol consumption

| Symbol | Gene description | p value | EtOH/N |
|--------|------------------|---------|--------|
| Vnnl1  | Visinin-like protein 1 | 0,0001  | 1,33   |
| Tmem130 | Transmembrane protein 130 precursor | 0,0005  | 1,27   |
| Pdia3  | Protein disulfide-isomerase A3 precursor | 0,0016  | 1,26   |
| Ipo7   | Importin-7 | 0,0001  | 1,24    |
| Camk2d | Calcium/calmodulin-dependent protein kinase type II delta chain | 0,0005  | 1,18    |
| Ranbp6 | Ran-binding protein 6 | 0,0016  | 1,18    |
| Atp6ap1 | Vacuolar ATP synthase subunit S1 precursor | 0,0081  | 1,18   |
| Ppap2b | Lipid phosphate phosphohydrolase 3 | 0,0083  | 1,17    |
| Ptprz2 | protein tyrosine phosphatase, receptor type Z, polypeptide 1 | 0,0004  | 1,17    |
| Zbtb2  | zinc finger and BTB domain containing 2 | 0,0005  | 1,16   |
| Prkar2b | cAMP-dependent protein kinase type II-beta regulatory subunit | 0,0051  | 1,16   |
| Zdhhc2 | Palmitoyltransferase ZDHHC2 | 0,0072  | 1,16   |
| Rab6   | Ras-related protein Rab-6A | 0,0099  | 1,16   |
| Slc6a1 | Sodium- and chloride-dependent GABA transporter 1 | 0,0016  | 1,16   |
| Arhgap5 | Rho GTPase activating protein 5 | 0,0029  | 1,16   |
| Glu    | glutaminase isomerase 1 | 0,0028  | 1,15    |
| Gabrg1 | Gamma-aminobutyric acid receptor subunit gamma-1 precursor | 0,0049  | 1,15   |
and diverse binding partners [46,47]. These include transcription factors and signaling molecules such as PKCs [48] and ionotropic glutamate receptors [49] that have previously been implicated in alcoholism [30,44,50–53]. Therefore, 14-3-3 proteins are well positioned to integrate signaling inputs and influence alcohol addiction. Indeed, previous studies reported gene and protein expression changes for 14-3-3 in the nucleus accumbens and amygdala after prolonged alcohol use [54,55] and a recent study has shown reduced 14-3-3 in brain tissue of human alcoholics [56]. Because we found that amygdala 14-3-3 levels were upregulated during the escalation of alcohol intake, we investigated whether amygdala 14-3-3 contributes to the escalation of alcohol intake in mice. For this purpose, lentiviral vectors expressing 14-3-3 specific shRNA sequences (1222 and 1854 bp, Fig. 3A) were generated to reduce the expression of 14-3-3 in vivo. As a control, a shRNA sequence that did not recognize any known mammalian gene in a BLAST search was used. Of the two shRNA constructs tested, the 1854 construct was most effective in knocking down 14-3-3 in vivo (Fig. 3B). Densitometry and slope analysis revealed lower 14-3-3 protein levels in Neuro2A cells that were transfected with both 14-3-3 shRNA constructs. Knockdown efficiency, calculated from the ratio of the slopes for 14-3-3 shRNA over those for untreated cells, was greater for the 1854 construct (71%) as compared to the 1222 shRNA construct (38%). In contrast, the scrambled construct did not affect 14-3-3 protein expression (2.5–3.7% change from untreated cells). *In situ* hybridization after unilateral infusion of the 1854 14-3-3 shRNA expressing lentivirus in the CeA and infection with the scrambled shRNA expressing lentivirus in the contralateral CeA confirmed effective knockdown of 14-3-3 in vivo in the CeA using this same 14-3-3 shRNA construct (1854 bp, Fig. 3C). Comparison of adjacent sections stained for GFP, to localize the infection site, with sections stained for 14-3-3 revealed that 14-3-3 mRNA was completely absent in the CeA that was infected with the 1854 14-3-3 shRNA expressing lentivirus while 14-3-3 was expressed in the contralateral CeA that was infected with the scrambled shRNA expressing lentivirus.

The effects of 14-3-3 knockdown on escalation of alcohol intake were determined by bilaterally infusing either the 1854 14-3-3 or the control lentivirus into the CeA and allowing the mice to consume alcohol in the limited access choice paradigm. The mice with CeA knockdown of 14-3-3 using the 1854 14-3-3 shRNA construct showed increased alcohol intake (45–53% increase from control in weeks 3 and 4; Fig. 4A, 10% v/v; rep. measures ANOVA: Ftime x group(18,216) = 2.6, P<0.01), demonstrating a gene-dosage effect of amygdala 14-3-3 on alcohol intake. We next confirmed the involvement of amygdala 14-3-3 in alcohol intake by examining the effects of knocking down 14-3-3 in the amygdala on alcohol intake.

Table 2. Cont.

| Symbol | Gene description | p value | EtOH/N |
|--------|------------------|---------|---------|
| Olf779 | Olfactory receptor | 0.0010 | 0.85 |
| Ap4e1 | AP-4 complex subunit epsilon-1 | 0.0003 | 0.83 |
| Tesc | Tescalcin | 0.0070 | 0.83 |
| Rpl27a | ribosomal protein L27a | 0.0007 | 0.83 |
| Itpka | Inositol-trisphosphate 3-kinase A | 0.0099 | 0.82 |
| Cck | Cholecystokinin precursor | 0.0072 | 0.81 |

4 weeks alcohol consumption

| Symbol | Gene description | p value | EtOH/N |
|--------|------------------|---------|---------|
| Pum2 | Pumilio homolog 2 | 0.0061 | 1.24 |
| Igt1 | Insulin-like growth factor 1 receptor precursor | 0.0030 | 1.21 |
| Psd | Phosphatidylserine decarboxylase proenzyme | 0.0021 | 1.20 |
| Pki | cAMP-dependent protein kinase inhibitor alpha | 0.0033 | 1.19 |
| Ptpz1 | protein tyrosine phosphatase, receptor type Z, polypeptide 1 | 0.0021 | 1.19 |
| Prepl | Prolyl endopeptidase-like | 0.0039 | 1.18 |
| Peg3 | Paternally-expressed gene 3 protein (ASF-1) | 0.0058 | 1.17 |
| Knp1 | Importin subunit alpha-1 | 0.0003 | 1.17 |
| Prkacb | cAMP-dependent protein kinase, beta-catalytic subunit | 0.0015 | 1.17 |
| Atp6ap1 | Vacuolar ATP synthase subunit S1 precursor | 0.0030 | 1.17 |
| Homer1 | Homer protein homolog 1 | 0.0032 | 1.16 |
| Ncdn | neurochondrin | 0.0032 | 1.16 |
| Dnm1 | Dynamin-1 | 0.0085 | 1.16 |
| Rasgp1 | RAS guanyl releasing protein 1 | 0.0003 | 1.16 |
| Tmem40 | Transmembrane protein 40 | 0.0059 | 0.84 |

*for the 1 week group only those genes are shown that display a change in expression of at least +/− 20% from naive controls. EtOH/N = fold change of the EtOH group (1 wk, 2 wk or 4 wk) to naive controls. Hypothetical genes were excluded from this list.

doi:10.1371/journal.pone.0037999.t002
consumption in a separate batch of mice using a higher alcohol concentration (15% v/v). In agreement with our initial experiment, CeA 14-3-3ζ knockdown using the 1854 shRNA increased intake of the 15% alcohol solution (63–73% increase from control in weeks 1–3; Fig. 4C; rep. measures ANOVA: F group(1,11) = 5.1, P < 0.05, F time x group(13,143) = 1.0, N.S.). Furthermore, to evaluate the relevance of our findings for alcoholism-like behavior, we next determined the effects of amygdala 14-3-3ζ knockdown on the development of inflexible alcohol drinking [33]. For this purpose, the alcohol solution was adulterated with graded concentrations of the bitter substance quinine. The mice with CeA 14-3-3ζ knockdown using the 1854 shRNA showed a persistent high preference for the quinine-adulterated alcohol solution, indicative of inflexible alcohol drinking (Fig. 4D; F quinine x group(5,50) = 2.37, P = 0.053).

Finally, we also determined specificity of these findings for alcohol and potential confounding effects of 14-3-3ζ knockdown on taste sensitivity. Mice treated with the 14-3-3ζ 1854 shRNA showed equal intake of the caloric sweet tastant sucrose (Fig. 5A; F group(1,11) = 0.002, N.S.; F sucrose x group(1,11) = 1.04, N.S.) and the non-caloric sweet tastant saccharin (Fig. 5B; F group(1,11) = 2.5, N.S.; F saccharin x group(1,11) = 1.6, N.S.) as compared to control mice. The groups also showed similar preference for these sweet solutions (not shown). Mice with 14-3-3ζ knockdown also did not differ from control mice in aversion to bitter quinine solutions (Fig. 5C; F group(1,11) = 0.001, N.S.; F quinine x group(1,11) = 0.21, N.S.). Taken together, the increase in alcohol intake in mice with CeA 14-3-3ζ knockdown does not generalize to natural rewards and can not be explained by altered taste sensitivity.

Bilateral infection of the CeA was confirmed by post-mortem immunohistochemistry for GFP with the main infection site located between 20.9 and 21.5 mm from bregma (Figure 4E) [36]. Mice that showed unilateral infection were excluded from further analysis; one mouse was excluded after histology revealed hydrocephalus. There was occasional infection of the basolateral nucleus of the amygdala and in the caudate putamen along the injection tract.

**Discussion**

The development of alcoholism is a progressive process that invariably starts with casual, social drinking, which escalates to heavy drinking, problem drinking and ultimately alcohol addiction. Consistent with its role in processing negative emotional stimuli, the amygdala contributes to alcohol consumption in human alcoholics and alcohol-dependent animals that display enhanced negative affect [19–22]. However, the amygdala is also important for perception of positive emotions [17] and it may therefore also be involved in the reinforcing properties of alcohol [57] and in escalation of alcohol intake in non-dependent animals [30]. Here, we combined gene expression analysis, RNA interference and a murine model for escalation of voluntary alcohol intake to identify amygdala 14-3-3ζ as a novel key modulator in the development of high alcohol intake.

**Amygdala 14-3-3ζ and escalation of alcohol intake**

In the limited access choice paradigm, C57BL/6J mice show a rapid increase in alcohol intake in 7–10 days time. This rapid acquisition of alcohol consumption is likely driven by the positive subjective properties of alcohol. After two weeks of daily alcohol consumption, the mice reach their highest levels of alcohol intake and by that time, C57BL6/J mice also display inflexible alcohol intake in that they are insensitive to quinine adulteration of the alcohol solution when this is the sole source of alcohol [33]. In subsequent weeks, C57BL/6J mice maintain high levels of alcohol intake and develop indifferent alcohol drinking, indicated by persistent intake from aversive, quinine-adulterated alcohol despite the availability of non-adulterated alcohol [33]. Although the level of alcohol exposure achieved in our rodent model may not be sufficient to induce a genuine state of alcohol addiction, the inflexible and indifferent alcohol drinking patterns displayed by our mice show remarkable similarities to compulsive alcohol drinking in human alcoholics [5860]. The limited access choice paradigm therefore models important aspects of alcoholism-like behavior.
The current microarray analysis compared gene expression levels at three stages of the development of alcoholism-like behavior: initial escalation of alcohol intake (1 week), the stage where high alcohol intake is reached and signs of alcoholism-like behavior emerge (2 weeks) and the stage of stable high alcohol intake (4 weeks). Our analysis revealed that gene expression changes in the amygdala occur predominantly during the initial rapid escalation of alcohol intake (1 week). These data suggest that the amygdala contributes to the positive subjective properties of alcohol, which is consistent with the known involvement of the amygdala in the generation and perception of stimuli with positive emotional valence [16–18].

One of the genes that stood out from our analysis of alcohol-regulated amygdala genes is 14-3-3\(\alpha\). Up-regulation of this gene during initial escalation of alcohol intake was confirmed by qPCR analysis in an independent batch of mice. Moreover, using RNA interference we demonstrate functional involvement of amygdala 14-3-3\(\alpha\) in the development of high alcohol intake and alcoholism-like behavior. Local knockdown of 14-3-3\(\alpha\) in the amygdala lead to increased alcohol intake and a greater propensity to develop inflexible alcohol intake. Together with the observed up-regulation of 14-3-3\(\alpha\) in the amygdala, these findings suggest that 14-3-3\(\alpha\) may serve to restrict alcohol intake.

The observed increase in alcohol intake after 14-3-3\(\alpha\) knockdown in the amygdala was behaviorally specific for alcohol, as we did not observe alterations in preference for natural rewards, i.e. sucrose and saccharin or in sensitivity to the aversive taste of quinine. The enhanced alcohol intake after amygdala 14-3-3\(\alpha\) knockdown, together with the up-regulation of amygdala 14-3-3\(\alpha\) expression during the initial increase in alcohol intake suggests that the rewarding properties of alcohol are increased in the absence of 14-3-3\(\alpha\). This implies that 14-3-3\(\alpha\) may be a protective factor against the development of alcoholism; up-regulation of 14-3-3\(\alpha\) would be required to demonstrate this role. The augmentation of alcohol intake after amygdala 14-3-3\(\alpha\) knockdown may also reflect impaired behavioral control. In fact, the increase in inflexible alcohol intake that we found is indicative of loss of control over alcohol intake. However, it is also possible that mice with amygdala 14-3-3\(\alpha\) depletions consume more alcohol and as a consequence develop inflexible alcohol intake more rapidly than control mice.

### Table 3. Gene Enrichment analysis in STEM.

| Transport | Ion transport | Ligand-gated ion channels | Synaptic transmission | Cytoplasm | Protein transport |
|-----------|---------------|---------------------------|-----------------------|-----------|------------------|
| Acd5      | Apc           |                           |                       |           |                  |
| Atp2a2    | Atp2a2        |                           |                       |           |                  |
| Atpbap1   | Atpbap1       |                           |                       |           |                  |
| Camk2a    | Camk2a        |                           |                       |           |                  |
| Ctnnd2    | Gatm          |                           |                       |           |                  |
| Exoc2     |               |                           |                       |           |                  |
| Gabra1    | Gabra1        | Gabra1                    |                       |           |                  |
| Gabrb3    | Gabrb3        | Gabrb3                    |                       |           |                  |
| Gabrg2    | Gabrg2        | Gabrg2                    | Gabrg2                | Mgea5     |                  |
| Gopc      |               |                           |                       |           |                  |
| Kif3a     |               |                           |                       |           | Prkacb           |
| Kpnas     |               |                           |                       | Rab18     |                  |
| Lrp11     |               |                           |                       | Sel1L     |                  |
| Rab10     |               |                           |                       | Sfrs1     |                  |
| Slc38a1   | Slc38a1       |                           |                       | Sto7      | Sto7             |
| Synj1     |               |                           |                       | Tmed2     | Tmed2            |
| Trappc3   |               |                           |                       | Ugc9      |                  |
| Trim9     | Trim9         |                           |                       | 14-3-3\(\eta\) | 14-3-3\(\eta\) |
| Tsg101    |               |                           |                       | 14-3-3\(\theta\) | 14-3-3\(\theta\) |

Gene Ontology analysis for the significant time profiles 45 and 47 in STEM revealed significant enrichment of genes involved in transport, ion transport, ligand-gated ion channel activity, synaptic transmission, cytoplasm and protein transport. Highlighted are the selected 8 top candidate genes.

doi:10.1371/journal.pone.0037999.t003
The present findings demonstrate an important role of amygdala 14-3-3ζ in the escalation of alcohol intake: 14-3-3ζ levels in the amygdala are enhanced and 14-3-3ζ knockdown causes profound increases in alcohol intake in mice. Alcohol has previously been shown to alter 14-3-3ζ levels, although in contrast to our current findings only after extended alcohol exposure. For example, prolonged alcohol use in rats and in alcohol dependent mice induced increased 14-3-3ζ gene and protein expression in nucleus accumbens and amygdala [34,55]. Moreover, a recent study showed reduced 14-3-3ζ in the motor cortex of human alcoholics, i.e. after extended alcohol abuse [56]. The apparent contrast in the directional changes in expression of 14-3-3ζ may reflect brain region dependency of 14-3-3ζ regulation by alcohol: while 14-3-3ζ is consistently up-regulated by alcohol use in limbic brain regions, the same gene may be down-regulated in cortical regions. Although gene and protein expression changes may not necessarily cause behavioral changes, these studies suggest involvement of 14-3-3ζ in alcohol intake after prolonged alcohol use. In conclusion, 14-3-3ζ pathways in the amygdala constitute important mechanisms that are engaged during the descent of casual alcohol intake into alcoholism-like behavior.

Biological function of 14-3-3ζ in relation to alcohol consumption

14-3-3 proteins are adapter proteins, that have multiple and diverse binding partners [46,47]. Upon binding, they can regulate the activity or subcellular localization of other proteins and thereby influence multiple cellular processes including signal transduction or the cell cycle. 14-3-3 proteins are most widely studied for their involvement in cancer [47,61,62], but they have also been associated with neurological diseases such as Parkinson’s disease [63–65]. The binding partners of 14-3-3 proteins are diverse and include transcription factors and signaling molecules. The molecular mechanisms through which 14-3-3ζ influences alcohol consumption are currently unknown. However, several binding partners of 14-3-3 proteins have been implicated in alcoholism and may contribute to 14-3-3 modulation of alcohol intake.

For example, 14-3-3ζ is known to interact with protein kinase C isoforms (PKCs) including PKCε [48]. In fact, 14-3-3 was first identified as a PKC inhibitor [66], although later reports showed that 14-3-3 may enhance PKC activity [48,67]. PKCs, including PKCδ and PKCζ, are critically involved in alcohol sensitivity [52,53,68]. In fact, amygdala PKCζ is important for alcohol intake [30]. Interactions of 14-3-3ζ with PKCs may contribute to these effects.

14-3-3 proteins may also affect GABAergic neurotransmission. Interactions of GABA-B receptor subunits with 14-3-3 proteins were reported [69]. Further, 14-3-3 can affect the phosphorylation of GABA-A receptor subunits [70] and GABA, in turn, can regulate 14-3-3 proteins [71]. Multiple studies have shown an association of alcohol dependence to genes encoding GABA-A receptor subunits alpha1 [45,72–76] and alcohol is known to regulate GABA-A receptor subunits alpha1 [45,72–76] and is known to regulate GABA-A receptor subunits alpha1 [45,72–76] and is known to regulate GABA-A receptor subunits alpha1 [45,72–76] and is known to regulate GABA-A receptor subunits alpha1 [45,72–76] and is known to regulate GABA-A receptor subunits alpha1 [45,72–76] and is known to regulate GABA-A receptor subunits alpha1 [45,72–76]. In fact, 14-3-3ζ may contribute to these effects.

Interactions of 14-3-3 proteins with ionotropic glutamate receptors [49] are another candidate mechanism through which 14-3-3 proteins may affect alcoholism. AMPA receptors have been associated with alcoholism and alcohol intake in animal models [44,50,51]. 14-3-3ζ may modulate alcohol consumption by interacting with and affecting the activity of glutamate receptors.

Our microarray analysis revealed that, in addition to 14-3-3ζ, the GABA-A β3 subunit (Gabbr3) and the AMPA receptor 2ζ subunit (GRIA3) were also up-regulated in the amygdala during initial escalation of alcohol intake, suggesting that GABA-A and AMPA receptors in the amygdala are also involved in escalation of alcohol intake, possibly through interactions with 14-3-3ζ. Clearly, the elucidation of the molecular mechanisms and binding partners involved in 14-3-3ζ control over alcohol intake, both during initiation and maintenance of alcohol use, should be addressed in future research. In fact, the recent development of drugs that...
Figure 4. Effects of local knockdown of 14-3-3ζ in the CeA on alcohol consumption.  

A Local knockdown of 14-3-3ζ in the CeA using the 1854 shRNA increased intake of a 10% alcohol solution (v/v).  

B Infection with the less effective 1222 shRNA also increased alcohol intake of a 10% alcohol solution (v/v), but less prominently so than the 1854 14-3-3ζ shRNA.  

C In a separate batch of mice, local knockdown of 14-3-3ζ in the CeA using the 1854 shRNA increased alcohol intake of a 15% alcohol solution (v/v) and local knockdown of 14-3-3ζ in the CeA using the 1854 shRNA caused persistent preference for the alcohol solution despite adulteration with the bitter tastant quinine.  

E The sites of viral infection in the brain are summarized. The black ellipses show the core of the infection site that was consistently targeted across all animals. The areas marked in grey represent less frequent infected sites that include the anterior amygdala, the basolateral amygdala and part of the caudate putamen, along the injection tract.  

Control mice; 14-3-3ζ-specific shRNA treated mice. * P<0.05 from controls; # P<0.05, ## P<0.01 from 0 μM quinine for mice treated with control lentivirus; $ P<0.05 from 0 μM quinine for mice treated with the 1854 14-3-3ζ shRNA expressing lentivirus by t-test.  

doi:10.1371/journal.pone.0037999.g004
specifically interact with certain 14-3-3 complexes [82,83] underscores the need to identify the 14-3-3ζ-protein complex that governs the escalation of alcohol intake.

**Conclusion**

This study identifies amygdala 14-3-3ζ as a novel key modulator of alcohol intake. Interactions of 14-3-3ζ with signaling proteins such as PKCε [48] or neurotransmitter receptors [49] may contribute to these effects. The recent development of 14-3-3 complex specific drugs provides exciting opportunities to develop innovative treatment strategies for alcoholism.

**Acknowledgments**

We thank J.P.H. Burbach and M. Joels for critical reading of the manuscript.

**Author Contributions**

Conceived and designed the experiments: HMBL FCPH LJMJV. Performed the experiments: HMBL JMH MG-K. Analyzed the data: HMBL JMH MG-K. Contributed reagents/materials/analysis tools: HMBL JMH MG-K. Wrote the paper: HMBL FCPH LJMJV.
29. Roberto M, Cruz MT, Gilpin NW, Sabino V, Schweitzer P, et al. (2010) Corticosterone releasing factor-induced amygdala gamma-amino-butyric acid release plays a key role in alcohol dependence. Biol Psychiatry 67: 831-839.

30. Lesscher HMB, Wallace MJ, Zeng L, Wang Y, Deitchman JK, et al. (2009) Amygdala yamagata C epsilon controls alcohol consumption. Genes Brain Behav 8: 495-729.

31. Ahmed SH (2011) The science of making drug-addicted animals. Neuroscience DOI: 10.1016/j.neuroscience.2011.08.014.

32. Lesscher HMB, Kieft WM, van ES, van Lith BA, Vanderschuren LJM (2009) A grandparent-influenced locus for alcohol preference on mouse chromosome 2. Pharmacogenet Genomics 19: 719-729.

33. Lesscher HMB, van Kerkhof LWM, Vanderschuren LJM (2010) Inhibitory and indolent alcohol drinking in male mice. Alcohol Clin Exp Res 34: 1219-1225.

34. Ford MM, Yoneyama N, Strong MN, Ferrett A, Tanchuck M, et al. (2008) Inhibition of saliva-reduced alpha-synuclein biosynthesis impedes acquisition of ethanol drinking in male C57BL/6J mice. Alcohol Clin Exp Res 32: 1408-1416.

35. Rhodes JS, Best K, Belnap JK, Finn DA, Crabbe JC (2005) Evaluation of a simple model of ethanol drinking to intoxication in C57BL/6J mice. Physiol Behav 84: 53-63.

36. Uhl A, Berg D, Hofmann C, Kruger B, Berger K, et al. (2002) 14-3-3 protein. Eur J Biochem 191: 421-429.

37. Hodge CW, Mehmert KK, Kelley SP, McMahon T, Haywood A, et al. (1999) Haplotype-analysis of genes regulated in the mouse extended amygdala by excessive ethanol administration in PKCepsilon-deficient mice. Eur J Neurosci 12: 4131-4140.

38. Haugbol SR, Ebert B, Ulrichsen J (2005) Upregulation of glutamate receptor beta3 subunit genes and haplotypic association of GABA(A) receptor phosphorylation in cerebellar pumice cells. Neuroscience 2011: 34-45.

39. van Heusden GP (2009) 14-3-3 proteins: Insights from genome-wide studies in mouse. Neurosci Biobehav Rev 33: 130-148.

40. Ernst J, Bar-Joseph Z (2006) STEM: A tool for the analysis of short time series gene expression data. BMC Bioinformatics 7: 191.

41. Schmittgen TD, Livak KJ (2008) Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc 3: 1158-1166.

42. Kabuki KM, Surmiak J, Robinson WA, Carter JS, Kastin AJ, et al. (1992) Protein kinase C inhibitor modulates alcohol intake in C57BL/6J mice. Alcohol Clin Exp Res 16: 615-619.

43. Ford MM, Yoneyama N, Strong MN, Ferrett A, Tanchuck M, et al. (2008) Inhibition of saliva-reduced alpha-synuclein biosynthesis impedes acquisition of ethanol drinking in male C57BL/6J mice. Alcohol Clin Exp Res 32: 1408-1416.

44. Solomon PR, Deschepper E, Li N, Tamminga CA, Aronson SB, et al. (2004) Differential neuroprotective effects of 14-3-3 proteins in models of Parkinson’s disease. Cell Death Dis 1:e2.

45. von Heusden GP (2009) 14-3-3 isoforms in human alcoholic brain. Alcohol Clin Exp Res 35: 1041-1049.

46. van Heusden GP (2009) 14-3-3 isoforms in human alcoholic brain. Alcohol Clin Exp Res 35: 1041-1049.

47. Kerns RT, Ravindranathan A, Hassan S, Cage MP, York T, et al. (2005) A grandparent-influenced locus for alcohol preference on mouse chromosome 2. Pharmacogenet Genomics 19: 719-729.

48. Lesscher HMB, van Kerkhof LWM, Vanderschuren LJM (2010) Inhibitory and indolent alcohol drinking in male mice. Alcohol Clin Exp Res 34: 1219-1225.

49. Ford MM, Yoneyama N, Strong MN, Ferrett A, Tanchuck M, et al. (2008) Inhibition of saliva-reduced alpha-synuclein biosynthesis impedes acquisition of ethanol drinking in male C57BL/6J mice. Alcohol Clin Exp Res 32: 1408-1416.

50. Rhodes JS, Best K, Belnap JK, Finn DA, Crabbe JC (2005) Evaluation of a simple model of ethanol drinking to intoxication in C57BL/6J mice. Physiol Behav 84: 53-63.