Supplementary Information for

Single-dose ethanol intoxication causes acute and lasting neuronal changes in the brain

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Protein candidates identified by mass spectrometry

Overall, 72 significantly changing proteins were detected of which 32 proteins were affected in both young and old mice (Table S1). These changes in abundance may be a consequence of distinct cellular mechanisms including changes in protein turnover dynamics, newly synthesized proteins or protein trafficking in and out of the synapse. Note that we did not intend to characterize the underlying causes for the abundance change but rather aimed to identify molecular candidates that mediate some of the complex alcohol-related effects in the brain.

Of the 72 proteins, 39 changed significantly in 2 independent experiments (in young or old animals; p<0.0025), 24 proteins changed significantly in 3 independent experiments (p<0.000125), and 9 proteins changed significantly in all 4 independent experiments (p<6.25*10^-6). This high reproducibility of the ex vivo approach therefore suggests that it is suitable for identification of proteins that change their synaptic abundance upon ethanol exposure.

Unlike chronic ethanol consumption, acute ethanol exposure predominantly manifests itself in complex behavioral responses that depend on alterations of neuronal transmission. We detected an increase in Clathrin light chains A and B, both are key components of the recycling synaptic vesicle, and an increase in the kinase SAD1 which was found to regulate synaptic transmitter release (1). In addition, ethanol led to an increase of two vacuolar ATP synthase subunits involved in acidification of synaptic vesicles which in turn aids loading the vesicles with neurotransmitter via the proton gradient. Ethanol also induced an increase of the Freud-1 repressor at the synapse, which was found to inversely regulate dopamine D2 receptor (2) and serotonin 5-HT1A receptor levels (3). The dopamine D2 receptor has been linked to genetic predisposition for alcoholism while the serotonin receptor is being implicated in mood regulation and psychiatric disorders. Several proteins linked to antidepressant treatment were also affected including cAMP-specific 3',5'-cyclic phosphodiesterase 4D (PDE4D), a key target of the antidepressant Rolipram (4). In addition, we discovered an ethanol-dependent elevation in monoamine oxidase A, deficiencies of which are associated with heightened aggressiveness (5).
pronounced change (2.19) was seen with MAP6 which elicits various cognitive impairments in null mutant mice (6).

Acute consumption of ethanol has long been known to diminish memory formation both in humans as well as animal models and pharmacologically relevant concentrations (50-100 mM) of ethanol inhibit long-term potentiation (LTP) (7). The underlying molecular and cellular mechanisms of this impairment are not well understood and only a few proteins implicated in the inhibition of LTP have been described (8). We identified six additional synaptic proteins that could potentially play a role in the acute ethanol-mediated impairment of LTP. These include Tenascin-R (9), GAP-43 (10), MAP6 (6), SNAP-25 (11), Phosphacan (12), and Ankyrin-G (13). In addition, a reproducible, but not statistically significant decrease of a key plasticity protein, the NMDA receptor 2B (0.91) was also detected at P30.

Ethanol is also known to elevate GABA-mediated synaptic inhibition but the molecular basis for this is not fully understood (14). We found a decrease of GABA transaminase, which is necessary for GABA catabolism, and a decrease of GABA transporter 4 levels (Fig. S2B), a protein involved in recycling released GABA transmitter from the synapse. The combination of both could at least partly explain the observed inhibitory action of ethanol.

We detected an increase of C-terminal binding protein 1, which positively affects expression of the multi-drug resistance protein (15), a finding that potentially has important medical implications. Similarly, expression of beta tubulin III, a pan-neuronal marker for differentiated neurons, was increased in all four experiments while overexpression of beta tubulin III in tumors has been associated with low chemotherapy response rates when using tubulin-binding agents (16). Also to our surprise, the moderate ethanol exposure was sufficient to affect synaptic abundance of proteins connected to apoptosis (Apoptosis inducing factor (AIF) (17), Adenylyl cyclase-associated protein 1 (CAP1) (18), asparagine synthetase (19), thioredoxin reductase 2 (20)). Ethanol has often been linked to apoptosis in the developing brain, especially in case of the fetal alcohol syndrome (FAS), but little is known about the apoptotic effects of acute exposure in the adult brain and even less is known about the underlying mechanisms. Our data highlight potential molecular pathways that could lead to programmed cell death in mature, differentiated neurons.
We detected a total of 19 ATP synthase proteins in the hippocampal synaptic proteome and 13 of these were significantly changing upon ethanol treatment. For the mitochondrial ATP synthase, we found an almost stoichiometric increase ($\bar{x}=1.46$) with a remarkably low variance (STD=0.05) for all 8 identified subunits suggesting that this ATP synthase complex is affected in its entirety rather than each protein changing independently. As expected, the change in abundance was inverse for certain proteins after acute versus chronic ethanol exposure (e.g. malate dehydrogenase).

Overall, there is a tremendous disconnection between our detailed understanding about the various forms of neuronal plasticity and our lack of knowledge about the changes in molecular compositions that mediate it. On a molecular level, we show here that high accuracy detection of minute, stimulus-dependent changes of synaptic protein abundance is now possible, including protein changes that potentially relate to synaptic plasticity. Prior to this study, the protein correlate of synaptic plasticity may have been imagined anywhere from a handful of ‘Master’ proteins to a large fraction of proteins. Our data provide a first benchmark for the number of stimulus-dependent protein abundance changes with 3.5% of the total synaptic proteome.
Supplementary Methods

Ethical approval and age of mice for in vivo experiments
Animal experiments were carried out in accordance with local guidelines for the care and use of laboratory animals. Priority was given to avoid or minimize animal suffering, while promoting animal welfare and reducing the number of animals using the 3R principle, for example through longitudinal experiments. Mice were housed singly in an individually-ventilated cage system (ZOONLAB) at a 12 h dark/light cycle. The animal room was tempered to 21-23 °C with a relative humidity of 55 %. Mice had access to water and chow ad libitum, except for experimental sessions. Behavioral tests were performed in the dark cycle. Mice used for behavioral tests were handled daily before starting the experiments and were habituated to the behavioral testing environments. For all in vivo ethanol experiments, we used adult mice about 2-6 months old. For two-photon imaging, we waited 5-8 weeks after surgery and virus injection to ensure high rAAV expression and to ease the inflammatory glial reaction (21). Therefore, the in vivo ethanol experiments were limited to adult animals.

Rationale for Ethanol treatments:
As the in vivo imaging experiments were complex and the availability of SILAC mice limited, we were not afforded the opportunity to conduct dose-response curves for different ethanol concentrations. Instead, we focused on one ethanol treatment that was well within the range of previously published ethanol concentrations while also providing sufficient stimulation to allow for detection of acute and lasting ethanol-dependent changes. Acute ex vivo hippocampal slices were treated with 50 mM ethanol, a standard concentration at which LTP is substantially blocked (22). The rationale was to be able to capture further plasticity related events with this concentration. With mice in vivo, we injected 3.5 g/kg ethanol, a standard concentration at which the loss-of-righting reflex is lost (23, 24) and thus we expected to see ethanol-dependent changes in cells in vivo. To compensate for the rapid ethanol metabolism of mice, we furthermore injected pyrazole (1 mmole/per kg mouse) for in vivo experiments. Since in vivo two-photon spine imaging did not show any ethanol-dependent changes in spine density, we additionally kept mice in an ethanol vapor
chamber (25) after the i.p. injection of ethanol. With such a high, constant ethanol exposure, we could ultimately rule out an effect of ethanol on spine density in vivo. Pilot experiments for the in vivo imaging of mitochondria and DCVs showed that trafficking was affected at lower ethanol concentrations and therefore we used 2.5 g/kg and no pyrazole. For Drosophila experiments, 70 % ethanol in air was used, a standard ethanol concentration at which flies show signs of intoxication, specifically, ethanol-induced sedation after about 10 min of exposure (26).

| Experiment                             | Brain Samples         | Sex   | Age           | Ethanol                      |
|----------------------------------------|-----------------------|-------|---------------|------------------------------|
| Quantitative synaptic proteomics       | Hippocampal slices    | female| P30, P210     | 50 mM                        |
| Synaptic protein dynamics in vivo      | Living mice           | male  | P60 - P90     | 3.5 g/kg (i.p.)              |
| In vivo spine analysis                 | Living mice           | male  | P60-P120      | 3.5 g/kg (i.p.) + vapor chamber |
| Imaging of spine dynamics              | Living mice           | male  | P60-P90       | 3.5 g/kg (i.p.)              |
| In vivo AIS analysis                   | Living mice           | male  | P60-P120      | 3.5 g/kg (i.p.)              |
| Imaging of mitochondria + boutons      | Living mice           | male  | P60-P120      | 2.5 g/kg (i.p.)              |
| Imaging of DCVs                        | Living mice           | male  | P60-P120      | 2.5 g/kg (i.p.)              |
| Drosophila experiments                 | Flies                 | male  | P3-5          | 70 % Eth. in air             |

SILAC mice
The generation of fully labeled SILAC mice was described previously (27). Briefly, a SILAC-diet was prepared by adding $^{13}$C$_6$-lysine to a customized lysine-free mouse diet (Harlan) to a final concentration of 1% according to standard mouse nutritional requirements. After feeding two generations of female C57BL/6N mice with the ‘heavy’ diet, complete in vivo labeling (>97 %) was achieved in the F2 offspring which contained virtually no unlabeled peptides (27). Only females were maintained from each litter and fed with the ‘heavy’ diet so that the offspring also fully incorporated the $^{13}$C$_6$-lysine. One animal was available for the P30 experiments, one for P210. The experiments were performed with animals from the F2 or F3 generation and labeled animals showed normal breeding behavior and motor activity.
In-gel digest and peptide extraction

In-gel digest was done according to standard procedures (27). Synaptic proteins were separated on a 4-12 % NuPage Novex Bis-Tris gel (Invitrogen), the gel stained using the Colloidal Blue Staining Kit (Invitrogen) and cut into 10 slices. The in-gel digest was performed with an overnight digestion at 37° C using 12.5 nmol endopeptidase LysC. Each sample was loaded on C18 StageTips, washed, and eluted.

LC-MS/MS

All LC-MS/MS experiments were performed by standard procedures (27). Briefly, peptides were separated using an Agilent 1100 or 1200 nanoflow LC-System. The HPLC system was coupled to an LTQ-Orbitrap mass spectrometer (ThermoFisher Scientific). Survey full scan MS spectra (m/z 300-2000) were acquired in the orbitrap analyzer. The five most intense ions from the survey scan were sequenced by collision induced dissociation in the LTQ. Data were acquired using Xcalibur software.

Bioinformatic analysis

Mass spectra were analyzed using the software MaxQuant (version 1.0.12.5) (28). The data were searched using Mascot (version 2.2.04, Matrix Science) against the Mouse International Protein Index protein sequence database (IPI, version 3.37) supplemented with frequently observed contaminants and concatenated with reversed copies of all sequences (2 x 51,467 entries). Initial maximum allowed mass deviation was set to 5 ppm for monoisotopic precursor ions and 0.5 Da for MS/MS peaks. The required false positive rate was set to 1% at the peptide level, the required false discovery rate was set to 1% at the protein level and the minimum required peptide length to 6 amino acids. In addition to the protein false discovery rate threshold, for protein identification we required at least two unique sequenced peptides, while for protein quantitation, at least two independently sequenced and quantified SILAC pairs, one ‘forward’ and one ‘reverse’ in the respective cross-over experiment were necessary. We allowed three missed cleavages and the enzyme specificity was set to Lys-C. We included carbamidomethylcysteine as a fixed modification, and N-acetylation and methionine oxidation as variable modifications. Contaminants such as keratins were discarded. The analysis of protein significance is
described in detail elsewhere (28). Briefly, the significance of protein ratios was computed taking into account the summed peptide intensity (‘Significance B’ of the MaxQuant software). To assess if the ethanol-dependent change of a given protein was significant, the complementary error function was used to produce a probability measure (‘p’) of the fold change. Statistically, only proteins that displayed a significant change in both, forward and reverse ratios (P < 0.05; P × P < 0.0025) were included. Furthermore, with a minor extension of our statistical requirements (P < 0.075; P × P < 0.0025) 17 additional proteins that merit consideration based on their biological roles also became statistically significant, including GAP-43 and Fyn kinase, both reported to be affected by ethanol (29, 30). Those were included in the list of 72 proteins that changed significantly upon ethanol exposure.

**Hippocampal slice preparations and synaptic protein extraction**

Acute hippocampal slices were prepared according to standard procedures (31). Briefly, we fed only female mice with ‘SILAC labeling chow’ to maintain labeling across generations (27). Animals (SILAC or wild-type C57/BL6 mice; P30 or P210) were anesthetized, rapidly perfused with ice-cold saline, and the brains quickly transferred to ice-cold carbogenated (95% O₂, 5% CO₂) artificial cerebral spinal fluid (ACSF in mM: 124 NaCl, 5 KCl, 1.25 Na₂PO₄, 26 NaCO₃, 2.5 CaCl₂, 2 MgSO₄, 10 Glucose). Hippocampal slices (400 µm) were allowed to recover in ACSF at room temperature for 1 h and were then transferred to ACSF with or without 50 mM ethanol (29-31° C; 4 h). Slices were combined in a cross-over fashion and homogenized in 0.32 M sucrose, 5 mM HEPES pH7.4, protease inhibitors (Roche tablets, EDTA free). The homogenate was centrifuged (10 min/1000 g) and the supernatant re-centrifuged (20 min/ 12000 g). The pellet was resuspended in homogenization buffer, layered onto a sucrose gradient (0.85 M, 1.0 M, 1.25 M; all solutions contained 5 mM HEPES pH7.4, protease inhibitor), and centrifuged (2 h/ 82500 g). Synaptosomes were collected at the 1.0/1.25 M interface and adjusted to a final concentration of 20 mM Tris pH6 and 1 % Triton-X (32). Synaptosomes were extracted for 30 min and subsequently centrifuged (15 min/ 15000 g). Pellets were re-extracted in 20 mM Tris pH6/ 1 % Triton-X and re-centrifuged (15 min/ 15000 g). The resulting pellet was dissolved in Laemmli buffer and directly loaded on a 1D SDS gel.
Immunofluorescence for detection of synaptic protein dynamics \textit{in vivo}

300 nm cryosections were washed in PBS 3 x 10 minutes. The cryosections were blocked in 0.5 % inactivated fetal calf serum (FCS). The primary antibody was added at the desired concentration in 0.5 % inactivated FCS. Primary antibodies were incubated for 45 minutes at 4 °C followed by three washing steps in PBS for 10 minutes. The secondary antibody was added at the desired concentration in 0.5 % inactivated FCS for 1 hour at 4 °C. CS were washed in PBS 3 x 10 minutes. Subsequently, CS were mounted with Polymount. For the immunofluorescence co-localization analyses, cryosections were stained for β–Actin (Alexa488), Synapsin (Alexa647), and either MAP6, Ankyrin-G, or PCCA (Alexa594). The resulting single 8–bit Tiff images were thresholded individually. Both, the Synapsin and the Actin signals were used to identify putative synaptic structures within the image, but the Synapsin signal was ultimately used for normalization of MAP6, Ankyrin-G, or PCCA signals. To quantify the overlapping regions between the Synapsin and the MAP6/PCCA/Ankyrin-G staining, the Synapsin channel was used as a ‘template-mask’ for the MAP6/PCCA/Ankyrin-G staining, i.e. candidate staining was only quantified in regions with a Synapsin signal. The resulting image now contained only binary ROIs of overlapping Synapsin and MAP6/PCCA Ankyrin-G regions. Those ROIs were plotted on the unprocessed images and the fluorescence values in these regions was measured with ImageJ. Expression ratios were then calculated from the intensity of the protein of interest divided by the Synapsin intensity in all overlapping ROIs found in each image. Synapsin (1:100, Synaptic Systems, Company ID: 106 004), β-Actin (1:200, Santa Cruz, Company ID: sc-28561), MAP6 (1:200, Santa Cruz, Company ID: sc-53513), PCCA (1:200, Santa Cruz, Company ID: sc-374341) and Ankyrin G (1:200, Santa Cruz, Company ID: sc-28561).

Spine analysis following ethanol exposure \textit{in vivo}

2-3 month old male Thy1-GFP mice (33) were co-injected with pyrazole (Sigma) in saline at 1 mmoles/kg mouse (23) and ethanol at 3.5 g/kg (from a 20% solution) or a corresponding volume of saline for control mice. Ethanol treated mice were additionally kept in an ethanol vapor chamber (La Jolla Alcohol Research, California, USA) (34). A
peristaltic pump delivered 98% alcohol at a flow rate of 0.44 ml/min to a heated round-bottom flask so that the alcohol quickly vaporized. Pressure controlled airflow to the flask carried the alcohol vapor to the individual chambers which were connected to a vacuum for constant air exchange. The airflow was adjusted to about 6 l/min to maintain the ethanol concentration of 10-15 mg/l air in the chamber. After 4-6 hours, mice were perfused with 4% PFA and the brains sliced. Dendritic spines on cortical dendritic stretches of GFP-positive neurons were imaged with a confocal microscope and manually quantified by a blinded observer.

**Immunofluorescence for AIS length analysis**

2-3 month old wild-type C57/BL6 male mice were transcardially exsanguinated under deep anesthesia with Ketamine (120 mg/kg BW) / Xylazine (16 mg/kg BW) with 0.9% saline followed by ice-cold 2% paraformaldehyde (Roth; in 0.1 M PBS, pH 7.4) and brains removed and cryoprotected in 30% sucrose in PBS at 4°C overnight. Samples were then trimmed to a block containing S1, embedded in Tissue Tek® (Sakura Finetek) and frozen in liquid nitrogen-cooled isopentane (Roth). Cryosections (30 µm) were cut using a cryotome (Microm HM 550, Thermo Fischer) and immediately processed for free-floating immunofluorescence staining. After a brief rinse in PBS, slices were blocked in fish skin gelatin blocking buffer (1% BSA, 0.2% fish skin gelatine, 0.1% Triton in 0.1 M PBS) for at least 90 min and subsequently incubated in primary antibody diluted in blocking buffer (4°C overnight) using antibodies against the AIS scaffolding protein βIV-spectrin (rabbit polyclonal antibody against amino acids 2237-2256 of human βIV-spectrin; 1:500) (35) and the neuronal nuclei marker NeuN (mouse monoclonal, Millipore; 1:500). Omission of the primary antibodies did not yield specific immunolabeling. After primary antibody incubation, slices were rinsed 4 x 10 min in PBS at room temperature and then incubated in secondary antibodies (goat anti mouse Alexa Fluor 568, 1:1000 and goat anti rabbit Alexa Fluor 488, 1:1000; Molecular Probes, Thermo Fisher) for 120 min in the dark. Slices were then rinsed 4 x 15 min in PBS using the nuclear counterstain TO-PRO-3 Iodide (1:2000, Thermo Fisher). For preservation of immunofluorescence, slices were mounted in a mounting medium with anti-fading effect (Roti-Mount FluorCare, Carl Roth) and stored at 4°C.
Image acquisition and analysis of AIS length

Confocal analysis was carried out on a C1 Nikon confocal microscope with a 60 x objective (oil immersion, numerical aperture of 1.4; Nikon Instruments). To increase the number of in-focus immunoreactive AIS, maximum intensity projections were saved and processed. Thickness of single optical sections was 0.5 µm in stacks of 10 - 20 µm total depth. Confocal x-y-resolution was kept at 0.21 µm per pixel. Images for qualitative analysis were evaluated and enhanced for contrast in Photoshop (Adobe Systems, USA). AIS length was measured using the software AISuite (36, 37), which is available online (github.com/jhnnsrs/aisuite2). This tool extends the well-established and widely used method of defining AIS start and end points as points where a predefined fluorescence threshold (relative to the maximum fluorescence intensity along a line drawn over an individual AIS) is surpassed (38). Using AISuite, the threshold was adjusted depending on the individual staining quality in each section and ranged from 10 - 30% of maximum fluorescence intensity.

Immunofluorescence of mitochondria and dense core vesicles

At different time points after the mice were imaged with two-photon microscopy, animals were transcardially perfused with 4 % PFA fixative and subsequently the brains were carefully removed from the skull. For immunofluorescence stainings, 80 µm coronal sections of the S1/M1 cortex were cut with a microtome (Leica VT100S, Leica). Brain sections were first blocked in PBS containing 5 % natural goat serum (NGS) and 1 % Tritum-X100 for 2 h, before incubating the slices in PBS containing 1 % NGS, 0.2 % Triton-X100 and the primary antibody over night at 4 °C. Slices were washed in PBS containing 2 % NGS. Secondary antibodies were incubated for 2-3 h at room temperature. Primary antibodies: α-Translocase of the outer mitochondrial membrane (TOM20, 1:500; Santa Cruz), α-Synaptophysin 1 (SyPhy1, 1:500; Synaptic Systems), α-Chromogranin A (Chr-A, 1:500; Synaptic Systems). Alexa Fluor antibodies (1:500; Invitrogen) were used as secondary antibodies. Slices were mounted with Mowiol (SigmaAldrich) and imaged on a Leica DM6000 wide-field epifluorescence light microscope with 10x/0.4 (magnification/NA) or 63x/1.30
glycerol-immersion objectives or on a Leica SP8 scanning confocal microscope using a 63x/1.40 oil-immersion objective.

In vivo two-photon imaging of synaptic turnover, mitochondria and DCVs in mice

Genetic labelling of mitochondria, DCVs and presynaptic boutons

Recombinant adeno-associated viruses (rAAVs) of the chimeric 1/2 serotype were used in order to label axonal organelles and presynaptic terminals in neurons in vivo. Mitochondria were labelled with GFP fused to the first 35 amino acids of the mitochondria-targeting sequence of cytochrome c oxidase subunit VIII leading to enrichment of GFP in the mitochondrial matrix (Plasmid was kindly provided by Dr. Ken Nakamura, (39)). Mitochondria-targeted GFP (mitoGFP) was expressed under the control of the EF-1 alpha promoter. As the construct contained a Cre-inducible double-floxed inverse open reading frame (DIO), Cre-recombinase was necessary for expression of the insert (EF-1α-DIO-mitoGFP). rAAVs encoding for Cre-recombinase under the control of the synapsin promoter (Syn-Cre) were always co-injected leading to a restricted expression of mitoGFP in neurons. For the labelling of dense-core vesicles (DCV), the DCV cargo protein neuropeptide-Y (NPY) fused to the fluorescent protein Venus was used under the control of the Synapsin promoter. The plasmid for Syn-NPY-Venus was kindly provided by Dr. Matthijs Verhage. DCV labelling was combined with axonal labelling by expressing mCherry under the control of the CAG promoter (Syn-mCherry). Labelling of presynaptic boutons was achieved by expressing synaptophysin (SyPhy) fused to mCherry under the control of the CAG promoter (CAG-SyPhy-mCherry).

In vivo two photon microscopy of anesthetized mice and ethanol injection

Two photon imaging (40) was conducted as described in (41) using an upright TriM Scope II microscope (LaVision BioTec GmbH) equipped with a pulsed Ti:Sapphire laser (Chameleon Ultra II; Coherent). A wavelength of 960 nm was used to excite Thy1-GFP OR mitoGFP OR simultaneously NPY-venus and cytosolic mCherry. Imaging of mitoGFP with 960 nm and SyPhy-mCherry with 1050 nm was serially done. The emitted signals were separated using a 560 nm dichroic mirror (Chroma, Bellows Falls) and appropriate filter sets (GFP, venus: 535/70 nm emission filter (Chroma, Bellows Falls); mCherry:
645/75 nm emission filter (Semrock, Rochester)). Imaging was done using a 25x water immersion objective (1.1 NA, Nikon) and the emitted fluorescence was collected with photo multiplier tubes (PMTs, H7422-40-LV 5M; Hamamatsu). Mice were anesthetized in an acrylic box with 5% isoflurane (Henry Schein) in medical O₂ (Air Liquid Medical GmbH) using a vaporizer (Vapor 2000, Dräger). The animal was head-fixed into a custom-built holder screwed onto the intravital microscope stage. Vessels were used for orientation and relocating to the same imaging site during every imaging session. During the imaging session, isoflurane was maintained at 0.8 – 1.5%. Breathing rate was under constant monitoring using an infra-red camera (ELP 1080P 2.0 Megapixel USB camera, Ailipu Technology Co.) and an infra-red LED light source (48 LED Illuminator, Sonline). Body temperature of the animal was kept at 37 °C by a heating pad (ExoTerra, HAGEN Deutschland GmbH).

For imaging spines in Thy1-GFP mice, imaging sites were chosen in such a way that enough dendritic stretches in S1/M1 layer II/III were visible and feasible for post hoc spine counting. Since Thy1-GFP mice show very dense labeling in this layer we aimed for sparsely labeled regions. 3D-stacks covering 436 x 436 x 50 µm, at 2731 x 2730 pixel resolution (0.16 µm/pixel resolution) and 1 µm z-steps were acquired starting from the pial surface. ImSpector (LaVision BioTec GmbH) was used for microscope control and image acquisition. Mice were imaged on three consecutive days (day 1 = pre-ethanol, day 2 = ethanol, day 3 = post-ethanol). Each day included 4 time points: 0, 2, 4 and 8 h. Mice were kept in their cages between the imaging sessions to minimize stress. On day 2, mice received an i.p. injection of ethanol (3.5 g/kg) at time point 0 h, and a second ethanol injection at 2 h. On the pre-ethanol and post-ethanol days mice received a saline injection. For imaging mitochondria, DCVs or pre-synaptic boutons, layer 1 of motor cortex, 50-100 µm away from the pial surface was imaged. When imaging mitochondria, frames were taken from an area covering 436 x 436 µm, at 1024 x 1024 pixel resolution (0.426 µm/pixel resolution) with a frame rate of 0.61 Hz using a galvanometer scanner. During acquisition of mitochondrial transport, the focal plane was rapidly alternated using a piezo-motor (LaVision BioTec GmbH) allowing the acquisition of three focal planes at each time point resulting in an effective frame rate of 0.2 Hz per image plane. When imaging DCVs, frames were taken from an area covering 200 x 200 µm, at 1071 x 1071
pixel resolution (0.19 μm/pixel resolution) with a frame rate of 0.94 Hz using the
galvanometer scanner. Each acquired time-lapse of mitochondrial transport was of 10 min
duration and 5 min of DCV transport. Baseline time-lapses were recorded at three time
points before i.p. injection of 500 µl of saline or 2.5 g/kg ethanol. Time-lapses up to 4 h
post-injection were acquired. Three additional time-lapses were acquired 24 h post-ethanol
injection. 3D-stacks covering 436 x 436 x 50 μm, at 2731 x 2730 pixel resolution (0.16
μm/pixel resolution) and 1 μm z-steps were acquired pre, 4 h and 24 h post saline and
ethanol injection in order to dissolve structural changes of mitochondria and presynaptic
terminals.

Craniectomy, viral injection, and chronic cranial window implantation

Protocol was adapted from (21) as well as (42). Mice were deeply anaesthetized by
intraperitoneally (i.p.) injecting a mixture of 0.48 µl fentanyl (1 mg/ml; Janssen), 1.91 µl
midazolam (5 mg/ml; Hameln) and 0.74 µl medetomidin (1 mg/ml; Pfizer) per gram body
weight each and mounted in a stereotaxic apparatus (EM70G, David Kopf Instruments).
Mice were kept on 35°C by a feedback-controlled heating pad throughout the surgery. The
eyes were covered with eye ointment (Bepanthen with 5% Dexpanthetol, Bayer Vital
GmbH) to prevent drying-out. Standard aseptic procedures were followed during all
surgeries. The local anesthetic Xylocain (1%, AstraZeneca) was injected subcutaneously
(s.c.) at the site of surgery and the cranium was exposed by removing a flap of the scalp,
approximately 1 cm², as well as the subjacent gelatinous periosteum. Levelling of the
mouse skull to a coordinate system centered at bregma was performed by eye using a
monocle and a digital indication (both David Kopf Instruments). To target the
somatosensory cortex of the right hemisphere in Thy1-GFP mice (33) following
coordinates where used relative to the bregma (in mm): -2.5 AP, 2 ML. Since Thy1-GFP
mice endogenously express GFP in neurons, those mice were only implanted with cranial
windows, without viral injections. In contrast, in wt mice a small hole was drilled into the
skull with a dental drill (drill: EXL-40, Osada Inc.; boring head: 1104005, Komet Dental)
and the right-hemisphere medio-dorsal nucleus of the thalamus (MD thalamus) was
targeted using the coordinates 0.82 AP, 1.13 ML and 3.28 DV (Paxinos and Franklin 2013)
mm relative to bregma. Approximately 800 nl of a mixture of rAAVs (1:1:1 mitoGFP, Cre,
SyPhy-mCherry OR 1:1 NPY-venus, mCherry) was slowly injected. For cranial window
implantation, a circular part of the skull with a diameter of 6.5 -7 mm (centered above the injection site in wt mice) was removed by drilling a deep groove into the outline of the circle. The dura mater was carefully removed with very fine forceps (straight Dumont #5, Fine Science Tools Inc.). A glass coverslip (6 mm diameter, #0) was placed inside the opening and was, together with a 3D-printed round plastic holder, cemented to the scull will dental acrylic cement (glue: Cyano, Hager Werken; powder: Paladur, Heraeus). Mice received i.p. a mixture of 1.86 µl naloxon (0.4 mg/ml; Inresa), 0.31 µl flumazenil (0.1 mg/ml; Fresenius Kabi), 0.31 µl antipamezole (5 mg/ml; Pfizer) in 3.72 µl saline (0.9 %; Braun) each per gram body weight to antagonize the anesthesia. For pain treatment, mice received an s.c. injection of 150 µl Carprofen (50 mg/ml; Bayer Vital GmbH) diluted in saline every 12 h for the next two days.

Post hoc analysis of synaptic turnover
3D-stacks of all time points were registered by manual selection of corresponding points and calculation of the resulting transformation in Matlab using a customized script. For analyzing spine turnover, 5 dendritic stretches of approx. 50 µm length were chosen randomly for analysis in each mouse. For analyzing the structural plasticity of mitochondria and presynaptic terminals axons clearly visible in all time points (pre, 4 h and 24 h post) were chosen. Image processing was done in the open-source image analysis software Fiji. Dendrites and axons were analyzed in 3D and searched for spines/boutons manually. 3D stacks covering 436 x 436 x 500 µm ROIs where cropped in x, y spanning the dendritic stretches of interest. In z, the image was cropped to 25 µm above and below the center of the dendrite. Spine labeling, counting and calculation of the spine turnover ratio (TOR) was performed according to previously published procedures (43): Spines were considered lost when they retracted into the dendrite (length < 5 pixels) or considered gained when they formed clear protrusions from the dendrite (length > 5 pixels). TOR was calculated for each day individually as \((N_{\text{gained}} + N_{\text{lost}})/(2 \times N_{\text{total}})\) where \(N_{\text{gained}}\) is number of spines newly formed between two consecutive time points (eg. Time point 0 h and 2 h, 2 h and 4 h, 4 h and 8 h), whereas \(N_{\text{lost}}\) is the number of spines lost between the consecutive time points. \(N_{\text{total}}\) represents the absolute number of spines counted in one time point. This results in 3 TOR values for each day, corresponding to the spine turnover from 0 to 2 h, 2
to 4 h and 4 to 8 h, respectively. Mean TOR for each dendritic stretch was calculated per day taking the mean out of the 3 TOR values for each of the 3 imaging days. All 3 days where compared statistically with each other. Spine density was calculated as spines/µm. The mean spine densities of the 4 time points (0, 2, 4 and 8 hours after injection of ethanol) were calculated for each dendritic stretch and statistically compared using a repeated measures ANOVA and a post hoc Bonferroni's test for multiple comparisons. For boutons, signals 1.5 times higher than the background of the axon were counted as mitochondria or presynaptic terminals, respectively. Turnover rate of boutons was calculated by comparing numbers of lost/gained boutons 4 h and 24 h post ethanol/saline to existing boutons in the pre ethanol/saline time point.

**Analysis of mitochondrial and DCV transport**

For image processing and analysis, the open-source image analysis software Fiji was used. Time-lapse images were registered in \(xy\) using the Fiji plugin “moco” (44). Average intensity projections of time-lapses were used to show stationary cell organelles as well as the outlines of axons, while maximum intensity projection was used to identify axons displaying active cell organelle trafficking. For mitochondrial transport, only active axonal stretches (ROIs) for analysis were selected. A total of 15 ROIs was analyzed per focal plane. Kymographs were generated with the Multi Kymograph tool (line width = 3) in Fiji. Kymographs revealed the number of stationary and mobile mitochondria, from which a mobility ratio was calculated. Kymographs were used to calculate the mean velocity of a mitochondrial track by using a Fiji macro written by Alessandro Moro. DCV transport was automatically tracked (see next section) and analyzed using self-written routines in Matlab (MathWorks). For mobility, DCV tracks were defined as mobile, if the total movement distance was above or equal to 5 µm and stable if the movement distance was below 5 µm. Mobility was calculated as mobile-fraction / (stable fraction + mobile fraction). Mean velocity was calculated on the mobile fraction only.

**Automatic tracking of dense core vesicles**

To quantify the mobility of vesicles in the mouse brain data, an automatic tracking approach was used (45). In each image frame of a video, vesicles were detected by applying
the spot-enhancing filter (44). Two different size thresholds yielding two sets of detections at each time point were used. Detected spots of small and large size were used for tracking, while for initialization of trajectories only large spots were used. A Kalman filter (46) was employed to determine predictions about the state of vesicles using a two motion model, comprising random walk and directed motion. For finding associations between the predictions and the detections at subsequent time points, a multi-frame approach was used, which is based on a graph theoretical formulation and supports one-to-one associations, many-to-one and one-to-many associations. The optimal associations were computed by solving a linear program (45). The state of vesicles was computed by the Kalman filter based on the resulting associations and the selected motion model.

Data processing and analysis
For image processing and analysis, the open-source image analysis software Fiji was used. Time-lapse images were registered in $xy$ using the Fiji plugin “moco” (44). Average intensity projections of time-lapses was used to show stationary cell organelles as well as the outlines of axons, while maximum intensity projection was used to identify axons displaying active cell organelle trafficking. For mitochondrial transport, only active axonal stretches (ROIs) for analysis were selected. A total of 15 ROIs was analyzed per focal plane. Kymographs were generated with the Multi Kymograph tool (line width = 3) in Fiji. Kymographs revealed the number of stationary and mobile mitochondria, from which a mobility ratio was calculated. Kymographs were used to calculate the mean velocity of a mitochondrial track by using a Fiji macro written by Alessandro Moro. DCV transport was automatically tracked (see next section) and analyzed using self-written routines in Matlab (MathWorks). For mobility, DCV tracks were defined as mobile, if the total movement distance was above or equal to 5µm and stable if the movement distance was below 5µm. Mobility was calculated as mobile-fraction / (stable fraction + mobile fraction). Mean velocity was calculated on the mobile fraction only.

The mitoGFP and SyPhy-mCherry 3D-stacks were registered by manual selection of corresponding points and calculation of the resulting transformation in Matlab. For analyzing the structural plasticity of mitochondria and presynaptic terminals axons clearly visible in all time points (pre, 4 h and 24 h post) were chosen. Signals 1.5 times higher than
the background of the axon were counted as mitochondria or presynaptic terminals, respectively.

**Statistical analysis**

Statistical analysis was performed in Prism (GraphPad Software). For parametric tests, normal distribution was tested using D’Agostino & Pearson normality test. For analysis either goodness of fit, Mann-Whitney test, two-way repeated measures ANOVA, or Kruskal-Wallis were performed. For avoiding α-error accumulation, results were adjusted using post-hoc Sidak’s multiple comparisons test (two way repeated-measures ANOVAs) or Dunn’s multiple comparison test (Kruskal-Wallis). F values as well as degrees of freedom (DF; F (DFn, DFd)) are provided for ANOVAs. Statistical significance was assumed for P < 0.05. All data are displayed as mean ± standard deviation (STD) if not indicated otherwise (e.g. as mean ± standard error of the mean (SEM)).

**Behavioral testing in mice - Go/NoGo task**

The Go/No-go task assesses the ability of mice to appropriately perform or withhold a lever pressing depending on the cues presented. Mice were trained for the Go/NoGo task in six operant chambers (TSE Systems). Each chamber was equipped with two ultrasensitive levers (required force, 1 g) on opponent sides: one functioning as the active and one as the inactive lever. Next to each lever, a front panel containing the visual stimulus was installed above a drinking microreservoir. When the programmed ratio requirements were met on the active lever, 10 μl of the sucrose (5%) solution was delivered into the microreservoir, and the visual stimulus was presented via a light located on the front panel. Responses on the inactive lever were recorded but had no programmed consequences. These responses were recorded as a measure of nonspecific behavioral activation. A microcomputer controlled the delivery of fluids, presentation of visual stimuli, and recording of the behavioral data.

The Go/NoGo task was adapted from Gubner and colleagues (47) and consisted of two training phases and one experimental phase. Briefly during each trial there was a variable duration pre-cue period (9-24 seconds) during which the house light was illuminated followed by a 5- seconds cue period, where one of two distinct cues (light of different colors in opposite walls) was used to differentiate Go trials from No-go trials. During a Go
trial, a lever pressing terminated the Go cue and was reinforced with sucrose solution. Mice were permitted 3 s to consume the reinforcer, and then the 10-s inter-trial interval (ITI) began, during which the house light was off. During a No-go trial, a no response was reinforced followed by the 3-s consumption period and a 10-s ITI.

We measured the following variables: the active and inactive lever pressings, the pre-cue period duration, total active pressings during the pre-cue phase, number of right responses to go (correct go), number of missing the chances when they should go (missed go), number of right responses of no go (correct NoGo), number of going when they should not go (false alarm), efficiency (total number of reinforcers earned/total number of lever presses), pre-cue response rate, number of correct go in all sessions (go rate) and learning performance index (correct go/active lever pressings). False alarms and pre-cue response rates were the two main dependent measures of behavioral inhibition for the Go/NoGo task.

During the experimental phase, i.e. after achieving a stable performance, mice were injected intraperitoneally (i.p.) with appropriate volumes of a 20% (v/v) ethanol solution to attain a dose of 3.5 g/kg body weight and confined to the operant boxes 4-6 h later. To test lasting effects, the test was repeated 24 and 48h later.

Statistics. Statistical analyses were performed by Student's t test using Statistica 10 (StatSoft). All values are given as mean ± STD, and statistical significance was set at $p < 0.05$.

**Drosophila experiments**

*Fly strains*

The following fly lines have been used: TH-Gal4 (48); UAS-milton-RNAi$^{GD8116}$, UAS-milton-RNAi$^{TRiPJF03022}$ and UAS-dmiro-RNAi$^{TRiPJF02775}$. RNAi–mediated knockdown of milton-RNAi$^{TRiPJF03022}$ reduced milton RNA levels and changed the axonal distribution of mitochondria similar to knock downs using the UAS-milton-RNAi$^{GD8116}$ and UAS-dmiro-RNAi$^{TRiPJF02775}$ showing that the transgenes are functional (49). We used male flies for the experiments because female flies vary in size depending on mating and feeding states (50). Male flies have a more constant body size and consequently more reproducible ethanol absorption.
For every experiment, 35 female virgins were crossed to 15 male flies and raised on an ethanol-free standard cornmeal/molasses/yeast/agar medium on 12-h/12-h light/dark cycle at 25°C with 60% humidity. The transgenes were crossed to \( w^{1118} \) to control for putative effect of the P-element insertion site of the transgene. The \( TH\)-Gal4 and \( UAS\)-milton-RNAi\(^{GD8116}\) transgenes were at least backcrossed for 5 generations to the \( w^{1118} \) stock of the Scholz lab to isogenize genetic background. To perform learning and memory experiments 50 zero to one day old male flies were collected under CO\(_2\) sedation and for recovery of sedation placed for 2 days on 25°C. Thus, 3 - 5 day-old male flies were tested.

**Conditioned preference for an odor associated with ethanol**

The olfactory conditioned preference was performed according to (26). Briefly, a population of 50 flies were exposed to odor A (IA) in humidified air for 10 min followed by a second exposure to odor B (EA/AA) reinforced with ethanol for 10 min. After 50 min rest, flies were trained again. The animals were trained in three cycles and the memory was tested 30 min later in a T-maze with a binary choice between odor A and odor B after two minutes. The training was also done reciprocally with a different group of flies. The odor/odor was diluted in mineral oil. The dilution for IA (VWR Life Science, 0944-1L) was 1:36 and the dilutions for EA 1:36 and AA 1: 400 were mixed in a 2:1 ratio of EA (Sigma-Aldrich, 58958-5ML) and AA (Sigma-Aldrich, 71251-5ML-F). The ethanol vapor was generated by bubbling 95% EtOH. The vaporized EtOH was combined with air that was streamed over the odor cups. The flow rate was 294 U for vaporized ethanol and 117 U for humidified air (where 100 U is equal to 1.7 l/min at 20°C) to get an approximately 70% Ethanol ratio in the air. At this ethanol concentration flies showed signs of intoxication, specifically, ethanol-induced sedation after 10 min of exposure. The training set up was similar to Nunez et al., 2018 (26). To analyze whether flies learnt to associate ethanol intoxication with an odor, they were tested in a Tully-Quinn paradigm (51) consisting of an odor choice paradigm using a T- shaped assay.

The preference index was calculated as follows: (number of flies in paired odor vial – number of flies in unpaired odor vial) / total number of flies. The average of the preference indexes of two reciprocally trained fly groups is used as conditioned odor preference or aversion (CPI). A positive CPI indicates a positive association with ethanol for the animals.
The preference index (PI) was calculated as follows: (number of flies on the odor side – number of flies on the paraffin oil side) / total number of flies. A negative PI indicates an initial avoidance towards the odor.

The sensory acuity was performed by exposing flies in a T-maze under similar condition as the test phase of the learning and memory experiment. However, in this case flies were not previously exposed to an odor. To analyse whether flies perceive the odor, flies were exposed in the choice situation only to one odor on one site. The second site contained the solvent paraffin oil of the odor.

Sucrose preference
The sucrose preference assay was determined using 2M sucrose dried on a filter paper in the tubes on one side of the T-maze and a non-sucrose filter paper on the other side for two minutes.

Training for Short Term Memory (STM)
Before training, flies were starved for 16 to 18 h in vials containing humidified Whatman paper. For conditioning, male flies were exposed to odor A (3-Oct) for two minutes in a tube. Next, flies were transferred to second tube containing 2 M sucrose and the odor B (MCH) for two min. After training, the flies were directly tested in a T-maze assay with a choice between odor A and odor B for two min. The procedure was repeated with a second independent set of flies in a reciprocally manner.

Training for Long Term Memory (LTM)
For LTM training, flies were starved for 16 to 18 h prior the training in vials containing humidified Whatman paper. For the conditioning, flies were at first exposed to odor A (3-Oct) for two minutes and afterwards to 2M sucrose and odor B (MCH) for two min. After 15 min of rest, the training was repeated. Flies were trained five times in total and after a 24 h rest period tested for their odor preference in a T-maze with the choice between odor A and odor B. The assay was done reciprocally with a second group of naïve flies.
Statistics:
The One-sample sign test was used to determine difference from random choice and significant differences with $P < 0.05$ were indicated with the letter “a”. Differences between more than two test groups were determined using ANOVA post-hoc Tukey-Kramer Honestly Significant Difference (HSD). $P^* < 0.05$, $P^{**} < 0.01$ and $P^{***} < 0.001$. The errors are given as s.e.m.
Fig. S1.

Proteomic analyses of synaptic proteins.
A. Cross-over experimental scheme.
B. Multi-step, small scale synapse purification procedure (P = pellet, S = supernatant).
A

| Function                                      | GO ID   | Enriched Proteins | P-value |
|-----------------------------------------------|---------|-------------------|---------|
| Electron-transferring flavoprotein            | GO:0004174 | 2/2 (2)            | **      |
| Dehydrogenase activity                        |         |                   |         |
| Propionyl-CoA carboxylase activity            | GO:0046588 | 2/2 (2)            | **      |
| Adenylyl cyclase binding                       | GO:0008179 | 2/2 (2)            | **      |
| Hydrogen ion transporting ATPase activity     | GO:0046981 | 13/19 (40)         | ***     |
| Biotin binding                                | GO:0009374 | 2/4 (4)            | **      |
| 4 iron, 4 sulfur cluster binding              | GO:0051539 | 2/6 (17)           | *       |
| Pyridoxal phosphate binding                   | GO:0030170 | 3/11 (45)          | **      |
| FAD binding                                   | GO:0050660 | 3/17 (59)          | *       |
| Metal ion binding                             | GO:0048872 | 19/333 (3388)      | *       |

B

GABA transporter 4: peptide LTVPSADLK

Forward P30

Reverse P30

C

Spine density [spines/µm]

| Condition   | Pre | EtOH | 24h Post |
|-------------|-----|------|----------|
|             |     |      |          |
|             |     |      |          |
|             |     |      |          |
|             |     |      |          |
Fig. S2. GO analysis, GAT4 spectra, and in vivo spine densities

A. Gene Ontology enrichment analysis of the 72 significantly changing proteins vs. detected hippocampal synaptic proteome. The numbers indicate: significantly changing protein of GO term / total number of GO term proteins detected in synaptic proteome (total number of GO term proteins in genome). The enrichment of ATP synthase proteins was highly significant as were a few other molecular functions such as biotin binding, indicating alterations in mitochondrial functions.

B. MS spectra of a unique peptide (LTVPSADLK) of the GABA transporter 4 protein. In both forward and reverse ethanol treated samples (P30), there was a significant reduction of the peptide/protein.

C. The distribution of spine densities during longitudinal in vivo 2-Photon imaging of Thy1-GFP mice revealed no significant changes before, during, or after ethanol exposure. (spine density in spines/µm: day 1: baseline - pre Ethanol: 0.8525, day 2: Ethanol: 0.8514, day 3: post Ethanol: 0.8549; repeated measures ANOVA p = 0.0001; Bonferroni's multiple comparisons test p > 0.44)
Fig. S3: Visualizing axonal organelle trafficking under saline and ethanol conditions using in vivo two-photon microscopy.

A. Two-photon imaging was accomplished by implanting a chronic cranial window. The 3D-printed crown, needed for head-fixation of the animal, was attached to the window glass coverslip.

B. Tile scan of thalamic injection site with projections reaching up to the cortex (wide field epifluorescence, scale bar 1 mm).

C. GFP-labelled mitochondria in thalamic projections imaged in upper layers of the cortex (two-photon, scale bar 25 µm).

D. Immunofluorescence staining of GFP-labelled mitochondria in neurons of the MD thalamus co-stained with the outer mitochondrial membrane marker TOM20 (single confocal plane, scale bar 5 µm).

E. Mean velocity under basal conditions was 0.30 ± 0.20 µm/s (n = 406 mitochondria from 4 mice). Data displayed as Box-Whisker Plot; mean indicated as +, whiskers show min-max.

F. Time course of mitochondrial velocity under basal conditions (black) and after ethanol injection (green). Mitochondrial velocity was unaffected by acute ethanol intoxication (p = 0.4601, two-way repeated measures ANOVA, n = 8 focal planes from 4 mice; mean ± STD).

G. Histogram of mitochondrial velocity with fit (R² = 0.9578) showing the sum of two Gaussian distributed populations of mitochondria transported at different mean speeds.

H. Mean DCV velocity under basal conditions was 0.48 ± 0.2 µm/s (n = 4657 DCVs from 3 mice). Data displayed as Box-Whisker Plot; mean indicated as +, whiskers show min-max.

I. Time course of DCV velocity under basal conditions (black) and after ethanol injection (green). DCV velocity increased after acute ethanol intoxication as well as in the saline condition; the increase was not significantly different between saline and ethanol injected mice (n = 3, mean ± STD).

J. Kymographs of the same axonal stretch before and 210 min post-ethanol injection. The number of transported DCVs remains stable.
K. Representative images of mitochondria (orange arrow heads) that delocalized from the imaged region 4 h after ethanol application (*in vivo*, scale bar 5 µm).
**Fig. S4: Acute ethanol intoxication does not affect mitochondrial structure, but mitochondrial occupation of presynaptic boutons**

A. *In vivo* occupied and unoccupied presynaptic boutons (red), mitochondria depicted in grey (scale bar 5 µm)

B. 80.5 ± 9.1 % of presynaptic boutons (n = 80 axons from 4 mice) were occupied by mitochondria in thalamic long-projection neurons. 4 h after ethanol intoxication occupancy of presynaptic boutons was significantly reduced (P=0.0164). After 24 h occupancy returned to baseline level (P<0.0001) (Two-way repeated measures ANOVA with Bonferroni’s multiple comparisons test, F (1,78) = 0.1315).

C. Diameter of stable, lost and formed occupied (occ.) and unoccupied (unocc.) presynaptic boutons (n > 50 per group). Occupied stable presynaptic boutons were significantly larger than other boutons. Occupied boutons had on average a larger diameter (1.26 µm) than unoccupied boutons (0.89 µm) and also reached higher maximal diameters (3.03 µm) compared to unoccupied synapses (2.372 µm) (P<0.0001, Kruskal-Wallis test, Dunn’s multiple comparison test for differences between occupied boutons and all other boutons, differences between the rest are not significant).

D. Mean mitochondria length was 1.52 ± 0.73 µm (n = 1343 mitochondria from 4 mice). Data displayed as Box-Whisker Plot; mean indicated as +, whiskers show 1-99 percentile.

E. Mitochondrial length (n > 600 mitochondria per time point from 4 mice, mean ± STD) was unaffected by acute ethanol intoxication (p = 0.17, two-way repeated measures ANOVA).
Fig. S5: Dynamics of mitochondrial occupation of presynaptic boutons.

A-C. Longitudinal *in vivo* imaging of boutons (marked in red by SyPhy-mCherry) and mitochondria (white, mitoGFP) revealed the dynamics of bouton turnover with or without mitochondria.
Fig. S6: Dynamics of occupied vs. unoccupied boutons.

A. Bouton turnover at different time points (corresponds to Fig. 4F); each color represents one mouse (Fig. S6A-E).

B. Loss of unoccupied presynaptic boutons was significantly increased after 4 h and 24 h following ethanol injection (n = 40 axons from 4 mice, mean ± STD; corresponds to Fig. 4G).

C. Loss of occupied presynaptic boutons was unaffected by acute ethanol intoxication when comparing the saline to the ethanol condition (P=0.822), however, a significant increase after 24 h compared to 4 h could be detected in the ethanol condition (P=0.0072), but not in the saline condition (Two-way repeated measures ANOVA with Bonferroni’s multiple comparisons test, F(1,78) = 0.05087).

D. Formation of occupied presynaptic boutons was unaffected by ethanol intoxication (p = 0.95, two-way repeated measures ANOVA, F (1,78) = 0.004359).

E. Formation of unoccupied presynaptic boutons was unaffected by ethanol intoxication (p = 0.1433, two-way repeated measures ANOVA, F (1,78) = 2.186).
Fig. S7: Time course of behavioral inhibition after a single dose of ethanol

After achieving a stable performance for the Go/NoGo task in two training sessions, mice were retested for different variables in experimental sessions at 4-6 h, 24 h, and 48 h following injection with 3.5 g/kg ethanol.

A. Correct go: number of right responses to go (4-6 h: $t(5)=4.1$, $P<0.01$; 24 h: $t(5)=2.3$, $P=0.07$; 48 h: $t(5)=1.7$, $P=0.1$).

B. False alarm: number of going when they should not go (4-6 h: $t(5)=2$, $P=0.1$; 24 h: $t(5)=2.1$, $P=0.148$; 48 h: $t(5)=2.6$, $P<0.05$).

C. Efficiency: total number of reinforcers earned/total number of lever presses (4-6 h: $t(5)=3.2$, $P<0.05$; 24 h: $t(5)=2.7$, $P<0.05$; 48 h: $t(5)=1.6$, $P=0.2$)

D. Performance rate: correct go/active lever pressings (4-6 h: $t(5)=4.9$, $P<0.01$; 24 h: $t(5)=3.8$, $P<0.05$; 48 h: $t(5)=2.2$, $P=0.1$). All values are given as mean ± SEM, and statistical significance was set at $P < 0.05$. 

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Fig. S8: Appetitive short term (STM) and long term (LTM) learning with sucrose as a reinforcer following knock-down of Miro/Milton

A. Olfactory associative learning and memory paradigm with ethanol as a reinforcer.

B. Following one-cycle sucrose training resulting in STM, the CPI did not differ between control flies and experimental groups. **Left:** Miro: The mean CPI was for TH-Gal4/+: 0.21 ± 0.02; UAS-dmiro-RNAi/+: 0.22 ± 0.04; TH-Gal4/UAS-dmiro-RNAi: 0.22 ± 0.04. **Right:** Milton: The mean CPI was for TH-Gal4: 0.24 ± 0.03; UAS-milton-RNAi/+: 0.25 ± 0.04; TH-Gal4/UAS-milton-RNAi: 0.17 ± 0.03

C. Following 5-cycle spaced sucrose training resulting in LTM, the CPI did not differ between control flies and the experimental groups. **Left:** Miro: The mean CPI was for TH-Gal4/+: 0.38 ± 0.05; UAS-dmiro-RNAi: 0.25 ± 0.04; TH-Gal4/UAS-dmiro-RNAi: 0.50 ± 0.05. **Right:** Milton: The mean of the CPI was for TH-Gal4/+: 0.34 ± 0.05; UAS-milton-RNAi/+: 0.23 ± 0.04; TH-Gal4/UAS-milton-RNAi: 0.31 ± 0.06.

The letter “a” indicates significant differences from random choice as determined with One-sample sign test. Differences between groups were determined using ANOVA post-hoc Tukey-Kramer HSD. Errors SEM.

**Dataset S1:**

2089 identified proteins of the hippocampal synaptic proteome including their average MS intensity.
Table S1: Proteins whose synaptic abundance changed significantly after ethanol

Ethanol-dependent changes in synaptic abundance for all significantly changing proteins. Ratios are given as fold change compared to controls. Depicted ratios are the average of forward and reverse experiments for young (P30) or old (P210).

| Synaptic proteins exhibiting ethanol-dependent changes | PND 30 | PND 210 |
|--------------------------------------------------------|--------|---------|
| 60S ribosomal protein L5                               | 0.72   |         |
| Adenyl cyclase-associated protein 1 (CAP1)             | 0.72   |         |
| Adenyl cyclase-associated protein 2 (CAP2)             | 2.17   | 0.57    |
| Ankyrin 2                                              |        |         |
| Apoptosis-inducing factor 1                            | 1.18   | 1.33    |
| Asparagine synthetase                                   | 1.52   | 1.34    |
| Aspartate aminotransfer                                 | 1.93   | 2.49    |
| ATP synthase subunit B                                  | 1.25   | 1.49    |
| ATP synthase subunit D                                  | 1.22   | 1.5     |
| ATP synthase subunit ε                                  | 1.34   | 1.44    |
| ATP synthase subunit epsilon                            | 1.38   |         |
| ATP Synthase subunit G                                  | 1.28   | 1.4     |
| ATP synthase subunit gamma                              | 1.53   |         |
| ATP synthase subunit O                                 | 1.26   | 1.49    |
| ATP synthase-coupling factor 6                          | 1.59   | 1.52    |
| C1-tetrahydrofolate synthase                            | 0.72   | 0.8     |
| cAMP-specific 3',5'-cyclic phosphodiesterase 4D/PDE4D   | 1.68   |         |
| Carnitine O-acetyltransferase                           | 0.74   | 0.83    |
| CD98 heavy chain                                        | 0.73   |         |
| Citrate synthase                                        | 0.77   | 0.85    |
| Clathrin light chain A                                  | 1.28   | 1.42    |
| Clathrin light chain B                                  | 2.28   | 1.41    |
| Creatine kinase                                         | 0.81   | 0.86    |
| CRMP-5/Dihydropyrimidinase-related protein 5            | 1.32   | 2.09    |
| C-terminal-binding protein 1                            | 1.41   |         |
| Cytoplasmic dynein 1 light intermediate chain 1         | 1.33   | 1.14    |
| Diacylglycerol lipase, alpha                            | 0.73   |         |
| Electron transfer flavoprotein-ubiquinone oxidoreductase| 1.59   |         |
| Freud-1 (Coiled-coil and C2 domain-containing protein 1A)| 1.66   | 1.19    |
| Fyn kinase                                              | 1.78   |         |
| GABA-transaminase                                       | 0.8    |         |
| GAP-43                                                  | 1.64   |         |
| Glycogen phosphorylase                                  | 1.92   | 1.45    |
| Heterogeneous nuclear ribonucleoprotein L               | 1.44   | 1.24    |
| Hydroxacyl-coenzyme A dehydrogenase                     | 0.73   |         |
| Kir4.1/ATP-sensitive inward rectifier potassium channel 10 | 0.73   |         |
| Long-chain specific acyl-CoA dehydrogenase              |        | 1.35    |
| Malate dehydrogenase                                    | 0.69   | 0.76    |
| Methylcrotonoyl-CoA carboxylase subunit alpha           | 1.32   |         |
| Methylglutaconyl-CoA hydratase                          | 0.82   |         |
| Methylmalonic aciduria (cobalamin deficiency) type B homolog | 0.67   |         |
| Microtubule-associated protein-6/STOP protein           | 2.19   | 1.17    |
| Mitochondrial transcription factor A                    | 0.79   |         |
| Monoamine oxidase A                                     | 1.25   |         |
| Myosin-11                                               | 0.76   |         |
| Neuronal cell adhesion molecule/Nr-CAM                  | 1.55   |         |
| Phosphacan                                              | 0.73   |         |
| Phosphoglucomutase-2-like 1                             | 2.1    | 2.13    |
| Programmed cell death 6-interacting protein/ALIX        | 0.76   |         |
| Propionyl Coenzyme A carboxylase, alpha                 | 2.28   | 2.11    |
| Propionyl Coenzyme A carboxylase, beta                  | 2.65   | 2.21    |
| Protein FAM126B                                         | 1.45   |         |
| Rabphilin-3A                                            | 1.26   |         |
| Receptor expression-enhancing protein 5                 |        | 1.36    |
| Reticulon 1 isofrom RTN1-C                              | 1.28   | 1.28    |
| Ribosome recycling factor                               | 0.71   | 0.75    |
| SAD1 kinase                                             | 1.74   |         |
| Serine hydroxymethyltransferase                         | 0.68   | 0.79    |
| Sodium- and chloride-dependent GABA transporter 4/GAT4  | 0.67   |         |
| Succinate dehydrogenase [ubiquinone] iron-sulfur subunit |        | 1.32    |
| Synaptosomal-associated protein 25/SNAP-25              | 1.33   |         |
| Tenascin-R                                              | 0.81   |         |
| Thioredoxin reductase 2                                 | 0.72   |         |
| Transportin-3/importin 12                               | 1.98   |         |
| Tubulin beta-2A                                         | 1.45   |         |
| Tubulin beta-3                                          | 1.4    | 1.27    |
| Ubiquitin-activating enzyme E1 X                        | 1.59   |         |
| Vacuolar ATP synthase catalytic subunit A               | 0.86   | 0.82    |
| Vacuolar ATP synthase subunit D                         | 1.73   | 1.6     |
| Vacuolar ATP synthase subunit E                         | 1.36   | 1.41    |
| Vesicle-associated membrane protein 4/VAMP-4            | 1.81   |         |
Table S2: Odours/odors preferences of trained adult flies.

The RNAi transgenes used are UAS-milton-RNAi\textsuperscript{GD8116}, UAS-milton-RNAi\textsuperscript{TRiPJF03022} and UAS-dmiro-RNAi\textsuperscript{TRiPJF02775}.

Odor preferences for ethanol experiments in Table A, for sucrose experiments in Table B. The errors are SEM.

* The values differ significantly from random choice as determined using One-sample sign test. ** None of the values differ from random choice as determined using One-sample sign test and they did not differ between the controls and experimental groups as determined using ANOVA post-hoc Tukey-Kramer HSD.
Movie S1: Mitochondrial trafficking *in vivo* under baseline conditions.
Imaging frame rate 0.2 Hz, sped up to 12 frames/s.

Movie S2: Mitochondrial trafficking *in vivo* after ethanol injection.
Imaging frame rate 0.2 Hz, sped up to 12 frames/s.

Movie S3: Dense core vesicle trafficking *in vivo* with automated tracking overlay.
Imaging frame rate 0.94 Hz, sped up to 25 frames/s.
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