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ORIGINAL ARTICLE

Disrupted circadian expression of β-arrestin 2 affects reward-related μ-opioid receptor function in alcohol dependence

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Abstract

There is increasing evidence for a daily rhythm of μ-opioid receptor (MOR) efficacy and the development of alcohol dependence. Previous studies show that β-arrestin 2 (bArr2) has an impact on alcohol intake, at least partially mediated via modulation of MOR signaling, which in turn mediates the alcohol rewarding effects. Considering the interplay of circadian rhythms on MOR and alcohol dependence, we aimed to investigate bArr2 in alcohol dependence at different time points of the day/light cycle on the level of bArr2 mRNA (in situ hybridization), MOR availability (receptor autoradiography), and MOR signaling (Damgo-stimulated G-protein coupling) in the nucleus accumbens of alcohol-dependent and non-dependent Wistar rats. Using a microarray data set we found that bArr2, but not bArr1, shows a diurnal transcription pattern in the accumbens of naïve rats with higher expression levels during the active cycle. In 3-week abstinent rats, bArr2 is up-regulated in the accumbens at the beginning of the active cycle (ZT15), whereas no differences were found at the beginning of the inactive cycle (ZT3) compared with controls. This effect was accompanied by a specific down-regulation of MOR binding in the active cycle. Additionally, we detect a higher receptor coupling during the inactive cycle compared with the active cycle in alcohol-dependent animals. Together, we report daily rhythmicity for bArr2 expression linked to an inverse pattern of MOR, suggesting an involvement for bArr2 on circadian regulation of G-protein coupled receptors in alcohol dependence. The presented data may have implications for the development of novel bArr2-related treatment targets for alcoholism.

KEYWORDS
β-arrestin 2, β-arrestin 2 overexpression virus, μ-opioid receptor, animal model of alcoholism, circadian regulation, receptor autoradiography

Abbreviations: AAV, adeno-associated virus; Acb, nucleus accumbens; AcbC, nucleus accumbens core; AcbS, nucleus accumbens shell; AUD, alcohol use disorder; BAC, blood alcohol concentration; bArr1, bArr2, β-arrestin 1 and β-arrestin 2; CCGs, clock controlled genes; CIE, chronic intermittent ethanol vapor exposure; CNS, central nervous system; Damgo, MOR agonist (D-Ala(2)-mephe(4)-gly-ol(5))enkephalin); GPCR, G-protein-coupled receptor; MOR, μ-opioid receptor; SD, sprague-dawley; ZT, zeitgeber.

Marcus W. Meinhardt and Francesco Giannone share first authorship.

1 | INTRODUCTION

Excessive alcohol consumption remains to be a serious public health issue accounting for over 3 million deaths per year globally (WHO, 2018). Alcohol has a strong ability to induce neuroadaptations that promote its incentive salience, formation of strong consumption habits, and addictive behaviors, often leading to the development of alcohol use disorder (AUD). The condition is highly prevalent, especially in industrialized countries; nevertheless, AUD is among the most under-recognized and under-treated health conditions (Shield & Rehm, 2019). Only a few medications are widely approved for the treatment of AUD, among them the non-selective opioid antagonists naltrexone and nalmefene, which in fact demonstrated proof-of-concept for a neuropharmacologic intervention to relapse prevention (Volpicelli et al., 1992). However, these medications have a modest overall effect size, with a wide range of individual treatment responses (Jonas et al., 2014). So far, the source of this high heterogeneity of clinical outcomes in AUD is not well understood and generates a strong incentive for research to better understand the mechanisms of action of existing medications to improve pharmacotherapeutic treatment strategies (Heilig et al., 2019).

Among the numerous physiologic effects that alcohol consumption has, and which it shares with most other drugs of abuse, the disruption of the circadian rhythmicity that is observed at different levels such as sleep disturbances, for example, changes in latency (the time needed for the initiation of the REM phase after falling asleep), density (the number of REM phases during a sleeping session), and percent REM sleep, leading to anticipated awakening and an increased amount of total time spent awake (Feige et al., 2006; MacLean & Cairns, 1982; Williams et al., 1983), body temperature alterations, such as a shift in rhythmicity and a reduction of the amplitude of the body temperature changes throughout the day (Danel et al., 2001), and hormonal secretions (Rachdaoui & Sarkar, 2013). Similar effects have been observed also for other drugs of abuse (Schierenbeck et al., 2008; Vescovi et al., 1992).

Such disruption of the circadian rhythm is most probably caused by the effects that alcohol has on the so-called "clock genes"; these are a series of genes that code for transcriptional factors (e.g., Cryptochromes 1 and 2 [Cry1, Cry2], Periods 1, 2, and 3 [Per1, Per2, Per3], Rev-Erbα and Rev-Erbβ) that in turn regulate the rhythmic expression of several targets "clock-controlled genes" (CCGs), both in the CNS and in the periphery (Partch et al., 2014). It has been demonstrated that exposure to drugs of abuse profoundly affects the expression of clock genes in the brain of rodents and humans (Halbout et al., 2011; Logan et al., 2014; Perreau-Lenz & Spanagel, 2008), while the modulation of clock gene expression has a strong impact on the manifestation of drug-related responses. In turn, altered circadian rhythmicity can affect the expression of genes in the central nervous system (Manev & Uz, 2006).

Together, there is an extensive body of evidence in both animals and humans indicating bidirectional relationships between the circadian system and drugs of abuse (for review, see Spanagel et al., 2005), ultimately leading to profound health consequences, including the development and progression of addiction. Patients with AUD display disrupted rhythms (Conroy et al., 2012; Fakier & Wild, 2011; Kovanen et al., 2010; Sjoholm et al., 2010; Vescovi et al., 1992), and chronic disruption has been found to increase the risk for substance abuse and relapse (Brower, 2003; Brower et al., 2001).

A key brain region for the control of motivated behaviors is the nucleus accumbens (Acb), where many signals for various sources are integrated including interactions of dopamine and opioid systems. These interactions play a particular role in the mediation of alcohol reward and are profoundly disturbed in alcohol use disorder (for review, see Hansson et al., 2019). Experiments in animals and humans have shown that opioid peptides with activity at μ-opioid receptors (MORs) are released by alcohol intake (Mitchell et al., 2012) and contribute to alcohol reward by activating the mesolimbic dopamine system and leading to dopamine release in the Acb (Hansson et al., 2019; Imperato & Di Chiara, 1986; Johnson & North, 1992; Ramchandani et al., 2011; Spanagel & Weiss, 1999).

MOR is a G-protein-coupled receptor (GPCR) shown to be circadian regulated, with varying expression levels throughout the day (Mitchell et al., 1998; Takada et al., 2013). As a GPCR, MOR levels and function are modulated, among the others, by a series of protein–protein interactions with adaptors or scaffolding proteins such as β-arrestins (Bjork & Svenningsson, 2011; Bockaert et al., 2010; Porter-Stransky & Weinshenker, 2017; Shenoy & Lefkowitz, 2011). β-arrestins are strongly expressed in the Acb and have been shown to facilitate rapid MOR endocytosis upon stimulation in striatal neurons (Haberstock-Debic et al., 2005). From the two known variants of β-arrestins (bArr1 and bArr2), bArr2 has a higher affinity for MOR, making it a likely candidate for modulating the effects of drugs of abuse (Oakley et al., 2000).

Indeed, several lines of evidence link bArr2 to alcohol reward. For instance, rats selectively bred for alcohol preference show elevated levels of bArr2 mRNA in the Acb, dorsal striatum, and hippocampus compared with alcohol-avoiding rats, while bArr2-knockout mice voluntarily consume less alcohol (Bjork et al., 2008). High levels of bArr2 thus lead to increased alcohol intake, whereas eliminating the protein reduces alcohol consumption. When specifically assessing the rewarding properties of alcohol, bArr2-KO mice also display an enhanced conditioned place preference for low doses of alcohol (Li et al., 2013), a sensitized dopamine release in the Acb, an increased reward upon low doses of alcohol (Bjork et al., 2013), and altered excitability of accumbal D2 receptor medium spiny neurons (Porter-Stransky et al., 2020). Despite these observations, the role of bArr2 in the development of AUD is less clear.

Arrestin in the visual system is a member of the same bArr2 family and is known for having a diurnal and possibly a circadian regulation (Battelle et al., 2000; Bowes et al., 1988; Craft et al., 1990; McGinnis et al., 1992). This indicates a possible circadian regulation also for the non-visual bArr2 and therefore a possible effect on the regulation of MOR levels. Furthermore, bArr1 was also found to show a circadian expression pattern in humans (Tomita et al., 2019). However, it is not known whether the circadian rhythm regulates the expression of bArr2 in the brain and

if this mechanism is altered in alcohol dependence, which might have an impact on MORs, and consequently on dependence and treatment effects.

Therefore, the present study aimed to evaluate possible circadian effects on bArr2 and MOR transcription in alcohol-dependent and non-dependent Wistar rats. We used a well-established rodent model of alcohol dependence that leads to intoxication levels similar to those seen in clinical AUD by chronic intermittent ethanol vapor exposure (CIE) (Goldstein & Pal, 1971; O'Dell et al., 2004; Rimondini et al., 2002; Rogers et al., 1979). The procedure induces long-lasting behavioral and pronounced molecular changes in all major domains of the addiction circuitry, that is, in motivational, emotional, and cognitive neuronal circuitries (Meinhardt & Sommer, 2015). Three-week abstinent rats were used to study bArr2 transcripts as well as MOR availability and MOR G-protein coupling.

2 | MATERIALS AND METHODS

2.1 | General experimental procedures

Experiments 1–3 were conducted in male Wistar rats (RRID:RGD_737929) obtained from Charles River (Sulzfeld). Rats were 8 weeks of age at the beginning of the experimental procedures (initial weight 220–250 g). For Experiment 4, Sprague-Dawley (SD) rats were used ($n = 4$, male, adult, mean body weight 440 g).

All rats were group-housed (four animals per Makrolon type 4 cage) under a 12-h light/dark cycle with ad libitum access to water and food.

Brain collection was performed after decapitation, the brains were then extracted and snap-frozen by immersion in −40°C liquid isopentane, then stored at −80°C until further processing.

The complete study was designed according to the 3R animal welfare principles, and all experiments were approved by the institutional Committees on Animal Care and Use and by the Regierungspräsidium Karlsruhe (AZ 35–9185.81/G-183/09 and AZ 35–9185.81/G-245/12) and were performed in accordance with the European and German national guidelines.

2.2 | Experiment 1. Circadian regulation of bArr2 in the nucleus accumbens

2.2.1 | Animals and general experimental procedures

For this experiment, a total of 24 rats ($N = 6$ per time point) were used. The animals were kept in their home cages for 8 weeks after which they were sacrificed at different time points throughout the day according to the Zeitgeber (ZT) notation (lights on from 00 until 12 h and lights off from 12 until 24 h). The time points used were 05, 11, 17, and 23 h referred to as ZT05, ZT11, ZT17, and ZT23, respectively. Collections at time points ZT17 and ZT23 were performed under red light to maintain the circadian rhythm. Six rats per time point were used (Figure 1).

2.2.2 | Microarray experiment to evaluate circadian effects for bArr2

The frozen rat brains were cut into 120-µm-thick coronal slices at an ambient temperature of −20°C in a Leica CM 3000 Cryostat (Leica). The nucleus accumbens was identified according to images from a rat brain atlas (Paxinos & Watson, 1998) and extracted with 0.75–1.5 mm diameter tissue punches (Stoelting). RNA was isolated by phenol–chloroform extraction (Chomczynski & Sacchi, 1987). One milliliter of TRIzol Reagent (Life Technologies), a monophasic solution of phenol, and the chaotropic agent guanidinium thiocyanate were added to the punched tissue samples, and the suspensions were homogenized by multiple passages through a 22-gauge needle. Samples were filled up with 200 µl of chloroform, mixed, and centrifuged to obtain a separation of the aqueous upper and the organic lower phase. The RNA-containing upper phases were carefully collected and purified with an RNeasy MinElute Cleanup Kit (Qiagen) according to the manufacturer's instructions. The concentration and purity of the RNAs were analyzed with a Nanodrop 1000 Spectrophotometer (Peqlab). All samples had a ratio of absorption at 260 nm versus 280 nm in the range of 1.8–2.2, signifying low contamination with leftover proteins. RNA integrity was further analyzed with an Agilent 2100 Bioanalyzer (Agilent Technologies). All samples had RNA integrity number (RIN) values above eight. RNA samples were treated with the Illumina TotalPrep RNA Amplification Kit (Life Technologies) following the manufacturer's protocol. The cRNA was hybridized on the microarrays using the RatRef-12 Expression BeadChip Kit (Illumina) following the manufacturer's protocol. In brief, 750 ng cRNA per sample were hybridized on RatRef-12 Expression BeadChips and incubated for 14 h on a rocker mixer. BeadChips were subjected to two washing steps according to the manufacturer's protocol to remove unbound cRNA. BeadChips were then stained with Cyanine 3 coupled to streptavidin which binds to the biotinylated UTPs of the cRNA. This was followed by another washing step. BeadChips were scanned on an Illumina BeadArray Reader using Bead Scan Software (Illumina) and read out as text files. The most up-to-date Illumina annotation for the RatRef-12 Bead Array was used (V1 0 R5 11222119 A).

2.3 | Experiment 2. Effects of alcohol dependence on bArr2 and MOR during the active cycle

2.3.1 | Induction of alcohol dependence

To induce alcohol dependence, 16 rats were exposed to chronic intermittent alcohol vapor (CIE) as described previously (Rimondini et al., 2002). Briefly, rats were exposed to ethanol vapor for a total exposure period of 7 weeks. Every week the animals were

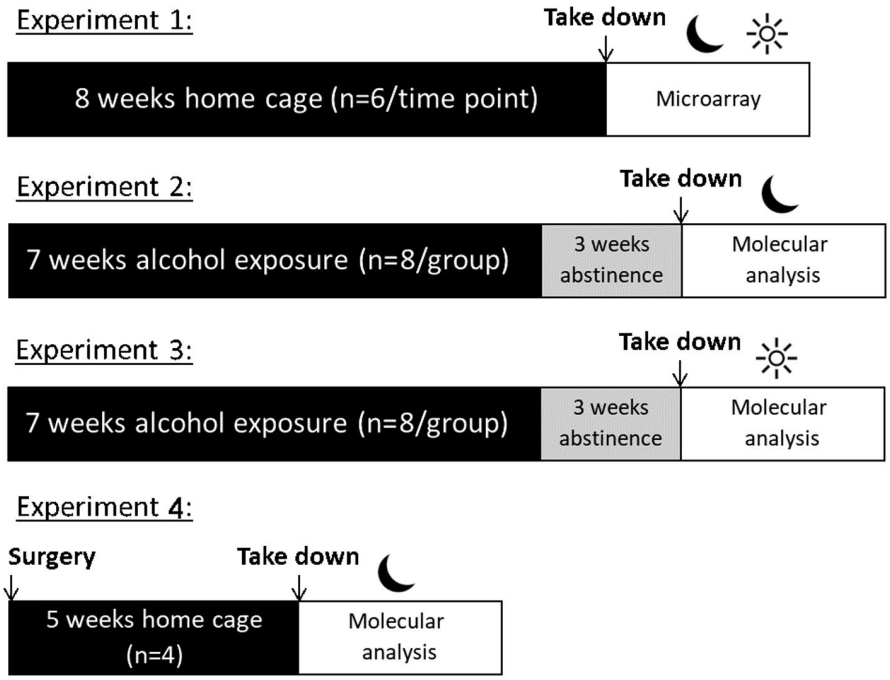

FIGURE 1 Timeline presentation of conducted experiments

exposed to alcohol vapor for 5 days (from Monday to Friday) combining daily intoxication sessions of 14 h of ethanol vapor with 10 h of withdrawal sessions (in which no alcohol vapor was delivered), daily intoxication sessions started at the beginning of the active cycle. No alcohol vapor was delivered during the weekends. Vapor exposed animals did not receive any loading dose or pyrazole treatment throughout the procedure. Control rats were instead exposed to normal airflow for the entire period, obtaining in this way two independent groups. The alcohol was delivered by dosing pumps (Knauer) into electrically heated stainless-steel coils (60°C) connected to an airflow of 18 L/min. Blood alcohol concentration (BAC) was controlled twice per week immediately after the end of the intoxication session and maintained between 150 and 250 mg/dl. These induced BAL values have been shown appropriate to induce dependence in male rats (Hirth et al., 2016; Meinhardt & Sommer, 2015; Rimondini et al., 2002). Signs of mild withdrawal, such as tail stiffness and piloerection, were observed during the off-intervals by the end of the 7-week exposure period, but withdrawal intensity never reached seizure levels (Hirth et al., 2016; Rimondini et al., 2002). After the last exposure cycle, exposed rats were kept in abstinence for 3 weeks. Finally, both dependent (n = 8) and control (n = 8) groups were sacrificed by decapitation at the beginning of the active cycle (ZT15–ZT16) (Figure 1). Brains were snap-frozen in −40°C isopentane and stored at −80°C until further processing.

G*Power analysis (two-tailed unpaired t test; α = 0.05; power = 0.80) conducted in previous CIE rats (Hirth et al., 2016; Meinhardt et al., 2021; Sommer et al., 2008; Uhrig et al., 2017) suggested a total of 16 rats (N = 8/group) with an effect size of Cohen's d = 1.5.

2.3.2 | In situ hybridization on rat brain sections

Fixation

For fixation, 12 µm coronal brain sections were warmed to room temperature and incubated in 4% paraformaldehyde (PFA) in PBS for 15 min, washed for 10 min in PBS, and twice in sterile water for 5 min. After treatment with 0.1 M HCl for 10 min and two times 5 min with PBS, brain sections were incubated in 0.1 M triethanolamine (pH 8) and 0.25% acetic anhydride for 20 min in order to acetylate proteins. Subsequently, sections were washed twice in PBS for 5 min, once in sterile water for 1 min, and dehydrated in a graded series of ethanol (70%, 80%, and 99%; 2 min each). After air-drying, sections were stored at −80°C in sealed boxes with silica gel to avoid moisture.

Probe generation

Gene-specific riboprobes for *bArr2* were designed from RefSeq NM_012911.1 in from the position 1238 to 1679 are described in Bjork et al. (2008) and were generated by PCR. Radioactively labeled riboprobes were generated by in vitro transcription. For this, 200 ng DNA was incubated with 1x transcription buffer, 12.5 nmol ATP, CTP, GTP, 50 pmol UTP, and 125 pmol Uridine 5-(α-thio)triphosphate-[^{35}S] (Perkin Elmer #NEG739H001MC, 2015), 1 U RNase inhibitor, and 1 U polymerase for 90–120 min at 37°C. Afterward, the DNA template was digested by DNAase (20 min, 37°C), and riboprobes were purified using IllustraTM MicrospinTM S-200 HR Columns.

Probe hybridization and washing

Fixed tissue sections were incubated in prehybridization buffer (100 mM Tris–HCl, pH 7.6, 5 mM EDTA, 5x Denhardt's solution,

1.25 mg/ml yeast tRNA, 40 mM NaCl) diluted 1:1 with deionized formamide for 2–4 h at 37°C followed by incubation with hybridization mix containing 10 000 CPM/µl at 55°C overnight. The hybridization mix consisted of 50% deionized formamide, 150 mM DTT, 330 mM NaCl, and 10% dextran sulfate, 1x basic mix (10x basic mix: 200 mM Tris–HCl, pH 7.6, 10 mM EDTA, 10x Denhardt's solution, 5 mg/ml yeast tRNA, 1 mg/ml polyadenylic acid). Sections were washed once for 40 min followed by two washing steps for 30 min in 1x SSC at 42°C. If necessary, sections were incubated in formamide (1:1 diluted with 1x SSC) for 1 h followed by two times 1x SSC. RNase treatment (2 mg/100 ml RNase buffer) was carried out at 37°C for 1 h. The enzyme reaction was stopped by washing the sections in 1x SSC at 55°C twice for 30 min. Sections were dipped in water for 2 min and dehydrated in a graded series of ethanol (70%, 80%, 99%; 2 min each). Fujifilm BAS imaging plates were exposed to sections for 1 week.

2.3.3 | Receptor autoradiography

Receptor autoradiographies with [^{3}H]-Damgo were performed under saturated conditions as described by Bork et al. (2013) and Hermann et al. (2017). K_d values, the dissociation equilibrium constant describing the affinity for a specific receptor, and B_{max} values, describing the maximum density of the receptor, were as follows: K_d 0.7 ± 0.1 nM and B_{max} 10.3 ± 1.8 fmol/mg (Sharif & Hughes, 1989). Sections were preincubated in 50 mM Tris, pH 7.4, 5 mM $MgCl_2$, and 1 mM EDTA twice for 15 min. Afterward, incubation buffer containing 1 nM or 8 nM [^{3}H]-Damgo (Damgo, [Tyrosyl-3,5-^{3}H(N)]-, spec. activity 50–51 Ci/mmol, Perkin Elmer #NET902250UC, 2015) was applied onto sections and incubated for 2 h at 30°C. Incubation buffer consisted of 50 mM Tris, pH 7.4, 5 mM $MgCl_2$, 1 mM EDTA, 0.1 mM Bacitracin, and 0.1% bovine serum albumin. For measuring non-specific binding on adjacent sections, 1 µM CTOP (Tocris, PubChem ID: 90479805) was added. Sections were washed three times for 2 min at 4°C in 50 mM Tris–HCl, pH 7.4, dipped in ice-cold water and dried in a cold air stream.

2.3.4 | G-protein coupling of MOR assessed by [^{35}S]-GTPγS assays

Sections were washed in 20 mM Tris–HCl, pH 7.4, 5 mM $MgCl_2$, and 1 mM EDTA twice for 15 min and then pretreated in incubation buffer containing 1 mM GDP. Incubation buffer contained 20 mM Tris–HCl, pH 7.4, 5 mM $MgCl_2$, 1 mM EDTA, 100 mM NaCl, 1 mM DTT, and 0.1% bovine serum albumin. G-protein coupling of MOR was determined by adding 10 mM GDP, 80 pM [^{35}S]-GTPγS (Perkin Elmer #NEG030H250UC, Massachusetts, 2016), and the MOR-specific agonist Damgo (1 µM, Tocris). Basal G-protein coupling was measured in the absence of Damgo but in the presence of the vehicle (acetonitrile). Incubation took place at 30°C for 1 h. Sections were then washed in 20 mM Tris–HCl and 100 mM NaCl two times for 2 min, rinsed in ice-cold water and air-dried.

2.3.5 | Autoradiographic image analysis

After performing in situ hybridizations, receptor bindings or [^{35}S]-GTPγS autoradiographies, Fujifilm BAS imaging plates (Fujifilm) were exposed to the sections. The plates were then scanned with a phosphoimager (Typhoon FLA 700, GE Healthcare). Mean density values were measured using the MCID software (MCID Image Analysis Software Solutions for Life Sciences). For in situ hybridization experiments, a sense probe was generated to measure unspecific binding that was subtracted from antisense signals. In [^{35}S]-GTPγS assay studies, basal and stimulated (in the presence of a specific agonist) was measured on adjacent sections, and the percentage of stimulated after agonist application was calculated for every sample. Total and non-specific binding (in the presence of a specific blocker) were determined for receptor-binding assays on adjacent sections and the non-specific signal was subtracted from the total signal (Bjork et al., 2013; Hermann et al., 2017). Based on the known radioactivity in ^{14}C standards, image values of in situ hybridization and [^{35}S]-GTPγS assay measurements were converted to nanocurie per mg tissue (nCi/mg). Values of measurements of the autoradiographies were converted to femtomolar per mg tissue (fmol/mg) based on ^{3}H standard values and the specific activity of the tritiated ligand, its K_d and B_{max} values as described previously (Hermann et al., 2017; Hirth et al., 2016; Sommer et al., 2014). These values were used for statistical analysis.

2.4 | Experiment 3. Effects of alcohol dependence on bArr2 and MOR during the inactive cycle

Everything was repeated exactly as in Experiment 2, the only difference was the time point of the day in which the alcohol-dependent (n = 8) and the control (n = 8) groups were sacrificed, which was at the beginning of the inactive cycle (ZT2-3) (Figure 1).

2.5 | Experiment 4. Site-specific effects of bArr2 overexpression on MOR availability in the nucleus accumbens core

2.5.1 | AAV vector injection

Injection of bArr2 overexpressing adeno associated viral vector (AAV) was performed on naïve SD rats (n = 4) in the nucleus accumbens core (AcbC) (Figure 1). To minimize animal suffering, rats were treated with Carprofen (5 mg/kg, s.c.) 1-h prior surgery, lidocaine (1–2%) was applied on ear bars during surgery and metamizole (3 g/L) in the drinking water for 3 days post-surgery. Isoflurane was used as an anesthetic during surgery because of its speed of induction and recovery. Briefly, anesthesia was induced via a 4% isoflurane–oxygen mixture inhalation, after the induction the animals were kept under anesthesia with 2–2.5% isoflurane–oxygen mixture throughout the operation (flow rate: 1 l/min).

A WPI microinjection pump with a 33-gauge blunt needle was used to deliver 1 µl of bArr2 overexpressing virus (1540 µg/µl) into the AcbC of the left hemisphere and 1 µl of control virus (1564 µg/µl) into the AcbC of the right hemisphere following the atlas of Paxinos and Watson (Paxinos 1998) coordinates (M/L[x]: +1.6 and −1.6 mm; A/P[y]: +1.6 and +1.6 mm; D/V[z]: −7.4 and −7.4 mm; relative to Bregma), at a speed rate of 100 nl/min and leaving the needle in place for 5 min after the end of the injection to avoid backflow.

After surgery, the rats were kept single housed for 5 weeks, they were then sacrificed by decapitation during the active cycle. Brains were finally extracted and snap-frozen by immersion in −40°C liquid isopentane, then stored at −80°C until further processing.

bArr2 mRNA levels and MOR availability were assessed as already described under Experiment 2.

2.5.2 | AAV vector construction

The injected bArr2 overexpressing AAV1/2 was created based on the vector AAV phSyn1(S)-FLEX-EGFP-WPRE, which was a kind gift from Hongkui Zeg (Addgene plasmid # 51504; http://n2t.net/addgene:51504; RRID:Addgene_51504); a synthetic Arrb2-T2A fragment produced by Invitrogen (containing Rattus norvegicus Arrb2 cDNA; NIH_MGC_254) was inserted, and a FLEX switch of the Arrb2-T2A-eGFP sequence was induced in vitro allowing for constitutive neuronal expression under the control of the synapsin promoter, shortly: frozen EL350 cells induced for Cre expression by prior growth in an arabinose-containing medium were used for inverting the floxed expression cassette of the FLEX AAV vector. A 10-mL overnight culture of EL350 cells was added to 500 ml of LB broth in a 2 L flask. The culture was placed in a water bath shaker at 32°C until OD600 = 0.4 (2.0 h, 180 rpm). Then 5 ml of 10% L(+)arabinose (Sigma A-3256) in H_2O was added to the culture to a final concentration of 0.1% and shaken at 32°C for another hour. Cells were collected, cell pellets were washed and frozen. Next, 1 ng of plasmid DNA was electroporated into 50 µl of frozen competent cells. Then 1.0 ml of LB broth was added to the electroporation cuvette; 10–100 µl of the cells were subsequently plated on an ampicillin plate and incubated at 32°C overnight. Single colonies were picked from the plate and screened for Cre recombination/inversion of the floxed expression cassette by restriction digestion.

The packaging of the AAV vector was performed in HEK 293T cells, which were co-transfected with the mentioned Arrb2-T2A containing plasmid, and with the three helper plasmids pFdelta6, pNLrep, pH21, via calcium phosphate; cells were then harvested, enzymatically lysed, and AAV1/2 vectors were finally purified with heparin columns (Monory et al., 2006). The control virus was created in the same fashion but the Arrb2-T2A sequence was not inserted. The construction of the AAV construct and the packaging were performed at the Institute of Psychopharmacology at the Central Institute of Mental Health (ZI), Mannheim.

2.6 | Statistics

For Experiment 1, microarray data were statistically analyzed using LibreOffice 3 and R statistical programming language version 2.15.0. For Experiments 2 and 3, data were analyzed by two-way ANOVA using region (AcbC/AcbS) and treatment (control/dependence) as independent factors (using Statistica11 [StatSoft] program), and after verification of normal distribution by Shapiro-Wilk test. Additionally, the G-protein-coupling data of the dependent animals were analyzed separately by two-way ANOVA after normalization over the inactive cycle using region (AcbC/AcbS) and cycle (active/inactive) as independent factors.

The alpha level was set to $\alpha < 5\%$. For visualization purposes, data in Figure 3 are expressed in percentage. The levels of control rats are expressed as 100% and changes in the dependent (CIE) group are presented in relation to the control group. Experiment 4 data were analyzed by a two-tailed unpaired *t* test.

We used box plots to describe the distribution of the data and to identify outliers. In detail, we defined the 25th (lower quartile "Q1") and 75th (upper quartile "Q3") percentiles as well as the interquartile range (IQ). Data points were considered as outliers in case they were found beyond the lower (Q1−3*IQ) or upper outer fence (Q3 + 3*IQ). Using this procedure, the following data point were excluded: bArr2 in situ: two controls for AcbS-inactive, one CIE for AcbC-active and AcbS-active; MOR binding: two controls for AcbC-active and AcbS-active, two CIE for AcbC-active and AcbS-active, three CIE for AcbS-inactive; [^{35}S]-GTPγS: two controls for AcbC-inactive, AcbC-active AcbS-inactive, AcbS-active, three CIE for AcbC-active and AcbC-inactive, one CIE for AcbS-inactive.

3 | RESULTS

To investigate a possible circadian rhythm for β-arrestins in the Acb under normal physiologic conditions, we assessed the expression levels at four different time points throughout 1 day to obtain time-dependent gene expression profiles (ZT05, ZT11, ZT17, and ZT23) (Figure 2). The terminology of time points is according to a standardized notation, the so-called "zeitgeber time" where ZT00 is the beginning of the light phase and ZT12 is the beginning of the dark phase (Gerstner & Yin, 2010). These time points were selected to present the mid of the light phase, the end of the light phase, the mid of the dark phase, and the end of the dark phase. All time points were 1 h before the actual mid-time points (ZT06, ZT12, ZT18, and ZT24) to allow for the consecutive termination of six animals before the start of the next quarter of the day (Figure 2a). bArr2 undergoes a diurnal expression pattern with significantly increased expression in the active cycle at ZT17 in the Acb (repeated measure ANOVA $F_{3,15} = 30.43$, $p < 0.001$). bArr1 by contrast did not show such a pattern $F_{3,15} = 1.33$, $p = 0.31$ (Figure 2b, c).

Next, we determined if the expression of bArr2 is altered in alcohol dependence (Figure 3b and d). Alcohol dependence in rats was induced by 7 weeks of CIE and brains were analyzed after 3 weeks

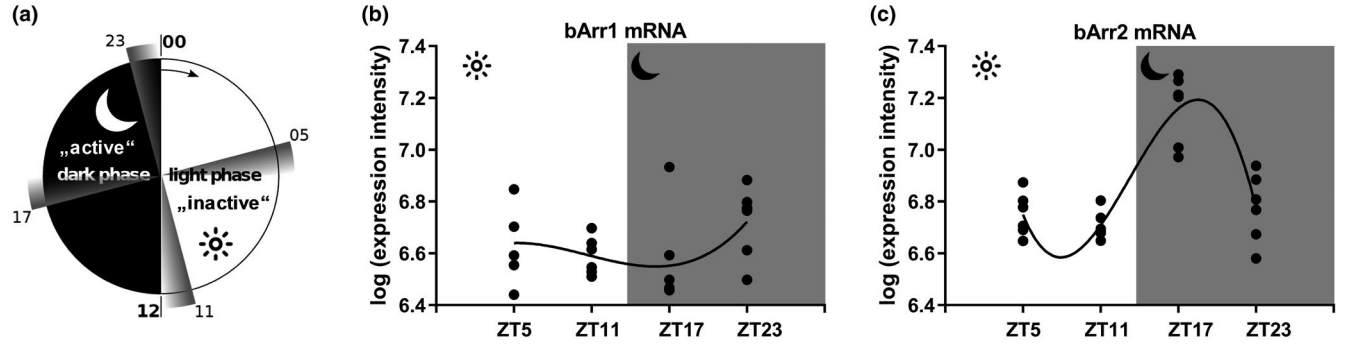

FIGURE 2 bArr2 transcript levels show a diurnal expression pattern in the nucleus accumbens. (a) Time points of brain collection from rats throughout the circadian rhythm in zeitgeber time (ZT) notation with lights on from 00 h to until 12 h and lights out from 12 h to until 24 h. The gray-shaded areas correspond with the four time slots for collection starting at 05, 11, 17, and 23 h. Daily expression patterns of *bArr1* (b) and *bArr2* (c) mRNA levels in the Acb were measured by Illumina microarray and are presented as logarithmic values of the normalized expression intensities ($N = 6$ rats/ZT). Each black dot represents one animal. bArr2 shows opposed to bArr1 a diurnal expression pattern in the accumbens region

of abstinence in the active and inactive cycle. Interestingly, transcript levels of bArr2 studied by in situ hybridization were significantly up-regulated in ventral striatal brain region by 52% (nucleus accumbens shell [AcbS]: $F_{1,13} = 33.41$, $p < 0.001$) and 54% (nucleus accumbens core [AcbC]: $F_{1,13} = 12.97$, $p < 0.01$) during the active cycle of alcohol-dependent rats compared with controls, whereas no significant differences were found in the inactive phase (Figure 3d). Since the adaptor protein bArr2 is known to influence MOR function and trafficking (Bjork et al., 2013; Li et al., 2013), MOR cell surface receptor availability was assessed in alcohol-dependent rats. MOR binding sites as measured by saturated [^{3}H]-Damgo receptor autoradiography were significantly reduced in the active cycle by about 10% specifically in regions with increased bArr2 transcript levels (AcbS: $F_{1,10} = 6.24$, $p < 0.05$ and AcbC: $F_{1,10} = 7.15$, $p < 0.05$, Figure 3e). In accordance with bArr2, striatal MOR-binding sites in the inactive cycle were not altered compared with controls (mean control: 80.4 ± 4.54; mean alcohol dependent: 69.4 ± 5.37; $F_{1,10} = 2.46$, $p = 0.15$), confirming a circadian-regulated mechanism for both bArr2 and MOR.

Next, we studied the MOR function again in both the active and the inactive cycle. When comparing the accumulation of [^{35}S]-GTPγS, representing coupling of the receptor to intracellular G-proteins, the DAMGO stimulated [^{35}S]-GTPγS signal was increased in dependent animals compared with controls in both the active (249%, $F_{1,21} = 16.71$, $p < 0.001$) and the inactive (67%, $F_{1,20} = 136.91$, $p < 0.001$) cycle (Tables 1–2). In both cycles, we found increased coupling in the AcbS compared with the AcbC (main effect of region: active [$F_{1,21} = 17.97$, $p < 0.001$], inactive [$F_{1,20} = 52.89$, $p < 0.001$]). When analyzing the G-protein coupling data specifically in dependent animals, we found significantly lower coupling during the active cycle compared with the inactive cycle (main effect of cycle [$F_{1,21} = 11.24$, $p < 0.01$], Figure 3g), thus confirming the results observed with DAMGO receptor autoradiography. We also found a significant main effect of region ($F_{1,41} = 24.14$, $p < 0.001$) with higher coupling within the AcbC of alcohol-dependent animals, and no cycle x treatment interaction.

To test the hypothesis whether bArr2 could have a direct impact on MOR levels, we locally injected a recombinant AAV virus overexpressing bArr2 in the AcbC of naïve rats ($n = 4$). After a period of 5 weeks to allow for the expression of the transgene, rats were sacrificed in the active cycle (ZT 14) and measured for both the levels of bArr2 mRNA and MOR availability (Figure 4) with the same techniques (in situ hybridization and saturated [^{3}H]-DAMGO-binding assay) used for the previous measurements. As expected, we found a strong increase of bArr2 mRNA (172%, $p = 0.001$, Figure 4c) compared with the contralateral injected control virus site. In the same area, we observed a significant down-regulation of MORs (−44%, $p = 0.006$) after overexpressing Arrb2 (Figure 4c and d).

4 | DISCUSSION

The presented data suggest a so far unrecognized diurnal rhythm of bArr2 in the brain, resulting in increased transcript levels within the ventral striatum during the active cycle. This circadian bArr2 is disrupted in alcohol dependence showing an up-regulation in the active phase. The altered expression of bArr2 in alcohol dependence is accompanied by a down-regulation of MOR availability and functions. Using a genetic overexpression technique, we established a causal link between bArr2 abundance and the internalization of MOR.

Here we show that bArr2, but not bArr1, has a daily oscillating expression, with a peak at the beginning of the active cycle, along with several other core clock genes (published in Stählin, 2013). So far, it has not been known that bArr2 is regulated by the circadian clock. Both bArr1 and bArr2 genes could potentially be modulated by some of the clock genes' products, for example, Bmal1 (Arntl), which binds to an E-box motif (sequence: CACGTG). Using the Eukaryotic Promoter Database (EPD) and JASPAR (a database of transcription factors binding motifs), we predicted at least two possible Bmal1-binding sites in both the Arrb1 promoter (at −262 and at −240 bp) and Arrb2 promoter (at −430 and −17 bp) of the

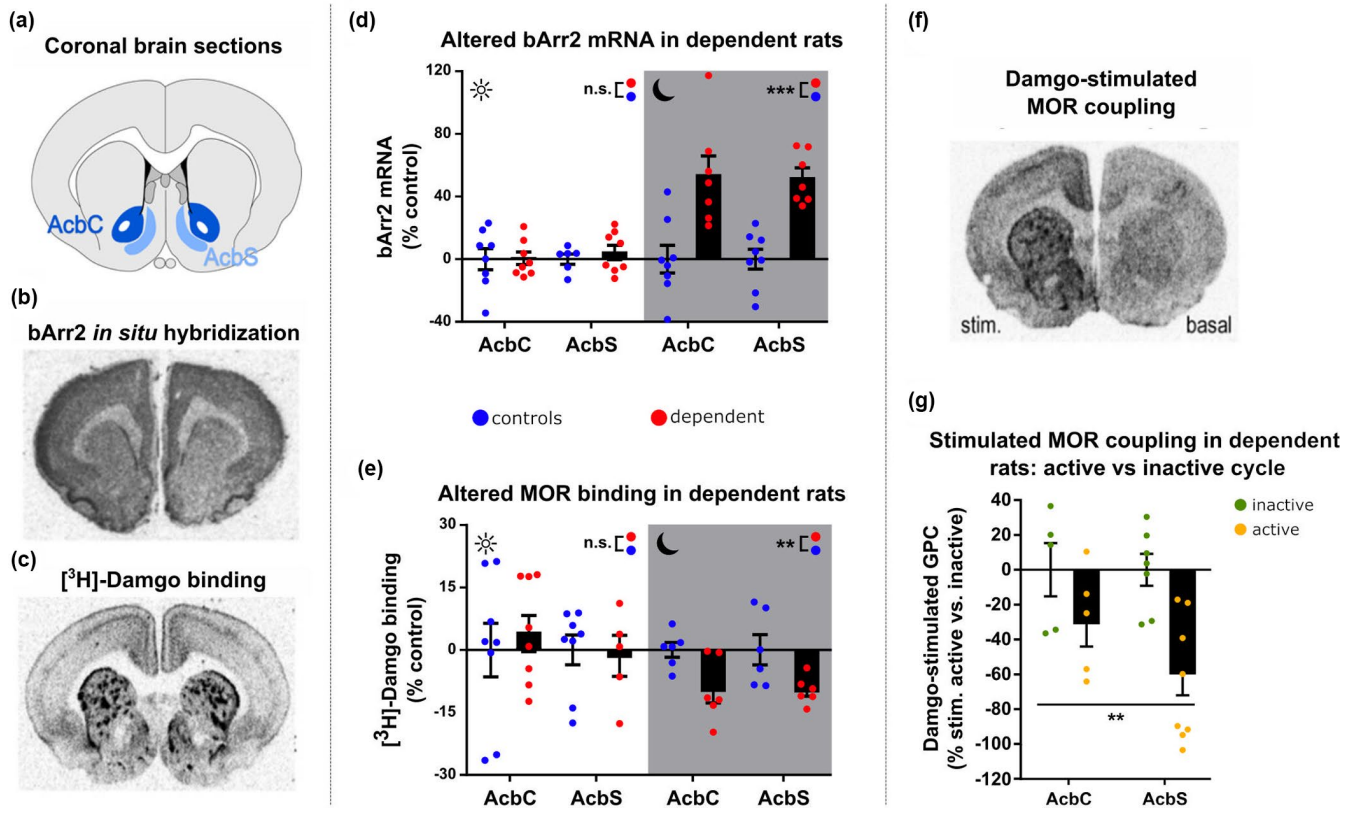

FIGURE 3 MOR-binding sites are significantly decreased in regions with increased bArr2 transcript levels in the active cycle along with decreased coupling of MOR in the active cycle within the nucleus accumbens. (a) Schematic illustration of coronal rat brain sections with regions analyzed according to Paxinos and Watson (1998). Representative autoradiogram showing the expression pattern of bArr2 mRNA (b) and MOR-binding sites (c) in the striatal region. (d) bArr2 mRNA levels were measured by in situ hybridization and are presented as % control ± SEM ($N = 8$/group). Levels are strongly increased in the nucleus accumbens shell (AcbS) and core (AcbC) of alcohol-dependent animals. In the same region, a significant reduction of MOR (e) was detected indicating a role of bArr2 in MOR regulation. MOR-binding sites were measured by [^{3}H]-Damgo receptor autoradiography and are here represented as normalized data compared with control animals (% control ± SEM). (f) The specific distribution of [^{35}S]-GTPγS accumulation in the presence of Damgo stimulation in the striatum (left image) and without stimulation (right image) is shown in the representative autoradiogram. (g) G-protein coupling of the MOR is significantly lower in the active cycle compared with the inactive cycle in dependent rats. Graphs in d and e: dots colored in blue (●) represent the control group, dots colored in red (●) represent dependent rats; in (g): dots colored in green (●) represent alcohol-dependent rats in the inactive cycle; dots colored in orange (●) represent dependent rats in the active cycle. Asterisks indicate the main effect of treatment analyzed by two-way ANOVA (**$p < 0.01$, ***$p < 0.001$)

rat genome (Rattus norvegicus), using a cut-off (p value) of 0.001. The E-box in position −262 of Arrb1 promoter and the E-box in position −430 of Arrb2 promoter were also identified as possible binding sites for Clock protein using the same cutoff. Considering that Bmal1 and Clock often work together forming the heterodimer CLOCK::BMAL1 (Huang et al., 2012), these two E-box motifs represent potential binding sites for Bmal1 and Clock. Clearly, the mere presence of such motifs does not mean that CLOCK::BMAL1 actually recognizes and binds them *in vivo*, this needs to be verified via specific experimental procedures (e.g., Chip-Seq), as a prediction of transcription factor binding has a high rate of false positives. It is also important to consider that CLOCK::BMAL1 can also bind to different regions than promoters (e.g., cis-acting elements localized within introns or far away from the promoter) and can recognize and bind non-canonical E-box sequences (Yoshitane et al., 2014). Furthermore, CLOCK::BMAL1 complex has been recently proposed to be mainly involved not in direct activation of target genes but rather in indirect activation/repression of target genes through modification of the chromatin state, allowing for "secondary" modulatory proteins to intervene (Trott & Menet, 2018). This would explain why many CCGs, even though they are bound by CLOCK::BMAL1 within the same time window in almost all tissues, are differentially regulated and, in our specific case, it would give a plausible explanation for why, even though both Arrb1 and Arrb2 seem to have E-box motifs, only bArr2 shows a diurnal expression pattern in the Acb. This is not new, as other studies have found the same protein to have or not have a diurnal expression pattern depending on the tissue analyzed: in a recent study by Mavroudis et al. (2018), for example, in which a mathematical tool (JTK_CYCLE algorithm) used to recognize circadian patterns was applied on microarray data taken from four different rat tissues (lung, muscle, adipose, and liver), both bArr1 and bArr2

TABLE 1 DAMGO-stimulated MOR signaling in the nucleus accumbens in dependent and non-dependent rats

Region	Cycle	Treatment group	[35S]-GTPgS baseline (nCi/g)	[35S]-GTPgS % stimulated	*N*
AcbC	Active	Control	590.68 ± 30.19	14.40 ± 1.25	6
		Dependent	964.85 ± 50.47	49.04 ± 6.82	5
	Inactive	Control	558.94 ± 10.31	6.30 ± 0.53	6
		Dependent	516.82 ± 29.26	27.90 ± 3.22	5
AcbS	Active	Control	584.14 ± 17.99	50.31 ± 1.54	6
		Dependent	656.09 ± 21.73	84.12 ± 11.87	8
	Inactive	Control	573.95 ± 12.57	17.55 ± 1.55	6
		Dependent	550.51 ± 25.33	50.63 ± 2.92	7

Data are expressed as mean ± SEM, *N* = 5–8/group. For details of treatment, see Materials and Methods. AcbC, nucleus accumbens core; AcbS, nucleus accumbens shell; SEM, standard error of the mean; *N*, sample size.

TABLE 2 Statistical analysis of DAMGO-stimulated MOR signaling in the nucleus accumbens in dependent and non-dependent rats

Cycle	Factor	df	MS	*F*	*p*	Partial η^2
Active	Region	1	7652.9	17.97	0.0004	0.46
	Treatment	1	7115.66	16.71	0.0005	0.44
	Region × Treatment	1	1.04	0.002	1.0	0.0001
Inactive	Region	1	1707.99	52.89	0.0000	0.73
	Treatment	1	4420.83	136.91	0.0000	0.87
	Region × Treatment	1	194.51	6.02	0.02	0.23

The overall effect of two-way ANOVA (treatment, region) for each cycle. Df, degrees of freedom; MS, mean square; partial η^2, the measure of effect size.

were found to show a circadian expression pattern in the lung tissue but not in the others.

We could then demonstrate that bArr2 does not only show a diurnal rhythm but that bArr2 is up-regulated in the first half of the active cycle in alcohol dependence compared with controls. We focused on this time period since previous large-scale microarray data in the nucleus accumbens, investigating the circadian rhythm in short- and long-term alcohol-drinking rats (Stählin, 2013), showed that this was the main period of alcohol-induced alterations. The finding of up-regulated bArr2 is accompanied by a down-regulation of MOR binding in the active cycle as well as a decreased Damgo-stimulated [^{35}S]-GTPγS accumulation, suggesting a role of bArr2 in the development and maintenance of these neuroadaptations.

Unexpectedly, however, we found MOR G-protein coupling to be generally increased in alcohol-dependent rats compared with controls, suggesting a possible not yet characterized hypersensitization state of the MORs during alcohol abstinence which may lead to an enhanced MOR response or signaling upon an opioidergic stimulation (arising, for example, from an alcohol challenge), even with reduced MOR availability on the cell surface.

Here we studied alcohol-induced dysregulations in rats with a history of alcohol dependence, that is, by exposure to daily cycles of intermittent alcohol vapor intoxication and withdrawal, a paradigm that produces high intoxication with brain alcohol levels above 200 mg/dl and induces behavioral and molecular changes relevant for the pathophysiology of alcoholism in both rats and mice (Becker & Lopez, 2004; Hansson et al., 2008; Melendez et al., 2012; O'Dell et al., 2004; Rimondini et al., 2002, 2003, 2008; Roberts et al., 2000; Rogers et al., 1979; Sommer et al., 2008). Animals derived from this procedure are termed "post-dependent" to emphasize the fact that neuroadaptations induced through a history of alcohol dependence remain even in the absence of continued ethanol intoxication. Notwithstanding the apparent lack of face validity, this procedure has consistently shown to produce long-lasting "addiction-like" behaviors as well as pronounced molecular changes in all major domains of the addiction circuitry, that is, in motivational (Hirth et al., 2016), emotional (Sommer et al., 2008), and cognitive circuits (Meinhardt et al., 2013, 2021). In this sense, post-dependent animals may model the increased propensity to relapse in abstinent alcoholic patients reviewed in Meinhardt and Sommer (2015).

In our study, we used for the molecular analysis the 3-week abstinence time point. In numerous well-cited review articles, we have chosen the same time point for both behavioral and molecular analyses (Hirth et al., 2016; Meinhardt et al., 2013; Sommer et al., 2008; Uhrig et al., 2017) and thus can compare present results with a wealth of data.

β-Arrestins (bArrs) are known for mediating the desensitization and internalization processes of MORs, but the underlying mechanism seems to be dependent on the ligand. For example, DAMGO (a selective MOR agonist) can strongly induce rapid internalization of MOR, while morphine (also a MOR agonist) does not (Keith et al., 1998). Our understanding of the molecular role played by bArrs

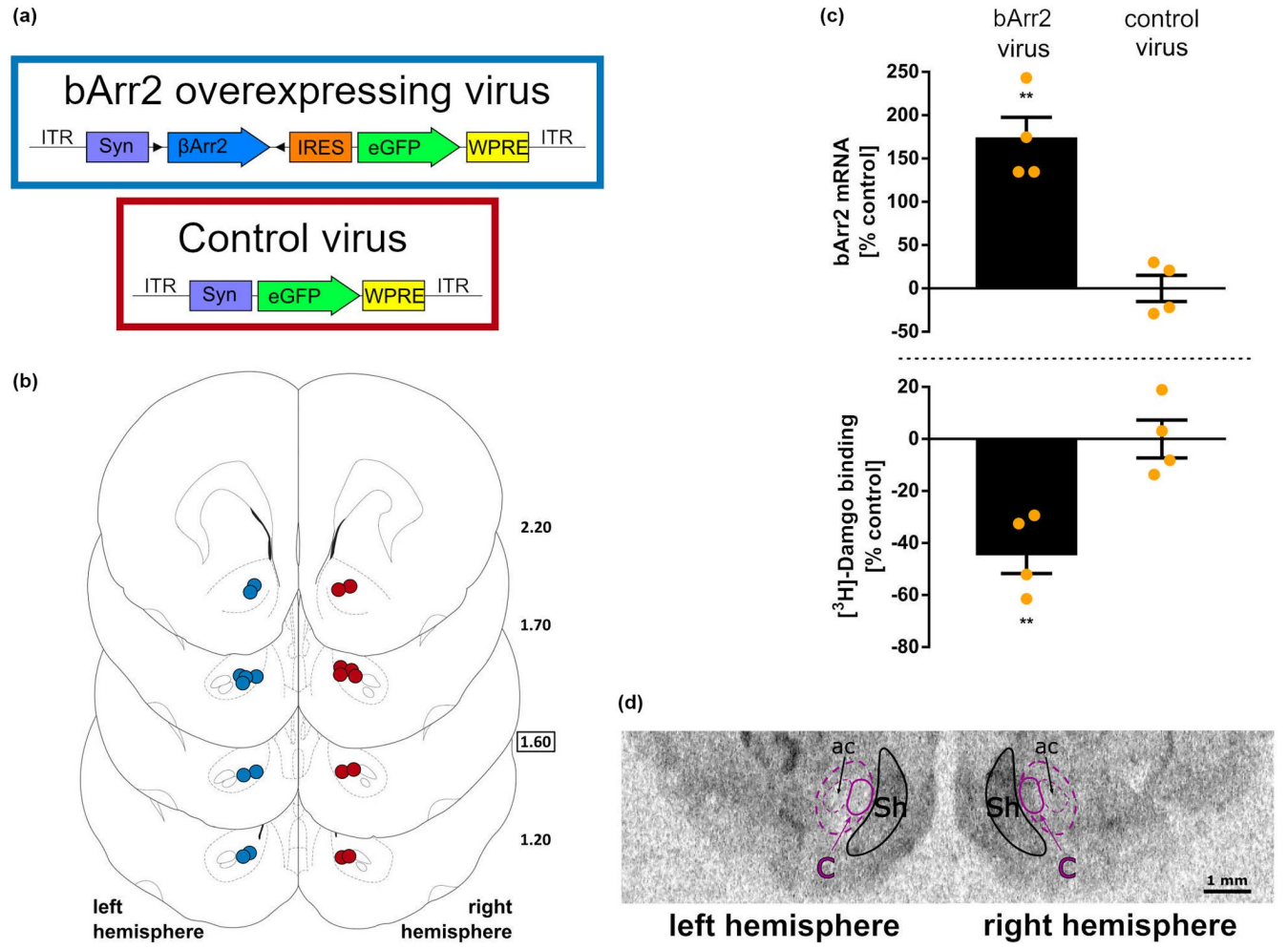

FIGURE 4 AAV-induced bArr2 overexpression causes a reduction of MOR levels. Two different viruses were injected in the right and left AcbC of naïve SD rats ($n = 4$): (a) bArr2-overexpressing AAV with the bArr2 open-reading frame fragment under the control of a synapsin promoter (left hemisphere), and a control virus lacking the bArr2 fragment (right hemisphere). (b) Schematic illustration showing the average hole punch locations. (c) Data of bArr2 mRNA levels (upper graph) and MOR-binding sites (lower graph) are presented as percentage over control ± SEM, $^{**}p < 0.01$. Dots in orange indicate single data points. (d) A decrease in MOR density is shown in the left hemisphere compared with the right hemisphere. ac, anterior commissure; Sh, nucleus accumbens shell; C, nucleus accumbens core

mostly comes from in vitro studies on cell lines, these have shown that bArrs can induce MOR internalization into endocytotic vesicles upon opioid agonists stimulation (Barak et al., 1997; Ferguson et al., 1996; Lowther et al., 2013; Whistler & von Zastrow, 1998); we also know that bArr1, when overexpressed, can enhance the internalization process of MORs upon DAMGO stimulation and even induce internalization upon morphine stimulation (Whistler & von Zastrow, 1998), which (as mentioned) does not recruit bArrs under normal circumstances; finally, we know that bArr2 has a higher affinity for MORs than bArr1 (Oakley et al., 2000). Our bArr2 overexpression experiment is the first, to our knowledge, where bArr2–MOR interaction was specifically assessed in situ on brain sections. We did not expect major differences in bArr2–MOR interaction in the subregion of the accumbens and thus focused on AcbC. Our findings confirm that bArr2 mediates MORs internalization in the AcbC, as local bArr2 overexpression causes a reduction (1.8 fold) of MOR-binding sites in this region. Importantly, the study of Björk et al. (2008) showed that elevated bArr2 transcript levels potentially translate into increased bArr2 protein. This suggests that besides bArr2 transcript levels also bArr2 protein levels are increased in alcohol-dependent animals. Elevated bArr2 protein availability might facilitate rapid MOR desensitization and internalization upon receptor activation, thereby resulting in reduced cell surface receptors; a potential molecular mechanism that triggers excessive alcohol drinking and dependence. This conclusion is supported by the evidence that high levels of bArr2 have been shown to lead to increased alcohol intake, and eliminating the protein reduces alcohol consumption (Bjork et al., 2008). However, future studies need to investigate whether modulating accumbal bArr2 has an impact on MOR availability and alcohol-related phenotypes in animal models of alcohol dependence such as the CIE model.

Increased MOR internalization has also been linked to elevated bArr2 immunoreactivity in rats after natural reward (Garduno-Gutierrez et al., 2013). However, the regulation of GCPRs by Arrb2 is

highly complex. For example, it has been shown that both the opioid and dopamine receptor signaling is influenced by multidimensional triggers from various potential signaling cascades (Zhou & Bohn, 2014). Also, we previously found that MOR availability is reduced in 3-week abstinent rats (Hansson et al., 2019). Thus, regulation of MOR on protein level via bArr2 seems plausible. In the same study from Hansson et al. (2019), we found that MOR is dynamically regulated in the course of the addiction cycle. We thus assume that bARR2 is similar but opposite regulated to MOR.

The observed results of down-regulated MOR are in line with recent clinical data. Hermann and colleagues observed a reduction of MOR-binding sites in post-mortem striatal tissue of alcoholics as well as a significant association between low [^{11}C]carfentanil positron emission tomography signal in the ventral striatum and higher relapse risk of alcoholics (Hermann et al., 2017). The same study also provides a meta-analysis of rodent data, including 233 rats showing a striatal down-regulation of MOR binding during the first 3 days of alcohol withdrawal and in protracted abstinence. The reduction of MOR binding can be interpreted as an adaptive mechanism of the brain in response to increased frequency and strength of opioidergic neurotransmission because of chronic alcohol consumption. The decreased MOR availability may be responsible for tolerance to the rewarding effects of alcohol and thus leading to further enhanced alcohol intake. In withdrawal states, the release of endogenous opioids because of alcohol consumption is suddenly interrupted, whereas MOR function is still down-regulated. This deficient μ-opioid system may then contribute to the anhedonic state in early abstinence, characterized by dysphoria, increased anxiety, and depressiveness (Heilig et al., 2010). However, the striking finding of our study is that MOR binding as well as a function varies within a day and follows a circadian rhythm. MOR binding and function were only found to be down-regulated in the active phase. Interestingly, alcohol consumption in rodents is modulated by the animals' internal clock so that animals drink more in the active phase (Spanagel et al., 2005) and this circadian rhythm of the behavior is lost during the development of addictive drinking (Spanagel et al., 2005). Together, increased circadian amplitude of MOR expression and decreased coupling of MOR at the beginning of the active cycle can be hypothesized as an adaptive mechanism, leading to a desensitization of alcohols' rewarding effects, which may further enhance excessive alcohol intake to maintain the level of hedonic response.

Other previous studies suggested an interaction between alcohol, clock genes, and the μ-opioid system. For example, in ß-endorphin-expressing neurons, ß-endorphin being the primary endogenous ligand for MOR, prenatal alcohol exposure was able to alter the expression of Per2 (Chen et al., 2006). In addition, Per2 mutant mice are impaired in ethanol-induced ß-endorphin neuron activation (Agapito et al., 2010) and mice exposed to alcohol prenatally show altered Per2 driven ß-endorphin release during stress exposure (Sarkar et al., 2007).

There are several limitations in the current study. One limitation is that we have separately performed experiments during the dark and light cycles in Experiments 2 and 3, respectively. Combining experiments for both cycles would have been most appropriate in order to directly compare cycle effects by three-way ANOVA. Another limitation is that only male rats were used. Since preclinical and clinical evidence suggests that sex influences disease trajectories and interventions in alcohol-dependent patients, it is mandatory to compare both sexes in preclinical research (Hansson & Spanagel, 2021). We also used a small sample size (N = 6–8/group) in the absence of a replication experiment of a different cohort. Finally, one potential limitation of the viral injection experiment is that injections were not counterbalanced between the hemispheres. Although here a small sample size (N = 4) has been used, we found convincing evidence on a direct link between bArr2 levels and MOR availability.

Future experiments may involve a behavioral battery exploring how the modulation of bArr2 expression might affect MOR in the context of alcohol dependence. In addition to our finding on circadian regulation of both bArr2 and MOR, future experiments may involve naltrexone treatment responses in abstinent rats during different times of the day. This may explain—at least in part—different treatment efficacies in human alcoholics.

Taken together, we conclude that alcohol seems to have a strong impact on bArr2, which in turn regulates the availability and activity of the MORs, adding further to the complexity of MOR as a target for pharmacotherapeutic interventions in alcoholism and is likely source of the highly variable outcomes of naltrexone. In fact, naltrexone and nalmefene have been reported to have detrimental effects on sleep affecting the clinical outcome of AUD treatment (Panin & Peana, 2019). Altogether, these data describe a new molecular mechanism, suggesting a critical role for bArr2 within the mesocorticolimbic system in alcohol dependence which may have implications for the development of novel bArr2-related treatment targets for alcoholism.

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CONFLICT OF INTEREST

The authors declare no competing financial interests. Rainer Spanagel is a former Handling Editor for the *Journal of Neurochemistry*.

AUTHOR CONTRIBUTIONS

RS, WHS, SMS, and ACH were responsible for study design and procured study funding. FG and NH performed in situ hybridization, receptor binding, and receptor coupling experiments. RS provided microarray data. DB generated bArr2 overexpression construct, WK provided AAV virus. MWM, FG, NH, and ACH analyzed data. MWM, FG, WHS, and ACH wrote the manuscript.

OPEN RESEARCH BADGES

This article has earned an Open Materials badge for making publicly available the components of the research methodology needed to reproduce the reported procedure and analysis. All materials are available at: https://doi.org/10.5281/zenodo.5769565.

DATA AVAILABILITY STATEMENT

Data available on request from the authors.

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REFERENCES

Agapito, M., Mian, N., Boyadjieva, N. I., & Sarkar, D. K. (2010). Period 2 gene deletion abolishes beta-endorphin neuronal response to ethanol. *Alcoholism, Clinical and Experimental Research*, 34, 1613–1618.

Barak, L. S., Ferguson, S. S., Zhang, J., & Caron, M. G. (1997). A beta-arrestin/green fluorescent protein biosensor for detecting G protein-coupled receptor activation. *Journal of Biological Chemistry*, 272, 27497–27500.

Battelle, B. A., Williams, C. D., Schremser-Berlin, J. L., & Cacciatore, C. (2000). Regulation of arrestin mRNA levels in Limulus lateral eye: Separate and combined influences of circadian efferent input and light. *Visual Neuroscience*, 17, 217–227.

Becker, H. C., & Lopez, M. F. (2004). Increased ethanol drinking after repeated chronic ethanol exposure and withdrawal experience in C57BL/6 mice. *Alcoholism, Clinical and Experimental Research*, 28, 1829–1838. https://doi.org/10.1097/01.ALC.0000149977.95306.3A

Bjork, K., Rimondini, R., Hansson, A. C., Terasmaa, A., Hyytia, P., Heilig, M., & Sommer, W. H. (2008). Modulation of voluntary ethanol consumption by beta-arrestin 2. *FASEB Journal*, 22(7), 2552–2560. https://doi.org/10.1096/fj.07-102442

Bjork, K., & Svenningsson, P. (2011). Modulation of monoamine receptors by adaptor proteins and lipid rafts: Role in some effects of centrally acting drugs and therapeutic agents. *Annual Review of Pharmacology and Toxicology*, 51, 211–242. https://doi.org/10.1146/annurev-pharmtox-010510-100520

Bjork, K., Tronci, V., Thorsell, A., Tanda, G., Hirth, N., Heilig, M., Hansson, A. C., & Sommer, W. H. (2013). Beta-Arrestin 2 knockout mice exhibit sensitized dopamine release and increased reward in response to a low dose of alcohol. *Psychopharmacology (Berl)*, 230(3), 439–449.

Bockaert, J., Perroy, J., Becamel, C., Marin, P., & Fagni, L. (2010). GPCR interacting proteins (GIPs) in the nervous system: Roles in physiology and pathologies. *Annual Review of Pharmacology and Toxicology*, 50, 89–109. https://doi.org/10.1146/annurev.pharmtox.010909.105705

Bowes, C., van Veen, T., & Farber, D. B. (1988). Opsin, G-protein and 48-kDa protein in normal and rd mouse retinas: Developmental expression of mRNAs and proteins and light/dark cycling of mRNAs. *Experimental Eye Research*, 47, 369–390. https://doi.org/10.1016/0014-4835(88)90049-8

Brower, K. J. (2003). Insomnia, alcoholism and relapse. *Sleep Medicine Reviews*, 7, 523–539. https://doi.org/10.1016/S1087-0792(03)90005-0

Brower, K. J., Aldrich, M. S., Robinson, E. A., Zucker, R. A., & Greden, J. F. (2001). Insomnia, self-medication, and relapse to alcoholism. *American Journal of Psychiatry*, 158, 399–404. https://doi.org/10.1176/appi.ajp.158.3.399

Chen, C. P., Kuhn, P., Advis, J. P., & Sarkar, D. K. (2006). Prenatal ethanol exposure alters the expression of period genes governing the circadian function of beta-endorphin neurons in the hypothalamus. *Journal of Neurochemistry*, 97, 1026–1033. https://doi.org/10.1111/j.1471-4159.2006.03839.x

Chomczynski, P., & Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analytical Biochemistry*, 162, 156–159. https://doi.org/10.1016/0003-2697(87)90021-2

Conroy, D. A., Hairston, I. S., Arnedt, J. T., Hoffmann, R. F., Armitage, R., & Brower, K. J. (2012). Dim light melatonin onset in alcohol-dependent men and women compared with healthy controls. *Chronobiology International*, 29, 35–42. https://doi.org/10.3109/07420528.2011.636852

Craft, C. M., Whitmore, D. H., & Donoso, L. A. (1990). Differential expression of mRNA and protein encoding retinal and pineal S-antigen during the light/dark cycle. *Journal of Neurochemistry*, 55, 1461–1473. https://doi.org/10.1111/j.1471-4159.1990.tb04927.x

Danel, T., Libersa, C., & Touitou, Y. (2001). The effect of alcohol consumption on the circadian control of human core body temperature is time dependent. *American Journal of Physiology: Regulatory, Integrative and Comparative Physiology*, 281, R52–55. https://doi.org/10.1152/ajpregu.2001.281.1.R52

Fakier, N., & Wild, L. G. (2011). Associations among sleep problems, learning difficulties and substance use in adolescence. *Journal of Adolescence*, 34, 717–726. https://doi.org/10.1016/j.adolescence.2010.09.010

Feige, B., Gann, H., Brueck, R., Hornyak, M., Litsch, S., Hohagen, F., & Riemann, D. (2006). Effects of alcohol on polysomnographically recorded sleep in healthy subjects. *Alcoholism, Clinical and Experimental Research*, 30, 1527–1537. https://doi.org/10.1111/j.1530-0277.2006.00184.x

Ferguson, S. S., Downey, W. E. 3rd, Colapietro, A. M., Barak, L. S., Menard, L., & Caron, M. G. (1996). Role of beta-arrestin in mediating agonist-promoted G protein-coupled receptor internalization. *Science*, 271, 363–366.

Garduno-Gutierrez, R., Leon-Olea, M., & Rodriguez-Manzo, G. (2013). Different amounts of ejaculatory activity, a natural rewarding behavior, induce differential mu and delta opioid receptor internalization in the rat's ventral tegmental area. *Brain Research*, 1541, 22–32. https://doi.org/10.1016/j.brainres.2013.10.015

Gerstner, J. R., & Yin, J. C. (2010). Circadian rhythms and memory formation. *Nature Reviews Neuroscience*, 11(8), 577–588. https://doi.org/10.1038/nrn2881

Goldstein, D. B., & Pal, N. (1971). Alcohol dependence produced in mice by inhalation of ethanol: Grading the withdrawal reaction. *Science*, 172, 288–290. https://doi.org/10.1126/science.172.3980.288

Haberstock-Debic, H., Kim, K. A., Yu, Y. J., & von Zastrow, M. (2005). Morphine promotes rapid, arrestin-dependent endocytosis of mu-opioid receptors in striatal neurons. *Journal of Neuroscience*, 25, 7847–7857.

Halbout, B., Perreau-Lenz, S., Dixon, C. I., Stephens, D. N., & Spanagel, R. (2011). Per1(Brdm1) mice self-administer cocaine and reinstate cocaine-seeking behaviour following extinction. *Behavioural*

Pharmacology, 22, 76–80. https://doi.org/10.1097/FBP.0b013e328341e9ca

Hansson, A. C., Grunder, G., Hirth, N., Noori, H. R., Spanagel, R., & Sommer, W. H. (2019). Dopamine and opioid systems adaptation in alcoholism revisited: Convergent evidence from positron emission tomography and postmortem studies. *Neuroscience and Biobehavioral Reviews*, 106, 141–164. https://doi.org/10.1016/j.neubiorev.2018.09.010

Hansson, A. C., Rimondini, R., Neznanova, O., Sommer, W. H., & Heilig, M. (2008). Neuroplasticity in brain reward circuitry following a history of ethanol dependence. *European Journal of Neuroscience*, 27, 1912–1922. https://doi.org/10.1111/j.1460-9568.2008.06159.x

Hansson, A. C., & Spanagel, R. (2021). No changes in the oxytocin system in alcohol-dependent female rodents and humans: Towards a sex-specific psychopharmacology in alcoholism. *Addiction Biology*, 26, e12945. https://doi.org/10.1111/adb.12945

Heilig, M., Augier, E., Pfarr, S., & Sommer, W. H. (2019). Developing neuroscience-based treatments for alcohol addiction: A matter of choice? *Translational Psychiatry*, 9, 255. https://doi.org/10.1038/s41398-019-0591-6

Heilig, M., Egli, M., Crabbe, J. C., & Becker, H. C. (2010). Acute withdrawal, protracted abstinence and negative affect in alcoholism: Are they linked? *Addiction Biology*, 15, 169–184. https://doi.org/10.1111/j.1369-1600.2009.00194.x

Heinz, A., Kiefer, F., Smolka, M. N., Endrass, T., Beste, C., Beck, A., Liu, S., Genauck, A., Romund, L., Banaschewski, T., Bermpohl, F., Deserno, L., Dolan, R. J., Durstewitz, D., Ebner-Priemer, U., Flor, H., Hansson, A. C., Heim, C., Hermann, D., … Spanagel, R. (2020). Addiction research consortium: Losing and regaining control over drug intake (ReCoDe)-From trajectories to mechanisms and interventions. *Addiction Biology*, 25, e12866. https://doi.org/10.1111/adb.12866

Hermann, D., Hirth, N., Reimold, M. et al (2017). Low mu-opioid receptor status in alcohol dependence identified by combined positron emission tomography and post-mortem brain analysis. *Neuropsychopharmacology*, 42, 606–614.

Hirth, N., Meinhardt, M. W., Noori, H. R., Salgado, H., Torres-Ramirez, O., Uhrig, S., Broccoli, L., Vengeliene, V., Roßmanith, M., Perreau-Lenz, S., Köhr, G., Sommer, W. H., Spanagel, R., & Hansson, A. C. (2016). Convergent evidence from alcohol-dependent humans and rats for a hyperdopaminergic state in protracted abstinence. *Proceedings of the National Academy of Sciences*, 113, 3024–3029. https://doi.org/10.1073/pnas.1506012113

Huang, N., Chelliah, Y., Shan, Y., Taylor, C. A., Yoo, S. H., Partch, C., Green, C. B., Zhang, H., & Takahashi, J. S. (2012). Crystal structure of the heterodimeric CLOCK:BMAL1 transcriptional activator complex. *Science*, 337, 189–194. https://doi.org/10.1126/science.1222804

Imperato, A., & Di Chiara, G. (1986). Preferential stimulation of dopamine release in the nucleus accumbens of freely moving rats by ethanol. *Journal of Pharmacology and Experimental Therapeutics*, 239, 219–228.

Johnson, S. W., & North, R. A. (1992). Opioids excite dopamine neurons by hyperpolarization of local interneurons. *Journal of Neuroscience*, 12, 483–488. https://doi.org/10.1523/JNEUROSCI.12-02-00483.1992

Jonas, D. E., Amick, H. R., Feltner, C., Bobashev, G., Thomas, K., Wines, R., Kim, M. M., Shanahan, E., Gass, C. E., Rowe, C. J., & Garbutt, J. C. (2014). Pharmacotherapy for adults with alcohol use disorders in outpatient settings: A systematic review and meta-analysis. *JAMA*, 311(18), 1889–1900. https://doi.org/10.1001/jama.2014.3628

Keith, D. E., Anton, B., Murray, S. R. et al (1998). mu-Opioid receptor internalization: Opiate drugs have differential effects on a conserved endocytic mechanism in vitro and in the mammalian brain. *Molecular Pharmacology*, 53, 377–384.

Kovanen, L., Saarikoski, S. T., Haukka, J., Pirkola, S., Aromaa, A., Lonnqvist, J., & Partonen, T. (2010). Circadian clock gene polymorphisms in alcohol use disorders and alcohol consumption. *Alcohol and Alcoholism*, 45, 303–311. https://doi.org/10.1093/alcalc/agq035

Li, H., Tao, Y., Ma, L., Liu, X., & Ma, L. (2013). Beta-Arrestin-2 inhibits preference for alcohol in mice and suppresses Akt signaling in the dorsal striatum. *Neuroscience Bulletin*, 29(5), 531–540.

Logan, R. W., Williams, W. P. 3rd, & McClung, C. A. (2014). Circadian rhythms and addiction: Mechanistic insights and future directions. *Behavioral Neuroscience*, 128, 387–412. https://doi.org/10.1037/a0036268

Lowther, K. M., Uliasz, T. F., Gotz, K. R., Nikolaev, V. O., & Mehlmann, L. M. (2013). Regulation of constitutive GPR3 signaling and surface localization by GRK2 and beta-arrestin-2 Overexpression in HEK293 cells. *PLoS One*, 8, e65365.

MacLean, A. W., & Cairns, J. (1982). Dose-response effects of ethanol on the sleep of young men. *Journal of Studies on Alcohol*, 43(5), 434–444. https://doi.org/10.15288/jsa.1982.43.434

Manev, H., & Uz, T. (2006). Clock genes: Influencing and being influenced by psychoactive drugs. *Trends in Pharmacological Sciences*, 27, 186–189. https://doi.org/10.1016/j.tips.2006.02.003

Mavroudis, P. D., DuBois, D. C., Almon, R. R., & Jusko, W. J. (2018). Daily variation of gene expression in diverse rat tissues. *PLoS One*, 13, e0197258. https://doi.org/10.1371/journal.pone.0197258

McGinnis, J. F., Whelan, J. P., & Donoso, L. A. (1992). Transient, cyclic changes in mouse visual cell gene products during the light-dark cycle. *Journal of Neuroscience Research*, 31, 584–590. https://doi.org/10.1002/jnr.490310325

Meinhardt, M. W., Hansson, A. C., Perreau-Lenz, S., Bauder-Wenz, C., Stahlin, O., Heilig, M., Harper, C., Drescher, K. U., Spanagel, R., & Sommer, W. H. (2013). Rescue of infralimbic mGluR2 deficit restores control over drug-seeking behavior in alcohol dependence. *Journal of Neuroscience*, 33, 2794–2806. https://doi.org/10.1523/JNEUROSCI.4062-12.2013

Meinhardt, M. W., Pfarr, S., Fouquet, G., Rohleder, C., Meinhardt, M. L., Barroso-Flores, J., Hoffmann, R., Jeanblanc, J., Paul, E., Wagner, K., Hansson, A. C., Köhr, G., Meier, N., von Bohlen und Halbach, O., Bell, R. L., Endepols, H., Neumaier, B., Schönig, K., Bartsch, D., … Sommer, W. H. (2021). Psilocybin targets a common molecular mechanism for cognitive impairment and increased craving in alcoholism. *Science Advances*, 7(47), 1–13. https://doi.org/10.1126/sciadv.abh2399

Meinhardt, M. W., & Sommer, W. H. (2015). Postdependent state in rats as a model for medication development in alcoholism. *Addiction Biology*, 20, 1–21. https://doi.org/10.1111/adb.12187

Melendez, R. I., McGinty, J. F., Kalivas, P. W., & Becker, H. C. (2012). Brain region-specific gene expression changes after chronic intermittent ethanol exposure and early withdrawal in C57BL/6J mice. *Addiction Biology*, 17, 351–364. https://doi.org/10.1111/j.1369-1600.2011.00357.x

Mitchell, J. M., O'Neil, J. P., Janabi, M., Marks, S. M., Jagust, W. J., & Fields, H. L. (2012). Alcohol consumption induces endogenous opioid release in the human orbitofrontal cortex and nucleus accumbens. *Science Translational Medicine*, 4, 116ra116. https://doi.org/10.1126/scitranslmed.3002902

Mitchell, V., Prevot, V., & Beauvillain, J. C. (1998). Distribution and diurnal variations of the mu opioid receptor expression in the arcuate nucleus of the male rat. *Neuroendocrinology*, 67, 94–100. https://doi.org/10.1159/000054303

Monory, K., Massa, F., Egertová, M., Eder, M., Blaudzun, H., Westenbroek, R., Kelsch, W., Jacob, W., Marsch, R., Ekker, M., Long, J., Rubenstein, J. L., Goebbels, S., Nave, K. A., During, M., Klugnann, M., Wölfel, B., Dodt, H. U., Zieglgänsberger, W., … Lutz, B. (2006). The endocannabinoid system controls key epileptogenic circuits in the hippocampus. *Neuron*, 51(4), 455–466.

Oakley, R. H., Laporte, S. A., Holt, J. A., Caron, M. G., & Barak, L. S. (2000). Differential affinities of visual arrestin, beta arrestin1, and beta arrestin2 for G protein-coupled receptors delineate two

major classes of receptors. *Journal of Biological Chemistry*, 275, 17201–17210.

O'Dell, L. E., Roberts, A. J., Smith, R. T., & Koob, G. F. (2004). Enhanced alcohol self-administration after intermittent versus continuous alcohol vapor exposure. *Alcoholism, Clinical and Experimental Research*, 28, 1676–1682. https://doi.org/10.1097/01.ALC.0000145781.11923.4E

Panin, F., & Peana, A. T. (2019). Sleep and the pharmacotherapy of alcohol use disorder: Unfortunate bedfellows. A Systematic Review with Meta-Analysis. *Frontiers in Pharmacology*, 10, 1164. https://doi.org/10.3389/fphar.2019.01164

Partch, C. L., Green, C. B., & Takahashi, J. S. (2014). Molecular architecture of the mammalian circadian clock. *Trends in Cell Biology*, 24, 90–99. https://doi.org/10.1016/j.tcb.2013.07.002

Paxinos, G., & Watson, C. (1998). *The rat brain in stereotaxic coordinates* (4th ed., pp 1–237). Academic Press.

Perreau-Lenz, S., & Spanagel, R. (2008). The effects of drugs of abuse on clock genes. *Drug News & Perspectives*, 21, 211–217. https://doi.org/10.1358/dnp.2008.21.4.1213350

Porter-Stransky, K. A., Petko, A. K., Karne, S. L., Liles, L. C., Urs, N. M., Caron, M. G., Paladini, C. A., & Weinshenker, D. (2020). Loss of beta-arrestin2 in D2 cells alters neuronal excitability in the nucleus accumbens and behavioral responses to psychostimulants and opioids. *Addiction Biology*, 25, e12823.

Porter-Stransky, K. A., & Weinshenker, D. (2017). Arresting the development of addiction: The role of beta-Arrestin 2 in drug abuse. *Journal of Pharmacology and Experimental Therapeutics*, 361, 341–348.

Rachdaoui, N., & Sarkar, D. K. (2013). Effects of alcohol on the endocrine system. *Endocrinology and Metabolism Clinics of North America*, 42, 593–615. https://doi.org/10.1016/j.ecl.2013.05.008

Ramchandani, V. A., Umhau, J., Pavon, F. J., Ruiz-Velasco, V., Margas, W., Sun, H., Damadzic, R., Eskay, R., Schoor, M., Thorsell, A., Schwandt, M. L., Sommer, W. H., George, D. T., Parsons, L. H., Herscovitch, P., Hommer, D., & Heilig, M. (2011). A genetic determinant of the striatal dopamine response to alcohol in men. *Molecular Psychiatry*, 16, 809–817. https://doi.org/10.1038/mp.2010.56

Rimondini, R., Arlinde, C., Sommer, W., & Heilig, M. (2002). Long-lasting increase in voluntary ethanol consumption and transcriptional regulation in the rat brain after intermittent exposure to alcohol. *The FASEB Journal*, 16, 27–35. https://doi.org/10.1096/fj.01-0593com

Rimondini, R., Sommer, W. H., Dall'Olio, R., & Heilig, M. (2008). Long-lasting tolerance to alcohol following a history of dependence. *Addiction Biology*, 13, 26–30. https://doi.org/10.1111/j.1369-1600.2007.00079.x

Rimondini, R., Sommer, W., & Heilig, M. (2003). A temporal threshold for induction of persistent alcohol preference: Behavioral evidence in a rat model of intermittent intoxication. *Journal of Studies on Alcohol*, 64, 445–449. https://doi.org/10.15288/jsa.2003.64.445

Roberts, A. J., Heyser, C. J., Cole, M., Griffin, P., & Koob, G. F. (2000). Excessive ethanol drinking following a history of dependence: Animal model of allostasis. *Neuropsychopharmacology*, 22, 581–594. https://doi.org/10.1016/S0893-133X(99)00167-0

Rogers, J., Wiener, S. G., & Bloom, F. E. (1979). Long-term ethanol administration methods for rats: Advantages of inhalation over intubation or liquid diets. *Behavioral and Neural Biology*, 27, 466–486. https://doi.org/10.1016/S0163-1047(79)92061-2

Sarkar, D. K., Kuhn, P., Marano, J., Chen, C., & Boyadjieva, N. (2007). Alcohol exposure during the developmental period induces beta-endorphin neuronal death and causes alteration in the opioid control of stress axis function. *Endocrinology*, 148, 2828–2834.

Schierenbeck, T., Riemann, D., Berger, M., & Hornyak, M. (2008). Effect of illicit recreational drugs upon sleep: Cocaine, ecstasy and marijuana. *Sleep Medicine Reviews*, 12, 381–389. https://doi.org/10.1016/j.smrv.2007.12.004

Sharif, N. A., & Hughes, J. (1989). Discrete mapping of brain Mu and delta opioid receptors using selective peptides: Quantitative autoradiography, species differences and comparison with kappa receptors. *Peptides*, 10, 499–522. https://doi.org/10.1016/0196-9781(89)90135-6

Shenoy, S. K., & Lefkowitz, R. J. (2011). beta-Arrestin-mediated receptor trafficking and signal transduction. *Trends in Pharmacological Sciences*, 32(9), 521–533.

Shield, K. D., & Rehm, J. (2019). Alcohol and the global burden of disease. *Lancet*, 393(10189), 2390. https://doi.org/10.1016/S0140-6736(19)30726-3

Sjoholm, L. K., Kovanen, L., Saarikoski, S. T., Schalling, M., Lavebratt, C., & Partonen, T. (2010). CLOCK is suggested to associate with comorbid alcohol use and depressive disorders. *Journal of Circadian Rhythms*, 8, 1. https://doi.org/10.1186/1740-3391-8-1

Sommer, W. H., Costa, R. M., & Hansson, A. C. (2014). Dopamine systems adaptation during acquisition and consolidation of a skill. *Frontiers in Integrative Neuroscience*, 8, 87. https://doi.org/10.3389/fnint.2014.00087

Sommer, W. H., Rimondini, R., Hansson, A. C., Hipskind, P. A., Gehlert, D. R., Barr, C. S., & Heilig, M. A. (2008). Upregulation of voluntary alcohol intake, behavioral sensitivity to stress, and amygdala crhr1 expression following a history of dependence. *Biological Psychiatry*, 63, 139–145. https://doi.org/10.1016/j.biopsych.2007.01.010

Spanagel, R., Rosenwasser, A. M., Schumann, G., & Sarkar, D. K. (2005). Alcohol consumption and the body's biological clock. *Alcoholism, Clinical and Experimental Research*, 29, 1550–1557. https://doi.org/10.1097/01.alc.0000175074.70807.fd

Spanagel, R., & Weiss, F. (1999). The dopamine hypothesis of reward: Past and current status. *Trends in Neurosciences*, 22, 521–527. https://doi.org/10.1016/S0166-2236(99)01447-2

Stählin, O. (2013). Characterisation of transcriptome changes caused by alcohol addiction. Ruperto-Carola University of Heidelberg, Dissertation Thesis, 1-151.

Takada, T., Yamashita, A., Date, A. et al (2013). Changes in the circadian rhythm of mRNA expression for micro-opioid receptors in the periaqueductal gray under a neuropathic pain-like state. *Synapse (New York, N. Y.)*, 67(5), 216–223.

Tomita, T., Mori, T., & Onishi, Y. (2019). Beta-Arrestin 1 (ARRB1) serves as a molecular marker of the peripheral circadian rhythm. *International Journal of Oral Science*, 11, 32.

Trott, A. J., & Menet, J. S. (2018). Regulation of circadian clock transcriptional output by CLOCK:BMAL1. *PLoS Genetics*, 14, e1007156. https://doi.org/10.1371/journal.pgen.1007156

Uhrig, S., Vandael, D., Marcantoni, A., Dedic, N., Bilbao, A., Vogt, M. A., Hirth, N., Broccoli, L., Bernardi, R. E., Schönig, K., Gass, P., Bartsch, D., Spanagel, R., Deussing, J. M., Sommer, W. H., Carbone, E., & Hansson, A. C. (2017). Differential roles for L-type calcium channel subtypes in alcohol dependence. *Neuropsychopharmacology*, 42, 1058–1069. https://doi.org/10.1038/npp.2016.266

Vescovi, P. P., Coiro, V., Volpi, R., & Passeri, M. (1992). Diurnal variations in plasma ACTH, cortisol and beta-endorphin levels in cocaine addicts. *Hormone Research*, 37, 221–224. https://doi.org/10.1159/000182316

Volpicelli, J. R., Alterman, A. I., Hayashida, M., & O'Brien, C. P. (1992). Naltrexone in the treatment of alcohol dependence. *Archives of General Psychiatry*, 49(11), 876–880. https://doi.org/10.1001/archpsyc.1992.01820110040006

Whistler, J. L., & von Zastrow, M. (1998). Morphine-activated opioid receptors elude desensitization by beta-arrestin. *Proceedings of the National Academy of Sciences of the United States of America*, 95, 9914–9919.

WHO (2018) In Vladimir P. & Dag R. (Eds.), *Global status report on alcohol and health* (pp. 1–450). https://www.who.int/substance_abuse/publications/global_alcohol_report/en/

Williams, D. L., MacLean, A. W., & Cairns, J. (1983). Dose-response effects of ethanol on the sleep of young women. *Journal of Studies on Alcohol*, 44, 515–523. https://doi.org/10.15288/jsa.1983.44.515

Yoshitane, H., Ozaki, H., Terajima, H., Du, N. H., Suzuki, Y., Fujimori, T., Kosaka, N., Shimba, S., Sugano, S., Takagi, T., Iwasaki, W., & Fukada, Y. (2014). CLOCK-controlled polyphonic regulation of circadian rhythms through canonical and noncanonical E-boxes. *Molecular and Cellular Biology*, 34(10), 1776–1787.

Zhou, L., & Bohn, L. M. (2014). Functional selectivity of GPCR signaling in animals. *Current Opinion in Cell Biology*, 27, 102–108. https://doi.org/10.1016/j.ceb.2013.11.010

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