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Study of the neurophysiological properties of Ventral Tegmental Area dopamine neurons following moderate alcohol drinking in the mouse

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CONTENTS

ABSTRACT

Alcohol is the most commonly used substance of abuse worldwide, with 250 million people worldwide affected by an alcohol use disorder (AUD). Dopaminergic (DA) projections from the ventral tegmental area (VTA) to the ventral striatum are strongly implicated in the reward and reinforcing properties of alcohol and other drugs of abuse. Permanent changes in this pathway underlie the formation of compulsive drug-seeking behavior. Unfortunately, neural alterations induced by voluntary ethanol (EtOH) consumption are not yet entirely understood, and current treatments for AUD are hardly available and not very effective. In this respect, a better understanding of the neural modifications marking the early phases of alcohol experience may unravel mechanisms that lead to the development of dependence in advanced stages. On these premises, the main goal of the present project was to investigate the functional remodeling of the reward system induced by EtOH in an animal paradigm of voluntary drinking. To achieve this objective, we first implemented and characterized the 2-bottle choice paradigm (3, 6, 12% v/v EtOH vs water) with the assessment of preference and total intake dynamics, and we evaluated anxiety levels and relapse after EtOH deprivation. We then investigated persistent alterations in VTA DA neurons induced by voluntary alcohol intake and following withdrawal, and the response to acute 40 mM EtOH application, by performing electrophysiological recordings in acute slices obtained from both EtOH-exposed and naive TH (tyrosine hydroxylase)–GFP (green fluorescence protein) mice. Finally, nociceptin/orphanin FQ (N/OFQ), applied in the bath during recordings, was tested on electrophysiological alterations in the VTA identified in this project. Our results demonstrated that voluntary drinking does not lead to behavioral escalation, and EtOH-exposed mice do not show withdrawal syndrome, such as increased anxiety and alcohol deprivation effect (ADE), following EtOH deprivation. However, we found a significant reduction in the basal firing rate of VTA DA neurons from exposed mice, accompanied by a greater response to acute application. In line with this, chronic EtOH increases the frequency of GABAergic, but not glutamatergic, inputs onto VTA DA neurons, while reducing the response to acute EtOH perfusion. Nevertheless, after 5 days of EtOH deprivation, the strength of the inhibitory synaptic activity onto DA neurons and response to acute EtOH is normalized. Together these changes may precede the onset of a hypodopaminergic condition and an addictive state. Finally, we obtained evidence that N/OFQ, long studied as an endogenous able to control addictive behavior in animal models, abolishes the acute EtOH-induced enhancing effect on the firing rate of VTA DA neurons in both groups without affecting their basal activity.

INTRODUCTION

1. ADDICTION

1.1 Substance Use Disorder

Drug addiction is defined as a chronic and relapsing brain disease characterized by compulsive drug-seeking despite severe negative subsequences (Nestler 2001), with similar relapse rates to other chronic medical illnesses such as diabetes and hypertension. This disease affects a person's brain and behavior who is not able to control the use of a drug or medication anymore, driven by pleasure-seeking, succumbing to peer-pressure, performance improvement, attempting to alter one's state, and self-medication for a mental disorder (Volkow and Warren 2014) but leading to the emergence of a negative emotional state when access to the drug is prevented (Koob and Volkow 2010). Substances such as tobacco, cannabis, hallucinogens, inhalants, opioids, sedatives, hypnotics, anxiolytics, stimulants, and alcohol have a high probability of leading to addiction (American Psychiatric Association 2013).

Clinically, addiction is not a diagnostic term. The Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-V) instead uses "Substance Use Disorders" (SUD). SUD can be diagnosed as mild if a patient has 2 to 3 of 11 criteria (Table 1) within 12 months; moderate if it has 4 to 5 criteria, and severe with 6 or more (American Psychiatric Association 2013). Depending on the substance, these symptoms may vary. Moreover, SUD can be diagnosed even without the presence of the physical symptoms of tolerance and withdrawal.

Overall Criteria Group	Individual Criterion
Impaired control	1. The substance may be taken in larger amounts or over a longer period than was originally intended
	2. Persistent desire to cut down or regulate substance use, and potential unsuccessful attempts to decrease or discontinue use
	3. Large amount of time spect obtaining and using the substance, and/or recovering from the effects of using the substance
	4. Craving (the intense desire or urge for the drug). It may present at any time, but is most likely to surface in an environment associated with the drug
Social impairment	5. Failure to fulfill duties at home, work, or school due to substance use
	6. Continued substance use despite persistent or recurrent social or interpersonal problems, as a result of substance use
	7. Reduced activity in social, occupational or recreational events in order to use the substance
Risky use	8. Recurrent substance use in physically hazardous situations
	9. Continued substance use despite knowing that a physical or psychological problem is either caused or exacberated due to substance use
Pharmacological criteria	10. Tolerance which is signaled by requiring an increasing dose of a substance in order to achieve the desired effect
	11. Withdrawal occurring when blood or tissue concentrations of a substance decline after prolonged heavy use

Table 1: DSM-5 Substance Use Disorder Criteria (American Psychiatric Association 2013).

We often confuse “dependence” with “addiction”, but the distinction between them is important to note. Dependence is characterized by physical symptoms as tolerance and withdrawal. Changes induced during a state of dependence are different than the adaptations shown in an addicted person. In addiction the drug’s actions change the physiological synaptic connections which can persist for a long time, even after the drug has been removed from the body (Volkow and Warren 2014). Addiction develops when a person uses the drug chronically and intermittently with a progressive increase in frequency and intensity of use (Volkow and Warren 2014). An addicted subject modifies his behavior because of the biochemical changes induced by continued substance abuse and he acts irrationally when he does not have the substance.

1.2 Addiction as a motivational disorder

Addiction develops chronically passing from occasional use, to recreational, regular, and finally compulsive (Koob 2017; D. B. Kandel 2002). Drug addiction has been described, in a heuristic framework, as a cycle composed of three stages: binge/intoxication in which a person consumes an intoxicating substance and feels associated rewarding effects, withdrawal/negative affect in which a subject senses a negative emotional state during the removal of the substance, and preoccupation/anticipation during which a person seeks the substance after an abstinence period (Koob and Le Moal 2001; Koob and Le Moal 1997) (Figure 1). Probably, dysregulation of three functional domains (incentive salience/habit, negative emotional states, and executive function), mediated by three brain areas (basal ganglia, extended amygdala, and prefrontal cortex, respectively), is just represented by the three-stage cycle. Then, dysregulation becomes more intense and finally, the pathological condition known as addiction occurs (Figure 1).

Addiction can be also defined as a motivational disease; indeed, it has components of impulsive control disorders and compulsive disorders. In the first ones, tension or excitement increase and lead to an impulsive action which brings a sense of gratification, pleasure or relief (Horseman and Meyer 2019). In this case, positive reinforcement plays a large part (Koob and Volkow 2010). A positive reinforcement is a stimulus that increases the probability of a response, for example it is a drug that produces gratification so that increases the probability to use it again. On the other hand, in compulsive disorders, stress and anxiety drive to a compulsive and repetitive action that brings relief once this behavior is completed. In this case, negative reinforcement is the major component. A negative reinforcement removes an aversive stimulus

that enhances the probability of a response (Koob 2017), for example a pleasant drug taken during the negative state of withdrawal (Koob 2017, Horseman and Meyer 2019) (Figure 1).

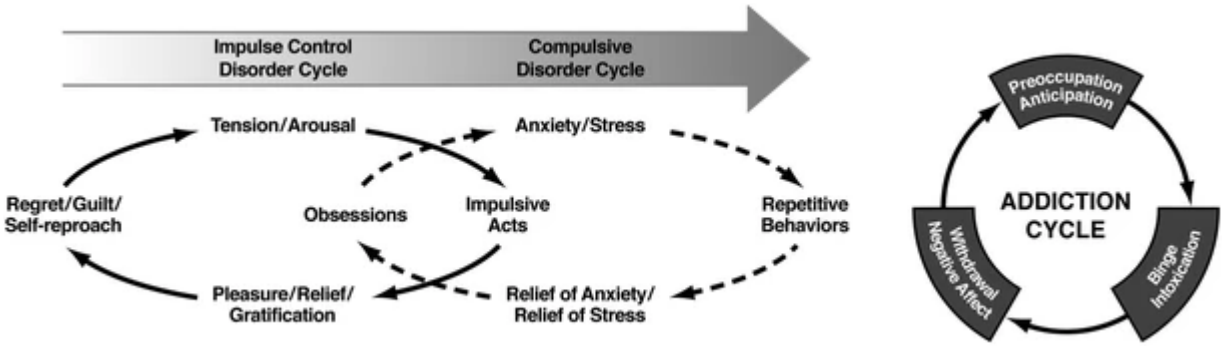


Figure 1: Diagram shows the stages of impulse control disorder and compulsive disorder cycles related to the sources of reinforcement. In impulse control disorders, tension or excitement increase and lead to an impulsive action which brings a sense of gratification, pleasure or relief. Then, there may be regret or guilt. In compulsive disorders, stress or anxiety, caused by obsessions, are followed by repetitive behaviors (compulsions) in order to prevent or reduce distress (American Psychiatric Association 1994). In impulse control disorders, positive reinforcement (pleasure/gratification) plays a large part, while negative reinforcement (relief of anxiety/stress) is more closely associated with compulsive disorders (left). The failure of the cycles of impulsivity and compulsivity results in the addiction cycle, conceptualized as three major components: preoccupation/anticipation, binge/intoxication, and withdrawal/negative affect (right). Figure adapted from (Koob 2017).

In particular, some psychobiological models think that a transition between impulsive and compulsive behavior occurs in addiction (Dalley, Everitt, and Robbins 2011)(Koob and Volkow 2010). Impulsivity is a strong mechanism during the early stages of addiction because it is responsible for the achievement of short-run rewards given by addictive substances and behavior (Fernández-Serrano et al. 2012); Verdejo-García, Lawrence, and Clark 2008). If this behavior continues to happen, impulsivity develops into compulsive habits (Zou et al. 2017).
In summary, addiction is a condition where pleasure is produced as well as a reduction of painful or negative affects, losing control of the behavior (Goodman 2008). Moreover, it is a heterogeneous disorder, with different ways to manifest based on the subjects, affected by a genetic component, environmental factors, and developmental processes. Since during adolescence some crucial brain areas controlling motivation are still developing, this period of life is very delicate and is often the moment in which addiction starts (Volkow and Warren 2014).

1.3 Impact of drug use on health

Drug use has an important impact on health and well-being. Drug use disorders are often associated with psychiatric comorbidities, indeed mental health disorders and drug use disorders have common risk factors (Ross and Peselow 2012; Mueser et al. 2012; Armstrong and Jane Costello 2002). In addition, people affected by drug use disorders often undergo adverse health consequences of drug use, such as non-fatal overdoses, and premature death and they are quite often affected by infectious diseases, like hepatitis C, and HIV (Kandel 2002) whose prevalence is disproportionally high among this group and represents a significant percentage of new cases of HIV and hepatitis C infections in the world (UNAIDS 2018; World Health Organization 2018).

SUD also has a negative impact on individual users social life. A person with this disorder often has a social disadvantage, like difficulty in remaining in employment, low level of education, and financial instability and poverty (Volkow et al. 2014).

People who take substances of abuse (PWID) are always left behind in almost all circumstances and just 12.5% of them have access to the treatment of drug use disorders (UNODC 2020).

Moreover, the negative consequences of drug use affect not only the single users but also their families, friends and anyone around them (UNODC 2020), just like they have an important influence on harmful patterns of drug use and dependence (Lander, Howsare, and Byrne 2013; Duncan, Palamar, and Williams 2014; Spooner and Hetherington 2005; Yangyuen, Kanato, and Mahaweerawat 2018; Edalati and Krank 2016; Dube et al. 2003).

2. ALCOHOL USE DISORDER

The alcohol use disorders (AUD) consist of alcohol dependence, alcohol abuse, and harmful use (American Psychiatric Association 2013; World Health Organization 1992). These are common and potentially lethal disorders often associated with several additional medical and psychiatric conditions, thereby reducing the lifespans of affected people by more than a decade (Gerevich 2007). However, people affected by AUD are difficult to identify, because they often have a job and a family, and they present just general medical problems, such as insomnia, anxiety, illness, and sadness (Schuckit 2009).

2.1 Ethanol and its metabolism

The type of alcohol that we find in beverages like wine and beer is chemically defined as ethanol (EtOH). Once ingested, it is ubiquitously distributed throughout total body water. It is a low-weight and nonionic molecule that diffuses and distributes in all body tissues and fluids with very high efficiency, easily reaching also the central nervous system (CNS) (Heckmann and Silveira 2009) where it has direct and indirect targets. This wide distribution is due to its chemical structure: EtOH is composed of a chain of two carbons and a hydroxyl group (-OH), which confers a perfect ratio between hydrophilic and hydrophobic components (Maher 1997; Costardi et al. 2015).

Once ingested, 2-10% (v/v) of alcohol is excreted through the urine, lungs and sweat, and the remainder 90-98% is metabolized by alcohol dehydrogenase (ADH) to acetaldehyde. Then, acetaldehyde is quickly converted to acetate, mainly by aldehyde dehydrogenase (ALDH) (Figure 2).

ADH exists in multiple forms (class I – class V) in the human liver and its function is to oxidize endogenous and exogenous ethanol and to oxidize substrates involved in steroid and bile acid metabolism. The class I ADH forms are mainly responsible for the oxidation of alcohol (Cederbaum 2012). The wild-type forms of ADH decrease the concentration of alcohol in the blood by about 4·5 mmol/L ethanol per h (this is the equivalent of about one drink per h) (Gerevich 2007). In particular, two variations of ADH genes (ADH1B*2 and ADH1C*1) produce a more rapid metabolism of alcohol and therefore faster production of acetaldehyde (Duranceaux et al. 2006).

ALDH*2 is the main form for acetaldehyde metabolism and if a person has the inactive mutation of this enzyme, ALDH*2*2, like in about 40% of Asian people, much more acetaldehyde than normal will be generated after drinking (Duranceaux et al. 2006; Dickson et

al. 2006). People who are homozygous for this inactive form become sick after drinking alcohol and have few possibilities to develop alcohol-use disorders.

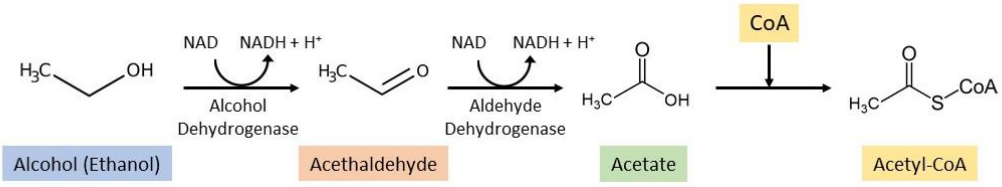


Figure 2: Alcohol metabolism by alcohol dehydrogenase and aldehyde dehydrogenase

2.2 Clinical characteristic of AUD

In ancient times, EtOH has been known for its intoxicating effects.

Short-term positive effects of moderate alcohol consumption include a general improvement of mood and enhanced sociability and self-confidence. However, EtOH consumption also brings numerous negative effects, like cardiovascular disease, risk of several disorders, some forms of cancer, liver cirrhosis, neuropsychiatric conditions, and, very important, it has a very high addictive potential. Despite its adverse effects, absolutely comparable with those caused by other drugs of abuse defined as “hard drugs”, alcohol is legal in all western countries (Nutt et al. 2007).

Blood EtOH levels (BECs) of alcohol below 0.08% v/v (~17 mM) are sufficient to produce first signals of intoxication such as motor incoordination, cognitive alteration, and slowed reaction times (Ando 1975; Schechter 1980). If higher BECs (~50 mM) are achieved, locomotor and cognitive impairment and sedation aggravate. Respiratory depression, strong sedation, anesthesia, coma, and death occur if 190 mM of EtOH in the blood is reached (Alifimoff, Firestone, and Miller 1989).

The clinical course of AUD is as predictable as most psychiatric disorders, with all the differences for example due to sex and age. In general, women with AUD become to have problems and ask for help earlier than men, and they are less violent than men (Gerevich 2007; Mann et al. 2005). Moreover, older people with AUD have more medical problems, are less violent and have less chance to be employed compared to younger people (Lemke et al. 2005). Therefore, people who started early suffering from AUD and are characterized by antisocial personalities, often have additional comorbidities include use, abuse and dependence on illicit drugs (Gerevich 2007; Babor et al. 1992), and almost 80% of alcohol-dependent people are smokers. These comorbidities make AUD more dangerous and the treatment of alcoholism becomes harder (Hughes and Kalman 2006).

2.3 Diagnosis of AUD

Criteria for diagnosis of alcohol dependence are almost the same for DSM and ICD: tolerance to alcohol, withdrawal syndrome, desire to use alcohol and inability to control use, devotion of a large proportion of time to getting and using alcohol, recovering from alcohol use, neglect of social, work, or recreational activities, and continued alcohol use despite physical or psychological problems (Schuckit et al. 2005).

However, harmful use and alcohol abuse have not the same meaning as dependence. Harmful use is defined by ICD10 as a mental and/or physical problem associated with alcohol in a 12-month period. Alcohol abuse is defined by DSM-IV as one or more problems (alcohol use in dangerous situations, continued use even in the presence of social or interpersonal problems and frequent legal problems) that are present in a person without dependence in a 12-month period. These two diagnostic systems have a low agreement, though (Schuckit et al. 2005; Hasin et al. 1997). Moreover, people with EtOH abuse drink smaller quantities compared to people with dependence, and just about 10% of them become dependent (Schuckit et al. 2005; Hasin et al. 1996).

In addition to this, other two criteria are used in order to diagnose alcohol dependence: questionnaires and blood tests.

The questionnaire most used is the CAGE (Cut down on drinking, feel Annoyed if criticized about EtOH use, feel Guilty after drinking, and need an Eye-opener drink in the morning). Based on the number of positive responses, patients are diagnosed as heavy drinkers (53% of affirmative answers) or having an alcohol dependence (77% of positive responses) (Saremi et al. 2001). This short test is very reliable, especially for heavy drinkers if combined with blood tests.

Another questionnaire is the ten-item Alcohol-Use Disorders Identification Test (AUDIT) in which a score of eight or more indicates both heavy drinkers and people with AUD (Reinert and Allen 2007).

Finally, another short test is the TWEAK questionnaire, which stands for Tolerance, Worry about drinking, Eye-opener drink early in the day, Amnesia about drinking, and need to Cut down (Schuckit 2009).

Blood tests are useful to diagnose patients who drink a dangerous amount of alcohol (Gerevich 2007; Hietala et al. 2006), if the veracity of the history is not secure, and to explain the patients the alcohol adverse effects on their health (Parker, Marshall, and Ball 2008). Although blood tests are not as sensitive as questionnaires, they can have very great sensitivity and specificity for people not overweight, smokers and diabetic (Conigrave et al. 2002). One marker used is

the activity of γ glutamyl transferase in the serum, which is important for aminoacid transport. This inexpensive test has 60% specificity and sensibility in men and lower in women (50%) (Conigrave et al. 2002). Another marker is carbohydrate-deficient transferrin, which is present following heavy drinking for a long period. The sensitivity for recognition of heavy drinking and AUD goes from 30% to 75%, even if results could be hard to understand in the case of iron deficiency (Conigrave et al. 2002; Alte et al. 2003; Niemelä 2007). There are also blood tests of liver function that can identify heavy drinking and AUD with sensitivities between 25% and 45%, in which the amount of alanine and aspartate aminotransferases is detected. Finally, alcohol dependence is strongly suspected if very high blood alcohol (eg. 35 mmol/L or more) is found (Parker et al. 2008).

2.4 Epidemiology

Alcohol addiction is often considered in society as one of the least problematic substance addictions, but numbers tell a different story. Data recently described by the World Health Organization (WHO) displays that approximately 2.1 billion people (around 43% of adults globally) drink alcohol, that 76.3 million people suffer from AUD worldwide, and that alcohol abuse causes 3 million deaths every year, which is equal to 5.3% of all deaths. Also, the harmful use of alcohol provokes more than 200 diseases and injury conditions. Moreover, alcohol use brings disability and death quite early in life, indeed in the age range of 20-39 years about 13.5% of all deaths are caused by alcohol (World Health Organization 2018).

The age when one first starts drinking is usually 15 years old, even if it is influenced by environmental and cultural factors. Of course, the earlier the subject starts drinking regularly, the greater the chances to develop subsequent problems (Gerevich 2007; Kuperman et al. 2005). The range between 18 and 22 years is usually the period in which heavy drinking occurs in both people with future AUD and the normal population (Gerevich 2007)(Kuperman et al. 2005). Alcohol abuse and dependence often develop in the first mid-20s (Gerevich 2007; Clark 2004), when people begin to have more responsibilities.

2.5 Therapeutic approaches for AUD

Despite alcoholism has been existing for several millennia, the efficacy of psychological, behavioral, and pharmacological treatment is almost non-existent. Independently of the modality, the high resistance of alcoholism to successful treatment is often due to the inadequate access to it, non-performance of it, limited personal resources, and neuroadaptive changes that occur within the brain following chronic alcohol consumption (Breese, Sinha, and Heilig 2011; Spanagel 2009).

So far, the specific neurochemical circuitry responsible for alcohol reinforcement and craving are not entirely clear, as well as all the neuroadaptations induced by chronic heavy alcohol drinking. For this reason, only three pharmacological medications have been approved by both the Food and Drug Administration (FDA) and the European Medications Agency (EMA) and are available for the treatment of alcoholism.

The first drug to be developed and approved by the FDA (1950s) was disulfiram, which acts by inhibiting the activity of ALDH. When the drug is taken, the concomitant use of alcohol results in the accumulation of acetaldehyde in the body leading to generalized malaise, nausea, vomit, headache, and flushing. These aversive reactions should induce patients to stop drinking.

The second medication to be approved by the FDA (1994) was the nonselective opioid receptor antagonist naltrexone. This drug induces patients to decrease drinking or to stop it by reducing the rewarding effects of alcohol.

The third medication to be approved by the FDA (2004) for alcoholism, whereas it was already approved for marketing by the EMA (1989), was the anti-craving drug acamprosate. Most evidence suggests that it reduces the desire to drink alcohol and the likelihood of relapse by restoring the imbalance between excitatory and inhibitory neurotransmission altered by chronic alcohol consumption (Kiefer and Mann 2010), despite both the efficacy and the neurochemical mechanism of action are still debated.

In addition, two more drugs have been approved and used for the treatment of AUD in some European countries only: nalmefene and gamma-hydroxybutyrate.

Nalmefene is an opioid antagonist but, in contrast to naltrexone, it has partial agonist activity at the κ-opioid receptor and brings less hepatotoxicity. There are contrasting data about its efficacy, though. In some studies, nalmefene, together with psychosocial treatment, has been described to have good efficacy in decreasing heavy alcohol consumption (Mason et al. 1994, 1999). Two other studies showed that this drug is able to reduce the quantity of alcohol drunk and the number of drinking days (Gual et al. 2013; Mann et al. 2013). In contrast, a study reported that the effect of nalmefene is not different from that of placebo (Anton et al. 2004).

Gamma-hydroxybutyrate (GHB) is a sedative medication that potentiates the inhibition mediated by both $GABA_A$ and $GABA_B$ receptors. It is used in clinical practice as a treatment for narcolepsy but it is also effective in AUD (Addolorato et al. 1996). Indeed, the sodium oxybate form of GHB is sold in several countries of Europe, even if it can have serious side-effects. It seems acting as an alcohol substitute decreasing alcohol seeking and preventing withdrawal. Because of its abuse potential, GHB is registered in a few countries where is given

only to selected patients who are carefully monitored during its administration (Addolorato et al. 2000; Caputo 2011).

A novel and promising approach for AUD, and in general for addictive behaviors, is transcranial magnetic stimulation (TMS). This technique consists of electrodes that are fixed on the scalp. They generate a continuous weak electric current to control and modulate the membrane potential through brain regions, like the cortex. The modulation of the dorsolateral prefrontal cortex (dlPFC) is usually used to treat alcohol-addicted behaviors (den Uyl et al. 2018), as well as the decrease of the activity of the medial prefrontal cortex (mPFC) via theta burst stimulation. It has been reported that mPFC could be an effective target to mitigate craving (Hanlon et al. 2015).
Another technique that showed promising results is the deep brain stimulation (DBS) of the NAc (Müller et al. 2016).

2.6 Animal models of alcoholism

Despite numerous uncertainties about the neurochemical and neurostructural plasticity induced by chronic alcohol exposure, what we know today is thanks to the development of animal models of alcoholism (Ripley and Stephens 2011). Models with the greatest face validity for human alcoholism are paradigms in which animals consume alcohol voluntarily. Among these, there are home cage tests such as the *two-bottle choice test* and the *drinking-in-the-dark paradigm*. Additional tests consist of *operant alcohol self-administration* that is conducted in specific chambers.
Since animals do not usually consume enough alcohol to produce physical dependence, genetically inbred strains of rats and mice that show innate propensity to take higher levels of alcohol consumption have been developed (Ripley and Stephens 2011; Crabbe, Phillips, and Belknap 2010). Moreover, *alcohol vapor inhalation* methods have been added to the voluntary consumption paradigm to burst alcohol consumption. In the alcohol vapor inhalation methods animals are exposed to cycles of intoxicating levels of EtOH vapors (~16 h/day) alternating with withdrawal periods (~8 h/day), in which withdrawal symptoms occur (Ripley and Stephens 2011).
Furthermore, the *alcohol deprivation effect* and *reinstatement* paradigms are used to study relapse symptoms. Several studies showed that these methods have good predictive validity if used to test an anti-relapse drug, like acamprosate (Spanagel and Kiefer 2008).

The *conditioned place preference* is a very simple and short-term model used to measure the motivational effects of substances, objects, or experiences (Tzschentke 2007). This paradigm can also be used to measure aversive stimuli; in this case, it is called *conditioned place aversion*. Moreover, *delayed discounting* and the *five-choice serial reaction time task* are methods used to study the impulsivity of alcoholism.

Finally, there are paradigms to study negative affective states, like anhedonia, that always occur in alcoholism, such as *intracranial self-stimulation* and *reduced preference for sweetened solutions*.

Despite all these models that mimic some precise aspects of alcoholism, a true model where animal consumes alcohol in a similar way and quantity to human, in which transition from normal drinking to compulsive alcohol use and continuous drinking despite negative consequences has not been found yet.

3. NEURAL CIRCUITS INVOLVED IN SUBSTANCE USE DISORDER: THE MESOCORTICOLIMBIC PATHWAY AND THE VENTRAL TEGMENTAL AREA

The ventral tegmental area (VTA) is located in the midbrain between several other major areas. The mammillary bodies and the posterior hypothalamus, both localized in the diencephalon, extended rostrally from the VTA. The red nucleus is situated laterally and oculomotor fibers are situated ventromedially to the VTA (Vitošević et al. 2013). The substantia nigra is lateral to the VTA (Coenen et al. 2018). Finally, the pons and the hindbrain are caudal to the VTA.

3.1 The mesocorticolimbic pathway

In the human, brain dopaminergic (DA) neurons are located in the mesencephalon, diencephalon, and olfactory bulb (Arias-Carrión and Pöppel 2007; Björklund and Dunnett 2007), even if most of them are situated in the ventral mesencephalon.

The VTA sends DA projections rostrally to the nucleus accumbens (NAc) forming the mesostriatal pathway, and also to the prefrontal cortex (PFC) forming the mesocortical pathway (Haber et al. 1990; Haber and Knutson 2010; Wise 2009) (Figure. 3). Because of the overlap between these two pathways, they are often indicated as the mesocorticolimbic system (Wise 2004, 2005). In particular, NAc mediates motor functions, PFC is important for planning and attention, and VTA mediates compulsive drug seeking and promotes behaviors associated with the reinforcing effects of drugs of abuse (Gatto et al. 1994; Gessa et al. 1985; Rodd et al. 2005). Indeed, the mesocorticolimbic system is well known to be involved in the reward and reinforcing properties of alcohol and other drugs of abuse (Diana 2011; Söderpalm, Löf, and Ericson 2009).

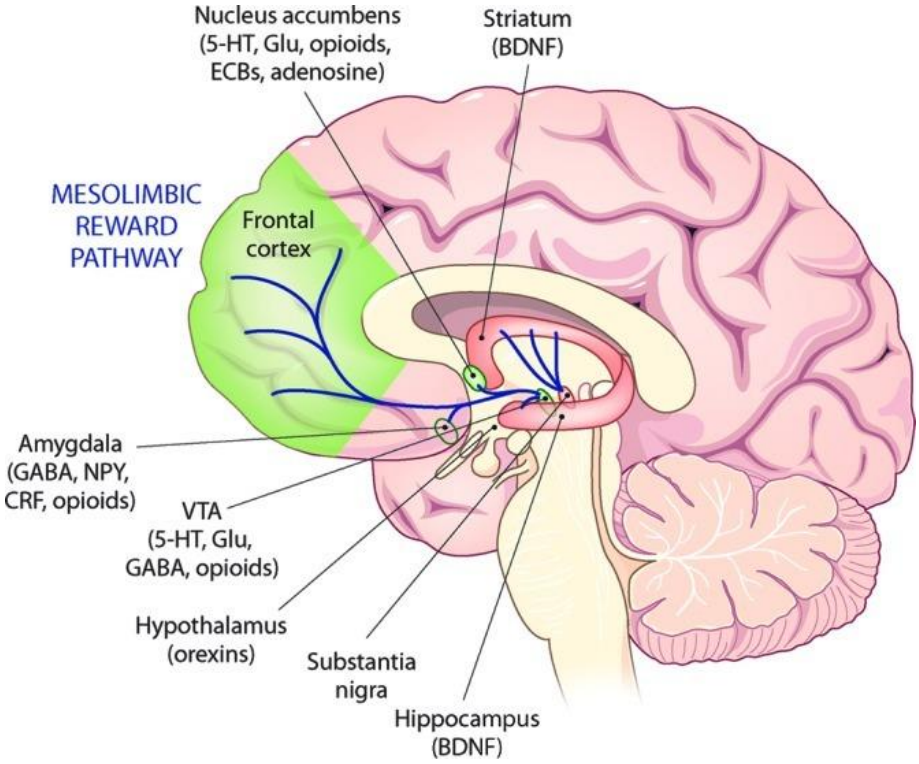


Figure 3: Graphic representation of the human mesocorticolimbic reward pathway and alcohol-modulated neurotransmitters that influence this system. Figure adapted from (Gass and Olive 2012).

VTA DA neurons, so defined because expressing tyrosine hydroxylase (TH) enzyme and releasing dopamine, are involved in both positive and negative reinforcement, decision making, memory formation, incentive salience, stimulus salience, and aversion (Adcock et al. 2006; Berridge 2007; Brischoux et al. 2009; Bromberg-Martin, Matsumoto, and Hikosaka 2010; Salamone and Correa 2012; Schultz 2002).

The activity of VTA DA neurons is regulated by various inputs, even by local GABAergic neurons and glutamatergic neurons (see chapter 3.2 for a more detailed description).

In addition, different functions of VTA are mediated by different subpopulations of VTA DA neurons, even if sometimes VTA GABA neurons (Berrios et al. 2016; Stamatakis et al. 2013; Tan et al. 2012; Van Zessen et al. 2012) or VTA glutamatergic neurons (Qi et al. 2016; Root, Mejias-Aponte, Qi, et al. 2014; Wang et al. 2015) can bring motivated behavior without the action of VTA DA neurons. Some VTA neurons can even release multiple neurotransmitters (DA, GABA or glutamate) from a single axon or the same vesicle (Root, Mejias-Aponte, Zhang, et al. 2014; Zhang et al. 2015).

3.2 VTA connectivity

Initially, the brain areas that innervate the VTA were mapped to study the connectivity. Then, with the discovery of more specific tract tracers and antibodies, even the specific afferents projecting to DA neurons and the distribution of VTA DA efferents were possible (Morales and Pickel 2012; Yetnikoff et al. 2014, 2015).

Lately, viral vector and transgenic animals have allowed exploring the discovery inputs onto and outputs from VTA neurons.

Furthermore, a complex microcircuitry has been revealed within the VTA itself through which interactions among local DA, GABA, and glutamate neurons are mediated.

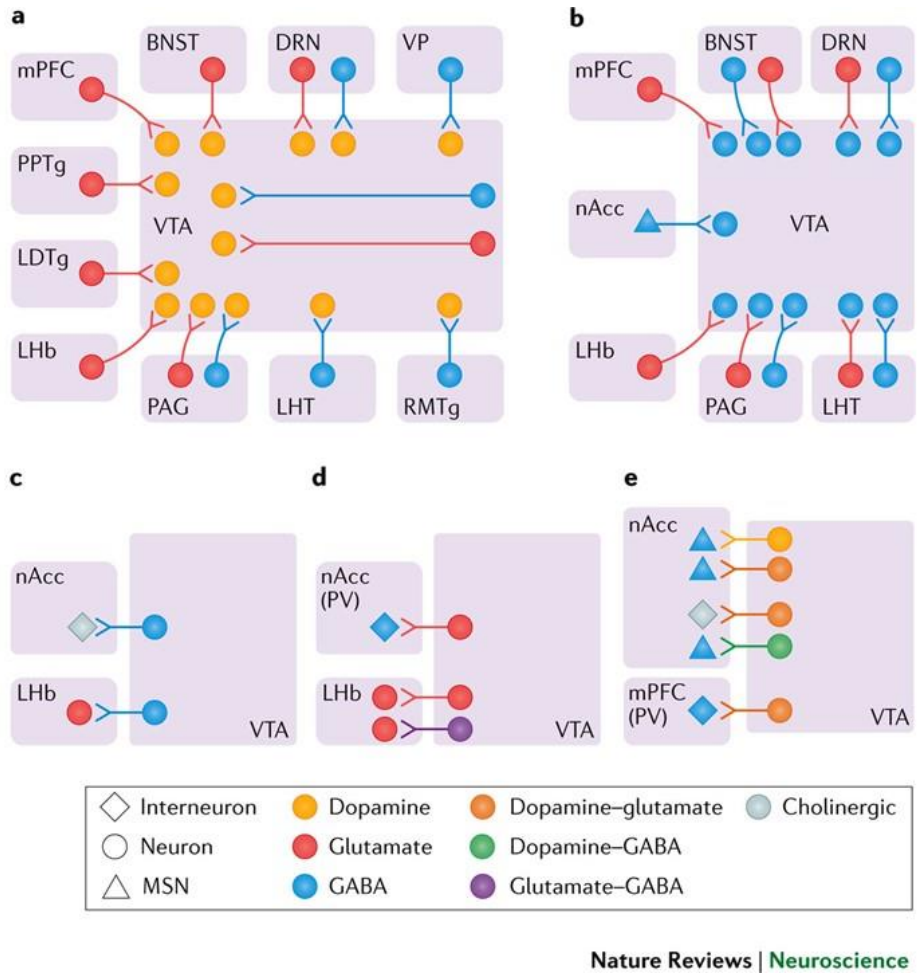


Figure 4: Afferents to and efferents from VTA neurons. (a) Inputs onto VTA DA neurons. (b) Inputs onto VTA GABA neurons. (c) Outputs from VTA GABA neurons. (d) Outputs from VTA glutamate and glutamate-GABA neurons. (e) Outputs from VTA DA, DA-glutamate, and DA-GABA neurons. Figure adapted from (Morales and Margolis 2017).

3.2.1 Afferents to VTA neurons

Despite the many advanced techniques we have available, there are no data on the source of afferents to VTA glutamate neurons, and few works have determined the source of glutamatergic or GABAergic inputs specifically onto VTA DA or GABA neurons.

Since the VTA is dense of neurons, an unintentional viral infection of the wrong neurons can happen and lead to ambiguous results.

Moreover, since discordant findings have been reported using different approaches, multiple techniques have to be used to confirm the synaptic network. For example, if on one hand anatomical data suggest that NAc sends afferents to VTA DA neurons in mice (Beier et al. 2015; Faget et al. 2016; Watabe-Uchida et al. 2012), on the other hand electrophysiological findings show that the main input from NAc goes to VTA GABA neurons in mice and rats (Bocklisch et al. 2013; Xia et al. 2011). Also, while the cTRIO (cell- type-specific tracing of the relationship between inputs and outputs) method shows a connection from mPFC to VTA DA neurons that project to the lateral NAc (Beier et al. 2015), electron microscopy and molecular tracer suggest that VTA DA neurons innervated by mPFC project to mPFC and not to NAc (Carr and Sesack 2000).

So far, what is sure is that "VTA DA neurons receive glutamatergic inputs from: mPFC (Carr and Sesack 2000), pedunculopontine tegmentum (PPTg) (Charara, Smith, and Parent 1996), laterodorsal tegmentum nucleus (LDTg) (Omelchenico and Sesack 2005), lateral habenula (LHb) (Omelchenko, Bell, and Sesack 2009), periaqueductal grey (PAG)(Omelchenko and Sesack 2010), bed nucleus of the stria terminalis (BNST) (Georges and Aston-Jones 2001), and dorsal raphe nucleus (DRN) (Qi et al. 2014). Also, VTA DA neurons receive GABAergic inputs from: rostromedial mesopontine tegmental nucleus (RMTg) (Jhou et al. 2009; Kaufling et al. 2010), PAG (Omelchenko and Sesack 2010), DRN (Beier et al. 2015), lateral hypothalamus (LHT) (Nieh et al. 2015), and ventral pallidum (VP) (Hjelmstad et al. 2013) (Figure 4a).

VTA GABA neurons receive glutamatergic afferents from: LHb (Omelchenko et al. 2009) and mPFC (Carr and Sesack 2000), and GABAergic inputs from NAc medium spiny neurons (MSNs) expressing the dopamine receptor D1 (Bocklisch et al. 2013). They also receive both glutamatergic and GABAergic afferents from: PAG (Omelchenko and Sesack 2010), DRN (Qi et al. 2014), LHT (Nieh et al. 2015), and BNST (Georges and Aston-Jones 2001) (Figure 4b).

Finally, VTA DA neurons receive even local inputs from both GABA and glutamate neurons (Tan et al. 2012; Wang et al. 2015; Van Zessen et al. 2012); Dobi et al. 2010; Omelchenko and Sesack 2009)" (Morales and Margolis 2017) (Figure 4a).

In particular, early electrophysiological studies in rats claimed that the inhibitory effect of local GABA neurons on DA neurons occurred through $GABA_A$ receptors (Johnson and North 1992a). Then, the same mechanism was confirmed in mice (Tan et al. 2012; Van Zessen et al. 2012). But what is not known, yet, is if the axon collaterals of these local GABA neurons project outside the VTA or just to local neurons.

VTA neurons also receive inputs from local glutamate neurons and VTA DA neurons activated by them have been reported projecting to the NAc (Wang et al. 2015).

3.2.2 Efferents from VTA neurons

The main targets of VTA DA neurons are MSNs of the NAc (Morales and Pickel 2012; Pickel et al. 1988) (Figure 4e), but actually, they also project to the amygdala, cortex, hippocampus, VP, PAG, BNST, olfactory tubercle, and locus coeruleus (LC).

Basing on the target region, VTA neurons have different characteristics. For example, DA neurons innervating the mPFC seem to display low levels of DAT compared to neurons projecting to other areas (Lammel et al. 2008; Sesack et al. 1998).

Also, despite the TH-expression of VTA neurons projecting to the LHb, these neurons do not possess VMAT2 and they do not release dopamine (Root et al. 2015; Stamatakis et al. 2013). For this reason, despite their TH-expression, it is difficult to figure out whether they have to be considered as DA neurons or not.

Moreover, using retrograde tracers in rats, it has been demonstrated that VTA DA neurons individually project to one brain region only (Swanson 1982), while using viral vector tracers in mice, subsets of VTA neurons seem to arrive in multiple brain areas (Aransay et al. 2015).

As for VTA GABA neurons, it has been displayed that they are connected to cholinergic interneurons of the NAc (Brown et al. 2012) and glutamate neurons of the LHb (Root, Mejias-Aponte, Zhang, et al. 2014) (Figure 4c). These last neurons receive synapses even from VTA combinatorial glutamate-GABA neurons (Root, Mejias-Aponte, Zhang, et al. 2014), and from glutamate-only neurons that innervate also parvalbumin-expressing GABA interneurons in the NAc (Qi et al. 2016) (Figure 4d).

In addition, VTA neurons releasing both glutamate and dopamine send innervations to the NAc and mPFC (Kabanova et al. 2015; Stuber et al. 2010; Tecuapetla et al. 2010; Yamaguchi et al. 2011) (Figure 4e). In particular within the mPFC, the release of glutamate leads to a fast excitation of parvalbumin-expressing GABA interneurons that lead to the inhibition of mPFC pyramidal cells (Kabanova et al. 2015). Finally, VTA combinatorial DA-GABA neurons project to NAc MSNs (Figure 4e).

3.3 Cellular organization of the VTA

The VTA includes a large percentage of DA neurons (60%) and smaller proportions of GABA (35%) and glutamatergic (2-5%) neurons.

3.3.1 Dopamine neurons

Dopamine is produced from L-tyrosine, then converted to L-DOPA by TH. Once synthesized or re-uptaken by the dopamine transporter (DAT), dopamine is stored in vesicles by vesicular monoamine transporter 2 (VMAT2) until it is released.

Every cellular compartment of VTA DA neurons expresses TH, indeed antibody anti-TH is often used for the identification of DA neurons. Interestingly, not all TH-expressing neurons in the VTA have detectable levels of VMAT2 or DAT, as it has been shown for a subset of TH-expressing neurons in the medial VTA (Li et al. 2013).

From an electrophysiological point of view, DA neurons generally are characterized by slow regular firing of large action potentials, a broad hyperpolarization-activated cation current (I_h), and are hyperpolarized by DA through D2 receptors but not by μ-opioid agonists (Figure 5).

However, VTA DA neurons can differ in their electrophysiological properties. Indeed, while a subpopulation of DA neurons located mainly in the most lateral VTA, laterally to the substantia nigra pars compacta (SNc), has the classical properties of DA neurons, other confirmed TH-expressing neurons in rodents have different electrophysiological and pharmacological properties (Berthet et al. 2014; Lammel et al. 2008; Margolis et al. 2006, 2012). Nevertheless, it seems that the response mediated by $GABA_B$ receptor is a common feature (Labouèbe et al. 2007; Margolis et al. 2012).

DA neurons can be either silent or spontaneously active and possess a firing. Silent DA neurons seem to be constantly hyperpolarized and inactivated by the ventral pallidum (VP) (Floresco et al. 2003). Spontaneously firing DA neurons can have either tonic activity with an irregular pattern or phasic activity with bursts of action potentials. Interestingly, when reinforcement learning occurs the neuron changes from tonic to phasic firing. In particular, when an unanticipated reward is presented for the first time, DA neurons show phasic bursts (Schultz 1997b; Zweifel et al. 2011), but after repeated stimulus-reward pairing, burst firing occurs already when the reward-predicting stimulus is given (Lammel et al. 2011; Schultz 1997a; Zweifel et al. 2011). If the reward is not presented anymore after the stimulus, DA neurons stop firing.

DA neurons are spontaneously active thanks to their intrinsic pace-maker membrane properties. Indeed, tonic activity remains even in brain slices where most of the afferents are cut off

(Deister et al. 2009; Shepard and Bunney 1988). Instead, since burst firing is stopped by the local application of glutamate receptor antagonists, burst activity seems to need synaptic inputs to be generated (Grace and Bunney 1984; Smith and Grace 1992).

3.3.2 GABA neurons

GABA is produced from glutamate by glutamate decarboxylase (GAD) 1 or 2, and accumulated in vesicles by vesicular GABA transporter (VGAT).

GABA neurons are distributed throughout the VTA of rat (Margolis et al. 2012; Olson and Nestler 2007), and are usually identified by the presence of GAD or VGAT.

Moreover, rat VTA GABA neurons have not always the same composition. For example, some of them contain cholecystokinin (Olson and Nestler 2007) or corticotropin-releasing factor-binding protein (Wang and Morales 2008), others respond only to the activation of DA receptor D2 or μ-opioid receptor (Margolis et al. 2012). Instead, a bigger homogeneity has been reported in VTA GABA neurons identified in GAD-green fluorescence protein (GFP) transgenic mice (Chieng et al. 2011a).

Electrophysiologically, GABA neurons often exhibit high-frequency firing, show a short duration of action potentials, do not display (or just small) I_h (Grace and Onn 1989; Johnson and North 1992b), and are inhibited by μ-opioid agonists but not DA (Figure 5).

While in the SNc the physiological and pharmacological properties of DA and GABA neurons are well distinct (Chieng et al. 2011b), in the VTA there is overlap among these properties (Ford, Mark, and Williams 2006; Lammel et al. 2008; Margolis et al. 2006, 2008). Hence, the distinction between DA and GABA neurons is just based on action potential properties (frequency and duration), I_h current, and DA-induced effects could be imprecise (Margolis et al. 2006).

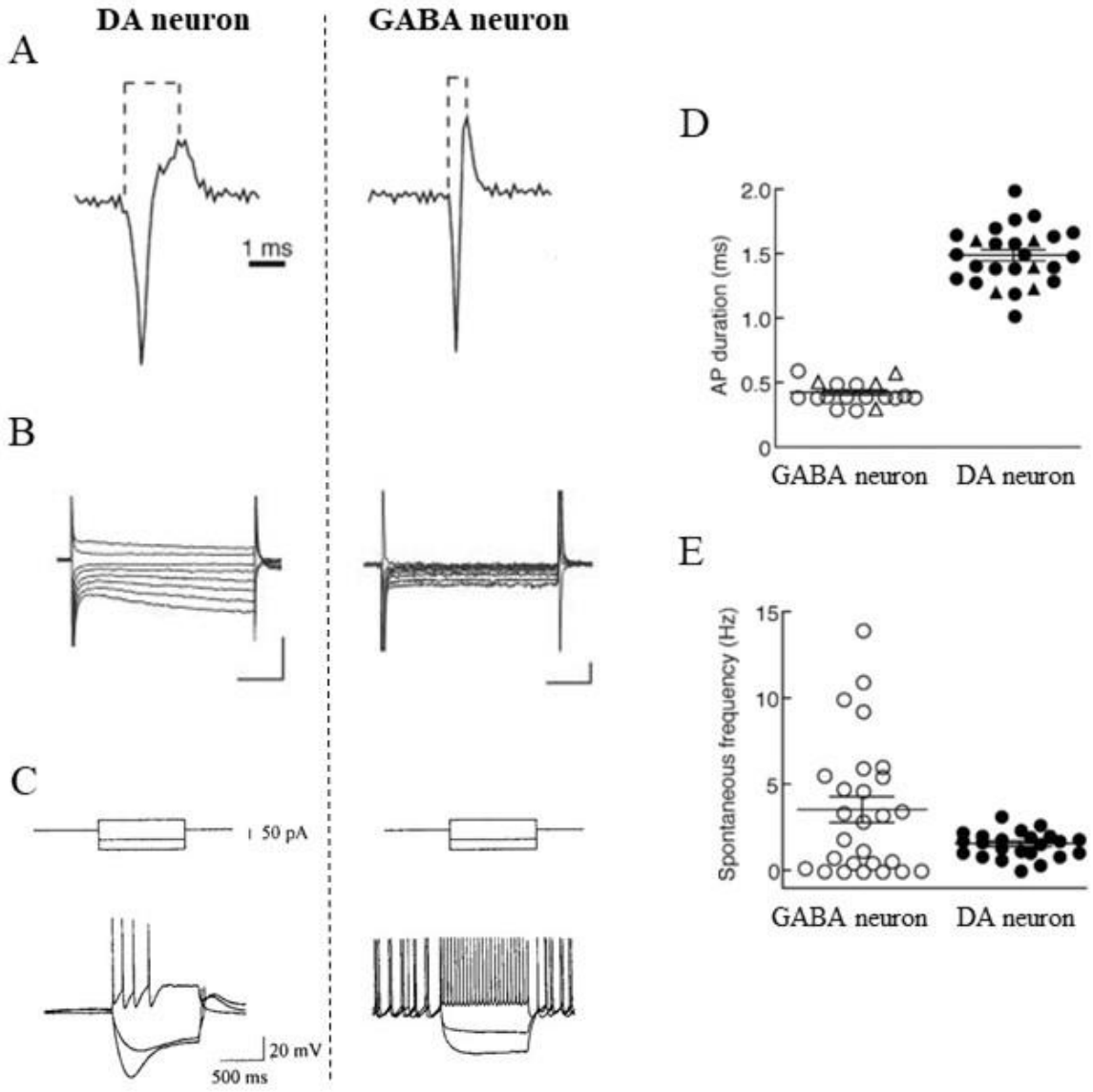


Figure 5: Electrophysiological properties of VTA DA and GABA neurons. (A) Representative traces of action potentials of a DA neuron (*left*) and a GABA neurons (*right*). (B) Representative trace of I_h in a DA neuron (*left*) and GABA neuron (*right*). Scale bars: 20 pA and 50 ms). (C) Representative changes in membrane potential in response to hyperpolarizing and depolarizing current pulses in a DA neuron (*left*) and GABA neuron (*right*). (D) Action potential duration of DA and GABA neurons. (E) Spontaneous action potential frequency for DA and GABA neurons sampled for 60 s. A, D, and E panels adapted from (Chieng et al. 2011b). B panel adapted from (Margolis et al. 2006). C panel adapted from (Marino et al. 2001).

3.3.3 Glutamate neurons

Glutamate is produced from glutamine by glutaminase that is present in all neurons and glia. The presence of glutamate neurons in the VTA was established about 10 years ago with

the demonstration that in rat some VTA neurons possess mRNA encoding vesicular glutamate transporter 2 (VGLUT2) (Kawano et al. 2006; Yamaguchi et al. 2011; Yamaguchi, Sheen, and Morales 2007).

Moreover, the prevalence of these neurons has been demonstrated to be especially in the midline nuclei (Yamaguchi et al. 2011, 2015, 2007). Indeed, the number of VGLUT2-expressing neurons is higher in the rostral and medial VTA compared to TH-expressing neurons (Yamaguchi et al. 2011, 2015, 2007).

Even the VTA of non-human primates and humans contains glutamate neurons (Root et al. 2016), and this is important because it allows performing translational studies to further investigate the role of this type of neurons in human behavior.

4. NEUROCHEMICAL AND ELECTROPHYSIOLOGICAL ALTERATIONS INDUCED BY ACUTE AND CHRONIC ALCOHOL EXPOSURE

The main molecular targets of alcohol within the brain are either voltage-gated ion channels, such as L-type Ca^{2+} channels and G protein-coupled inwardly-rectifying potassium (GIRK) channels, or ligand-gated ion channels, like the γ-aminobutyric acid type A ($GABA_A$) receptor, the N-methyl-D-aspartate (NMDA) receptor for glutamate, the 5-hydroxytryptamine type 3 (5-HT3) receptor for serotonin, nicotinic receptors for acetylcholine (nAChRs), and glycine receptors (Lovinger and Roberto 2011).

$GABA_A$ and NMDA receptors are those that arouse much scientific interest since chronic alcohol consumption leads to the most adaptive changes in these types of receptors. Moreover, they are affected by both low and moderate concentrations of alcohol in the CNS (Lovinger and Roberto 2011).

However, there are also neurochemical systems altered by chronic alcohol which are indirect targets of alcohol itself. Neurotransmitters, for example, are very important in modulating numerous activities in specific brain areas, and changes in their concentration bring to severe diseases. In addition to the disruption of the function of neurotransmitter receptors, ion channels, and intracellular signaling proteins (Yoshimura et al. 2006; Pany and Das 2015; Ron and Barak 2016; Mohamed et al. 2018), low to intermediate concentrations of EtOH also alter membrane lipids (Cui and Koob 2017).

Dopamine (DA), GABA, glutamate, serotonin (5HT), and acetylcholine (Ach) are the main neurotransmitters involved in AUD.

4.1 Dopamine

Dopamine is the main modulatory neurotransmitter of the mesocorticolimbic pathway involved in reward-related behavior.

Among neurotransmitter systems, not only GABA and glutamate, but also opioid peptides, acetylcholine, endocannabinoids, and other modulators, are responsible for the activation of the mesocorticolimbic reward system at different anatomical regions (Figure 3). Acute and chronic alcohol alters DA signaling.

Acute EtOH leads to a massive release of DA in the VTA and NAc, and consequently, neurons are overstimulated (Kegeles et al. 2018). In particular, DA in the NAc seems to be crucially involved in EtOH addiction development. Indeed, if a DA antagonist is administered in the NAc of rodents, a drastic decrease in alcohol drinking is noted (Jayaram-Lindström et al.

2016). Furthermore, it has been reported that this pathway is also activated by alcohol-associated cues when presented to alcoholics or EtOH-exposed animals (Jupp et al. 2011; Myrick et al. 2008). This suggests that alcohol also intervenes in maladaptive reward-related learning (Di Chiara 1999).

Also, acute EtOH application enhances the spontaneous firing rate of VTA DA neurons both *in vivo* and *in vitro* (Brodie, McElvain, et al. 1999; Brodie and Appel 1998; Brodie, Pesold, and Appel 1999; Brodie, Shefner, and Dunwiddie 1990; Gessa et al. 1985). This happens even when VTA DA neurons have not synaptic or glial connections (Brodie, McElvain, et al. 1999; Brodie, Pesold, and Appel 1999), and when glutamate, Ach, or GABA antagonists are present (Nimitvilai et al. 2016).

The I_h current has been discovered to be another direct target of alcohol (Harris and Constanti 1995; Neuhoff et al. 2002). I_h is known to be involved in the pacemaker firing contribution of DA neurons, probably because EtOH can facilitate the cyclic adenosine monophosphate (cAMP)-dependent voltage-gating (Okamoto, Harnett, and Morikawa 2006), even if it has been shown only in mice (Appel et al. 2003; McDaid, McElvain, and Brodie 2008). The application of an I_h blocker, ZD7288, inhibits the firing rate irreversibly and diminishes the EtOH-induced increase of the firing frequency. Also, EtOH seems to increase barium-sensitive potassium currents in VTA DA neurons (McDaid, McElvain, and Brodie 2008), which can slow down the firing rate only if I_h is blocked.

EtOH can also modulate M-current (I_m) (Hansen et al. 2008) which is a potassium current modulating the generation of action potentials (AP) in a negative sense (Aiken, Lampe, and Brown 1995; Brown and Adams 1980). In particular, it has been demonstrated that *in vitro* EtOH decreases I_m leading to a minor inhibition and direct enhancement of the firing frequency of VTA DA neurons (Koyama, Brodie, and Appel 2007). Kv7.4 channels are the main components of the slow voltage-gated M-channel (Brown and Passmore 2009). Interestingly, it has been recently discovered that Kv7.4 channels are modulators of the EtOH intake and dependence in rodents (McGuier et al. 2018).

EtOH also acts on both small and big conductance calcium-activated potassium channels (SK and BK) (Brodie et al. 2007), that are expressed in VTA DA neurons. These channels activate during the membrane depolarization and calcium influx during an AP, leading to fast after-hyperpolarization (AHP) and the end of the AP (Faber and Sah 2007; Lee and Cui 2010). Acute EtOH decreases SK channel function and increases the probability of channel open of BK (Dopico, Bukiya, and Martin 2014; Mulholland et al. 2009). The quantity of SK current seems to indicate the sensitivity of VTA DA neurons to EtOH-induced excitation (Brodie, McElvain,

et al. 1999); however, if this channel is pharmacologically inhibited, it does not reduce EtOH-induced excitation of VTA DA neurons. Of course, further studies are needed to establish the role of these channels in VTA DA mediated EtOH reinforcement.

Other alcohol targets are GIRK channels, through which EtOH modulates the excitability of DA neurons. These channels are all about maintaining the resting membrane potential and the switch from tonic to burst firing modes of DA neurons (Lalive et al. 2014). EtOH can acts on GIRK channels both directly and through a G-coupled protein (Aryal et al. 2009; Beckstead et al. 2004; Kobayashi et al. 1999; Labouèbe et al. 2007; Lewohl et al. 1999). By activating GIRK channels, EtOH can enhance the inhibition of VTA DA neurons induced by the $GABA_B$ receptor (Federici et al. 2009). So, it seems that EtOH potentiates the function of GIRK channels on VTA DA neurons leading to inhibitory effects and stabilizing the firing of VTA DA neurons.

Preclinical studies, in which chronic alcohol exposure has been used, explain compensatory mechanisms, abnormal learning, and long-term plasticity changes leading to the long-lasting effects of EtOH in the brain (Weiss and Porrino 2002). For example, it has been demonstrated that after *in vivo* chronic EtOH administration (3.5 mg/kg twice a day, intraperitoneal (i.p.), for 21 days), VTA DA neurons appear sensitized to the bath application of EtOH, although no changes have been seen in the basal firing rate (Brodie 2002). Also, another change induced in mice by *in vivo* repetitive EtOH administration (2 g/kg once a day, i.p., for 5 days) has been found in the I_h, whose density is reduced, as well as the degree of EtOH-induced stimulation of firing *in vitro* (Okamoto et al. 2006). Another group of researchers found that, after continuous and voluntary alcohol consumption through a two-bottle choice paradigm, VTA DA neurons projecting to NAc have dramatically decreased I_h current in high alcohol drinking mice and increased I_h current in low alcohol drinking mice compared to control mice (Juarez et al. 2017). Also, they found, in the same experimental conditions, that low alcohol drinking mice have significantly higher VTA DA neuron firing and burst activity. Unexpectedly, the activity of VTA DA neurons in high alcohol-drinking mice is not different from alcohol naive mice (Juarez et al. 2017).

Furthermore, it has been shown that microinjections of quinpirole (a DA D_2/D_3 agonist) in the VTA in rats reduces 10% EtOH-reinforced responding, suggesting that DA activity in the VTA is essential for the regulation of EtOH reinforcement (Hodge et al. 1993).

Moreover, Bailey et al. demonstrated that, after chronic EtOH consumption, the basal firing rate of VTA DA neurons recorded *in vitro* is decreased (Bailey et al. 2001). Importantly, an altered DA transmission has also been revealed in the ventral striatum of alcohol-dependent people (Martinez et al. 2005; Volkow, Wang, et al. 2007). Probably, as a consequence of this

DA reduction, the expression of the D_3 DA receptor in alcohol-preferring rats has been found to be up-regulated after long alcohol consumption (Vengeliene et al. 2006). Moreover, a D_3 antagonist and a D_3 partial agonist suppressed the alcohol deprivation effect (ADE) and the cue-induced reinstatement of alcohol-seeking behavior (Vengeliene et al. 2006). For this reason, the D_3 receptor has been thought to be a new target for the treatment of alcoholism.
On the other hand, an enhanced level of D_2 receptor in the striatum seems to be a protective factor against alcoholism in humans (Volkow et al. 2006). Thus, unfortunately, DA alterations within the mesolimbic system are too complex to be considered a possible target for alcoholism treatment (Diana 2011). Moreover recently, it has been reported that a substance that impairs DA signaling in the mesolimbic pathway, like 6-hydroxydopamine, does not cancel the alcohol-seeking tendency. This suggests that DA is not the only responsible for the development of an addictive state for alcohol (Rassnick, Stinus, and Koob 1993).
GIRK channels are also affected by chronic alcohol exposure. In particular, electrophysiological recordings revealed that, after 3 injections a day of EtOH 2 mg/Kg i.p. for a week, the inhibition mediated by D_2/GIRK is increased while no changes affect the inhibition mediated by $GABA_B$/GIRK.
Also, the function of SK channel is reduced 7 days after chronic EtOH exposure (2 g/kg i.p. twice a day, for 5 days) (Hopf et al. 2007). So, it has been assumed that this alteration, together with other ones, could contribute to increasing burst activity of DA neurons leading to an enhancement of EtOH reinforcement and thus the development of addicted behaviors.

Following a long alcohol exposure that causes some neuroadaptations, several studies demonstrate a decrease in the function of the mesolimbic reward system and the occurring of a "hypodopaminergic state" (Melis, Spiga, and Diana 2005). Indeed, in alcohol-dependence rats, the spontaneous firing rate (both tonic and phasic firing) of DA neurons within the VTA is drastically reduced after stopping EtOH administration (Diana et al. 1993, 1995), leading to a decreased DA transmission that returns to normal levels after 2 months of EtOH withdrawal (Bailey et al. 2001). This hypodopaminergic state is responsible for drug seeking and relapse.
A decrease in tonic DA levels has also been shown in the NAc in animals withdrawn after repetitive EtOH exposure (Rossetti et al. 1992; Diana et al. 1993; Weiss et al. 1996). On the contrary, a hyperdopaminergic state has been found in animals treated with chronic vapor exposure after a prolonged withdrawal (Hirth et al. 2016). The fact that a hypodopaminergic state occurs during withdrawal and a hyperdopaminergic one follows could maybe be responsible for the neuroadaptations shown in advanced-stage alcoholics.

4.2 Glutamate

Glutamate is an excitatory neurotransmitter that binds to ionotropic and metabotropic receptors. Its ionotropic receptors, the N-methyl-D-Aspartate (NMDA) and the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, are inhibited by alcohol in a concentration-dependent manner (Lovinger and Roberto 2011; Lovinger, White, and Weight 1989). Generally, when NMDA and AMPA receptors are activated, the firing and the burst activity of VTA DA neurons *in vivo* increase (Chergui et al. 1993; Johnson, Seutin, and North 1992; Zweifel et al. 2009). Notably, it has been reported an enhancement in the AMPA receptor-mediated transmission *in vitro* 24 h after an EtOH i.p. injection (20 mg/Kg) in EtOH-preferring C57BL/6J mice (Saal et al. 2003), while in EtOH-non preferring DBA/2J mice a reduction of AMPA and NMDA receptor function has been shown 24 h after a single EtOH i.p. injection (2 g/Kg) (Wanat et al. 2009), suggesting a difference in EtOH sensitivity based on the strain.

Moreover, acute EtOH can also bind to glutamate presynaptic receptors leading to an increase in the frequency but not in the amplitude of spontaneous excitatory postsynaptic currents (sEPSCs) (Xiao et al. 2009; Deng et al. 2009).

Consequently, chronic alcohol brings to an up-regulation of the NMDA receptor subunit expression and its functionality. This increase leads to hyperexcitability within the CNS after EtOH withdrawal which produces excitotoxicity.

Also, alcohol inhibits the function of the AMPA and kainic acid (KA) receptors that, together with an increase in the extracellular level of glutamate (Licata and Renshaw 2010) and a decrease in the function of the GABAergic transmission (Licata and Renshaw 2010; Lovinger and Roberto 2011), may exacerbate the CNS hyperexcitability during withdrawal.

Furthermore, it seems that alcohol, which is a predominantly inhibitory molecule, can activate the mesolimbic reward pathway by potentiating glutamatergic transmission to DA neurons of the VTA (Xiao et al. 2009).

Glutamate acts on NMDA receptors, mediating synaptic plasticity, but beyond that, glutamate also acts on G-protein coupled metabotropic glutamate receptors (mGluRs), modulating synaptic transmission. Indeed, numerous studies show that mGluR1 or mGluR5 antagonists decrease alcohol drinking in animals, as well as mGluR2 and mGluR3 agonists (Duncan and Lawrence 2012; Olive 2009). However, it has been shown that mGluR2/3 or mGluR5 receptor modulation is changed in alcohol-dependent rodents (Kufahl, Martin-Fardon, and Weiss 2011; Sidhpura, Weiss, and Martin-Fardon 2010). For this reason, mGluR-based ligands could potentially have a therapeutic application.

Importantly, it has been reported that chronic alcohol exposure can enhance synaptic plasticity of NMDA receptors in VTA DA neurons by increasing long-term potentiation (LTP) of glutamate transmission, especially the NMDA-mediated transmission (Bernier, Whitaker, and Morikawa 2011).

However, two groups of research demonstrated that chronic EtOH injections (2–3.5 g/kg, i.p. once/twice a day for 5–21 days) do not alter the function of NMDA receptor (Brodie 2002; Hopf et al. 2007).

Moreover, voluntary and very long EtOH exposure (up to 50 days) has been found to increase AMPA-mediated transmission onto VTA DA neurons in rats (Stuber et al. 2008).

Finally, Ortiz et al. showed that, after 12 weeks of alcohol administration, levels of two glutamate receptor subunits, the NMDAR1 and GluR1, are increased in the VTA (Ortiz et al. 1995).

4.3 GABA

GABA is the most important inhibitory neurotransmitter that binds to $GABA_A$ and $GABA_B$ receptors (Gilpin and Koob 2008).

Acute alcohol consumption modulates GABAergic transmission, both pre- and postsynaptically. This means that the presynaptic GABA release can be changed, as well as the function of the postsynaptic $GABA_A$ receptor.

Acute EtOH acts on inhibitory GABA transmission with different effects on GABA release at VTA DA synapses (Burkhardt and Adermark 2014; Abrahao, Salinas, and Lovinger 2017). For instance, a single alcohol administration *in vivo* has been reported to suppress VTA GABA neurons firing (Steffensen et al. 2000; Stobbs et al. 2004).

Moreover, GABA neurons express dopamine D2 and l-opioid receptors which are both targets of EtOH. Their activation leads to a decrease of GABA firing and an increase in the DA release in the VTA (Ludlow et al. 2009; Xiao et al. 2007).

However, it has been demonstrated that acute EtOH, either i.p. injected (2 g/Kg) *in vivo* or applicated on a slice (50 mM) *in vitro*, increases inhibitory transmission and GABA release in the VTA DA neurons (Theile et al. 2011, 2008).

Also, it has been shown that acute EtOH, i.v. injected in low concentration (0.01–0.1 g/kg), increases VTA GABA neurons activity in rats (Steffensen et al. 2009).

So, it has been assumed that the mechanism behind the increase in the DA neurons firing is the suppression of local inhibitory transmission that leads to a disinhibition of VTA DA neurons

(Xiao et al. 2007). However, further studies are needed to clarify if only local GABA neurons or even GABA neurons coming from other brain areas are involved in this mechanism.

As for chronic EtOH exposure, it seems to decrease the sensitivity of GABA-induced inhibition of VTA DA neuron firing (Brodie 2002; Stobbs et al. 2004; Burkhardt and Adermark 2014; Adermark, Söderpalm, and Burkhardt 2014).

Also, 2-3 weeks of 5% EtOH (liquid diet) has been reported to decrease the expression of D_2 receptors on VTA GABA neurons, probably due to the continual release of DA on GABA neurons (Ludlow et al. 2009).

Interestingly, chronic EtOH has been also seen to inhibit LTP of GABA neurons through μ-opioid receptors (Guan and Ye 2010).

Chronic EtOH drinking induces neuroadaptations in which the presynaptic GABA release is decreased (Lovinger and Roberto 2011) as well as changes in the expression levels and surface expression of $GABA_A$ receptor proteins, especially in some region like the hippocampus, cerebellum and cortex (Uusi-Oukari and Korpi 2010), leading to an imbalance between excitatory and inhibitory system within the brain. Moreover, these alterations may either persist or revert after alcohol deprivation (Uusi-Oukari and Korpi 2010). Importantly, it seems that alteration in GABA transmission is responsible for the tolerance phenomenon and many psychological and physiological effects of alcohol (Lovinger and Roberto 2011).

With regards to the $GABA_B$ receptor, which is also present in the presynaptic elements where acts as an autoreceptor, it has been reported to be decreased in functionality by alcohol. Indeed, in chronically alcohol-exposed rodents, the function of the presynaptic $GABA_B$ receptor in the hippocampus is declined (Frye et al. 1991; Peris et al. 1997). Even in humans, it has been shown that alcohol brings an impairment in the expression of the $GABA_B$ R1 receptor subunit in the hippocampus (Enoch et al. 2012).

So, we can affirm that alcoholism significantly perturbs GABAergic transmission. Indeed, benzodiazepines that activate $GABA_A$ receptors are sometimes used for withdrawal symptoms, just like baclofen, a $GABA_B$ agonist, that is used for the treatment of alcoholism (Addolorato et al. 2012).

4.4 Serotonin

Serotonin is another important neurotransmitter that mediates happiness, anxiety, and moodlike behaviors in the brain (Tops et al. 2014). It also mediates the motivational and emotional behaviors, and focus on a specific task (Uhl, Koob, and Cable 2019).

The receptors of serotonin regulate the state of mood by activating second messengers and intracellular cascades (Patkar, Belmer, and Bartlett 2016). Serotonin is produced in the brain from the raphe nucleus (RN) only. From the RN, the serotoninergic projection arrives at the amygdala, a brain region involved in drug addiction pathophysiology, where controls the neuronal outputs.

It has been shown that selective serotonin reuptake inhibitors (SSRIs) reduce alcohol intake in animals (Ferraguti, Pascale, and Lucarelli 2015). However, their efficacy is milder in human alcoholics because of the high rate of comorbidity in alcohol-dependent people and mutations in the serotonin transporter (SERT) gene (Sari, Johnson, and Weedman 2011).

Importantly, greater effectiveness in the reduction of EtOH drinking and relapse has been shown with the use of ondansetron, a 5-HT_3 antagonist (Sari et al. 2011), precisely because these receptors are molecular targets of alcohol. Indeed, chronic alcohol consumption leads to hypersensitivity of 5-HT_3 receptors function (Yoshimoto et al. 1996). In particular, 5-HT_3 receptors in the VTA seem to be important in the serotoninergic modulation of alcohol preference (Sari et al. 2011).

4.5 Opioid peptides

The involvement of the endogenous opioid peptides (endorphins, enkephalins, and dynorphins) in alcohol reinforcement effects and craving has been suggested from the efficacy of naltrexone, a non-selective opioid antagonist, in both EtOH-dependent animals and humans (Gianoulakis 2009; Roth-Deri, Green-Sadan, and Yadid 2008; Trigo et al. 2010). Naltrexone seems to act with a mechanism that could modify the pleasing effect of alcohol in chronic users only. Indeed, it decreases the rewarding effects of alcohol not by blocking the acute release of DA in the VTA of EtOH-naïve rats but by reducing the DA releasing time (Valenta et al. 2013). Alcohol brings the release of opioid peptides in some reward-related brain regions (Marinelli et al. 2006; Olive et al. 2001) and chronic EtOH consumption alters the receptors densities and effector systems of opioid peptides (Gianoulakis 2009; Walker and Koob 2008). The manipulation of opioid receptors, for example by using μ or δ antagonists, decreases alcohol drinking (Méndez and Morales-Mulia 2008).

For many years, μ and δ receptors have been the most studied in order to figure out their role in alcoholism. Then, the interest expanded even to the κ opioid receptor and its endogenous ligand, showing that the stimulation of this receptor causes aversive effects.

From this point, it has been hypothesized that repeated alcohol intake brings an increased activation of the κ receptor. This alteration would lead to the occurrence of a discomfort state

which could be responsible for further alcohol intake, acting as a negative reinforcement to alleviate the illness (Bruchas, Land, and Chavkin 2010; Wee and Koob 2010). Indeed, it has been demonstrated that κ antagonist administration especially suppresses alcohol consumption in EtOH-addicted rats (Walker and Koob 2008).

4.6 Nociceptin/orphanin FQ

Interestingly, even another neuropeptide seems to be involved in alcohol abuse. It is nociceptin/orphanin FQ (N/OFQ) whose receptor is opioid-receptor-like 1 (ORL1, also called NOP) (Murphy 2012). N/OFQ is a 17 amino acid peptide, deorphanized in 1995, that shows high structural homology with dynorphin, just like NOP receptor with κ opioid receptor. For this reason, the N/OFQ-NOP system is considered another member of the opioid family (Cox et al. 2015; Toll et al. 2016). Since its discovery, N/OFQ has attracted the attention of many researchers for its potential role in drug abuse.

Initially, the anti-addictive properties of NOP agonists have been described in alcohol studies. Later these effects have been reported also for psychostimulants and opioids. Strangely, NOP agonists seem to have the opposite effect on nicotine consumption.

Intriguingly, if on the one hand NOP activation leads to the reduction in the consumption of substance of abuse (except for nicotine), on the other hand it has been reported that animals that overexpress NOP receptors have a higher probability of abuse drug whereas NOP knockout rats self-administer less alcohol, cocaine, and heroin (Sommer et al. 2008; Aujla et al. 2013; Kallupi et al. 2017).

Surprisingly, even NOP antagonists decrease alcohol consumption and nicotine self-administration as well, leading to an apparent paradox (Rorick-Kehn et al. 2016; Post et al. 2016).

4.6.1 The N/OFQ system and NOP agonism

The first scientific paper describing the role of N/OFQ in alcohol abuse demonstrated that repeated intracerebroventricular (ICV) administration of nociceptin decreases voluntary EtOH drinking (two bottle-choice with EtOH 10% and water) in Marchigian Sardinian alcohol-preferring (msP) rats (Ciccocioppo et al. 1999). After that, numerous other studies confirmed this finding, in different experimental conditions and with different NOP agonists. For example, it has been shown that in mice NOP activation by N/OFQ peptidic analogues or synthetic agonists attenuates the reinforcing effects of alcohol, measured with conditioned place preference (CPP) experiment (Kuzmin et al. 2003, 2007; Zaveri et al. 2018) as well as EtOH self-administration and relapse in rats (Aziz et al. 2016; Ciccocioppo et al. 2002, 2003;

Economidou et al. 2006, 2008; Kuzmin et al. 2007; Martin-Fardon et al. 2000). These studies were performed in either msP or Wistar rats. However, if compared with each other, msP rats showed bigger sensitivity to the effects of NOP agonists compared to Wistars (Economidou et al. 2008; De Guglielmo et al. 2015; Martin-Fardon et al. 2010). The msP line is reported to be more anxious, show depression-like symptoms and are more sensitive to stress compared to unselected Wistar. All of these features are meliorated by alcohol consumption (Ciccocioppo 2012; Ciccocioppo et al. 2006; Egervari et al. 2018). Notably, msP rats display overexpression of the corticotropin-releasing factor (CRF) system, due to polymorphism at CRF1 receptor locus (Ayanwuyi et al. 2013; Cippitelli et al. 2015; Hansson et al. 2006; Logrip et al. 2018). Two weeks of voluntary alcohol consumption are enough for reducing this overexpression suggesting that msP rats could drink to self-medicate negative symptoms induced by an overactive stress system (Hansson et al. 2007).

Interestingly, msP rats have been found to exhibit overexpression of the N/OFQ-NOP system in various brain areas, like the CeA. This could be a compensatory mechanism that counterbalances the overactivity of the CRF system (Economidou et al. 2008). This overexpression may explain why these animals have a higher sensitivity to NOP agonists. Indeed, the injection of NOP agonists directly in the CeA has been demonstrated to reduce both anxiety and alcohol consumption (Aujla et al. 2013; Economidou et al. 2008).

Interestingly, chronic alcohol exposure induces the same neuroadaptive changes described for msP rats in Wistar rats: enhanced anxiety, sensitivity to stress, sensitivity CFR1 antagonist, and increase in the number of CRF1 receptors (Ciccocioppo et al. 2009; Gehlert et al. 2007; Sommer et al. 2008). Moreover, it has been demonstrated that NOP agonists reduce acute withdrawal symptoms in alcohol-dependent rats (Economidou et al. 2011).

In addition, electrophysiological studies performed in CeA slices showed that nociceptin increases paired-pulse facilitation ratio of inhibitory postsynaptic currents (IPSCs) and that this is significantly marked in alcohol-dependent and msP rats (Cruz et al. 2012; Herman et al. 2013; Roberto and Siggins 2006). Also, if on one hand NOP agonism in the CeA decreases glutamatergic excitatory transmission, on the other hand it impedes that alcohol inhibits glutamate (Kallupi et al. 2014).

In conclusion, it seems that chronic and intoxicating doses of EtOH induce overexpression and overactivity of the N/OFQ-NOP system and that activation of the NOP receptor has positive effects, especially in anxious and stress-sensitive animals.

4.6.2 NOP antagonism

Curiously, reduction of alcohol drinking has reported also following administration of NOP antagonists.

For example, LY2940094, a potent and selective NOP antagonist, reduced alcohol self-administration and alcohol-seeking in msP rats and nonselected heterogeneous rats (Rorick-Kehn et al. 2016). This molecule seems to have positive effects even in patients who suffer from AUD where it decreases the number of heavy drinking days, suggesting that NOP antagonism could be a potential treatment for alcohol dependence (Post et al. 2016).

The therapeutic potential of NOP antagonism is indirectly supported by studies performed in NOP-knockout rats. Indeed, these genetically modified animals, although exhibit a normal motivation for saccharin, are more resilient to develop drug addiction, consuming a significantly smaller quantity of alcohol, heroin, and cocaine compared to wild-type animals (Kallupi et al. 2017).

To explain this apparent paradoxical effect with both NOP agonists and antagonists showing similar effects on alcohol drinking, few hypotheses have been proposed. First of all, it has been suggested that this effect is mediated by NOP receptor desensitization. In pharmacological studies, exogenous administration of NOP agonists may produce a nonphysiological overactivation of the system that could lead to rapid receptor desensitization. If this is true, the effect of the agonist would result from a paradoxical functional antagonism. This hypothesis is supported by data showing that NOP receptors can undergo rapid desensitization following chronic agonist administration or a high dose of an agonist (Toll et al. 2016).

Furthermore, Ciccocioppo et al. recently demonstrated that the potent and selective NOP agonist MT-7716 does not affect alcohol intake if administrated acutely, while reduces it when given chronically and, also, the decreased consumption persists for several days after suspension of the treatment (Ciccocioppo et al. 2014). Moreover, another study reported a significant increase in EtOH consumption after ICV infusions of a low dose of N/OFQ for 7 consecutive days in msP rats (Cifani et al. 2006). In the same rat's line, it has been demonstrated that another potent and selective NOP agonist, Ro64-6198, leads to drinking more if administered once but reduces alcohol intake after repeated administrations (Economidou et al. 2006).

There are also other studies supporting the possibility that NOP agonism increases alcohol drinking. For instance, after repeated exposure to intoxicating concentrations of alcohol, Wistar

rats drink higher amounts of alcohol, but they also exhibit upregulation of NOP receptors in the CeA and BNST (Sommer et al. 2008; Aujla et al. 2013). Perhaps, this overexpression of the NOP system is the result of a physiological attempt to compensate the overactivity of the CRF system that is innate in msP rats and occurs in Wistars following dependence induction (Hansson et al. 2006; Gehlert et al. 2007; Sommer et al. 2008; Ciccocioppo et al. 2009; Aujla et al. 2013; Ayanwuyi et al. 2013; Cippitelli et al. 2015).

However, it has been shown that stimulation of NOP receptors by injecting N/OFQ in the VTA decreases DA release in the NAc. The onset of a hypodopaminergic and hypohedonic state could be the cause of increased motivation for drugs of abuse (Murphy and Maidment 1999).

In conclusion, it is now clear that the modulation of the N/OFQ- NOP receptors system plays a crucial role in modulating alcohol abuse-related behaviors. However, the direction in which activation of the system modulates these behaviors is still unclear, as it is unclear whether therapeutic approaches should be based on agonists or antagonists.

5. AIM OF THE THESIS

Today, substance use disorder is one of the most serious health problems worldwide. Every year, addiction to substances like alcohol and tobacco causes the death of millions of people (Wittchen et al. 2011). Unfortunately, current treatments are hardly available and not very effective. In this respect, a better understanding of the neural modifications marking the early phases of alcohol experience may unravel mechanisms which drive behavioral reinforcement and lead to the development of dependence in advanced stages. These early mechanisms may represent potentials targets for more effective pharmacological therapy.

On these premises, the main goal of the present project was to investigate the functional remodeling of the reward system induced by ethanol in an animal paradigm of voluntary drinking. This goal was pursued through the accomplishment of three main objectives.

a) Implementation and characterization of a voluntary drinking paradigm with assessment of preference and total intake dynamics. The emotional and behavioral response to alcohol deprivation was also evaluated by testing anxiety levels and relapse.
b) Electrophysiological investigation of persistent alterations in the midbrain dopaminergic reward system induced by voluntary alcohol intake and following withdrawal.
c) Preliminary validation of therapeutics able to target the early steps of neurobiological mechanisms eventually leading to the development of an addictive state.

6. METHODS

6.1 ANIMALS

All procedures required for ex vivo experiments were conducted in compliance with the Council Directive of the European Community (2010/63/EU), Decreto Legislativo Italiano 26 (13/03/2014), and approved by the Animal Care Committee of the Department of Neurofarba, University of Florence (authorization number 545/2018). B6.Cg-Tg (TH-GFP) mice (Matsushita et al. 2002) were obtained from Riken BioResource Center, Japan, and housed in a temperature-controlled (24 °C) animal colony under a 12 h light/dark cycle with food and water available ad libitum. In these mice, a green-fluorescent protein (GFP) expression is driven by the TH promoter. In order to avoid gender bias, both male and female at postnatal day 40-60 mice were used and randomly assigned to either experimental group in this study. "Exposed" mice were exposed to a voluntary alcohol drinking paradigm (3, 6, 12% v/v EtOH) while "naive" mice had access to water only. Finally, behavioral and electrophysiological experiments were carried out on both naïve and exposed groups.

6.2 ALCOHOL DRINKING PARADIGM AND BEHAVIORAL TESTS

6.2.1 Voluntary alcohol drinking paradigm

At 6-8 weeks of age, mice were individually housed in standard nonventilated Plexiglas mouse cages with a mouse house (Tecniplast), bedding and nesting materials. Mice had access to two 50 mL tubes filled with water and equipped with rubber stoppers with a metal sipper tube for at least 5 days of acclimatization. After that, mice were divided into "naive" and "exposed" groups.

The water in one tube of the exposed group was replaced with a 3% EtOH v/v solution and placed close to the water tube.

Every day, mice were weighed, and the position of the two bottles switched in order to avoid the development of a side preference.

After 4 days of choice between water and alcoholic solution, exposed mice were weighed, the 3% EtOH solution was replaced with a 6% EtOH v/v solution as well as the old water with a new one, and both EtOH and water tubes were weighed before to be placed into the cage. Again, after 4 days of continuous access to water and 6% EtOH, the water was refreshed and the EtOH

concentration was increased to 12% EtOH for 10 days, during which mice and tubes were weighed and bottles switched every day.

Naive mice were kept in the same condition but they had only access to two tubes of water. Both groups had unlimited access to food for the entire duration of the 2-bottle choice paradigm. At the end of the drinking paradigm, alcohol drinking behaviors were determined: alcohol preference was calculated as ((EtOH intake / total fluid intake) × 100), while alcohol consumption was determined as g of EtOH consumed in 24 hours, calculated based on EtOH concentration / mouse weight.

6.2.2 Sucrose Preference Test

The sucrose preference test was performed just on the first experimental sets of animals in order to evaluate the hedonic tone of TH-GFP mice. The percent sucrose intake is usually used as a benchmark for the natural reward (sucrose) (Russo and Nestler 2013).

Before the alcohol drinking paradigm, mice were subjected to a sucrose preference test where, in their home cage, one tube filled with water was still replaced with a 5% sucrose solution, weighed, and placed next to the water-filled bottle for 4 days.

Sucrose preference was calculated in percentage as ((intake of sucrose solution / total fluid intake) × 100).

6.2.3 Elevated Plus Maze (EPM)

Following the first 18 days of the drinking paradigm, animals were deprived of alcohol solution which was placed with water for 5 days. On the last day of EtOH suspension, the EPM was used in order to assess the anxiety behavior of mice that could be increased for alcohol abstinence.

Before this test, mice were trained to walk on a smooth and elevated surface for 5 minutes a day for 4 days. The EPM was performed in a sound-attenuated room under dim light conditions. The EPM relies on mice's propensity for enclosed and dark spaces and an unconditioned fear of open and heightened spaces (Brain 1976). Moreover, this test studies the behavior, in particular the activity in the open arms of the EPM, as the result of a conflict between the innate motivation of mice to explore a new environment and their preference for closed areas, just like the closed arms of EPM.

Briefly, mice were placed in the intersection of the 4 arms of the EPM with their face facing one of the two open arms and, starting from that moment, their activity and movement were recorded for 5 minutes (Montgomery 1955).

The measures that were recorded are the time spent and the number of entries made on the open and closed arms of the EPM.

6.2.4 Alcohol Deprivation Effect (ADE)

After the first 18 days of the drinking paradigm followed by the 5 days of EtOH deprivation, the ADE was measured. Mice were re-exposed to EtOH 12% v/v for 2 days, just like during the 2-bottle choice paradigm, in order to evaluate the EtOH consumption and preference and compare them to those obtained before the EtOH deprivation.

6.3 *EX VIVO* ELECTROPHYSIOLOGY

6.3.1 The patch-clamp technique

The patch-clamp technique, invented by Erwin Neher and Bert Sakmann in 1976, consists of tight sealing the tip of a pipette filled with a physiological solution to the membrane of a cell, clamping it to a specific voltage to measure currents flowing through ion channels (voltage-clamp). Alternatively, the experimenter can control the current injected into the cell through the recording electrode, measuring potential dynamics (current-clamp). Two recording configurations were used in this project: the "whole-cell" and the "cell-attached" configurations. The whole-cell configuration relies on the rupture of the seal, allowing the pipette solution to diffuse inside the cell. With this configuration, the experimenter may control the cell's internal ionic content. During whole-cell recordings, an irreversible washout of diffusible intracellular constitutes (e.g. ATP, phosphorylating molecules, etc) into the much larger volume of the pipette occurs. As a consequence, the properties and functions of ion channels can be impaired, leading to a decrease in ionic currents over time. In the cell-attached configuration, the patch electrode is sealed to the membrane (reaching a GΩ resistance). As the membrane integrity is preserved, this configuration is more conservative as compared to the whole-cell, thus allowing more stable recordings and physiological responses.

6.3.2 Reagents

Unless otherwise specified, reagents were purchased from Sigma-Aldrich (Saint-Louis, MO, USA).

During cell-attached recordings, the firing rate of VTA DA neurons was measured both freely and in the presence of a complete synaptic block by using NBQX (10 µM; Tocris bioscience,

Bristol, UK), D,L-APV (50 µM; Tocris bioscience, Bristol, UK), gabazine (10 µM; Tocris bioscience, Bristol, UK) and CGP (1 µM; Tocris bioscience, Bristol, UK), the $GABA_B$ blocker. During whole-cell recordings, sEPSCs of VTA DA neurons were isolated by inhibiting the $GABA_A$ component with gabazine (SR95531, 10 µM; Tocris bioscience, Bristol, UK), while sIPSCs of VTA DA neurons were isolated by blocking AMPA and NMDA currents with NBQX (10 µM; Tocris bioscience, Bristol, UK) and D,L-APV (50 µM; Tocris bioscience, Bristol, UK), respectively.

Nociceptin/orphanin FQ (30 nM; provided by Professor Girolamo Calò, University of Ferrara) was used as pharmacological treatment.

All drugs were added at the final concentration to the recording aCSF media.

6.3.3 Preparation of midbrain slices

40-60 days old TH-GFP mice were anesthetized with isoflurane and decapitated. The brain was quickly removed and blocked, attached to a slicing dish with cyanoacrylate glue in the slicing chamber of a vibroslicer (Leica VT 1000S, Leica Microsystem, Wetzlar, Germany). Midbrain horizontal slices (200 µm) containing the VTA were cut in an ice-cold slicing solution, composed of (in mM): N- methyl-D-glucamine (92), 4-(2-hydroxyethyl)-1-piperazine-1- ethanesulfonic acid (20), glucose (25), NaHCO3 (30), NaH2PO4 (1.25), KCl (2.5), MgSO4 (10), CaCl2 (0.5). NMDG is a substituent of sodium used during slicing in order to reduce neuronal activity and improve viability. Low calcium is also used to prevent neuronal death. The slicing solution was kept cold and saturated with a 95% O_2 + 5% CO_2 gas mixture. Immediately after cutting, slices were allowed to recover in a low calcium artificial cerebrospinal fluid (aCSF) solution, composed of (in mM): NaCl (130), KCl (3.5), NaH2PO4 (1.25), NaHCO3 (25), glucose (10), CaCl2 (1) and MgSO4 (2), maintained at 35-37°C with constant oxygenation for about 1 hour before the experiment. During the recordings, a single slice was transferred to a flow chamber positioned under the microscope objective and continuously perfused with warm, carbo-oxygenated aCSF solution, composed of (in mM): NaCl (130), KCl (3.5), NaH2PO4 (1.25), NaHCO3 (25), glucose (10), CaCl2 (2) and MgSO4 (1).

6.3.4 Cell-attached patch-clamp recordings

Patch pipettes were made from thin-walled borosilicate capillaries (Harvard Apparatus, London, UK) with a vertical puller (Narishire PP830, Narishire International Ltd, London, UK) and back-filled with the following intracellular solutions containing (in mM): K^+ gluconate

(120), KCl (15), HEPES (10), EGTA (1), $MgCl_2$ (2), Na_2Pcreatina (5), NaGTP (0.3), MgATP (4), pH 7.3-7.4, 295–305 mOsm and resulting in a bath resistance of 3-5 MΩ.

VTA was identified by its position relative to the medial terminal nucleus of the accessory optic tract (MT) (Neuhoff et al. 2002; Margolis et al. 2006) using infrared microscopy. VTA DA neurons were identified with fluorescence microscopy based on GFP expression, and, during the recordings, with the frequency of the firing. Indeed, it has been reported that DA neurons have a slower firing rate (~ 0.5-3 Hz) compared to GABAergic ones (~ 2-10 Hz) (Margolis et al. 2006).

Recordings of VTA DA and GABAergic neurons firing were performed in the absence of synaptic blockers, except for VTA DA neurons which were also recorded in the presence of $GABA_A$, $GABA_B$, AMPA and NMDA receptors blockers: gabazine (SR95531) 10 µM, CGP 1 µM, NBQX 10 µM and D,L-APV 50 µM, respectively.

As for free firing rate recordings, after 5 minutes of basal recording, EtOH 40 mM was bath-applied for up to 10 minutes, and the washout was recorded for the other 10 minutes in which bath-applied EtOH was removed from the slice and replaced with aCSF. In the case of isolated firing rate, 5 minutes of basal recording was first performed, followed by 5 minutes of application of all synaptic blockers. Once they worked, EtOH 40 mM was added to aCSF for 10 minutes, followed by its washout.

Once EtOH or blockers were added to the bath, they worked on the entire slice. For this reason, after the recording of a cell, the slice had always to be discarded.

Moreover, at the end of the recording, when possible, the surface of the neuron was broken to pass to the whole-cell configuration and confirm the identity of the recorded neuron. To do this, its firing pattern was assessed as well as the presence of I_h (see 6.5.2).

Recordings were performed at 34 °C, creating a loose seal (< 100 MΩ), which is particularly adequate for recording action potential currents, imposing 0 mV as voltage holding (V_{hold}). Signals were sampled at 10 kHz and low-pass filtered at 3 kHz with an Axon Multiclamp 700B (Molecular Devices, Sunnyvale, CA, USA).

6.3.5 Whole-cell patch-clamp recordings

Patch pipettes were made from thin-walled borosilicate capillaries (Harvard Apparatus, London, UK) with a vertical puller (Narishire PP830, Narishire International Ltd, London, UK) and back-filled with the following intracellular solutions containing (in mM):

(a) K^+ gluconate (120), KCl (15), HEPES (10), EGTA (1), $MgCl_2$ (2), Na_2Pcreatina (5), NaGTP (0.3), MgATP (4), pH 7.3-7.4, 295–305 mOsm and resulting in a bath resistance of 3-5 MΩ.

(b) KCl (140), $MgCl_2$ (1.6), MgATP (2.5), NaGTP (0.5), EGTA (2), HEPES (10), pH=7.3-7.4, 295–305 mOsm and resulting in a bath resistance of 3-5 MΩ.

The (a) intracellular solution was used for the recording of spontaneous excitatory postsynaptic currents (sEPSCs), the (b) solution, which is a high KCl (140 mM) intracellular solution was used for the recording of spontaneous inhibitory postsynaptic currents (sIPSCs). In these experimental conditions, E_{Cl} (~ 0mV) > V_{hold} (-60 mV), therefore, GABA current is an outward current mediated by negative chloride ions, which is read by the amplifier as a signal of equal amplitude and kinetics, but with opposite polarity.

VTA was identified by its position relative to the medial terminal nucleus of the accessory optic tract (MT) (Neuhoff et al. 2002; Margolis et al. 2006) using infrared microscopy. VTA DA neurons were identified with fluorescence microscopy based on GFP expression, and using a standard electrophysiological hallmark. that is the presence of a prominent I_h (Faleiro, Jones, and Kauer 2004; Liu, Pu, and Poo 2005; Ye et al. 2004). During voltage-clamp, I_h current was obtained by imposing 2 s-voltage steps with amplitude ranging from 0 mV to -60 mV, using -60 mV as V_{hold}. During current-clamp recordings, the activation of I_h is responsible for the so-called "voltage sag" (VS). The typical DA and/or GABAergic firing patterns were made by a series of hyperpolarizing and depolarizing current steps with amplitude ranging from -100 pA to +100 pA with 50 pA increments, immediately after breaking into the cell. I_h-mediated VS was measured as the difference between the voltage at the bottom of the sag and that at the end of the pulse.

Excitatory synaptic activity during recordings of sEPSCs was isolated by using the $GABA_A$ receptor blocker, gabazine (SR95531) 10 µM.

On the contrary, inhibitory activity during recordings of sIPSCs was isolated by using the D,L-APV 50 µM to block NMDA receptors, and NBXQ 10 µM to block AMPA receptors.

After 5 minutes of basal recording, $GABA_A$ receptors blocker or AMPA and NMDA receptors blockers were added to aCSF and recorded for 5 minutes. After that, EtOH 40 mM was applied together with the blockers for up to 10 minutes, and finally, the washout of EtOH in the blockers was recorded for 10 minutes.

Once blockers and EtOH were added to the bath, they worked on the entire slice. For this reason, after the recording of a cell, the slice had always to be wasted.

In addition to the spontaneous synaptic activity of VTA DA neurons, passive membrane properties were saved. In particular, membrane capacitance (Cm), input resistance (Ri) and resting membrane potential (RMP) were recorded.

In voltage-clamp recordings, access resistance was monitored for the entire duration of the experiment with brief test pulses (-10 mV, 500 ms). Recordings were performed at 34 °C. Recordings undergoing a drift in access resistance ≥ 10% were discarded. No whole-cell compensation was used. Signals were sampled at 10 kHz and low-pass filtered at 3 kHz with an Axon Multiclamp 700B (Molecular Devices, Sunnyvale, CA, USA).

6.4 DATA ELABORATION AND STATISTICAL ANALYSIS

The mean frequency and amplitude of sIPSCs and sEPSCs were calculated offline based on 1-min bins before and at the end of synaptic blockers or EtOH 40 mM bath application by using Clampfit 10.7 (Molecular Devices, Sunnyvale, CA, USA).

Cell-attached and whole-cell patch-clamp recordings were collected and analyzed using Clampfit 10.7 (Molecular Devices, Sunnyvale, CA, USA) and Origin 2019 (OriginLab, Northampton, MA, USA).

Passive membrane properties were measured from current transient elicited by 10 mV hyperpolarizing pulses from the holding potential of −60 mV in voltage-clamp. Membrane capacitance (Cm) was measured from the capacitive charge (the area underlying current transients), and cell input resistance (Ri) was calculated by dividing the steady-state voltage response by the current pulse amplitude (Rinput =$\Delta V/\Delta I$). Resting membrane potential (RMP) was measured immediately after whole-cell formation during the current-clamp protocol.

All data were tested for normality. Data are presented as individual and mean values ± standard error of the mean (SEM) of “n” cells obtained from “N” animals. Statistical analysis of data was performed either with GraphPad Prism 7.0 whether Student’s t-test (paired or unpaired samples) was used, or with STATISTICA 7 whether a two-way ANOVA was the test most indicated, and with StatPac 16 when a two-sample t-test had to be performed between percents.

When achieved, the significance at the $p < 0.05$, 0.01, 0.001, and 0.0001 level is indicated with *, **, ***, and **** respectively, in figures. Graphs were generated with GraphPad Prism 7.0, and representative traces were generated with Origin 2019. Example traces represent typical observations.

7. RESULTS

7.1 TH-GFP MICE SHOW NORMAL HEDONIC STATE

The drinking paradigm used in this project was a 2-bottle choice. This type of paradigm is based on the rewarding properties of EtOH and the motivation that drives mice to consume alcohol which may be affected by their basal hedonic state. To this aim, we first performed a sucrose preference test on EtOH-naïve, TH-GFP mice, in which the preference for a 5% w/v sucrose solution versus water is measured over 4 days (**figure 6A**).

We found that mice strongly prefer the sweet solution compared to water during the entire test (day 1 = 89.27 ± 1.21 %, day 2 = 90.00 ± 0.90 %, day 3 = 87.55 ± 0.92 %, day 4 = 92.25 ± 0.74 %. **Figure 6B**). Sucrose is a standard natural reward (Russo and Nestler 2013), thus this finding suggests that our TH-GFP mice have a normal hedonic state.

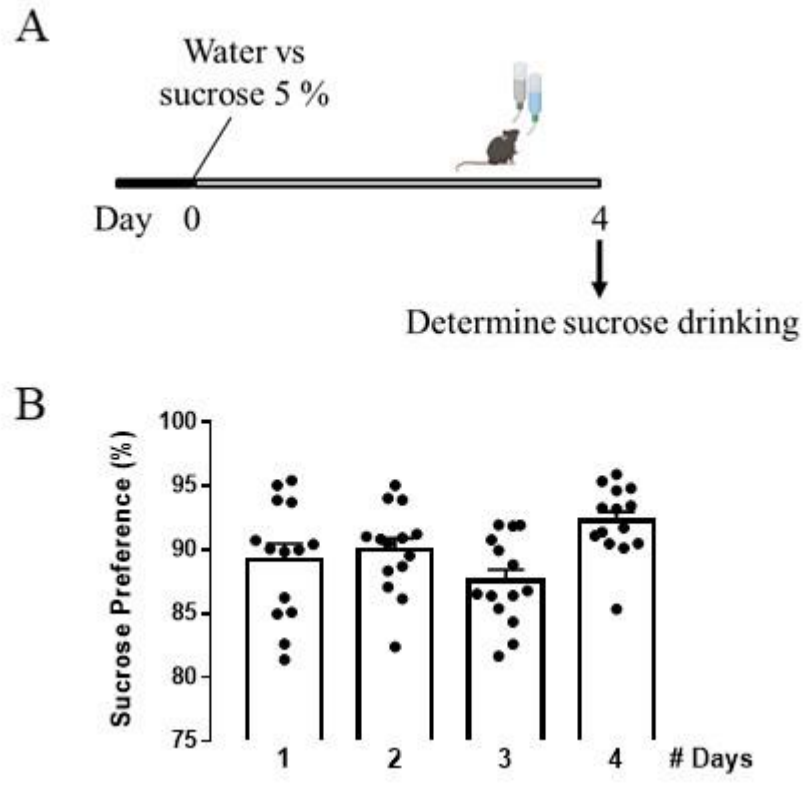


Figure 6: Sucrose preference test. (A) Timeline of the sucrose preference test. Mice had access to both water and a sucrose solution 5% w/v for 4 days. On the last day, sucrose preference was determined. (B) Quantification of the preference for the sucrose solution for each day. Data are reported as individual (dots) and mean values ± SEM (bars). N = 14.

7.2 EtOH INTAKE AND PREFERENCE VS WATER IN THE 2-BOTTLE CHOICE PARADIGM

During the 2-bottle choice paradigm animals had free and continuous access to both water and EtOH solution (EtOH 3% for the first 4 days, EtOH 6% for 4 days, and EtOH 12% for the last 10 days; **figure 7A**).

Data shown in Figure 7B demonstrate that the EtOH intake increases with concentration. Of note, when exposed to the highest EtOH concentration, values of intake start to disperse (3% EtOH = 3.84 ± 0.20 g/Kg, 6% EtOH = 7.65 ± 0.51 g/Kg, 12% EtOH = 11.69 ± 0.69 g/Kg. N = 25. **Figure 7B**, *left*). Importantly, mice show a preference for EtOH solution compared to water, especially for EtOH 6% (3% EtOH = 55.6 ± 2.28 %, 6% EtOH = 59.9 ± 3.34 %, 12% EtOH = 55.74 ± 3.37 %. N = 25. n.s. Student's t-test. **Figure 7B**, *right*).

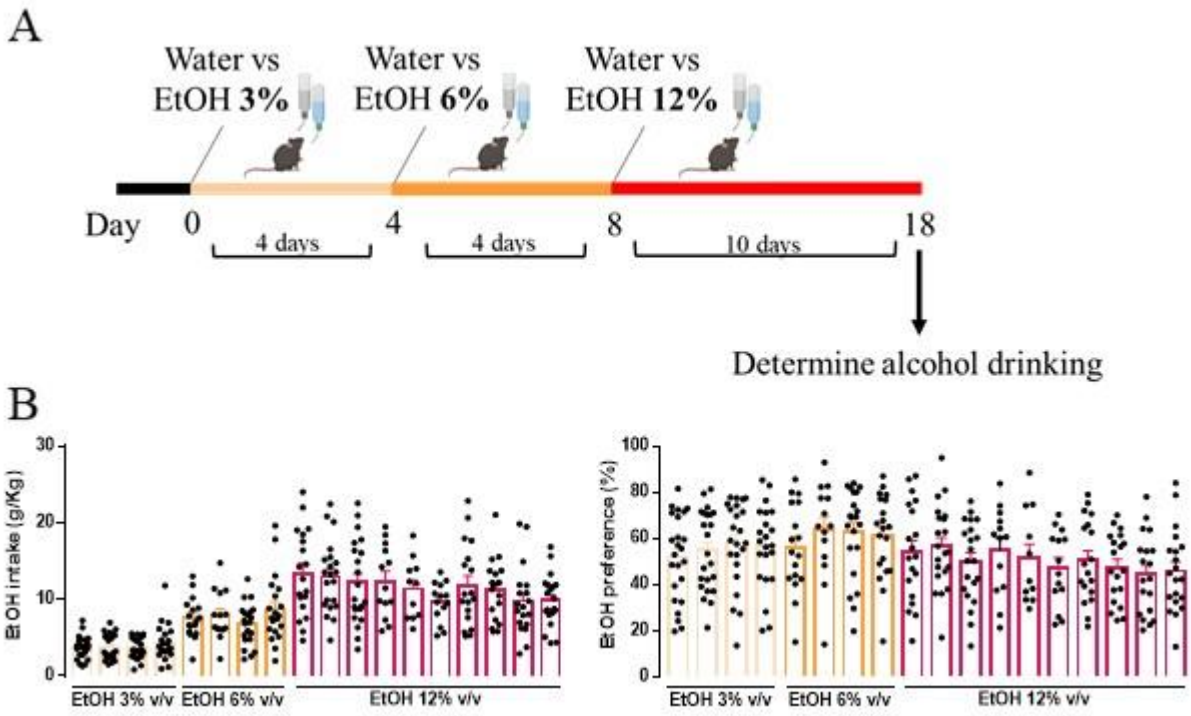


Figure 7: (A) Timeline of the 2-bottle choice paradigm. Exposed mice had access to a 3% v/v EtOH solution for 4 days which was then replaced with a 6% solution for the same period. Finally, 6% EtOH was changed for a solution at 12% for 10 days. On the last day of the drinking paradigm, alcohol drinking behavior was determined. (B) The two diagrams report the raw (black dots) and mean ± SEM (bars) values of EtOH consumption (g/Kg, left), and preference (%, right), during exposure to EtOH 3%, 6%, and 12%.

7.3 THE 2-BOTTLE CHOICE PARADIGM DOES NOT INCREASE ANXIETY LEVEL AFTER ALCOHOL DEPRIVATION

In the 2-bottle choice paradigm animals voluntary drink moderate levels of EtOH compared to other paradigms used in the field to induce an addictive state (Becker 2012). Once alcohol dependence is established, withdrawal occurs following abrupt discontinuation of drinking. Abstinence, in addition to somatic symptoms, is associated with increased anxiety, depressive-like behavior and a transient increase in EtOH intake upon re-exposure. This latter phenomenon is called alcohol deprivation effect (ADE) (Vengeliene, Bilbao, and Spanagel 2014; Fleming et al. 2019). Aware of this, we tested whether EtOH-exposed mice showed signs of abstinence following EtOH deprivation. Thus, we performed an EPM test to verify the anxiety level of drinking mice (in this case called 'withdrawn mice') compared to naive mice on the last day of 5-day EtOH deprivation (**Figure 8A**). The number of total entries (open + closed arms), the number of entries in open arms, and the time spent in open arms were measured.

There was no difference between naive and withdrawn mice in the total number of arm entries and entries in open arms (total entries: naive = 10.3 ± 2.24 entries, N = 10; withdrawn = 7.03 ± 1.23 entries, N = 14, n.s. Mann-Withney; entries in open arms: naive = 1.1 ± 0.46 entries, N = 10; withdrawn = 1.07 ± 0.32 entries, N = 14; n.s. Student's t-test. **Figure 8B,** *left* and *center*). Also, we found that withdrawn mice spent the same time in open arms compared to naive mice (time spent in open arms: naive = 11.30 ± 3.64 % time spent, N = 10; withdrawn = 13.23 ± 2.78 % time spent, N = 14; n.s. Student's t-test**. Figure 8B**, *right*).

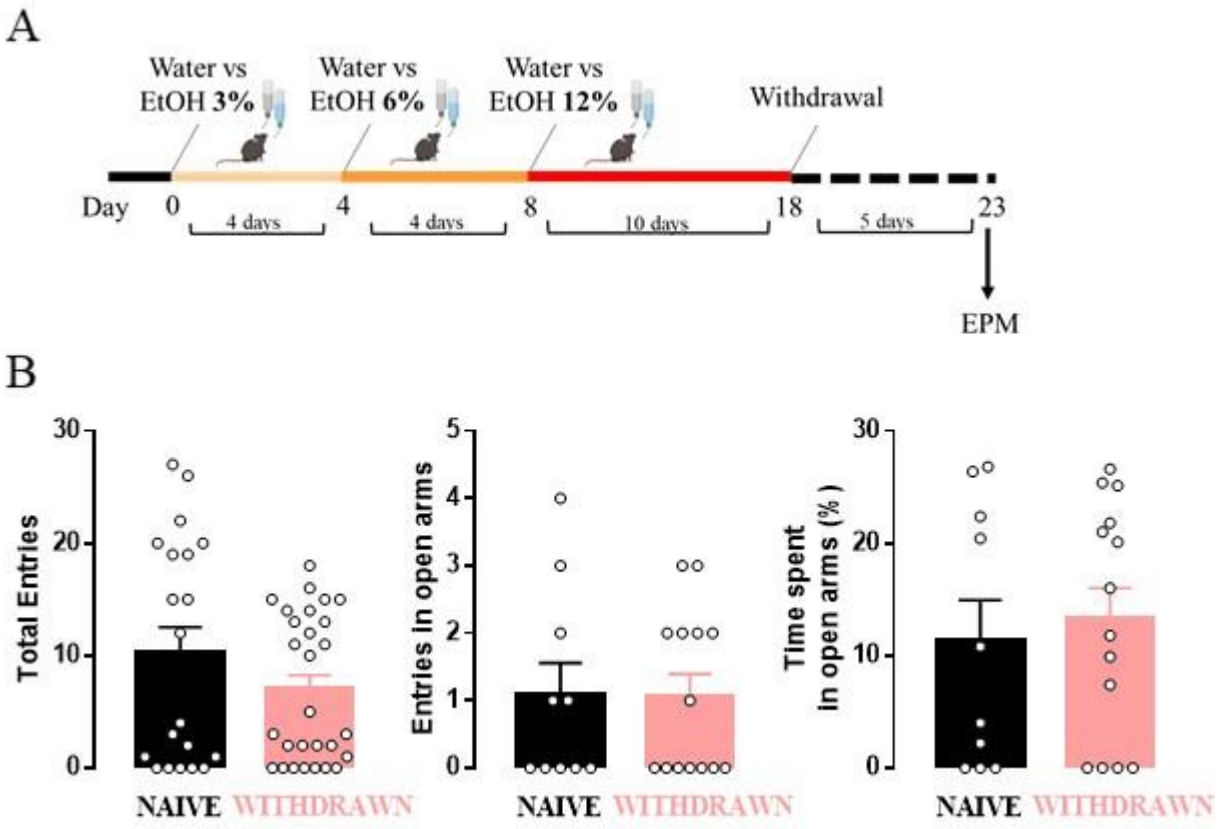


Figure 8: Effect of withdrawal from chronic alcohol exposure on the EPM test. (A) Timeline of the 2-bottle choice paradigm followed by 5-day EtOH deprivation. (B) Average number of total entries in both open and closed arms (left), open arms only (center), and time spent in open arms (right) for naive (black) and withdrawn (pink) mice. Data represent individual (dots) and mean values ± SEM (bars) (Mann-Withney test and Student's t-test).

7.4 MICE WITHDRAWN FROM VOLUNTARY DRINKING DO NOT EXHIBIT ADE

ADE is a phenomenon that replicates in experimental animals the reinstatement of alcohol dependence in individuals when re-exposed to alcohol following a period of abstinence. Since relapse is defined as the repetition of a past condition, that is excessive and uncontrolled drinking after an abstinence phase, ADE is considered a good operational measure to evaluate it. It has been reported that animals subject to voluntary access to alcohol for a prolonged time and then deprived for some days or weeks could temporally increase their alcohol intake over average pre-withdrawal intake (Sinclair and Senter 1967).

Following the drinking paradigm and the 5-day EtOH deprivation, mice were re-exposed to the 12% EtOH solution for 2 days (**Figure 9A**) in order to compare EtOH preference and total intake before and after withdrawal. Data show a trend to increase in EtOH intake during the

first day of re-exposition, returning to the pre-withdrawal level the following day (exposed = 4.76 ± 0.21 g/Kg before withdrawal and 5.48 ± 0.35 g/Kg after withdrawal; N = 9; n.s. Student's t-test. **Figure 9B**, *left*), while there are no differences in EtOH preference (exposed = 47.96 ± 2.54 % before withdrawal and 46.29 ± 2.06 % after withdrawal; N = 9; n.s**.** Student's t-test**.** **Figure 9B**, *right*).

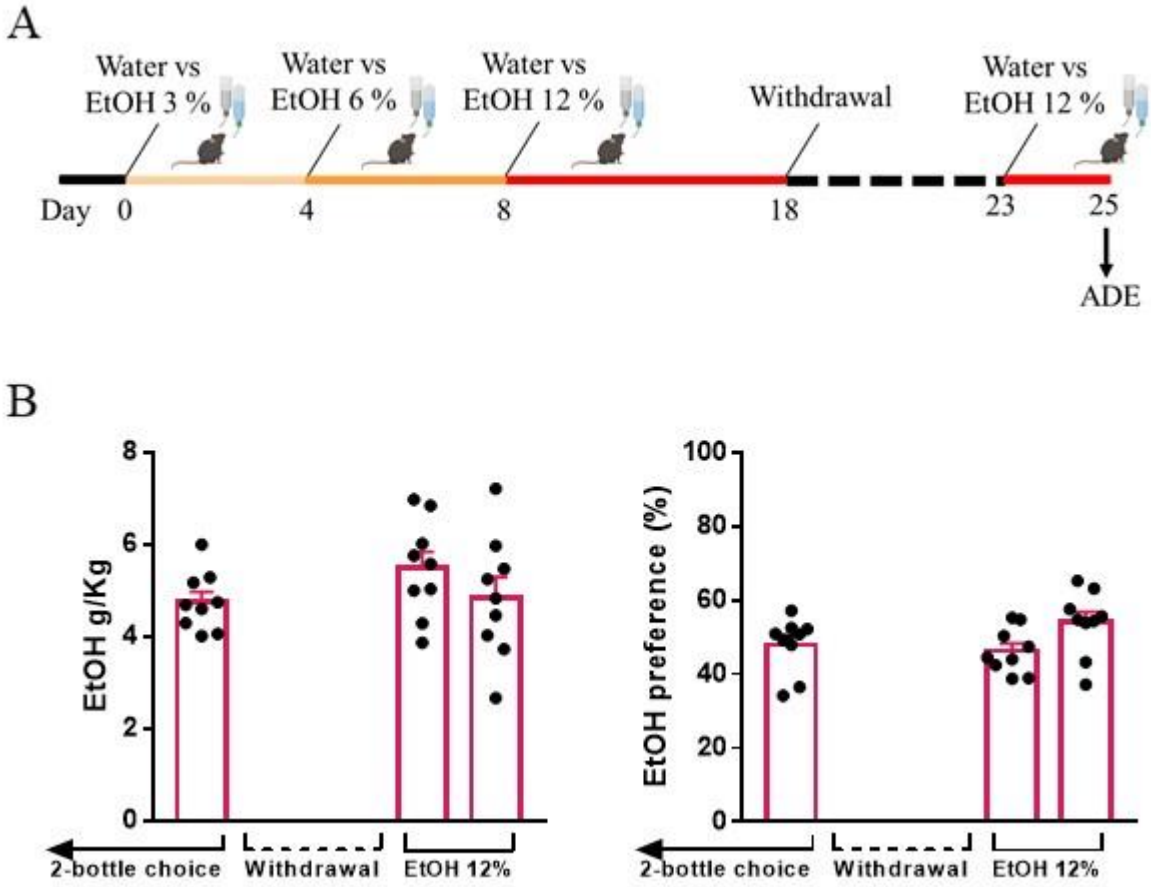


Figure 9: Effect of EtOH deprivation on alcohol intake and preference. (A) Timeline of the 2-bottle choice paradigm followed by 5-day EtOH deprivation and 2-day re-exposition. (B) Plotted averaged absolute EtOH intake (left) and EtOH preference (right) one day before and after the alcohol suspension. Data represent individual (dots) and mean (bars) values ± SEM(Student's t-test).

7.5 CHRONIC EtOH EXPOSURE DOES NOT AFFECT MEMBRANE PROPERTIES OR I_h CURRENT IN VTA DA NEURONS

Once characterized the 2-bottle choice paradigm as a mild animal model not leading to the development of alcohol dependence, we sought to investigate the electrophysiological properties of VTA neurons after chronic EtOH exposure. Cell-attached and whole-cell patch-clamp recordings were performed in acute horizontal slices prepared from EtOH-naive and

exposed mice on the last day of the 2-bottle choice paradigm (**Figure 10A**). The passive membrane properties, such as membrane capacitance (C_m), input resistance (R_i), and resting membrane potential (V_r) were studied as well as I_h current (**Figure 10B**).

Data shown in **figure 10** show that membrane properties of VTA DA neurons are not altered by chronic alcohol exposure (C_m: naive = 91.94 ± 13.68 pF, N/n = 2/3; exposed = 111.62 ± 10.62 pF, N/n = 3/7; n.s. ; R_i: naive = 484.89 ± 78.45 MΩ, N/n = 2/3; exposed = 430.00 ± 30.43 MΩ, N/n = 3/7; n.s.; V_r: naive = -51.00 ± 1.70 mV, N/n = 2/3; exposed = -49.39 ± 1.46 mV, N/n = 3/7; n.s. Student's t-test. **Figure 10A**). In addition, we found that I_h current is not affected by voluntary EtOH drinking (naive = -365.77 ± 57.35 pA, N/n = 3/9; exposed = -396.11 ± 72.79 pA, N/n = 7/20; n.s**.** Student's t-test. **Figure 10C**).

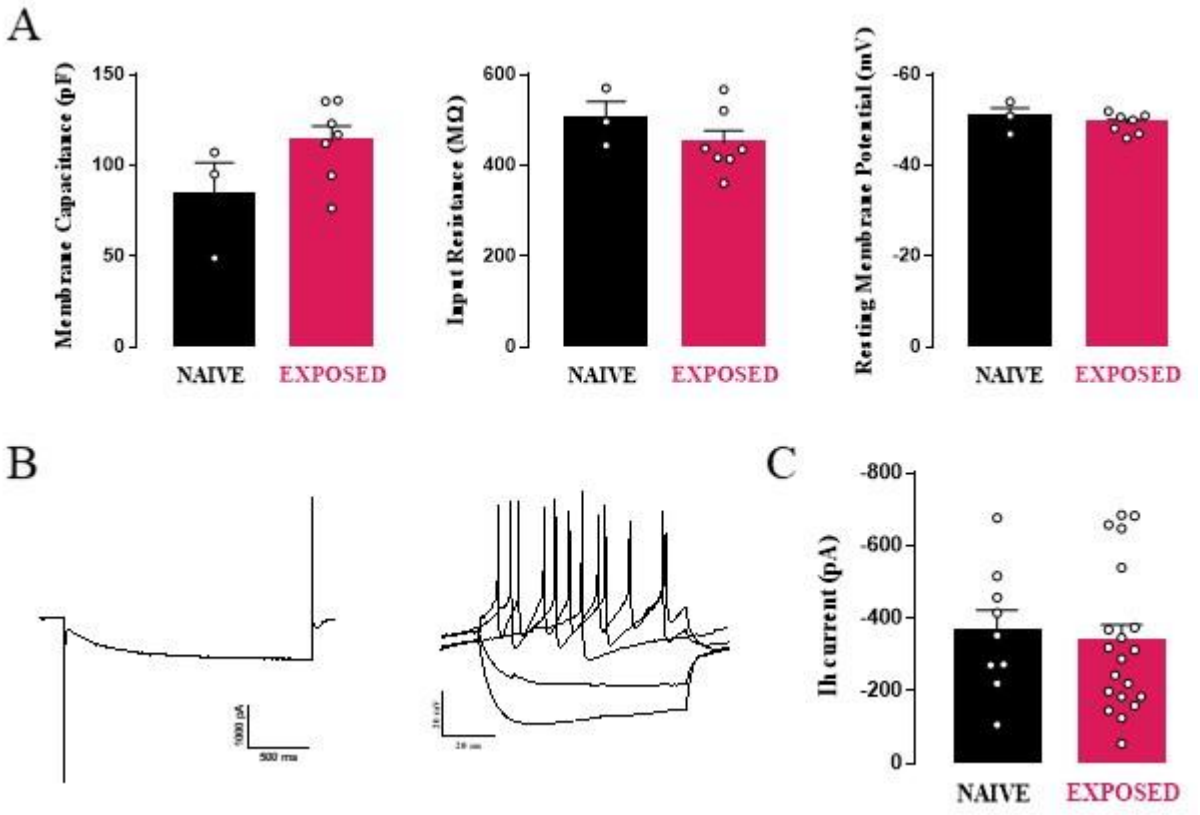


Figure 10: Electrophysiological properties of VTA DA neurons from naive (black) and exposed (red) mice. (A) Analysis of passive membrane properties: membrane capacitance (left), input resistance (center), and resting membrane potential (right). (B) Representative current trace evoked by imposing a 2 s-,-60 mV voltage step from holding potential (-60 mV) (left), and voltage traces evoked applying current pulses ranging from -100 pA to +100 pA in 50 pA increments, where I_h determines the voltage sag (right) in response to hyperpolarization. (C) Analysis of Ih current. Data represent individual (dots) and mean (bars) values ± SEM (Student's t-test).

7.6 CHRONIC EtOH ALTERS THE BASAL FIRING RATE OF VTA DA NEURONS AND THEIR RESPONSE TO ACUTE EXPOSURE

The firing rate of VTA DA neurons was measured by performing voltage-clamp cell-attached recordings in slices from both naive and exposed mice. First, the basal firing rate was recorded during aCSF perfusion (10 minutes) before application of 40 mM EtOH (10 minutes, **Figure 11A**).

Results indicate that basal firing frequency is significantly reduced in VTA DA neurons from EtOH-exposed as compared to naive mice (baseline: naive = 2.22 ± 0.20 Hz, N/n = 10/20; exposed = 1.29 ± 0.13 Hz, N/n = 10/13; p = 0.002. Student's t-test. **Figure 11B**). However, the response to acute application is intact, as perfusion with 40 mM EtOH increases the firing frequency in all neurons tested from both groups (acute EtOH: naive = 2.76 ± 0.21 Hz, N/n = 10/20, $p < 0.001$ vs its baseline, two-way ANOVA, Newman-Keuls post-hoc test); exposed = 2.09 ± 0.20 Hz, N/n = 10/13, $p < 0.0001$ vs its baseline, two-way ANOVA, Newman-Keuls post-hoc test. **Figure 11B**). Interestingly, the effect of acute EtOH application is greater in the exposed group. Indeed, the fold increase graph, which represents the ratio between the firing rate measured during EtOH perfusion and basal condition, indicates that a statistical difference between the two groups exists (naive = 1.31 ± 0.05, N/n = 10/20; exposed = 1.64 ± 0.06, N/n = 10/13; p = 0.0003 Student's t-test. **Figure 11C**).

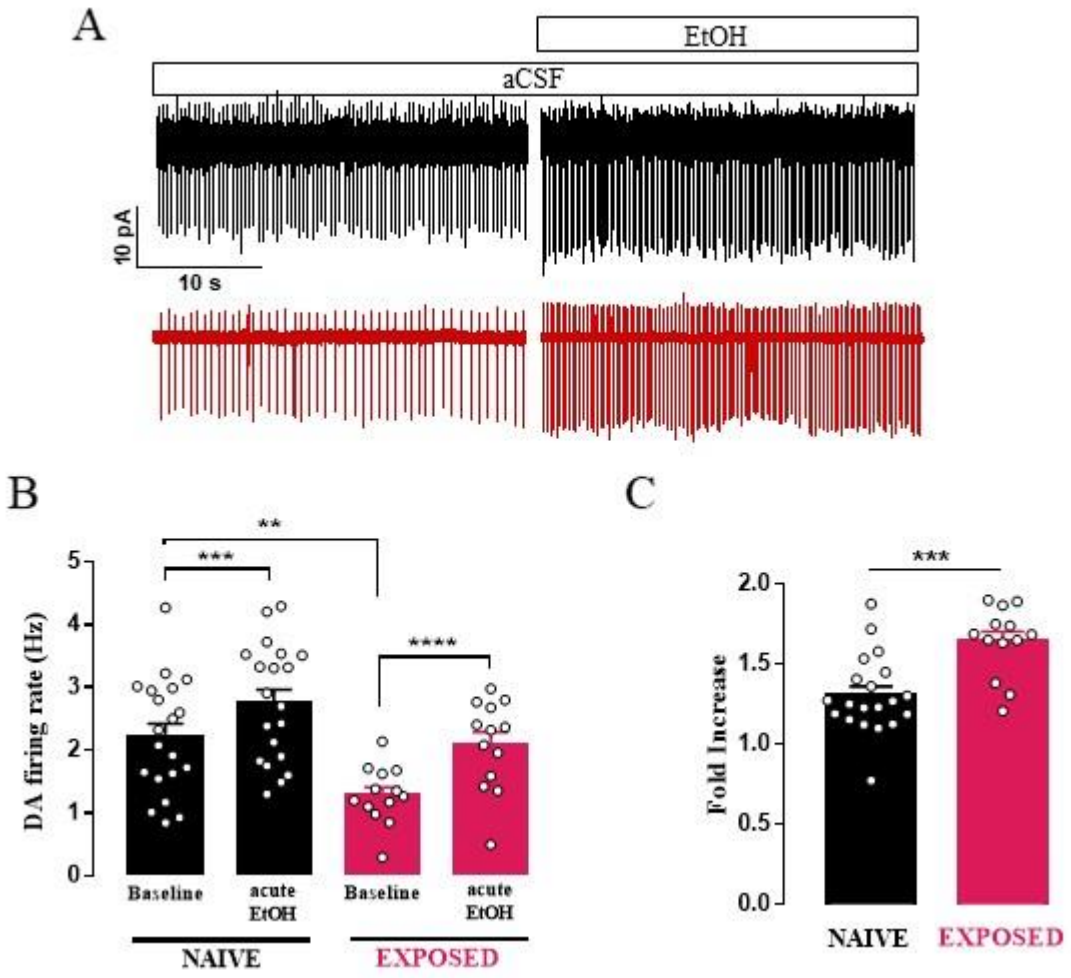


Figure 11: Effect of chronic and acute EtOH on firing rate of VTA DA neurons. (A) Representative traces obtained from cell-attached recordings of the firing rate from naive (black) and exposed (red) mice before (left) and after (right) 40 mMEtOH perfusion. (B) Plotted averaged frequency of the firing rate of VTA DA neurons of naive and exposed mice before (baseline) and after (acute EtOH) EtOH application. (C) EtOH-induced increase of firing rate in naive and exposed mice. Data represent individual (dots) and mean (bars) values ± SEM (Student's t-test and two-way ANOVA, Newman-Keuls post-hoc test).

7.7 BOTH CHRONIC AND ACUTE EFFECTS OF EtOH ON FIRING RATE ARE NETWORK-DEPENDENT

To assess the relative contribution of intrinsic versus network mechanisms of the effects of EtOH on neuronal activity, cell-attached recordings of spontaneous firing were repeated in conditions of synaptic isolation. In these experimental conditions, the recorded basal firing frequency and response to acute EtOH (40 mM) are largely dependent on intrinsic neuronal mechanisms. Basal firing rate was first recorded (10 minutes) in the presence of D/L-APV 50 µM, NBQX 10 µM, gabazine (SR95531) 10 µM and CGP 1 µM to block, respectively NMDA-

, AMPA-, GABA-A and GABA-B mediated neurotransmission. Then, the effect of acute 40 mM EtOH was assessed (10 minutes, **figure 12A**).

Results suggest that the effects of chronic EtOH experience disappear when synaptic transmission onto VTA DA neurons is blocked (baseline: naive = 2.61 ± 0.38 Hz, N/n = 6/15; exposed = 2.08 ± 0.25 Hz, N/n = 9/21; n.s. Student's t-test. **Figure 12B**). In these conditions, also the effects of acute EtOH application are abolished (acute EtOH: naive = 2.88 ± 0.41 Hz, N/n = 6/15, n.s; exposed = 2.11 ± 0.23 Hz, N/n = 9/21, n.s**.** Student's t-test. **Figure 12B**).

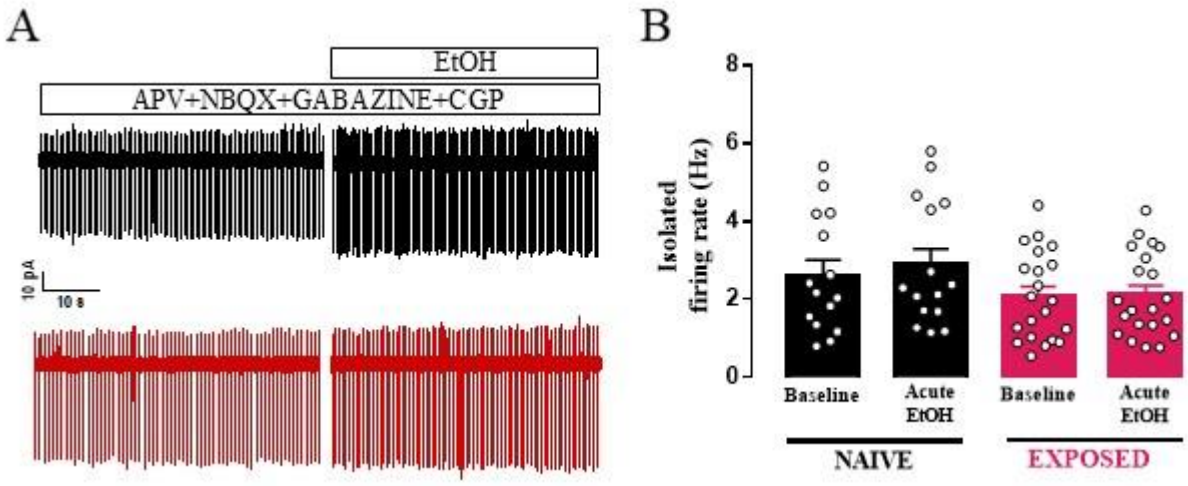


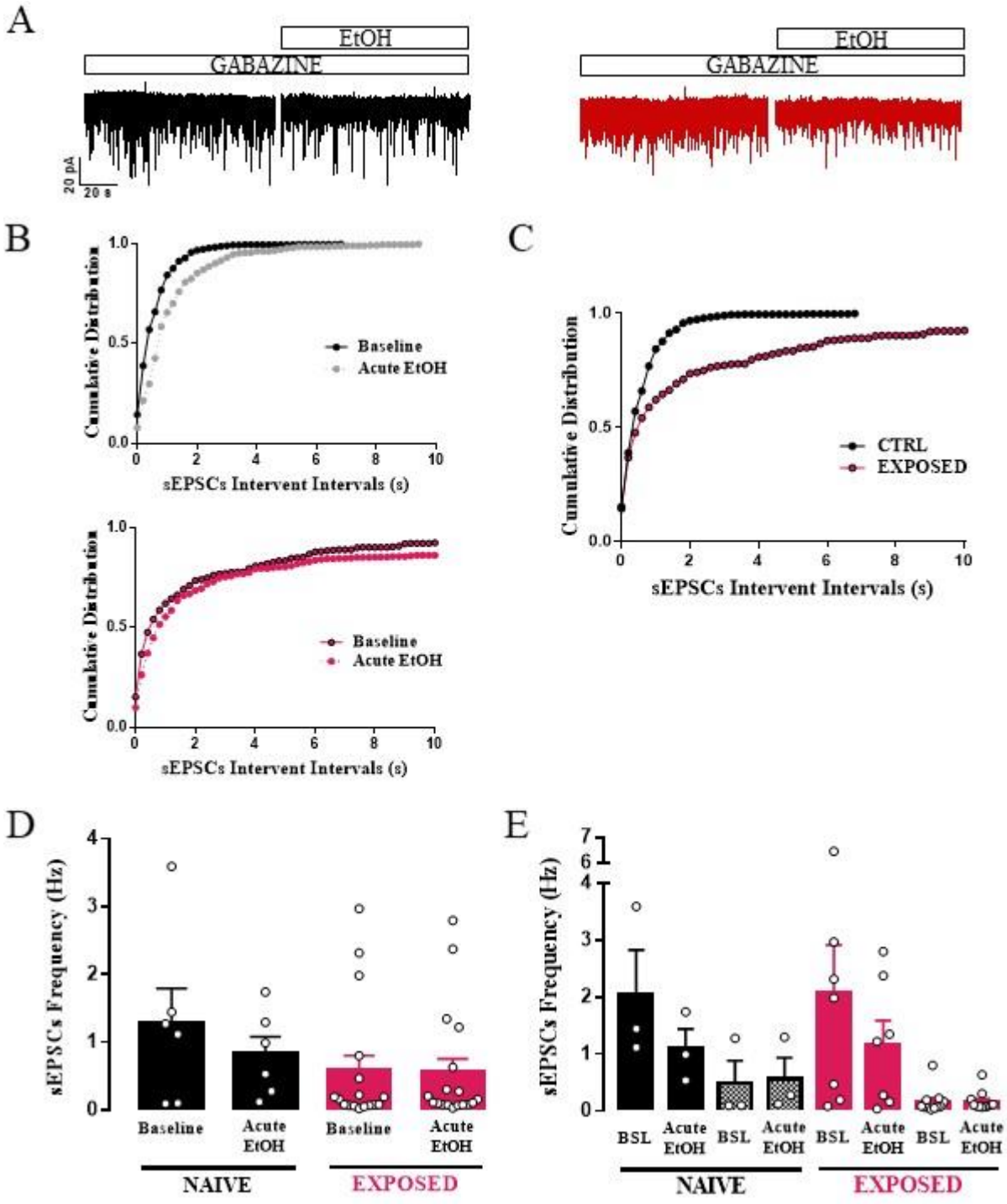
Figure 12: Effect of chronic and acute EtOH on the synaptically isolated firing rate. (A) Representative traces from cell-attached recordings of the firing rate from naive (black) and exposed (red) mice before (left) and after (right) acute 40 mM EtOH perfusion. (B) Plotted averaged frequency of the synaptically isolated firing rate of naive and exposed mice before (baseline) and after (acute EtOH) EtOH application. Data represent individual (dots) and mean (bars) values ± SEM (Student's t-test).

7.8 CHRONIC AND ACUTE EtOH DO NOT INTERACT WITH RESPECT TO THE EFFECT ON GLUTAMATERGIC TRANSMISSION

Based on our results showing that the effects of chronic EtOH are largely dependent on extrinsic, network mechanisms, we decided to examine the possible interactions between chronic and acute EtOH on excitatory neurotransmission. To this aim, spontaneous excitatory postsynaptic currents (sEPSCs) were measured during whole-cell voltage-clamp recordings with a standard internal solution (see 6.3.5 in methods, solution (a)) and in the continuous presence of gabazine (SR95531) 10 μM to block GABAergic currents. Basal sEPSCs were recorded in aCSF (5 minutes), then following application of $GABA_A$ blocker (5 minutes), finally during 40 mM EtOH perfusion (10 minutes, **figure 13A**).

We found that chronic EtOH causes a non significant reduction in the basal frequency of sEPSCs (naive = 1.27 ± 0.52 Hz, N/n = 4/6, n.s.; exposed = 0.90 ± 0.39 Hz, N/n = 9/18, n.s. Two-way ANOVA, Newman-Keuls *post-hoc* test. **Figure 13D**). Acute EtOH application leads to a non significant reduction of sEPSCs frequency in both groups (naive = 0.83 ± 0.26 Hz, N/n = 4/6, n.s.; exposed = 0.56 ± 0.20 Hz, N/n = 9/18, n.s. Two-way ANOVA, Newman-Keuls *post-hoc* test. **Figure 13D**). Interestingly, we noticed that VTA DA neurons from both groups are divided in two distinct subsets based on the response to acute EtOH. Although the percentage of neurons responding to acute EtOH is significantly reduced in exposed mice (naive = 50.0%; exposed = 38.9%), re-analyzing the entire population according to sensitivity to acute EtOH did not unmask differences in baseline (EtOH-responding neurons: naive = 2.05 ± 0.78 Hz, n = 3, n.s.; exposed = 2.07 ± 0.85 Hz, n = 7, n.s.; EtOH-not responding neurons: naive = 0.49 ± 0.39 Hz, n = 3, n.s.; exposed = 0.16 ± 0.07 Hz, n = 11, n.s. Two-way ANOVA, Newman-Keuls *post-hoc* test. **Figure 13E**) or responsiveness (EtOH-responding neurons: naive = 1.09 ± 0.35 Hz, n = 3, n.s.; exposed = 1.17 ± 0.41 Hz, n = 7, n.s.; EtOH-not responding neurons: naive = 0.56 ± 0.37 Hz, n = 3, n.s.; exposed = 0.17 ± 0.05 Hz, n = 11, n.s. Two-way ANOVA, Newman-Keuls *post-hoc* test. **Figure 13E**) between the two groups.

Finally, sEPSC amplitude does not change in response to either chronic or acute EtOH (baseline: naive = -17.70 ± 1.71 pA, N/n = 4/6, n.s.; exposed = -22.36 ± 3.56 pA, N/n = 9/18, n.s.; acute EtOH perfusion: naive = -18.75 ± 1.51 pA, N/n = 4/6, n.s.; exposed = -17.67 ± 1.14 pA, N/n = 9/18, n.s. Two-way ANOVA, Newman-Keuls *post-hoc* test. **Figure 13H**).


A
EtOH
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20 pA
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Cumulative Distribution
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Baseline
Acute EtOH
sEPSCs Intervent Intervals (s)
C
Cumulative Distribution
CTRL
EXPOSED
sEPSCs Intervent Intervals (s)
D
sEPSCs Frequency (Hz)
Baseline
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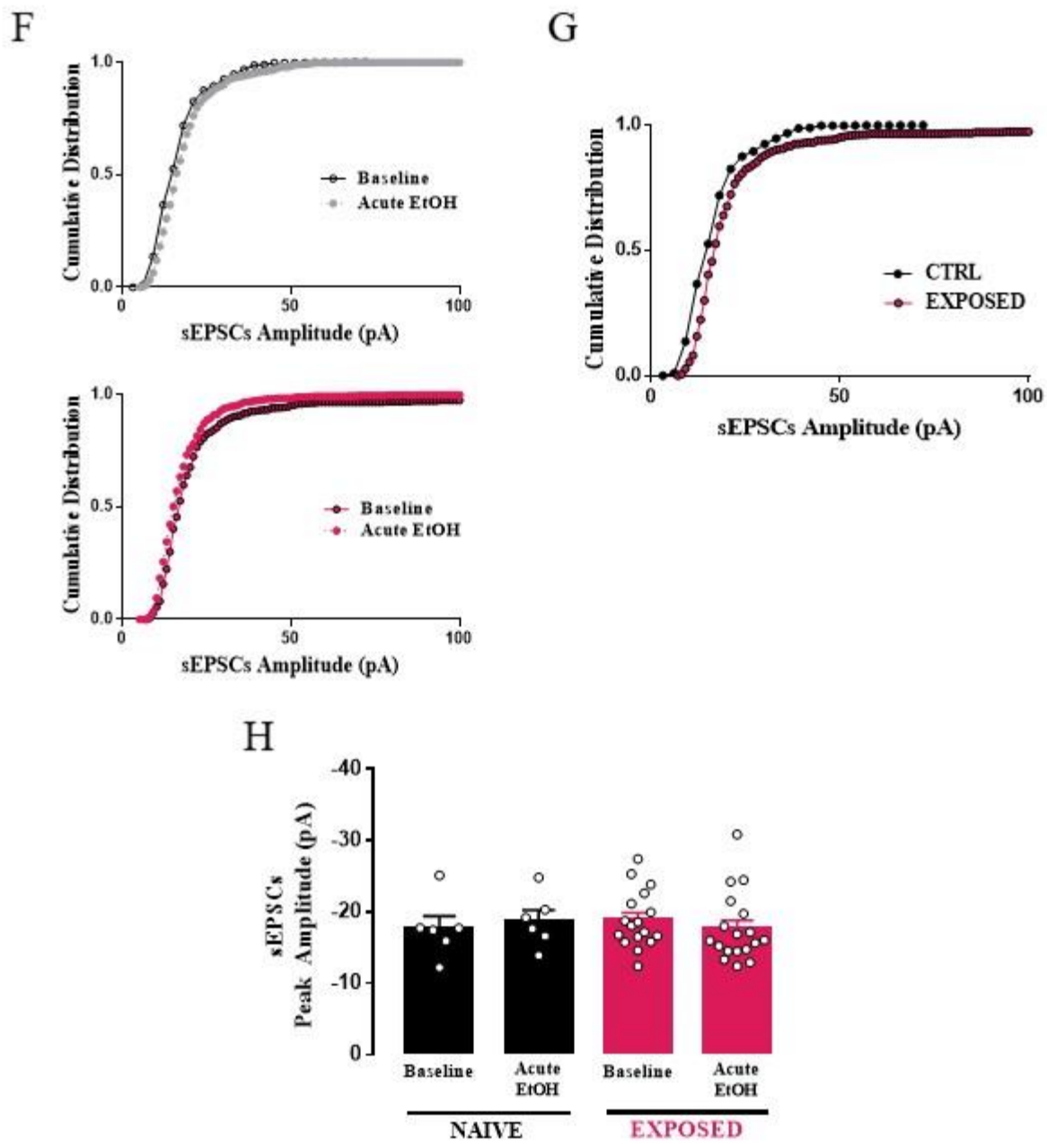


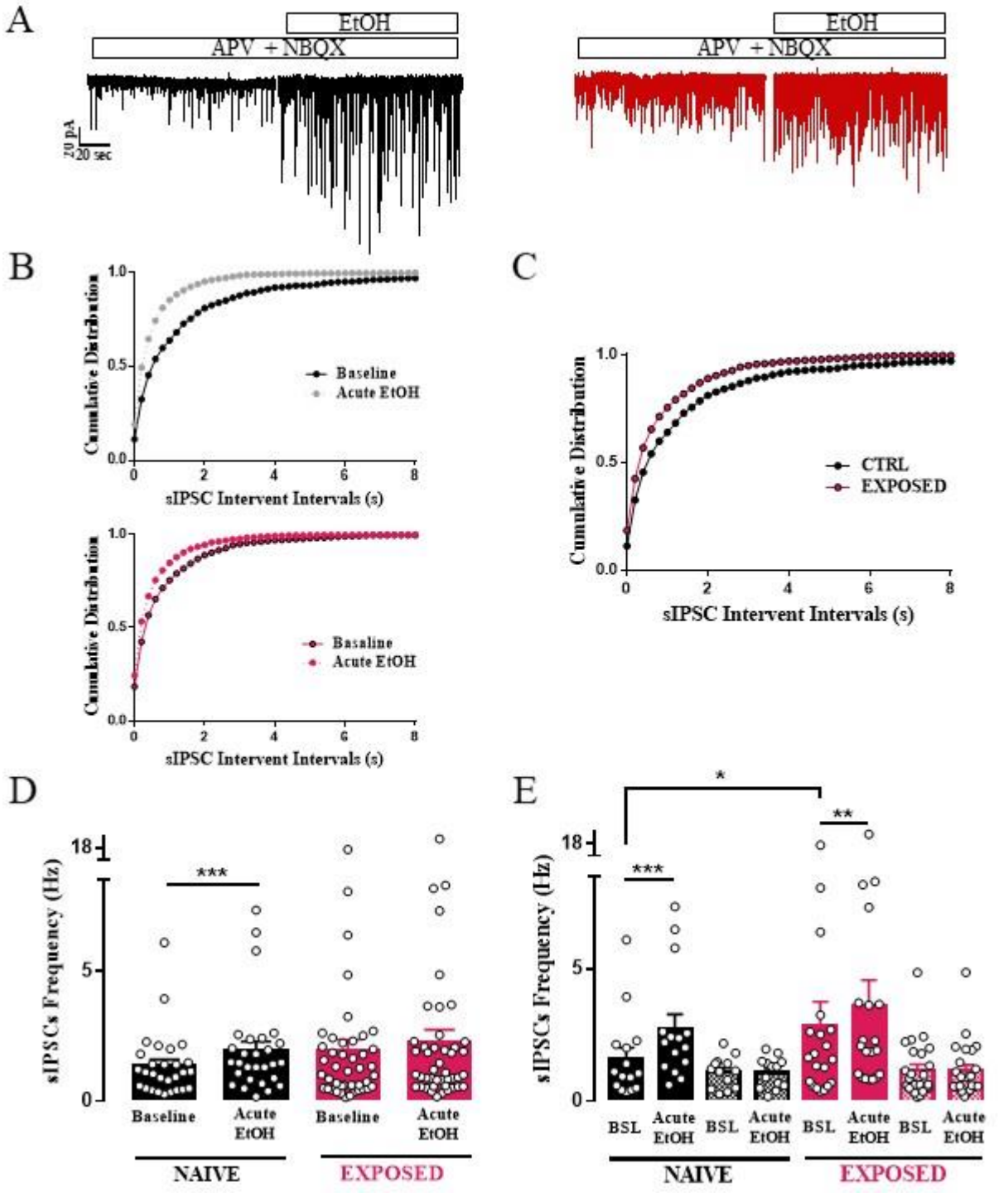
Figure 13: Excitatory neurotransmission of VTA DA neurons. (A) Representative traces from whole-cell voltage-clamp recordings of sEPSCs from naive (black) and exposed (red) mice during GABAergic isolation (gabazine) and addition of acute 40 mM EtOH perfusion. (B) Cumulative distribution plots of sEPSC interevent interval demonstrating the effect of acute EtOH 40 mM on the baseline in naive (top) and exposed (bottom) mice. (C) Cumulative distribution plots on sEPSC interevent interval demonstrating the effect of chronic EtOH exposure on the baseline of both naive (black) and exposed (red) mice. (D) Plotted averaged frequency of sEPSCs from naive and exposed mice before (baseline) and after (acute EtOH) EtOH application. (E) Same as (D) but with recorded neurons split between EtOH-responding (full bars) and EtOH-non responding (chequered bars). (F-G) Same as (B, C) but showing amplitude of sEPSCs. (H) Same as (D) but showing amplitude of sEPSCs. Data represent individual (dots) and mean (bars) values ± SEM (Kolgomorov-Smirnov test and two-way ANOVA, Newman-Keuls post-hoc test).

7.9 CHRONIC EtOH ALTERS BASAL INHIBITORY TONE ONTO DA NEURONS AND THE RESPONSE TO ACUTE APPLICATION

Based on our results showing that VTA DA neurons in slices from EtOH-exposed mice have lower firing activity, we then decided to study the inhibitory tone onto VTA DA neurons. To this purpose, spontaneous inhibitory postsynaptic currents (sIPSCs) were recorded during whole-cell voltage-clamp recordings from both groups using a high KCl (140 mM) intracellular solution (see chapter 6.3.5 in methods for a theoretical discussion of this experimental expedient). Recordings were performed in presence of NBQX 10 μM and DL-APV 50 μM to block AMPA and NMDA receptors. Basal sIPSCs were recorded in aCSF (5 minutes), then during application of blockers (5 minutes), finally during EtOH 40 mM perfusion (10 minutes, **figure 14A**).

In agreement with results from firing rate recordings, we found a non significant increase in the basal frequency of inhibitory tone onto VTA DA neurons in EtOH-exposed mice (baseline: naive = 1.36 ± 0.23 Hz, N/n = 10/28, p = 0.0007 vs acute EtOH naive mice; exposed = 1.96 ± 0.33 Hz, N/n = 16/44, n.s. Two-way ANOVA, Newman-Keuls *post-hoc* test. **Figure 14D**). In naive mice, acute application of EtOH leads to a significant increase in the frequency of sIPSCs (acute EtOH: naive = 1.93 ± 0.44 Hz, N/n = 10/28, p = 0.0007 vs baseline naive mice. Two-way ANOVA, Newman-Keuls *post-hoc* test. **Figure 14D**). The effect of acute EtOH on this parameter is not significant in VTA DA neurons from exposed animals (acute EtOH: exposed = 2.26 ± 0.48 Hz, N/n = 16/44, n.s. Two-way ANOVA, Newman-Keuls *post-hoc* test. **Figure 14D**). Again, we noticed that VTA DA neurons from both groups are divided in two distinct subsets based on the response to acute EtOH (EtOH-responding neurons: naive = 53.6%; exposed = 45.4%). We then re-analized the entire datasets and measured baseline sIPSC frequency and magnitude of the response to acute EtOH based on the sensitivity to acute application. With this approach, a significant increase in the basal sIPSCs frequency in the exposed group is unmasked (EtOH-responding neurons – baseline: naive = 1.60 ± 0.41 Hz, N/n = 9/15, p = 0.0002 vs acute EtOH perfusion and p = 0.039 vs exposed mice baseline; exposed = 2.84 ± 0.91 Hz, N/n = 12/20, p = 0.006 vs EtOH 40 mM perfusion and p = 0.039 vs naive mice baseline; EtOH-not responding neurons – baseline: naive = 1.08 ± 0.16 Hz, N/n = 8/13, n.s.; exposed = 1.17 ± 0.22 Hz, N/n = 13/24, n.s.; EtOH-responding neurons – acute EtOH: naive = 2.73 ± 0.54 Hz, N/n = 9/15, p = 0.0002 vs baseline; exposed = 3.60 ± 0.96 Hz, N/n = 12/20, p = 0.006 vs baseline; EtOH-not responding neurons – acute EtOH: naive = 1.07 ± 0.16 Hz, N/n = 8/13, n.s.; exposed = 1.14 ± 0.21 Hz, N/n = 13/24, n.s**.** Student's t-test and Two-way ANOVA, Newman-Keuls *post-hoc* test. **Figure 14E**).

On the amplitude of sIPSCs, no difference was detected between the two groups in baseline conditions (baseline: naive = -30.28 ± 4.07 pA, N/n = 10/28, p = 0.0001 vs acute EtOH perfusion; exposed = -26.94 ± 1.84 pA, N/n = 16/44, n.s. Two-way ANOVA, Newman-Keuls *post-hoc* test. **Figure 14H**). The increase induced by acute EtOH in the naive groups is lost in exposed mice (acute EtOH perfusion: naive = -44.04 ± 7.44 pA, N/n = 10/28, p = 0.0001 vs its baseline; exposed = -32.75 ± 2.15 pA, N/n = 16/44, n.s Two-way ANOVA, Newman-Keuls *post-hoc* test. **Figure 14H**).



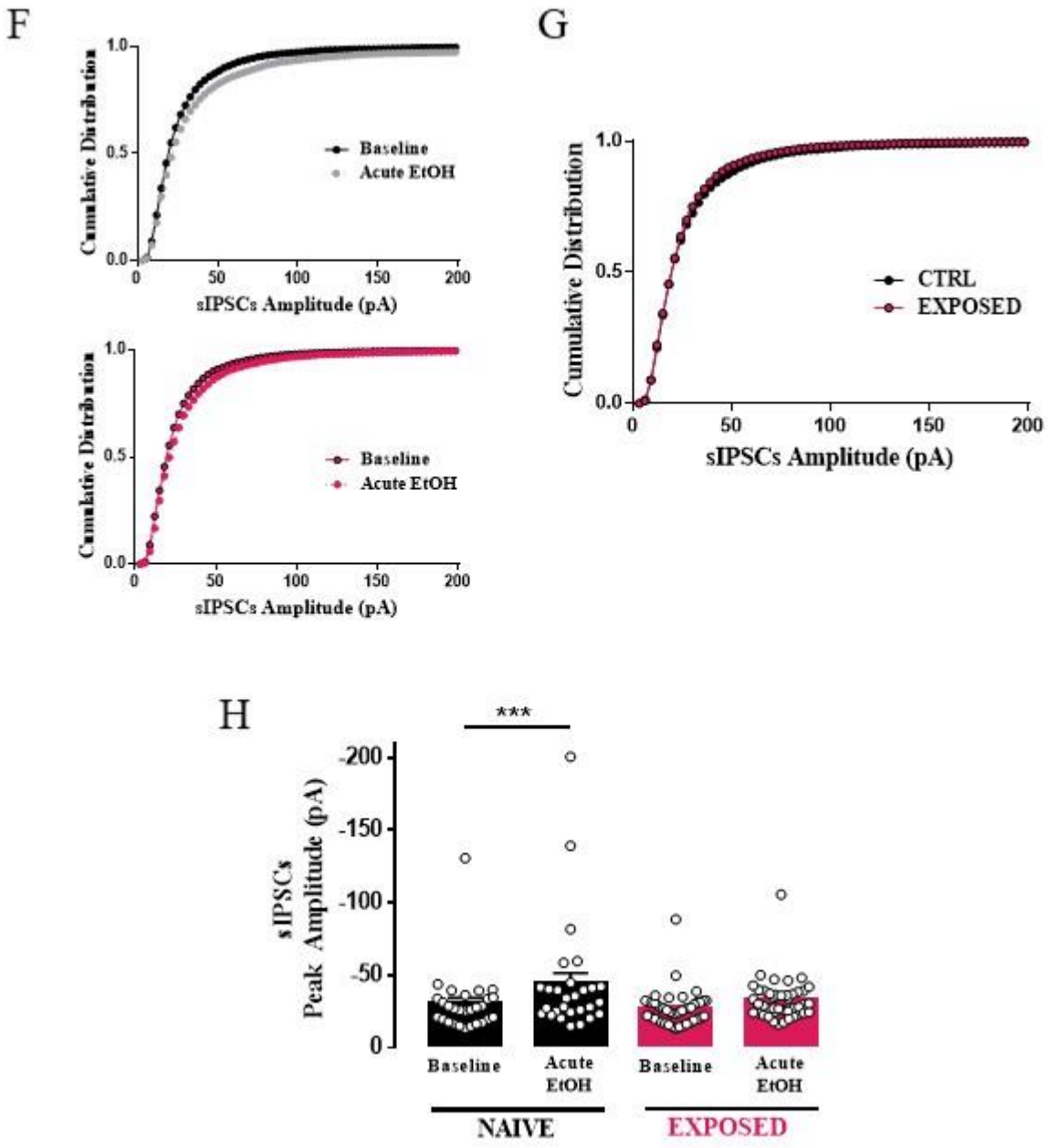
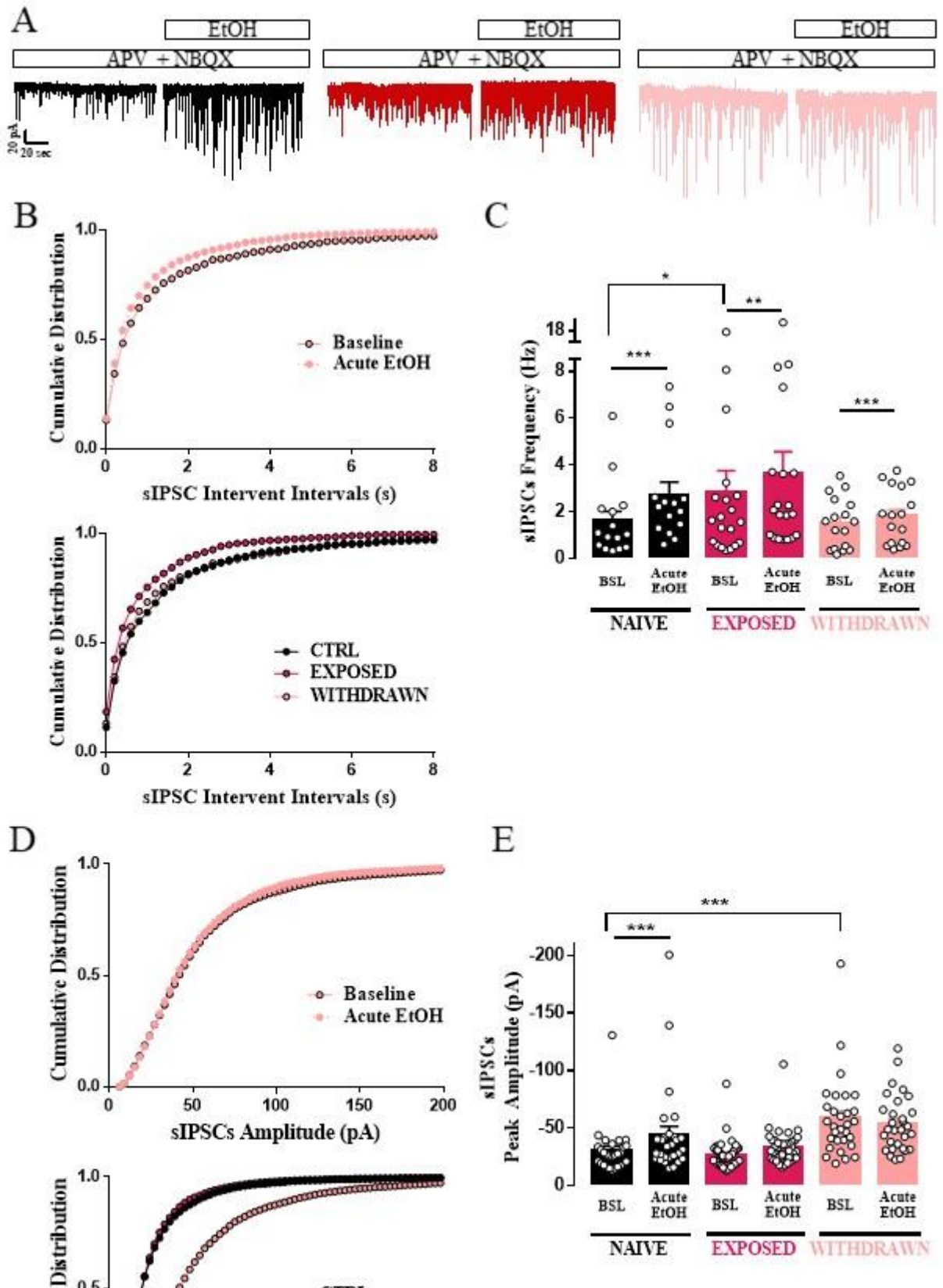


Figure 14: Inhibitory neurotransmission in VTA DA neurons. (A) Representative traces from whole-cell voltage-clamp recordings of sIPSCs from naive (black) and exposed (red) mice during glutamatergic isolation (APV + NBQX) and addition of acute 40 mM EtOH perfusion. (B) Cumulative distribution plots of sIPSC interevent interval demonstrating the effect of acute EtOH 40 mM on the baseline in naive (top) and exposed (bottom) mice. (C) Cumulative distribution plots on sIPSC interevent interval demonstrating the effect of chronic EtOH exposure on the baseline of both naive (black) and exposed (red) mice. (D) Plotted averaged frequency of sIPSCs from naive and exposed mice before (baseline) and after (acute EtOH) EtOH application. (E) Same as (D) but with recorded neurons split between EtOH-responding (full bars) and EtOH-not responding (chequered bars). (F-G) Same as (B, C) but showing amplitude of sIPSCs. (H) Same as (D) but showing amplitude of sIPSCs. Data represent individual (dots) and mean (bars) values ± SEM (Kolgomorov-Smirnov test, student's t-test and two-way ANOVA, Newman-Keuls post-hoc test).

7.10 ALTERATIONS INDUCED BY CHRONIC ALCOHOL EXPOSURE ARE PARTIALLY NORMALIZED AFTER 5 DAYS OF DEPRIVATION

After the drinking paradigm, mice were deprived of EtOH for 5 days. Then, we performed electrophysiological experiments in order to test whether the intrinsic and extrinsic alterations in the activity of VTA DA neurons, induced by chronic EtOH, could be reverted by a 5-day deprivation. We focused on the parameter showing the highest responsiveness to chronic exposure, i.e., the inhibitory synaptic frequency. Recordings were obtained in the same conditions as 7.8. (**Figure 15A**).

Results indicate that, after deprivation, basal sIPSCs frequency matches that of of naive mice (baseline: naive = 1.60 ± 0.41 Hz, N/n = 9/15; exposed = 2.84 ± 0.91 Hz, N/n = 12/20; withdrawn = 1.52 ± 0.26 Hz, N/n = 11/17, $p < 0.0001$ vs acute EtOH perfusion**.** Student's t-test**. Figure 15C**). Also, the response to acute EtOH application equals that of the EtOH-naive group (acute EtOH application: naive = 2.73 ± 0.54 Hz, N/n = 9/15; exposed = 3.60 ± 0.96 Hz, N/n = 12/20; withdrawn = 1.81 ± 0.29 Hz, N/n = 11/17, $p < 0.0001$ vs its baseline**.** Student's t-test**. Figure 15C**). Interestingly, EtOH deprivation leads to a significant increase of basal IPSC amplitude, compared to both naive and exposed mice (baseline: naive = -30.28 ± 4.08 pA, N/n = 10/28; exposed = -26.94 ± 1.84 pA, N/n = 15/44; withdrawn = -58.66 ± 6.29 pA, N/n = 12/30, $p = 0.0004$ vs naive baseline. **Figure 15E**) while the response to acute EtOH perfusion is lost (acute EtOH application: naive = -44.03 ± 7.44 pA, N/n = 10/28; exposed = -32.75 ± 2.15 pA, N/n = 15/44; withdrawn = -54.13 ± 4.48 pA, N/n = 12/30 n.s. **Figure 15E**).


A
EtOH
APV + NBQX
20 pA
20 sec
B
Cumulative Distribution
Baseline
Acute EtOH
sIPSC Intervent Intervals (s)
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WITHDRAWN
C
sIPSCs Frequency (Hz)
BSL
Acute EtOH
NAIVE
EXPOSED
WITHDRAWN
D
sIPSCs Amplitude (pA)
E
sIPSCs Peak Amplitude (pA)

Figure 15: Inhibitory neurotransmission in VTA DA neurons after 5-day EtOH deprivation. (A) Representative traces from whole-cell voltage-clamp recordings of sIPSCs from naive (black), exposed (red), and withdrawn (pink) mice during glutamatergic isolation (APV + NBQX) and addition of acute 40 mM EtOH perfusion. (B, top) Cumulative distribution plots of sIPSC interevent interval demonstrating the effect of acute EtOH 40 mM on the baseline in withdrawn (top) mice. (B, bottom) Cumulative distribution plots on sIPSC interevent interval demonstrating the effect of chronic EtOH exposure on the baseline of naive (black), exposed (red) and withdrawn (pink) mice. (C) Plotted averaged frequency of sIPSCs from naive, exposed, and withdrawn mice before (BSL) and after (acute EtOH) EtOH application. (D) Same as (B) but showing amplitude of sIPSCs. (E) Same as (C) but showing amplitude. Data represent individual (dots) and mean (bars) values ± SEM (Kolgomorov-Smirnov test, student's t-test and two-way ANOVA, Newman-Keuls post-hoc test).

7.11 EFFECT OF CHRONIC EtOH ON THE FIRING ACTIVITY OF VTA GABAERGIC INTERNEURONS

Building on the effect of chronic EtOH consumption on the presynaptic inhibitory tone onto VTA DA neurons, we performed cell-attached patch-clamp recordings from both naive and exposed mice in order to directly measure the firing rate of local VTA GABAergic interneurons. First, the basal firing rate was recorded during aCSF perfusion (10 minutes) before application of 40 mM EtOH (10 minutes, **figure 16A**).

Chronic EtOH does not seem to change the basal firing rate of VTA GABAergic neurons (baseline: naive = 3.51 ± 1.00 Hz, N/n = 3/4, n.s.; exposed = 4.07 ± 1.22 Hz, N/n = 7/9, n.s. Two-way ANOVA, Newman-Keuls *post-hoc* test. **Figure 16B**). However, acute EtOH application causes a not significant increase in the firing frequency in both groups (acute EtOH perfusion: naive = 4.11 ± 1.00 Hz, N/n = 3/4, n.s.; exposed = 4.92 ± 1.39 Hz, N/n = 7/9, n.s. Two-way ANOVA, Newman-Keuls *post-hoc* test. **Figure 16B**). Interestingly, exclusion of neurons not responding to acute EtOH from the data set unmasks a not significant elevation in basal firing rate of VTA GABAergic neurons in the exposed group (baseline: naive = 4.37 ± 0.71 Hz, N/n = 2/3; exposed = 7.27 ± 1.64 Hz, N/n = 2/4, n.s.; acute EtOH: naive = 4.93 ± 0.80 Hz, N/n = 2/3; exposed = 8.46 ± 1.94 Hz, N/n = 2/4, n.s. Two-way ANOVA, Newman-Keuls *post-hoc* test. **Figure 16C**).

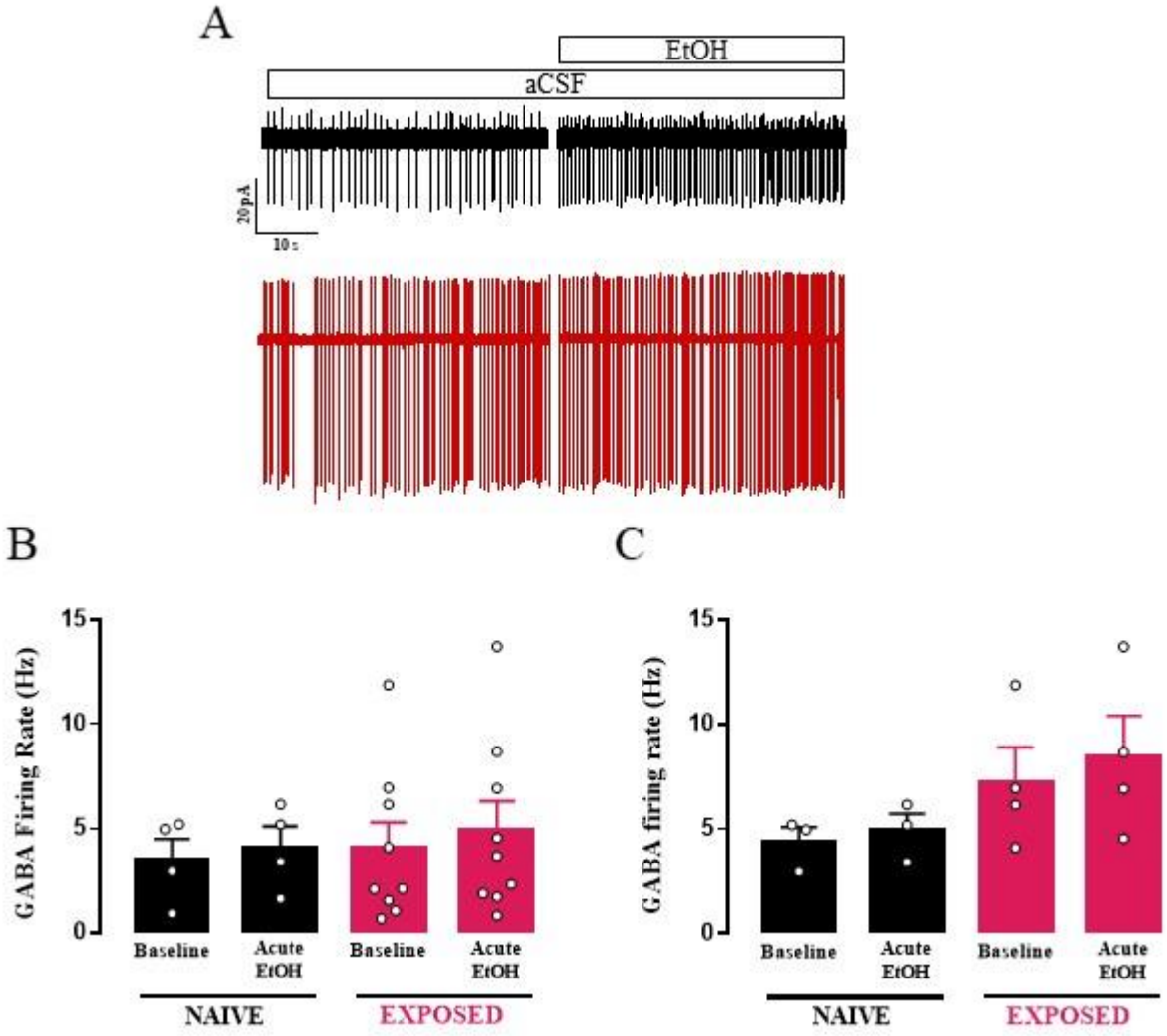

Figure 16: Effect of chronic and acute EtOH on firing rate of VTA GABAergic neurons. (A) Representative traces from cell-attached recordings of firing rate from naive (black) and exposed (red) mice before (left) and after (right) acute 40 mM EtOH perfusion. (B) Plotted averaged frequency of firing rate from naive and exposed mice before (baseline) and after (acute EtOH) EtOH application. (C) Same as (B) but considering neurons with the basal frequency > 3 Hz and EtOH-responding neurons only. Data represent individual (dots) and mean (bars) values ± SEM (Two-way ANOVA, Newman-Keuls post-hoc test).

7.12 N/OFQ ABOLISHES THE ACUTE EFFECT OF EtOH ON THE FIRING RATE OF VTA DA NEURONS

The action of the endogenous neuropeptide nociceptin/orphanin FQ (N/OFQ) and NOP receptors has a crucial role in regulating alcohol abuse-related behaviors. For 20 years, the anti-addictive properties of NOP agonists have been described in alcohol studies. In particular, nociceptin was repeatedly demonstrated to reduce voluntary EtOH drinking, EtOH self-

administration and relapse in msP and Wistar rats (Ciccocioppo et al. 1999, 2002, 2003). Based on these arguments, we decided to investigate the responsiveness of electrophysiological alterations observed in the earlier experiments from EtOH-exposed mice to nociceptin. For this purpose, cell-attached patch-clamp recordings from both naive and exposed mice were performed. Firstly, the basal firing rate was recorded during aCSF perfusion (5 minutes), before the acute N/OFQ (30 nM) application (5 minutes), followed by the addition of 40 mMEtOH (5 minutes, **figure 17A**).

Results indicate that nociceptin slows the firing rate of VTA DA neurons in both naive and exposed group, although the effect does not achieve statistical significance (**Figure 17B**). More importantly, nociceptin abolishes the enhancing effect of acute EtOH on firing rate in both naive and exposed mice (BSL: naive = 100.00 ± 13.63 %, N/n = 4/8, n.s.; exposed = 100.00 ± 12.16 %, N/n = 4/16, n.s.; acute noci: naive = 89.16 ± 8.00 %, N/n = 4/8, n.s.; exposed = 95.38 ± 4.58 %, N/n = 4/16, n.s.; acute noci + acute EtOH: naive = 92.07 ± 13.65 %, N/n = 4/8, n.s.; exposed = 93.98 ± 10.78 %, N/n = 4/16, n.s. Two-way ANOVA, Newman-Keuls *post-hoc* test. **Figure 17B**). Indeed, acute EtOH significantly increases the firing rate when applied alone (baseline: naive = 100.00 ± 16.91 %, N/n = 5/5, p = 0.003 vs its acute EtOH; exposed = 99.1 ± 6.29 %, N/n = 5/5, p = 0.004 vs its acute EtOH; acute EtOH: naive = 174.83 ± 14.26 %, N/n = 5/5, p = 0.003 vs its baseline; exposed = 171.78 ± 5.40 %, N/n = 5/5, p = 0.004 vs its baseline. Two-way ANOVA, Newman-Keuls *post-hoc* test. **Figure 17C**).

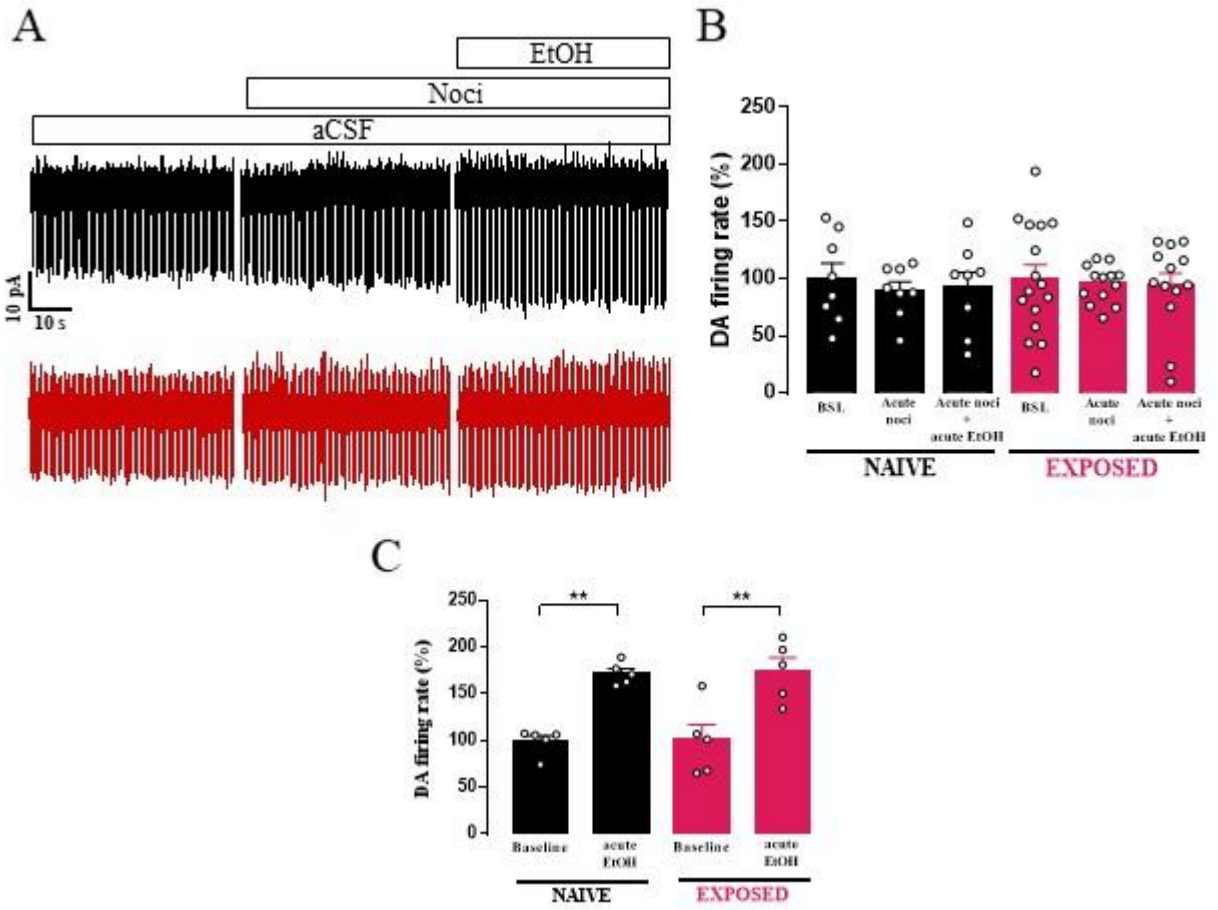

Figure 17: Effect of nociceptin on the firing rate of VTA DA neurons. (A) Representative traces from cell-attached recordings of the firing rate from naive (black) and exposed (red) mice before and during 30 nM noci application (left and center), and after the addition of acute 40 mM EtOHperfusion (right). (B) Plotted averaged frequency of the firing rate from naive and exposed mice before (BSL), during nociceptin application (acute noci), and during EtOH application (acute noci + acute EtOH). (C) Plotted averaged frequency of the firing rate from naive and exposed mice before (baseline) and after EtOH application (acute EtOH). Data represent individual (dots) and mean (bars) values ± SEM (Student's t-test and Two-way ANOVA, Newman-Keuls post-hoc test.)

8. DISCUSSION

Alcohol addiction develops over time. Initially, alcohol is consumed in small amounts for recreational use to experience its positive rewarding properties. Occasionally, consumption occurs in binges leading to episodic intoxications. Over time, some individuals progressively increase their alcohol consumption ending in its abuse and in some cases experiencing dependence. This latter stage is characterized by excessive drinking that if stopped leads to the expression of somatic and affective withdrawal signs. At this stage, alcohol intoxication and occasional abstinences are usually alternated leading to relapse episodes characterized by a compulsive resumption of alcohol use largely triggered by negative reinforcement mechanisms.

The aim of this research was to investigate the early stages of the process preceding the development of dependence in mice exposed to alcohol in a home cage two-bottle choice condition. This protocol was used to determine if voluntary intake of low alcohol concentration, mimicking the early stage of drinking, is sufficient to trigger plastic changes in the mesocorticolimbic DA transmission. It is well known that molecular mechanisms mediating the positive reinforcing effects of alcohol involve activation of the VTA DA system (Diana 2011; Söderpalm et al. 2009). However, it is thought that once dependence is established, maladaptive plastic changes in the VTA activity occur leading to a hypodopaminergic state (Melis, Spiga, and Diana 2005). This reorganization of VTA transmission may then contribute to the expression of negative mood occurring during drug abstinence (Diana et al. 1993, 1995; Bailey et al. 2001; Melis, Spiga, and Diana 2005; Rossetti et al. 1992; Weiss et al. 1996). To date, it is unclear when this switch in the mesolimbic DA transmission actually happens. Our hypothesis is that voluntary consumption of low doses of alcohol, occurring during early drinking experiences, is sufficient to elicit adaptive changes of this catecholaminergic system that ultimately may contribute to the motivation to consume alcohol.

Behavioral results showed that during the drinking acquisition phase mice progressively increased their consumption when alcohol concentration was elevated from 3% to 12%. At the end of the 18-day of voluntary drinking, mice showed an average intake of 11.69 ± 0.69 g/Kg and a 51.08 ± 3.12 % preference for the 12% solution. Of note, the highest level of EtOH preference was detected at the concentration of 6% (3% EtOH = 55.6 ± 2.28 %, 6% EtOH = 59.9 ± 3.34 %, 12% EtOH = 55.74 ± 3.37 %). Our recorded values of EtOH preference and intake are in agreement with those reported by other groups with continuous access protocol (Juarez et al. 2017; Yoneyama et al. 2008) and compatible with non-toxic, blood levels (Lê et al. 1994). Consistently, when mice were subjected to forced abstinence periods they did not show withdrawal symptoms or the expression of negative affective states. For instance, no differences between exposed and naive mice were revealed by the EPM, a standard test used to

measure basal anxiety levels. This finding is in line with the results of earlier work conducted in other laboratories (Pang et al. 2013; Younis et al. 2019). We also observed that, when access to alcohol was resumed after five days of deprivation, mice did not show the rebound of drinking (alcohol deprivation effect, ADE), which is classically accepted as an index of compulsive drinking. In this respect, while ADE is often reported in forced drinking models (vapor chamber inhalation, systemic administration, etc.), it has not been consistently reported following deprivation after voluntary consumption (Younis et al. 2019; Vengeliene, Bilbao, and Spanagel 2014; Tomie, Azogu, and Yu 2013). Together, these findings demonstrate that the drinking paradigm used here models the moderate intake of alcohol occurring during the early, reversible stage of the process leading to alcoholism.

When we looked at the spontaneous firing of VTA DA neurons we observed that the incubation of alcohol significantly increased it. This is consistent with earlier work showing that acute EtOH accelerates VTA DA firing in brain slices (Brodie, McElvain, et al. 1999; Brodie and Appel 1998; Brodie, Pesold, and Appel 1999; Brodie, Shefner, and Dunwiddie 1990; Gessa et al. 1985), and increase DA release *in vivo* (Foddai et al. 2004; Gessa et al. 1985; Mereu, Fadda, and Gian Luigi Gessa 1984). Enhanced VTA DA transmission elicited by alcohol is considered on the basis of its rewarding and reinforcing effects as well as that of other substances and natural rewards (Gatto et al. 1994; Gessa et al. 1985; Rodd et al. 2005). On the other hand, it is less clear what are neural alterations caused by voluntary drinking of low doses of alcohol as compared to passive administration of toxic EtOH concentrations (Brodie 2002; Okamoto, Harnett, and Morikawa 2006; Gallegos et al. 1999; Ludlow et al. 2009). Here, by performing cell-attached recordings from individual VTA DA neurons of naive and alcohol-drinking mice, we identified some important effects associated with EtOH exposure. Specifically, we found a significant reduction in the basal firing rate in brain slices from EtOH-exposed mice, accompanied by an augmented response to the acute application of the substance. This is consistent with the "hypodopaminergic theory" (Melis et al. 2005) stating that sustained stimulation with substances activating the reward system triggers a homeostatic mechanism leading to a negative shift in the basal level of activation. Of note, experimental support to this theory comes from imaging studies on alcoholics and animal models of forced consumption, mimicking advanced stages of the disorder (Volkow et al. 2010; Volkow, Fowler, et al. 2007). In this condition, ingestion of alcohol transiently relieves the negative affective state experienced by the individual, thus acting as self-medication. In agreement with this postulate, our data indicate that alcohol drinking leads to a hypodopaminergic state, but in our case, this

effect was observed at the very early stages and before the appearance of addictive-like behaviors.

Once ascertained that voluntary drinking of low amounts of alcohol is sufficient to affect VTA DA transmission we sought to determine if this phenomenon was linked to readaptation of presynaptic circuitries or postsynaptic mechanisms. For this purpose, we recorded DA activity in the presence of excitatory and inhibitory synaptic blockers. Interestingly, results showed that in the presence of presynaptic blockade the effect of alcohol on DA firing activity was abolished both in naive and alcohol-exposed mice. This observation clearly demonstrated that neurophysiological response to chronic and acute EtOH relies on network mechanisms rather than direct effects on DA cells in this model. Building on this evidence we then examined the strength of excitatory and inhibitory synaptic inputs with whole-cell recordings. Indeed, it was reported that acute EtOH increases the excitatory tone onto VTA DA neurons (Saal et al. 2003; Xiao et al. 2009; Deng et al. 2009). While we found no differences between groups in the frequency or amplitude of excitatory inputs, the frequency of inhibitory inputs in basal conditions was significantly elevated and the response to acute EtOH was reduced in VTA DA neurons from exposed mice. This is in good agreement with the firing recordings reported above, showing changes of the opposite polarity. Of note, not all neurons revealed an increased inhibitory tone during acute EtOH and this fraction is greater in exposed mice, although not significantly. Furthermore, the evidence that response to acute EtOH is predictive of increased basal inhibitory tone suggested the existence of two synaptic pathways within the VTA showing distinct response to EtOH. In particular, it seems that responsive GABAergic neurons adapt to chronic EtOH by increasing their tonic firing activity. The functional and synaptic heterogeneity internal to the GABAergic VTA population in basal conditions and following challenge with psychoactive substances has been largely demonstrated (St. Laurent et al. 2020; St. Laurent and Kauer 2019; Morales and Margolis 2017). A more in-depth characterization of EtOH-responding GABA neurons and the reconstruction of their connectivity (for example using retrograde and anterograde neuronal tracing) could be of enormous value for further interpretation of our results.

The electrophysiological alterations induced by chronic EtOH in our voluntary drinking paradigm are reversible. When we tested the strength of the inhibitory synaptic activity onto VTA DA neurons from mice after 5 days of EtOH deprivation we found a complete normalization of basal IPSC frequency and response to acute EtOH. The significant increase in basal IPSC amplitude, which reaches values exceeding baseline levels of naive animals, was an unexpected finding which remains, at present, unanswered. The expression levels and subunit

composition of $GABA_A$ receptors should be examined in order to test for possible remodeling induced by chronic EtOH intake and withdrawal, a phenomenon demonstrated previously in similar studies (Enoch 2008; Nelson et al. 2018; Ortiz et al. 1995; Charlton et al. 1997).

The evidence that the effects on the firing rate of VTA DA neurons induced by chronic and acute EtOH are abolished in synaptically-isolated recordings and that acute EtOH application increases the frequency of action potential-dependent IPSCs suggests the existence of a population of local GABAergic interneurons responding to EtOH both chronically and acutely. Preliminary cell-attached recordings of firing rate from putative GABAergic neurons reveal an increase in both basal and EtOH-stimulated firing frequency which, however, does not reach statistical significance. Although preliminary, this finding could explain the response to chronic and acute EtOH in the inhibitory synaptic activity recorded from DA neurons.

Collectively, our experiments demonstrate that a population of VTA GABAergic neurons undergo functional remodeling following voluntary consumption of moderate doses of EtOH. *Ex vivo*, this modification is detected as an increased inhibitory tone onto the DA population. The inhibitory activity is, however, less responsive to the enhancing effect of acute EtOH, possibly due to ceiling effects. In agreement, the basal firing rate of DA neurons is lower than normal, while the enhancing action of EtOH is elevated. Of note, alterations in GABAergic synaptic frequency are normalized by five days of abstinence when behavioral tests reveal no signs of withdrawal syndrome at that time point. Nonetheless, our results indicate that alteration in VTA transmission in the direction of a hypodopaminergic state precedes the onset of overt alcohol dependence as demonstrated in earlier studies.

Finally, after characterizing the response of DA VTA neurons to alcohol we decided to determine the effect of N/OFQ in naive and EtOH drinking mice. N/OFQ is an opioid-like neuropeptide that decreases voluntary EtOH drinking, self-administration and relapse, and that is known to blunt the reinforcing effects of EtOH in several behavioral paradigms (Ciccocioppo et al. 1999, 2002, 2003; Kuzmin et al. 2003, 2007; Zaveri et al. 2018; Aziz et al. 2016; Economidou et al. 2006; Economidou et al. 2008; Martin-Fardon et al. 2000). Of relevance, earlier work demonstrated that activation of NOP by N/OFQ or by synthetic receptor ligands produce a much stronger effect in post-dependent models or genetically-selected alcohol-preferring rats. Moreover, electrophysiological studies demonstrated that activation of NOP receptor in the VTA leads to a reduction in the frequency of spontaneous IPSCs and amplitude of IPSCs evoked by electrical stimulation of the midbrain slice. N/OFQ also increases the paired-pulse ratio in evoked sIPSCs suggesting the inhibition in the GABA release onto DA neurons by acting presynaptically (Zheng, Grandy, and Johnson 2002). Furthermore, a

concentration-dependent hyperpolarization of both DA and putative-GABAergic neurons has been reported after NOP receptor activation (Driscoll et al. 2020; Hernandez et al. 2020; Zheng et al. 2002). However, whether neuronal inhibition is a consequence of the enhancement of $GABA_A$-mediated or K^+ currents, remains to be determined. Interestingly, it was also shown that N/OFQ effects on the activity of VTA neurons vary based on their projection area. Indeed, N/OFQ hyperpolarizes VTA cells projecting to both PFC and NAc while slightly exciting those projecting to the posterior-anterior cingulate cortex (pACC) (Driscoll et al. 2020).

Bearing this in mind, we determined how NOP stimulation could affect EtOH-induced changes in VTA DA activity. Results from cell-attached recordings revealed that bath application of N/OFQ abolishes the enhancing effect of acute EtOH on the firing rate of VTA DA neurons of both exposed and naive mice. Interestingly, nociceptin had no effect on the basal firing rate of either group.

In conclusion, the main findings of this work can be summarized as follows:

1) Voluntary, moderate EtOH consumption does not lead to the development of an addictive-like state but induces measurable, significant, partially reversible alterations in basal and EtOH-induced electrophysiological properties of VTA DA neurons, which are reminiscent of a hypodopaminergic state.

2) Neurophysiological adaptations to EtOH are expressed as both basal and dynamic changes in the frequency of GABAergic transmission onto a subset of distinct DA neurons, which in turn suggests the existence of separate populations of local GABAergic neurons with differential response to EtOH.

3) N/OFQ, a neuropeptide with anti-addictive properties is able to abolish the stimulating effects of EtOH on VTA DA firing activity.

9. ABBREVIATIONS

5HT:	5- hydroxytryptamine or serotonin
5-HT3:	5-hydroxytryptamine type 3 receptor
ADE:	Alcohol Deprivation Effect
ADH:	Alcohol Dehydrogenase
AHP:	After-hyperpolarization
ALDH:	Aldehyde Dehydrogenase
AMPA:	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AP:	Action Potential
AUD:	Alcohol Use Disorders
BECs:	Blood Ethanol Concentrations
BK:	Big conductance Potassium Channel
BNST:	Bed Nucleus of the Stria Terminalis
cAMP:	Cyclic Adenosine Monophosphate
CeA:	Central Amygdala
CNS:	Central Nervous System
CPP:	Conditioned Place Preference
CRF:	Corticotropin-Releasing Factor
cTRIO:	cell-type-specific Tracing of the Relationship between Inputs and Outputs
DA:	Dopamine or Dopaminergic
DAT:	Dopamine Transporter
DBS:	Deep Brain Stimulation
dlPFC:	Dorsolateral Prefrontal Cortex

DRN:	Dorsal Raphe Nucleus
DSM-V:	Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition
EMA:	European Medications Agency
EPM:	Elevated Plus Maze
EtOH:	Ethanol
FDA:	Food and Drug Administration
GABA:	γ-aminobutyric acid
GABAA:	γ-aminobutyric acid type A channel
GAD:	Glutamate Decarboxylase
GFP:	Green Fluorescence Protein
GHB:	Gamma-Hydroxybutyrate
GIRK:	G-protein activated inwardly rectifying potassium channel
i.p.:	Intraperitoneal
ICV:	Intracerebroventricular
Ih:	Hyperpolarization-activated ation current
KA:	Kainic Acid
LC:	Locus Coeruleus
LDTg:	Laterodorsal Tegmentum nucleus
LHb:	lateral habenula
LHT:	Lateral Hypothalamus
LTP:	Long-Term Potentiation
mGluRs:	G-protein coupled metabotropic glutamate receptors
mPFC:	Medial Prefrontal Cortex

MSN: Medium Spiny Neuron

msP: Marchigian Sardinian alcohol-Preferring

NAc: Nucleus Accumbens

nAChRs: nicotinic receptors for acetylcholine

NMDA: N-methyl-D-aspartate receptor

NMDA: N-methyl-D-Aspartate

ORL 1: Opioid-Receptor-Like 1

PAG: Periaqueductal Grey

PFC: PreFrontal Cortex

PPTg: Pedunculopontine Tegmentum

PWID: People Who Inject Drugs

RMTg: Rostromedial Mesopontine Tegmental nucleus

RN: Raphe Nucleus

sEPSC: Spontaneous Excitatory Post-Synaptic Current

SERT: Serotonin Transporter

sIPSC: Spontaneous Inhibitory Post-Synaptic Current

SK: Small conductance Potassium Channel

SNc: Substantia Nigra pars Compacta

SSRIs: Selective Serotonin Reuptake Inhibitors

SUD: Substance Use Disorder

TH: Tyrosine Hydroxylase

TMS: Transcranial Magnetic Stimulation

VGAT: Vesicular GABA Transporter

VGLUT2: Vesicular Glutamate Transporter 2

V_{hold}: Voltage Holding

VMAT2: Vesicular Monoamine Transporter 2

VP: Ventral Pallidum

VS: Voltage Sag

VTA: Ventral Tegmetal Area

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11. APPENDIX

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ORIGINAL ARTICLE

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Ethanol neurotoxicity is mediated by changes in expression, surface localization and functional properties of glutamate AMPA receptors

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Abstract

Modifications in the subunit composition of AMPA receptors (AMPARs) have been linked to the transition from physiological to pathological conditions in a number of contexts, including EtOH-induced neurotoxicity. Previous work from our laboratory showed that EtOH withdrawal causes CA1 pyramidal cell death in organotypic hippocampal slices and changes in the expression of AMPARs. Here, we investigated whether changes in expression and function of AMPARs may be causal for EtOH-induced neurotoxicity. To this aim, we examined the subunit composition, localization and function of AMPARs in hippocampal slices exposed to EtOH by using western blotting, surface expression assay, confocal microscopy and electrophysiology. We found that EtOH withdrawal specifically increases GluA1 protein signal in total homogenates, but not in the post-synaptic density-enriched fraction. This is suggestive of overall increase and redistribution of AMPARs to the extrasynaptic compartment. At functional level, AMPA-induced calcium influx was unexpectedly reduced, whereas AMPA-induced current was enhanced in CA1 pyramidal neurons following EtOH withdrawal, suggesting that increased AMPAR expression may lead to cell death because of elevated excitability, and not for a direct contribution on calcium influx. Finally, the neurotoxicity caused by EtOH withdrawal was attenuated by the non-selective AMPAR antagonist 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[*f*]quinoxaline-7-sulfonamide disodium salt as well as by the selective antagonist of GluA2-lacking AMPARs 1-naphthyl acetyl spermine. We conclude that EtOH neurotoxicity involves changes in expression, surface localization and functional properties of AMPARs, and propose GluA2-lacking AMPARs as amenable specific targets for the development of neuroprotective drugs in EtOH-withdrawal syndrome.

Abbreviations: AMPARs, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors; CpAMPARs, Ca^{2+}-permeable AMPA receptors; EtOH, ethanol; GluA, AMPA receptor subunit; GRIP, glutamate receptor-interacting protein; NASPM, 1-naphthyl acetyl spermine; NBQX, 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[*f*]quinoxaline-7-sulfonamide disodium salt; PI, propidium iodide; PICK1, protein interacting with C kinase-1; PSD, postsynaptic density; REST, repressor element-1 (RE1) silencing transcription factor gene; SAP97, synapse-associated protein 97; TIFs, Triton insoluble fraction.

Elisabetta Gerace and Alice Ilari equally contributed to the manuscript.

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KEYWORDS
AMPA receptors, CA1 injury, ethanol withdrawal, organotypic hippocampal slices, scaffold proteins

1 | INTRODUCTION

Ethanol (EtOH) abuse causes persistent structural and functional alterations in the brain by mechanisms that are not yet fully understood. Many studies have shown permanent impairment in synaptic function and neuronal damage caused by EtOH exposure and withdrawal in multiple neuronal areas, including the hippocampus, with both in vivo and in vitro models of neurotoxicity (Gerace et al., 2016, 2019; Läck et al., 2007; Marty & Spigelman, 2012). AMPA receptors (AMPARs) mediate the fast component of excitatory neurotransmission, and the dynamic regulation of these receptors, both at transcriptional and posttranscriptional level, is a crucial factor in the transition from physiological to pathological conditions. The homeostasis of GluA1-2-containing AMPARs is controlled by several scaffold and adhesion complex proteins that capture, retain and promote surface expression of AMPARs, thus, regulating physiological neuronal functions and, in certain conditions, cell fate. AMPARs are localized mostly in the postsynaptic density and their trafficking is tightly regulated by interactions with scaffold molecules controlling the distribution of AMPARs in the synaptic, intracellular and extrasynaptic pools (Newpher & Ehlers, 2008). Several AMPAR interacting proteins have been identified, including glutamate receptor-interacting protein (GRIP) and protein interacting with C kinase-1 (PICK1), which have been shown to specifically interact with GluA2 subunit (Daw et al., 2000; DeSouza et al., 2002; Perez et al., 2001; Seidenman et al., 2003), the transmembrane protein stargazin, which interacts directly with AMPAR (Chen et al., 2000) and Synapse-associated protein 97 (SAP97), which specifically binds GluA1 subunit (Howard et al., 2010; Leonard et al., 1998).

Great attention has been focused on the GluA2 subunit as a result of its profound effects on AMPAR assembly, trafficking and ionic selectivity. A relative decrease in GluA2 subunit increases calcium permeability of AMPARs, an event that has been linked to the transition from physiological to pathological conditions in a number of contexts. For example, one of the earliest biological manifestations of dementia in Alzheimer disease (AD) is owing to a reduction in synaptic AMPARs (Shankar et al., 2008). Moreover, prolonged decreases in surface GluA2-containing AMPARs have been described as a causal factor for cell death of hippocampal CA1 pyramidal neurons in models of toxicity (Anzai et al., 2003; Gerace et al., 2014) and in pathological conditions including global ischemia (Pellegrini-Giampietro, 1997). Interestingly, dysfunctional Q/R editing in GluA2 occurs in the motor neurons of patients with amyotrophic lateral sclerosis (ALS, Hideyama & Kwak, 2011). Calcium-permeable AMPARs (CpAMPARs) have also been demonstrated to play an essential role in substance use disorders (SUD) models, as exposure to substances lead to AMPAR molecular switch and formation of CpAMPARs (Mameli et al., 2011; Pascoli et al., 2014). For example, it was recently shown that cocaine-induced potentiation of VTA excitatory synapses is mediated by the insertion of CpAMPARs to the synaptic membranes (Mills et al., 2017). Moreover, cadherin adhesion complex proteins have been shown to contribute to the stabilization of AMPARs into the synaptic membranes (Saglietti et al., 2007; Tai et al., 2008) and may contribute to the susceptibility to cocaine addiction by the stabilization of GluA1-2-containing AMPARs into synaptic membranes (Mills et al., 2017).

A recent paper from our laboratory has demonstrated that EtOH withdrawal induces cell death in mature organotypic hippocampal slices and that glutamate receptors are mediators of EtOH withdrawal–induced toxicity (Gerace et al., 2019). In particular, we found that EtOH withdrawal increases the AMPA-mediated spontaneous excitatory postsynaptic currents (EPSCs) as well as the expression of GluA1 but not GluA2 AMPAR subunits, suggesting that the toxicity induced by EtOH withdrawal could be caused by the formation of GluA2-lacking, calcium-permeable AMPARs.

On the basis of these considerations, here we sought to investigate the role of GluA2-lacking AMPARs in the toxicity induced by EtOH withdrawal.

2 | MATERIALS AND METHODS

Male and female Wistar rat pups (94 animals, 7–9 days old, weight 16 ± 3 g) were obtained from Charles River. Animals were housed at 23 ± 1°C under a 12 hr light–dark cycle (lights on at 07:00) and were fed a standard laboratory diet with ad libitum access to water. The experimental protocols were approved by the Italian Ministry of Health (Aut. 176; 17E9C.N.VAS) and the European Communities Council Directive of 2010/63/EU.

The authors further attest that all efforts were made to minimize the number of animals used and their suffering, as reported in the Guidelines McGill Module-1. The present study was NOT pre-registered. NO subjects were excluded in the present study. No blinding for experiments was performed.

2.1 | Materials

Ethanol (EtOH, CAS No: 64-17-5), 1-Naphthylacetyl spermine trihydrochloride (NASPM, catalogue number: #N193) and propidium iodide (PI, CAS No: 25535-16-4) were purchased from Sigma. Tissue culture reagents were obtained from Gibco-BRL and Sigma. 2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[*f*]quinoxaline-7-sulfonamide disodium salt (NBQX, catalogue number: #1044/1), tetrodotoxin Citrate (TTX, catalogue number: #1069), DL-2-Amino-5-phosphonopentanoic acid (APV, catalogue number: #0105) and

pictotoxin (catalogue number: #1128) were purchased from Tocris. (R,S)-AMPA (CAS No: #74341-63-2) was purchased from Abcam.

2.2 | Preparation of rat organotypic hippocampal slice cultures

Organotypic hippocampal slice cultures were prepared as previously reported (Gerace et al., 2012, 2015; Landucci et al., 2018). Briefly, after decapitation hippocampi were isolated and removed from the brains of Wistar rat pups, transverse slices (420 μm) were prepared using a McIlwain tissue chopper and then transferred onto 30-mm-diameter semi-porous membranes inserts (Millicell-CM, catalogue number: #PICM03050; Millipore), which were placed in six well tissue culture plates containing 1.2-ml medium per well. Slices were maintained at 37°C in an incubator in an atmosphere of humidified air and 5% CO_2 for 10 days. Before experiments all the slices were screened for viability by phase-contrast microscopy analysis; slices displaying signs of neurodegeneration were discarded from the study (exclusion criteria).

2.3 | Ethanol exposure and Drug Treatment in organotypic hippocampal slices

The experiments were conducted as previously described in Gerace et al. (2016) and Gerace et al. (2019). Briefly, hippocampal slice cultures were exposed for 7 days to 150 mM of EtOH. The medium was changed every day adding EtOH to the fresh culture medium. For control slices, the medium was changed every day by adding fresh culture medium. After 7 days of EtOH treatment, some of the slices were incubated in EtOH fresh culture medium, EtOH-free medium or in EtOH-free medium plus the non-selective AMPA antagonist NBQX (10 μM) and the selective blocker of GluA2-lacking AMPARs NASPM (10 μM) for 24 hr before they were assessed for neuronal injury using PI fluorescence.

2.4 | Assessment of CA1 pyramidal cell injury

PI (5 μg/ml) was added to the medium either at the end of the 7-day EtOH incubation period or 24 hr after it was removed from the medium. Thirty minutes later, fluorescence was viewed using an inverted fluorescence microscope (Olympus IX-50; Solent Scientific) equipped with a xenon-arc lamp, a low-power objective (4×) and a rhodamine filter. Images were digitized using a video image obtained by a CCD camera (Diagnostic Instruments Inc.) controlled by software (InCyt Im1™; Intracellular Imaging Inc.) and subsequently analysed using the Image-Pro Plus morphometric analysis software (Media Cybernetics). In order to quantify cell death, the CA1 hippocampal subfield was identified and encompassed in a frame using the drawing function in the image software (ImageJ; NIH) and the optical density of PI fluorescence was detected. There was a linear correlation between CA1 PI fluorescence and the number of injured CA1 pyramidal cells as detected by morphological criteria (Pellegrini-Giampietro et al., 1999).

2.5 | Western blot analysis

The experiments were conducted as previously described in Gerace et al. (2015) and Gerace et al. (2019). Cultured slices were washed with cold 0.01 M PBS and dissolved in 1% SDS. Total protein levels were quantified using the BCA (bicinchoninic acid) Protein Assay (catalogue number: # 23,225) (Pierce). Forty μg of proteins were resolved by electrophoresis on SDS-polyacrylamide gel and transferred onto nitrocellulose membranes using the transblot TURBO (Bio-Rad). Blots were probed overnight at 4°C with primary antibodies, all diluted 1:1,000. Immunodetection was performed with secondary antibodies conjugated to horseradish peroxidase. The reactive bands were detected using chemiluminescence (ECLplus; Euroclone). Quantitative analysis was performed using the QuantityOne analysis software (Bio-Rad).

2.6 | Fluorescence immunohistochemistry and quantitative analysis

At the end of experiments, the organotypic slices were harvested and fixed O/N in ice-cold paraformaldehyde, 4% in PBS buffer. The day after, slices were rinsed in PSB and placed for at least 2 days in 18% sucrose in PBS. Immunostaining was performed with the free-floating method reported in Lana et al. (2014). Day 1: Organotypic hippocampal slices were placed in a multiwell and washed 3 times for 5 min in PBS-TX, then blocked for 60 min with BB containing 10% Normal Goat Serum. Slices were then incubated overnight at 4°C under slight agitation with a combination of two different primary antibodies (anti-NeuN (catalogue number: #ABN78), 1:400; Millipore; GluA1-AMPA subunit (catalogue number: #AGC-004), 1:100 or GluA2-AMPA subunit (catalogue number: #AGC-005), 1:100 (Alomone Labs) dissolved in BB.

Day 2: Sections were incubated for 2 hr at room temperature in the dark with AlexaFluor 488-conjugated donkey anti-rabbit IgG secondary antibody diluted in BB and then for 2 hr at room temperature in the dark with AlexaFluor 488-conjugated donkey anti-rabbit IgG (catalogue number: #A32790) secondary antibody plus AlexaFluor 555 goat anti-mouse (catalogue number: #A-21422; Invitrogen, Thermo Fisher) both diluted 1:400 in BB. After three washings, slices were mounted onto gelatin-coated slides using Vectashield hard set mounting medium with DAPI (catalogue number: #H-1200–10; Vector Laboratories).

Slices were observed under a LEICA TCS SP5 confocal laser scanning microscope (Leica Microsystems CMS GmbH) equipped with a 63× objective (z step of 0.3 μm). Confocal scans were acquired keeping all parameters constant. Image analyses were conducted on z-stacks projections which contained the region of interest, corresponding to

the CA1 area, using Image J (National Institute of Health, http://rsb.info.nih.gov/ij). Quantification of GluA1 and GluA2 AMPA subunit immunoreactivity in CA1 was performed separately using Image J. Forty consecutive z-scans (0,3 μm each, total 12 μm) were stacked, starting at 10 μm inside the slice and an appropriate and constant threshold level was selected. Quantitation of immunofluorescence was then obtained from the ratio between positive pixels above threshold and total pixels in each region of interest.

2.7 | Real-time PCR for REST and GluA2 mRNA expression

Real-time PCR was performed as previously reported in Llorente et al. (2015) and Lapucci et al. (2017). Total ribonucleic acid (RNA) was extracted using Trizol Reagent (catalogue number: #15596018; Life Technologies). Any contaminating DNA in the RNA samples was removed by incubation with DNase (Sigma Aldrich). Total RNA concentrations were determined using a NanoDrop ND-3300 spectrophotometer (NanoDrop Technologies). Isolated RNA was maintained at 80°C until further processing. One μg of RNA was reverse transcribed using iScript (Bio-Rad). Real-Time PCR was performed in duplicate using Rotor-Gene 3,000 (Qiagen) and the Rotor-Gene TM SYBR® Green PCR Kit (Qiagen), the reactions were run at 95°C for 30 s, 95°C for 5 s and 60°C for 15 s for 45 cycles. As an internal control for normalization, PCR reactions were performed concurrently with the amplification of a reference gene, 18S ribosomal RNA (rRNA).

The following primers were used:

REST: Forward primer (5′–3) GACGGAGAGCAAACCCAAGACCAG, Reverse primer (5′–3′) CTTGAGCCATCTCCGAGGAGGGTTC;

GluA2: Forward primer (5'–3') CCAAGGACTCGGGAAGTAAGG, Reverse primer (5′–3′) CCCCCGACAAGGATGTAGAA;

RNA 18S: Forward primer (5'–3') GATTAAGTGCCCTTTGTA, Reverse primer (5′–3′) GATCCGAGGGCCTCACTAAAC.

2.8 | Preparation of protein extracts and western blot analyses

Proteins from hippocampal slices were extracted as previously described with minor modifications (Caffino et al., 2018; Fumagalli et al., 2008). Briefly, hippocampal slices were homogenized in a teflon-glass homogenizer in cold 0.32 M sucrose buffer pH 7.4 containing 1 mM HEPES, 1 mM MgCl2, 1 mM NaHCO3 and 0.1 mM PMSF, in presence of commercial cocktails of protease (Roche) and phosphatase (Sigma-Aldrich) inhibitors and an aliquot of each homogenate was then sonicated. The remaining homogenate was centrifuged at 13,000 *g* for 15 min obtaining a pellet. This pellet was resuspended in a buffer containing 75 mM KCl and 1% Triton X-100 and centrifuged at 100,000 *g* for 1 hr. The resulting pellet, referred as post-synaptic density (PSD) or Triton X-100 insoluble fraction (TIF), was homogenized in a glass–glass potter in 20 mM HEPES, protease and phosphatase inhibitors and stored at −20°C in presence of glycerol 30%. Total proteins have been measured in the total homogenate and in the TIF fraction according to the Bradford Protein Assay procedure (Bio-Rad Laboratories, Italy), using bovine serum albumin as calibration standard.

Equal amounts of proteins of the homogenate (6 μg) and of TIF fraction (5 μg) were run on criterion TGX precast gels (Bio-Rad Laboratories) under reducing conditions and electrophoretically transferred onto nitrocellulose membrane (Bio-Rad Laboratories). Blots were blocked 1 hr at room temperature with I-Block solution (catalogue number: # T2015, Life Technologies, Italia, Italy) in TBS + 0.1% Tween-20 buffer and incubated with antibodies against the proteins of interest.

The conditions of the primary antibodies were the following: anti N-Cadherin (catalogue number: #13116, 1:1,000; Cell Signaling Technology); anti mGluR5 (1:2000, Chemicon, USA); anti GRIP (catalogue number: #ABN27; 1:1,000; Sigma Aldrich); anti GluA1 (catalogue number: #13185; 1:2000; Cell Signaling Technology); anti GluA2 (catalogue number: #13607; 1:1,000; Cell Signaling Technology); anti SAP97 (catalogue number: #ab2057181:1,000; Abcam); anti PSD-95 (catalogue number: #2507; 1:4,000, Cell Signaling Technology), anti β-catenin (catalogue number: # sc-7963; 1:1,000, Santa Cruz Biotechnology) and anti β-Actin (catalogue number: # A5316; 1:10,000; Sigma-Aldrich). Results were standardized using β-actin as the control protein, which was detected by evaluating the band density at 43 kDa. Immunocomplexes were visualized by chemiluminescence using the Chemidoc MP Imaging System (Bio-Rad Laboratories). Gels were run three times each and the results represent the average from three different western blots.

2.9 | Electrophysiological recordings

Whole-cell voltage-clamp recordings were performed in CA1 pyramidal neurons from organotypic hippocampal slices as previously described (Gerace et al., 2019). The slices were chronically treated with 150 mM of EtOH or under control conditions, followed by 4–6 hr of EtOH withdrawal before recordings. A single slice was removed from the culture insert, placed in a flow chamber positioned under the microscope objective and continuously perfused with warm (34–35°C) artificial Cerebrospinal Fluid (aCSF), composed of (in mM) 130 NaCl, 3.5 KCl, 1.25 NaH_2PO_4, 24 $NaHCO_3$, 10 glucose, 2 $CaCl_2$ and 1 $MgSO_4$ and saturated with a 95% O_2 + 5% CO_2 gas mixture. Whole-cell pipettes were pulled from thin-walled borosilicate capillaries (Harvard Apparatus) with a vertical puller (Narishige PP830, Narishige International Limited) back-filled with the following intracellular solution (in mM): K^+ Methanesulfonate (120), KCl (15), HEPES (10), EGTA (0.1), $MgCl_2$ (2), Na_2PhosphoCreatine (5), Na_2GTP (0.3) and MgATP (2), resulting in a bath resistance of 3–5 MΩ. For coupled recordings of electrical and fluorescent calcium signals, 0.1 mM of Fluo 4 pentapotassium salt (Molecular Probes) was added. Signals were sampled at 10 kHz and low-pass filtered at 3 kHz with an Axon Multiclamp 700B (Molecular Devices, Sunnyvale,

CA, USA). After establishing a Giga-Ohm seal, a whole-cell configuration was achieved by rupturing the neuronal membrane. Access resistance was monitored during voltage clamp recordings with brief test pulses (−10 mV, 500 ms), throughout the experiment. All data were analysed using pCLAMP (Axon Instruments) and GraphPad Software (San Diego, CA). AMPA-mediated electrical and optical responses were studied by obtaining sequences of 30 × 30-s recording frames from individual CA1 neurons in whole-cell voltage clamp ($V_{HOLDING}$ = −65 mV) configuration. After the achievement of stable baselines, a mix of inhibitors was bath applied which included the voltage-dependent sodium channel blocker TTX (1 μM), the NMDA receptor antagonist DL-2-Amino-5-phosphonopentanoic acid (APV, 50 μM) and the $GABA_A$ receptor antagonist picrotoxin (PTX, 50 μM). After 5 min, 0.3 μM AMPA was applied, in presence of inhibitors, in order to record pharmacologically isolated cellular responses.

2.10 | Microfluorometric determination of calcium responses

AMPA-induced calcium responses were studied as described in Carbone et al. (2017) with some modifications. During electrophysiological recordings, fluorescence signal was collected from a square-shaped window comprising the cell body of the neuron under investigation loaded with 100 μM of the high-affinity, non-ratiometric calcium dye Fluo 4 pentapotassium salt (Molecular Probes). Fluorescence was elicited with a 488 nm LED and collected with a photomultiplier tube (PMT; Cairns Research) with a 10 kHz sampling rate. LED excitation was triggered with the electrophysiological protocol and consisted of 1 s of LED ON at the end of each 30-s gap-free recording. Somatic calcium responses (SCRs) are reported as $\Delta F/F_0$, where F_0 signal was the baseline emission of the loaded neuron at rest, and ΔF was defined as $F_{AMPA} - F_0$ where F_{AMPA} was taken at the end of AMPA application. Background fluorescence was obtained by measuring the emission of a Fluo 4-free area of the slice and subtracting the obtained value from F_0. Off-line analysis was performed with Clampfit 10 (Molecular Devices) and Origin 9.1.

2.11 | Statistical analysis

Data are presented as means ± *SEM* of n experiments from independent cell preparations. Statistical significance of differences between PI fluorescence intensities, immunostaining or Western blot optical densities was evaluated by performing one-way ANOVA followed by Tukey's w test for multiple comparisons. For electrophysiological

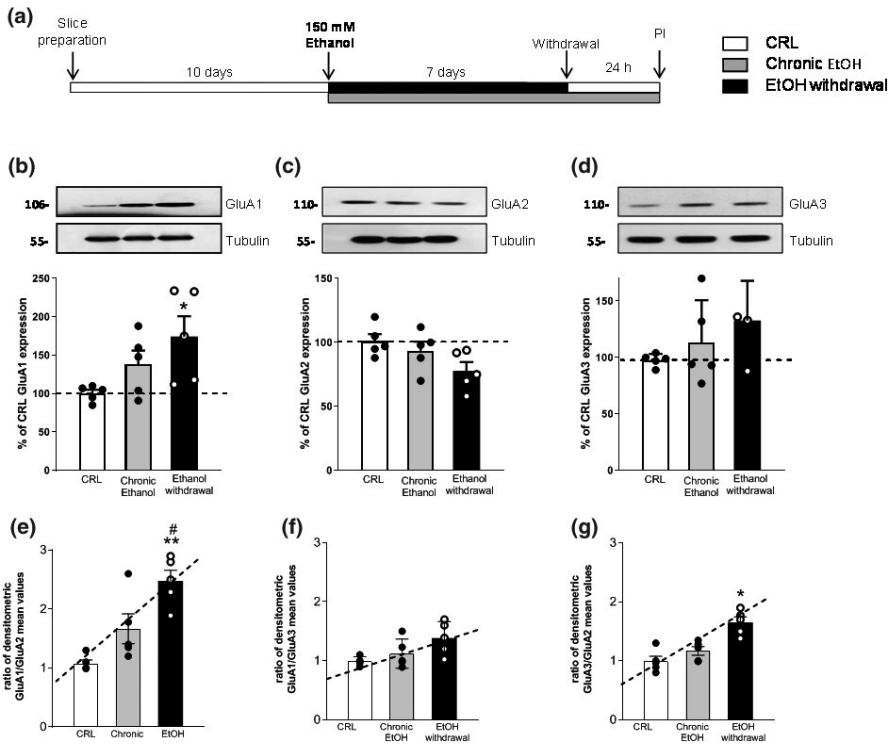

FIGURE 1 Ethanol withdrawal induces changes in α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) subunit composition. Top: experimental protocol (a). Middle: representative Western blots using antibodies directed against the AMPAR subunit GluA1, GluA2 and GluA3 (b, c, d) and quantitative analysis of immunoreactive bands (e, f, g), showing that EtOH withdrawal increases the expression of the AMPA subunit GluA1. Tubulin was used as loading control. Bottom: analysis of the ratio between GluA1/GluA2, GluA1/GluA3 and GluA3/GluA2 AMPA subunits expression in mature slices after chronic EtOH and EtOH withdrawal suggesting that EtOH withdrawal modifies AMPA subunit composition leading to GluA2-lacking and Ca^{2+}-permeable AMPARs. Data are expressed as a percentage of control. Bars represent the mean ± *SEM*of at least five experiments from independent cell preparations (about ≥8 slices for each experimental point). *$p < .05$, **$p < 0.1$ versus. CRL and $^{\#}p < .05$ versus. Chronic EtOH (ANOVA + Tukey's test)

experiments and determination of calcium response, statistical significance was evaluated by performing a Student's *t* test for unpaired samples. Differences were considered significant for $^{*}p < .05$, $^{**}p < .01$ and $^{***}p < .001$. All statistical calculations were performed using GRAPH-PAD PRISM v.5 for Windows (GraphPad Software). No sample calculation was performed to predetermine the sample size. Data were not assessed for normality. NO test for outliers was conducted.

3 | RESULTS

We used rat organotypic hippocampal slice cultures exposed to 150 mM EtOH for 7 days (chronic) to mimic chronic EtOH consumption and then we incubated the slices in EtOH-free medium for the subsequent 24 hr to mimic EtOH withdrawal in vitro. In these experimental conditions, EtOH withdrawal led to a dose-dependent CA1

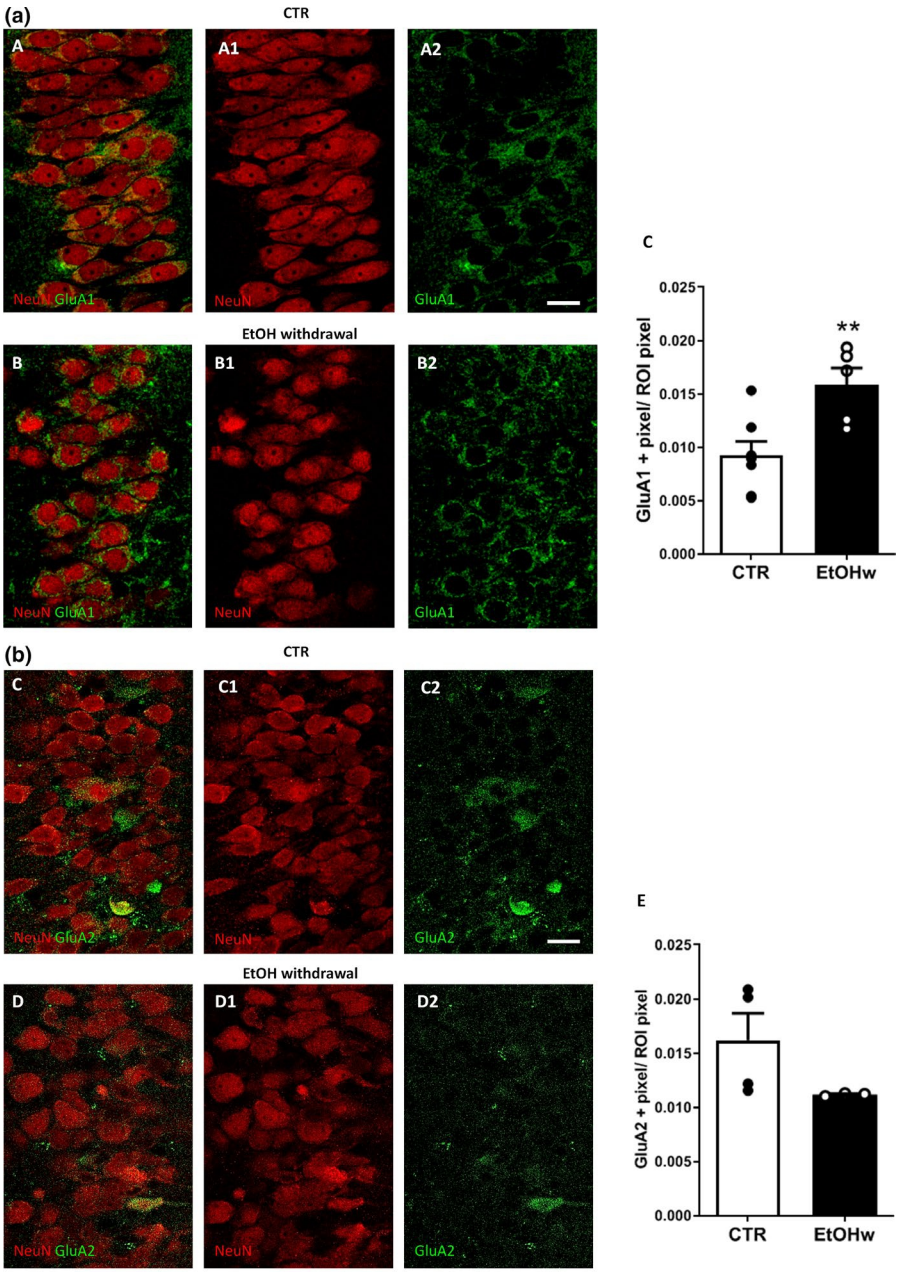

FIGURE 2 Ethanol withdrawal increases GluA1 AMPAR subunit expression in CA1 Stratum pyramidale of organotypic hippocampal slices. (a–b2) Representative confocal microscopy images showing CA1 immunostaining of neurons (NeuN, red, a1,b1), and GluA1-AMPAR subunit (GluA1, green a2,b2) in CA1 pyramidal neurons of organotypic hippocampal slices in control (a–a2) or after 24h of EtOH withdrawal (b–b2). Scale bar: 10 μm. (c) Quantitative analysis of GluA1 immunoreactivity in CA1 Stratum pyramidale of control (CRL) (white column,$n = 6$), and EtOH-withdrawal slices (black column, $n = 6$) ($^{**}p < .05$ EtOH withdrawal versus. CRL, Student'sttest). (c–d2) Representative confocal microscopy images showing CA1 immunostaining of neurons (NeuN, red, c1,d1), and GluA2 AMPAR subunit (GluA2, green, c2, d2) in CA1 Stratum pyramidale of organotypic hippocampal slices in control (c–c2) or after 24h of EtOH withdrawal (d–d2). Scale bar: 10 μm. (e): Quantitative analysis of GluA2 immunoreactivity in CA1 Stratum pyramidale of control (CRL) (white column, $n = 4$), and EtOH-withdrawal slices (black column, $n = 3$). The decrease was not statistically significant $^{**}p< 0.1$ versus. CRL (Student's *t*test)

pyramidal cell injury examined using propidium iodide fluorescence as previously demonstrated and published (Gerace et al., 2016, 2019). With the aim to study the mechanism of EtOH-withdrawal toxicity, we firstly performed western blotting experiments to characterize the composition of AMPA receptor tetramers after EtOH withdrawal. We used specific antibodies directed against GluA1, GluA2 and GluA3 AMPAR subunits (Figure 1a–c). We found that EtOH withdrawal induced a significant increase in the expression of GluA1 (Figure 1a), no change was observed for GluA3 (Figure 1c), while a relative trend in a reduction was observed in the expression of GluA2 (Figure 1b) subunit. To this matter, the analysis of GluA1/GluA2 was significant after EtOH withdrawal as compared to control and to chronic EtOH and GluA3/GluA2 ratio expression was significant only versus control after EtOH withdrawal, while no changes was noticed for GluA1/GluA3 ratio. These data suggest that EtOH withdrawal induces relevant modification of AMPAR subunit composition leading to formation of GluA2-lacking AMPARs (Figure 1d–f). We confirmed the data obtained by western blot analysis with immunofluorescence technique. Figure 2a shows that GluA1 expression is significantly increased while GluA2 appears to be decreased (Figure 2b) in CA1 neurons after 24h of EtOH withdrawal compared to control slices. In order to elucidate the mechanisms of GluA2 lacking AMPA formation, we considered another type of evidence resulting from the analysis of mRNA expression levels. In particular, we used real-time PCR to study the transcriptional repressor element-1 (RE1) silencing transcription factor (REST) gene, which was demonstrated to repress the transcription of GluA2 AMPAR subunit (Noh et al., 2012). The results showed that EtOH withdrawal induces the up-regulation of REST gene, conversely GluA2 mRNA gene was down-regulated (Figure 3), indicating that GluA2-lacking AMPARs formation could be caused by a transcriptional process. Furthermore, in order to study the trafficking of AMPARs, we measured the expression levels of GluA1 and GluA2 in enriched postsynaptic membranes (PSD, Figure S1) under control, chronic EtOH or EtOH withdrawal (Figure 4). In contrast with the previously shown increase in GluA1 subunit in total homogenates, these data showed that EtOH down-regulates the expression of both GluA1 and GluA2 AMPA subunits in PSD, suggesting that AMPARs should be relocated in the intracellular and/or extrasynaptic membrane compartments after EtOH treatment. Since cadherin adhesion complex proteins have been recently shown to contribute to the stabilization of GluA1/2-containing AMPARs into the synaptic membranes (Mameli et al., 2011; Mills et al., 2017), we analysed the levels of the scaffold proteins SAP97, GRIP and of N-cadherins in total homogenate and PSD preparations of organotypic slices under control, chronic EtOH or EtOH-withdrawal conditions (Figure 5). Our results showed that in total homogenate there is no modification of the expression level of the proteins examined in any experimental conditions. On the contrary, a clear reduction in their expression was present in PSD. Collectively, these results indicate that chronic EtOH induces profound alterations in the molecular composition and surface expression of AMPARs. We then sought evidence that EtOH-induced molecular rearrangements result in increased calcium conductance through GluA2-lacking AMPARs. To directly test this hypothesis, we simultaneously measured electrophysiological and fluorescence calcium responses induced by AMPA stimulation in organotypic hippocampal slices during EtOH-withdrawal and in control conditions. These experiments were performed during early withdrawal (4–6 hr), when neurons are still viable and amenable to functional investigation. CA1 pyramidal neurons

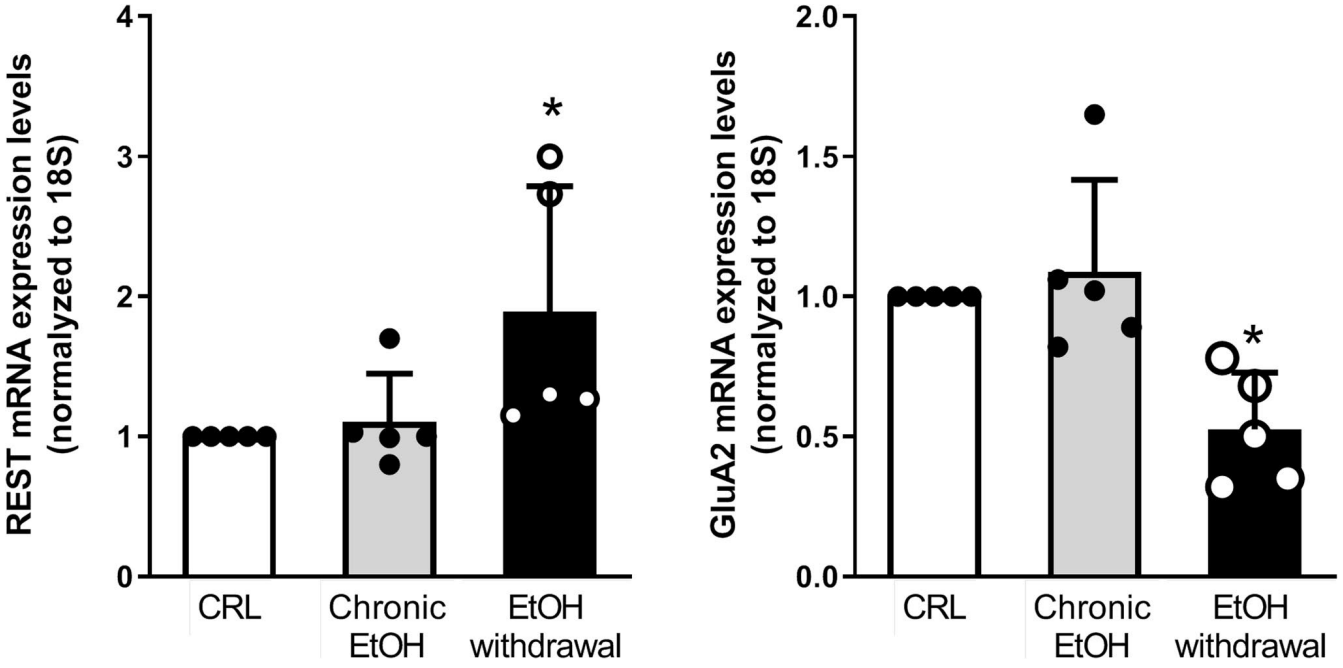

FIGURE 3 Ethanol withdrawal induces the up-regulation of the transcriptional repressor element-1 (RE1) silencing transcription factor (REST). Real-time PCR assessed mRNA expression levels of REST and of the AMPA subunit GluA2 gene after 24h of EtOH withdrawal. Data are expressed as percentage of control protein levels (white column). Bars represent the mean ± *SEM*of four experiments from independent cell preparations (about ≥ 4 slices for each experimental point). *$p < .05$ versus. CRL (ANOVA + Tukey's w test)

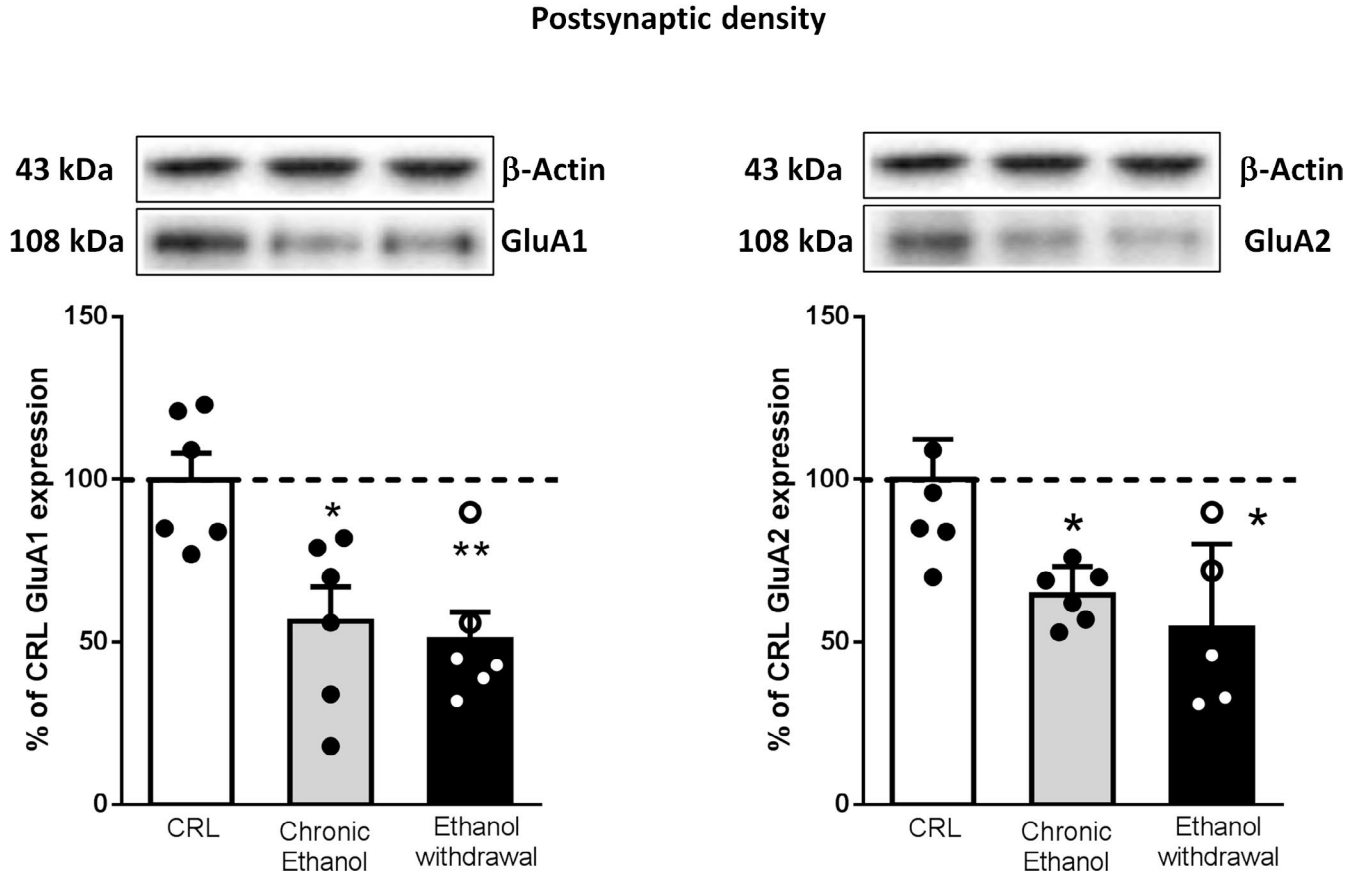

FIGURE 4 EtOH induces a significant reduction in GluA1 and GluA2 AMPA receptor subunit expression in the postsynaptic densities (PSD). The experiments were conducted as described in Figure 1. Representative immunoblots showing that the main AMPA receptor subunits GluA1 (left panel) and GluA2 (right panel) are significantly decreased after chronic EtOH and EtOH-withdrawal treatment in the PSD of organotypic hippocampal slices. Data are expressed as percentage of control protein levels (white column). Bar represent the mean ± *SEM* of six experiments from independent cell preparations (about ≥ 12 slices for each experimental point). $^{**}p < .01$ and $^{*}p < .05$ versus. CRL (ANOVA + Tukey's w test)

were individually loaded with the non-ratiometric calcium dye Fluo4 and then AMPA-mediated electrical and fluorescence responses were elicited by agonist application. Our results show that AMPA reliably induces an inward cationic current in both controls and EtOH-treated slices. Moreover, both peak value and area under the curve (AUC) of AMPA-induced inward currents are significantly increased in CA1 neuron after EtOH withdrawal compared to control slices (Figure 6). These findings confirm that the EtOH-dependent rearrangements in expression and localization of AMPARs that we observed lead to functional changes. Of note, the increase in AMPA current is reflective of an actual increase in current density, since no differences in whole-cell capacitance (a proxy for soma size) were observed between the two conditions (Figure S5). AMPA application also induced detectable elevations in intracellular calcium in both experimental groups but, quite unexpectedly, the magnitude of AMPA-induced calcium transients was significantly smaller in CA1 neurons from EtOH-treated slices compared to controls (Figure 6d). Our data suggest that GluA2-lacking AMPARs may lead to excitotoxicity and neuronal death by driving network hyperexcitability rather than by a direct contribution of GluA2-lacking AMPARs-mediated calcium influx. To selectively assess the contribution of GluA2-lacking AMPARs in EtOH-induced hyperexcitation, we tested the neuroprotective efficacy of 1-naphthyl acetyl spermine (NASPM), a selective blocker of GluA2-lacking AMPARs, to protect CA1 neurons from EtOH-withdrawal toxicity, compared to NBQX (a non-selective AMPA antagonist). Our data showed that incubation of NASPM (10 μM) during the 24h of EtOH withdrawal significantly attenuated EtOH withdrawal–induced injury as well as NBQX, suggesting that the neurotoxicity induced by EtOH withdrawal is largely mediated by GluA2-lacking AMPARs (Figure 7).

4 | DISCUSSION

We have recently demonstrated that EtOH withdrawal induces CA1 pyramidal cell death in organotypic hippocampal slices. In our experimental condition, EtOH neurotoxicity was largely prevented by the non-selective AMPAR blocker NBQX and preceded by an increase in the amplitude of spontaneous EPSCs and in GluA1/GluA2 expression ratio, thus, pointing to GluA2-lacking AMPARs and to AMPAR-mediated calcium currents, as a possible death mechanism involved in EtOH toxicity (Gerace et al., 2019). Building on this hypothesis, we investigated the implication of AMPARs and, in particular of

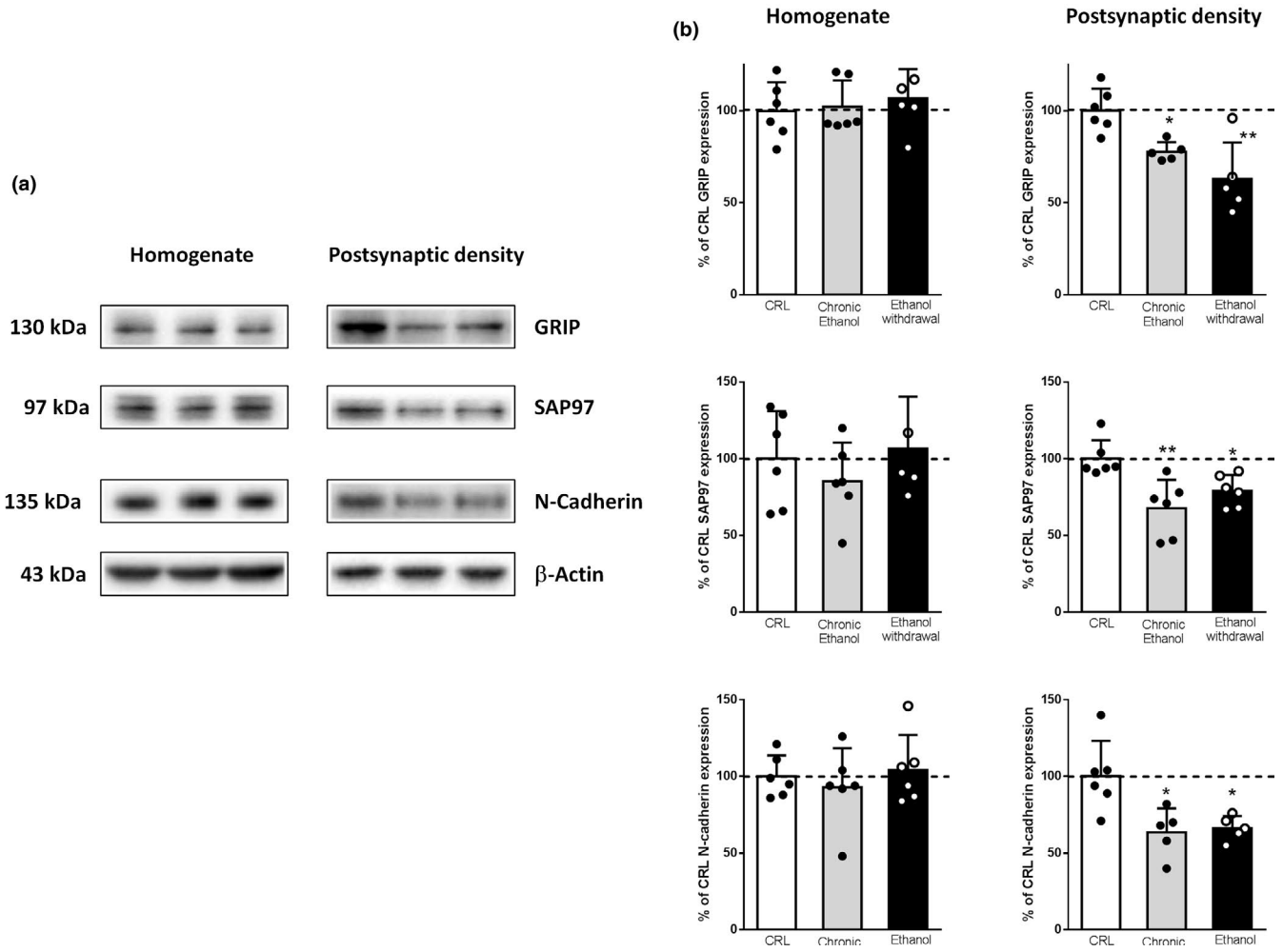

FIGURE 5 Effects of EtOH withdrawal on glutamate receptor-interacting protein (GRIP), synapse-associated protein 97 (SAP97) and N-cadherin expression in the whole homogenate and post-synaptic densities (PSD). (a) Representative immunoblots showing that GRIP, SAP97 and N-cadherin proteins expression are significantly decreased after chronic EtOH and EtOH-withdrawal treatment in the PSD, but not in the homogenate of organotypic hippocampal slices. Panel (b) shows the protein levels of GRIP1 (upper panels), SAP97 (middle panels) and N-cadherin (lower panel). Data are expressed as percentage of control protein levels (white column). Bar represent the mean ± *SEM*of six experiments from independent cell preparations (about ≥12 slices for each experimental point). $^{**}p < .01$ and $^{*}p < .05$ versus. CRL (ANOVA + Tukey's w test)

GluA2-lacking AMPARs, in EtOH toxicity by using biochemistry, molecular biology, microscopy and physiology. We first examined the expression levels of the main AMPAR subunits (GluA1, GluA2, GluA3) in the total homogenate of hippocampal slices chronically exposed to EtOH for 7 days followed by 24-hr withdrawal. We found that EtOH withdrawal leads to a significant increase in overall GluA1, but not GluA2 and GluA3, expression, thus, pointing to an increase in the number of functional AMPARs (Figure 1b). Additionally, the significant increase in GluA1/GluA2 ratio suggests the molecular switch to GluA2-lacking AMPARs (Figure 1e), in line with previous works (Acosta et al., 2012; Jin et al., 2014; Li et al., 2017). Remarkably, the switch to GluA2-lacking AMPARs has been implicated in the alterations associated with EtOH consumption, such as behavioural reinforcement (Mameli et al., 2011; Marty & Spigelman, 2012). In addition, it has been reported that repeated systemic administration of EtOH causes facilitation of LTP that is mediated by the insertion of AMPAR into the synaptic membrane and in a long-lasting increase in the GluA1 and GluA2 AMPAR subunits expression in the dorsomedial striatum of rats (Wang et al., 2012).

In the attempt to reveal the mechanism underlying the formation of GluA2-lacking AMPARs, as a consequence of EtOH withdrawal, we examined the mRNA expression of REST gene, one of the repressors involved in the regulation of the AMPAR subunit GluA2 (Noh et al., 2012). The activation of REST is clinically relevant in a model of ischemic stroke in vivo where it was reported to bind a subset of transcriptionally responsive genes, including GluA2 (Noh et al., 2012). Consistently, we found that REST gene expression is significantly up-regulated as a consequence of withdrawal and, at the same time, GluA2 gene expression is significantly reduced.

In order to get further insights into the modulation of AMPAR expression and function, we investigated the proteins regulating the trafficking and localization of these receptors to the post-synapse.

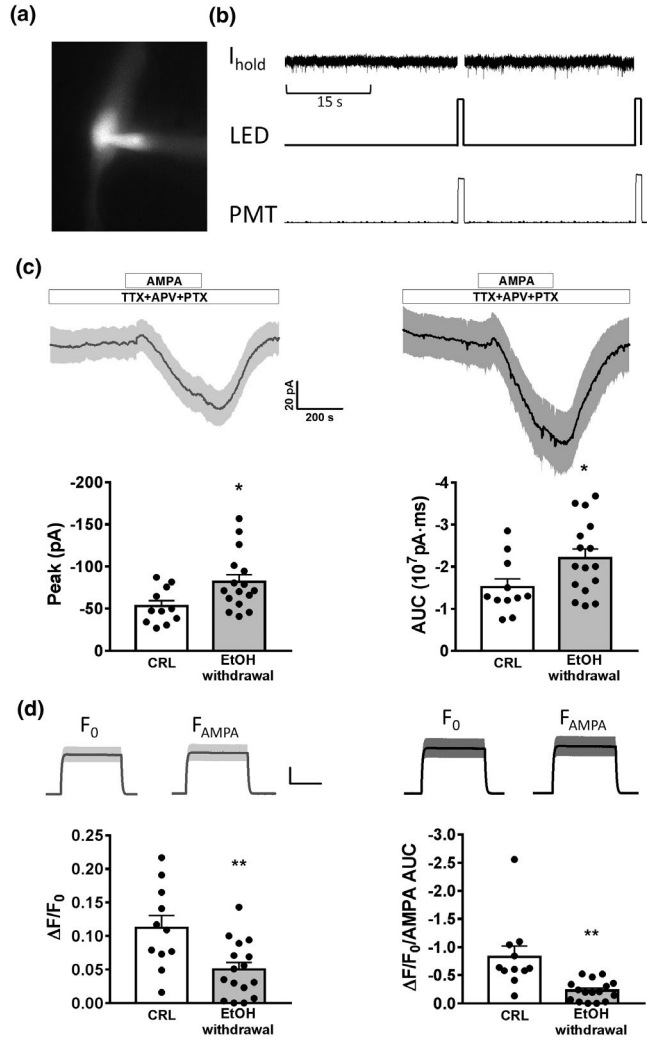

FIGURE 6 Electrophysiological and fluorescence calcium responses induced by AMPA stimulation in organotypic hippocampal slices. (a) CA1 pyramidal cell filled with Fluo-4 excited with blue LED light. (b) Experimental paradigm.*Top*: Electrophysiological protocol consisted of 30×, 30-s gap-free recordings.*Middle and bottom*: Optical protocol consisted of 1-s LED pulse and fluorescence collection with PMT at the end of each 30-s gap-free recording. (c)*Top*: time course of isolated AMPA current reported as average of "n" superimposed recordings ± *SEM*(gray shading) from CRL (left,$n = 11$) and EtOH-withdrawal group (right,$n = 16$).*Bottom*: quantification of AMPA-mediated current expressed as peak amplitude (*left*; CRL: −53.11 ± 6.38 pA,$n = 11$; EtOH withdrawal: −81.82 ± 8.58 pA,$n = 16$) and AUC (*right*; CRL: −1.51 ± 0.20 107pA·ms,$n = 11$; EtOH withdrawal: −2.20 ± 0.22 107pA·ms,$n = 16$). (d)*Top*: averaged fluorescence responses to pulsed LED excitation before (F0) and after (F_{AMPA}) agonist application, from CRL (left,$n = 10$) and EtOH-withdrawal group (*right*,$n = 14$). Scale bar,$x = 500$ ms,$y = 0.2$ ΔF/F0.*Bottom*: quantification of absolute ΔF/F0 (*left*, CRL: 0.11 ± 0.02,$n = 11$; EtOH withdrawal: 0.05 ± 0.01,$n = 16$) and ΔF/F_0normalized to total AMPA current (*right*, CRL: −0.83 ± 0.19,$n = 11$; EtOH withdrawal: −0.23 ± 0.04,$n = 16$). All histograms report data as mean values ± *SEM*versus. CRL group. *$p < .05$ and **$p < .01$ versus. CRL group (Student's*t*-test)

To this end, we examined the levels of GluA1 and GluA2, as well as scaffold proteins SAP97, GRIP, N-cadherin and beta catenin in total homogenate and PSD-enriched fractions from organotypic slices in control, chronic EtOH or EtOH withdrawal. We observed a clear reduction in the expression of all the proteins analysed (GRIP, SAP97, N-cadherin) in the PSD preparation. Together with the previously shown increase in GluA1 subunit in total homogenates, this result suggests a re-localization of AMPARs to extrasynaptic compartments. The extrasynaptic distribution of AMPARs have particularly relevant consequences for synapse function and the lateral distribution of AMPARs within the PSD provides an effective mechanism for modulating synaptic strength (MacGillavry et al., 2011; Newpher & Ehlers, 2008). For example, hippocampal neurons exposed to TNFα treatment display an increased surface level of AMPARs after 15 min, the majority of which are relocalized to extrasynaptic sites. This mechanism has been proposed to contribute to excitotoxic neuronal death and is prevented by GluA2-lacking AMPAR antagonist NASPM (Leonoudakis et al., 2008), suggesting a role for AMPAR-mediated calcium in neurotoxicity. Functional determination of AMPA-induced calcium influx and whole-cell current during early EtOH withdrawal confirms that increased expression and subunit remodelling of AMPARs involve deep and complex changes at functional level. The most striking finding was that AMPA-induced, AMPAR-mediated calcium entry was reduced in CA1 neurons of EtOH-treated slices during early withdrawal. This result, which came unexpected, based on biochemical data, seems to exclude a direct involvement of AMPAR-mediated calcium inflow in EtOH-withdrawal neurotoxicity. The paradoxical reduction in AMPA-induced calcium influx in presence of increased GluA2-lacking AMPAR levels is possibly caused by post-translational modulation (Ivanova et al., 2020), and requires further investigation which goes beyond the scope of the present study. In contrast, we found that AMPA-induced whole-cell currents in CA1 neurons subject to EtOH withdrawal were enhanced. This is consistent with the increased GluA1 levels and EPSCs amplitude that we previously reported in this model following EtOH withdrawal (Gerace et al., 2019). Furthermore, early single-channel recordings reported larger unitary conductance of GluA2-lacking AMPARs in heterologous expression systems (Swanson et al., 1997).

In light of these results, we propose increased network excitability, promoted by simultaneous increase in AMPAR function and removal of EtOH-mediated potentiation of inhibition, as the main mechanism of EtOH excitotoxicity, in agreement with the protective effect exerted by AMPAR blockers. In this regard, the evidence that the non-selective blocker NBQX and the selective GluA2-lacking AMPAR blocker NASPM show equal neuroprotective action suggests that, at molecular level, GluA2-lacking AMPARs are the main molecular entities accounting for the increased AMPA-induced current and thereby potentially represent a specific target for the development of neuroprotectants against the neurotoxic effects of EtOH withdrawal.

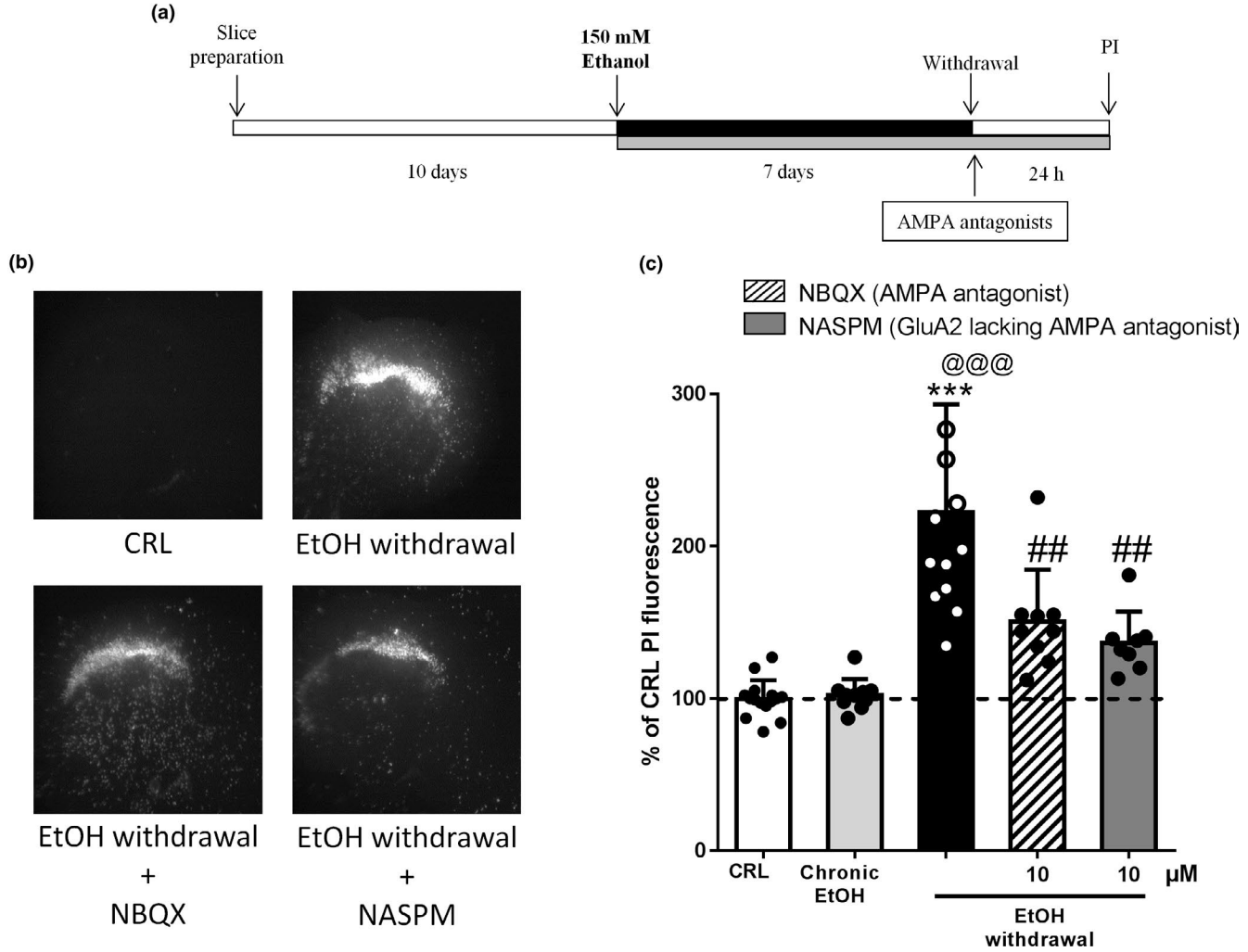

FIGURE 7 Neuroprotective effects of the selective GluA2-lacking α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) antagonist 1-naphthyl acetyl spermine (NASPM) on EtOH-withdrawal toxicity. (a) Experimental protocol. (b) Hippocampal slices displaying background levels of propidium iodide (PI) fluorescence under control conditions, an intense PI labelling in the CA1 subregion 24 hr after EtOH withdrawal and a reduction in CA1 PI fluorescence when incubated with non-selective AMPA antagonist 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[*f*]quinoxaline-7-sulfonamide disodium salt (NBQX) and the selective blocker of GluA2-lacking AMPARs NASPM. (c) Quantitative analysis of CA1 region expressed as percentage of CRL PI fluorescence. Values represent the mean ± *SEM*of at least five experiments from independent cell preparations (about ≥ 8 slices for each experimental point). $^{***}p < .001$ versus. CRL Chronic EtOH, $^{\#\#}p < .01$ AMPA antagonists versus. CRL and Chronic EtOH and $^{@@@}p < .001$ versus. EtOH withdrawal alone (ANOVA þ Tukey's w test)

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All experiments were conducted in compliance with the ARRIVE guidelines.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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Neurotoxicity of Unconjugated Bilirubin in Mature and Immature Rat Organotypic Hippocampal Slice Cultures

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Keywords
Bilirubin · Neurotoxicity · Albumin · Neuroprotection · Brain · Organotypic slice

Abstract
Background: The physiopathology of bilirubin-induced neurological disorders is not completely understood. ***Objectives:*** The aim of our study was to assess the effect on bilirubin neurotoxicity of the maturity or immaturity of exposed cells, the influence of different unconjugated bilirubin (UCB) and human serum albumin (HSA) concentrations, and time of UCB exposure. ***Methods:*** Organotypic hippocampal slices were exposed for 48 h to different UCB and HSA concentrations after 14 (mature) or 7 (immature) days of in vitro culture. Immature slices were also exposed to UCB and HSA for 72 h. The different effects of exposure time to UCB on neurons and astrocytes were evaluated. ***Results:*** We found that 48 h of UCB exposure was neurotoxic for mature rat organotypic hippocampal slices while 72 h of exposure was neurotoxic for immature slices. Forty-eight-hour UCB exposure was toxic for astrocytes but not for neurons, while 72-h exposure was toxic for both astrocytes and neurons. HSA prevented UCB toxicity when the UCB:HSA molar ratio was ≤1 in both mature and immature slices. ***Conclusions:*** We confirmed UCB neurotoxicity in mature and immature rat hippocampal slices, although immature ones were more resistant. HSA was effective in preventing UCB neurotoxicity in both mature and immature rat hippocampal slices.

Introduction

Hyperbilirubinemia is the most frequent clinical problem that neonatologists must deal with during the newborn period because under certain circumstances high levels of bilirubin may be potentially toxic to the central nervous system, both in term and preterm infants. In fact, sufficiently elevated levels of bilirubin can lead to acute bilirubin encephalopathy (ABE), bilirubin-induced neurological disorders (BINDs), and subsequently kernicterus, with devastating, permanent neurodevelopmental handicaps or death [1]. Therefore, recommendations

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have been developed to prevent ABE and BINDs by establishing levels of total serum bilirubin (TSB) to indicate risk of neurological damage at which to start treatment with phototherapy, the first-line treatment for neonatal hyperbilirubinemia and, eventually, with intravenous immunoglobulin or exchange transfusions [2].

Jaundice is considered physiologic when TSB level ranges from 5–6 to 7–17 mg/dL in term infants, whereas when TSB is >18 mg/dL, jaundice is considered frankly pathologic [3]. A TSB of 20 mg/dL should not be exceeded in the first 96 h of life in term newborns. After this period, a TSB of 25 mg/dL should be avoided since the efficacy of the blood-brain barrier increases with postnatal age [2]. The safe bilirubin level is lower for preterm infants: 12 mg/dL for infants with a gestational age of ≤30 weeks and 15 mg/dL for infants with a gestational age of 31–36 weeks [4, 5]. However, to establish individual safe TSB levels remains a big challenge and reported numbers have limited evidence base.

The pathophysiology of ABE and BINDs has been investigated in several studies. At autopsy of kernicteric infants, bilirubin is macroscopically visible within the globus pallidus, hippocampus, lateral ventricular walls, cerebellum, and subthalamic nuclei, reflecting its preferential deposition in specific brain areas [6, 7]. However, other evidence has shown that bilirubin can also accumulate in other brain areas, such as the cortex, striatum, midbrain, hypothalamus, cerebellum, and medulla [8]. Bilirubin neurotoxicity occurs by multiple mechanisms including cell membrane perturbation, DNA damage, changes in synaptic transmission, increase in cytokine release, inhibition of neurotrophin-mediated protective signals, and induction of cell apoptosis and necrosis [8].

However, some potential determinants of bilirubin neurotoxicity are particularly interesting from a clinical point of view, such as the maturity or immaturity of exposed cells, the influence of different unconjugated bilirubin (UCB) and human serum albumin (HSA) concentrations, and time of UCB exposure. Thus, we hypothesized that: mature cells might be less susceptible to bilirubin neurotoxicity than immature cells; increasing HSA concentration might decrease bilirubin neurotoxicity; and prolonged time of UCB exposure might increase bilirubin neurotoxicity. To assess these hypotheses, we carried out this in vitro study in rat organotypic hippocampal slice cultures, since brain slice models offer unique advantages over other in vitro platforms in that interactions between neurons or between neurons and glial cells are fundamentally preserved replicating many aspects of the in vivo context [7].

Table 1. Concentrations of unconjugated bilirubin (UCB) and human serum albumin (HSA) in culture media of organotypic hippocampal slices used for experiments (expressed as μM/L, molar ratio, mg/dL) and related calculated unbound bilirubin (nM/L)

UCB/HSA, μM/L/μM/L	UCB/HSA ratio	UCB/HSA, mg/dL/mg/dL	Calculated unbound bilirubin, nM/L
10/100	0.1	0.5850/0.665	6
100/10	10	5.850/0.067	1,140
100/100	1	5.850/0.066	215
100/200	0.5	5.850/1.329	160
200/100	2	11.700/0.665	10,074
200/200	1	11.700/1.329	288

Methods

Organotypic hippocampal slice cultures from the brains of 7- to 9-day-old Wistar rat pups (Harlan, Milan, Italy) were prepared as previously reported [9]. All experiments were performed in primary slice cultures obtained from different litters. Before the experiments, all slices were screened for viability by incubating them for 30 min with propidium iodide (PI: 5 μg/mL); slices displaying signs of neurodegeneration were discarded from the study. All the experiments with UCB were performed under light protection to avoid photodegradation.

We decided to study mature and immature organotypic hippocampal slices to mimic what may occur in term and preterm infants. According to recent studies, the first postnatal week in the rat corresponds to 19–28 weeks of gestation in humans [10]. Thus, we used hippocampal slices after 14 days of culture in vitro, considering them as mature slices, and hippocampal slices after 7 days of culture in vitro considering them as immature slices.

The first set of experiments regarded mature cells. Rat organotypic hippocampal slices were exposed for 48 h to 10–200 μM (0.585–11.700 mg/dL) of UCB (Sigma, St. Louis, MO, USA) and 10–200 μM of HSA (0.067–1.329 g/dL) without changing the medium to assess its possible neuroprotective effect (Table 1).

Under our experimental conditions, the theoretically calculated unbound fraction of UCB concentrations ranged from 6 (UCB:HSA = 0.1) to 1,140 nM (UCB:HSA = 10.0), and was 215 and 288 nM, respectively, at 100 and 200 equimolar concentrations of UCB and HSA, according to the model: $K'_f = B_t - B_f / B_f$ (HSA – $B_t + B_f$), where K'_f is the binding constant of albumin; B_t is the total bilirubin concentration; and B_f is the free bilirubin concentration, as proposed by Weisiger et al. [11] and Ostrow et al. [12]. Thus, our model generally reproduced concentrations of unbound bilirubin which are associated with the development of kernicterus in preterm (15–34 nM) and in term (>68 nM) infants [13].

The effects of glutamate receptor activation as mediators of bilirubin toxicity were evaluated by exposing mature hippocampal slices to 100 μM of UCB alone or plus HSA (100 μM) or AMPA antagonist NBQX (10 μM) or NMDA antagonist MK-801 (10 μM) or mGluR5 antagonist MPEP (30 μM) for 48 h; they were then assessed for neuronal injury using PI fluorescence.

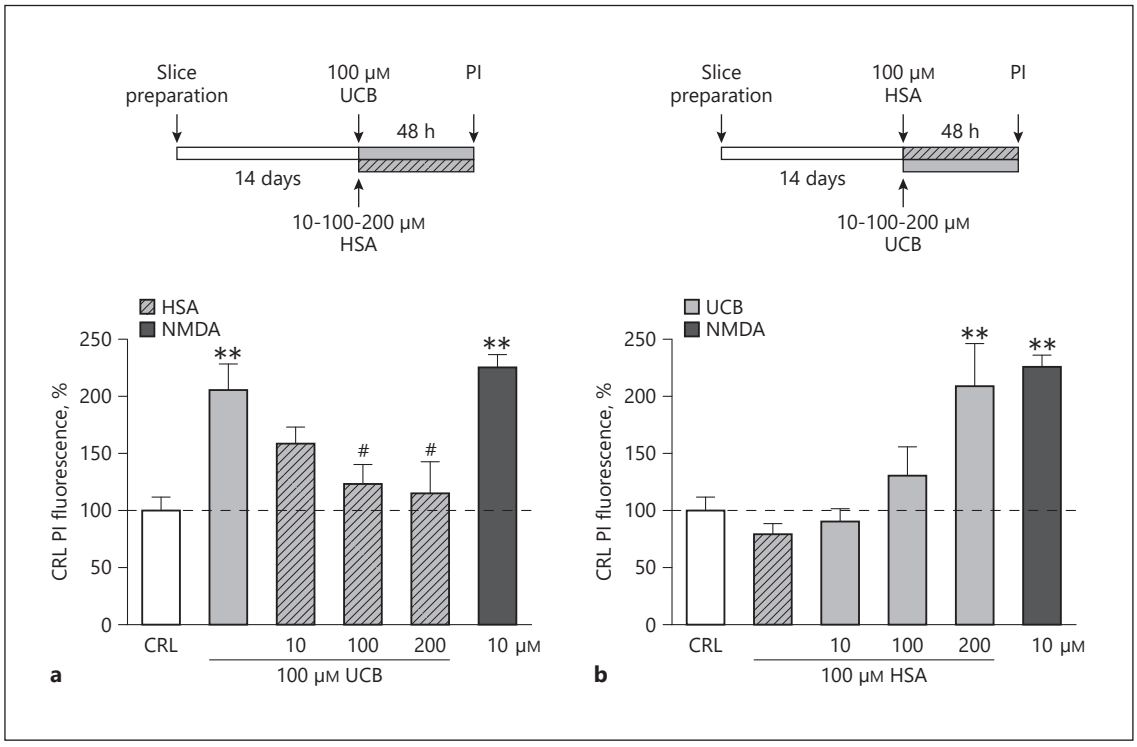

Fig. 1. Neuroprotective effects of human serum albumin (HSA) on unconjugated bilirubin (UCB)-induced toxicity in mature organotypic hippocampal slices. **a** Experimental protocol showing hippocampal slices cultured for 14 days (mature) and then exposed to 100 μM of UCB alone or plus HSA (10–200 μM) for 48 h. Quantitative analysis shows that HSA is neuroprotective against UCB toxicity at equimolar concentration. Quantitative analysis is expressed as percentage of control (CRL) PI fluorescence. Bars represent the mean ± SEM of at least 7 experiments (about ≥28 slices for each experimental point). ** $p < 0.01$ versus CRL, # $p < 0.05$ versus 100 μM UCB (ANOVA + Tukey's *w* test). **b** Experimental protocol showing hippocampal slices cultured for 14 days (mature) in vitro and then exposed to 100 μM of HSA alone or plus UCB (10–200 μM) for 48 h. Quantitative analysis shows that UCB is neurotoxic when present in concentration higher than HSA. Quantitative analysis is expressed as percentage of CRL PI fluorescence. Bars represent the mean ± SEM of at least 5 experiments (about ≥20 slices for each experimental point). ** $p < 0.01$ versus CRL (ANOVA + Tukey's *w* test).

The second set of experiments regarded immature cells. Rat organotypic hippocampal slices were exposed for 48 h to 1–450 μM of UCB after 7 days of in vitro culture without changing the medium. Neuroprotective effects of HSA and effects of glutamate receptor activation were not measured since UCB did not injure immature cell slices. However, to explore other less evident injuries, further experiments were performed only in hippocampal slices of immature cells. Thus, we investigated effects of UCB exposure in promoting cell apoptosis by incubating hippocampal slices for 48 h with 1–300 μM of UCB to measure expression of BAD, cleaved PARP-1, and AIF apoptotic proteins by Western blot analysis. Possible different effects of UCB on neurons and astrocytes were evaluated in CA1 pyramidal stratum in immature organotypic hippocampal slices exposed to 100 μM UCB for 48 h using immunostaining methods and confocal microscopy for quantitative analysis of regions of interest. Electron microscopic evidence for intraneuronal effects of UCB were assessed in CA1 pyramidal cells after incubation for 48 h with 100 μM of UCB. Moreover, further experiments assessed the effect on neurotoxicity of chronic (72 h) exposure to UCB of immature cells to better mimic what occurs in clinical practice. In these experiments, slices were exposed for 72 h to 100 μM UCB and the possible protective effect of 100 μM HSA was evaluated, with or without daily change of culture medium. Effects of chronic exposure to UCB on neurons and astrocytes was evaluated in CA1 pyramidal stratum in immature organotypic hippocampal slices using immunostaining methods and confocal microscopy for quantitative analysis of regions of interest. Slices were exposed for 72 h to 100 μM UCB with or without daily change of culture medium, alone or plus HSA (100 μM). The possible role of glutamate transporters in chronic UCB neurotoxicity was evaluated measuring mRNA expression of glutamate transporter GLAST, Glt1, EAAC1, vGluT1, and vGluT2 genes using the real-time polymerase chain reaction (PCR) and specific primers (online suppl. Table S1; for all online suppl. material, see www.karger.com/doi/10.1159/000494101).

Neuronal cell viability injury was evaluated in CA1 pyramidal cells using the PI method (5 μg/mL; Sigma, St Louis, MO, USA).

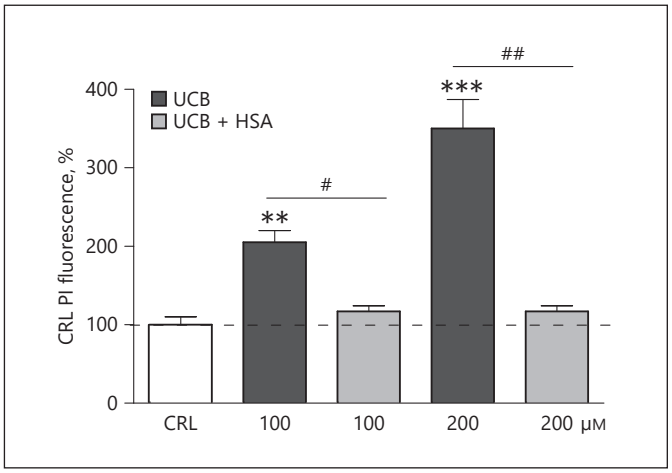

Fig. 2. Human serum albumin (HSA) is neuroprotective against unconjugated bilirubin (UCB)-induced toxicity at equimolar concentration. Mature slices were exposed to equimolar concentration (200 μM) of UCB plus HSA for 48 h and then incubated with propidium iodide (PI) to assess neuronal injury. Quantitative analysis shows that HSA is neuroprotective against UCB toxicity when present at equimolar concentration. Quantitative analysis is expressed as a percentage of CRL PI fluorescence. Bars represent the mean ± SEM of at least 6 experiments (about ≥24 slices for each experimental point). ** $p < 0.01$ versus CRL, *** $p < 0.001$ versus CRL, # $p < 0.05$ versus 100 μM UCB, ## $p < 0.01$ versus 200 μM UCB (ANOVA + Tukey's *w* test).

Statistical Analysis

Data are presented as means ± SEM of *n* experiments. In toxicity, Western blot, and immunofluorescent experiments, each experimental point consisted of 8 hippocampal slices. Statistical significance of differences between PI fluorescence intensities or Western blot optical densities was evaluated by performing one-way analysis of variance (ANOVA) followed by Dunnett's and Tukey's *w* test for multiple comparisons. Statistical significance of differences between immunofluorescence was evaluated by performing ANOVA and Newman-Keuls multiple comparison test. All statistical calculations were performed using GraphPad Prism v.5 for Windows (GraphPad Software, San Diego, CA, USA). A probability value (p) of <0.05 was considered statistically significant.

Results

Mature organotypic hippocampal slices were exposed to increasing concentrations of UCB (10–200 μM) and HSA (10–200 μM) for 48 h to resemble clinical TSB and unbound bilirubin in term and preterm infants (Table 1). We observed that UCB induces a selective CA1 pyramidal cell injury when the UCB/HSA ratio is at >1:1 (UCB/HSA 200/100 μM), whilst it is not neurotoxic when UCB and HSA were equimolar both at 100 (Fig. 1) and 200 μM (Fig. 2). Among glutamate receptor antagonists, we found that NBQX (10 μM) and MPEP (30 μM), but not MK-801 (10 μM), are neuroprotective against 100 μM UCB. This protective effect was similar to that induced by 100 μM HSA (see online suppl. Fig. S1).

Immature organotypic hippocampal slices were exposed to increasing concentrations of UCB (1–450 μM) for 48 h. Toxic effects were not found with the PI method (Fig. 3) nor with immunofluorescence. Moreover, Western blotting experiments demonstrated that UCB (100 μM for 48 h) does not affect the expression levels of the apoptotic proteins BAD, cleaved PARP-1, and AIF (see online suppl. Fig. S2), suggesting that UCB activated neither the necrotic nor apoptotic pathways in neurons. Immunostaining methods and confocal microscopy confirmed that UCB (100 μM, 48 h) is not toxic for immature neurons but evidenced a toxic effect on astrocytes (Fig. 4). However, electron microscopic evidence for intraneuronal effects of UCB reveals that CA1 pyramidal cells exposed to 100 μM UCB for 48 h displayed abnormal swollen mitochondria, smaller Golgi apparatus, swollen dendrites, and reduction of synapses, some of which are depleted of synaptic vesicles, in comparison to healthy CA1 pyramidal cells (see online suppl. Fig. S3). The chronic exposure (72 h) to 100 μM UCB of immature organotypic hippocampal slices was found neurotoxic when a change of culture medium and UCB addition were performed every day. This detrimental effect was prevented by an equimolar amount of HSA (100 μM) (Fig. 5). Chronic exposure to UCB was toxic for both neurons and astrocytes (Fig. 6). Indeed, we performed real-time PCR by measuring the levels of mRNA expression of glutamate transporter GLAST, Glt1, EAAC1, vGluT1, and vGluT2 and we found that 100 μM UCB chronic exposure induces a significant decrease in some important genes involved in gliotransmission (see online suppl. Fig. S4) confirming the detrimental effect of UCB on astroglial cells.

Discussion

In this study, we evaluated for the first time the susceptibility of mature and immature rat organotypic hippocampal slices to UCB neurotoxicity, the possible neuroprotective effects against UCB of HSA, and the effect of UCB exposure duration on its neurotoxicity in immature slices. We found that 48-h exposure to 100–200 μM UCB is neurotoxic for mature rat organotypic hippocampal slices when the UCB:HSA molar ratio is >1, while HSA is

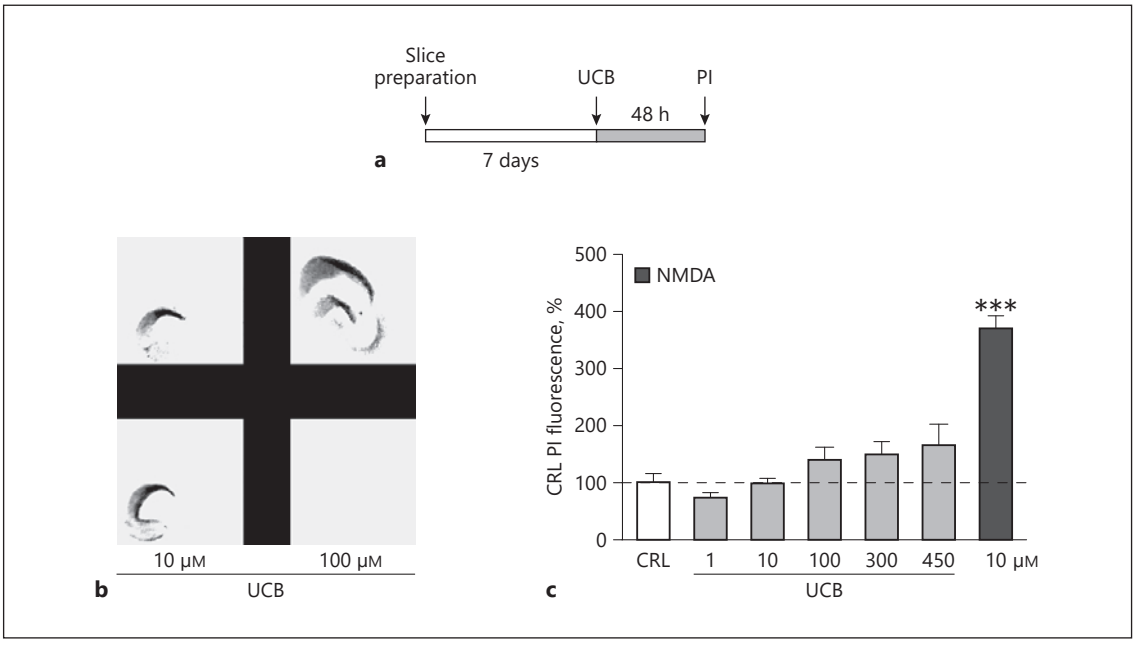

Fig. 3. Unconjugated bilirubin (UCB) does not induce damage in immature organotypic hippocampal slices. **a** Experimental protocol showing hippocampal slices cultured for 7 days (immature) and then exposed to 1–450 μM of UCB for 48 h. At the end of this period, the fluorescent dye propidium iodide (PI) was added to the medium to assess neuronal injury. **b** Qualitative analysis shows that UCB does not induce toxicity. **c** Quantitative analysis is expressed as a percentage of control (CRL) PI fluorescence. Bars represent the mean ± SEM of at least 5 experiments (about ≥20 slices for each experimental point). *** $p < 0.001$ versus CRL (ANOVA + Tukey's *w* test).

protective when the UCB:HSA molar ratio is ≤1. This toxicity was abolished by some glutamate receptor antagonists, such as NBQX and MPEP. We then demonstrated that 48-h exposure to 1–450 μM UCB does not induce necrosis or apoptosis in immature rat hippocampal slices. We observed that 48-h exposure to 100 μM UCB is not toxic for neurons but is toxic for astrocytes, although electron microscopic analysis showed that 48-h exposure to 100 μM UCB induced pathologic changes in CA1 pyramidal cells. However, a chronic exposure (72 h) to 100 μM UCB was toxic for both neurons and astrocytes and this toxicity is abolished by an equimolar amount of HSA (100 μM). We found that UCB chronic toxicity involves glutamate transporter GLAST, Glt1, EAAC1, vGluT1, and vGluT2, since their mRNA expression decreased.

Although the better potential of organotypic hippocampal slices compared to isolated cell cultures in investigating UCB-induced injuries has been well recognized [6], few studies were performed using this method [14–16], being mainly focused on UCB-induced modification of synaptic transmission. These studies are heterogeneous: donor rats were 6–8 days old [14], or 7–10 days old [15], or 2 and 8 days old [16]; rat hippocampal slices were maintained in vitro for 3 [14, 15] or 6 [16] days prior to use (to allow tissues to recover from experimental trauma caused by the isolation procedure) and, according to Bayer et al. [10], represent models of immature tissues; slices were exposed to 1–10 μM UCB plus 2–20 μM HSA (molar ratio 0.5) for 24 or 48 h [14], or 50 μM UCB plus 100 μM HSA (molar ratio 0.5) for 24 h [15], or to 70, 140, or 300 nM of unbound bilirubin for 24 h [16]. Therefore, our findings on the neurotoxic effect of UCB in mature slices when the UCB:HSA molar ratio is >1 cannot be compared to previous studies [14–16], because these had a different design and, mainly, were carried out in immature slices [10]. However, in one study, UCB did not affect cell viability [14], while in others it induced neuronal necrosis [15, 16].

We found that glutamate non-NMDA and metabotropic receptor antagonists, such as NBQX and MPEP, abolished UCB neurotoxicity in mature slices, and this result confirms the reported pivotal role of glutamate in the pathogenesis of bilirubin-induced cell damage [7, 8]. However, this did not occur with MK-801 glutamate NMDA receptor antagonist. This finding is in agreement

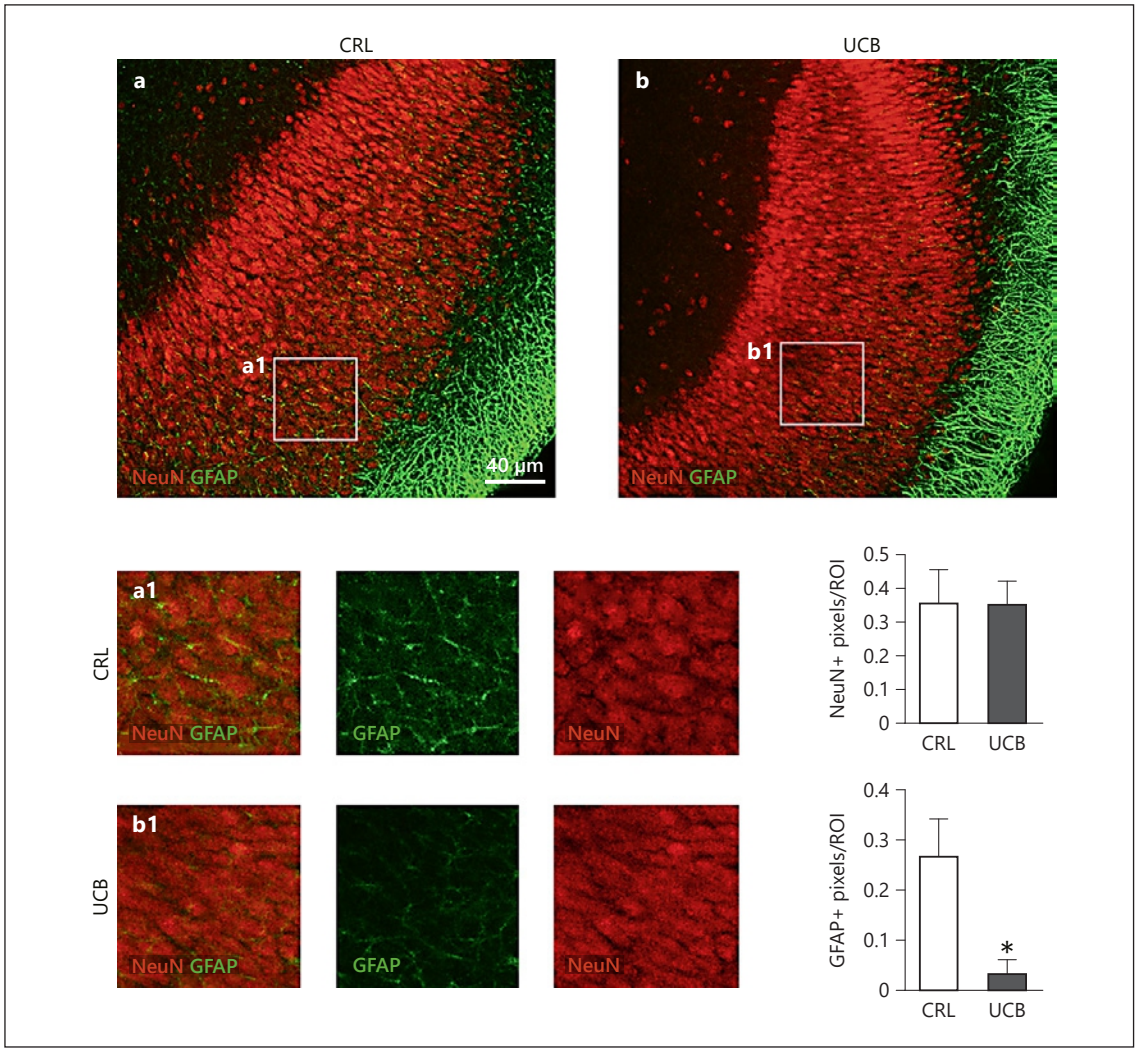

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Fig. 4. Effects of unconjugated bilirubin (UCB) on neurons and astrocytes in immature organotypic hippocampal slices immunostaining of neurons (NeuN, red), and astrocytes (GFAP, green) in CA1 stratum pyramidale of immature organotypic hippocampal slices after exposure to bilirubin (100 µM, 48 h). Representative confocal microscopy images showing CA1 NeuN- and GFAP-positive cells in the pyramidal cell layer of the three experimental groups. Scale bar: 40 µm. Quantitative analysis of NeuN-positive neurons (upper panel) and GFAP-positive astrocytes (lower panel) of control (CRL) (white column, $n = 12$), and bilirubin (black column, $n = 12$) slices (mean ± SEM; * $p < 0.01$ vs. CRL; one-way ANOVA and Newman-Keuls multiple comparison test).

with Shapiro et al. [17] who found in hippocampal neurons and in Gunn rat pups that bilirubin neurotoxicity is not mediated through NMDA receptor activation. On the other hand, our results are in disagreement with Hankø et al. [18] who demonstrated in NT2-N cells, a teratocarcinoma-derived cell line, that MK-801 can only delay UCB-induced cell death; and with Grojean et al. [19] who found in primary forebrain neuronal cultures that MK-801 is protective against bilirubin-induced apoptosis and necrosis. It is difficult to explain these different results, but one key difference between our study and that of Shapiro et al. [17] and those demonstrating neuroprotection with MK-801 [18, 19] is that the former were performed in neuronal slices or cultures from hippocampi, whereas the latter were performed in teratocarcinoma-derived cell line [18] or embryonic cortical neurons [19]. In fact, in human and rat studies of bilirubin encephalopathy, the hippocampus has been shown to be affected, along with brainstem and the basal ganglia, while no injury has been demonstrated in the cortex [6].

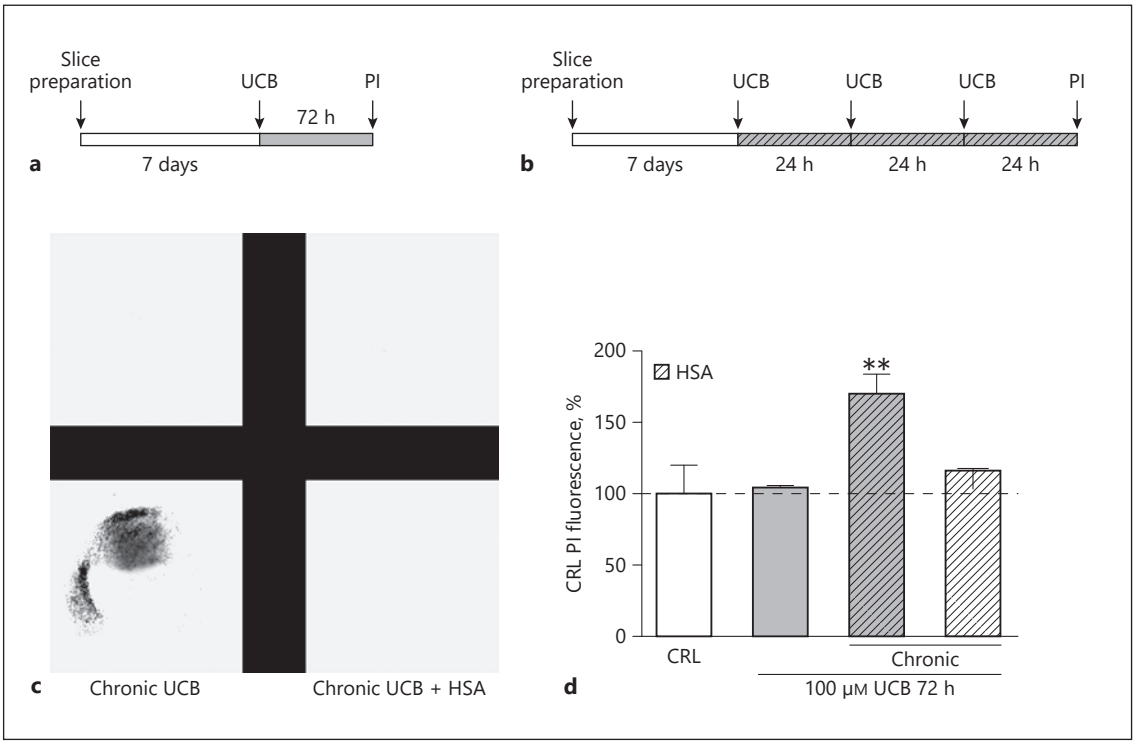

Fig. 5. The chronic application of UCB is neurotoxic in immature organotypic hippocampal slices. Experimental protocol showing immature organotypic hippocampal slices exposed to 100 μM UCB once (**a**) or exposed to a chronic application of UCB (72 h), changing the medium once a day with daily addition of 100 μM UCB, alone or plus human serum albumin (HSA: 100 μM) (**b**), and then incubated with propidium iodide (PI). **c** Qualitative analysis with observation under fluorescence optics to detect neuronal injury shows that chronic UCB exposure induces toxicity. **d** Quantitative analysis confirms UCB toxicity and shows HSA protective effect. Quantitative analysis is expressed as a percentage of control (CRL) PI fluorescence. Bars represent the mean ± SEM of at least 5 experiments (about ≥20 slices for each experimental point). ** $p <$ 0.01 versus CRL (white bar), single UCB exposure (gray bar), and plus HSA (white hutched bar) (ANOVA + Tukey's *w* test).

In our study, immature organotypic hippocampal slices were more resistant to UCB neurotoxicity than mature ones, as evidenced by the longer exposure to UCB (72 vs. 48 h) needed to induce cellular injuries. These results disagree with previous studies in cell cultures of neurons and astrocytes [20, 21], which report a higher vulnerability of immature than mature cells. However, previous studies used rat cortical astrocyte and neuron cultures, which were exposed to 50–100 μM of UCB for 4 h [20, 21], while we used organotypic hippocampal slices, which were exposed to 10–200 (mature slices) or 1–450 μM (immature) of UCB for 48 h. Thus, opposing results might depend on these different experimental conditions. On the other hand, some considerations support the result reliability of our model: brain lesions in kernicterus are usually in the hippocampus but not in cortex areas [6] since the hippocampus is the most vulnerable brain region to UCB injuries [22]; moreover, brain slice models represents a better in vitro model than cell cultures because they maintain interactions between neurons and glial cells [6] and mainly with microglia whose role in the physiopathology of UCB-induced neurotoxicity has been elucidated [15]. Moreover, our findings are in agreement with a recent study demonstrating a lower vulnerability of immature than mature hippocampal slices to noxious stimuli other than UCB (i.e., alcohol) [23]. However, it is important to note that an equimolar amount of HSA abolished UCB toxicity both in mature slices exposed for 48 h and in immature slices chronically exposed to UCB confirming the role of unbound bilirubin in the pathogenesis of kernicterus [13]. Electron microscopic analysis disclosed abnormal neuronal changes of mitochondria, Golgi apparatus, dendrites, and synapses after 48 h of UCB exposure of immature slices. We speculate that these anomalies probably prelude cellular death, as reported by many studies detailing neurotoxic effects of UCB [7, 8].

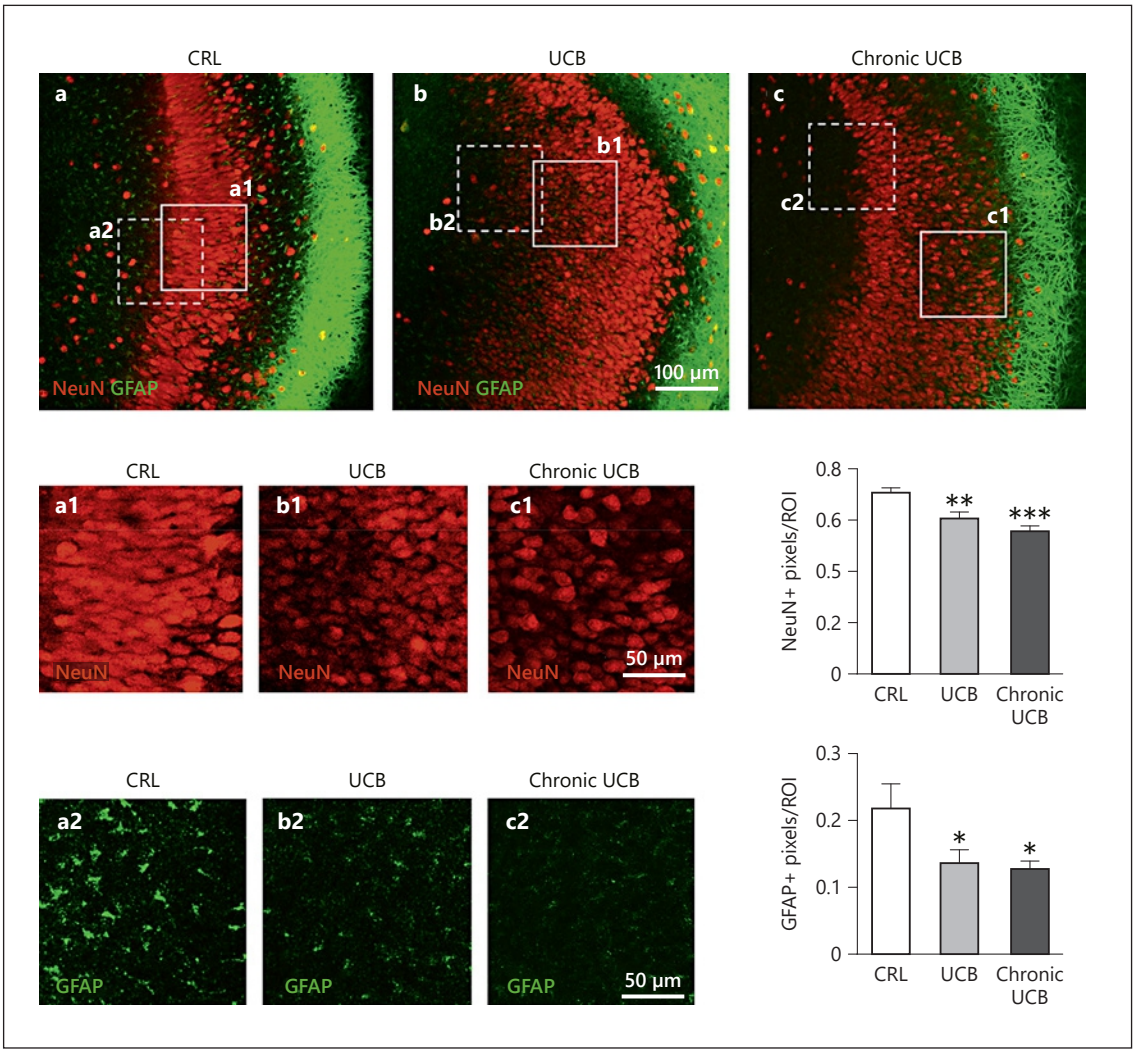

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Fig. 6. Effects of chronic UCB exposure on neurons and astrocytes in immature organotypic hippocampal slices. Immunostaining of neurons (NeuN, red), and astrocytes (GFAP, green) in CA1 stratum pyramidale of immature organotypic hippocampal slices after exposure to UCB (100 µM for 72 h). Representative confocal microscopy images showing CA1 NeuN- and GFAP-positive cells in the pyramidal cell layer of the three experimental groups. Scale bar: 40 µm. Quantitative analysis of NeuN-positive neurons (upper panel) and GFAP positive astrocytes (lower panel) of CRL (white column, $n = 12$), bilirubin (one exposition, gray column, n = 12) and chronic bilirubin (black column, $n = 12$) slices (mean ± SEM; * $p < 0.05$ vs. CRL, ** $p < 0.01$ vs. CRL slices, *** $p < 0.001$ vs. CRL; one-way ANOVA and Newman-Keuls multiple comparison test).

We observed that in immature hippocampal slices, 48 h of UCB exposure injured astrocytes but not neurons, while 72 h of UCB exposure injured both neurons and astrocytes. This observation supports previous studies in neuron and astrocyte cultures reporting that astrocytes were more vulnerable than neurons to UCB toxicity [20]. On the other hand, UCB exposure reduced mRNA expression of glutamate transporters in our immature hippocampal slices and this might favor cellular death by excitotoxicity prolonging cellular exposure to glutamate. This finding confirms previous studies demonstrating that UCB exposure induces excitotoxic cellular death in rat primary cortical neurons and astrocyte cultures [21, 24] and in 7-day-old Gunn rat pups [25].

In conclusion, we found that 48 h of UCB exposure was neurotoxic for mature rat organotypic hippocampal slices, while 72 h of UCB exposure was neurotoxic for immature rat hippocampal slices. However, HSA was able to prevent UCB toxicity when the UCB:HSA molar ratio was ≤1 in both mature and immature slices.

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Disclosure Statement

The authors declare no conflicts of interest.

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