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EFFECTS OF 3,4-METHYLENEDIOXYMETHAMPHETAMINE (MDMA) ON BDNF PATHWAY, HDAC EPIGENETIC ENZYMES AND NEUROFILAMENT PROTEINS

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ABSTRACT

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3,4-methylenedioxymethamphetamine (MDMA) is a psychoactive substance used for recreational purposes, especially by young people, because of its ability to increase extroversion, empathy and self-confidence. It acts as a mood enhancer, increasing feeling of well-being and extroversion and enhancing communication skills; for these pro-social acute effects, MDMA was investigated in psychotherapy during the early 1980s but studies were stopped after MDMA was classified as a drug of abuse by Drug Enforcement Administration (DEA) in 1985. The possible clinical use of MDMA, in combination with psychotherapy, has been recently reconsidered for the treatment of Post-Traumatic Stress Disorders (PTSD), given that this substance seems to facilitate trauma processing and communication, working as a catalyst.

However, it is well known that MDMA causes neurotoxic effects, involving particularly on serotoninergic neurons. In fact, MDMA chronic use has been associated with psychiatric symptoms, memory and cognitive deficits have been observed after MDMA assumption.

Histone deacetylase (HDAC) inhibitors are currently under investigation in the field of neuropsychiatric and neurodegenerative disorders due to their ability to increase the transcription of regulatory genes involved in synaptic plasticity like brain-derived neurotrophic factor (BDNF). Alteration of this neurotrophin has been reported both in different brain regions of animals treated with psychoactive substances and in the blood of addictive substances abusers. Moreover, BDNF seems to be altered also in psychiatric disease. This suggests that substance use disorders might share common pathways with mental disorders.

In the field of neurodegenerative and neuropsychiatric disorders, a particular attention has been recently devoted to neurofilaments (NFs) and their potential role as promising biomarkers after a neuronal injury.

Based on this evidence, we aimed to investigate:

1) the level of BDNF, its receptor tropomyosin receptor kinase B (TrkB) and HDAC enzymes in prefrontal cortex (PFCX) and hippocampus (HIPPO) of MDMA-treated rats and the effect of the HDAC inhibitor (HDACi) sodium butyrate (NaBut).
2) peripheral BDNF levels in serum and plasma of MDMA users and MDMA-naïve controls
3) the effects of MDMA treatment on neurofilaments in a differentiated serotonergic neuronal cell line (RN46A) and the potential protective role of BDNF.

Regarding point 1), data obtained in animals showed that after acute or repeated MDMA treatment, there has been an increase in BDNF/TrkB gene expression in the PFCX, while an opposite tendency was observed in HIPPO. These results are in agreement with the data reported in other studies and suggest that these alterations in PFCX could represent a compensatory mechanism to counteract serotonin depletion induced by MDMA (this mechanism is probably related to the alterations of tryptophan hydroxylase observed in PFCX). On the other hand, the downregulation of BDNF mRNA level in HIPPO followed by MDMA treatment could be related to the negative effect of MDMA on memory.

HDAC analysis showed an overall increase in the expression of this class of enzymes, suggesting an involvement of epigenetic modifications in MDMA effects. Considering these results, the possible protective role of the HDACi sodium butyrate on MDMA behavioral and molecular effects has been investigated. Gene expression analysis of BDNF and its receptor TrkB has shown that both MDMA and NaBut were able to induce a significant upregulation of BDNF and TrkB gene expression in PFCX. By contrast, in HIPPO, only the cotreatment NaBut+MDMA was able to cause a significant increase in gene expression. Behavioral results suggested that NaBut pretreatment is able to potentiate MDMA anxiolytic effect. In fact, HDACi have also been shown to potentiate some drug-induced behaviors, suggesting synergic interactions between HDACi and drugs of abuse. These data are also in line with other studies, indicating that MDMA could be useful for the treatment of psychiatric pathologies like PTSD during adulthood.

According to the second objective of this dissertation, we analyzed both serum and plasma BDNF protein levels in MDMA users and naïve controls.

Although we found only weak and non-significant group differences in BDNF blood levels between recently abstinent MDMA users and controls, the correlation between BDNF plasma levels and MDMA abstinence period may indicate that BDNF is impacted by MDMA use postacutely but also recovers with longer abstinence periods. Moreover, an analysis of BDNF blood levels confirmed that BDNF serum and plasma do not correlate but may reflect different biological pools of the neurotrophin. Finally, males seem to have higher peripheral BDNF levels than females, specifically in plasma.

Regarding the third point, data obtained from the study on neurofilaments showed that MDMA was able to induce alterations on NFs and in particular on neurofilament light (NF-L). These results are in agreement with previous studies, which have investigated NF-L as one of the most promising biomarkers of neuronal damage and have suggested its involvement in MDMA neurotoxicity.

In conclusion, data presented in this thesis showed that MDMA is able to alter the expression of different crucial genes and proteins that are involved both in substance use disorders and in neuropsychiatric conditions. Animal studies showed alterations both in BDNF pathways and in the class I HDACs, whereas human results suggested that peripheral BDNF does not seem to be significantly affected by MDMA use; however, obtained data also suggested that serum and plasma can reflect different types of this neurotrophin. Finally, our results on the differentiated serotonergic cell line highlight the useful role of NF-L as a biomarker of neuronal damage induced by MDMA.

1. INTRODUCTION

1.1 COMORBIDITY BETWEEN DRUG ABUSE AND MENTAL DISORDERS

The term "comorbidity" was defined for the first time by Feinstein in 1970 to indicate "*any distinct additional clinical entity that has existed or that may occur during the clinical course of a patient who has the index disease under study*" (Feinstein, 1970). The term "comorbidity" refers to the presence of more than one disorder in the same subject over a period of time and the particular condition of comorbidity called "dual diagnosis" is defined as "the coexistence of psychoactive substance use and another psychiatric disorder in the same person" (Drake et al., 1998; Hryb et al., 2007; World Health Organization., 1994).

Some epidemiological studies have found a large number of subjects in whom, after chronic exposure to substances of abuse and subsequent irreversible alteration of brain functions, the psychoactive substance has acted as a facilitator for the onset of a neurological disorder (Hambrecht and Hafner, 1996; Kleinman et al., 1990; Liraud and Verdoux, 2002; Niemi-Pynttari et al., 2013; Zhornitsky et al., 2015). On the other hand, other clinical studies have focussed on the use of substances after the onset of psychiatric symptoms, in the form of self-medication for coping with feelings of distress and mood disorders of the dysphoric or depressive type (Aharonovich et al., 2001; Khantzian, 1985; 1997; Phillips and Johnson, 2001). The US National Comorbidity Survey showed that about 50 % of subjects with a mental illness had a lifetime history of use of substances, and that about 50% of respondents with a lifetime substance dependence had a history of mental disorder (Kessler, 2004).

Epidemiological studies indicate that comorbid disorders can start during adolescence. In particular, non-substance related disorders seems to precede substance abuse. In fact, the estimated average of onset of mental disorders is about 11 years old compared to 21 years old, which is the estimated average of onset of substance disorders (Kelly and Daley, 2013; Kessler, 2004).

Moreover, individuals with schizophrenia are vulnerable to drug of abuse and the result from the Epidemiologic Catchment Area Study showed that 47% of them struggle with substance abuse (Regier et al., 1990).

Schizophrenia is a multifactorial disorder in which genetic factors may interact with epigenetic processes and environmental factors (Danese, 2006) and it is characterized by different symptoms such as auditory hallucinations, paranoia, disorganized speech and thought with alterations in different neurotransmitter systems. The alteration of some neurotransmitters such as glutamate and dopamine in the mesocorticolimbic system, leads individuals with

schizophrenia to overestimate the rewards induced by substances of abuse and to underestimate the negative consequences of addiction (Krystal et al., 2006).

Some authors have already discussed about the self-medication hypothesis also in schizophrenia, as it has been reported that patients with schizophrenia seem to use substances of abuse to reduce the severity of negative symptoms caused by antipsychotic medication (Kumari and Postma, 2005).

In fact, several studies have supported the hypothesis in which schizophrenic patients have an increased risk of developing drug dependence.

Moreover, it has been observed that subjects with damage to the ventromedial prefrontal cortex have impaired performance on the *Iowa Gambling Task*, a psychological test that requires the subject to choose between less attractive but less risky rewards and better but riskier rewards (Waters-Wood et al., 2012; Xiao et al., 2013). A group of alcohol and substance abusers but also schizophrenic patients showed altered performance during that test (Bechara et al., 2001; Sevy et al., 2007), supporting the possible presence of common circuits. However, Sevy et al. examined the gambling task in schizophrenic patients with or without concomitant cannabis use disorders and they have found no difference between the groups (Sevy *et al.*, 2007).

Depression is another psychiatric disorder characterized by a lowered mood, loss of interest, negative thoughts, and even suicide. These symptoms are very common among drug users, in particular during the withdrawal phase, including anhedonia, sadness, sleep disturbances, and cognitive deficits (Markou and Kenny, 2002; Quello et al., 2005). This suggests that the withdrawal phase of drug dependence and depression may share common mechanisms.

In general, the condition of mood and anxiety disorders with drug use disorders has been well-documented in the literature (Lai et al., 2015). Moreover, the use of substances to calm down negative symptoms has been shown to be related with an increased risk of developing drug dependence (Lazareck et al., 2012).

Regarding the co-occurrence of anxiety disorders and alcohol dependence, a common explanation of this frequent comorbidity is that alcohol is taken to calm down anxiety, so, it is used to self-medicate the symptoms of this disorder (Crum et al., 2013). In particular, Crum et al. observed that drinking to self-medicate anxiety symptoms is associated with the development of alcohol dependence, and that this dependence is persistent (Crum *et al.*, 2013). However, it is also known that alcohol may lead to anxiety disorders (George et al., 1990).

As mentioned above, many studies have already suggested a relationship between drug abuse and the development of psychiatric disorders. In particular, it seems that psychoactive substances could promote a series of molecular alterations favouring the onset of psychiatric

disorders and it has been observed that people with psychiatric disorders are at high risk for substance abuse.

This evidence also underlines that drug addiction and mental illness may share common pathways. Understanding the mechanisms by which psychiatric disorder and drug dependence co-occur is therefore fundamental to develop appropriate treatments. This is particularly important considering how difficult it is to treat subjects with comorbidities of substance abuse and mental illness. In fact, treatment outcomes tend to be worse for individuals with comorbidities and there is a high risk for relapse after treatment (McCarthy et al., 2005; Suter et al., 2011). The integrated treatment, in which both disorders are treated concurrently with the same doctor/team and in which combined treatments are used, such as psychotherapy and pharmacotherapy, is considered the standard of care for this co-occurrence condition. As the co-presence of two or more pathological conditions increases the complexity of the treatment and is usually associated with poor treatment outcomes (Carra et al., 2015; Grella et al., 2001; Mangrum et al., 2006), the integrated treatment approach has demonstrated its superiority in comparison to a single approach. Understanding the molecular mechanism shared by mental health conditions and substance abuse could help to understand the development of these co-occurent diseases and to improve the therapies used to date.

1.2 MDMA

3,4-methylenedioxymethamphetamine (MDMA), also known as “ecstasy”, is an amphetamine derivative and it was first synthesized by the German pharmaceutical company Merck which succeeded in obtaining the patent of MDMA in 1914 (Siegel, 1986). It is an *entactogen* substance able to increase the levels of empathy and closeness. The United Nations Office on Drugs and Crime reported that MDMA was used by 20 million of people in 2019 (United Nations Office on Drugs and Crime, 2021) and the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA, 2021) estimated that in the last year, in Europe, MDMA has been used by 2.6 million people (15-64 years old), 2 million of which are young adults between 15 and 34 years old (data updated June 2021). These data showed that MDMA is still a popular drug among adolescents and young adults.

MDMA acts as a releaser of neurotransmitters and its main mechanism of action is the inhibition of monoamine reuptake, binding neurotransmitter transporters. Thus, the main consequence after MDMA assumption is an acute increase in neurotransmitter levels, followed by a period of depletion (Hall and Henry, 2006). In particular, having high affinity for the

serotonin transporter, the main consequence is the release of serotonin into synaptic cleft but also affects dopamine and noradrenaline (Baumann et al., 2007; Verrico et al., 2007). The main acute effects of this psychostimulant are related to the serotonin release, which explains its recreational use; in particular, the acute effects of MDMA are euphoria, emotional warmth, increased self-confidence, empathy towards others. In fact, MDMA acts as a mood enhancer (Kalant, 2001) but it is also able to give negative effects on many physical functions and it might lead to persistent psychosis even after a single use (Henry, 1992; Patel et al., 2011; Potash et al., 2009; Van Kampen and Katz, 2001). For its ability to increase of self-confidence and empathy, this substance has been considered for psychiatric treatment since 1970s, and it has been discussed again recently, particularly for its potential use in the treatment of post-traumatic stress disorder (PTSD) (Mithoefer et al., 2011). On the other hand, the adverse effects have been associated to many neuropsychiatric symptoms and disorders like depression, panic attacks, memory and cognitive deficits, which can persist months after the cessation of substance use (Morgan, 2000; Parrott, 2002). In particular, the positive effects of this substance are produced at low doses and acutely, whereas high doses and repeated use seems to be associated only with adverse responses (Brunt et al., 2012; Kalant, 2001).

In addition, MDMA produces a release of oxytocin that has been shown to modulate emotion processing and behavior, and it could be linked to the increase levels of empathy (Dumont et al., 2009; Hysek et al., 2014; Kirkpatrick et al., 2014).

MDMA is available as a racemic mixture and, even if the two stereoisomers are different, both the S(+) and the R(-) isomers are pharmacologically active (Kalant, 2001; Schechter, 1987). It is usually consumed orally, formulated in a tablet containing 50-150 mg of MDMA (Parrott, 2004). It is important to mention that ecstasy tablets may contain other substances such as caffeine or ketamine which can contribute to the effects of MDMA (Parrott, 2004).

About 20% of a MDMA tablet is excreted unaltered in the urine (de la Torre et al., 2004a) while about 80% of this psychostimulant undergoes hepatic metabolism that can have 2 different metabolic ways, the O- demethylenation and the N-dealkylation, giving different metabolites (de la Torre et al., 2004b).

MDMA is detectable in the blood after 30 minutes and the elimination half-life of a common dose of MDMA is about 8 hours, while it reaches the highest concentration in blood occurs in about 2 hours (de la Torre et al., 2000; Kolbrich et al., 2008; Mas et al., 1999). Also, MDMA can be detected in body fluids like saliva or in the hair (de la Torre *et al.*, 2004a; Navarro et al., 2001). In this latter, the substance can be detected for several months after use (Pichini et al., 2006).

MDMA is able to give long-term plasticity (Lanteri et al., 2014) even if, whether MDMA is capable of being addictive in humans is still a matter of debate. The nature of addiction to MDMA seems to be different in comparison to other drugs (Degenhardt et al., 2010) and the reinforcement provided by MDMA has already been observed, despite it being less strong than other amphetamines (Degenhardt *et al.*, 2010). It has also been demonstrated that repeated use of MDMA induces behavioral sensitization and self-administration behavior in animals (Kalivas et al., 1998; Schenk et al., 2007).

1.2.1 MDMA, neurotoxicity and cognitive impairment

Severe hyperthermia is the most known acute effect produced by ecstasy since it is one of the causes leading to the death of the users. However, the long-term effects of MDMA are generally adverse due to neurotoxicity. The dysregulation of monoamine neurotransmitters causes neurotoxic effects as well as behavioral changes in animal models and humans (Sarkar and Schmued, 2010).

MDMA is known to cause different effects in distinct species. While in mice MDMA cause a damage mainly in the dopaminergic system (Colado et al., 2004; Costa et al., 2017; Iravani et al., 2000), in the majority of other species, including humans and rats, its toxicity mainly affects the serotonergic system (Baumann *et al.*, 2007; McCann et al., 1998; Parrott, 2001).

Focusing on the latter, serotonin regulates different functions linked to memory and cognition (Meneses et al., 2011) and it is also implicated in different psychiatric diseases like depression and anxiety (Cowen and Lucki, 2011). In the psychiatric field, a lot of studies suggest an association between MDMA exposure and psychopathology even if the nature of these relationships is still debated (Karlsen et al., 2008).

In fact, there is a wealth of evidence in which MDMA, following single or repeated administration, causes long-term depletion of serotonin content in different brain areas in animal models.

In this regard, some studies have been shown that MDMA causes a reduction in markers of serotonin in rats and in nonhuman primates (Baumann *et al.*, 2007; Stone et al., 1987) and it is also able to inhibit tryptophan hydroxylase (TPH) (Green et al., 2003; Schmidt and Taylor, 1987; Stone *et al.*, 1987), contributing to decreaseing the availability of serotonin.

In humans, MDMA has been found to be able to reduce serotonergic markers in abstinent ecstasy users (Benningfield and Cowan, 2013; Cowan, 2007; Kish et al., 2000). In one

postmortem study, it was observed that the level of serotonin and one of its metabolite were reduced by 50-80% in different brain regions, compared to controls (Kish *et al.*, 2000).

The first neuroimaging studies of humans were performed in the late 1990s and they showed reduced serotonergic markers in ecstasy users (McCann *et al.*, 1998; Parrott, 2012; Reneman et al., 2001; Semple et al., 1999). After implementing improved techniques such as PET and fMRI over the years, scientific studies have continued to observe a decrease in serotonergic markers (Benningfield and Cowan, 2013; Cowan, 2007; Reneman et al., 2006). On the other hand, in other studies on humans, only little alteration was found in the serotonergic system after the use of MDMA (Costa et al., 2020; Garg et al., 2015; Mueller et al., 2016). This is probably due to the variabilities in the the different studies: i.e. dosage taken, the duration of withdrawal, and the use of several substances of abuse taken simultaneously etc. Despite contrasting results, a decrease in serotonergic levels has been generally noted, and Cowan concluded that the most robust finding was indeed a reduction in the density of serotonin transporter in abstinent users (Cowan, 2007).

Roberts C.A. and colleagues (Roberts et al., 2018) have recently analyzed series of studies that linked serotonergic system changes with neurocognitive deficits in MDMA users and they came to the conclusion that research needs more powerful and clean studies to evaluate the connection between serotonin and cognitive changes in MDMA users.

On the other hand, an impairment of cognitive functions is reported both in animal and in human studies after MDMA administration (de la Torre and Farre, 2004; Parrott, 2013).

In other researches deficits in memory performance tasks were observed in rats after MDMA treatment (Arias-Cavieres et al., 2010; Camarasa et al., 2008) and in MDMA users (Bolla et al., 1998; Quednow et al., 2006), alongside deficits in associative learning (Montgomery et al., 2005).

In addition, a plethora of studies discussed about the "neurodegeneration hypothesis", hypothesis supported for example due to serotonergic axon terminals damaged after MDMA assumption but also to an increase of oxidative stress in the brain (Biezonski and Meyer, 2011; Yamamoto and Raudensky, 2008).

In fact, one of the mechanisms of MDMA neurotoxicity also involves the alteration of antioxidant enzymes and consequently of the MDMA metabolism itself. MDMA administration has been shown to increase the SOD activity in mice (Costa et al., 2021; Peraile et al., 2013) and the hyperthermia given by amphetamine compounds seems to increase the formation of reactive oxygen species and reactive nitrogen species (Chipana et al., 2008; Sharma and Ali, 2008; Shokry et al., 2019).

MDMA and its metabolites are thought to contribute to the formation of reactive oxygen species and this seems to be a key event in the loss of serotonin function that occurs following MDMA treatment in animals (de la Torre and Farre, 2004; Puerta et al., 2009; Sprague and Nichols, 2005).

Finally, MDMA is able to induce neuronal apoptosis and to increase apoptotic markers (Capela et al., 2013; Capela et al., 2007; Soleimani Asl et al., 2012) and to induce autophagy in cortical neurons (Li et al., 2014). All these effects contribute to increase MDMA-induced neurotoxicity.

Another important aspect to take into account is that MDMA is mainly consumed during adolescence and this period is known to be critical for brain development, e.g. for prefrontal cortex (PFCX). In fact, it is demonstrated that adolescent brain is particularly vulnerable to drug of abuse such as MDMA (Cadoni et al., 2015; Daza-Losada et al., 2009). For instance, Costa et al. observed that MDMA potentiates the damage caused by a toxin that is able to induce Parkinson's disease in different areas of adolescent mice brain (Costa et al., 2013), underlying that MDMA can amplify the effects of other substances.

1.2.2 MDMA in PTSD treatment

Post-Traumatic Stress Disorder (PTSD) is an anxiety disorder that can develop following the exposure to a single or to repeated traumatic experiences, and it is characterized by a high rate of comorbidity with depression, anxiety and drug addiction (Kessler et al., 1995; Mitchell et al., 2021).

During the early '80s, psychotherapists evaluated the potential use of MDMA in psychotherapy. Even though its use was forbidden after few years, MDMA actions have never ceased to be considered as potentially useful for the treatment of some pathological conditions (Greer and Tolbert, 1986; Greer and Tolbert, 1998).

The first clinical study, where the potential use of MDMA in combination with psychotherapy was investigated, was published in 2011 (Mithoefer *et al.*, 2011). This study was carried out in twenty patients with resistant PTSD. The stage I of the study demonstrated that 83% of patients treated with MDMA, in combination with psychotherapy, did not meet anymore the criteria for PTSD versus a percentage of 25% in the placebo group (Mithoefer *et al.*, 2011). In addition, there were no severe adverse events or adverse neurocognitive effects (Mithoefer *et al.*, 2011).

The aim of exposure-based therapies in the treatment of PTSD is to overcome the fear associated with the recall of trauma. Sertraline and paroxetine are the first-line therapeutic

options for PTSD next to psychotherapy. Although some patients show a reduction in PTSD symptoms, approximately 40-60% of patients do not respond adequately to therapy (Bradley et al., 2005; Feduccia and Mithoefer, 2018; Steenkamp et al., 2015). In fact, a widespread problem in this group of pathologies is the treatment resistance.

Given the typical symptoms of PTSD (hypervigilance, fear, evasive and isolating behaviors), the psychopharmacological characteristics of MDMA make it an ideal adjuvant in supporting psychotherapeutic treatment (Mithoefer *et al.*, 2011; Sessa et al., 2019), especially for treatment-resistant PTSD. In studies in animal models, it has been observed that MDMA modulates fear memory extinction and reconsolidation (Hake et al., 2019; Nardou et al., 2019; Young et al., 2015). In particular, MDMA facilitates the extinction of fear memory and this effect seems to be related to elevated BDNF levels in the amygdala (Young *et al.*, 2015; Young et al., 2017).

These phenomena could explain how MDMA helps psychotherapy sessions: MDMA seems to work as a catalyzer, facilitating trauma processing and helping patients to remember emotionally painful events which are usually avoided, thanks to MDMA's ability to increase empathy, allowing traumatic memories to be processed effectively. Nowadays MDMA is in phase 3 for the treatment of PTSD (Mitchell *et al.*, 2021).

1.3 BDNF

Brain derived neurotrophic factor (BDNF) is the most abundant neurotrophin in the brain and it is well known for playing a key role in synaptic plasticity, cell growth, differentiation, learning and memory (Autry and Monteggia, 2012; Kowianski et al., 2018).

BDNF is synthesized as proBDNF, which can be cleaved into the mature form (mBDNF). Both these forms can be detected in the brain (Ismail et al., 2020). The immature form is not inactive but it has the opposite cellular effect in comparison to the mature one. Whereas the main effect of proBDNF is inducing apoptosis, the functions of mature BDNF is promoting survival (Kowianski *et al.*, 2018; Lu et al., 2005). In addition, some studies have observed that a wrong cleavage of proBDNF can play a role in cognitive impairment (Fleitas et al., 2018; Gerenu et al., 2017).

BDNF and its receptor tropomyosin-related kinase B (TrkB) are both localized at pre- and postsynaptic levels, where BDNF is released in an activity-dependent manner (Waterhouse and Xu, 2009).

BDNF binds different receptors: the immature BDNF has high affinity for the p75NTR receptor and sortilin, whereas the mature form has high affinity for the TrkB receptor (Koshimizu et al., 2010; Kowianski *et al.*, 2018).

After BDNF-TrkB binding, the receptor undergoes dimerization and autophorsphorylation, and all of these phenomena can activate different intracellular signaling cascades (Figure 1): phosphatidylinositol 3-kinase (PI3K), mitogen-activated protein kinase (MAPK), phospholipase C-γ (PLC-γ), and guanosine triphosphate hydrolases (GTP-ases) of the Ras homolog gene family (Kowianski *et al.*, 2018).

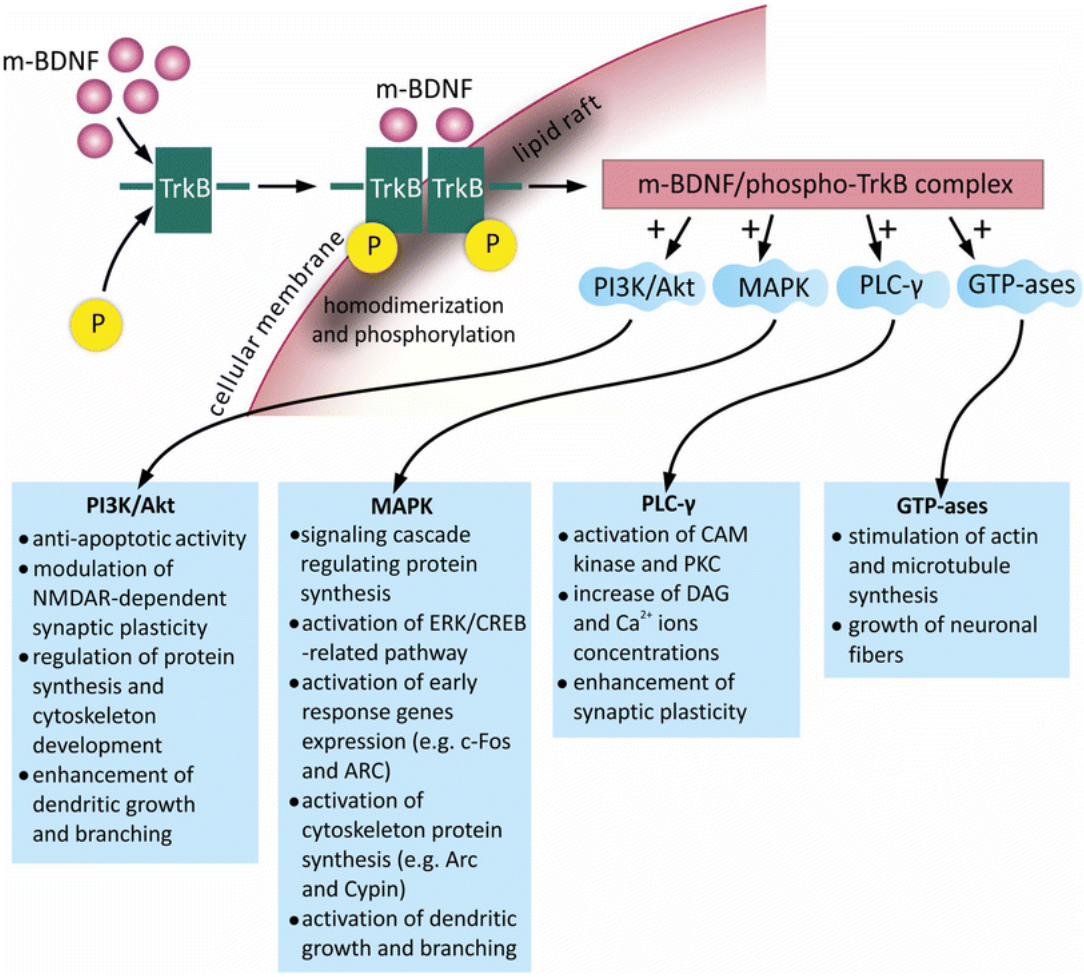


Figure 1. Activation of different intracellular signaling cascades given by the interaction of BDNF with its receptor TrkB (Kowianski *et al.*, 2018).

Given its involvement in brain development, maturation, plasticity and the activation of different signaling cascade, BDNF plays an important role in psychiatric diseases. In fact, it is often altered in many neuropsychiatric disorders such as depression, schizophrenia, anxiety and

addition (Autry and Monteggia, 2012). Since BDNF is crucial for memory and cognitive processes and it is involved in brain development, this neurotrophin is proposed as a biomarker of different pathological conditions, in particular in neuropsychiatric diseases (Peng et al., 2018).

BDNF seems to play a role also in schizophrenia, even if the relationship between this neurotrophin and this pathological condition is still unclear. It this regard, a postmortem study demonstrated an increase in BDNF levels in anterior cingulate cortex and in hippocampus (HIPPO) in patients with schizophrenia (Takahashi et al., 2000). By contrast, some studies have shown a downregulation of BDNF levels in the cortices of subjects with schizophrenia compared to controls (Hashimoto et al., 2005; Weickert et al., 2003).

Also, regarding the peripheral level of BDNF in schizophrenia there are studies that came to contrasting results: BDNF levels in serum of schizophrenic patients could have been decreased (Jindal et al., 2010; Toyooka et al., 2002) or increased (Jockers-Scherubl et al., 2004; Reis et al., 2008).

However, it is important to underline that these differences could be related to the various conditions of patients: treated vs untreated. Moreover, the different type of treatment followed by patients with schizophrenia should be taken into account. For example, clozapine seems to increase BDNF levels in serum compared to risperidone (Pillai, 2008) and in rats, haloperidol treatment seems to impact more on BDNF than risperidone (Angelucci et al., 2000).

1.3.1 BDNF in addiction and psychiatric disorders

BDNF is known for being fundamental for the proper growth and survival of serotonergic, dopaminergic, GABAergic and cholinergic neurons (Pillai, 2008) and it has been shown to play a central role in different kind of plasticity including drug dependence (Barker et al., 2015; Ornell et al., 2018). In fact, this neurotrophin affects a wide variety of neuronal functions.

Substances of abuse are able to modulate BDNF at central and/or peripheral levels, alcohol (Pandey, 2016), opioid (Akbarian et al., 2002), methamphetamine (Ren et al., 2016), cocaine (Li and Wolf, 2015) have been demonstrated to modulate the level of this neurotrophin in neural circuits responsible for addictive behaviors or at peripheral level (Autry and Monteggia, 2012).

With amphetamine administration, used as a pharmacological model for psychotic symptoms of schizophrenia (Featherstone et al., 2007; Pillai, 2008), it has been shown that amphetamine

chronic treatment downregulates BDNF protein levels in the hypothalamus and occipital cortex in rat brain (Angelucci et al., 2007).

Cocaine exposure generally causes an increase in BDNF levels both in the brain regions and in peripheral blood (Li and Wolf, 2015). This increase can persist during drug withdrawal and it has been associated with an enhanced synaptic plasticity that may underlie increased drug reactivity, drug seeking and relapse (Corominas et al., 2007; D'Sa et al., 2011; Grimm et al., 2003).

In fact, an increase in BDNF gene expression levels was observed in different brain areas of animals receiving cocaine (Filip et al., 2006; Fumagalli et al., 2007; Graham et al., 2007; Russo et al., 2009). Moreover, it has been observed that prenatal cocaine exposure augmented BDNF-TrkB signalling in rats (Stucky et al., 2016).

Graham et al. have shown that the regulation of BDNF synthesis and release in the NAc during prolonged cocaine use contribute to the development of cocaine dependence, but it also facilitates relapse, supporting the role of BDNF in rewarding (Graham *et al.*, 2007).

Mouri A. et al observed BDNF alterations in different brain areas after acute and repeated MDMA treatment and, using mice with heterozygous deletions of the BDNF gene, they discovered that BDNF is involved in MDMA-induced dependence and psychosis (Mouri et al., 2017). This study also suggested that BDNF alterations could be related to changes in serotonergic and dopaminergic transmission (Mouri *et al.*, 2017). Interestingly, it has been shown that MDMA modulates BDNF levels in animal models, in an opposite manner in PFCX and in HIPPO, (Hemmerle et al., 2012; Martinez-Turrillas et al., 2006) and these alterations seem to reflect the trend of rate-limiting enzyme of serotonin, TPH (Garcia-Osta et al., 2004; Martinez-Turrillas *et al.*, 2006).

Until now, BDNF levels after MDMA assumption in humans were analysed in only one study (Angelucci et al., 2010), and obtained results suggested an increase in BDNF in the blood of ecstasy users. This could reflect a response to MDMA neurotoxicity as a compensatory mechanism or the results of alterations in BDNF trafficking between periphery and central nervous system (Angelucci *et al.*, 2010).

Since several studies have already mentioned that BDNF is involved in memory and cognitive functions, while underlining its key role in brain development, BDNF involvement in mental illnesses as well as in substance use disorders comes as no surprise. Therefore, manipulating BDNF expression and related pathway seems to be a promising strategy to develop a new approach for treating different psychiatric illnesses.

1.3.2 Peripheral BDNF

The main limitation in the research on BDNF in humans is that it is not possible to measure BDNF levels in brain tissue in a non-invasive manner. For this reason, the level of BDNF in periphery and in particular most of the time in serum and plasma, is used as a proxy.

Some researches in animal models have shown that peripheral and central BDNF are linked to each other (Klein et al., 2011) and that the neurotrophin BDNF is able to cross the blood-brain barrier (BBB) (Molinari et al., 2020; Pan et al., 1998), although other studies did not support these findings (Kyeremanteng et al., 2012; Pardridge, 2007; Pardridge et al., 1998).

Thus, it is still unclear if in humans, peripheral BDNF levels can reflect central ones. Moreover, some authors are arguing if serum and plasma BDNF are correlated and can be used indiscriminately to measure this neurotrophin. While observing healthy volunteers, a strong correlation between serum and plasma BDNF concentrations has been reported on one side (Yoshimura et al., 2010) but, on the other side, further studies have shown no correlation between BDNF level in serum and plasma (Bocchio-Chiavetto et al., 2010; Gejl et al., 2019). Recently it has also been suggested that serum and plasma levels of BDNF might reflect different biological pools of this neurotrophin (Gejl *et al.*, 2019).

Serum has for instance a much higher amount of BDNF than plasma (Fujimura et al., 2002; Radka et al., 1996): the majority of BDNF is contained in platelets and consequentially in serum (Yamamoto and Gurney, 1990). In particular, BDNF is released from platelets to serum during the coagulation process and therefore clotting time is a factor that can influence analysis (Gejl *et al.*, 2019). In addition, recent findings have observed a positive correlation between platelet counts and BDNF levels in serum (Kronenberg et al., 2021; Naegelin et al., 2018; Ziegenhorn et al., 2007).

However, it is not yet clear if BDNF is able to cross the blood-brain barrier in humans (Klein *et al.*, 2011; Kyeremanteng *et al.*, 2012; Molinari *et al.*, 2020; Pardridge, 2007; Pardridge *et al.*, 1998). Since platelets do not seem to cross the BBB, it appears that free plasma BDNF, rather than serum BDNF, might reflect better central BDNF pool (Gejl *et al.*, 2019; Radka *et al.*, 1996). Geji et al. have also underlined other challenges in the study of peripheral BDNF: the level of BDNF contained in serum is affected by clotting time, whereas BDNF level in plasma is influenced by the centrifugation strategy (Gejl *et al.*, 2019).

Finally, studies revealed that BDNF in periphery can be modulated by other factors such as age, gender and body mass index (BMI) (Lommatzsch et al., 2005; Yang et al., 2019). In fact, estrogen can affect BDNF levels and postmenopausal or amenorrhoeic women have been

shown to have lower level of plasma BDNF than fertile women (Begliuomini et al., 2007) and high BMI seems to be associated with lower level of BDNF (Yang *et al.*, 2019).
For all these reasons it is still debated whether peripheral BDNF can reflect the central one.

1.4 EPIGENETICS

Epigenetic studies the dynamic changes that occur during cellular development that do not involve a change in the DNA base sequence; they are therefore changes that can be acquired but cannot be attributed to changes in the DNA sequence (Morange, 2002).
In particular, the word "epigenetic" refers to chemical modifications of the proteins around which there is packaged the DNA and DNA methylation and histone modifications being the most studied epigenetic modifications.
DNA and histones bound in the cell nucleus create a particular structure named chromatin. The unity of this structure is called nucleosome and it is formed by octamers of the four essential histones (H3, H4, H2A, and H2B) around which the DNA is wrapped (Kouzarides, 2007).
In recent years, many studies have focused their attention on epigenetic mechanisms to find new therapeutic targets considering that they regulate the access to the transcriptional machinery, they modulate gene expression and are therefore involved in many diseases.
Addiction or Substance Use Disorder (SUD, from DMS V) is a neuropsychiatric disorder that results from neuronal adaptations at the molecular, cellular and tissue levels following repeated exposure to substances of abuse. Current research on psychostimulant dependence is attempting to elucidate the mechanisms underlying such adaptive changes, as they may explain the transition from recreational drug use to addictive behavior.
As already mentioned, psychostimulants cause an increase in the release of neurotransmitters, but the increased level of neurotransmitters alone does not justify the long-lasting transcriptional and behavioral alterations. These long-lasting aspects of SUD suggest that epigenetic modifications could be the long-term modulators of substance-of-abuse-induced changes, as they can maintain durable structural adaptations at the chromatin level (Godino et al., 2015).
All things considered, epigenetic modifications may mediate long-term changes in gene expression and this might explain the transition from recreational drug use to drug abuse.

Most of the information available today on the epigenetic regulation associated with drug dependence concerns histone modifications, with histone acetylation being the most studied chromatin modification in animal models of addiction to date (Nestler, 2014).

1.4.1 HDACs

Histone acetylation is an epigenetic modification that can affect chromatin structure, modifying transcriptional activity. In particular, histone modifications regulate the chromatin state, making it more or less opened and consequentially more or less accessible to transcription factors (Kouzarides, 2007; Samantara et al., 2021) (Figure 2).

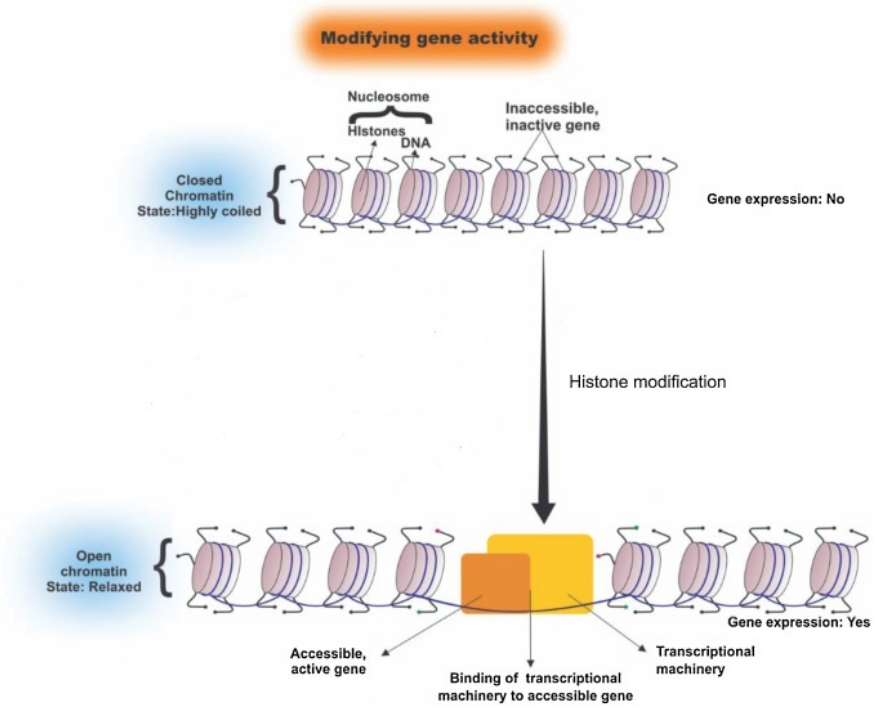


Figure 2. Gene expression is driven by histone modification. The chromatin state can be "closed" or "relaxed", reflecting an inactive or active transcriptional state (Samantara *et al.*, 2021).

Histone acetyltransferases (HATs) and histone deacetylases (HDACs) are two classes of enzyme that contribute to balancing the acetylation of the histone and then the transcriptional activity. In fact, increase levels of HAT, acetylating conserved lysine amino acids on histone

proteins, are associated with an increase in transcription, whereas an increase in HDACs levels is associated with a decrease in transcription by removing the acetyl group from lysine residue (Shahbazian and Grunstein, 2007; Shukla and Tekwani, 2020) (Figure 3).

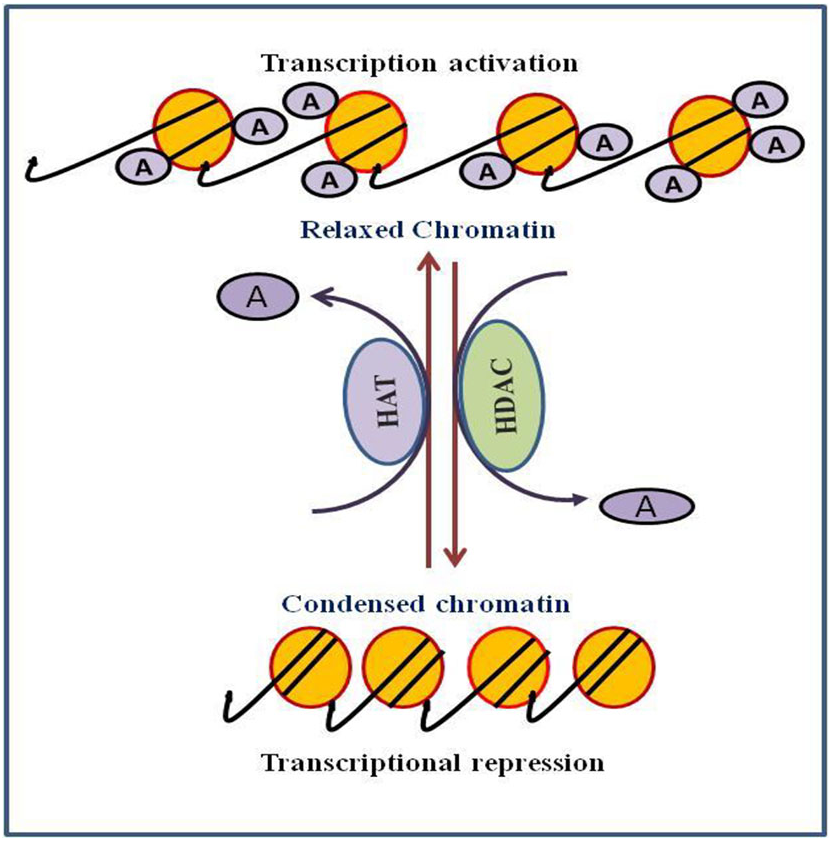


Figure 3. Involvement of HATs and HDACs in the regulation of transcription (Shukla and Tekwani, 2020).

Generally, HDAC enzymes are divided into four groups called classes (I, II, III, IV), based on homology to their analogue yeast (Park and Kim, 2020). The HDACs Class I is expressed ubiquitously and shares homology with yeast Rpd3. This class contained the HDAC1, 2, 3, and 8. The class II is divided in two subgroups (a and b) based on their domain composition and has a high degree of homology with yeast Hda1. Class IIa includes HDAC4, 5, 7, and 9, whereas class IIb containes HDAC6 and 10. The class III involves enzymes called sirtuins, which are most similar to the yeast Sir2. The class IV is composed by only the HDAC11 and it shares a similar sequence to yeast Hos3 (Park and Kim, 2020).

In the following studies, we will focus on the class I HDACs since most HDAC activity is catalyzed by this class and it is highly expressed in the brain (Covington et al., 2015; Kennedy et al., 2013).

The class I of HDACs is well researched and it is linked to learning and memory functions. This class is highly expressed in brain regions and a part from the HDAC3, the other enzymes are expressed exclusively in the nucleus (Park and Kim, 2020).

Since the balance of this class of enzyme is crucial in the control of gene expression, it is clear how its dysfunction can cause several problems in the cell regulation and consequentially in many pathologies. In the field of cognition and memory, HDAC1 and HDAC2 seems to play a crucial role.

The upregulation of HDAC1 seems to be associated with enhanced fear memory extinction and the inhibition of HDAC, with the inhibitor MS-275, showed an impaired fear memory extinction (Bahari-Javan et al., 2012).

HDAC2 is found to be the most promising target of HDAC inhibitors (HDACi) in improving memory and HDAC2 KO mice exhibited memory facilitation (Guan et al., 2009), whereas, memory impairment is associated with an overexpression of HDAC2 (Guan *et al.*, 2009).

HDAC3 has also been shown to be involved in memory regulation. In fact, the inhibition of this isoform has been shown to improve long-term object recognition memory in mice, demonstrating the negative role of HDAC3 in long-term memory formation (McQuown et al., 2011).

In addition, it has been observed that epigenetic mechanisms are playing a fundamental role in the regulation of neurotrophin gene expression such as BDNF gene expression. In fact, using HDACi it has been observed that can be modulate the transcription of BDNF (Bagheri et al., 2019; Bredy et al., 2007; Intlekofer et al., 2013; Sartor et al., 2019). In particular, some studies have shown that HDAC inhibitors are able to enhance BDNF expression, suggesting a neuroprotective effect of this class of inhibitors, which can be useful for treating central nervous system disorders (Chen et al., 2006; Koppel and Timmusk, 2013; Zeng et al., 2011).

Our lab group has already demonstrated that HDACs class I is altered in the caudate putamen of mice partially lacking BDNF (Caputi et al., 2015), observing that low levels of BDNF are associated with a low level of HDAC enzymes (Caputi *et al.*, 2015). Moreover, some authors have highlighted that animals with a low level of BDNF are more likely to become addicted to alcohol (Hensler et al., 2003; McGough et al., 2004).

1.4.2 HDAC inhibitors

Recent evidence suggested that HDACs are paying a key role in learning and memory processes and seem to be potential targets in neuropsychiatric disease (Figure 4)(Abel and Zukin, 2008). It has been shown that HDAC inhibitors improve memory processes and cognitive functions (Abel and Zukin, 2008; Fischer et al., 2010).

In particular, the hyperacetylation of histone through a direct inhibition of HDACs seems to correspond to a neuroprotective action (Shukla and Tekwani, 2020).

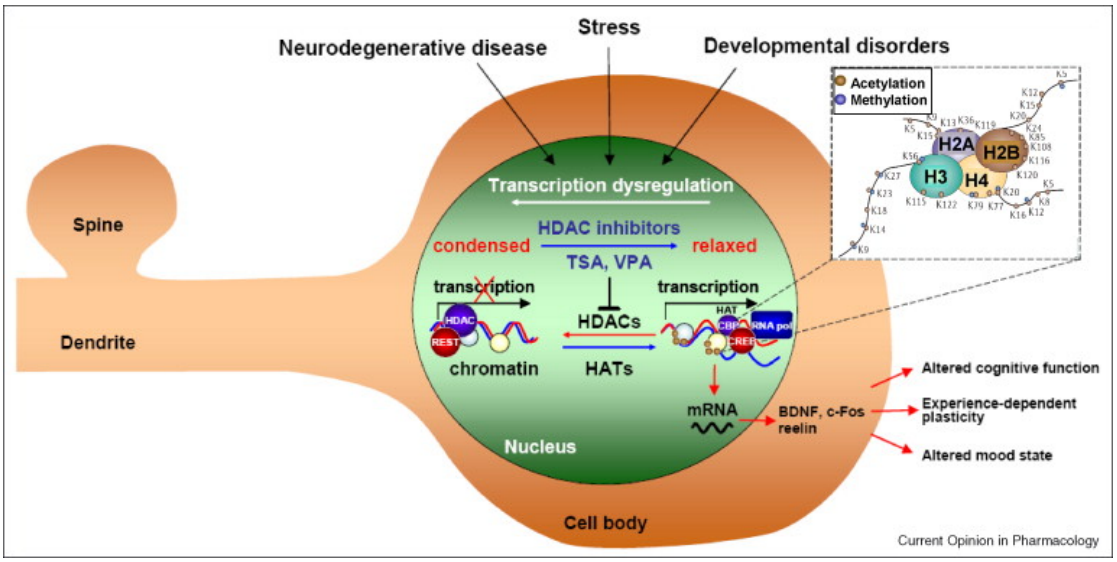


Figure 4. HDACi such as valproic acid (VPA) and trichostatin A (TSA) inhibit the activity of HDACs, leading to active transcription of regulatory genes, which is fundamental for plasticity and for improving cognitive deficits, such as BDNF (Abel and Zukin, 2008).

Moreover, HDACs are known to control processes such as differentiation and cell proliferation. For this reason, the is a growing interest in their use as therapeutic agents in neurological disorders and in cancer.

This family of inhibitors can be classified in different classes, based on the chemical structure of the molecules. In the class of short chain fatty acids there is the HDACi sodium butyrate and valproic acid, which can cross the BBB (Minamiyama et al., 2004; Silva et al., 2020).

The use of HDACi in neurological disorders, such as Alzheimer and Huntington disease, has been considered and it also seems promising in the treatment of several psychiatric disorders like schizophrenia and depression (Shukla and Tekwani, 2020).

In a non-pathological condition, the histone acetylation homeostasis is balanced and HAT e HDACs collaborate to maintain this balanced condition. However, in many neurodegenerative diseases, there is a shifting toward hypoacetylation, and HDACi are used to reestablish the homeostasis working as neuroprotective agent.

It has been observed that blocking HDAC1 in the nucleus accumbens of mice increases histone acetylation levels and it antagonizes cocaine-induced behavioral changes (Kennedy *et al.*, 2013).

Moreover, there is an increasing interest in HDACi for their possible use in tackling the behaviors associated with pathological alcohol (EtOH) dependence. In fact, HDACi have shown to alter the behavioral effects induced by EtOH. In particular, the HDACi sodium butyrate has been shown to prevent and reverse behavioral sensitization and gene expression alterations induced by EtOH, such as changes in BDNF expression levels in the striatum and PFCX (Legastelois et al., 2013); Sodium butyrate is also able to significantly attenuate excessive EtOH intake and to prevent relapse in rats (Simon-O'Brien et al., 2015).

In addition, the HDACi MS-275 is able to decrease EtOH self-administration (by about 75%), to reduce EtOH consumption motivation (by 25% reduction) and relapse (by approximately 50%) (Jeanblanc et al., 2015). These results confirm the increasing therapeutic interest on these specific class of enzymes.

Using TSA, an HDACi selective for class I and II, a reduction of alcohol consumption, anxiety behavior and HDACs activity has been observed, with a reduction in HDAC2 protein levels (Sakharkar et al., 2014), suggesting that the HDAC2 isoform might play an important role in the epigenetic alterations observed in the comorbidity between alcohol dependence and anxiety.

Another study has shown as SAHA, another HDACi, is able to decrease the motivation to consume alcohol (Warnault et al., 2013).

All these studies strengthen the hypothesis that treatment with HDACi might prevent anxious behavior and excessive alcohol intake.

Moreover, HDAC inhibitors have also been shown to increase behaviors induced by psychostimulant exposure, suggesting synergic interactions between HDAC inhibitors and drugs of abuse (Adachi and Monteggia, 2009). For instance, Sanchis-Segura et al. demonstrated that sodium butyrate potentiates cocaine, alcohol and morphine-induced locomotor sensitization (Sanchis-Segura et al., 2009).

Regarding the connection between HDACi and BDNF, recent evidence has shown an enhanced BDNF expression after the inhibition of HDAC enzymes. Hydroxamic-based HDAC inhibitors have proved to be able to induce BDNF expression in an in vitro model of human neural progenitor cells-derived neurons, both at gene expression and protein levels (Bagheri *et al.*, 2019). In another study an enhanced BDNF expression after HDAC2 knockdown in the cervical cord of a mouse model has been observed (Sada et al., 2020). All this evidence underlines that HDACi, enhancing BDNF expression, could be useful for the treatment of a pathology in which there is a downregulation of this neurotrophin.

The HDAC inhibitor sodium butyrate (NaBut) is able to cross the BBB and it exhibits anti-inflammatory, neuroprotective and antidepressant-like effect. Also, it is well known for improving memory functions, even in an advanced phase of disease progression (Fernando et al., 2020; Govindarajan et al., 2011; Schroeder et al., 2007; Valvassori et al., 2014). It has shown an antimaniac properties reverting the behavioral alteration induced by ouabain, such as some parameters related to locomotor activity and it is able to revert oxidative stress alterations in an animal model of mania induced by ouabain (Valvassori et al., 2016). All this evidence suggests that sodium butyrate may be used as a mood stabilizer and in pathologies characterized by impaired memory.

1.5 NEUROFILAMENTS

Neurofilaments (NFs) belong to the class of intermediate filament proteins and are neuronal cytoskeletal protein that exert structural support for axon maintaining the shape of neurons.

The true role of NFs has been debated for years, and it appears to be much more than just a structural role, suggesting that these cytoskeletal proteins are integral components of synapses and modulators of neurotransmission (Yuan et al., 2015).

Three main isoforms exist for the NFs (light (NF-L), medium (NF-M) and heavy (NF-H), according to the different molecular weight) in which the structure has some common features; a conserved central α-helical rod region, a variable head domain at the N-terminal end and a variable tail at the C-terminal (Yuan and Nixon, 2016).

These proteins are expressed exclusively in neurons (Yuan et al., 2017), and are considered as promising biomarkers for neuronal damage, since it has been observed that they are released in

the extracellular spaces and consequently into the cerebrospinal fluid (CSF) and in the blood after a neuron-axonal injury (Khalil et al., 2018).

Khalil et al, explained that first-generation (immunoblots) and second-generation (ELISA) immunoassays can detect neurofilaments in the CSF but these techniques have limited sensitivity for detection in the blood, while the third-generation (electrochemiluminescence) and, in particular, fourth-generation (single-molecule array, SiMoA) immunoassays can reliably measure blood levels of NF-L (Figure 5), allowing to detect changes in pathological conditions (Khalil *et al.*, 2018).

For this reason, NF-L is studied nowadays as a biomarker for different pathologies (Gaetani et al., 2019), such as multiple sclerosis (Teunissen and Khalil, 2012), Alzheimer's disease (de Wolf et al., 2020), drug abuse (Liu et al., 2021), schizophrenia and depression (Bavato et al., 2021).

In the field of addiction or SUD, chronic opioid abusers showed to have reduce levels of NFs in prefrontal cortex (Ferrer-Alcon et al., 2000; Garcia-Sevilla et al., 1997) and recently, in ketamine users higher blood levels of NF-L in comparison to controls were found (Liu *et al.*, 2021).

Regarding MDMA, García-Cabrerizo et al. have demonstrated that this substance decrease NFs levels in rat hippocampus (Garcia-Cabrerizo and Garcia-Fuster, 2015). The authors suggested that NFs reduction could reflect neural injury such as apoptotic mechanisms (Garcia-Cabrerizo and Garcia-Fuster, 2015).

Other substances have been shown to decrease NFs levels: cocaine and morphine decreased NFs in ventral tegmental area of rats (Beitner-Johnson et al., 1992), methamphetamine reduced NF-L in striatum of mice (Sanchez et al., 2003) and nicotine reduced NFs in rat ventral tegmental area (Bunnemann et al., 2000).

All this evidence underlines that NFs are involved in different psychiatric disorders and are modulated by different substances involved in drug dependence. Furthermore, little is known about the effect of MDMA on these neuronal proteins. Since MDMA is known to cause neurotoxicity and it is involved in the development of psychiatric symptoms, NFs could also be used as a biomarker not only in neurological disorders but also in the condition of neuronal damage following the use of this specific substance.

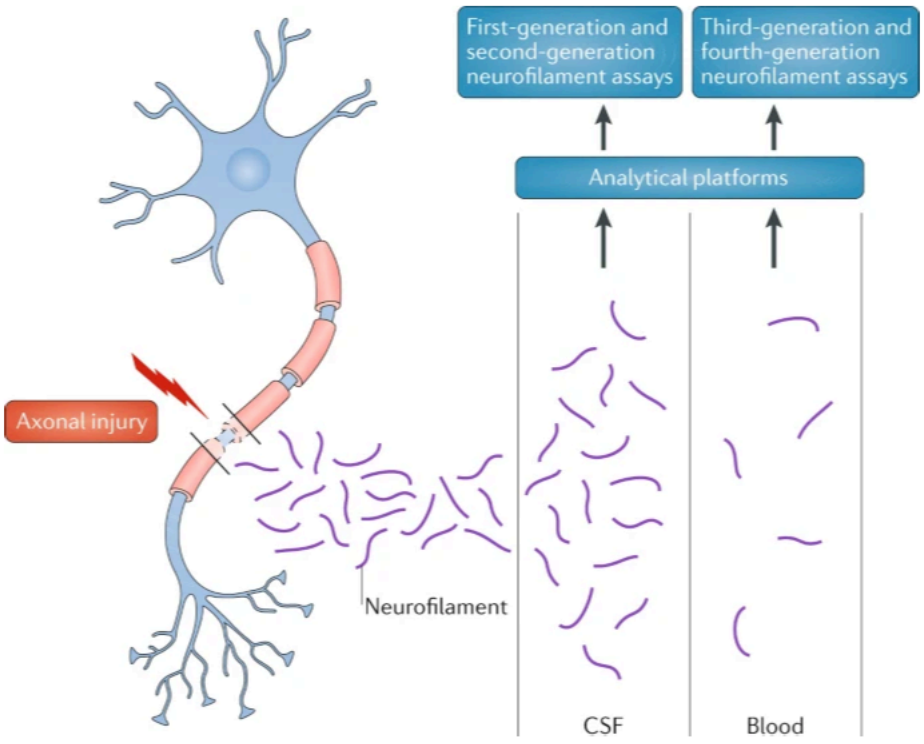


Figure 5. Axonal damage induces the release of NFs, which can be detected in the cerebrospinal fluid and blood. (Khalil *et al.*, 2018)

2. AIM OF THE RESEARCH

Based on the evidence reported in the introduction, the first objective of this PhD thesis was to investigate the effects of acute and repeated MDMA treatment on BDNF/TrkB gene expression and HDAC enzymes (gene expression and protein levels) in animal models. According to recent promising evidence about HDAC inhibitors, we decided to use the HDACi sodium butyrate to investigate the ability of this drug to affect MDMA-induced molecular and behavioral alterations.

In addition, considering that an alteration of BDNF has been reported in different brain regions of animals treated with psychoactive substances and in the blood of addictive substances abusers (Mouri et al., 2017; Hemmerle et al., 2012; Martinez-Turrillas et al., 2006; Angelucci et al., 2010; Ren et al., 2016; Autry and Monteggia, 2012; Ren et al., 2016; Li and Wolf,205), possible alterations of this neurotrophin levels were evaluated in blood samples from MDMA users, to better investigate the relationships between MDMA effects and BDNF. Since different BDNF pools appear to exist in plasma and serum (Gejl *et al.*, 2019), and their link with central BDNF it is not yet clarified, distinct determination of the neurotrophin were carried out in both matrices.

Recent evidence has shown that neurofilaments can represent valid biomarkers of neural damage. Taking into account the neurotoxic effects of ecstasy, we finally decided to investigate neurofilaments in an in vitro model of serotonergic neuronal cell line. In particular, we first assessed MDMA effects on neurofilament proteins in differentiated RN46A serotonergic neurons then, we investigated whether BDNF could protect serotonergic neurons from MDMA effects.

3. MATERIAL AND METHODS
3.1 ANIMAL STUDIES

3.1.1 ACUTE AND REPEATED MDMA STUDY

3.1.1.1 Animals

The experiments were carried out on male Sprague-Dawley rats weighing approximately 300g. The animals were kept in a controlled environment: lighting was automatically adjusted in cycles of 12 hours of light (7-19) and 12 hours of dark (19-7) and the temperature kept constant at 21±2°C. The animals were fed a standard pelleted diet and ad libitum water.
Prior to their experimental use, rats were handled once a day. After this period the animals were subjected to the treatments.
All experiments were carried out in accordance with the European Communities Council Directives on the protection of animals used for scientific purposes of (86/609/EEC – 2010/63/EU) and Italian National (Ministry of Health, Italy) laws and policies. This study has been approved by the "Ethic Scientific Committee for the Animal Experiments" of the University of Bologna.

3.1.1.2 Drugs and Treatments

MDMA was dissolved in 0.9% NaCl (saline) and it was supplied from the National Institute on Drug Abuse/National Institutes of Health (Research Triangle Institute, Research Triangle Park, NC, USA). The dose of MDMA was selected based on our previous study (Caputi et al., 2016; Di Benedetto et al., 2006).
Rats were divided into four experimental groups, and subjected to acute and repeated treatment with MDMA intraperitoneally (i.p.) according to the following scheme:

- acute control group: a single injection of saline solution
- acute treatment group: a single injection of MDMA (8 mg/kg, in a volume of 2 ml/kg)
- repeated control group: two injections of saline per day, for one week;
- repeated treatment group: two injections a day for 7 days of MDMA (8 mg/kg, in a volume of 2 ml/kg).

3.1.1.3 Tissue collection, RNA extraction and qRT-PCR

Two hours after the last administration, the animals were sacrificed and the brains were rapidly dissected on ice. The areas of interest, PFCX and HIPPO, were immediately frozen on dry ice and stored at -80°C until analysis.

The RNA extraction from the tissues was performed according to the method of Chomczynski and Sacchi (Chomczynski and Sacchi, 2006). Briefly, frozen tissue was homogenized in 1 mL of TRIZOL Reagent (Life Technologies, USA) and incubated at room temperature for 5 minutes. Then, 200 µL of chloroform were added to the homogenates, samples were transferred to 1.5 mL conical bottom tubes and incubated for 10 minutes at room temperature. The samples were then centrifuged at 12000 rpm for 15 minutes at 4°C. At the end of the centrifuge, were obtained three different phases: an underlying pink organic phase, a central lipid layer and finally at the top of the tube a colorless phase containing RNA. This latter is removed and transferred to a new 1.5 mL tube and about 500 µL of isopropanol was added. Samples are vortexed and incubated for 10 minutes at room temperature. After incubation, samples are centrifuged at 12,000 rpm for 10 minutes at 4°C. At the end of the centrifuge, a pellet is formed; the supernatant liquid is removed and the pellet is washed with 1 mL of 75% EtOH in a centrifuge at 8000 rpm for 10 minutes at 4 °C. After the complete removal of EtOH, the pellet is dissolved in 25 µL of nuclease-free water. The extracted RNA is stored at -20 °C.

The integrity of RNA was checked by 1% agarose gel electrophoresis and the amounts of RNA were determined by measuring optical densities. Only RNA samples with a ratio of OD260/OD280 from 1.8 to 2 were used. Total RNA was reverse transcribed using GeneAmp RNA PCR kit (ThermoFisher Scientific, USA), following the manufacturer's instructions. Quantitative real-time PCR was performed on a StepOne Real-Time PCR System (Life Technologies, USA) using SYBR Green (SYBR Green Master Mix, Applied Biosystem).

All samples were run in triplicate and were normalized to the endogenous reference gene glyceraldehyde- 3-phosphate dehydrogenase (GAPDH).

Relative abundance of each mRNA species was analyzed with the method of Delta–Delta Ct ($\Delta\Delta$Ct) and converted to relative expression ratio ($2^{-\Delta\Delta Ct}$) for statistical analysis.

The primers used were designed using Primer 3, and the sequences are reported in the table 1.

	Forward (5'- 3')	*Reverse (5'- 3')*
BDNF	AAGTCTGCATTACATTCCTCGA	GTTTTCTGAAAGAGGGACAGTTTAT
Trk-B	AAGTTCTACGGTGTCTGTGTG	TTCTCTCCTACCAAGCAGTTC
HDAC 1	GATCGGCTAGGTTGCTTCAA	CAGCACCGAGCGACATTAC
HDAC 2	GCTGTCCTCGAGCTACTGAAA	GTCATCACGCGATCTGTTGT
HDAC 3	ATGCTGAAGAGAGGGGTCCT	TCATAGAATTCATTGGGTGCTTC
HDAC8	TGCCCTGCATAAACAAATGA	GATAGGCATCAGTGTGGAAGG
GAPDH	AGACAGCCGCATCTTCTTGT	CTTGCCGTGGGTAGAGTCAT

Table 1 Primer sequences (Eurofins Genomics) of the selected gene used for qRT-PCR.

3.1.1.4 Protein extraction and Western Blot

Extraction of nuclear proteins from PFCX and HIPPO was performed according to the manufacturer's instructions suggested in the Histone Deacetylase (HDAC) Activity Assay Kit (Fluorometric) (Abcam, ab156064). Briefly, the brain tissue was then homogenized, using a dounce homogenizer, in 1 mL of Lysis Buffer (10 mM Tris-HCl pH 7.5, 10 mM NaCl, 15 mM MgCl2, 250 mM Sucrose, 0.5% NP-40, 0.1 mM EGTA). The homogenate thus obtained was transferred to microtube and vortexed for 10 seconds and then kept on ice for 15 minutes. After the incubation, the sample was centrifuged on a 4 mL sucrose cushion (consisting of: 30% sucrose, 10 mM Tris HCl pH 7.5, 10 mM NaCl, 3 mM MgCl2) at 1,300g for 10 minutes at 4°C. At the end of the centrifugation, the pellet obtained was washed using 1-2 mL of washing solution (10 mM Tris HCl pH 7.5, 10 mM NaCl). After washing, the solution was removed and the resulting pellet was resuspended in 200 µL of Extraction Buffer (50 mM HEPES KOH pH 7.5, 420 mM NaCl, 0.5 mM EDTA Na2, 0.1 mM EGTA, 10% glycerol). The resulting sample was sonicated for 30 seconds and then incubated for 30 minutes on ice. After incubation, the sample was centrifuged at 20,000g for 10 minutes and the supernatant, containing the nuclear extract, was collected and stored at -80 °C. Nuclear extract was then quantified by Bradford method and the absorbance was revealed by TECAN GENios instrument at a wavelength of 595 nm.

The amount of HDAC proteins belonging to class I (HDAC1, HDAC2) in the single extracts, were then investigated by Western Blotting. Samples were prepared by adding an appropriate volume of 4x Laemmli sample buffer (Bio-Rad Laboratories) and boiled for 5 minutes prior to electrophoresis to allow for protein denaturation. An equal amount of protein (20 µg) from each

sample was separated into appropriate Mini-PROTEAN® TGX Stain-FreeTM Protein Gels (BIORAD, #456-8034), placed in the electrophoretic chamber filled with running Buffer (Tris Base, Glycine and SDS). Together with the samples, 5 µl of marker (Thermo Fisher Scientific, LC5925) was loaded into each gel to allow identification of the molecular weights of the individual proteins analyzed. At the end of the electrophoretic run, the proteins were transferred onto nitrocellulose membranes (Thermo SCIENTIFIC, 88018). The membranes were recovered and incubated with a 0.1% (w/v) Ponceau red solution in 5% (v/v) acetic acid to assess the successful transfer of proteins.

After washing with PBS Tween, the blots were blocked for 1 hour at room temperature with 5% skim milk solution in PBS Tween and incubated overnight at 4 °C with the respective primary antibodies (Table 2).

At the end of the incubation, the membranes were washed three times with PBS Tween and incubated for 1 hour at room temperature with the HRP-conjugated secondary antibody (Table 2).

The signal was visualized by chemiluminescence using the Chemidoc MP Imaging System (Bio-Rad Laboratories). The intensity of the bands was quantified using Image J software and the incubation of the filter with the antibody directed towards the actin structural protein allowed the loading of the different protein extracts into the polyacrylamide gel to be assessed.

Name	**Code**	**Company**	**MW (kDa)**	**Diluition**
Anti-HDAC1	# 06-720	MILLIPORE	~65	1:500
Anti-HDAC2	ab16032	Abcam	~60	1:500
Anti-ACTIN	A2066	SIGMA-ALDRICH	~ 43	1:1000
Anti-Rabbit	NA934V	GE Healthcare UK Ltd	-	1:3000

Table 2. Antibodies used in western blot analysis

3.1.1.5. Statistical Analysis

Data are expressed as mean ± standard error (SEM) and analyzed by statistical analysis using Student t-test. The GraphPad Prism 7 program (GraphPad Software, San Diego California USA) was used for statistical analysis. The level of statistical significance was set at $p < 0.05$ compared to the control group.

3.1.2 SODIUM BUTYRATE AND MDMA STUDY

3.1.2.1 Animals

The experiments of this section were carried out in collaboration with Prof. Roberto Ciccocioppo of the University of Camerino.

For this study, male Wistar rats (CHARLES RIVER) weighing approximately 300g were used. Animals were kept in a controlled environment: lighting was automatically adjusted in cycles 12 h/12 h light/dark (lights off at 8:00 am) and the temperature was kept constant at 20±2°C. The animals were fed a standard pelleted diet and ad libitum water.

Prior to their experimental use, rats were handled once a day. After this period the animals were subjected to the treatments. All experimental sessions were conducted during the nocturnal phase of the light/dark cycle.

3.1.2.2 Drugs and Treatments

MDMA and Sodium butyrate (Sigma-Aldrich, Germany) were dissolved in saline solution (0.9% NaCl). The cumulative dose of NaBut was selected based on previous studies (Blank et al., 2015; Levenson et al., 2004; Reolon et al., 2011).

The experiments were conducted on male Wistar rats. The rats were divided into 4 experimental groups and received saline, MDMA (8 mg/kg twice a day for 7 days), NaBut (630 mg/kg twice a day for 7 days) or NaBut plus MDMA (twice a day for 7 days) intraperitoneally (i.p.) according to the following scheme (Figure 6):

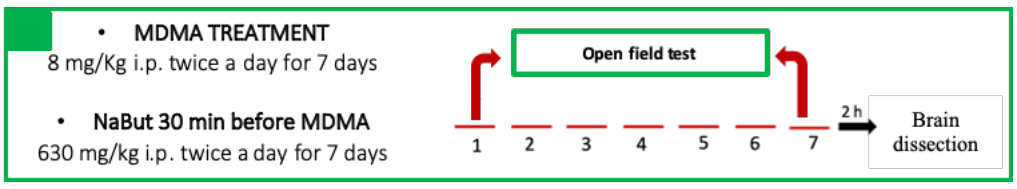


Figure 6. Treatment schedule

Sodium butyrate was administered 30 minutes before MDMA.

After the first drug administration (acute treatment) and after 7 days of treatment, behavioral and biochemical analysis were carried out.

3.1.2.3 Behavioral test (Open field)

The spontaneous locomotor activity was assessed by the open field test (OFT). For OFT, the rats were placed in an arena (44x44 cm) delimited by plexiglas wall. The movement of the animals was recorded by infrared photobeam interruptions to obtain different parameters such as the number of entries in the central zone of arena. The open field was conducted during the nocturnal phase in a room illuminated by a red light and the rats received an i.p. injection of the drug 15 min before the beginning of the test.

Wistar rats were placed in the center of the arena and their activity was recorded for 20 min. Ambulatory time, distance travelled, and number of entries in central zone (11.7x11.7 cm) were evaluated.

3.1.2.4 Tissue collection, RNA extraction and qRT-PCR

See section 3.1.1.3.

3.1.2.5. Statistical Analysis

All experimental results were expressed as mean ± SEM and data were analyzed by *t*-test or by one-way ANOVA followed by Newman-Keuls multiple comparison post hoc test, as appropriate. $P < 0.05$ were considered statistically significant. Statistical analysis was performed using GraphPad Prism software package version 7.

3.2 HUMAN STUDIES

3.2.1 Recruitment and inclusion criteria

The recruitment of participants, 25 MDMA users (10 males and 15 females, mean age 29.5±7.0 years) and 25 MDMA-naïve controls (10 males and 15 females, mean age 29.7±6.4 years), was performed by the group of Experimental and Clinical Pharmacopsychology of Boris B Quednow, at the Department of Psychiatry, Psychotherapy, and Psychosomatics of the Psychiatric Hospital of the University of Zurich, by the PhD student Josua Zimmermann. The participants were recruited via online media and in cooperation with local institutions involved in drug prevention/ information and harm reduction (e.g. Saferparty, Streetwork Zurich). For each subject, samples of serum and plasma were collected to study the BDNF levels, and the analysis were performed in both samples.

The general inclusion criteria were enrollment of women and men with an age range from 18 to 40 years old, able to read, understand and write. In addition, women had to be on stable use of contraceptives to minimize the hormonal effects.

Regarding the group of drug users, they had to satisfy the criteria for a current substance use disorder according to DSM-5 (at least presence of two symptoms occurring within a 12-month period) and they had been using MDMA > 50 occasion and have an abstinent period inferior to 3 months.

In addition, subjects had no history of heroin injections, no polytoxic drug use, no DSM-IV/5 Axis I adult psychiatric disorders - except for alcohol, cannabis, nicotine or stimulant use disorders -, no attention deficit hyperactivity disorder, and no previous depressive episodes.

The control group inclusion criteria were no illegal drug use: less than 15 occasions per substance (including MDMA and METH) and not during the past 12 months (with the exception of cannabis) and no DSM-IV/5 Axis I adult psychiatric disorders (except for nicotine use disorder).

The general exclusion criteria were family history of genetic mediated (h^2> 0.5) psychiatric disorders according to DSM-IV/5, presence of severe neurological disorder or brain injury, current diagnoses of infectious diseases or severe somatic disorder. In addition, intake of medication with potential action at the central nervous system during the last seven days, daily use of cannabis and, for women, no pregnancy or breastfeeding were added as general exclusion criteria.

Each participant self- reported the MDMA abstinence period, resulting in the variable named “MDMA days since last consumption”, and this variable was used for correlation analysis.

Additional criteria related for discontinuation from inclusion were: non-compliance of abstinence for illegal drugs assessed by self-reporting and urine analysis (>72 hours), alcohol (>24 hours), and caffeine containing drinks (>1 hour) previous to measurement onset.

3.2.2 Samples preparation

Serum and plasma samples were prepared from whole blood samples that were collected in tubes with anticoagulant for the preparation of plasma and in tubes with non-additive for the preparation of serum.
To obtain plasma, whole blood was centrifuged at 2000rpm for 15 min at 20°C, while to obtain serum before the centrifuge with the same parameter, it was allowed samples to clot for 30 minutes.

3.2.3 BDNF Elisa assay

BDNF levels of serum and plasma from 25 MDMA users and 25 MDMA naïve controls subjects were measured via Enzyme-Linked Immunoassorbent Assay (ELISA). Samples were run in triplicate. The assay was performed using the RayBio Human BDNF ELISA Kit (code ELH-BDNF, RayBiotech) following the manufacture's instructions. Briefly, samples were diluted property and standards were loaded into the wells and the BDNF present in the samples were bound to the wells by the immobilized antibody. The wells were washed 4 times and then the biotinylated anti-BDNF antibody was added. After washing away the biotinylated antibody, the HRP-conjugated streptavidin was added to the wells. After another wash, the TMB substrate was added to the well and a blue color developed in proportion to the amount of BDNF. The stop solution was added to end the reaction and the color changed from blue to yellow. The intensity of the color was measured at 450nm with the ELISA reader Mithras^2 LB 943 and a five-parameter logistic curve was used to create the standard curves. The total protein amount in the samples, to normalize BDNF measurements, was quantified using the Bradford method and bovine serum albumin as a standard.

3.2.4 Statistical Analysis

All statistical analyses were performed using SPSS STATISTICS v.27. BDNF levels were compared using t test and gender differences were determined using 2*2 (group*sex) ANOVAs. Possible associations between the variables (serum BDNF levels, plasma BDNF levels and MDMA days since last consumption) were analyzed by Pearson's correlations. Results with $p < 0.05$ were considered statistically significant.

Two subjects (1 in MDMA group for serum BDNF levels, 1 in MDMA group for plasma BDNF levels) were excluded as outliers (>mean + 3SD).
Because the distribution in the total levels of BDNF PLASMA (MDMA group + control group) and in the MDMA days since last consumption were not normally distributed, the log-transformed values (log_{10}) were used for statistical analysis.

3.3 IN VITRO STUDIES

3.3.1 Reagents

DMEM/F-12 (11320033, Gibco™), FBS (16000036, Gibco™), poly-D-lysine (P0899, Sigma Aldrich), laminin (code 23017015, Gibco™), trypsin-EDTA 0.25% (25200056, Gibco™), neurobasal medium (21103049 Gibco™), B27 (17504044, Gibco™), PBS with calcium and magnesium (14040133, Gibco™), PBS, pH 7.4 w/o Calcium and magnesium (10010015, Gibco™), LookOut Mycoplasma PCR Detection kit (Thermofisher). D,l-MDMA.HCl (Lipomed) and BDNF (B3795, Thermofisher), Cell Proliferation Kit I (MTT) (11465007001, Roche), MAP-2 (diluition 1:1000, 188 004 Synaptic Systems), Nestin (MERKMAB5326, Merk millipore), anti NF-L antibody (2835, Cell signaling) ,anti NF-H antibody(2836, Cell signaling), anti NF-M antibody (ab254125, abcam), DAPI (ab228549, abcam), Alexa Fluor® 647 AffiniPure Donkey Anti- Guinea Pig IgG 800X (Jackson ImmunoResearcj), DAKO Fluorescence Mounting Medium (S3023, Agilent).

3.3.2 Cell culture

Rat brain raphe nucleus RN46A cell line was obtained from the laboratory of Prof. Whittemore (Kentucky Spinal Cord Injury Research Center).
The cells (passage 21-27) were maintained in culture a 33°C in DMEM/F-12 (11320033, Gibco™) medium supplemented with 10% of FBS (16000036, Gibco™) in six-well cell culture plates (83.3920, Sarstedt). For new passages, at approximately 80% of confluency, the cells were removed by adding Trypsin-EDTA (25200056, Gibco™). To induce differentiation, RN46A cells (10000 cells/well) were seeded for 24h with DMEM/F-12 in 96-well plate (Nunc 167008, Thermofisher) coated with poly-D-lysine (P0899, Sigma Aldrich) and laminin (code

23017015, Gibco™). Then, the medium was changed with neurobasal medium (21103049 Gibco™) supplemented with B27 (17504044, Gibco™) and after an adaptation period of 24h at 33°C, the temperature was shifted to 37°C, to initiate the differentiation. All the experiments were performed after 8 days of differentiation induction. The neurobasal medium was changed every 3 days and the cells were checked for mycoplasma contamination with the LookOut Mycoplasma PCR Detection kit (Thermofisher).

3.3.4 Cell viability assay

D,l-MDMA.HCl (Lipomed) and BDNF (B3795, Thermofisher) were dissolved in sterile water to create a stock solution at a concentration of 20 mM and 2000ng/ml, respectively. For each experiment, fresh stock solution was diluted in culture medium (supplemented by B27) to obtain the final concentration of MDMA (1.3mM) and BDNF (100 ng/ml).
The neurotoxicity and the 50% growth inhibitory concentration (IC_{50}) of RN46A was measured after exposing RN46A differentiated cells to different concentration of MDMA (0.25-0.5-1-1.5-2 mM) for 24h and 48h. For the pre-treatment studies, the cells were pre-treated with 100ng/ml BDNF 1h prior to the MDMA treatment that was set to 1.3mM (dose ~ IC_{50}). After 24h and 48h of the treatments, cell viability was evaluated by MTT assay (see details under). All the experiments were performed at least in quadruplicates.

3.3.4.1 MTT assay

The MTT assays were performed following the Cell Proliferation Kit I (MTT) (11465007001, Roche) manufacture's protocol. Briefly, after the treatments, the cells were incubated with the MTT solution (final concentration 0.5 mg/ml) at 37°C for 4 hours. Then, the solubilisation buffer was added and the well plate was allowed to stay in the incubator overnight to ensure that the formazan crystals were dissolved.
The optical density was measured at 570 nm with a reference wavelength of 670 nm, using the Mithras2 LB 943 Multimode Reader (Berthold Technologies). Cell viability was reported as a percentage of control.

3.3.5 Immunofluorescence assay

For immunocytochemistry experiment, RN46A cells (10000cells/well) were seeded in 96-wellplates (Nunc 96 wellplate 167008, Thermofisher) coated with poly-d-lysin and laminin. After 8 days under differentiation conditions, the cells were pretreated with 100 ng/ml BDNF or vehicle 1h prior to 1.3mM (IC50) of MDMA or vehicle for 24h and 48h. At the end of the treatment, the cells were washed with PBS and fixed with 4% of paraformaldehyde for 20 minutes at room temperature, washed three times with PBS and permeabilized with blocking buffer solution (0.1%of Triton X-100, 1% BSA in PBS).

The cells were incubated with MAP-2 (dilution 1:1000, 188 004 Synaptic Systems), NF-L (1:50), NF-H (1:100), NF-M (1:100) overnight at 4°C. After 3 washing with PBS, cells were incubated with secondary Donkey anti-guinea pig IgG Alexa Fluor 647 800X (Jackson ImmunoResearch) and Goat anti-mouse IgG Alexa Fluor 555 (Life Technologies A21127), for 30 min at RT and mounting with medium containing 4′,6-diamidino-2-phenylindole (DAPI, 1:100) (abcam). The dilution of secondary antibody was the same of the primary antibody respectively.

Immunofluorescence was detected with the cellSens Dimension software on an Olympus fluorescent microscope. For each antibody, 2 independent experiments, performed in triplicate (five pictures per each well), were analyzed.

3.3.6 Statistical analysis

All statistical analyses were performed using Prism version 7.0 (GraphPad, San Diego, CA, USA). To normalize the data of the effect of BDNF and MDMA treatment of RN46A serotonergic neurons, it was used the mean of the control values of two independent experiments resulting similar in OD values. Quantitative data were expressed as mean ± SD. After testing for assumption of normality distribution, One-Way ANOVA followed by Tukey's multiples comparison or Kruskal-Wallis followed by Dunn's multiples comparison test were used to analyze the data. Results with $p < 0.05$ were considered statistically significant.

4. RESULTS

4.1 Animal studies

ACUTE AND REPEATED MDMA STUDY

4.1.1 Gene expression analysis of class I HDACs following acute and repeated treatment with MDMA in PFCX

In PFCX, gene expression analysis of animals acutely treated with MDMA showed a significant reduction in the gene encoding for HDAC1, HDAC2 and HDAC3 compared to vehicle (Acute MDMA: HDAC1, 0.66 ± 0.07 vs Vehicle, 1.00 ± 0.07, $p < 0.01$; HDAC2, 0.67 ± 0.09 vs Vehicle, 1.00 ± 0.08, $p < 0.05$; HDAC3 0.70 ± 0.07 vs Vehicle, 1.00 ± 0.10; $p < 0.05$) (Fig.7 a, c and e). No significant gene expression alteration was found for HDAC8 after acute MDMA treatment (Fig.7g). By contrast, animals receiving MDMA repeated treatment showed a statistically significant increase in HDAC1, HDAC2, HDAC3 and HDAC8 gene expression levels compared to the control group (Repeated MDMA: HDAC1, 1.65 ± 0.11 vs. Vehicle, 1.00 ± 0.08, $p < 0.01$; HDAC2, 2.04 ± 0.19 vs Vehicle, 1.00 ± 0.16, $p < 0.01$; HDAC3 1.59 ± 0.17 vs Vehicle, 1.00 ± 0.12 $p < 0.05$; HDAC8, 2.01 ± 0.12 vs Vehicle, 1.00 ± 0.08; $p < 0.001$) (Fig. 7 b, d, f and h).

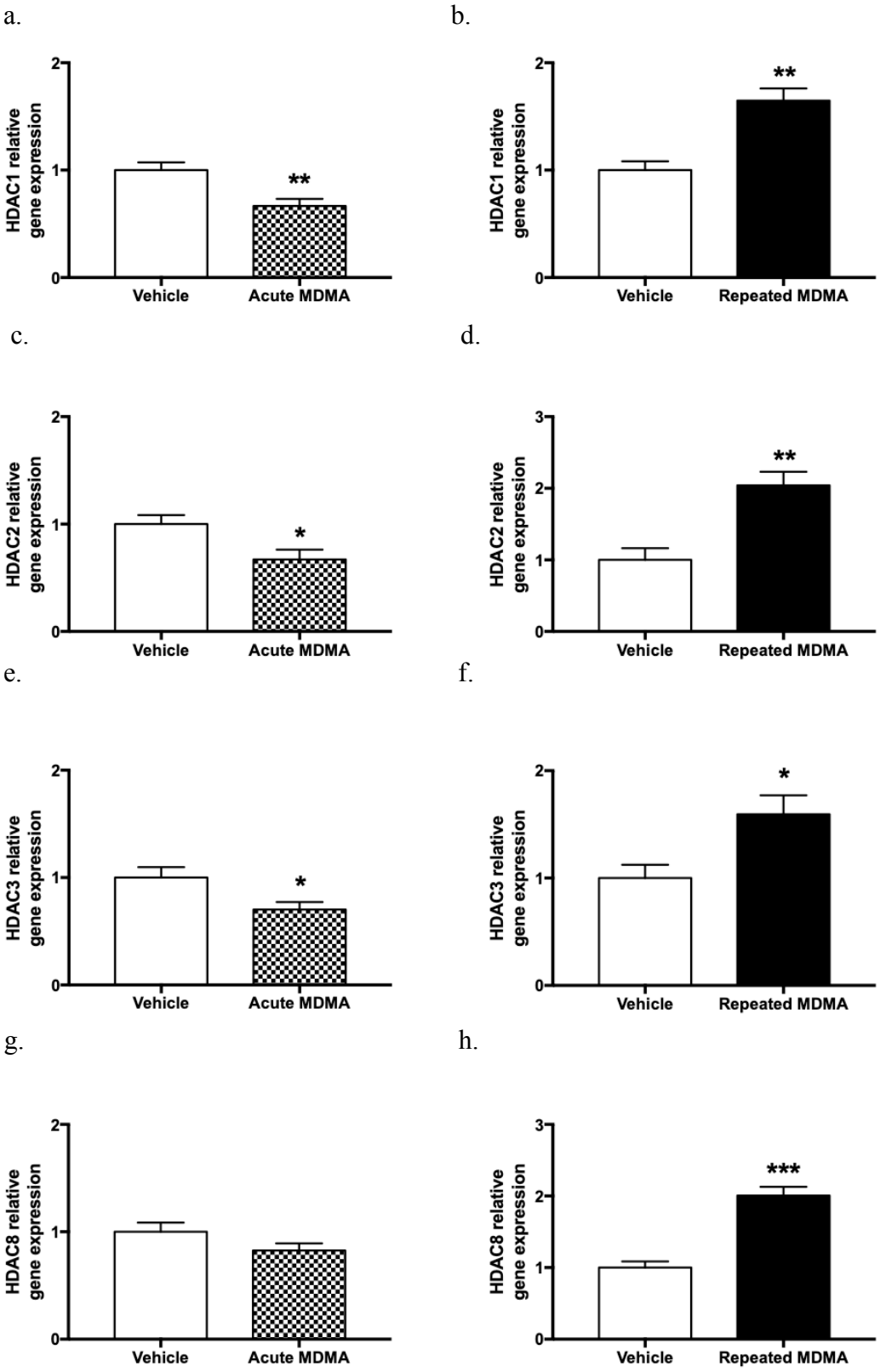


Figure 7. Relative gene expression of class I HDACs in PFCX after acute and repeated MDMA treatment. Data represent 2^{-DDCt} values calculated by DDCt method and are expressed as mean ± SEM (n=5/6 animals per group). *p < 0.05, ** p<0.01, ***p < 0.001 vs respective Vehicle; data analyzed by t test.

4.1.2 Protein levels of HDAC1 and HDAC2 in PFCX: Western Blot analysis

Statistical analysis revealed a significant increase in HDAC1 protein level in the PFCX of acute MDMA treated rats compared to vehicle group (Acute MDMA: HDAC1, 1.62 ± 0.1 vs Vehicle, 1.00 ± 0.23) (Fig.8a). On the other hand, no significant alteration of HDAC2 protein was observed in the group of animals receiving the same treatment (Fig.8c).
Student t test showed a significant decrease in HDAC1 protein level after repeated treatment with MDMA (Repeated MDMA: HDAC1, 0.38 ± 0.14 vs Vehicle, 1.00 ± 0.17) (Fig.8b), whereas no changes were observed for HDAC2 protein (Fig.8d).

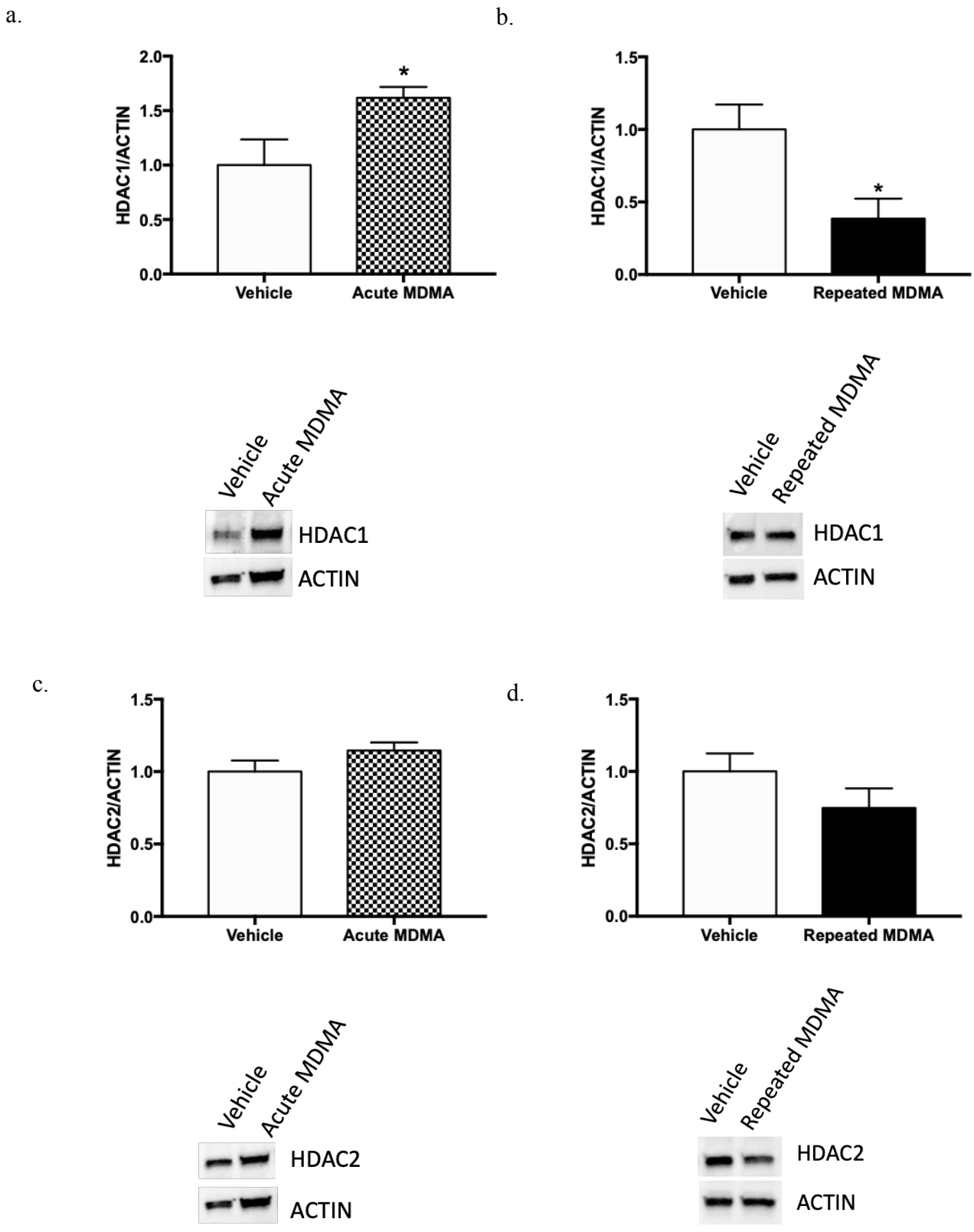


Figure 8. Nuclear contents of HDAC1 and HDAC2 protein levels in the PFCX after acute or repeated MDMA treatment: Western blot analysis. Under histogram bars, representative immunoblots are showed. Band intensities were quantified using ImageJ software and normalized to ACTIN protein levels. Data are expressed as mean ± SEM (n=5/6 animals per group). *$p<0.05$ vs Vehicle; data analyzed by t test.

4.1.3 Gene expression analysis of BDNF and TrkB following acute and repeated treatment with MDMA in PFCX

Gene expression analysis of BDNF after acute MDMA treatment showed a significant increase in the levels of the coding gene compared to control animals in PFCX (Acute MDMA: BDNF, 2.38 ± 0.43 vs Vehicle, 0.88 ± 0.07, $p < 0.01$) (Fig.9a), whereas no statistical differences were found in the level of the receptor TrkB (Fig. 9c).

After repeated MDMA treatment there was still an up-regulation of BDNF gene expression levels compared to controls and an increase mRNA levels of TrkB (Repeated MDMA: BDNF, 2.76 ± 0.49 vs. Vehicle, 1.00 ± 0.09, $p < 0.01$; TrkB, 1.78 ± 0.23 vs Vehicle, 1.00 ± 0.07, $p < 0.01$) (Fig. 9 b and d).

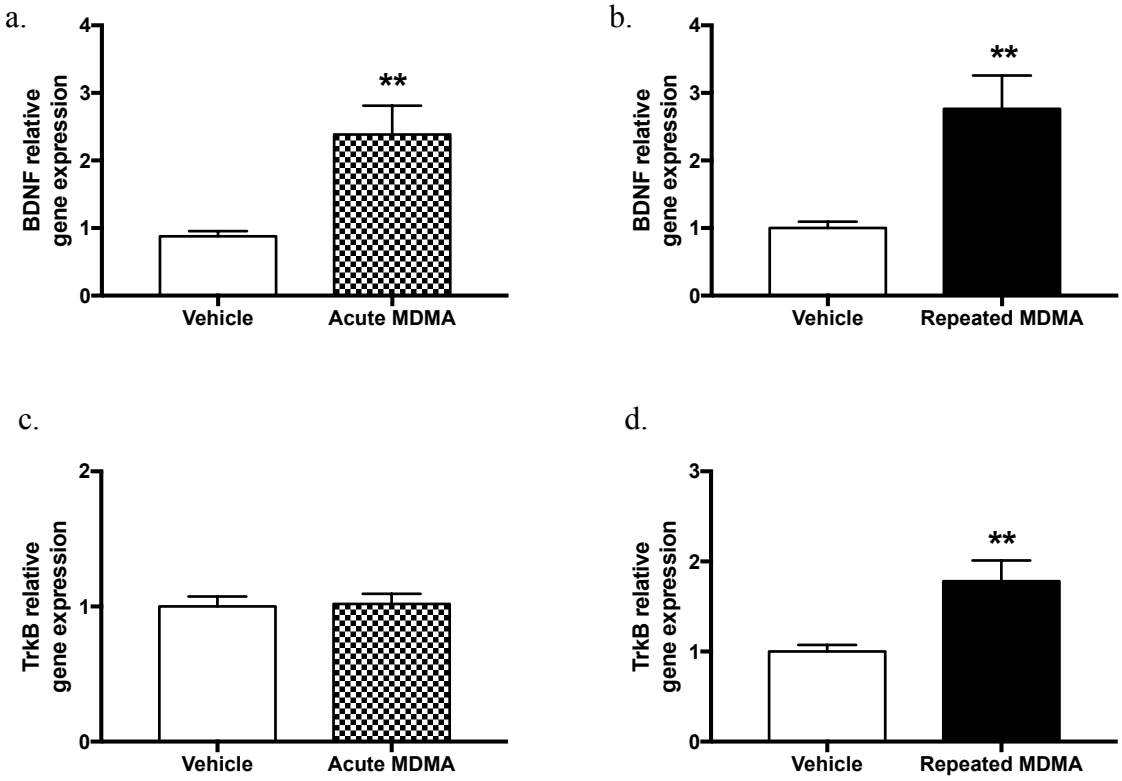


Figure 9. Relative gene expression of BDNF and TrkB in PFCX after acute and repeated MDMA treatment. Data represent 2^{-DDCt} values calculated by DDCt method and are expressed as mean ± SEM (n=4/6 animals per group). ** $p<0.01$ vs respective Vehicle; data analyzed by t test.

4.1.4 Gene expression analysis of class I HDACs following acute and repeated treatment with MDMA in HIPPO

In HIPPO, statistical analysis did not show any significant changes in the gene expression levels of HDAC1, HDAC2 and HDAC3 isoforms following acute MDMA treatment (Fig. 10 a, c and e). Differently, a significant downregulation was observed for HDAC8 isoform in animals receiving the same treatment (Acute MDMA: HDAC8, 0.70 ± 0.04 vs Vehicle, 1.00 ± 0.06, $p < 0.01$) (Fig. 10 g).

Data obtained after repeated MDMA treatment showed a significant down-regulation of the gene encoding for HDAC1 (Repeated MDMA: HDAC1, 0.74 ± 0.03 vs. Vehicle, 1.00 ± 0.09; $p < 0.05$) (Fig.10 b) and a significant up-regulation for the isoforms HDAC3 and HDAC8 (Repeated MDMA: HDAC3, 1.49 ± 0.11 vs Vehicle, 1.00 ± 0.06; $p < 0.01$; HDAC8, 1.37 ± 0.06 vs Vehicle, 1.00 ± 0.07, $p < 0.01$) (Fig.10 f and h). Differently, no significant alteration of HDAC2 gene expression was observed (Fig. 10 d)

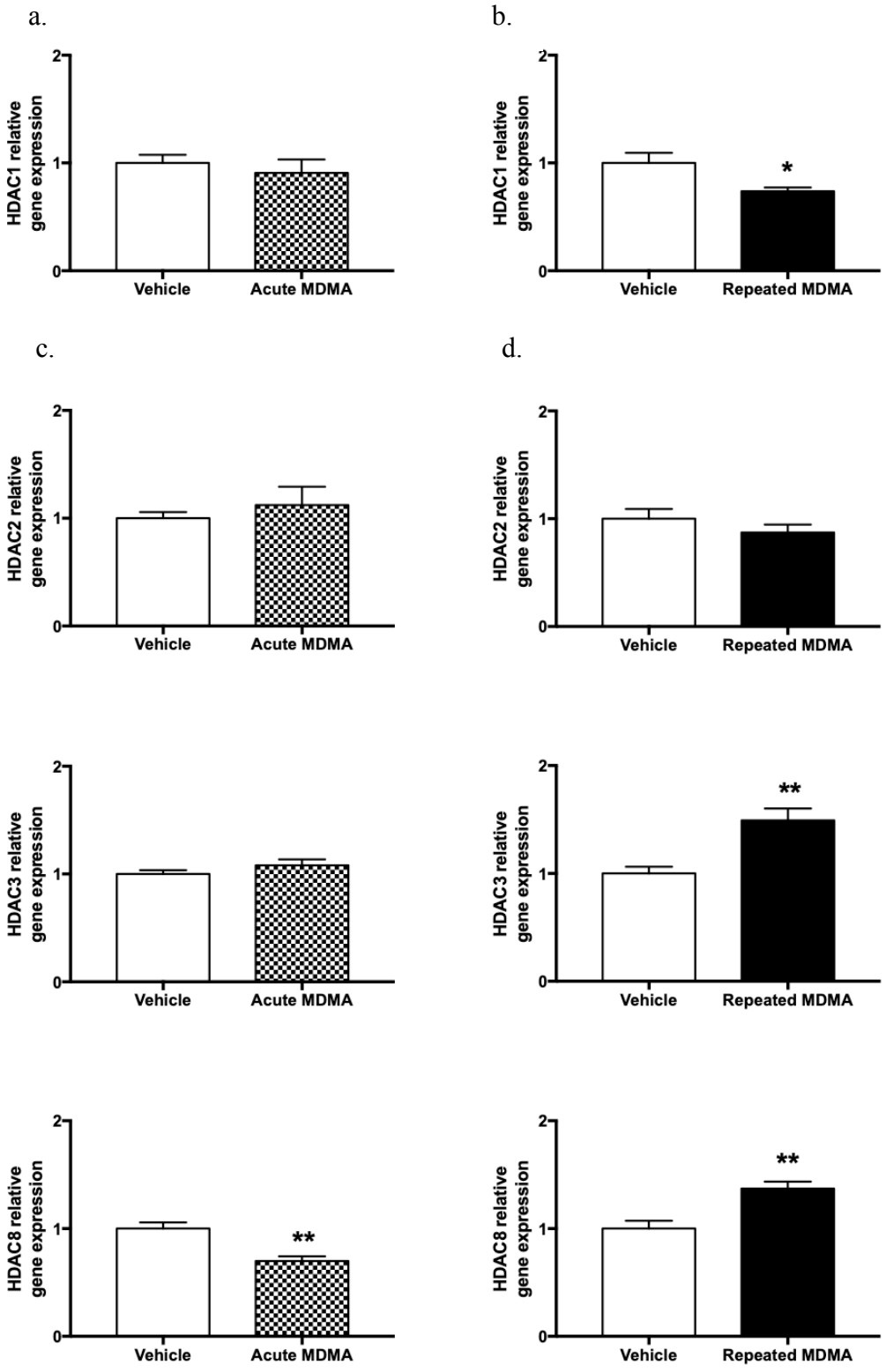


Figure 10. Relative gene expression of class I HDACs in HIPPO after acute and repeated MDMA treatment. Data represent 2^{-DDCt} values calculated by DDCt method and are expressed as mean ± SEM (n=5/6 animals per group). *p < 0.05, ** p < 0.01 vs respective Vehicle; data analyzed by t test.

4.1.5 Protein levels of HDAC1 and HDAC2 in HIPPO: Western blot analysis

In HIPPO, protein level analysis did not show any significant differences of both HDAC1 and HDAC2, after acute MDMA treatment (Fig. 11a and 11c). In the same way, no changes were observed for HDAC1 after a repeated treatment with MDMA (Fig.11b). Instead, a significant alteration of HDAC2 protein was observed in the group of animals receiving MDMA repeated treatment compared to vehicle group (Repeated MDMA: HDAC2 1.77 ± 0.33 vs Vehicle, 1.00 ± 0.13) (Fig.11d)

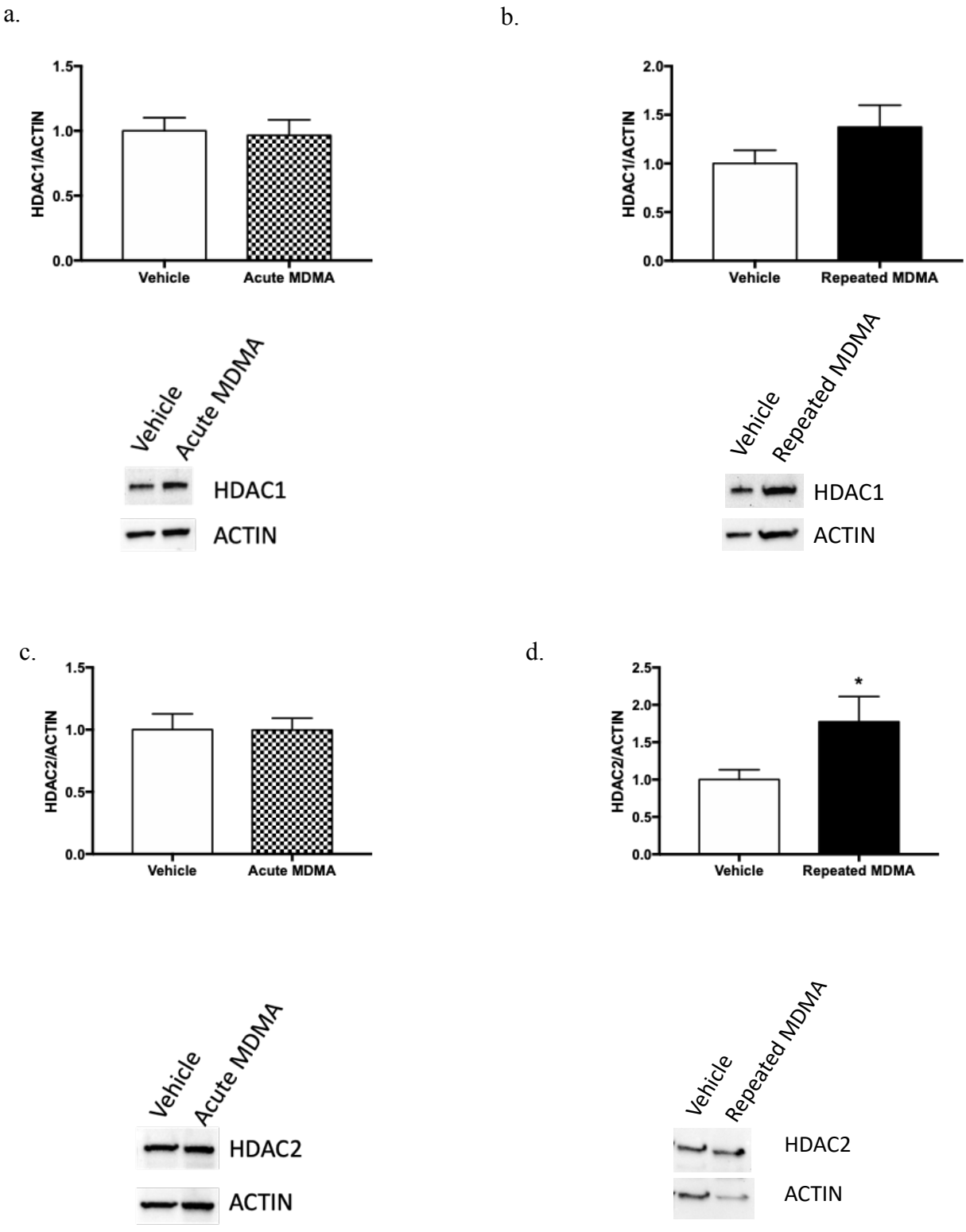


Figure 11. Nuclear contents of HDAC1 and HDAC2 protein levels in the HIPPO after acute or repeated MDMA treatment: Western blot analysis. Under histogram bars, representative immunoblots are showed. Band intensities were quantified using ImageJ software and normalized to ACTIN protein levels. Data are expressed as mean ± SEM (n=5/6 animals per group). *$p<0.05$ vs Vehicle; data analyzed by t test.

4.1.6 Gene expression analysis of BDNF and TrkB following acute and repeated treatment with MDMA in HIPPO

In HIPPO, t test analysis showed a significant decrease in the levels of BDNF mRNA after both acute and repeated MDMA treatment compared to control animals (Acute MDMA: BDNF, 0.60 ± 0.06 vs Vehicle, 1.00 ± 0.07, $p < 0.01$; Repeated MDMA: BDNF, 0.74 ± 0.08 vs Vehicle, 1.00 ± 0.07, $p < 0.05$), (Fig.12 a and b), whereas no statistical differences were observed in the level of the receptor TrkB after both acute and repeated MDMA treatment (Fig. 12 c and d).

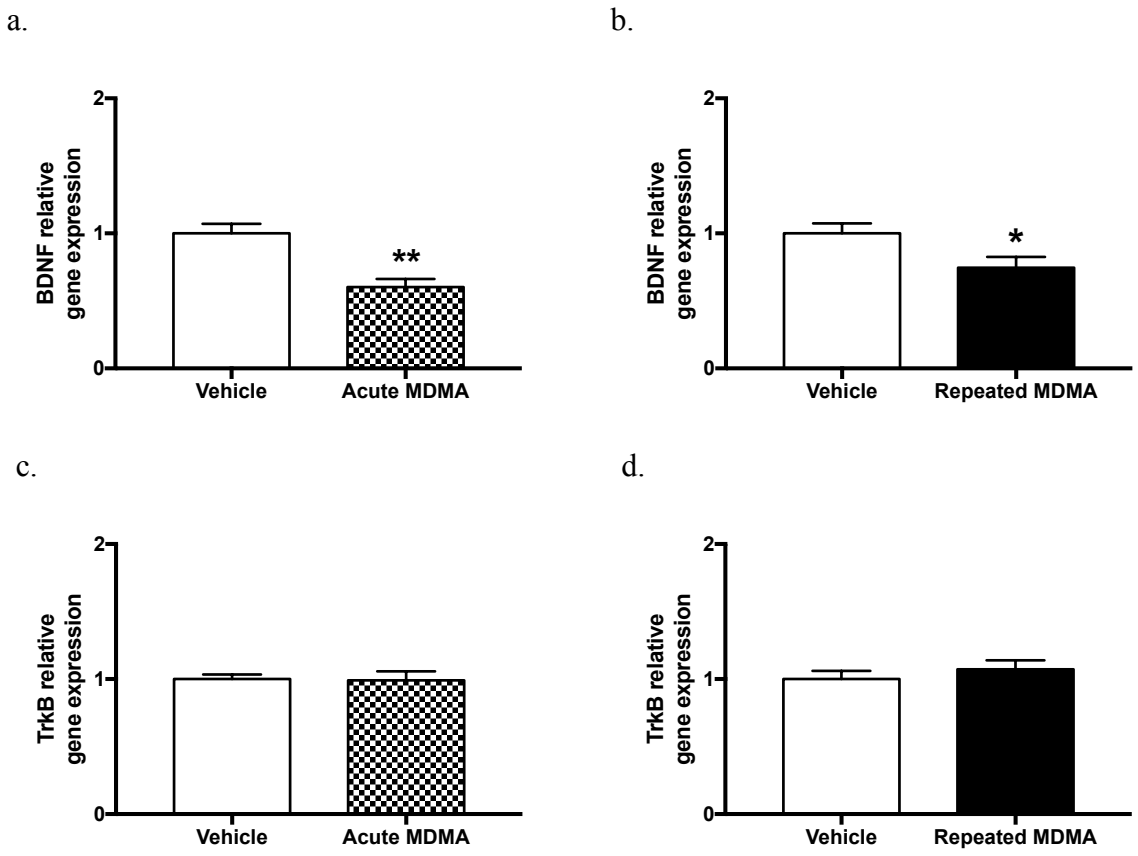


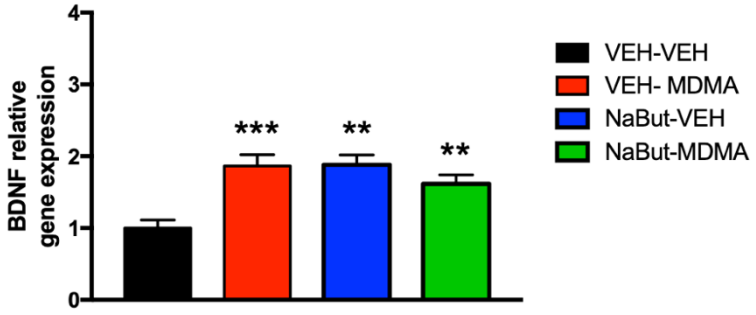
Figure 12. Relative gene expression of BDNF and TrkB in HIPPO after acute and repeated MDMA treatment. Data represent 2^{-DDCt} values calculated by DDCt method and are expressed as mean ± SEM (n=5/6 animals per group). *p<0.05, ** p<0.01 vs respective Vehicle; data analyzed by t test.

SODIUM BUTYRATE AND MDMA STUDY

4.1.7 Gene expression analysis of BDNF and TrkB following NaBut and MDMA treatment in PFCX

One way ANOVA followed by Newman Keuls multiple comparison test showed significantly changes of BDNF gene expression after MDMA treatment (VEH-MDMA, 1.87 ± 0.16 vs VEH-VEH, 1.00 ± 0.11, ***p<0.001) (Figure 13a). In the same way, an increase in mRNA levels of this neurotrophin was observed both in the group of animals that received NaBut only (NaBut-VEH, 1.88 ± 0.14 vs VEH-VEH, 1.00 ± 0.11, **p<0.01) and in the group treated with NaBut prior to MDMA (NaBut-MDMA, 1.62 ± 0.13 vs VEH-VEH, 1.00 ± 0.11, **p< 0.01) (Fig.13a). Similarly, an increase in TrkB gene expression, was observed after MDMA or NaBut administer alone or in combination (VEH-MDMA, 3.08 ± 0.20 vs VEH-VEH, 1.00 ± 0.14, NaBut-VEH, 2.70 ± 0.17 vs VEH-VEH, 1.00 ± 0.14, NaBut-MDMA, 3.25 ± 0.27 vs VEH-VEH, 1.00 ± 0.14, ****p<0.0001) (Fig.13b).

a.



b.

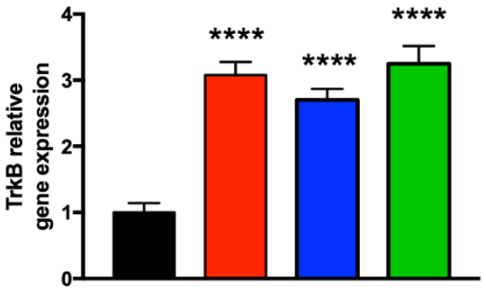


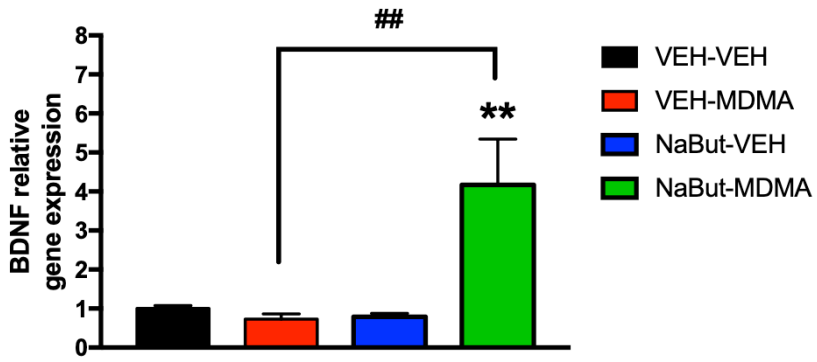
Figure 13. Relative gene expression of BDNF and TrkB in PFCX after treatment with MDMA, NaBut or NaBut prior to MDMA. Data represent 2^{-DDCt} values calculated by DDCt method and are expressed as mean ± SEM (n=5/6 animals per group). **p<0.01, *** p<0.001, ****p<0.0001 vs VEH-VEH group; data analyzed by One Way ANOVA followed by Newman keuls multiple comparison test.

4.1.8 Gene expression analysis of BDNF and TrkB following NaBut and MDMA treatment in HIPPO

In HIPPO, statistical analysis did not show any significant differences of BDNF gene expression after MDMA or NaBut treatment (Fig14a). By contrast, an increase in BDNF mRNA levels was observed in animals treated with NaBut prior to MDMA (NaBut-MDMA, 4.17 ± 1.17 vs VEH-VEH, 1.00 ± 0.08, **p<0.01; NaBut-MDMA, 4.17 ± 1.17 vs VEH-MDMA, 0.73 ± 0.13, ##p<0.01) (Fig.14a).

Similarly, the pretreatment with NaBut in the MDMA treated animals is able to induce an increase in TrkB gene expression while, no changes were revealed after MDMA or NaBut treatement (NaBut-MDMA, 4.09 ± 1.30 vs VEH-VEH, 1.00 ± 0.06, *p<0.05 vs VEH-VEH, NaBut-MDMA, 4.09 ± 1.30 vs VEH-MDMA, 0.80 ± 0.22, #p<0.05 vs VEH-MDMA) (Fig.14b).

a.



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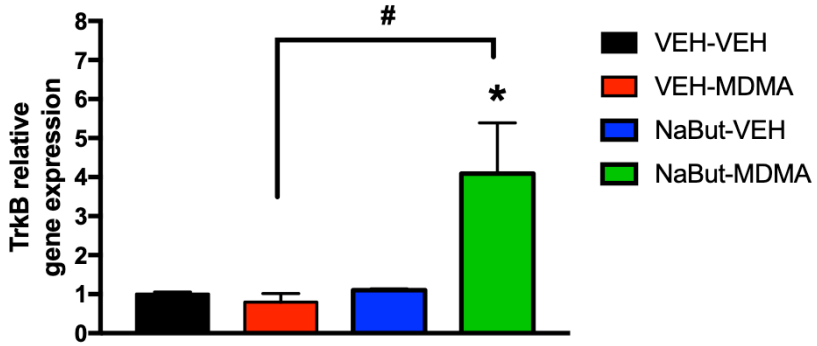


Figure 14. Relative gene expression of BDNF and TrkB in HIPPO after treatment with MDMA, NaBut or NaBut prior to MDMA. Data represent 2^{-DDCt} values calculated by DDCt method and are expressed as mean ± SEM (n=5/6 animals per group). *p<0.05, ** p<0.01, vs VEH-VEH group; #p<0.05, ##p<0.01 vs VEH-MDMA group; data analyzed by One Way ANOVA followed by Newman Keuls multiple comparison test.

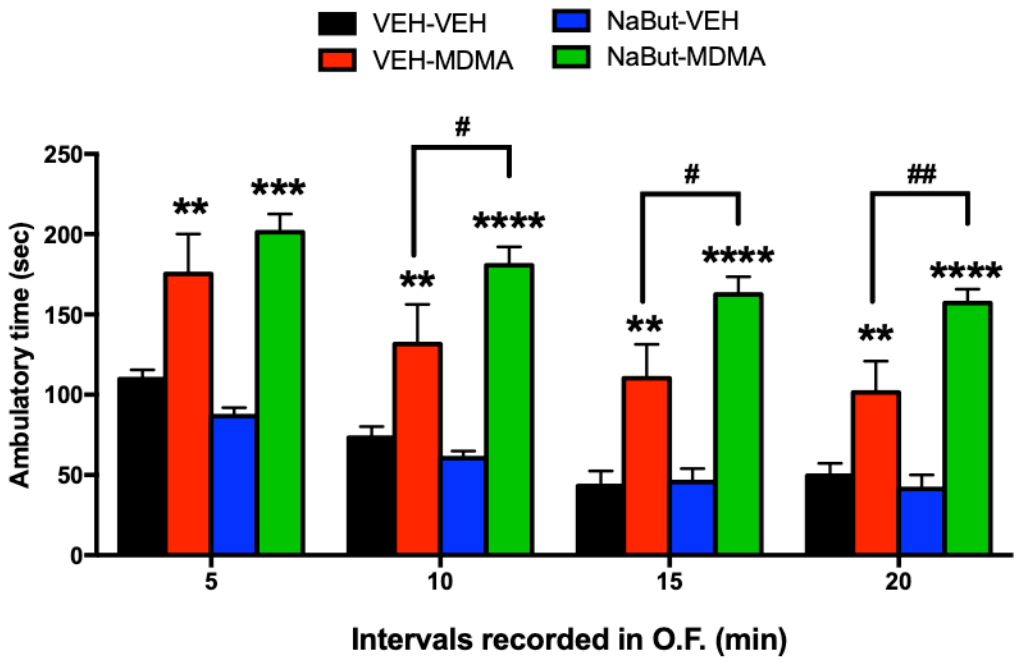
4.1.9 Effects of the HDACi NaBut on MDMA treatment (Open Field test)

DAY 1 - ZONE TOTAL

One way ANOVA followed by Newman Keuls multiple comparison test revealed that MDMA induced significant alteration in locomotor activity. In fact, an increase in ambulatory time was observed at all selected time point in the group of animals receiving MDMA treatment compared to control group (**p<0.01 vs VEH-VEH) (Fig.15a). A similar effect was observed in NaBut-MDMA group (***p<0.001, ****p<0.0001 vs VEH-VEH) (Fig 15a). Moreover, data here reported showed the ability of NaBut to potentiate MDMA at 10,15 and 20 minutes (#p<0.05, ##p<0.01 vs VEH-MDMA) (Fig.15a).

Concerning distance travelled, results displayed a significant upregulation of this parameter both in VEH-MDMA and in NaBut-MDMA group compared to control (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, vs VEH-VEH) (Fig.15b). Moreover, the pretreatment with NaBut enhanced the effect induced by MDMA on distance travelled (##p<0.01 vs VEH-MDMA) (Fig.15b).

a.



b.

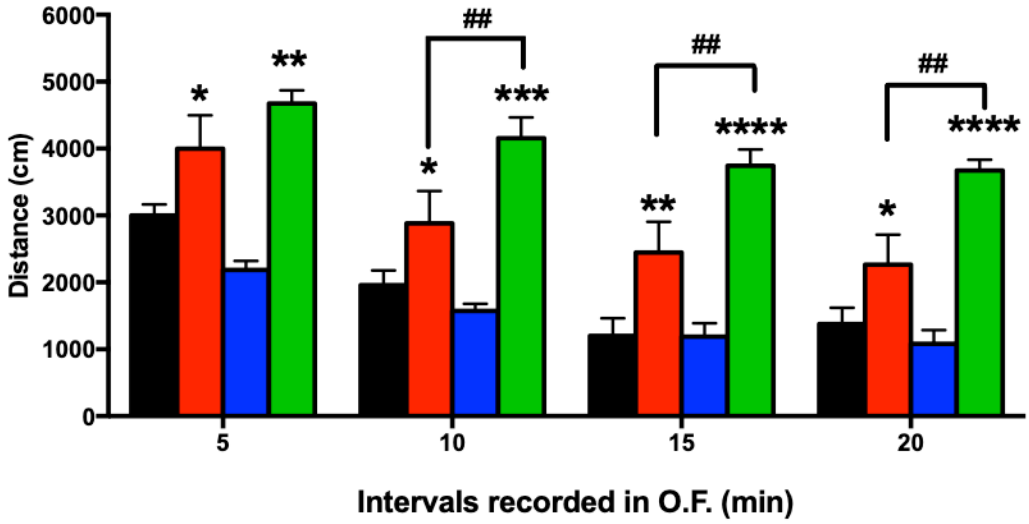


Figure 15. a) ambulatory time and b) distance travelled at day 1 after treatment with MDMA or NaBut alone or in combination at different time point. Each value represents the mean ± SEM of five/six animals per group (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 vs VEH-VEH group; #p<0.05, ##p<0.01 vs VEH-MDMA group). Data were analyzed by One Way ANOVA followed by Newman Keuls multiple comparisons test.

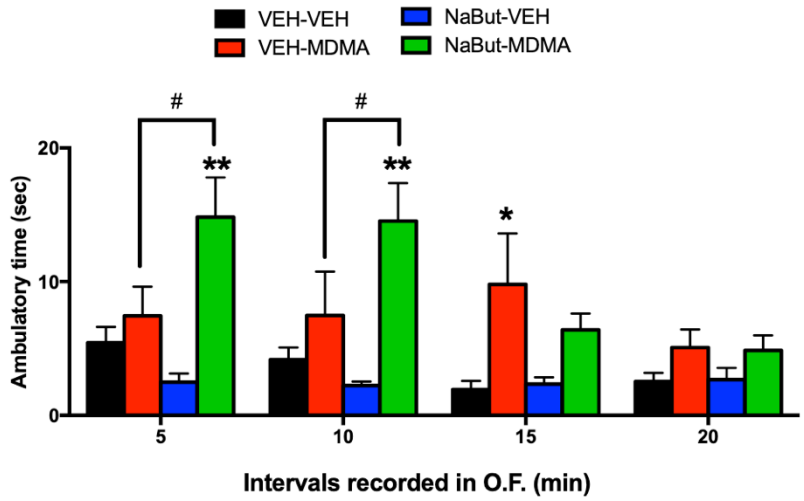
DAY 1-CENTRAL ZONE

In the central zone of arena (zone 1), data revealed a significant increase in ambulatory time in MDMA group at 15 min only (*p<0.05 vs VEH-VEH) (Fig.16a). In the same way, an upregulation of this parameter was observed in NaBut-MDMA group at 5 and 10 min (**p<0.01 vs VEH-VEH) (Fig.16a). In addition, the pretreatment with the HDACi, NaBut, induced a potentiation of MDMA effect on ambulatory time at 5 and 10 min (#p<0.05 vs VEH-MDMA) (Fig.16a).

Similarly, MDMA group showed an increase in the distance travelled at 15 min (*p<0.05 vs VEH-VEH) and an upregulation of this parameter was also revealed in NaBut-MDMA group at the selected time point (*p<0.05 vs VEH-VEH) (Fig.16b). In addition, results showed that the HDACi, NaBut, administered 30 min prior MDMA, potentiates the MDMA effect at 5 and 10 min (#p<0.05 vs VEH-MDMA) (Fig.16b).

Moreover, statistical analysis revealed that the group of animals treated with MDMA exhibited a statistically significant increase in the number of entries in the central zone (zone 1) at 5 min only (*p<0.05 vs VEH-VEH) and NaBut enhanced this effect (#p<0.05 vs VEH-MDMA) (Fig.16c).

a.



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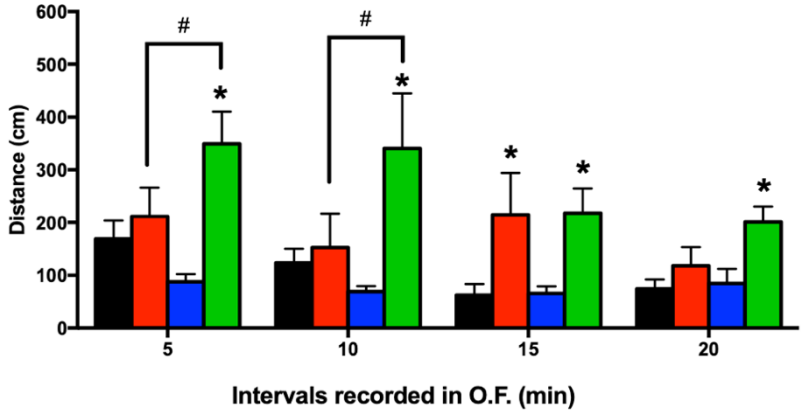


c.

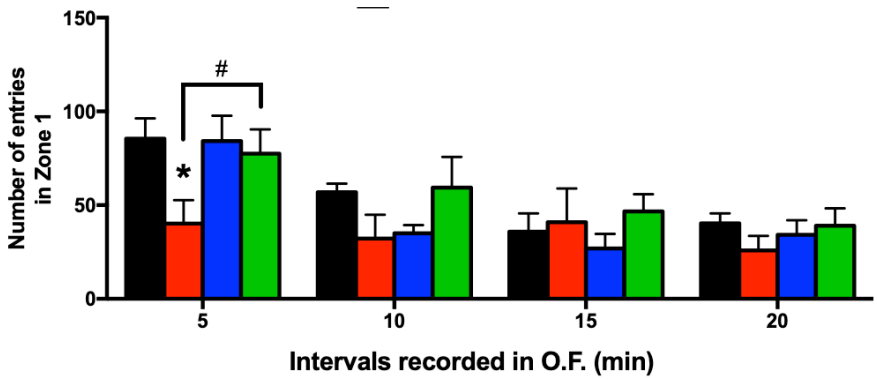


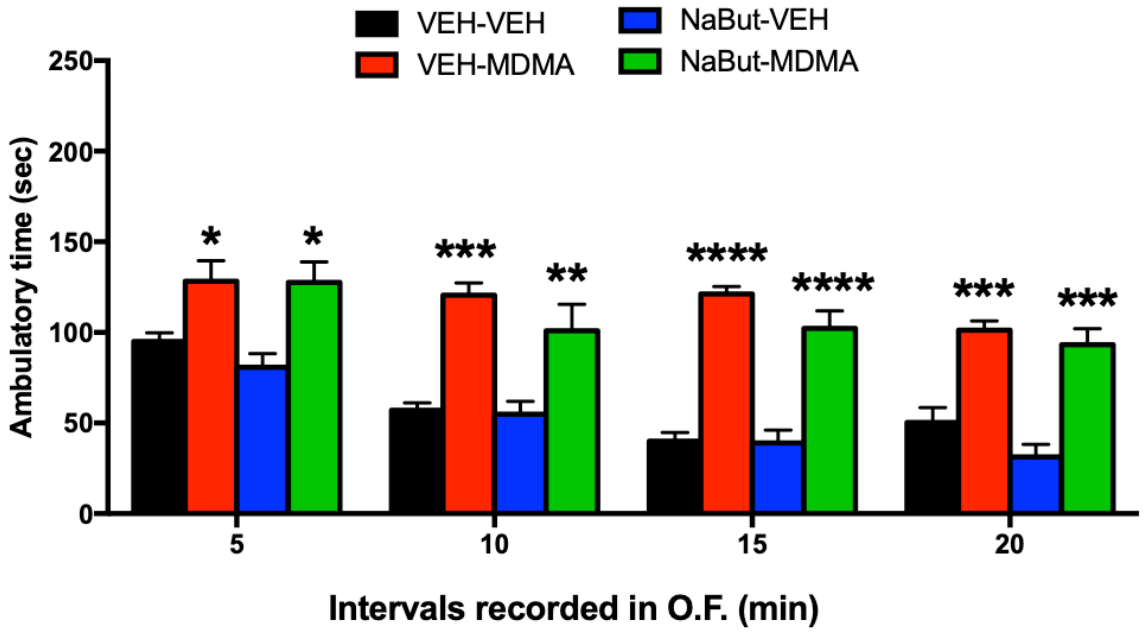
Figure 16. a) ambulatory time, b) distance travelled and c) number of entries in the central zone of arena at day 1, after treatment with MDMA or NaBut alone or in combination at different time point. Each value represents the mean ± SEM of five/six animals per group (*p<0.05, **p<0.01 vs VEH-VEH group; #p<0.05 vs VEH-MDMA group). Data were analyzed by One Way ANOVA followed by Newman Keuls multiple comparisons test.

DAY 7 - ZONE TOTAL

One way ANOVA analysis followed by Newman Keuls multiple comparison test showed a significant alteration in locomotor activity in VEH-MDMA and in NaBut-MDMA group. In particular, an increase in ambulatory time was observed in VEH-MDMA group compared to VEH-VEH group at all time point (*p<0.05, ***p<0.001, ****p<0.0001 vs VEH-VEH) (Fig.17a). A similar effect was also observed in NaBut-MDMA group (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 vs VEH-VEH) (Fig 17a).

In addition, data showed a significant upregulation of distance travelled both in VEH-MDMA and NaBut-MDMA groups compared to control at 10, 15 and 20 minutes (**p<0.01, ***p<0.001, ****p<0.0001, vs VEH-VEH) and a significant decrease in this parameter was observed in NaBut-MDMA group compared to MDMA group at 15 min only (#p<0.05 vs VEH-MDMA) (Fig.17b).

a.



b.

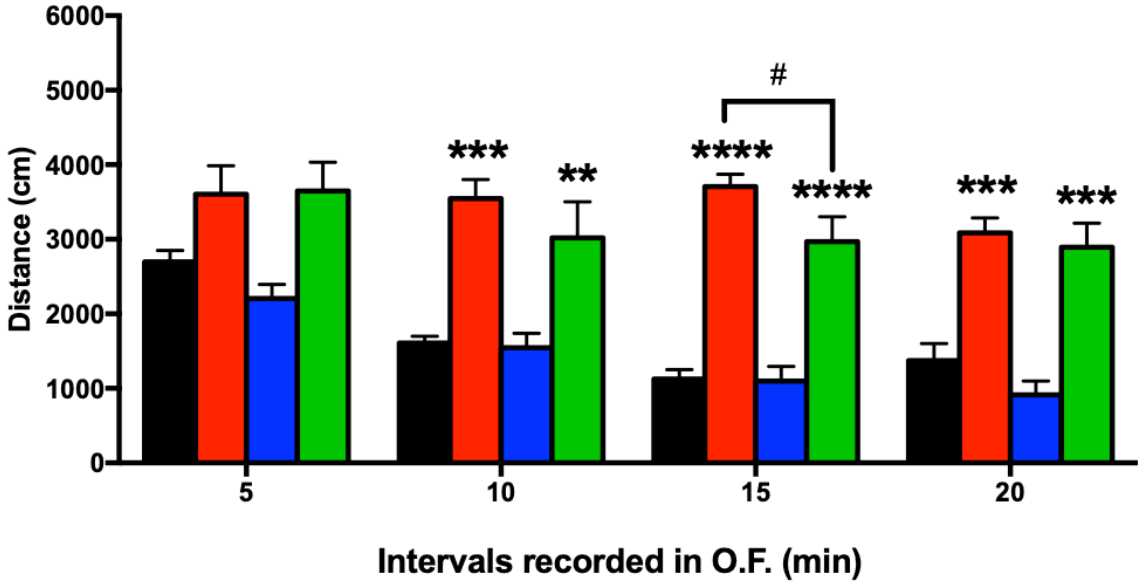


Figure 17. a) ambulatory time and b) distance travelled at day 7 after treatment with MDMA or NaBut alone or in combination at different time point. Each value represents the mean ± SEM of five/six animals per group (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 vs VEH-VEH; #p<0.05 vs VEH-MDMA). Data were analyzed by One Way ANOVA followed by Newman Keuls multiple comparisons test.

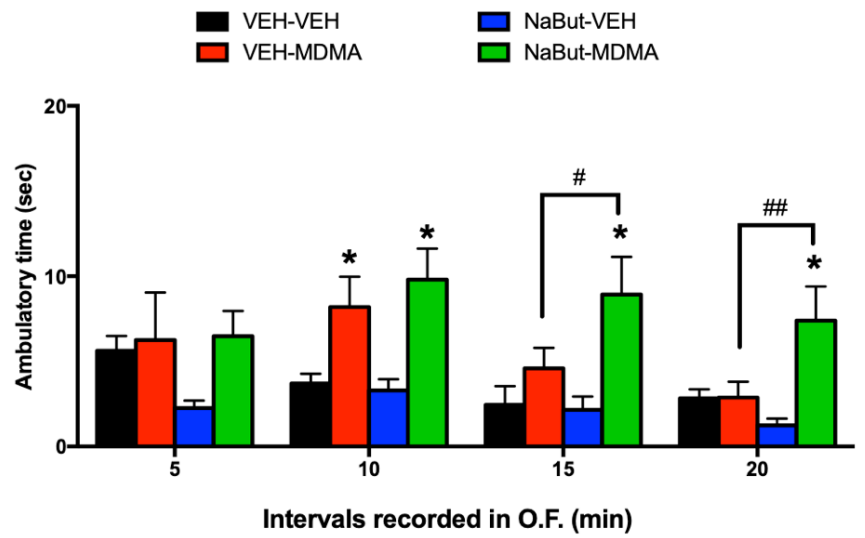
DAY 7-CENTRAL ZONE

In the central zone of arena (zone 1), data revealed a significant increase in ambulatory time in MDMA group at 10 min only (*p<0.05 vs VEH-VEH), whereas an increase in this parameter was observed in NaBut-MDMA group at 10, 15 and 20 min (*p<0.05 vs VEH-VEH). In addition, the pretreatment with the HDACi, NaBut, induced a potentiation of MDMA effect on ambulatory time at 15 and 20 min (#p<0.05, ##p<0.01 vs VEH-MDMA) (Fig.18a).

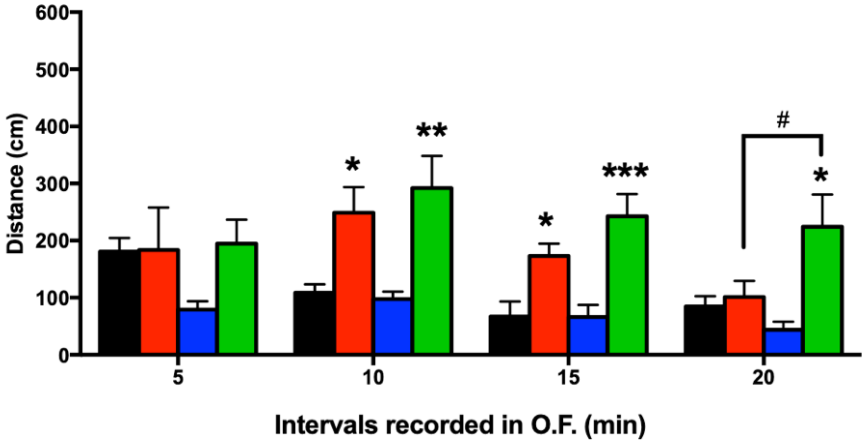
In a similar way, MDMA group showed an increase in the distance travelled at 10 and 15 min (*p<0.05 vs VEH-VEH) and an upregulation of this parameter was also revealed in NaBut-MDMA group at 10, 15 and 20 min (*p<0.05, **p<0.01, ***p<0.001 vs VEH-VEH). In addition, results showed that the HDACi, NaBut, administered 30 min prior MDMA, potentiate the MDMA effect at 20 min (#p<0.05 vs VEH-MDMA) (Fig.18b).

Moreover, the group of animals treated with MDMA showed a statistically increase in the number of entries in the central zone (zone 1) at 20 min only (*p<0.05 vs VEH-VEH), whereas in the group pretreated with NaBut there is an upregulation of this parameter at 10,15 and 20 minutes (**p<0.01, ***p<0.001, ****p<0.0001 vs VEH-VEH) and in particular, NaBut potentiated MDMA effect at 15 and 20 minutes (#p<0.05, ##p<0.01 vs VEH-MDMA) (Fig. 18c).

a.



b.



c.

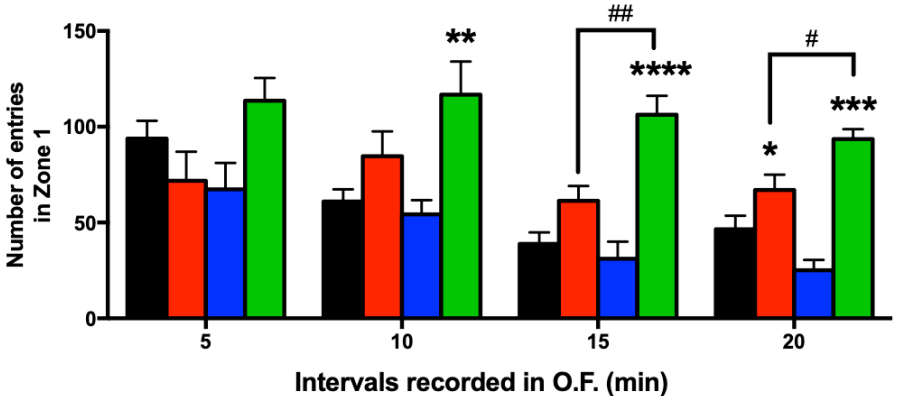


Figure 18. a) ambulatory time, b) distance travelled and c) number of entries in central zone of arena at day 1, after treatment with MDMA or NaBut alone or in combination at different time point. Each value represents the mean ± SEM of five/six animals per group (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 vs VEH-VEH; #p<0.05, ##p<0.01 vs VEH-MDMA). Data were analyzed by One Way ANOVA followed by Newman Keuls multiple comparisons test.

4.2 Human studies

BDNF levels in serum and plasma MDMA users in comparison to MDMA-naïve controls

We analyzed the BDNF levels in serum and plasma samples of MDMA users and MDMA-naïve controls.

As shown in Fig.19, t test showed no significant differences in the levels of serum (Fig.19a; Cohen's d=0.13) and plasma BDNF (Fig.19b; d=0.30) in MDMA users in comparison to controls.

As expected, no significant correlation between serum and plasma BDNF was found (Fig.20; r=0.21, p=0.15). However, a significant positive correlation was found between plasma BDNF level and the duration of MDMA abstinence days since last consumption (Fig.21b; r=0.46, $p<0.05$). Because the distribution in the total levels of BDNF PLASMA (MDMA group + control group) and in the MDMA days since last consumption were not normal distributed, the log-transformed values were used for statistical analysis.

In addition, 2*2 (group*sex) ANOVA with plasma and serum BDNF revealed that males displayed significant higher levels of BDNF than females in plasma ($p<0.05$; Fig.22b) and a similar trend in serum (p=0.07; Fig. 22a). By contrast, the group effects remained insignificant (both p-values >0.20) and no group*sex interaction effects occurred (both p-values >0.30).

In all the analysis, two subjects (1 male in MDMA group for serum, 1 female in MDMA group for plasma) were excluded as outliers (>mean of the group + 3SD).

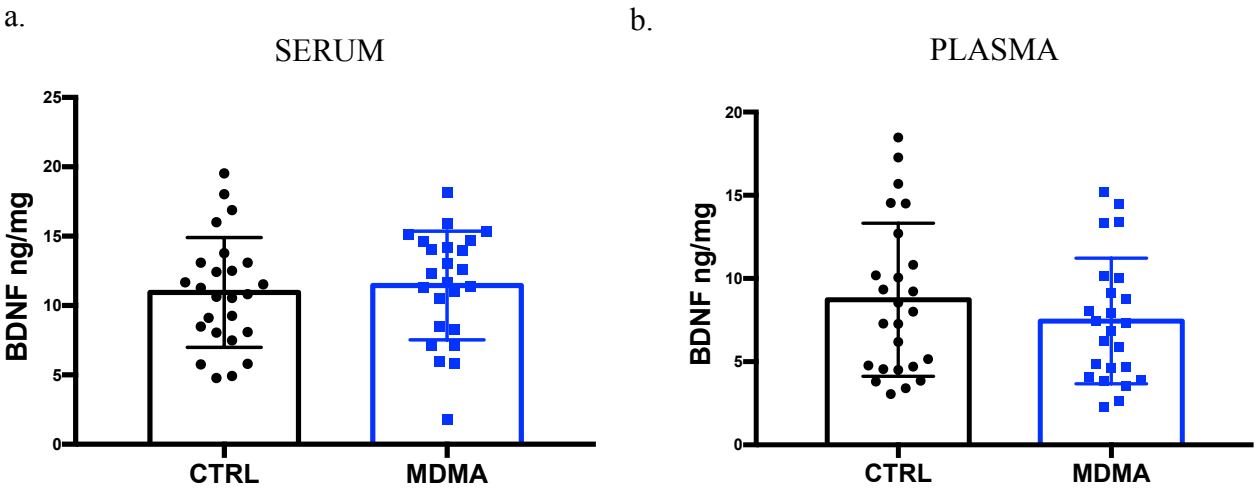


Figure 19. BDNF levels of serum (a; CTRL n=25, MDMA n=24) and plasma (b; CTRL n=25, MDMA n=24) are represented as mean ± SD and are analyzed by t test ($p>0.05$). The data were normalized with Bradford methods. Two subjects (1 in MDMA group for serum, 1 in MDMA group for plasma) were excluded as outliers (>mean + 3SD).

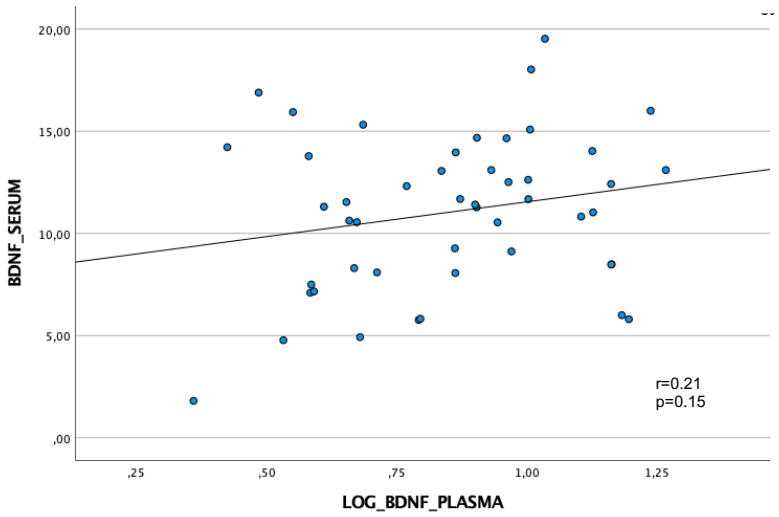
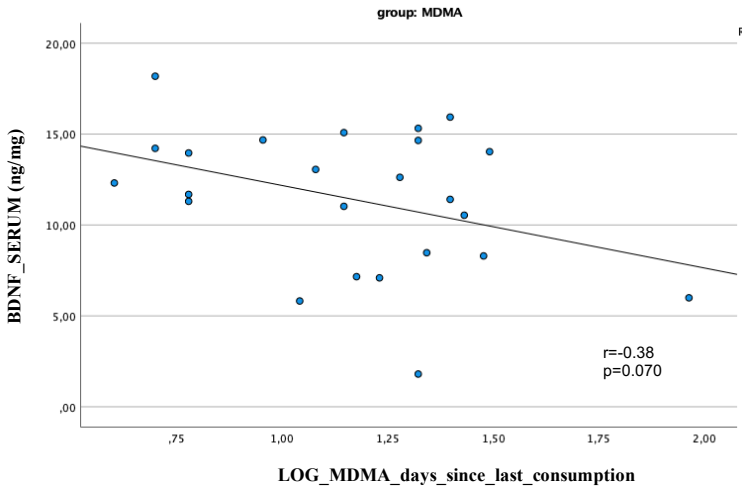


Figure 20. Pearson's correlation between serum (n=49) and plasma (n=49) BDNF levels. The log-transformed values (log_{10}) were used for plasma variable to obtain a normal distribution. Two subjects (1 in MDMA group for serum, 1 in MDMA group for plasma) were excluded.

a.



b.

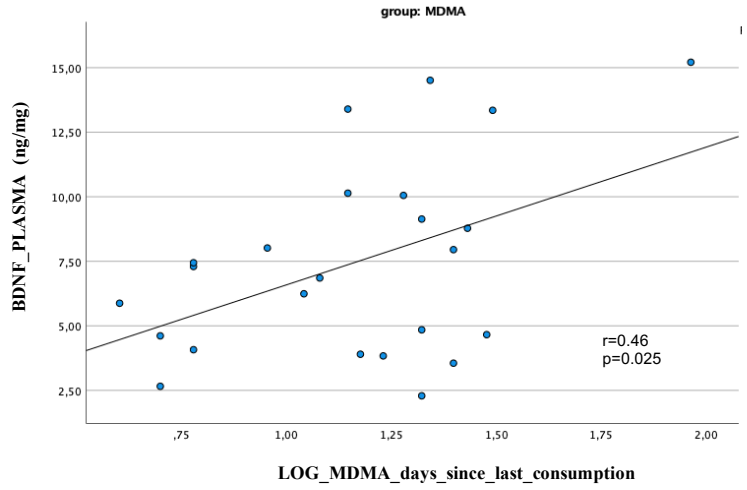
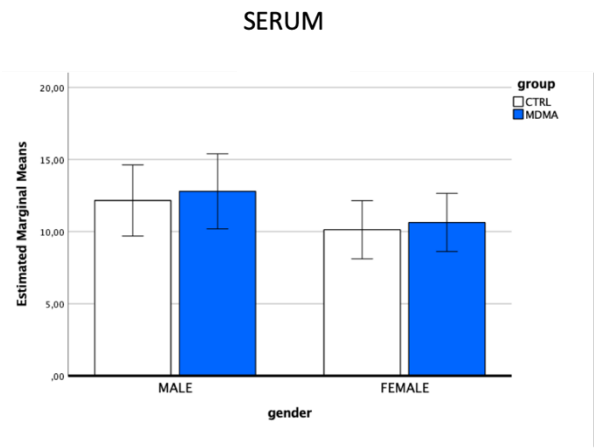
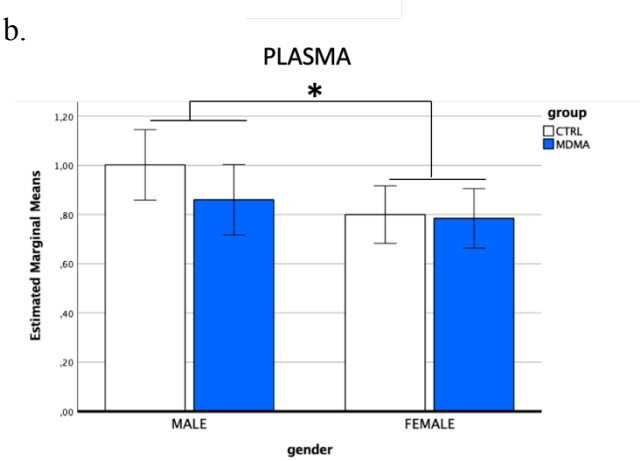


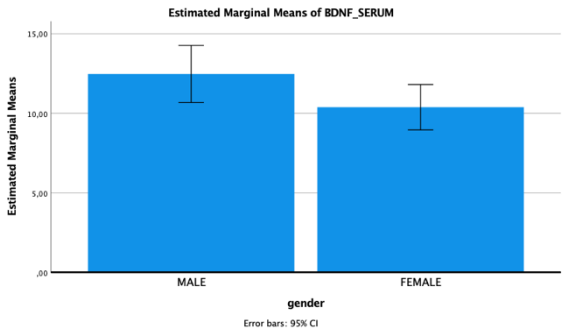
Figure 21. Pearson's correlation between BDNF serum (a; n=24), plasma (b; n=24) and MDMA days since last consumption (n=24). The log-transformed values (log_{10}) were used for the variable "MDMA days since last consumption" to obtain a normal distribution.

a.



b.





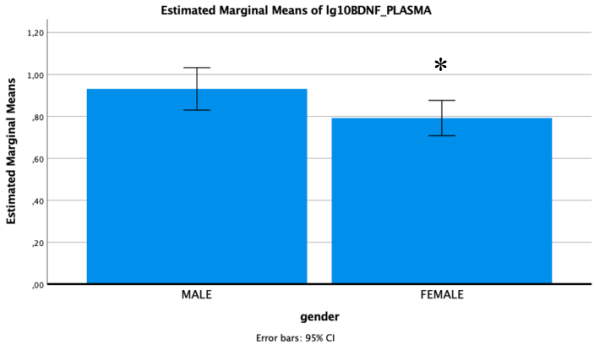


Figure 22. 2*2 (group*sex) ANOVA with serum (a) and plasma (b) BDNF revealed that males displayed significant higher levels of BDNF than females in plasma ($p<0.05$) and a similar trend in serum ($p=0.07$). Bars show the 95% confidence interval. *$p<0.05$ in comparison to male.

4.3 In vitro studies

4.3.1 Mycoplasma Contamination Assay

LookOut® Mycoplasma PCR Detection kit was used to check a possible mycoplasma contamination in the cell line. The PCR products were run on a 1.2% agarose gel. Mycoplasma positive samples were expected to show bands in the range of 260 ± 8 bp and the positive control was expected to show a distinct band at 259 bp. A negative control showed a band at 481 bp, indicating a successful performance of the reaction, was present in every run. The absence of a band at 259 bp in the samples of supernatant of RN46A line confirmed the absence of mycoplasma (Fig. 23).

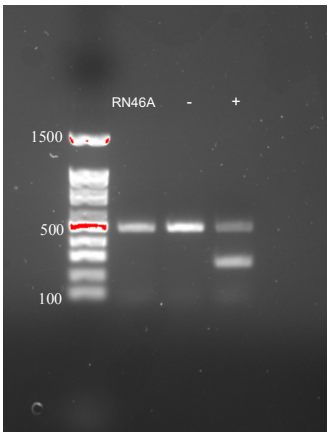


Figure 23. PCR products generated with a mycoplasma detection kit run on a 1.2% agarose gel. The positive control showed a band at 260 bp and the negative control sample at 480 bp. No mycoplasma contamination was detected in the RN46A sample.

4.3.2 Differentiation of RN46A in mature neurons

To characterize the RN46A cell line used in all of the experiments of this study, cells were maintained in culture at 33°C with DMEM/F12+10%FBS or were differentiated for 8 days at 37°C with neurobasal medium supplemented with B27 after a period of adaptation at 33°C. Differentiation success was checked with the microscope and using MAP-2 marker indicating mature neurons (Fig. 24b). The presence of MAP2 in the undifferentiated cells line showed that they start spontaneously to differentiate (Fig. 24a).

RN46A cells proliferating at 33 °C have a fibroblastic morphology, while at 37 °C, they cease dividing and take on a generally bipolar neuronal-like morphology (see Fig. 24).

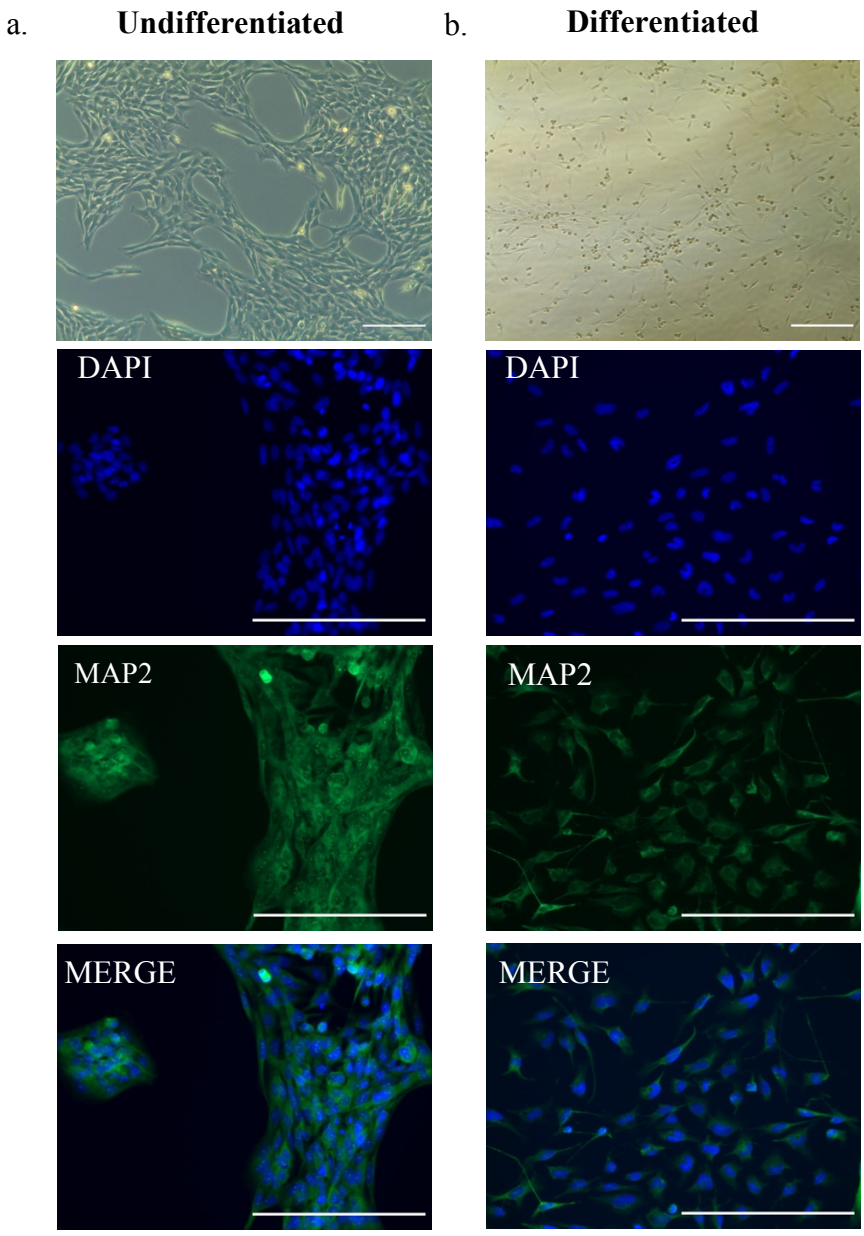


Figure 24. Exemplary immunofluorescence staining of undifferentiated (panel a) and differentiated (panel b) RN46A cell line showing the expression of MAP2 in green and cell nuclei are marked in blue (DAPI).

The merged image confirmed the expression of MAP2 in undifferentiated and differentiated cells, however under the light microscopy the morphology of the differentiated cells is distinct and neuronal like compared to the undifferentiated. Scale bar: 200 μm.

4.3.3 Cell viability

To determine the concentration of MDMA needed to cause 50% cell death/cell survival, differentiated RN46A cell line was exposed to different doses of MDMA for 24h and 48h. The $IC_{50,}$ defined as the dose able to inhibit 50% of the cell growth, was found to be 1.75 mM after 24h and 1.15mM after 48h of the treatment (Fig.25).

To compare the effect on MDMA both at 24h and 48h, the dose of MDMA used with 100 ng/ml BDNF pre-treatment was selected to be at 1.3 mM.

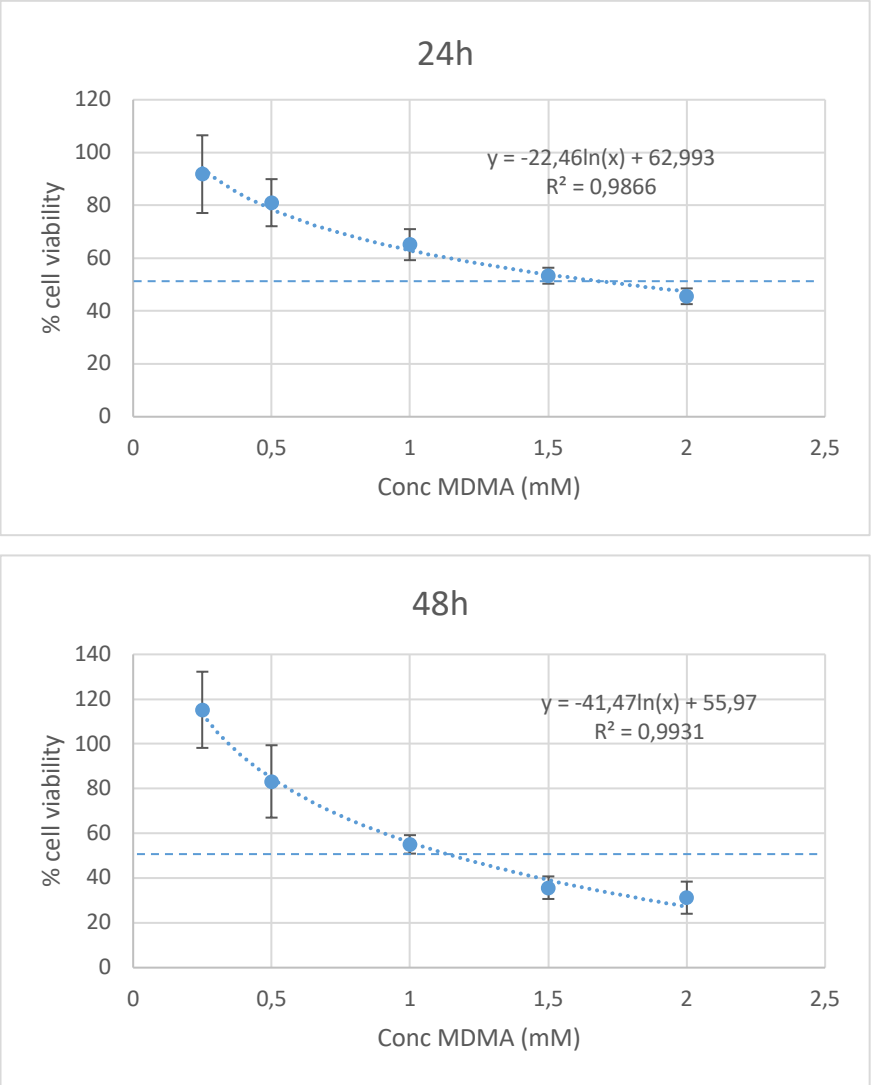


Figure 25. Graphs representing % cell viability curves and IC_{50} values (concentration responsible for 50% cells growth inhibition) of RN46A differentiated cell line versus the concentration of MDMA. Cell viability was determined using the MTT assay incubated for four hours. The values reported are the mean of two independent experiments (n=6 each experiment; Total n=12) ± SD.

The effect of BDNF pre-treatment on cell viability after MDMA was assessed using the MTT assay, at 24h and 48h, utilizing 100ng/ml of BDNF given 1h prior 1.3 mM of MDMA. One-way ANOVA revealed significant differences between treatment groups in comparison to control in both 24h and 48h, while only a significant difference between BDNF+MDMA relative to MDMA group was found at 24h (Fig.26).

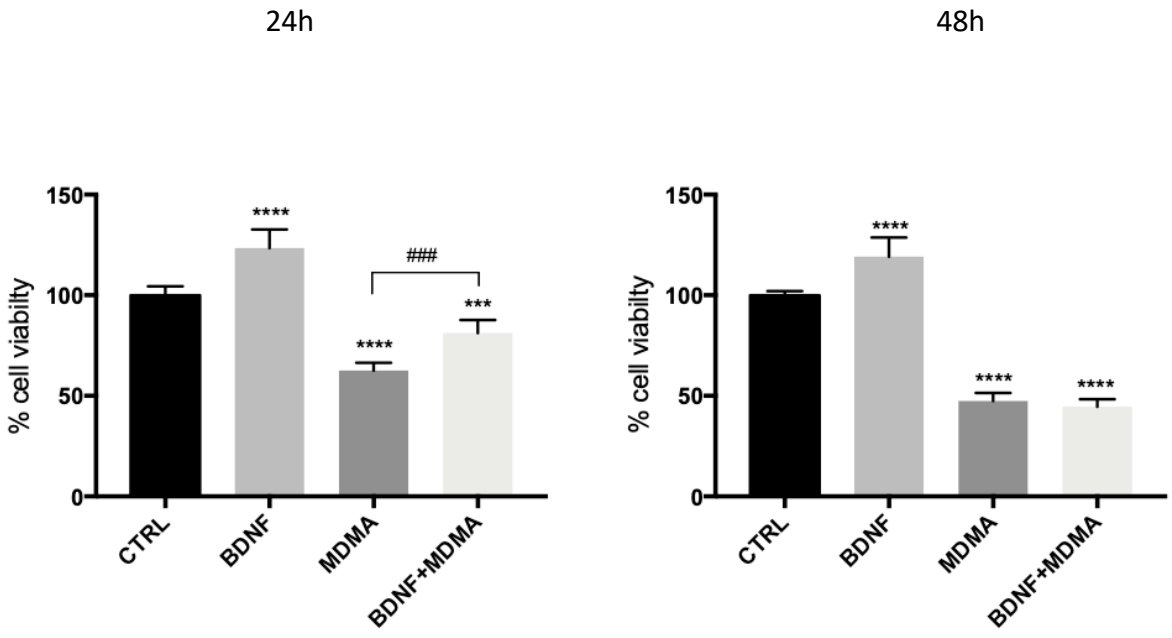


Figure 26. Effect of BDNF and MDMA treatment of RN46A serotonergic neurons. **A %** RN46A cell viability at 24h **B %** RN46A cell viability at 48h. *$p < 0.05$ compared to CTRL. $^{\#}$ $p < 0.05$ compared to MDMA. The values represent the mean ± SD of 3 independent experiments (n=5/9).

4.3.4 ICC Analysis of NFs

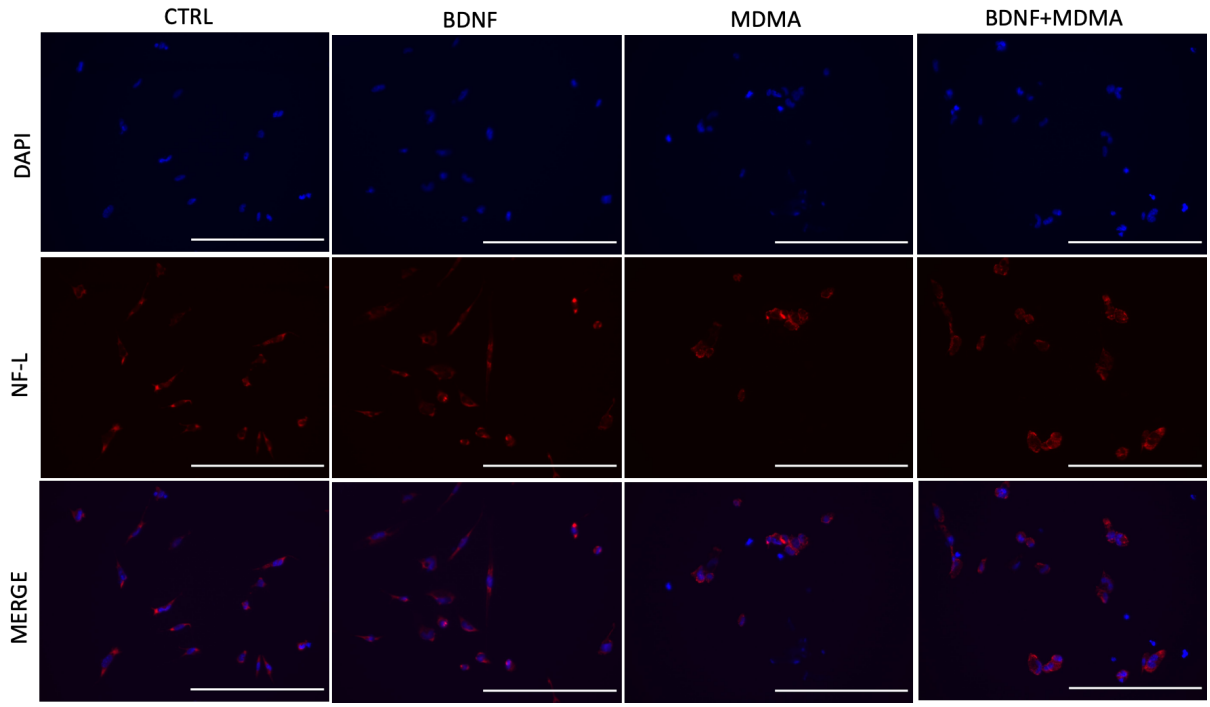
Two independent experiments, five pictures per well (3 wells per treatment), taken at 20X magnification, were analyzed for NF-L, NF-M, NF-H.
The positive cells for each NFs were counted manually and expressed in % relative to the total amount of cells.

NF-L: The statistical analysis of NF-L revealed that the positive cell % was significantly lower in MDMA and BDNF+MDMA groups compared to CTRL at 24h (Fig.27). In addition, the same effect was observed after MDMA exposure at 48h (Fig.28). Interestingly, as displayed in the graphs, the effect of MDMA was counteracted by the pre-treatment with BDNF at the two selected time point (Fig.27 and 28).

NF-M: For NF-M at 24h, the exposure of RN46A cells to different treatments resulted in no change in the % of positive cells (Fig.29). However, after 48h a slight reduction in % of positive cells in BDNF+MDMA group compared to CTRL was observed (Fig.30).

NF-H: Statistical analysis did not reveal any significant differences in the % of NF-H positive cells at all selected treatment both at 24h and 48h (Fig.31 and 32).

A.



B.

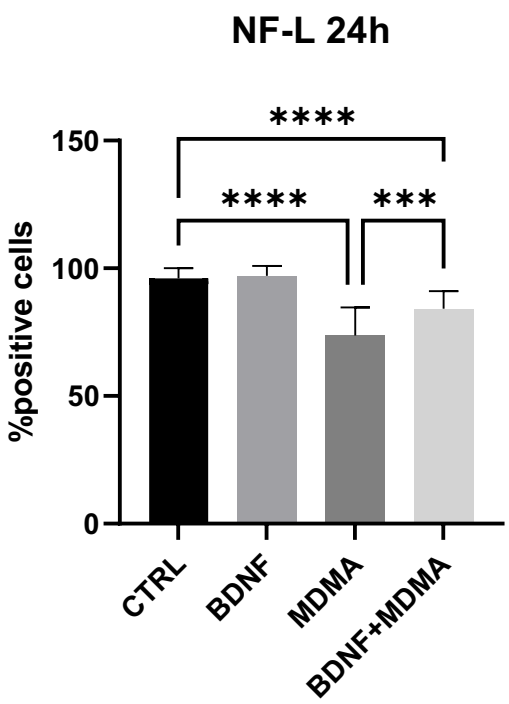
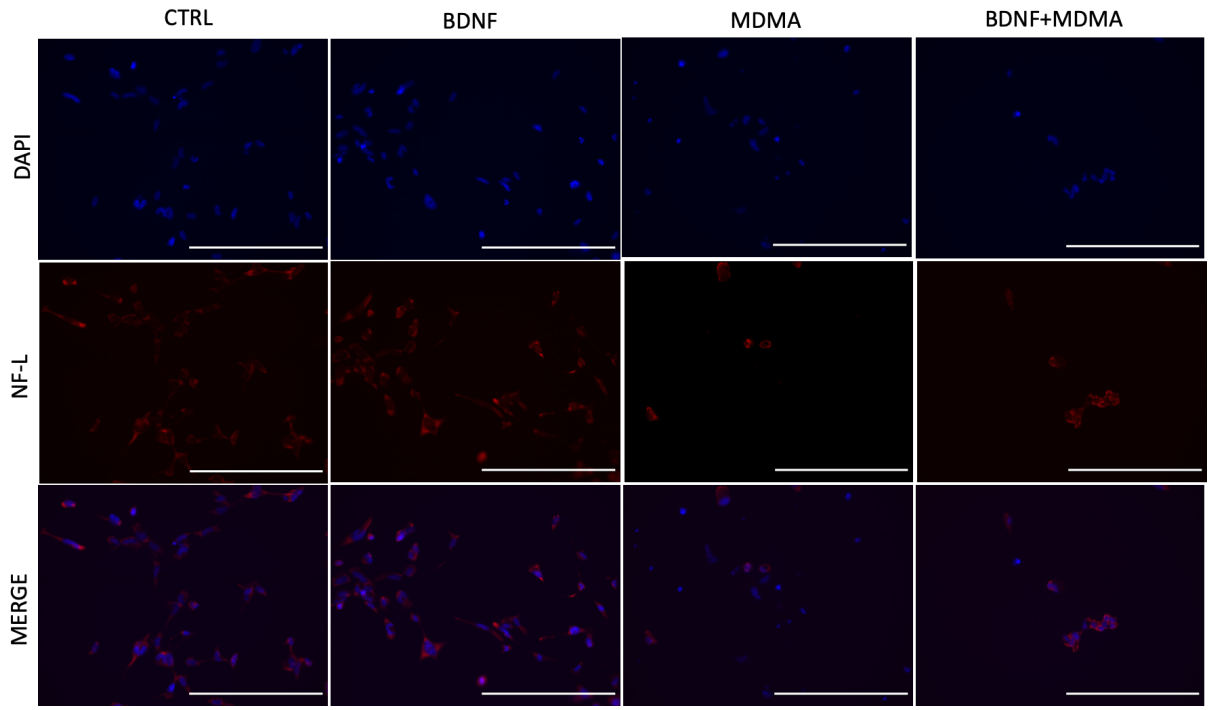


Figure 27. (Panel A and B): **A**: Exemplary immunofluorescence staining of NF-L in RN46A differentiated cells at 24h. NF-L is marked in red and cell nuclei are marked in blue (DAPI). Scale bar: 200 µm. **B**: The histograms show the percentage of NF-L-positive cells at 24h. The data are expressed with mean ± SD (CTRL n=27, BDNF n=27, MDMA n=17, BDNF+MDMA n=15) and are analyzed by One Way Anova followed by Tukey's multiple comparison test.

A.



B.

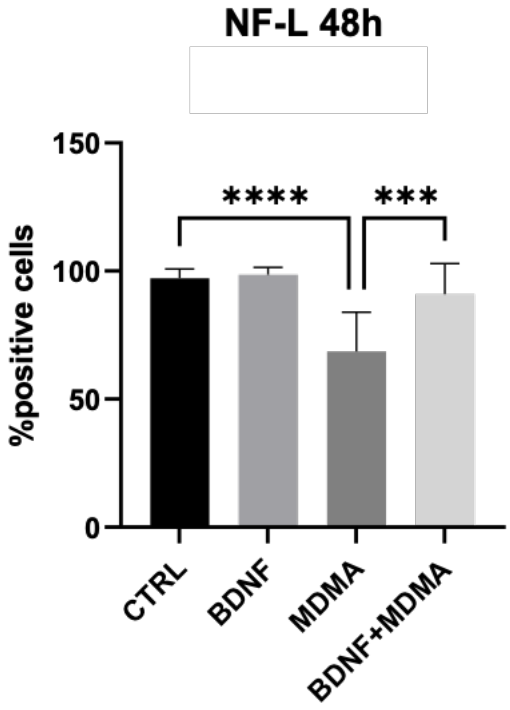
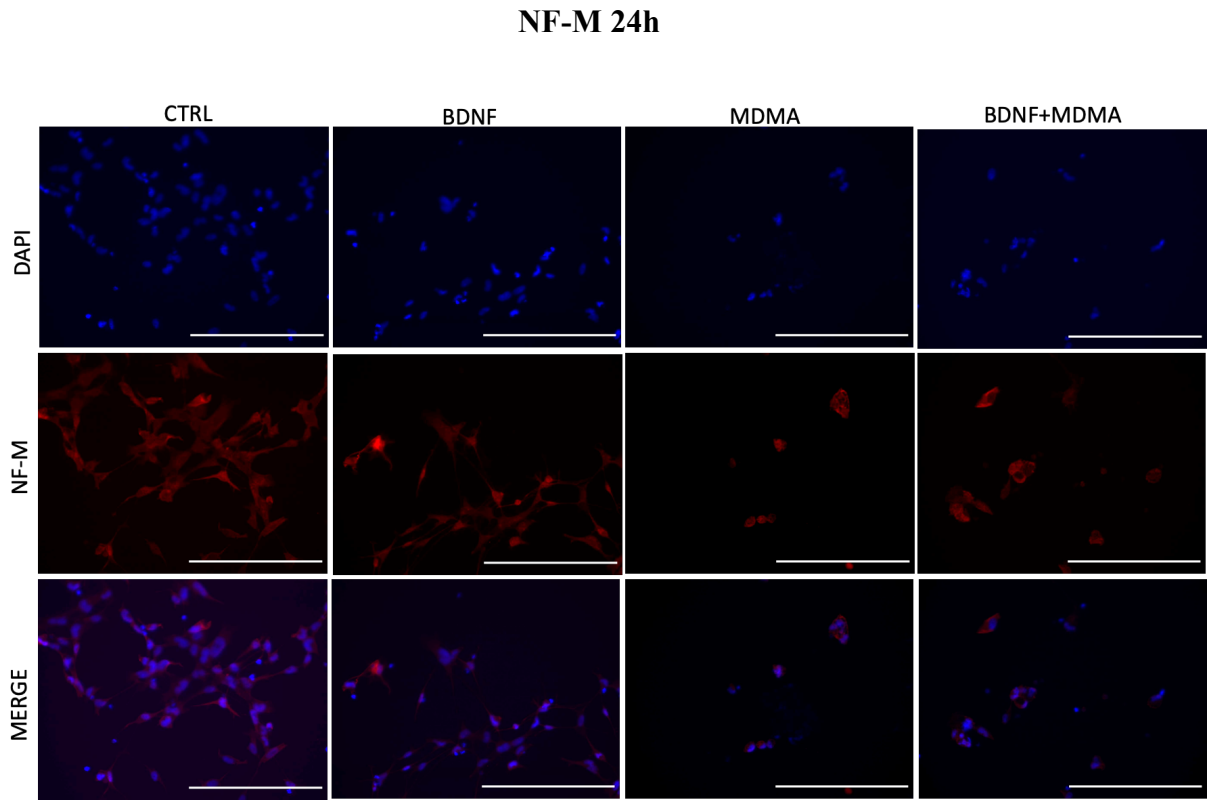


Figure 28. (Panel A and B): **A**: Exemplary immunofluorescence staining of NF-L in RN46A differentiated cells at 48h. NF-L is marked in red and cell nuclei are marked in blue (DAPI). Scale bar: 200 µm. **B**: The histograms show the percentage of NF-L-positive cells at 48h. The data are expressed with mean ± SD (CTRL n=29, BDNF n=27, MDMA n=16, BDNF+MDMA n=25) and are analyzed by Kruskal-Wallis followed by Dunn's test.

A.



B.

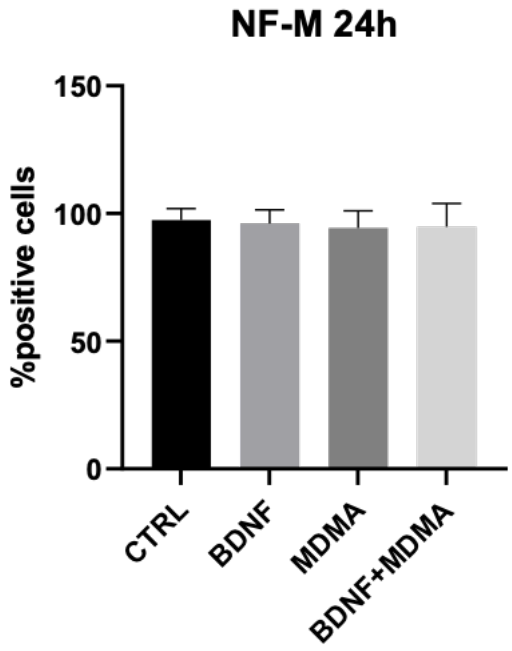
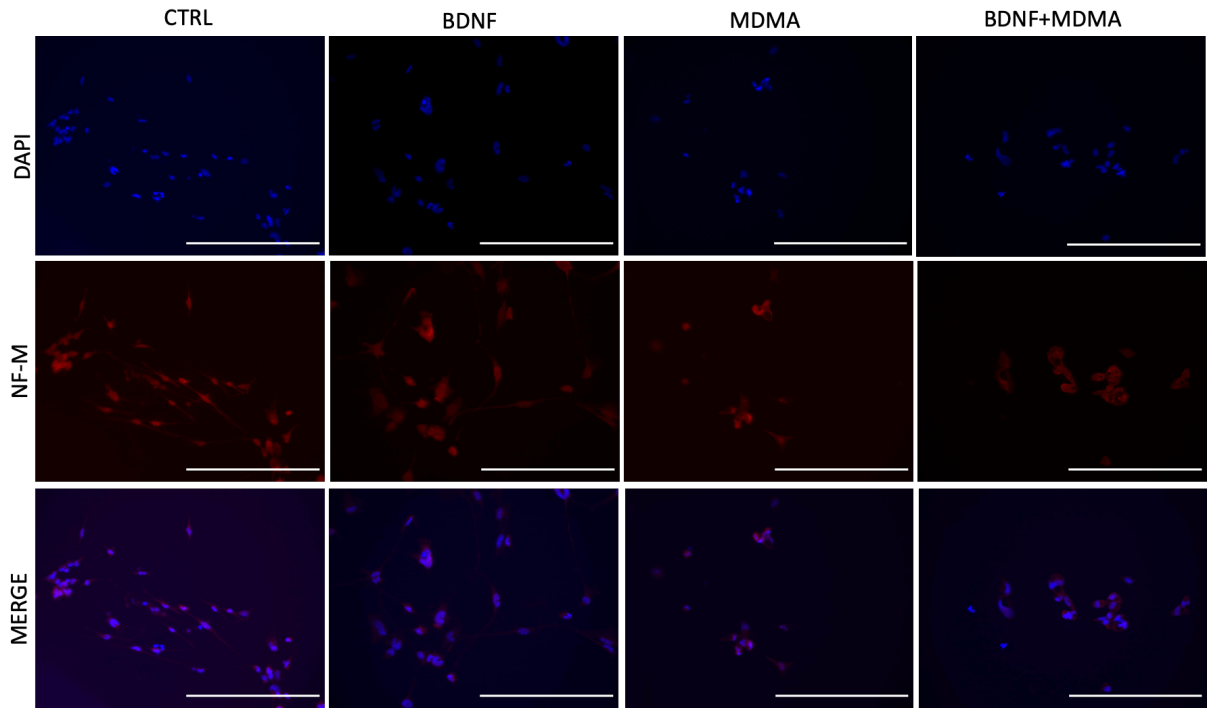


Figure 29. (Panel A and B): **A**: Exemplary immunofluorescence staining of NF-M in RN46A differentiated cells at 24h. NF-M is marked in red and cell nuclei are marked in blue (DAPI). Scale bar: 200 µm. **B**: The histograms show the percentage of NF-M-positive cells at 24h. The data are expressed with mean ± SD (CTRL n=25 BDNF n=30, MDMA n=27, BDNF+MDMA n=30) and are analyzed by Kruskal-Wallis followed by Dunn's test.

A.



B.

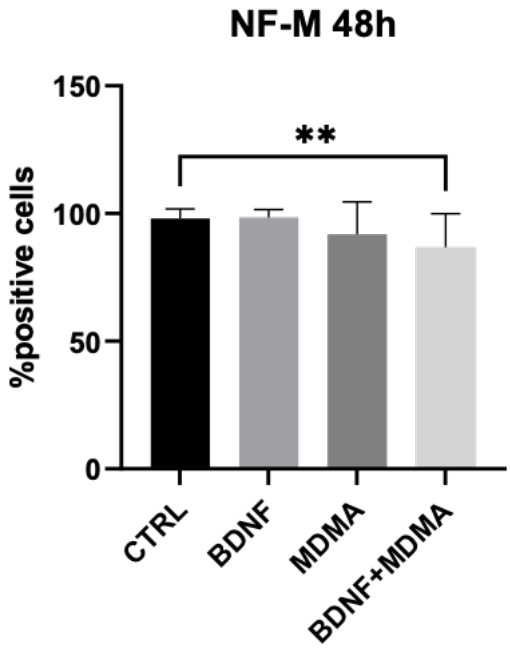
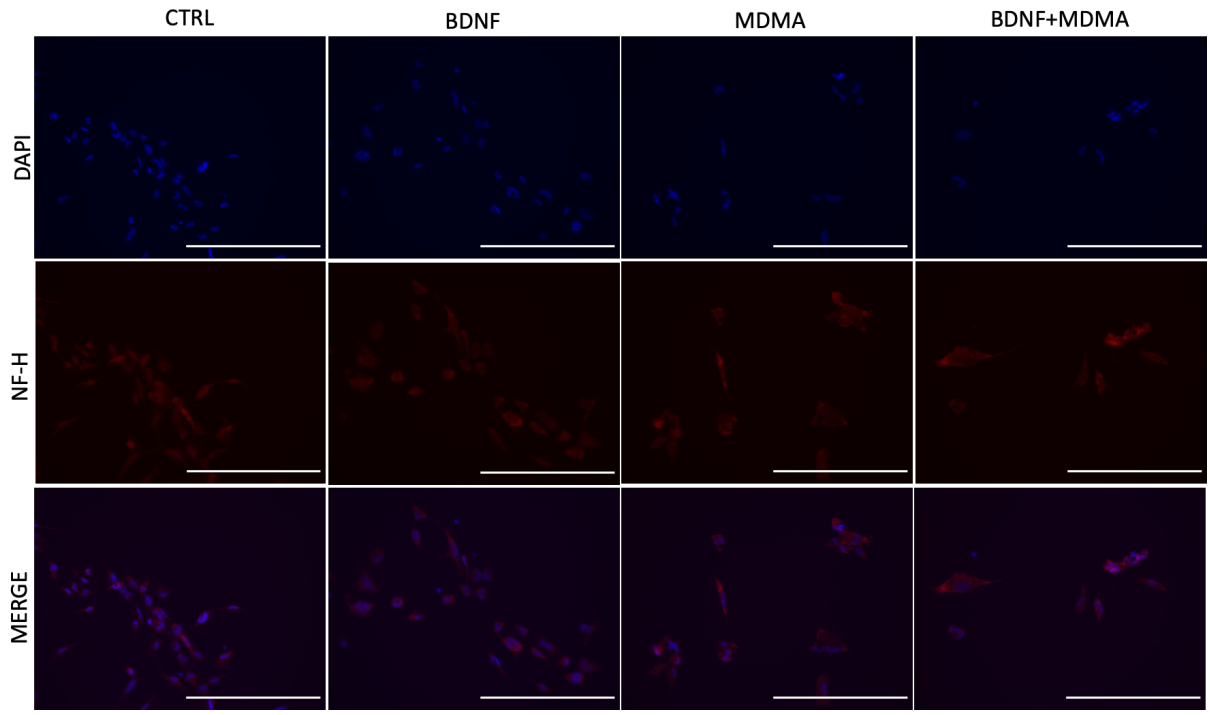


Figure 30. (Panel A and B): **A**: Exemplary immunofluorescence staining of NF-M in RN46A differentiated cells at 48h. NF-M is marked in red and cell nuclei are marked in blue (DAPI). Scale bar: 200 µm. **B**: The histograms show the percentage of NF-M-positive cells at 48h. The data are expressed with mean ± SD (CTRL n=30, BDNF n=30, MDMA n=26, BDNF+MDMA n=27) and are analyzed by Kruskal Wallis followed by Dunn's test.

A.



B.

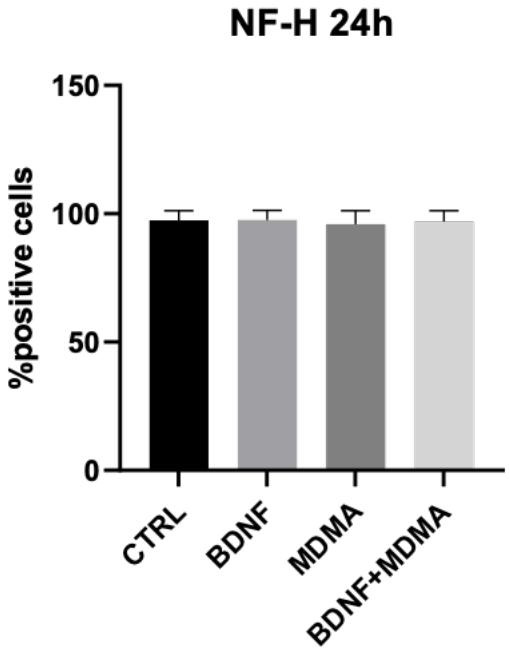
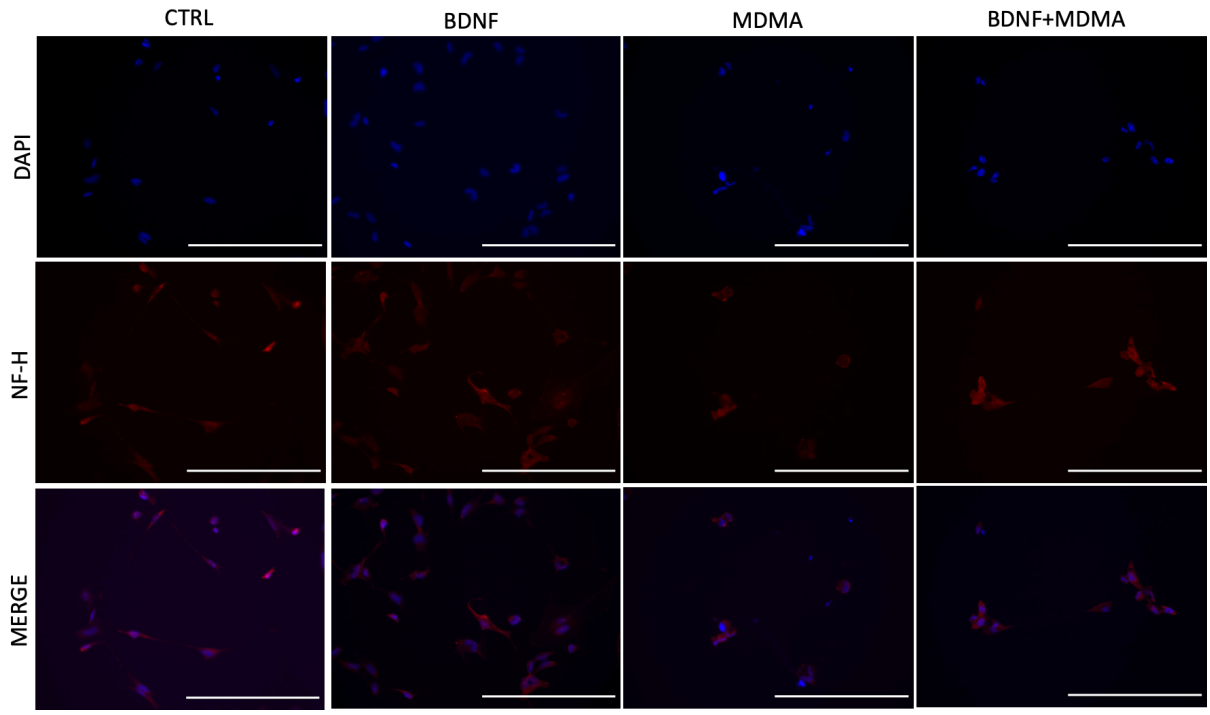


Figure 31. (Panel A and B): **A**: Exemplary immunofluorescence staining of NF-H in RN46A differentiated cells at 24h. NF-H is marked in red and cell nuclei are marked in blue (DAPI). Scale bar: 200 µm. **B**: The histograms show the percentage of NF-H-positive cells at 24h. The data are expressed with mean ± SD (CTRL n=29, BDNF n=30, MDMA n=25, BDNF+MDMA n=27) and are analyzed by Kruskal Wallis followed by Dunn's test.

A.



B.

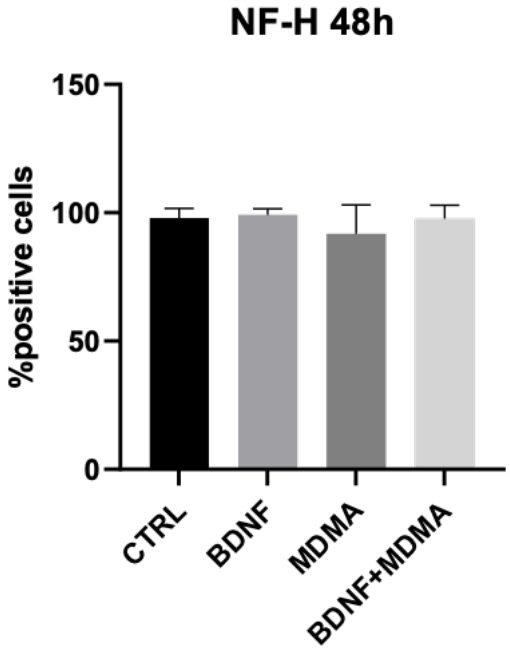


Figure 32. (Panel A and B): **A**: Exemplary immunofluorescence staining of NF-H in RN46A differentiated cells at 48h. NF-H is marked in red and cell nuclei are marked in blue (DAPI). Scale bar: 200 µm. **B**: The histograms show the percentage of NF-H-positive cells at 48h. The data are expressed with mean ± SD (CTRL n=30, BDNF n=30, MDMA n=26, BDNF+MDMA n=27) and are analyzed by Kruskal Wallis followed by Dunn's test.

5. DISCUSSION

Although MDMA has been recently proposed as an adjunct in psychotherapy to treat anxiety-related disorders and, in particular, PTSD (Mithoefer *et al.*, 2011), its 'dark side' as well as its neurotoxic effects, and its ability to promote the onset of psychiatric symptoms, have always been acknowledged and constantly investigated (Lieb et al., 2002; McGuire et al., 1994; Morgan, 2000; Parrott, 2002; Potash *et al.*, 2009; Thomasius et al., 2005). In particular, the dual diagnosis condition, i.e. the coexistence of substance use disorder and mental illness, underlines the urgency of understanding whether the alterations caused by psychedelic substances such as MDMA may reflect the alterations known to be also involved in mental illnesses.

As mentioned in the introduction, the discussion of this PhD thesis will focus on the below results:

1) the level of BDNF/TrkB and HDAC enzymes in PFCX and HIPPO of MDMA-treated rats and the effect of the HDACi sodium butyrate.
2) peripheral BDNF levels in serum and plasma of MDMA users and MDMA-naïve controls
3) the effects of MDMA treatment on neurofilaments in a differentiated serotonergic neuronal cell line (RN46A) and the potential protective role of BDNF.

With the present study it was observed that acute and repeated MDMA treatment, induced an increase in the neurotrophin BDNF and its receptor TrkB gene expression in the PFCX while an opposite direction was observed in HIPPO. These results are in agreement with previous studies (Hemmerle *et al.*, 2012; Martinez-Turrillas *et al.*, 2006), suggesting that these alterations could represent a compensatory mechanism to counteract serotonin depletion induced by MDMA in the PFCX, and they are probably related to an observed increase in TPH in the same area (Garcia-Osta *et al.*, 2004). In this regard, it has been shown that there is a cross regulation between BDNF and serotonin (Martinowich and Lu, 2008; Mattson et al., 2004). Another study has shown that BDNF is increased in KO mice for TPH 2 (TPH2 $^{-/-}$), suggesting that, in the absence of serotonin, the levels of BDNF are increased in the attempt to recover serotonergic synapses in this brain region (Kronenberg et al., 2016). The opposite alteration was observed in HIPPO following both acute and repeated MDMA treatment and it

could be related to the negative effect of MDMA on memory (Hemmerle *et al.*, 2012; Ros-Simo et al., 2013; Sprague et al., 2003). On the other hand, decreased BDNF levels seem to be also associated with the glucocorticoid levels (Schaaf et al., 1997; Smith et al., 1995). In fact, a decreased in this neurotrophin in the HIPPO was reported after corticosterone administration (Schaaf et al., 1998) and MDMA can increase corticosterone levels (Aguirre et al., 1997; Hemmerle *et al.*, 2012; Nash et al., 1988).

The analysis of class I HDACs in PFCX showed a reduction of mRNA levels of these genes following acute MDMA treatment and, conversely, an increase in their biosynthesis after repeated exposure. In the hippocampus, a similar overall trend was observed.

Since treatment with HDAC inhibitors has been shown to have a neuroprotective effect (Ziemka-Nalecz et al., 2018), the increased gene expression of class I HDACs following repeated treatment, which occurred especially in PFCX, could represent a predisposing factor for the onset of neuropsychiatric disorders. Moreover, in PFCX an opposite direction of HDAC1 protein was observed, increasing after acute exposure and decreasing after repeated treatment. Given the involvement of HDACs in the epigenetic regulation of BDNF gene expression and in the light of the data here reported, it is possible to speculate that the lower protein level of HDAC1 in PFCX after repeated treatment could enhance BDNF expression and facilitate a neuroprotective action. In the same way, the increase of HDAC2, known to be involved in the regulation of cognitive and memory processes, after repeated MDMA treatment in the HIPPO, could underline the higher vulnerability of this area to MDMA neurotoxicity. In support of this view, Yu Sun and colleagues showed that increased HDAC2 expression leads to repression of BDNF transcription in mouse hippocampal neurons, leading to memory impairment, and that inhibition of HDAC2 expression in the same hippocampal neurons restores BDNF levels preventing cognitive dysfunction (Sun et al., 2019).

Based on these results, showing that repeated MDMA exposure caused a predominant increase in HDAC levels and considering that HDAC inhibitors regulates crucial gene for brain development such as BDNF, the influence of a histone deacetylase inhibitor upon MDMA behavioral and molecular effects was investigated.

Gene expression analysis showed that both MDMA and NaBut induced a significant upregulation of BDNF and TrkB gene expression in the PFCX. In fact, some studies have shown that sodium butyrate is able to increase BDNF levels in different pathological conditions and to evoke an antidepressive-like effect (Barichello et al., 2015; Han et al., 2014; Schroeder *et al.*, 2007; Sun et al., 2016; Varela et al., 2015). In the HIPPO, a different situation was observed. In this area only the cotreatment NaBut+MDMA, but not MDMA or NaBut alone,

was able to cause a significant increase in BDNF gene expression and of its receptor TrkB, probably due to a synergistic effect on these two drugs. However, further detailed studies are required to clarify the possible reasons of this effect.

During the behavioral studies, we observed a significant increase in locomotor activity in the whole arena, which was induced by both acute and repeated MDMA treatment. In addition, we also observed that NaBut had no effect on overall locomotor activity but the pretreatment with this HDAC inhibitor induced locomotion potentiation that it is significant at day 1 only. After this time, this potentiation was no longer observed.

Considering the time spent and the distance travelled in the different zones of the open field arena, we observed that rats treated with MDMA spent more time than controls in the central zone, thus revealing a reduction in anxious behavior. NaBut pretreatment seems to potentiate this anxiolytic effect.

Regarding the number of entries in the central zone of arena, no significant changes were observed in the MDMA group in comparison with saline-treated animals, whereas the number of entries in the group pretreated with NaBut before MDMA was significantly increased. Moreover, the amount of time spent in the central zone by animals of the MDMA group was higher in comparison to controls (saline-treated rats) and this parameter was further increased by NaBut pretreatment. All in all, obtained data suggest that NaBut pretreatment was able to potentiate MDMA anxiolytic effect. In fact, HDAC inhibitors have also been shown to potentiate some drug-induced behaviors, suggesting synergic interactions between HDAC inhibitors and drugs of abuse (Adachi and Monteggia, 2009; Sanchis-Segura *et al.*, 2009). These data are also in line with other studies, indicating that MDMA could be useful for the treatment of psychiatric pathology like PTSD during adulthood (Sessa *et al.*, 2019). This hypothesis may be supported by data obtained in recent studies concerning the use of NaBut in a rat model of PTSD (Mohammadi-Farani et al., 2021; Mohammadi-Farani et al., 2020). In this regard, Mohammadi-Farani A. and colleagues showed that NaBut treatment is able to reverse single prolonged stress-induced extinction deficits and that it can prevent the development of a PTSD phenotype (Mohammadi-Farani *et al.*, 2021).

However, some further evidence should be also considered, which indicates that MDMA exposure during adolescence could cause different neurotoxic effects that are responsible for the later onset of psychiatric disorders (Cadoni et al., 2017; Chitre et al., 2020; Frau et al., 2016; Hernandez-Rabaza et al., 2010).

Thus, the possibility of a long-lasting neurotoxic effect of MDMA is not excluded, but additional studies are required to better clarify this aspect.

With respect to the human study, we found non-significant group differences in BDNF blood levels between recently abstinent MDMA users and controls. Although the alteration of BDNF

in serum showed only a weak increase, these data seem to be in line with Angelucci et al, who observed an increase in serum BDNF in ecstasy users, suggesting that the observed alteration of this neurotrophin can be a compensatory mechanism to recover MDMA-induced serotonin depletion (Angelucci *et al.*, 2010).

Several studies suggested that peripheral BDNF can be used as a proxy of brain BDNF (Klein *et al.*, 2011; Pan *et al.*, 1998; Sartorius et al., 2009). However, given that serum and plasma measurements do not correlate often (Bocchio-Chiavetto *et al.*, 2010; Gejl *et al.*, 2019; Tsuchimine et al., 2014) and they seem to reflect different pool of this neurotrophin (Gejl *et al.*, 2019), recently it has been widely debated whether plasma or serum analysis better reflects central BDNF.

In fact, serum BDNF derives from platelets and some critical methodological issue, such as the clotting time, has to be taken into account (Gejl *et al.*, 2019). In this regard, due to the small contribute of platelet-released to plasma BDNF, plasma BDNF measurement seems to reflect free BDNF, possible better related to central BDNF (Gejl *et al.*, 2019). However, BDNF in plasma can also be affected by pre-analytical conditions such as temperature and centrifugation strategy (Elfving et al., 2010; Gejl *et al.*, 2019). It is important to consider that platelets can also be present in plasma. In addition, several studies have found a positive correlation between platelet count and BDNF concentration (Kronenberg *et al.*, 2021; Naegelin *et al.*, 2018; Ziegenhorn *et al.*, 2007).

However, even if our data are not statistically significant, MDMA group has shown lower BDNF levels in plasma, which correlated with MDMA abstinence period. This result may indicate that BDNF is impacted by MDMA use post acutely but also recovers with longer abstinence periods. Moreover, as expected, no significant correlation between serum and plasma BDNF was found.

Finally, in our analysis, males seem to have higher peripheral BDNF levels than females specifically in plasma. This difference is in agreement with the similar trend observed in the study of Lommatzsch and colleagues (Lommatzsch *et al.*, 2005) whereas, other studies reported different results. It is in fact well known that there are many confounding factors in the measurement of peripheral BDNF such age, platelets count, BMI and for women, menstrual cycles (Glud et al., 2019; Golden et al., 2010; Lommatzsch *et al.*, 2005; Wei et al., 2017; Williams et al., 2016).

Considering these human results, our obtained data confirmed that BDNF serum and plasma do not correlate but may reflect different biological pools of the neurotrophin (Gejl *et al.*, 2019).

Regarding the in vitro model experiments, it is well known that MDMA is neurotoxic and that it mainly acts on serotonergic neurons. For this reason, we differentiated the serotonergic neuronal cell line RN46A. This cell line expresses NFs, neuronal intermediate filaments that are fundamental to maintain the shape of neurons and that have recently been shown to be released into the extracellular spaces after a neuronal damage and death.

After checking the positivity for neuronal markers MAP2 and the different morphology, the neuronal cells demonstrated, as it is already known, that BDNF enhance neuronal growth. Interestingly, BDNF was able to rescue from MDMA toxicity only after 24h. This effect is probably due to the capacity of BDNF to protect neurons from MDMA neurotoxicity. In fact, MDMA is known to induced apoptosis, necrosis and autophagy mechanisms (Capela *et al.*, 2007; Li *et al.*, 2014). By contrast, BDNF neuroprotection is not maintained over 24h, and this may be due to neurotrophin degradation in the cell culture medium.

In addition, we evaluated ICC for NFs and our data showed that there was a global decrease in NF-L levels after MDMA treatment. The current findings are in agreement with the literature, in which NFs and NF-L in particular, are considered the most promising biomarkers for pathology involving a neuronal injury. García-Cabrerizo and colleagues have already demonstrated that MDMA decrease NFs levels in rat hippocampus (Garcia-Cabrerizo and Garcia-Fuster, 2015), while other substances such as methamphetamine, morphine and cocaine were found to decrease NFs levels in animals brain (Beitner-Johnson *et al.*, 1992; Sanchez *et al.*, 2003). These results suggest substantial neuro-axonal alterations after MDMA use and the obtained data are in agreement with recent findings, which consider NF-L the most promising biomarker for all the pathologies characterised by neuronal damage, including substance use disorders.

6. CONCLUSION

Present data show that MDMA is able to alter different crucial genes as well as the proteins involved in both substance use disorders and psychiatric conditions. Animal studies showed alterations both in BDNF pathways and in the epigenetic enzymes HDAC.

Investigation in humans brought into view that peripheral BDNF could not reflect central BDNF and serum and plasma BDNF can express different types of this neurotrophin, so the use of different matrices appears critical in the study design. Moreover, data obtained in the differentiated serotonergic cell line highlight the useful role of NF-L as a biomarker of neuronal damage induced by MDMA, confirming the importance of studying NFs in the field of

neuropsychiatric disorders. Monitoring the levels of NF-L can be beneficial not only to evaluate the outcome of the disease activity, but also to consider the possible use of this protein as a biomarker for measuring the response to new therapeutic strategies.

However, further detailed studies are required to better clarify the damage caused by MDMA.

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