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Linking addiction-related behavior to synaptic efficacy and network activity in the prefrontal-accumbal pathway of behaving rats

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Mamã e papá,

Pelo amor maior

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PREFACE

The term will (to) power, *der Wille zur Macht*, was first coined by Nietzsche, referring to the essential driving force in humans. Free will, despite limited or *determined* by our human cognitive bias, as beautifully shown by Kahneman, underlies the nature of choice and decision and still allows for true freedom at times. The mere belief in free will, in which the *self* commands thought and action, causes humans to be more emphatic and compassionated. Skinner alludes to free will as delusive, due to the dependency of all actions on prior events. António Damásio stated, about the advantages of understanding the mechanisms underlying a decision-making mind, that "*Explaining the mental processes, conscious or unconscious, increases the possibility to strengthen our deliberation power*".

Addiction has the subversive ability to take away power of deliberation, or the illusion of it, and turn the willing self into a sightless chasing vessel of the unachievable at the cost of everything else. This very unique facet of addictive behavior of narrowing and striping human nature to a single instrument of pursuing a drug, in such a perverse and vicious manner, is what led me to research the biological mechanisms underpinning addiction.

Artists, writers, humans of scientific and creative endeavor have been drawn to psychoactive substances, and its perception and mind-altering effects. Would Hemingway or Fitzgerald's essence and identity been as singular if not afflicted by the struggle of alcoholism?

The most interesting questions are usually the more troubling ones. Neuroscience allows us to glimpse into the mind's "clockwork" and of the concerted timely wheels that run behavior. Thus, it is the perfect toolkit and framework to study neuropsychiatric illness such as addiction. Nobel laureate Saramago said, "Only if we stop to think about the small things will we come to understand the great ones". Being part of the common effort to build a body of knowledge that will take us further in decoding addiction through my research has been a sincere and fun privilege. The work here described was conducted with the hope to add a humble piece into the complex and edifying puzzle of substance use disorders.

Pursuing a PhD set me out on a voyage of discovery. Having the opportunity to be a part in the dissemination of new ideas and practices, excellence, interdisciplinary, interaction with talented and imaginative people worldwide and the constant flow of knowledge and intellectual challenge were all enticing aspects of doctoral work. The pursuit of knowledge, of knowing for the sake of knowing and the never ending will to learn were and are my drivers in research and life. With the same curiosity that glued me to the screens every

weekend morning to Sir David Attenborough for an hour-long voyage of knowledge and beauty.

Lastly, I believe to be a *Scientist* one needs to be, or attempt to be, a generalist. I certainly feel one, and did a conscious exercise to infuse this notion - of looking beyond - and having an englobing perspective in this dissertation. Thus, the small infusions of history and scientific 'digressions' are more than a mere ornament, but instead attempts to entice curiosity and sneak peek into the 'big picture'. Focusing solely on the very "own", specific and compact piece to which most time is dedicated is reductive and counterproductive. But rather to demonstrate interest and child-like curiosity to knowledge will shine light not only on the work being pursuit, but also on the ones alongside with whom it is being sought.

Using Einstein's voice, *"Intellectual growth should commence at birth and cease only at death",* thinking, talking and breathing science in this dissertation is just another step on a journey of continuous growth.

'The brain is a world consisting of a number of unexplored continents and great stretches of unknown territory.'

Santiago Ramón y Cajal

ABSTRACT

Addiction is a chronically relapsing brain disorder, involving compulsive drug seeking and taking. Enduring vulnerability to relapse is a challenging feature to manage in substance use disorder, with devastating effects to those who suffer from it, as well as at familial and public health levels. Incubation of drug craving characterized by gradual increases in cue-induced drug craving following halting of drug use, may contribute to heightened relapse risk, even after prolonged abstinence.

Addictive drugs act upon and usurp the mesolimbic circuit, with long-term drug abuse leads to reward processing, cognitive and decision-making deficits. Drug-driven neuronal plasticity within the Prefrontal cortex (PFC) to Nucleus Accumbens (NAc) pathway is a known key substrate and mediator of addictive behavior.

Here we performed longitudinal *in vivo* local field potential (LFP) recordings in freely behaving rats throughout an incubation of drug seeking paradigm. This approach proved suitable to assess both evoked and spontaneous LFP activity at distinct behavioral stages of the addiction cycle, in a within subject manner.

Chronic cocaine self-administration induced strengthening of the PFC-NAc pathway, accompanied by enhanced glutamate release, when compared to drug naïve conditions. Compellingly, the degree of synaptic adaptation correlated with the cocaine intake and incubation severity of individual rats. At the network level, accumbal oscillatory profile of rats that underwent CSA was also altered, with suppression of high gamma and enhanced alpha and beta waves.

Throughout withdrawal, persistent pre-synaptic release subsisted, while network changes proved to be transient. Yet, rats with history of cocaine intake did showed altered LFP patterns, upon a cocaine challenge, when compared to saline yoked counterparts, suggesting impaired corticostriatal network dynamics that endures after long-term abstinence. During reinstatement, *i.e.* relapse-like conditions, distinct frequency components were found to be differentially modulated by drug seeking behavior.

Drug-driven adaptations to synaptic transmission and concomitant alterations of oscillatory landscape of functionally connected areas, such as the PFC and NAc, represent multiple-leveled dysregulation exerted by addictive drugs. Concerted maladaptive changes may contribute to the development of a *de novo* homeostatic threshold that is both driven by and drives drug abuse, craving and relapse in a spiraling cycle of addiction.

ZUSAMMENFASSUNG

Sucht ist eine chronisch rezidivierende Gehirnerkrankung, die zwanghaftes Drogensuchen und -nehmen beinhaltet. Dauerhafte Anfälligkeit für einen Rückfall ist eine Herausforderung, nicht nur für die Betroffenen, sondern auch für die Angehörigen und das öffentliche Gesundheitswesen. Inkubation der Drogensucht, die durch einen allmählichen Anstieg des Drogenkonsums gekennzeichnet ist, kann zu einem erhöhten Rückfallrisiko auch nach längerer Abstinenz führen.

Suchtmittel modulieren das mesolimbische System, wobei langfristiger Drogenmissbrauch zu einer veränderten Belohnungsverarbeitung, kognitiven Defiziten und Entscheidungsdefiziten führt. Drogen-vermittelte neuronale Plastizität innerhalb der Projektion vom präfrontalen Kortex (PFK) zum Nucleus Accumbens (NAc) ist ein bekannter Mediator von Suchtverhalten.

In dieser Arbeit führten wir longitudinale in vivo lokale Feldpotential (LFP)-Aufzeichnungen in frei verhaltenden Ratten während einer Drogeninkubation durch. Dieser Ansatz erwies sich als geeignet sowohl evozierte als auch spontane LFP-Aktivität in verschiedenen Stadien des Suchtzyklus subjektbezogen zu erfassen.

Chronische Kokain-Selbstverabreichung (KSV) induzierte eine Verstärkung der PFK-NAc-Projektion durch eine erhöhte Glutamatfreisetzung im Vergleich zu Kochsalz. Interessanterweise korrelierte der Grad der synaptischen Anpassung mit der Kokainaufnahme und dem Ausmaß der Inkubation einzelner Ratten. Auf Netzwerkebene veränderte KSV die oszillatorische Netzwerkaktivität, wobei Alpha- und Betawellen verstärkt sowie Gammawellen im hohen Frequenzbereich unterdrückt wurden.

Während des Kokainentzugs persistierte die erhöhte präsynaptische Freisetzung, während sich die Netzwerkänderungen erholten. Eine nachfolgende Kokain-Provokation veränderte jedoch die Netzwerkaktivität im Vergleich zu einer Kochsalzlösung, was auf eine veränderte kortikostriatale Netzwerkdynamik hindeutete, die nach längerer Abstinenz bestehen blieb. Während der Wiederaufnahme des Kokainsuchverhaltens, *d.h.* unter rezidivähnlichen Zuständen, wurden verschiedene Frequenzkomponenten der Netzwerkaktivität differentiell moduliert.

Kokain-vermittelte Anpassungen der synaptische Übertragung und damit einhergehende Veränderungen der oszillatorischen Netzwerkaktivität funktionell verbundener Hirnareale, wie PFK und NAc, stellen eine mehrstufige Dysregulation durch Suchtmittel dar. Konzertierte maladaptive Veränderungen können zur Entwicklung einer *de novo* homöostatischen Schwelle beitragen, die sowohl durch Drogenmissbrauch, Verlangen und Rückfall in einem spiralförmigen Suchtzyklus angetrieben wird als auch diesen Vorschub leistet.

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AIM AND THESIS OUTLINE **26**

1 INTRODUCTION

The term Neuroscience was first coined in the 1960s, marking the dawn of a period in which *"disciplines would work together cooperatively, sharing a common language, common concepts, and a common goal—to understand the structure and function of the normal and abnormal brain"* (Squire *et al.*, 2008).

The multidisciplinary nature of neuroscience that encloses biological, clinical and psychological dimensions, allowed for a prompt knowledge buildup and a shift in understanding that profoundly transformed our view of psychiatric disorders.

Mental disorders are more impairing and disabling, among all disease groups, in terms of years lived in disability (YLD ,42%) than fatal, as measured by DALY (28%, disability adjusted life years). The high disability burden is due to early onset, chronic and relapsing nature the disease, specific impairment and poor treatment (Wittchen *et al.*, 2011). In Europe, brain disorders are the biggest contributor of disease burden, which reflects the currently inability to provide effective and long-lasting treatment solutions (Gustavsson *et al.*, 2011; Wittchen *et al.*, 2011). According to the World Health Organization (WHO), the 'non-economic' burden of neuropsychiatric illness accounts for 1/3 of the disease burden in developed countries (Gustavsson *et al.*, 2011). Substance dependence, in particular, is estimated to affect over 29 million people worldwide, with consequences spanning through personal, familial, criminal, societal and public health spheres (UNODC, 2016).

1.1 Addiction: Clinical definition, facts and figures

References of alcohol intoxication date back to the nascence of civilized humankind. The Chinese (10 000 BC) are known to have produced and consumed inebriating beverages (*e.g.* Jiahu, 10%), mostly derived from fermented fruit and grain.

Wine and beer intoxication and its deleterious behavioural effects are portrayed in numerous religious texts, as the Torah, Christian Bible and the Koran (Nathan *et al.*, 2016).

Concomitantly to the emergence of modern Psychiatry, Benjamin Rush (1745–1813), American psychiatrist and founding father, was a fierce proponent of compulsive drinking as a mental health condition, rather than a character flaw or a self-inflicted predicament. He founded the first treatment center for alcoholism in Boston (1812).

German psychiatrist Emil Kraepelin (1856-1926) is considered by many a pioneer and the "father" of modern psychiatry. His influential work, emphasized the physiological and

genetic factors in the etiology of neuropsychiatric disorders, the importance of meticulous nosology and diagnosis, as well as more humane and psychopharmacology-based treatments (Kraepelin, 1913). As such Kraepelin was also an advocate for the view of alcoholism as a medical disease (Nathan *et al.*, 2016).

The term addiction, as is used in the clinical setting nowadays, was first introduced in Anglophonic countries. Addiction, from the latin *addictus*, stands for 'enslaving'. Also, rooted in the Latin, the French used alternative terms such as *toxicomanie* or *assuétude* (habit). In German terminology includes *Abhängigkeit* (dependence) or *Sucht* (addiction) (Crocq, 2007).

Interestingly, not only the human species engages in binging and substance use. Even in natural ecosystem there is evidence of an evolutionary root of substance use, namely alcohol. In Malaysia's tropical forest, Wiens and colleagues discovered, for the first time, chronic alcohol consumption in mammals in the wild (Wiens *et al.*, 2008).

Nowadays, one in 20 adults is estimated to have used at least one drug in 2014 (UNODC, 2016). According to the latest European drug report, issued by the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA), more than quarter of people aged between 15 and 64 years old have tried illicit drugs during their life time.

Despite some alarming reports (*e.g.* US opioid epidemic), global trends in the estimated prevalence of drug use remain stable (2006-2014). Specifically, prevalence of drug use has stagnated at 5.2%, of which 0.6% refer to prevalence of individuals with drug use problems (UNODC, 2016). Worldwide, there were 113,700- 250,100 estimated drug-related deaths (*i.e.* 23.8-52.5 per million people aged 15-64, 2014). The societal impact of drug is a multi-leveled problem, comprising tangible (*e.g.* health care, productivity and crime) and intangible costs (*e.g.* loss of life, and quality of life). Economically, substantial variation is observed, with some estimations pointing towards costs ranging from 0.07 to 1.7 % of the GDP [with strong bias towards developed countries, where most studies were conducted] (UNODC, 2016).

Cocaine is the most frequently used illicit stimulant in Europe, being the second most tried drug following cannabis (EMCDDA, 2017). An estimated 17.5 million (5.2%, 15-64) Europeans have tried cocaine at least once. Among cocaine users, one important distinction regards route of administration (Figure 1.1). Whereas socially functional users tend to sniff powder cocaine (cocaine hydrochloride), more stigmatized users smoke crack or inject cocaine, often associated with polydrug use, such as opioids (EMCDDA, 2017).

Epidemiological studies like the National Comorbidity Survey in the US, have also revealed that only a small fraction of recreational drug users do eventually develop substance use disorder, as characterized by diagnostic criteria. In fact, only 15-16% of cocaine users, 8% of marijuana users and 12-13% of alcohol users transition to addiction, within 10 years of first use (Wagner & Anthony, 2002). This study evidently demonstrates that the majority of population will not get addicted, even when regularly using highly addictive substances (Lüscher, 2016).

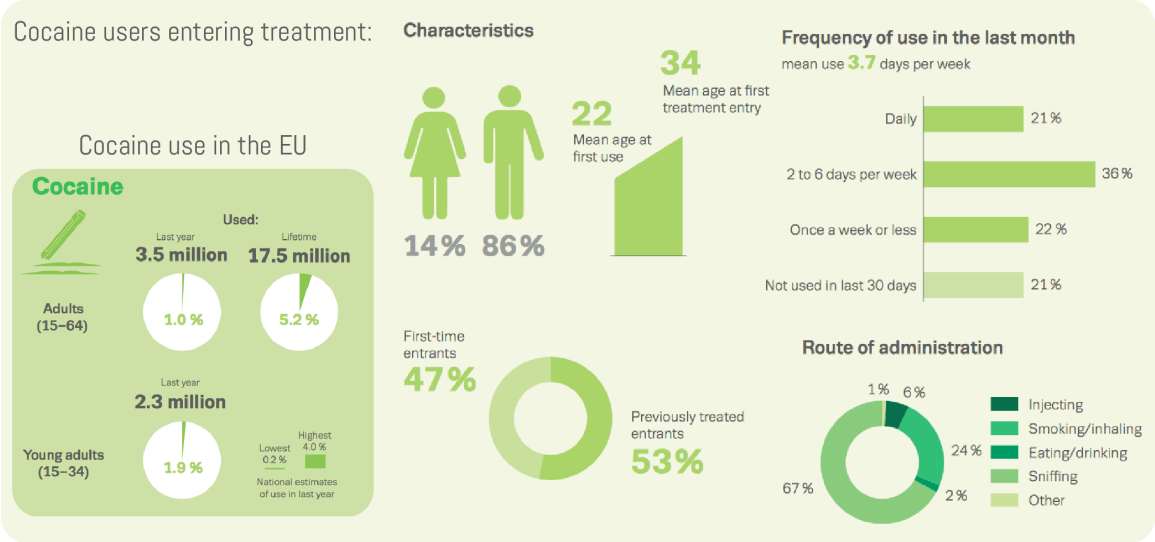

Figure 1.1 Estimates of cocaine use and demographics of users entering treatment in European Union (EU). Characteristics are for all treatment entrants with cocaine as primary drug (adapted from EMCDDA, 2017).

Over the last decades, concurrently to the development of neuroscience techniques, the view of addiction as a brain disorder has emerged and solidified.

'Addiction is a disease of altered behavior' (Lüscher, 2016). This seems an oddly broad definition of addiction. However, it reflects the still current difficulty in defining and diagnosing addiction, as well as reaching a consensus on the *"Addiction-ary"* (Kelly et al 2016).

The International Classification of Diseases (ICD), the standard diagnostic tool issued by WHO, defines substance dependence as *"A cluster of behavioral, cognitive, and physiological phenomena that develop after repeated substance use and that typically include a strong desire to take the drug, difficulties in controlling its use, persisting in its use despite harmful consequences, a higher priority given to drug use than to other activities and obligations, increased tolerance, and sometimes a physical withdrawal state"* (ICD-10, WHO, 2016).

According to ICD-10, a conclusive diagnosis of dependence should include three or more of the following criteria, having been present at some point during the past year (Table 1).

Table 1 Diagnostic criteria for substance dependence, according to ICD-10

1	A strong desire or sense of compulsion to take the substance;
2	Difficulties in controlling substance-taking behaviour in terms of its onset, termination and levels of use;
3	A physiological withdrawal state;
4	Evidence of tolerance
5	Progressive neglect of alternative pleasures or interests;
6	Persisting with substance use despite clear evidence of overtly harmful consequences.

However, even this fairly recent definition is already undergoing revision. In fact, the new draft diagnostic guidelines of ICD-11 for substance dependence include only three central features. The diagnosis requires *"two or more features to be present at the same time and to repeatedly occur over a period of 12 months, or continuously over a period of at least a month"* (Rehm & Poznyak, 2017).

Table 2 Diagnostic criteria for substance dependence, according to ICD-11 (for field testing)

1	**Impaired control over substance use**, be this its onset, level, circumstances or termination of use, often accompanied by a subjective sense of urge or craving to use the substance.
2	**Substance use is a priority in life** such that its use takes precedent over other interests or enjoyments, daily activities, responsibilities or health or personal care, and may continue despite harmful consequences.
3	**Physiological features** (indicative of neuroadaptation to the substance) as manifested by: (i) **Tolerance**, (ii) **withdrawal symptoms**, following cessation or reduction in substance use, or (iii) repeated **use** of the substance (or pharmacologically similar substance) where the use is to prevent or alleviate withdrawal symptoms.

Here, I made the deliberate choice of describing in detail the clinical guidelines, as presented by the ICD. This publication, developed by WHO (1948) and freely available, covers all health conditions, and countries report on health statistics using ICD codes.

Another very popular diagnostic tool is the Diagnostic and Statistical Manual of Mental Disorders 5 (DSM-5), which is US-based, only covers mental and behavioral disorders, and it is "owned" by the American Psychiatric Association. In DSM-5 addiction is the term used to describe the most severe and chronic stage of substance-use disorder. This stage is characterized by compulsive drug use, even when trying to quit.

BOX 1 THE CONCEPT OF DISEASE

Defining disease, at least at a first glance, seems quite straightforward. However, once one puts his/her mind into it, it is remarkably challenging to conceptualize and articulate a universal definition of disease. Even when stating that disease is the opposite of health the task does not get any simpler. The World Health Organization defines health as "a state of complete physical, mental and social well-being, not merely the absence of disease or infirmity" (WHO, 1946). Furthermore, ill-health labels do not stand in a vacuum, but are vastly dependent on sociocultural backgrounds, and "only exist in relation to people, and people live in varied cultural contexts" (Scully, 2004). The contextual dependency, weighs in complexity, especially when referring to mental illness, as drug and behavioral addictions.

Is there such a state of purely "unproblematic" drug use? Or how to differentiate between high involvement - passion - and dysfunctional involvement - behavioral addiction (Billieux et al., 2017).

Regardless, why should a scientist, who studies physiological mechanisms, be concerned with anthropological perhaps philosophical notions of experience that might not be fit into the current medical model of disease? Scientific enterprise takes place in a cultural framework that determines research trends and funding. Additionally, how science conceptualizes and 'pathologizes' conditions shapes public perception. Thus, disease models and diagnosis guidelines do not only impact research and corporate stakes but "embody profound ethical debates about in identity, human rights and tolerance of difference" (Scully, 2004).

The definition of addiction (see Box 1) is still an evolving concept and is still raising discussion nowadays, as reflected by continuing updates on diagnostic manuals as well as deep discussion in meetings (Rehm & Poznyak, 2017). One very interesting approach, as an attempt to reach consensus, arose from the Delphi method that aims to create a transdiagnostic dimensional approach towards neurocognitive assessment of addiction (Yücel, 2017). This method makes use of a panel of experts, ranging from basic investigators to psychological and clinical practitioners, to delineate on the essential cognitive-affective domains (from Research Domain Criteria, RDoc, NIH) shared in substance and behavioral additions. Seven essential domains gathered consensus: Reward valuation, Expectancy (reward prediction error), Reward learning, Response selection/inhibition, Action selection,

Habit and Compulsivity. These contribute to different extents to vulnerability and chronicity aspects of addiction (Yücel, 2017, personal communication).

One astonishing feature of addiction is its dependency on context. Patients usually relapse in environments previously associated with drug use. A remarkable 'historical experiment' – Operation Gold Rush (1971) – that clearly illustrates this effect, refers to the Vietnam War veterans suffering from heroin dependence (15-20%, Robins *et al.*, 2010). When returned to the United States (US), veterans had higher rates of treatment success when compared to local clients (Zinberg, 1986). One persuasive explanation lies in the fact that soldiers were no longer under psychologically straining circumstances, as in the battlefield, and were also reimmersed back into their civilian life. Therefore, contextual drug cues were also rarer and so was relapse (Zinberg, 1986; Lüscher, 2016). In fact only 12% relapsed within three years (Robins *et al.*, 2010), in contrast with the relapse rates following residential treatment in general population (≈60%, 6 month follow-up; Schuman-Olivier *et al.*, 2014).

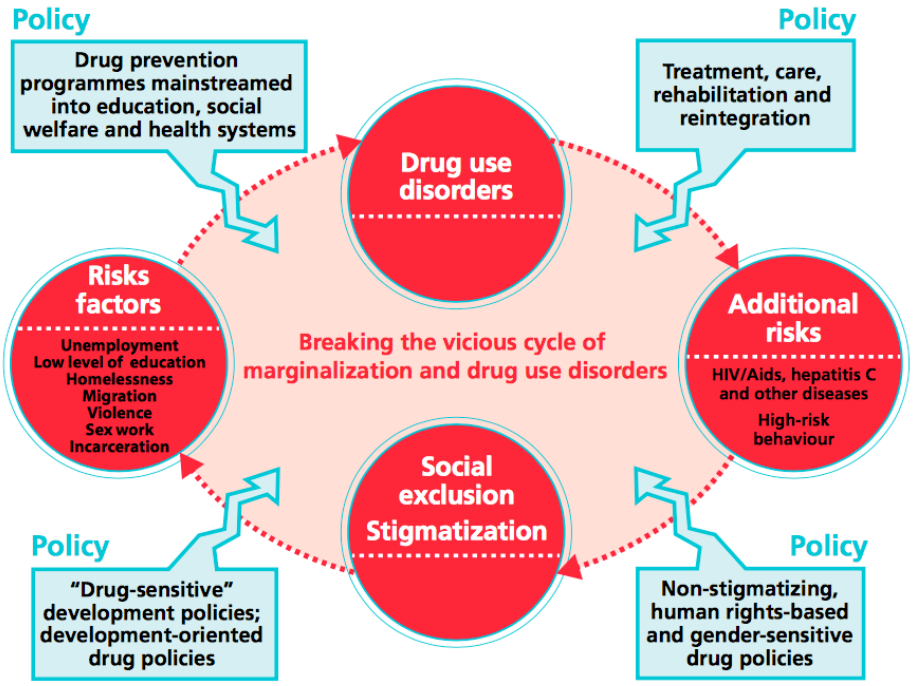

Figure 1.2 United Nations Office on Drug and Crime (UNODC) recommendation on integrated policy strategies to tackle drug use disorders. *"The world drug problem is intertwined with all aspects of sustainable development."* Both analysis and responses to the drug problem – policies – should reflect sustainable development goals – social and economic development and environmental sustainability, within a global partnership framework; Thus, aiming to maintain specialized drug policies alongside general developmental strategies (UNODC, 2016).

The impact that external and contextual stimuli can have on drug use behavior spans over a broader perspective than relapse and drug cues. Social inequality and poverty are a known (avoidable) risk factors of mental disorders, including addiction, as well as general welfare and success outcomes, as teenage births, lower literacy scores, obesity and imprisonment (Pickett & Wilkinson, 2015). Thus, despite compelling evidence on the neurobiological basis of addiction that should be further pursued, as researchers and citizens, we should also be keenly aware of socioeconomic factors, as determinants of substance use (WHO). As such, we should support and champion an integrated approach (Figure 1.2) that is likely to reduce wealth inequality and thus ameliorate on a multifaceted manner individuals' chances of developing problematic drug use, and eventually seeking treatment.

1.2 Theories of Addiction

Different conceptual and experimental frameworks have emerged to describe addiction, as a condition seeded in psychobiological processes.

A multistep general theory of transition to addiction was recently attempted but greeted with skepticism. Nonetheless, it conceptualizes addiction as a "true psychiatric" disorder by emphasizing individual vulnerability and how it interacts with drug exposure, in a 3-step process: (1) recreational use; (2) intensified escalated and sustained use; and (3) loss of control of drug intake (Piazza & Deroche-Gamonet, 2013). However, this theory does not attempt to explain relapse and associated reinstatement model, thus will not be further deepened.

Here, I'll briefly describe some of the contemporary and most influential theoretical frameworks of addiction: hedonic-allostasis, incentive-sensitization and aberrant-learning theories.

1.2.1 Hedonic-allostasis theory

Developed by Koob and LeMoal, it is based on the opponent-process theory of motivation (Solomon & Corbit, 1974). Addiction is represented as a spiraling cycle of dysregulation of neural systems mediating reward, that culminates in compulsivity and loss of control over drug intake. This cycle comprises of three stages: preoccupation-anticipation, binge-intoxication, and withdrawal-negative affect (Koob & Le Moal, 2001). This theory postulates that initial drug taking behavior is mediated by hedonically rewarding

properties of the drug - positive reinforcement. And, the emotional state of withdrawal functions as a negative reinforcer, since drug use is continued in order to avoid dysphoria and discomfort.

In contrast, chronic drug abuse is maintained by diminished drug rewarding effects and recruitment of stress systems that gives rise to a state of hedonic allostasis. Allostasis represents a pathological chronic maladaptive state characterized by a stable deviation of the normal reward setpoint or thresholds (Badiani *et al.*, 2011). Development of compulsive drug use is consequence of allostatic recruitment of cortico-striatal-thalamic loops (Koob & Le Moal, 2001).

1.2.2 Incentive-sensitization

From the Cambridge psychobiological school of thought, by Robinson and Berridge, incentive-sensitization encapsulates four essential premises (Robinson & Berridge, 1993; Badiani *et al.*, 2011). (1) First, the notion that addictive substances increase dopaminergic transmission. (2) It also postulates that the mesocorticolimbic dopaminergic system mediates incentive salience, as the process of attributing higher valence to cues associated with rewards, making them attractive or 'wanted'. (3) Repeated drug exposure induces enduring neural adaptations that make these brain circuits hypersensitive or 'sensitized' to drugs and drug-associated stimuli through associative learning. Therefore, weighed (incentive) motivation is devoted to 'drug wanting' that can explicitly manifest as drug craving. And finally, (4) sensitization of the neural systems controlling incentive salience ('drug wanting'), occurs independently of the pleasure elicited by the drug ('drug liking') and of the systems that mediate withdrawal.

One of the pitfalls of the incentive-sensitization theory of addiction is that sensitization in particular is extremely difficult to prove in human subjects. There is some evidence that repeated amphetamine induced enhanced behavioral responses, such as eye-blinking, vigor and energy ratings (Strakowski *et al.*, 1996; Robinson & Berridge, 2008).

1.2.3 Aberrant-learning

These theories emphasize the role of recurrent drug taking on shifting goal-directed actions (A-O) to stimulus-response processes (S-R, habits). The latter, emerge from enhanced instrumental conditioning to drug-associated cues and are characterized by

resistance to outcome devaluation, such as punishment (Belin *et al.*, 2013). Thus, addicts sustain drug taking behavior despite deleterious consequences.

Aberrant-learning processes are mediated by a gradual shift from ventral striatal regions, as the nucleus accumbens (NAc), and dopamine-dependent A-O mechanisms to dorsal striatal control of habitual drug seeking and taking.

Kalivas and O'Brien also build on the idea of addiction, as a disease of 'staged neuroplasticity'; highlighting the transient and stable forms of drug-induced plasticity (Kalivas & O'Brien, 2008a). Importantly, dysregulated glutamatergic neurotransmission plays a crucial role in the transition from declarative responses to loss of control and compulsive relapse.

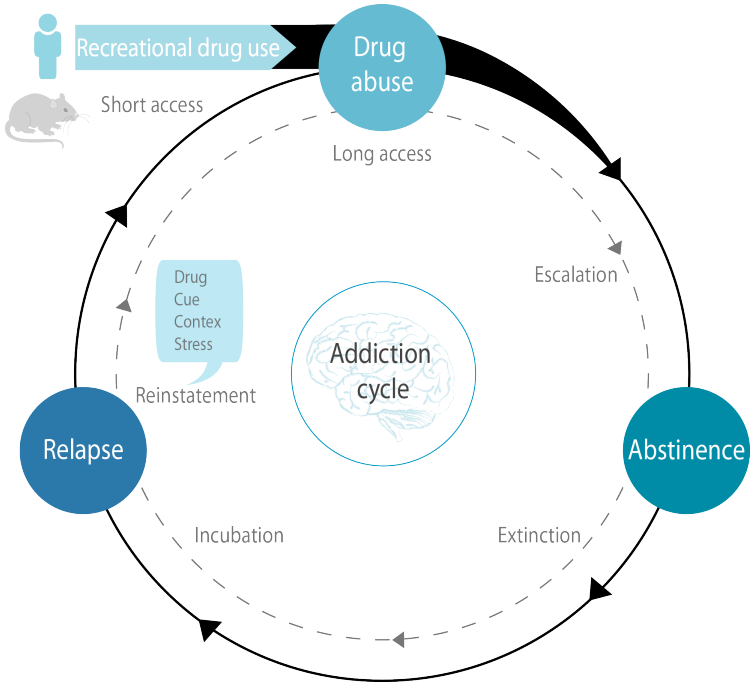

Figure 1.3 Addiction cycle: Stages of addiction. Stages of the development of addictive disorders. And experimental approaches to model human addiction specific components through distinct drug-exposure procedures and animal behavioral models (Catarina Luís, in accordance with Kourrich *et al.*, 2015).

In summary, despite the different neuropsychiatric constructs used to conceptualize drug addiction, all theories recognize addiction as a dynamic process in which different components emerge gradually making up for a developing cycle of pathophysiology (Figure 1.3). Addictive behavior can only develop in the presence of recurrent drug use – *recreational* followed by *drug abuse* – and addicts reiterate between *abstinence* periods and the enduring inability to restrain from drug taking, which can be influenced by environmental factors – *relapse*.

1.3 Animal Models: Self-administration

Detailed criteria and guidelines (section 1.1) and theoretical frameworks (section 1.2) are of importance when developing relevant research tools to determine the underlying neural mechanisms of addiction. Animal models are essential for the mechanistically understanding of addiction. Although there is no model that recapitulates all aspects of addiction (see Box 2), existent paradigms do model several atomic symptoms of the disorder (Figure 1.3).

The animal model for human substance abuse currently providing greater face validity is the self-administration (SA) model. One of the first accounts of self-administration was described by Weeks (1962) in unrestrained rats, which was soon followed by Denau and colleagues report in monkeys (Weeks, 1962; Deneau *et al.*, 1969). Since then, this model has been established not only in rats, but also in mice and non-human primates.

Typically, animals are implanted with chronic intravenous jugular vein catheters. Following recovery, animals are placed into an operant conditioning chamber (Skinner box) in which, upon instrumental response (lever press or nose poke) a contingent drug infusion is delivered. Infusions are usually paired with discrete cues, such as a cue light or a tone. Short-access (2h/day) versus long-access SA (6h/day) protocols, as well as varying durations (10-50 days) can be used to mimic distinct facets – *e.g.* recreational use, escalation or drug abuse (see Figure 1.3). Self-administration procedures also have different stages which allow for the study of specific aspects of addictive-like behavior. Acquisition of self-administration is a measure of the reinforcement potential of drugs, since animals obtain the reinforcer under fixed-ratio schedule, higher response rates indicate increased abuse liability. SA acquisition also allows to detect individual differences in the drug reinforcement sensitivity. During maintenance stage, responding rates are stable and different features can be assessed, for example: motivation for the drug – under progressive ratio schedule. Here, the number of instrumental responses needed to obtain the drug increases exponentially, until animals stop responding (*i.e.* break-point). Continued use despite negative consequences is examined by persistence of SA despite punishment by contingent electric foot-shocks (Panlilio & Goldberg, 2007).

DSM-IV/5-based multi-symptomatic model makes use of such criteria to determine addicted and non-addicted rats, despite equal cocaine intake. Three criteria are used to define addicted-like behavior: *(1) inability to refrain from drug seeking, (2) high motivation for the drug,* and *(3) compulsive drug use despite negative consequences* (Deroche-Gamonet, 2004).

It is remarkably predictive of individual vulnerability to develop addiction, since only a fraction of outbread rats (≈17%) loose control and transition to full addiction (Deroche-Gamonet, 2004). This figure fully aligns with human survey data, which reports that only ≈15% of recreational cocaine users eventually meet the diagnostic criteria for substance dependence (Wagner & Anthony, 2002).

BOX 2 ANIMAL MODELS OF NEUROPSYCHIATRIC DISORDERS

Validated experimental models of neuropsychiatric disorders, as substance dependence, are primary research tools to deepen our understanding of the pathophysiology of mental illness (Nestler and Hyman, 2010; Kaiser and Feng, 2015). Traditionally, three essential validators are used to assess a model's relevance and suitability:

Construct validity (or etiologic)

Degree to which the model is based on disease etiology, such as environmental or genetic risk conditions for developing the disease - meaningfullness;

Face validity

Degree to which the model replicates phenotypically the disorder - anatomical, physiological and behavioral features;

Predictive validity

Identical treatment response or outcome between the animal model and human patients

Nonetheless, as a researcher, one should keep in mind that a truly valid animal model of a psychiatric phenomenon would require the animal experience to be directly paralleled with the human condition, entailing "shared mechanisms for distress, despair, perspective, narrative and self-reflection" as well as "shared cultural or linguistic context" (Rollin and Rollin, 2014). This poses an ethical consideration, independent of validity, manifested by the 'psychologist's dilemma', which acknowledges the impossibility of a model to be simultaneously adequate and ethical (Rollin, 2010).

1.3.1 Reinstatement and incubation of drug seeking

Human abstinence or withdrawal, depending on the drug class, can be modeled in animals by forced abstinence, by either remaining in the home cage or undergoing extinction (Figure 1.3). During extinction, responding is no longer reinforced (no drug delivery) leading to decreased frequency of operant responses (Panlilio & Goldberg, 2007; Wolf, 2016).

Abstinence without extinction, after long-access SA, can produce incubation of drug seeking. This is a 'hypothetical motivational process' associated to progressive time-dependent increase in cue-induced drug seeking. Drug seeking in animals is used as a measure of incubation of drug craving in humans. Drug craving, an affective state in humans, can be triggered by drug-associated cues and may lead to drug taking, even after prolonged abstinence - relapse (Wolf, 2016).

Preventing relapse is the main goal of a successful treatment (see Box 3). Relapse, *i.e.* the resumption of drug-taking behavior following abstinence, is perhaps the most clinically challenging feature to manage in drug dependent patients.

Reinstatement models are used to replicate relapse-like behavior. In this paradigm, animals reinstate or recover a learnt response (*i.e.* drug seeking) when exposed to the unconditioned stimulus, such as non-contingent discrete or contextual drug cues, or drug-priming and stressors (Bossert *et al.*, 2013; Wolf, 2016).

BOX 3 TREATING SUBSTANCE DEPENDENCE

The first phase of treatment is **detoxification**. For drugs that cause withdrawal such as alcohol and opioids, is usually accompanied by pharmacotherapy like naltrexone, methadone and buprenorphine. To maintain abstinence there are several (or combination of) strategies.

Behavioral therapies are by far the most widespread approach to prevent relapse. These aid clients to be engaged in treatment, provide support and incentives to abstain, as well as learning to modify behavior, avoiding possible triggers, and resilience training for adverse circumstances and contextual cues (Carroll and Onken, 2005).

Substitution approaches are used for tobacco, like nicotine patches, and opioids with methadone and buprenorphine. **Anti-craving medication**, like naltrexone and acamprosate is also used to reduce craving in alcohol and opioids dependence (O'Brien, 2008). Despite behavioral and pharmacological approaches available, they have limited effectiveness, with only some patients responding and relapse rates remaining high.

Stimulant addiction therapies are even scarcer. Nonetheless, stemming from neurobiological research evidence (Chen et al., 2013), a pilot trial in Italy is paving the way by using **transcranial magnetic stimulation** (TMS) in the treatment of cocaine addiction. Patients on TMS reported less craving and had higher cocaine-free urine drug tests (Terraneo et al., 2016). Some clinical trials are also exploring the possible anti-relapse effects of **modafinil**, which is known to decrease impulsive behavior. Promising results were reported for cocaine and methamphetamine users, as well as alcohol-dependent patients with response control impairments (Dackis et al., 2005; Shearer et al., 2009; Joos et al., 2013).

1.4 Neurocircuitry of reward: implications for addiction

The first evidence of a brain "reward center" arose from Olds and Milner seminal study in 1954. Rats would voluntarily and repeatedly self-stimulate clearly defined brain areas, whose electrical activation acted as positive reinforcement. These mesoencephalic areas or afferent inputs were later described to comprise of dopaminergic neurons and projections (Olds & Milner, 1954; Crow, 1972).

The mesolimbic system attains its name from the Latin term *'meso'* or middle, and limbic or *'limbus'*, which means edge or border, firstly used by Paul Broca when describing *'le grand lobe limbique'* (1878). Nowadays, the mesocorticolimbic system refers to a group of cortical and limbic structures that are innervated by mesencephalic dopamine releasing neurons. The mesocorticolimbic dopamine system originates in the ventral tegmental area (VTA, A10)

and project to nucleus accumbens (NAc) dorsal striatum, lateral septum, bed nucleus of the stria terminalis, amygdala (Amg), hippocampus (Hip), olfactory tubercle and prefrontal (PFC), cingulate and enthorinal cortices (Sesack & Grace, 2010; Figure 1.4D). Besides the mesolimbic and mesocortical pathways, there are two additional dopaminergic systems the nigrostriatal and the tuberoinfundibular systems, originating in the substancia nigra (pars compacta, A9) and the hypothalamus, respectively.

Dopamine-releasing neurons are usually identified by tyrosine hydroxylase (TH) expression, an enzyme involved in dopamine synthesis. In rats, there are 40 000 to 45 000 TH-positive cells, half of which are located in the substancia nigra (SN). Humans have about ten times more dopaminergic neurons, 400 000 to 600 000, with about 70% in the SN (Björklund & Dunnett, 2007). Although formally segregated into distinct pathways – mesocorticolimbic and nigrostriatal - there is growing evidence of functional interaction between SN and VTA dopamine systems (see Wise, 2009).

Dopamine exerts its functions by binding to G-protein coupled receptors. They are divided in D1 (D1R and D5R) and D2 (D2R, D3R and D4R) classes of receptors. D1-class receptors are predominantly expressed postsynaptically and activate G□s/olf, which stimulates cAMP via adenyl cyclase (AC). D2-class receptors activate G□i/o to inhibit AC activity and cAMP production and are found both pre and postsynaptically (Beaulieu & Gainetdinov, 2011).

Over 40 years of research into dopamine enlightened the multitude of its physiological roles. Several studies, employing different methods, suggest a role in movement, positive and negative reinforcement, motivation, decision-making and incentive salience, among others (Nutt *et al.*, 2015). The range of sometimes contradictory functions might not be only dependent on the site but also the time course of dopamine's action. Namely, faster time courses playing a preferential role in reward and prediction error. At slower time scales, dopamine exhibits a 'steady-state function', alike hormonal signaling, which accounts for uncertainty, punishment and movement (Schultz, 2007).

Dopamine release can be induced by novel potentially salient stimuli, unpredictable natural rewards, and conditioned cues that predict reward, and when blocked was shown to attenuate rewarding effects of food, water and stimulants. Specifically dopaminergic projections to the ventral striatal complex have been associated with goal-directed behavior, response–reward and stimulus–reward associative learning (Wise, 2004).

Drugs of abuse directly (*e.g.* cocaine, amphetamine – monoamine uptake blockers) or indirectly (*e.g.* opioids, alcohol) act upon the dopaminergic system. In fact, addictive

substances acutely promote dopamine release (with different potencies and time courses), specially in the ventral striatum (Di Chiara & Imperato, 1988; Nestler, 2005; Keiflin & Janak, 2015). Thus, this brain region has been strongly implicated in mediating drug reward and neuroadaptations within the system are though to drive compulsive drug seeking and taking (Kreitzer & Malenka, 2008; Kasanetz *et al.*, 2010).

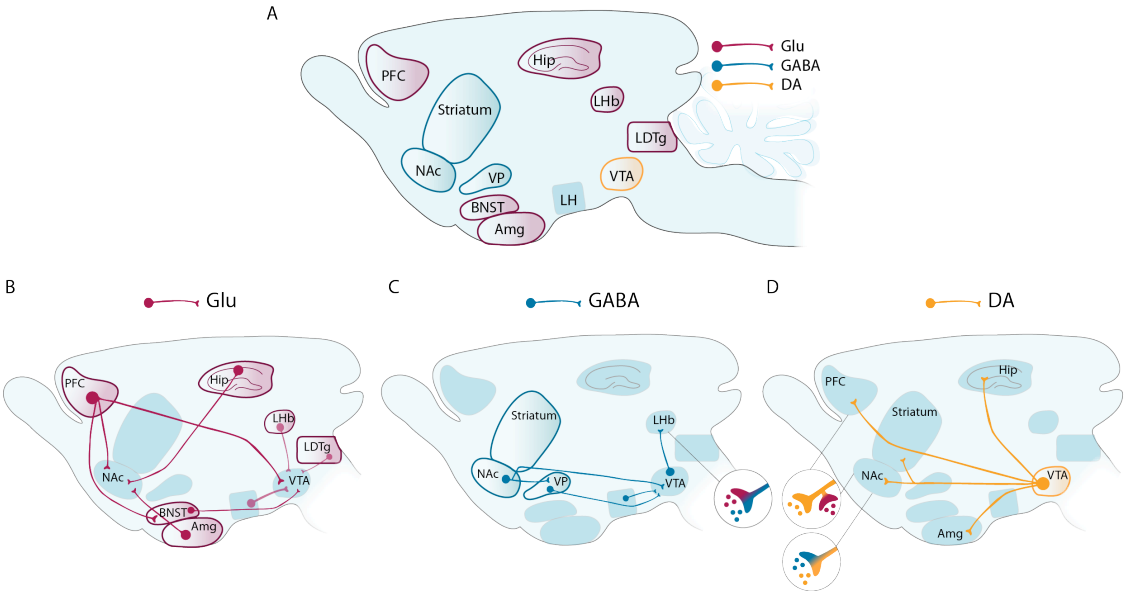

Figure 1.4 Representation of the mesocorticolimbic system*: Addiction-relevant circuits. A. Brain structures and neurotransmitter systems implicated in drug reward. B. Glutamatergic pathways; C. Gabaergic projections; and D. Major ascending dopaminergic pathways originating in the VTA. Inlets depict neurotransmitter co-release, specifically GABA-Glu, DA-Glu and DA-GABA synapses. Abbreviations: Glu, glutamate; GABA, γ-Aminobutyric acid; DA, dopamine; Amg, Amygdala; BNST, bed nucleus of the stria terminalis; Hip, hippocampus; LDTg, laterodorsal tegmental nucleus; LH, lateral hypothalamus; LHb, lateral habenula; NAc, nucleus accumbens; PFC, prefrontal cortex; Striatum, dorsal striatum (caudate-putamen); VP, ventral pallidum; VTA, ventral tegmental area. *Simplified scheme of the mesocorticolimbic system, for the purpose of visualization not all projections are depicted. (Catarina Luís, in accordance with Kauer & Malenka, 2007; Morales & Margolis, 2017).

The ventral striatal complex, more commonly known as the nucleus accumbens (NAc), was first described by Theodor Ziehen in 1904. It is located in the rostro-ventral area of the forebrain along the anterior commissure (AC). The striatum, including the NAc, is composed of mainly gabaergic projection neurons, the medium spiny neurons (MSN), which represent 95% of the neuronal population. These are characterized by high spine density, negative resting membrane potential (-80 to -90 mV) and low firing rates. The remaining neurons include different classes of interneurons, namely fast-spiking, low-threshold spiking and cholinergic interneurons (Kreitzer, 2009).

According to anatomical and morphological criteria, the NAc is classically subdivided into core and shell. Additionally, it also exhibits a patch-matrix organization of functional domains, striatosomes, defined by high density of μ-opiate receptors, substance P (SP) and D1R (Salgado & Kaplitt, 2015). In addition, a dorsal-ventral gradient view has also emerged, which emphasizes the input organization into the striatal complex (Voorn *et al.*, 2004). Functional and connectivity studies also differentiate between direct and indirect pathways, akin to the 'go' and 'no go' pathways of the dorsal striatum. Classic view is that striatonigral neurons, D1 and SP-expressing MSN, project directly to the ventral mesencephalon (VTA, SN); whereas the striatopallidal pathway consists of D2 and enkephalin-expressing MSN that mainly project to the ventral pallidum (VP, Figure 1.4C). However this does not seem to fully apply to the NAc, with both D1 and D2-MSN contributing to the NAc-VP pathway (Kupchik *et al.*, 2015).

Canonical understanding considers the NAc as a 'limbic-motor interface', integrating information between cognitive and limbic systems and generating behavioral motor output (Mogenson *et al.*, 1980). Thus, the NAc mediates survival drives essential for reproduction, foraging and feeding (Salgado & Kaplitt, 2015). The NAc core is also crucial for learning drug-reward associations, acquisition of drug taking and cue-induced drug seeking (detailed in section 1.5). As such, NAc core inactivation significantly impairs acquisition of cocaine self-administration (Ito *et al.*, 2004). Human neuroimaging studies, consistently show decreased D2R availability as well as blunted drug-induced dopamine release in the NAc of cocaine abusers (Volkow & Morales, 2015).

The NAc receives extensive glutamatergic innervation from multiple cortical and basal areas (Figure 0.4B), including prefrontal cortex (PFC), parahippocampal formation and basolateral amygdala. Excitatory transmission is mediated by glutamate receptors. These include, ionotropic, cation channels NMDA (N-methyl-D-aspartate) and AMPA (□ -amino-3-hydroxy-5-methyl-4-isoxazoleproprionic acid) receptors, as well as, metabotropic receptors (mGluR). mGluR1/5 are primarily found at the postsynapse, while mGluR2/3 are presynaptically located (Figure 1.5).

Cortical projections converge onto accumbal MSN, which have been show to support temporal integration of glutamatergic drive (McGinty & Grace, 2009). Medial PFC stimulation produces an excitatory postsynaptic potential within the NAc that is reduced by presynaptically located D2R (Brady & O'Donnell, 2004).

Stimulants, such as cocaine and amphetamine, induce glutamate release in the PFC and NAc, which is enhanced by repeated administration (Reid & Berger, 1996; Reid *et al.*, 1997).

Not only will animals self-stimulate the medial PFC but its activation induces conditioned place preference (CPP). These findings support PFC's role in regulating behavioral sensitivity to positive reinforcement (Routtenberg & Sloan, 1972; Moorman *et al.*, 2015).

The PFC, specifically the prelimbic region in rats has functional parallels with the human and primate dorsal lateral PFC (dlPFC), being primarily implicated in executive functions. These include working memory for objects and places, temporal processing, behavioural flexibility (reversal learning), delay discounting and uncertainty-decision making (Kesner & Churchwell, 2011). Inhibitory control in rats (Jonkman *et al.*, 2009; Moorman & Aston-Jones, 2015) and self-control in humans (Hare *et al.*, 2009) are mediated by the prelimbic cortex and dlPFC, respectively. Basal PFC activity during abstinence is reduced in addicts (Kalivas *et al.*, 2005). However, cocaine dependent patients display increased PFC activation (*e.g.* right dlPFC) to drug associated cues, which is positively correlated with self-reported craving (Bonson *et al.*, 2002; Moorman *et al.*, 2015).

The meticulous work of the Morales group, among others, has expanded the current understanding of how heterogeneous the DA system is. Specifically, VTA neurons projecting to the nucleus accumbens co-release dopamine and GABA and regulate motivational drive (Berrios *et al.*, 2016), as well as asymmetric co-release of dopamine and glutamate, which when abolished increases cocaine self-administration (Figure 0.4D; Hnasko *et al.*, 2010). The latter neuronal population also projects to the PFC (Figure 0.4D), and produced enhanced perseverative behavior when knocked-out (Kabanova *et al.*, 2015). Dual GABA- and glutamate-containing neurons in the VTA innervate the lateral habenula, and have been shown to be involved in promoting reward (Stamatakis *et al.*, 2013; Morales & Margolis, 2017) – see Figure 1.4C.

Drugs of abuse '*hijack*' neuronal circuits, namely the mesocorticolimbic system, known to be involved in natural reward processing as well as executive control.

1.5 Neurophysiological substrates of addiction

"Drugs leave a trace in the brain that outlasts their actual presence and that may be ultimately responsible for the pathological behavior" (Lüscher, 2016). The development of addiction is rooted in progressive maladaptive neurobiological mechanisms, implicating multiple brain areas and involving different neurotransmitter systems (Figure 1.4). Albeit drug-induced plasticity, other factors may also be implicated in the emergence of addictive disorders (see Box 4).

Here I'll be describing mainly drug-driven adaptions taking place in the prefrontal cortex to nucleus accumbens pathway. Other mesolimbic areas will be mentioned but not in great detail.

BOX 4 GENETIC MAKE-UP: ETIOLOGY OF VULNERABILITY?

Not only drug-induced adaptations drive the development of addictive behavior. Pre-existing genetic factors can confer vulnerability or susceptibility (traits) to drug use and dependence. In fact, heritability of cocaine dependence is the highest (0.72) among psychoactive substances (Goldman, Oroszi and Ducci, 2005). The following genes (or gene variants) have been associated with drug abuse and dependence:

Gene	Function	Drug phenotype
5HTT (or SERT) (5-Hydroxytryptamine transporter)	Neurotransmitter transport	Alcohol (i, d, c), cocaine (d,c), heroin (d), methamphetamine (d), nicotine (d)
CYP2A6 (Cytochrome P450)	Oxidation reduction	Alcohol (d), nicotine (i,d,c)
DAT1 (Dopamine transporter)	Neurotransmitter transport	Alcohol (d, c), cocaine (d), heroin (d), methamphetamine (d), nicotine (i,d,c)
DRD2 (Dopamine receptor 2)	Dopaminergic synaptic transmission	Alcohol (d, c), cocaine (d), heroin (d), nicotine (i,d,c)
BDNF (Brain-derived neurotropic factor)	Regulation of synaptic transmission	Alcohol (i, d, c), cocaine (d), methamphetamine (d), nicotine (d)

This is only a partial list of genes reported to be involved or associated with addictions. c, cessation or withdrawal; d, abuse or dependence; i, initiation. (Adapted from Li and Burmeister, 2009)

1.5.1 Synaptic neuroadaptations

The early days of synaptic plasticity in addiction research were considerably shaped by the memory and learning field, as to the first accounts of drug-induce plasticity focusing on long-term potentiation (LTP). Namely, acute non-contingent cocaine injection induced LTP in the VTA neurons (Ungless *et al.*, 2001). This early 'drug trace' seems to be mediated by native AMPAR being switched for, calcium permeable, GluA2-lacking AMPAR (CP-AMPAR; Mameli *et al.*, 2011); while NMDA receptors also being exchange for GluN3-contaning NMDA, which are magnesium insensitive and have very low calcium permeability (Yuan *et al.*, 2013). As a consequence, synaptic calcium signaling switches from NMDAR to

AMPAR, causing an inversion in activity-dependent synaptic plasticity - Anti-Hebbian LTP (Mameli *et al.*, 2011; Lüscher, 2016).

Although acute drug-evoked plasticity primarily takes place in the VTA, subsequent or delayed neuroadaptations, perhaps relevant for disease development, occur at excitatory afferents onto MSNs of the NAc.

I*n vitro* reports showed, one day after repeated non-contingent cocaine, decreased AMPA/NMDA ratio and occlusion of long-term depression (LTD) (Thomas *et al.*, 2001). During abstinence, this scenario was reversed with enhanced LTD, increased AMPA/NMDA ratio and LTP occlusion (Thomas *et al.*, 2001; Kourrich *et al.*, 2007). Re-exposure to cocaine leads to depression of synaptic transmission, as observed with initial administration (Kourrich *et al.*, 2007). Early decreased glutamatergic drive is associated with the emergence of silent synapses, *i.e.* lacking AMPAR, which are unsilenced during abstinence (Lee *et al.*, 2013; Terrier *et al.*, 2016).

Long-term regime of non-contingent cocaine (28 days) induced increased probability of pre-synaptic glutamate release (as measured by miniature EPSPs), increased AMPA/NMDA ratio and also induced morphological plasticity, with higher spine density (see Figure 1.5). These adaptations were observed in the NAc core D1-neurons and were not further potentiated by withdrawal (Dobi *et al.*, 2011).

Furthermore, optogenetic self-stimulation of VTA dopaminergic neurons causes the same plastic adaptations in D1-MSNs as following cocaine self-administration (CSA) (Pascoli *et al.*, 2015), suggesting dopamine-dependent mechanism of neuroadaptations in the NAc.

In vivo studies have also shown enhanced tonic and phasic firing associated with drug-cues, following extended CSA (Ghitza *et al.*, 2004; Fabbricatore *et al.*, 2009).

Different cocaine self-administration regimes, which predicate more insightful evidence, have also been shown to affect drug-driven adaptations during withdrawal. Some authors claim that CP-AMPAR insertion only takes place following CSA and mediates incubation of drug craving in the NAc core (Conrad *et al.*, 2008; Wolf, 2016). CP-AMPAR increase significantly from withdrawal day 30 (WD30) to WD90, which might increase MSN glutamate-mediated activation due to drug-paired cues causing an exacerbated cocaine seeking response (Wolf, 2016).

However recent study found insertion of CP-AMPAR to be a general response to cocaine exposure (Lüscher & Malenka, 2011; Terrier *et al.*, 2016). This plasticity was specific to D1-class MSN, only recruiting D2-neurons at high doses of cocaine (1.5 vs regular 0.75

mg/kg; Terrier *et al.*, 2016). In fact plasticity in D2 MSN might have a protective effect, as stimulating indirect pathway is associated with resilience to compulsive drug taking (Bock *et al.*, 2013).

Nonetheless, incubated rats exhibit more neurons encoding for cocaine-associated cues at WD30, when compared to early withdrawal (Hollander & Carelli, 2005, 2007). Concomitantly, the increase in number of neurons responding to drug associated cues was correlated with magnitude of incubation (Guillem *et al.*, 2014).

With the advent of optogenetics, and thus the ability to modulate a single region or a specific cell population, mapping of input-specific drug-induced neuroadaptations became possible, allowing for better interpretation of lesion and pharmacological studies.

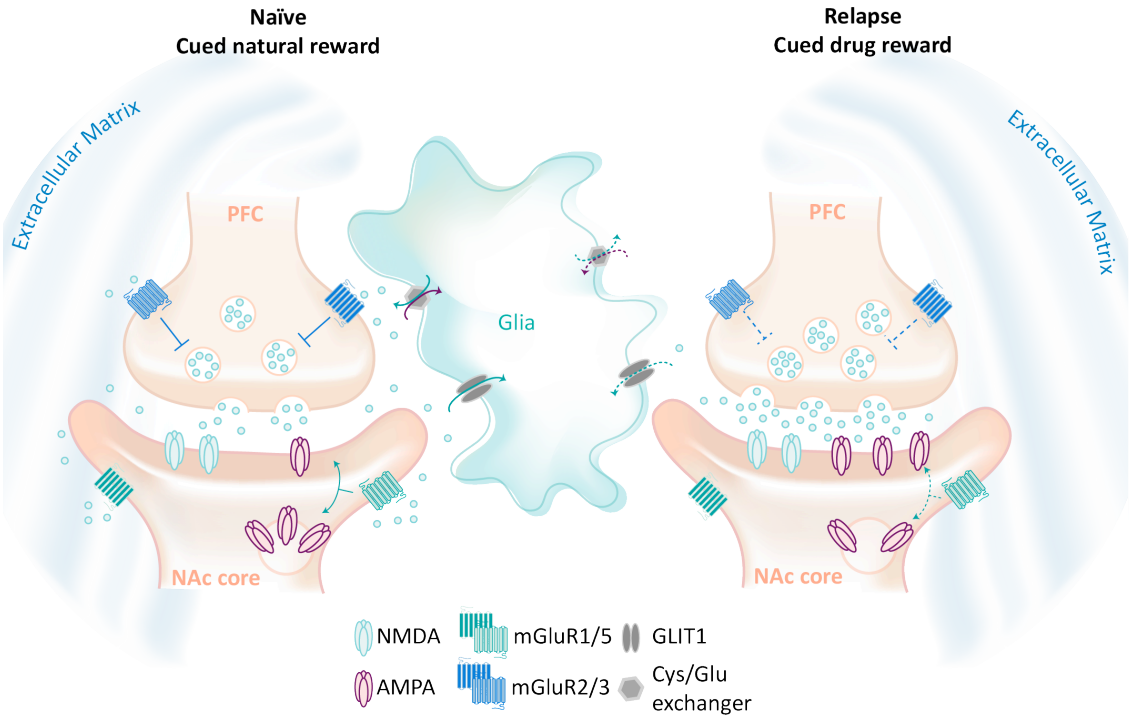

Figure 1.5 Drug-induced synaptic neuroadaptations: Glutamate dynamics in the tetrapartite PFC-NAc core synapse in relapse. Heightened excitatory activation towards drug cues, when compared to natural rewards. Cocaine exposure also leads to down-regulation of the glial glutamate transporter 1 (GLIT1) and cysteine/glutamate exchanger, reducing mGluR2/3 presynaptic release inhibition. Thus, glutamate levels are increased due to enhanced release and to impaired elimination from the extracellular space. Moreover, AMPAR are overexpressed, and spine density and diameter is enhanced (Catarina Luís, in accordance with Kalivas, 2009; Scofield *et al.*, 2016a)

Glutamatergic input from the prelimbic PFC to the NAc core has been shown to mediate relapse-like behaviour. Prefrontal glutamate is necessary for cocaine-induced reinstatement and inhibition of this pathway abolishes cocaine-primed reinstatement (McFarland *et al.*,

2003). In addition, the PL to NAc core pathway is also recruited to drive cue-induced cocaine seeking (McFarland & Kalivas, 2001; LaLumiere & Kalivas, 2008). Optogenetic depotentiation of PFC to NAc core projections reduces cocaine and cocaine/cue-primed reinstatement of cocaine-seeking behavior (Stefanik *et al.*, 2013). Moreover, activation of the prefrontal-accumbal pathway correlated with reinstatement behaviour, but not other excitatory inputs, such as the basal lateral amygdala (BLA) or ventral subiculum (McGlinchey *et al.*, 2016). Incubation of drug seeking was also reduced by selective inhibition of PFC-NAc core pathway (Ma *et al.*, 2014).

Prefrontal mGluR2/3 dependent LTD is abolished in addicted-like rats, revealing an inability to adapt (anaplasticity) and down regulate glutamate release during periods of neuronal (*e.g.* cue-induced) activity (Martin *et al.*, 2006; Kasanetz *et al.*, 2010, 2013). Presynaptic release enhancement was also observed following one and 30 days of withdrawal in the PFC-NAc synapse (Suska *et al.*, 2013).

The evidence described supports a scenario in which cocaine taking dyregulates glutamate transmission, specifically leading to a state of augmented and frozen glutamate release (see Figure 1.5). This state is hypothesized to underlie '*unmanageable motivation*' to seek drugs, and thus vulnerability to relapse (Kalivas *et al.*, 2005; Kalivas, 2009; Scofield *et al.*, 2016a).

Synaptic neuroadaptations are not the solely mechanisms mediating drug-related behaviors. Multiple adaptations have been reported, as altered intrinsic plasticity (Kourrich *et al.*, 2015) and also in other non-neuronal effectors, including astrocytes (Scofield *et al.*, 2015; 2016b), microglia (Brown *et al.*, 2017) and extracellular matrix (Wiggins *et al.*, 2011), highlighting the complexity of interactions at play (Figure 1.5).

1.5.2 Drug-driven network activity alterations

The first demonstration of behaviorally relevant human brain oscillations was recorded by Hans Berger, when recording from his own son (alpha rhythms, 1929). Historically, the potential generated via electric currents within a volume of neuronal tissue was first recorded from the scalp and termed electroencephalogram (EEG). When recorded from the surface of the cortex is referred as electrocorticogram (ECoG), and intracerebral recording is referred as local field potentials (LFP, Buzsáki *et al.*, 2012).

Generally, wideband electric fields can be recorded in a specific brain area or multiple brain areas simultaneously. Low frequencies (1-300 Hz) are used to detect slow membrane potential fluctuations in a large neural population.

There are several mechanisms thought to be involved in LFP signal generation, such as synaptic activity, calcium spikes, intrinsic currents and resonances, afterpolarizations, 'down' states, and gap junctions (detailed in Buzsáki *et al.*, 2012). Synaptic activity is generally considered the main *source of extracellular current flow* (IPSP and EPSP, van der Meer *et al.*, 2010; Buzsáki *et al.*, 2012). Neurons have arborized shape, with a conducting internal *milieu* and an insulating outer membrane. At the synapse, there are NMDA and AMPA channels, ionotropic receptors that mediate the inward cation flux (Na^+ and Ca^+). Influx of positive charge from the extracellular space into the neuron generates a *local extracellular sink*. In order to obey by the electroneutrality principle a *source*, in which current flows in the opposite direction, has to be generated as well (*i.e.* return current). As a result, a dipole or n-pole is generated, which creates a potential (V*e*) that is measured relative to a reference potential. This difference in potential forms an electrical field that can be measured by extracellular electrodes, with millisecond precision (Buzsáki *et al.*, 2012).

Classically, LFP have been categorized into distinct frequency bands: delta (1-4 Hz), theta (4-8 Hz), alpha (8-12 Hz), beta (12-30 Hz), and gamma (>30 Hz) - see Figure 1.6.

Rhythmic oscillations entail precise and concerted activity within a large area, which can be related to activity (spikes) and LFPs in other areas, and change in response to environmental stimuli sensory and cognitive events as well as different behavioral states (see Figure 1.6).

Spectral power, as the probabilistic strength of a specific frequency width, and coherence, as a measure of synchrony between two signals (LFP-spike or LFP-LFP) are two typically employed analytical methods to study LFP (van der Meer *et al.*, 2010).

Synchronization (of phase) of brain signals seems to support neuronal communication, as suggested by the *'communication through coherence'* hypothesis (Fries, 2015). CTC proposes that 'effective connectivity' across circuits is aided by the degree of LFP coherence between the regions involved (Catanese *et al.*, 2016); thus, allowing for synaptic inputs to arrive simultaneously and trigger a rapid and more efficient postsynaptic depolarization. As such, CTC may represent a mechanism that controls information flow in neuronal circuits (Fell & Axmacher, 2011). However, even without a mechanistic role for oscillatory rhythms, they can provide insights on the "temporal organization in meso-scale neural activity" (Friston *et al.*, 2015; Catanese *et al.*, 2016).

In the corticostriatal circuit, oscillations have been showed to be modulated by distinct behavioral states (Gruber *et al.*, 2009), by learning (Koralek *et al.*, 2013; Thorn & Graybiel,

2014) and in neuropsychiatric disorders, in humans and animal models (Hammond *et al.*, 2007; Dejean *et al.*, 2012; Zhang *et al.*, 2016).

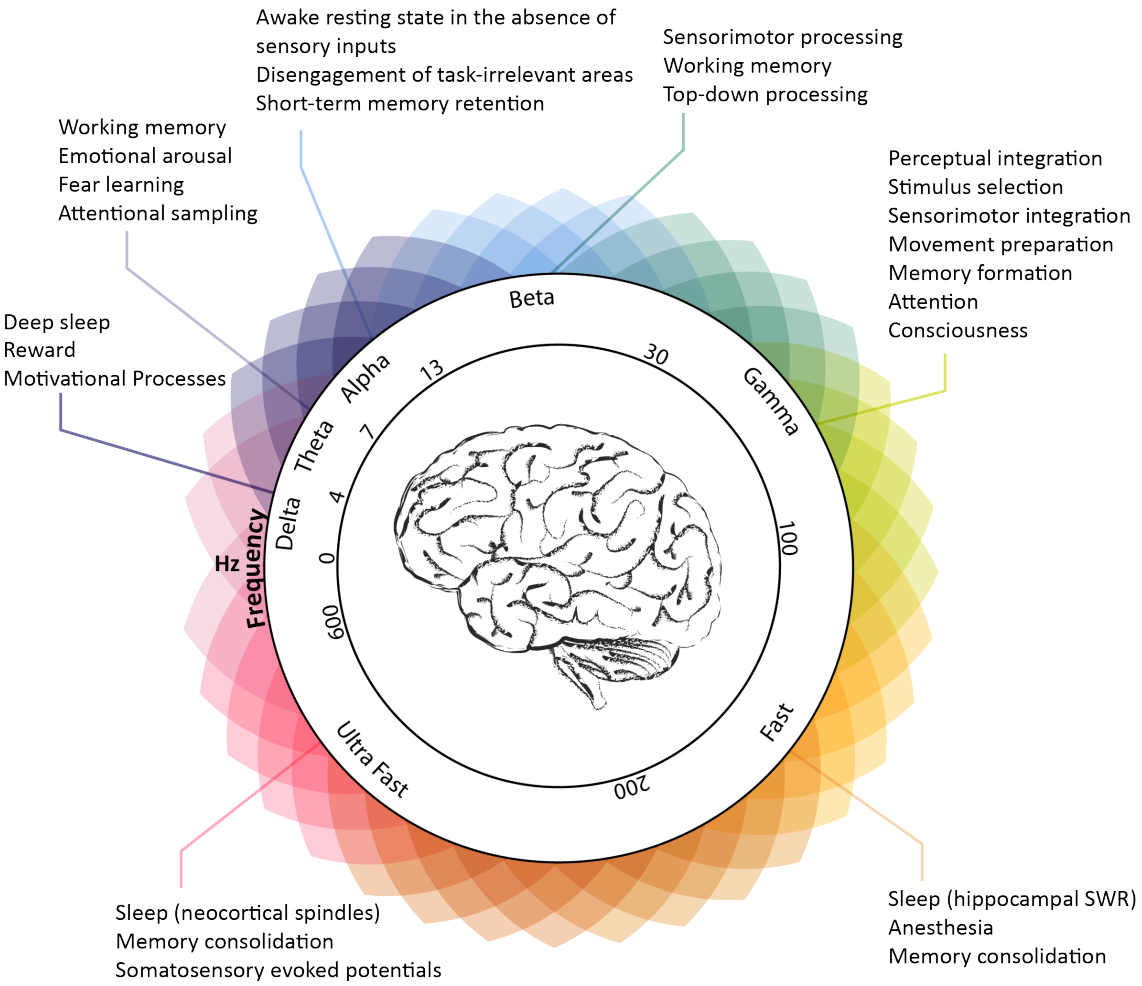

Figure 1.6 Network oscillatory activity and its described functions. Summary of distinct frequency bands and physiological processes in which they have been implicated. SWR, sharp wave-ripples (Catarina Luís, in accordance with Engels & Fries, 2015).

Reward and drugs of abuse also change LFP activity in the corticostriatal network. In fact, accumbal gamma has been related to reward. Distinct gamma sub-bands, low gamma 60 and gamma 80 have been linked to reward retrieval and reward uncertainty, respectively (van der Meer & Redish, 2009; Dejean *et al.*, 2017). Cortico-accumbal LFP activity also correlates with reward outcome and impulsivity, mainly associated with gamma 60 and theta changes (Donnelly *et al.*, 2014). In humans, accumbal gamma was shown to be modulated in a gambling task (Cohen *et al.*, 2009a).

Regarding addictive drugs, a sensitizing regime of amphetamine causes alterations in delta, theta/alpha and gamma, which become more prominent, after repeated exposure (Lapish *et al.*, 2012). Chronic morphine also induces a network shift, sustained by changes in delta and gamma oscillations in the PFC-NAc-BLA circuit (Dejean *et al.*, 2013).

Studying LFP may not only aid in understanding the neuronal dynamics underpinning drug-related behavior but can create tools for clinical practice. In fact, abnormal EEG activity in alcohol dependent patients has been used to predict likelihood of relapse, with an accuracy of 85%, as well as to screen and diagnose patients (Bauer, 2001; Mumtaz *et al.*, 2016).

Taken together, the present state of the art points out the advantages of characterizing drug-induced neuroadaptations, at the synaptic and circuit level, that alter cortico-accumbal integration. This understanding allows to elaborate and fine-tune the '*circuit model of addiction*' (Lüscher, 2016) that will hopefully yield more successful treatments.

AIM AND THESIS OUTLINE

The present project aimed to identify synaptic and network neuroadaptations associated with chronic cocaine use and withdrawal in the prefrontal-accumbal circuit.

The specific **objectives** pursued in the thesis were:

i) Establish and perform reliable and reproducible recording set up for long-term evoked and spontaneous local field potentials (LFP) in freely behaving rats (**Chapter 3**);
ii) Monitor cocaine-induced synaptic changes in the PFC-NAc pathway of awake rats, in a model of drug craving and relapse (**Chapter 3**);
iii) Assess whether chronic contingent cocaine modifies network activity in the NAc during spontaneous behavior in the absence of the drug. And to determine whether a cocaine challenge effect on PFC-NAc oscillatory dynamics depends on previous history of cocaine intake (**Chapter 4**);
iv) Test if incubation of drug seeking modulates PFC and NAc processing during extinction and reinstatement of drug seeking (**Chapter 5**);

The studies conducted to examine the latter are described in the upcoming chapters. General methods (**Chapter 2**) describe transversal techniques used across all chapters. Yet, each chapter describing ***results*****,** includes the ***methods*** that were specifically used in that section as well as a ***discussion*** of those findings. Conclusions (**Chapter 6**) summarizes main findings within a broader theoretical framework.

2. General Methods

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Chapter 2

GENERAL METHODS

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2 General Methods

2.1 Animal housing and husbandry

All experiments were conducted at the Central Institute of Mental Health in Mannheim (Germany). Experimental procedures were in accordance with the Directive 2010/63/EU guidelines for care and use of laboratory animals, and were approved by the local animal care committee (G-273/12; Regierungspräsidium Karlsruhe, Germany). Sprague-Dawley rats (Charles River, Germany), seven-week-old at their arrival, were single housed under 12 h dark/light reverse cycle in a temperature (22 ± 1 °C) and humidity (60 ± 5%) controlled room. Subjects were given access to food and water ad libitum throughout the experiment.

2.2 Surgery

Two weeks after arrival rats were implanted with a catheter (Micro-Renathane®) inserted in the right jugular vein under isoflurane anesthesia. Catheters were flushed daily with a heparinized solution (100 I.U./ml) containing enrofloxacin (1 mg/ml, Baytril®).

Self-made bipolar tungsten electrodes (52 μm, California Fine Wire, Grover Beach, California, USA) were chronically implanted in the prelimbic PFC for stimulation (AP +3.0, ML +0.6, DV –3.3 mm from brain surface) and in the NAc core for recording (AP +1.8, ML +1.3 to 1.5, DV –5.5 to –6.5 mm from brain surface), according to the Paxinos and Watson atlas. The final position of the electrodes was determined by online monitoring evoked field potentials, depth profiles and input-output (IO) curves under ketamine/xylazine anesthesia (65/14 mg/kg, Figure 3.1). Animals were given 7-9 days recovery before cocaine self-administration (CSA) sessions and electrophysiological recordings began. Four stainless steel screws were used, to fix the implant as well as to serve as reference and ground for electrophysiological recordings.

2.3 Drugs

Cocaine-HCl (Sigma-Aldrich, Germany) was dissolved in sterile saline and self-administered by the subjects via intravenous (i.v.) route.

2.4 Self-administration apparatus

Self-administration chambers (40 cm long x 30 cm wide x 52 cm high) were located in sound-attenuating cubicles equipped with exhaust fans to assure air renewal. Two poke

holes were on opposite walls of the chambers, 5 cm above the grid floor (Figure 3.3). When rats poked their snout in the holes, breaking an infrared beam, their instrumental responding was recorded. One hole was associated with cocaine delivery and designated as the active hole, while the other was designated as the inactive hole and served as control. A white cue light was located 9.5 cm above the active hole, and a blue light was on the left side of the opposite wall 33 cm above the grid floor. A speaker allowing presentation of a tone cue was located in the middle of the back wall 40 cm above the greed floor. Data were collected with Windows-compatible SK_AA software (Imetronic, France).

2.5 Self-administration training

Behavioral training and testing were adapted from Grimm and colleagues (2001). Cocaine self-administration (CSA) training (Figure 3.3) of seven rats consisted of 16 daily 6 h sessions during which nose-poking at the active hole under a fixed ratio 1 (FR1) of response was reinforced by 0.5 mg/kg/infusion of cocaine (infusion bolus: 40 μl). Cocaine availability was signaled by illumination of the blue light. Each earned infusion was coupled to the presentation of two discrete cues (light, 300 Hz; tone, 60 dB; each 5 s). All infusions were followed by a 40 s time-out, during which the blue light was turned off and drug was not available. Responding during time-out or poking at the inactive hole was recorded, but resulted in no programmed consequences. To avoid overdose risk, infusions were limited to 25 injections per hour. After the 25th infusion the blue light signaling cocaine availability was turned off and nose-poking in either hole was recorded but had no consequence until the beginning of the next hour.

2.6 Reinstatement of cocaine-seeking

Cue-induced reinstatement of cocaine seeking was tested at withdrawal day (WD) 1 and 30 during a 2 h session in the operant chamber. The first hour consisted of extinction of nose-poking behavior, in which active nose-poking was neither rewarded nor paired with the discrete light/tone cues, and the blue-light was turned off. The second hour comprised cue-induced reinstatement and started with turning on the blue light and one non-contingent presentation of the light/tone cues followed by contingent presentations of the light/tone cues in the absence of cocaine delivery.

2.7 Histology

Animals were deeply anesthetized with ketamine/xylazine (65/14 mg/kg). Electrical current was applied at 0.2 mA for 20 s to each tungsten wire individually. Subsequently, rats were transcardially perfused with phosphate buffered saline (PBS) followed by 4% paraformaldehyde (PFA). The brains were extracted and incubated in PBS over night. Coronal sections of 50 μm thickness were mounted onto glass slides. The location of the electrodes was confirmed by Nissl staining. Placement of the electrodes tip was determined by examining the relative position of observable marks to visual landmarks and anatomical organization in accordance with Paxinos and Watson.

3. Results

Persistent strengthening of the prefrontal cortex – nucleus accumbens pathway during incubation of cocaine-seeking behavior

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3

Persistent strengthening of the prefrontal cortex – nucleus accumbens pathway during incubation of cocaine-seeking behavior

Rationale "High rates of relapse after prolonged abstinence are often triggered by exposure to drug-associated cues that induce drug craving. Incubation of drug craving consists of time-dependent increases in cue-induced drug craving in humans and drug-seeking in animals during withdrawal. This augmented drive to seek and use drugs is thought to be rooted in long lasting drug-induced neuroadaptations, specifically in excitatory synaptic transmission's efficacy (Huang *et al.*, 2015; Lüscher, 2016; Wolf, 2016) as well as intrinsic plasticity (Kourrich *et al.*, 2015). Drug-dependent alterations of the glutamatergic system have been demonstrated to play a major role in mediating incubation of drug-seeking as well as in driving abnormal learning associated with progressively greater behavioral emphasis towards the drug and drug associated cues at the expense of natural rewards (Kalivas *et al.*, 2005). Namely, the prefrontal cortex (PFC) to nucleus accumbens (NAc) pathway emerges as instrumental in regulating drug-seeking responses (Bossert *et al.*, 2013; Li *et al.*, 2015). As such, both pharmacological inactivation of the NAc core as well as optogenetically blocking its PFC projections reduces reinstatement of cocaine-seeking behavior (McFarland & Kalivas, 2001; Stefanik *et al.*, 2013).

The PFC glutamatergic input to the NAc core has been well characterized regarding cocaine-evoked plasticity following non-contingent versus contingent exposure to cocaine or alternatively after protracted abstinence. Still, the synaptic strength during the course of withdrawal compared to drug-naïve condition is unknown, since electrophysiological characterizations are mainly performed in brain slices or focus on distinct time points during cocaine-evoked plasticity *in vivo*.

Here, we sought to identify electrophysiological neuroadaptations associated with cocaine self-administration and withdrawal. We conducted a longitudinal study that allowed us to monitor PFC-NAc core synaptic transmission, via *in vivo* field potential recordings, over time within individuals."

The scientific work described in the following chapter has been the subject of an article, which has been originally written by myself, here listed: Luís, C., Cannella, N., Spanagel, R., & Köhr, G. (2017). Persistent strengthening of the prefrontal cortex – nucleus accumbens pathway during incubation of cocaine-seeking behavior. Neurobiology of Learning and Memory 138; 281-290

3.1 Methods

3.1.1 Animal groups

Seven incubated rats underwent 16 days of self-administration followed by 30 days of withdrawal. During forced abstinence, rats remained in the home cage. Four age-control rats remained in their home cage throughout 46 days (Figure 3.3) except for 6 h in the operant chamber one day after the first home cage recording session in order to control for novelty/stress.

3.1.2 Evoked *in vivo* field potential recordings

"Recordings of evoked postsynaptic potentials (ePSPs) were carried out exclusively in the home cage on days without behavioral testing (Figure 3.3), *i.e.* one day before training started (naïve), the day after the first CSA (acute), the day after the 13th CSA session (chronic) and at withdrawal day (WD) 7, 20 and 28. Before the first recording, rats were left to habituate for 30 min to the recording apparatus, particularly being connected to the miniature headstage (1 g, npi electronic GmbH, Tamm, Germany). This device did not impair normal behavior, as experienced before in mice (Li *et al.*, 2016), since rats were able to move freely and sleep while connected (Commutator model SL-12-C, Dragonfly, Ridgeley, West Virginia, USA). For electrical stimulation, biphasic current pulses (0.25 ms) were delivered every 30 s, single pulses or paired pulses to determine paired-pulse ratios (PPR, ePSP2/ePSP1). During each recording session, four input-output (IO) curves (stimulation intensity, 0.1-1.5 mA) with paired pulses at 50 ms inter-stimulus interval (ISI) were acquired. In addition, 40-50% of stimulation intensity that evoked maximum field response was applied to acquire baseline ePSPs, either with single pulse stimulation or with paired-pulse stimulation at 40, 80 and 160 ms ISI. Differential ePSPs were amplified by an EXT-02F amplifier (npi electronic GmbH) and band-pass filtered at 1-500 Hz. Signals were digitized at 10 kHz (ITC-16, HEKA Elektronik, Lambrecht, Germany), and noise was filtered by a Hum Bug Noise Eliminator (AutoMate Scientific, Inc., Berkeley, CA)."

3.1.3 Analysis and statistics of evoked field potential recordings

"Matlab custom-made script was used to determine the amplitude of the ePSP. Individual ePSP amplitudes were determined by averaging the field response size over a 20 ms window encompassing the period of negative deflection of the signal. All electrophysiological

measurements were normalized within subject to the naïve condition. IO curves under naïve condition were normalized to the ePSP evoked with maximal stimulus intensity for each rat. IO curves acquired during the following sessions were normalized to the naïve condition for each stimulation intensity. In consideration of the predefined longitudinal study, repeated measures (RM) two-way ANOVA was used to compare IO curves. RM one-way ANOVA was used to compare PPRs between all time points relative to naïve condition within the same group. Multiple *post-hoc* comparisons were performed with Fisher LSD. In-between group comparisons were statistically analyzed by two-way ANOVA. Unpaired t-test was used to compare non-normalized PPR at the beginning of the experiment (*i.e.* naïve condition). Extinction and cue-induced reinstatement, each comprising 1h at WD1 and WD30, were divided in 20-min bins and analyzed by RM three-way ANOVA, with WD1/WD30, extinction/cue condition and 20-min time bins as repeated measures. Linear regression was used to determine the association between mean cocaine infusions during CSA and the change in ePSP amplitude and PPR (40 ms) as well as the association between degree of incubation (Cue poking WD30/WD1) and change in ePSP amplitude during IO recordings. Graphs and statistical analysis were performed in Graphpad Prism 5 and Adobe illustrator. All results are shown as mean ± s.e.m ($p<0.05$)."

3.2 RESULTS

3.2.1 Electrode implantation and optimization

Electrode implantation was performed in a two-stage approach with multimodal anesthesia. Anesthesia proved to be a key aspect during implantation. Isoflurane did not allow for the recording of PFC-evoked postsynaptic potentials (ePSPs) in the nucleus accumbens and ketamine/xylazine was not long lasting, hence not suitable for long procedures in adult male Sprague-Dawley rats. Thus, the first part of the surgery was performed under isoflurane anesthesia that allows for lasting and controllable unconsciousness. This stage comprised preparing the skull by cleaning and drying as well as drilling the skull holes over the electrode locations and screwing in the skull screws for the ground and reference. Subsequently, isoflurane anesthesia was ceased, the rat was let to recover and ketamine/xylazine was injected intra-peritoneally.

Rats were repositioned in the stereotaxic frame and the electrodes were slowly lowered to their positions relative to the brain surface. Specifically, the bipolar stimulation electrode was positioned in the prelimbic PFC and the recording electrode in the NAc core (Figure 3.1A). The final dorsal-ventral coordinate was determined through online monitoring of the characteristic evoked field potential (Figure 3.1B, bold trace), short-latency sink currents alike hippocampal CA1 field responses. PFC-evoked field potentials in the NAc core have been previously characterized in anesthetized rats, both anatomically and physiologically as primarily monosynaptic glutamatergic postsynaptic potentials by Moussawi and colleagues (2009).

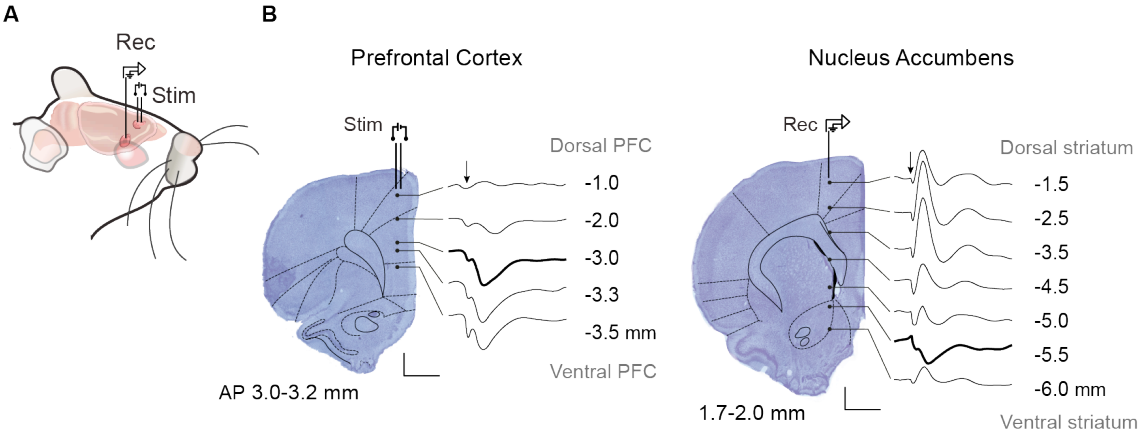

Figure 3.1 Recording layout and online monitoring of field responses during electrode implantation. (A) Scheme showing general electrode positioning, namely a bipolar stimulation electrode in the

prefrontal cortex (Stim) and recording electrode in the nucleus accumbens core (Rec). (B) Depth profiles acquired while lowering the stimulation electrode to the prelimbic PFC (left) and maintaining the recording electrode stationary in the NAc core (DV -5.5–6.5); or while lowering the recording electrode to the NAc core (right) and maintaining the stimulating electrode in the PFC unchanged (DV -3.3). Representative traces are averages of ten consecutive ePSPs (scale bar, 0.5 mV and 25 ms). Time of stimulation is indicated by arrow.

As exemplified in Figure 3.1B by the depth profiles, maximal amplitudes of ePSPs were observed when ventral prelimbic PFC and dorsomedial NAc core were targeted for stimulation and recording, respectively.

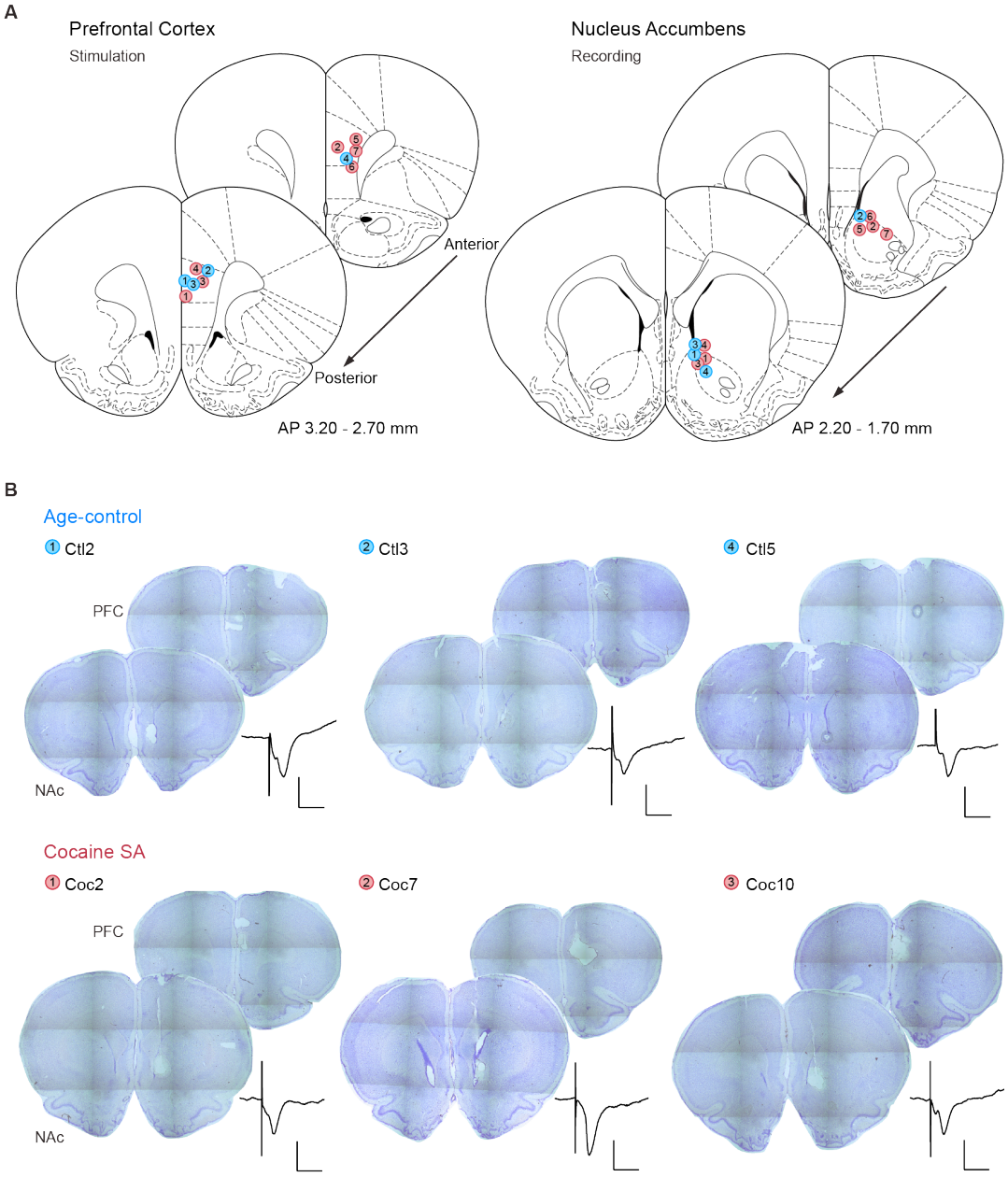

Figure 3.2 Placement of recording electrode in the nucleus accumbens (NAc) core and stimulation electrode in prefrontal cortex (PFC). (A) Left panel, two schematic representations of the anterior-posterior plane depict the respective recording sites in PFC. Right panel, schematic representations of

the anterior-posterior plane (arrow) depict the recording sites in NAc core from four age-control rats (blue) and seven cocaine self-administering (SA) rats (red). (B) Nissl-stained sections (50 μm) from three representative age-control (Ctl) and three cocaine SA (Coc) rats. For each animal, the section with a mark in the NAc overlaps the section with a mark in the PFC. Representative traces are averages of 15 to 20 consecutive ePSPs (scale bar, 0.5 mV and 25 ms).

The topographical organization of the cortical inputs into the NAc core supports this observation. Specifically since the medial NAc core is primarily innervated by the ventral region of the prefrontal cortex (Voorn *et al.*, 2004).

Post mortem anatomical confirmation of the location of the electrodes further corroborated these observations (Figure 3.2).

Figure 3.2A illustrates the final electrode position in the prelimbic PFC for stimulation and in the NAc core for recording sites. On panel A, all electrode positions are indicated for all individuals included in the study, namely age-controls in blue (1 to 4) and cocaine administering rats in red (1 to 7). The relative position of observable marks to visual landmarks and anatomical organization in accordance with Paxinos and Watson was used to determine electrode placement. In panel B, sections from three controls and three cocaine-administering rats exemplify the lesion sites that verified electrode coordinates.

As evidenced by the ePSP traces in Figure 3.2B all recording sites exhibit similar ePSP shape, although response amplitude varied moderately in between subjects. In order to account for inter-subject variability in ePSP amplitude, all responses were normalized within subject to the first recording session – naïve (for more detail see section 3.1.3).

3.2.2 Longitudinal PFC-evoked postsynaptic potentials in freely moving rats

"Since drug-induced neuroadaptations develop over time, a longitudinal study design proved to be a fitting approach.

To ensure stable recordings in awake animals over time an age-control group (n = 4) remained in their home cage, except for 6 h in the operant chamber one day after the first recording session in order to assess potential novelty/stress-driven effects. Recordings were acquired at 6 time points exclusively in their home cage to obtain a temporal profile of excitatory synaptic transmission longitudinally (Figure 3.3).

As previously stated, all sessions were normalized to the naïve condition, that is the first recording session acquired (D0, Figure 3.3)."

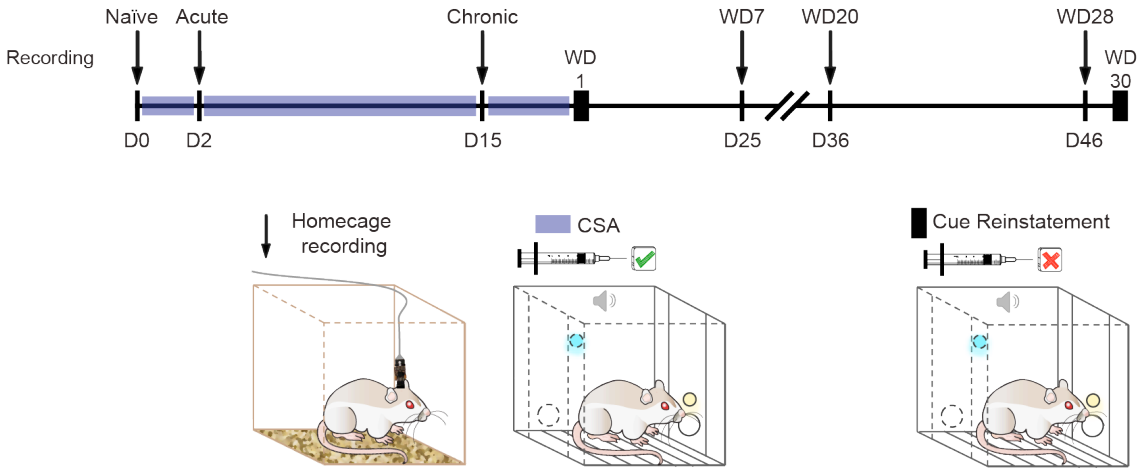

Figure 3.3 Experimental timeline illustrating cocaine self-administration (CSA) paradigm. Upper panel depicts the timeline indicating days of home cage recordings at distinct time points (arrow) both during CSA (purple) and 28 days of withdrawal (WD) for cocaine group, as well as for age-control group labeled D0 to D46. The lower panel depicts home cage recording setup, operant chamber during CSA (purple, green check) and during cue-induced reinstatement in the absence of reward (black, red cross), which took place at WD1 and 30.

"Field potentials during input-output recordings (IO, Figure 3.4A and B) revealed no statistically significant effect of time ($F_{(5,15)} = 0.31$, $p = 0.898$) or stimulation intensity × time ($F_{(70,210)} = 0.93$, $p = 0.632$; repeated measures (RM) two-way ANOVA) on synaptic transmission over the course of the 6 time points recorded. No effect was observed both at earlier time points (D2 and D15) as well as up to 46 days after the first recording session, as evidenced by the absence of significant amplitude changes across stimulation intensities (Figure 3.4C)."

"To further characterize the PFC-NAc pathway, paired-pulse stimulation was applied both during IO recording, with 50 ms inter-stimulus interval (ISI), as well as during additional recordings with 40, 80 and 160 ms ISI (Figure 3.4D and E, respectively). Here, ePSPs were evoked at 40–50% of maximum ePSP. No significant change was found both across time points and for all inter-stimulus intervals measured (40 ms, $F_{(2.6,7.9)} = 2.43$, $p = 0.144$; 50 ms, $F_{(1.9,5.7)} = 0.93$, $p = 0.441$; 80 ms, $F_{(1.7,5.0)} = 1.13$, $p = 0.380$; 160 ms, $F_{(2.8,19.4)} = 1.54$, $p = 0.236$, RM one-way ANOVA).

Since PFC-evoked ePSPs recorded in the NAc in behaving rats revealed to be stable up to 55 days after surgery, this was considered a suitable tool for long-term within subject study design to assess synaptic transmission in the PFC-NAc pathway."

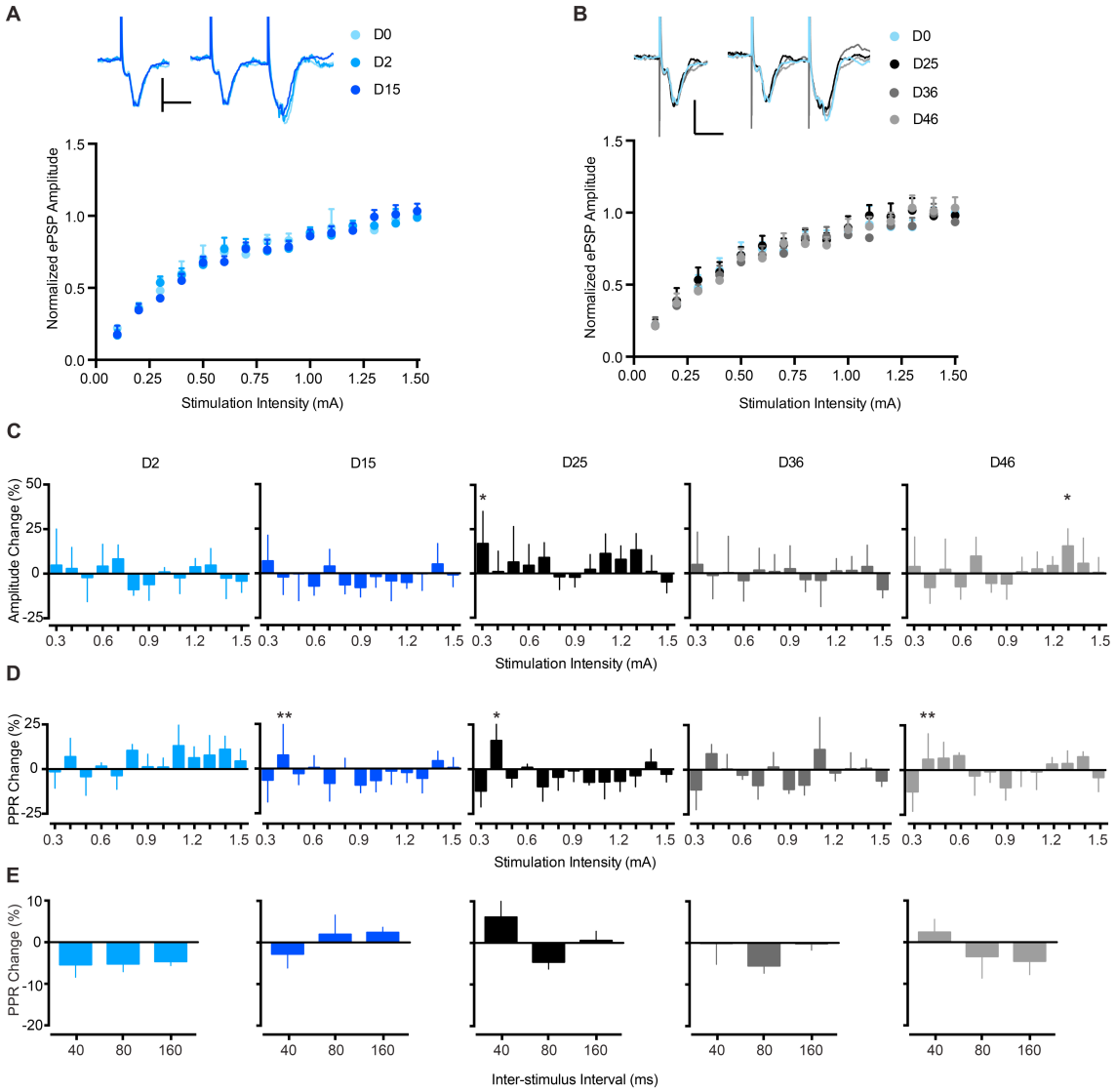

Figure 3.4 Field potentials in control group remain stable throughout longitudinal study. (A) Input-output (IO) curves at three time points: D0, D2 and D15. Upper panel shows representative traces of paired-pulse ePSPs (50 ms; 0.9 mA) for the respective time points. The first peaks are either overlaid to compare amplitudes across time points (left) or ePSPs are normalized to the first peak of D0 (right) to compare paired-pulse ratios (PPR). (B) IO curves at D25, D36 and D46 with D0 as reference. (C) Change in field potential amplitude relative to D0 for each stimulation intensity (RM two-way ANOVA, time $F_{(5,15)} = 0.37$, p= 0.865; time _ stimulation intensity $F_{(60,180)} = 0.77$, p = 0.878). (D) Change in PPR of two ePSPs with 50 ms inter-stimulus interval evoked during the IO curves and quantified for each stimulation intensity (RM two-way ANOVA, time $F_{(5,15)} = 1.01$, p= 0.446; time × stimulation intensity $F_{(60,180)}=0.92$, p = 0.643). (E) Change in PPR of two ePSPs with 40, 80 and 160 ms inter-stimulus intervals evoked at 40–50% of maximum ePSP (≈0.5 mA). All scale bars in the figures are 0.5 mV and 25 ms. All data in the figures are presented as mean ± s.e.m. Repeated measures two-way and one-way ANOVA with Fisher LSD *post hoc* was used to compare with D0. *p < 0.05; **p < 0.005.

"Therefore, extracellular field potentials were recorded from freely behaving rats (n = 7) to identify cocaine-driven neuroadaptations on excitatory synaptic transmission throughout drug exposure as well as abstinence (*i.e.* one day before training started (naïve), the day after

the first cocaine self-administration (CSA, acute), after 13 days of CSA (chronic) and at withdrawal day WD 7, 20 and 28; Figure 3.3)."

3.2.3 Incubation of drug-seeking behavior

"All animals of the cocaine group (n = 7) acquired cocaine self-administration (CSA) over a period of 16 days (Figure 3.3). Likewise, rats were able to discriminate between active and inactive holes, since number of active responses was significantly higher than pokes on the inactive hole throughout CSA training (active, 2729 ± 2258; inactive, 252 ± 134; $t_{(12)}$= 4.63, p = 0.0006 n = 7 paired t-test).

Furthermore, rats received a stable quantity of cocaine infusions during CSA training (113 ± 8, Figure 3.5A), which was close to the maximum number of infusions permitted during the daily 6 h sessions (n = 150). Escalation of cocaine intake is often observed with an extended schedule, which was not the case here, likely because there was a maximum of 25 infusions per hour. After cessation of CSA training all rats remained in their home cage for additional 30 days to study abstinence-driven neuroadaptations."

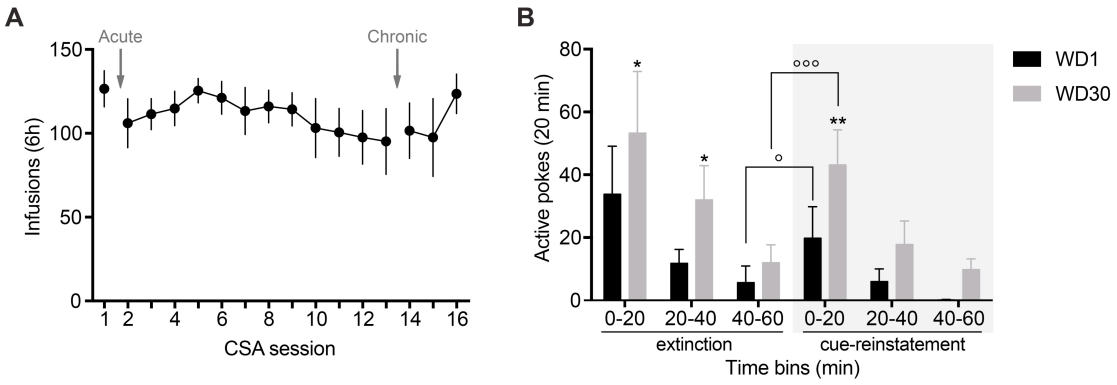

Figure 3.5 Incubation of cocaine-seeking behavior. (A) Average cocaine intake (0.5 mg/kg per infusion) received throughout CSA training (16 daily sessions of 6 h). (B) Active nose poking during cue-induced reinstatement test at WD1 and WD30. Data are presented in 20 min time bins, demonstrating that at both WD1 and WD30 cocaine seeking progressively extinguished during 1 h of extinction from time bin 0–20 to 40–60 and it was reinstated by presentation of cocaine-paired cues at reinstatement time bin 0–20. Reinstatement, as well as extinction responding in time bins 0–20 and 20–40, was higher at WD30 respect to WD1. Data are presented as mean ± s.e.m. °p < 0.05 and °°°p < 0.001 vs extinction time bin 40–60; *p < 0.05 and **p < 0.01 vs WD1 same time bin.

"At WD1 and 30, rats performed cue-induced reinstatement (Figure 3.3), preceded by one hour of extinction. ANOVA found an overall effect of withdrawal ($F_{(1,6)}$ = 7.22, p = 0.036) and time bins ($F_{(2,12)}$ = 6.11, p = 0.015). Detailed *post hoc* analysis revealed that at both WD1 and WD30 active nose-poking progressively decreased during 1 h of extinction from

time bin 0–20' to 40–60' and then it was reinstated by presentation of cocaine-associated cues (WD1, $p < 0.05$; WD30, $p < 0.001$; Figure 3.5B). As expected, reinstatement was significantly higher at WD30 respect to WD1 ($p < 0.01$). In line with incubation of cocaine seeking, at WD30 nose-pokes during extinction time bins 0–20'and 20–40'were higher than nose-pokes at WD1 ($p < 0.05$) suggesting increased cocaine-seeking, although extinction responding at time bin 40– 60' was comparable between the two withdrawal time points (Figure 3.5B). Inactive pokes remained unaffected by test conditions. The increment in cue-induced drug-seeking showed that animals did indeed develop incubation of drug-seeking."

3.2.4 Cocaine self-administration potentiates field potentials in the PFC-NAc core synapse

"The naïve condition, to which all other sessions were normalized to, was acquired prior to CSA start at day 0 (Figure 3.3). Age-matched controls and rats that self-administered cocaine were comparable at the start of the experiment, regarding input-output ($F_{(1,135)} = 0.12$, $p = 0.728$ two-way ANOVA). Extended access to cocaine (6 h) both after 1 day (acute) and 13 days (chronic) significantly increased PFC-evoked ePSPs (Figure 3.6A and B). RM two-way ANOVA revealed significant effect of drug ($F_{(5,30)} = 3.09$, $p = 0.023$) and stimulation intensity × drug ($F_{(70,420)} = 1.898$, $p < 0.0001$) in ePSP amplitude across the 6 recording sessions. Particularly, acute and chronic CSA significantly potentiated ePSPs in the PFC-NAc synapse, as indicated by a shift of the 'naïve' IO curve to the left at acute and chronic condition (Figure 3.6A). Compared with pre-cocaine (naïve), contingent cocaine intake increased ePSPs across several stimulation intensities (Figure 3.6C), both after 1 and 13 days of training. This indicates an enhanced glutamatergic transmission from the prelimbic cortex to the NAc during extended CSA.

When comparing IO changes between cocaine and age-matched control group across all 6 time points, intergroup difference was observed driven by drug/time ($F_{(11,810)} = 5.36$, $p < 0.0001$ $n = 11$). As with the within subject analysis, basal transmission as measured by IO was found to be potentiated at acute ($F_{(1,135)} = 5.63$, $p = 0.019$) and chronic time points ($F_{(1,135)} = 13.57$, $p = 0.0003$) relative to the control group.

To test whether any behavioral variable could predict the changes in ePSPs described above, we correlated the average of cocaine infusions during the CSA to the respective changes in ePSP amplitude."

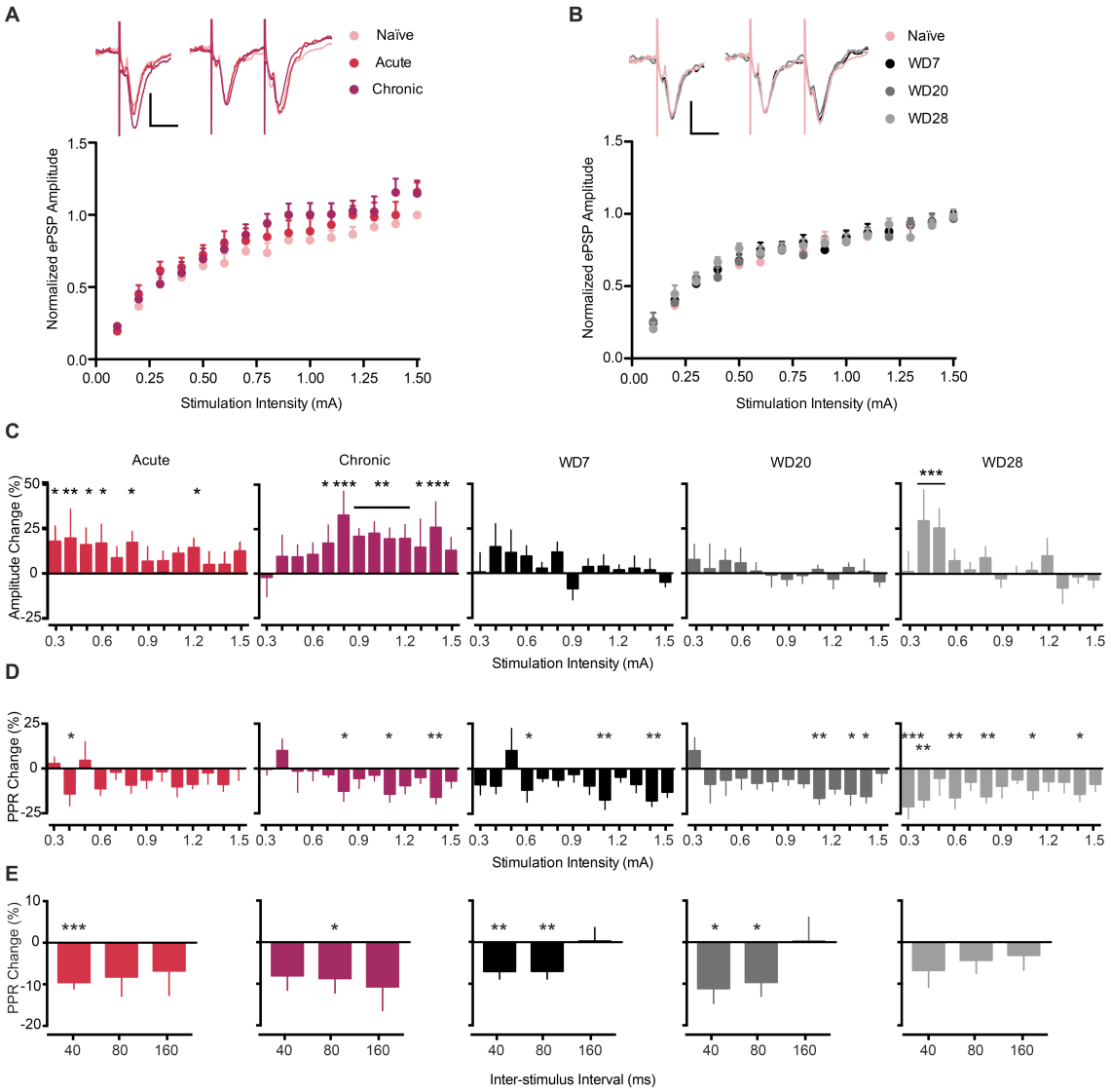

Figure 3.6 Field potentials in the NAc core are potentiated after chronic CSA and remain strengthened during WD. (A) IO curves at three time points: naïve, one day before CSA; acute, one day after the first CSA session; chronic, after 13 days of CSA. Upper panel shows representative traces of paired-pulse ePSPs (50 ms; 1.1 mA) for the naïve, acute and chronic time points. The first peaks are either overlaid to compare amplitudes across time points (left) or ePSPs are normalized to the first peak of the naïve condition (right) to compare paired-pulse ratios (PPR). (B) IO curves at WD7, D20 and D28 with naïve as reference. (C) Change in field potential amplitude relative to naïve for each stimulation intensity (RM two-way ANOVA, drug $F_{(5,30)}$ = 2.89, p = 0.030; drug _ stimulation intensity $F_{(60,360)}$ = 1.69, p = 0.002). (D) Change in PPR of two ePSPs with 50 ms inter-stimulus interval evoked during the IO curves and quantified for each stimulation intensity (RM two-way ANOVA, drug $F_{(5,30)}$ = 3.94, p = 0.007; drug × stimulation intensity $F_{(60,360)}$ = 1.61, p = 0.005). (E) Change in PPR of two ePSPs with 40, 80 and 160 ms inter-stimulus intervals evoked at 40–50% of maximum ePSP with around 0.5 mA. *p < 0.05, **p < 0.005, ***p < 0.001.

"A significant regression was found for the number of cocaine infusions and change in baseline ePSP amplitude (r^2 = 0.591; $F_{(1,5)}$ = 7.23, p = 0.043; Figure 3.7A). Thus, rats having higher number of cocaine infusions during CSA also had stronger potentiated ePSPs.

Interestingly, the degree of incubation, as measured by the ratio WD30/WD1, also positively correlated with the change in ePSP amplitude analyzed from IO measurements at chronic condition ($r^2 = 0.803$; $F_{(1,5)} = 20.31$, $p = 0.006$; Figure 3.7B). Thus, in-between subject variability in cocaine-driven potentiation related to individual responsiveness to drug-associated cues."

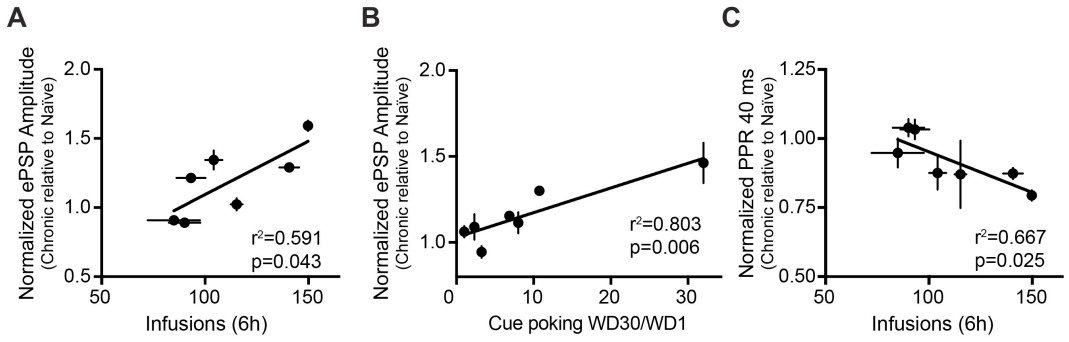

Figure 3.7 CSA performance and incubation of cocaine-seeking correlate with synaptic changes. (A) Linear regression showing positive correlation between average infusion per session during the first 13 CSA sessions and baseline ePSP amplitude recorded at chronic condition. (B) Linear regression showing positive correlation between degree of incubation (cue poking WD30/WD1) and ePSP amplitude analyzed from IO measurements at chronic condition. (C) Linear regression showing negative correlation between average infusion per session during the first 13 CSA sessions and paired-pulse ratio (PPR) with 40 ms inter-stimulus interval recorded at the chronic time point.

"Taken together, these findings suggest a potentiation of the glutamatergic postsynaptic potentials from pre-limbic PFC afferents onto the NAc core in animals that underwent chronic CSA. Indeed, correlation analysis shows that the more cocaine is taken over time the higher the synaptic potentiation."

3.2.5 A pre-synaptic mechanism contributes to NAc core potentiation

"PPRs did not differ between control and cocaine group at the start of the behavioral paradigm (naïve) at all ISIs investigated (40 ms, $t_{(9)} = 1.37$, $p = 0.205$; 50 ms, $t_{(9)} = 0.43$, $p = 0.674$; 80 ms, $t_{(9)} = 2.10$, $p = 0.065$; 160 ms, $t_{(9)} = 1.78$, $p = 0.109$). In behaving rats, the prelimbic PFC to NAc core synapse showed paired-pulse facilitation at 50 ms ISI with a paired-pulse ratio (PPR) larger than one (1.22 ± 0.05, $n = 11$; Figure 3.4A and Figure 3.6A). Paired-pulse facilitation was also observed at 40 ms (1.33 ± 0.04), and this PPR was significantly different from PPR at longer intervals ($F_{(2,30)} = 10.33$, $p = 0.004$ $n = 11$; 80 ms, 1.06 ± 0.05, $t_{(30)} = 4.28$, $p = 0.002$; 160 ms, 1.11 ± 0.04, $t_{(30)} = 3.47$, $p = 0.0002$). Comparable

PPRs at 80 and 160 ms ($t_{(30)} = 0.81$, $p = 0.423$) further indicated absence of facilitation at the latter intervals. For the subsequent analysis, we considered PPR changes relative to the naïve condition for both groups.

For the cocaine group, RM two-way ANOVA revealed a statistically significant difference in PPR at 50 ms, both across stimulation intensities (0.3–1.5 mA) and across all 6 time points monitored (drug, $F_{(5,30)} = 3.94$, $p = 0.007$; stimulation intensity × drug $F_{(60,360)} = 1.61$, $p = 0.0047$ n = 7; Figure 3.6D). Namely, when comparing overall change in PPR 50 ms ($F_{(3.3,20.0)} = 4.49$, $p = 0.012$, RM one-way ANOVA n = 7) PPR was found to be significantly decreased after chronic cocaine intake ($t_{(6)} = 2.65$, $p = 0.038$). PPR 40 ms was also decreased during cocaine exposure (acute, $t_{(6)} = 6.32$, $p = 0.0007$; chronic, $t_{(6)} = 2.34$, $p = 0.057$), although RM one-way ANOVA did not detect significance ($F_{(2.5,14.8)} = 2.04$, $p = 0.097$, n = 7). Decreases in PPR reflect increases in release probability, although the relation of PPR to release probability may be nonlinear (Branco & Staras, 2009).

To test whether individual differences in cocaine intake were associated with PPR changes, we correlated the average of cocaine infusions during the CSA to the respective changes in PPR. A significant linear regression was found, as the number of cocaine infusions inversely correlated with the change in PPR at 40 ms inter-stimulus interval ($r^2 = 0.667$; $F_{(1,5)} = 10.00$, $p = 0.025$; Figure 3.7C). This correlation suggests that differences in cocaine intake drive the degree of potentiation observed after 13 days of CSA likely via an increased pre-synaptic glutamate release."

3.2.6 PPR decrease persists throughout withdrawal

"Next, we examined whether enhanced glutamatergic postsynaptic potentials within the NAc core would persist during withdrawal."

"Evoked field potentials were recorded at distinct times across withdrawal, specifically 7, 20 and 28 days after CSA (Figure 3.3). In rats that underwent contingent exposure to cocaine (n = 7), no statistically significant effect on IO ePSP amplitude was found during withdrawal time points relative to naïve (Figure 3.6B). Consistently, relative changes of ePSP amplitudes (in comparison to naïve) for individual stimulation intensities were negligible during withdrawal (Figure 3.6C). This indicated that the ePSP amplitudes did not remain potentiated throughout withdrawal. Consistently, in-between group comparisons revealed that IOs of cocaine-seeking rats did not significantly differ from control rats both at earlier stages of withdrawal (WD7, $F_{(1,135)} = 2.08$, $p = 0.15$; WD20, $F_{(1,135)} = 0.0002$, $p = 0.99$) as well

as at the latest stage of withdrawal (WD28, $F_{(1,135)} = 0.09$, $p = 0.768$). PPR decreased during CSA and persisted throughout protracted abstinence for the cocaine group (drug, $F_{(5,30)} = 3.94$, $p = 0.007$; stimulation intensity × drug $F_{(60,360)} = 1.61$, $p = 0.0047$ n = 7). In respect to ISI of 50 ms (Figure 3.6D), PPR decreases became more prominent at later stages of withdrawal ($F_{(3.3,20.0)} = 4.49$, $p = 0.012$; WD7, $t_{(6)} = 2.45$, $p = 0.050$; WD20, $t_{(6)} = 3.32$, $p = 0.016$; WD28, $t_{(6)} = 5.00$, $p = 0.0002$). Consistent reductions in PPR were observed when stimulating with ISIs of 40 ms and 80 ms (40 ms: WD7, $t_{(6)} = 3.92$, $p = 0.008$; WD20, $t_{(6)} = 3.12$, $p = 0.021$; WD28, $t_{(6)} = 2.09$, $p = 0.082$; 80 ms: WD7, $t_{(6)} = 3.85$, $p = 0.008$; WD20, $t_{(6)} = 2.87$, $p = 0.028$; WD28, $t_{(6)} = 1.825$, $p = 0.117$; Figure 3.6E).

In summary, within subject longitudinal design revealed that chronic CSA potentiates the PFC-NAc pathway. Based on PPR analysis, a pre-synaptic increase in glutamate release might contribute to the increased synaptic strength, which is maintained and intensified towards longer periods of withdrawal. Importantly, individual differences in cocaine intake behavior predict the degree of potentiation observed."

3.3 Discussion

"The development and progression of drug-seeking behavior as well as susceptibility to relapse is rooted in gradual and persistent drug-driven neurobiological modifications (Kalivas & O'Brien, 2008b). These drug-induced alterations are seen in the reward system (Mameli & Lüscher, 2011). In particular, the NAc encodes for the motivational value of rewarding stimuli and the motor programs to obtain those rewards. In addition, the ability of excitatory synapses within the NAc to undergo adaptation is crucial to establish drug-cue memories (Knackstedt & Kalivas, 2009). NAc receives glutamatergic afferents from the ventral hippocampus, basolateral amygdala and prefrontal cortex (PFC) (Schmidt & Pierce, 2010). The latter exerts executive control over reacting upon drugs and drug-associated stimuli (Kalivas & Volkow, 2005). Therefore, the PFC-NAc pathway arises as key player in mediating drug-seeking and reinstatement.

The present study is one of the first using a longitudinal design that allows monitoring of synaptic adaptations within the same individual in a time-dependent manner, *i.e.* from a cocaine-naïve state, throughout cocaine self-administration and during abstinence.

In this study, we show (i) that chronic cocaine intake leads to a strengthening of the PFC-NAc core synapse, which based on paired-pulse ratio (PPR) analysis involves a pre-synaptic increase in glutamate release, and (ii) this strengthening persists throughout withdrawal and may drive incubation of cocaine-seeking behavior."

3.3.1 Effects of a single CSA session

"Acute motivational effects of cocaine are mostly mediated by dopamine (Berridge, 2007). Cocaine, as all addictive drugs, over time leads to an increase in net dopamine levels in the NAc (Di Chiara & Imperato, 1988; Frank *et al.*, 2008), a neurochemical event that contributes to the acquisition of drug- seeking behavior. Fewer studies focused on acute effects of self-administered cocaine on glutamatergic transmission.

Here, we report increased field potential amplitudes in the NAc core on the day after the first CSA session in the cocaine group relative to pre-cocaine exposure and relative to control group. This result contrasts with observations obtained with non-contingent cocaine. A single injection does not change input-output (IO) curves and PPR of field potentials in slices (Fourgeaud *et al.*, 2004), and has no influence on AMPA/NMDA ratio in the NAc (Kourrich *et al.*, 2007). The increased field potentials we observed could therefore

result from higher levels of brain cocaine reached following CSA (on average 113 times 0.5 mg/kg equals 56.5 mg/kg) than following a single injection (usually 15–20 mg/kg; 10 and 40 mg/kg in Kourrich et al. 2007), and/or from the fact that none of the studies aforementioned investigated specific inputs to the NAc. Alternatively, acute stress and environmental novelty, which constitutes a psychological stressor (Pfister, 1979), have been shown to enhance excitatory synaptic strength in the NAc (Rothwell *et al.*, 2011; Garcia-Keller *et al.*, 2015). During the first six-hour operant CSA session, field potential amplitudes unlikely increased due to novelty/stress, since synaptic responses remained unaffected in our control rats. We can however not exclude that field potential amplitudes increased because of the association of self-administered cocaine with novelty and/or stress."

3.3.2 Effects of chronic cocaine exposure

"Chronic contingent cocaine exposure decreases basal extracellular glutamate levels in the NAc (Pierce *et al.*, 1996; Moussawi *et al.*, 2011; Wydra *et al.*, 2013; Lutgen *et al.*, 2014). Still, heightened reactivity to cues in animal models of drug use depends on increased cue-evoked glutamate release (Hotsenpiller *et al.*, 2001). Non-contingent cocaine treatments and extended CSA strengthen AMPA receptor transmission in the NAc core as indicated from increases in both mEPSC frequency and AMPA/NMDA ratio after the last non-contingent cocaine treatment (Dobi *et al.*, 2011). In addition to synaptic changes, passive and active membrane properties as well as firing capacity of the core were found to increase following 1–3 days of non-contingent cocaine treatment (Kourrich & Thomas, 2009). These findings in slices are reflected *in vivo*, particularly in the NAc core. Tonic firing increases are more prevalent in NAc core (vs shell) during long access CSA (Fabbricatore *et al.*, 2009), and phasic firing to cocaine associated cues is enhanced after an extended CSA regimen (Ghitza *et al.*, 2004). Besides electrophysiological data, biochemical (*e.g.* increased surface expression of AMPA receptors) and morphological evidence (*e.g.* increased dendritic spine density) also point towards enhanced glutamatergic transmission in the NAc core following cocaine injections (Boudreau & Wolf, 2005; Dobi *et al.*, 2011)."

"In the present study, rats that chronically self-administered cocaine showed one day after the last session PFC-NAc potentiation both relative to the pre-cocaine state as well as when compared to the control group. Concomitantly, we observed a decrease in PPR, which can be indicative of pre-synaptic enhancement of glutamate release (Branco & Staras, 2009). An increased synaptic release of glutamate is of particular functional importance in the context of reduced basal levels of extracellular glutamate (Kalivas, 2009). Increased

glutamate release into the NAc had been described before, following contingent and non-contingent cocaine exposure one and 45 days after the last cocaine exposure (Suska *et al.*, 2013). Potential mechanisms underlying a disturbance in the probability of glutamate release could involve downregulation of the cystine-glutamate exchanger (Baker *et al.*, 2003; Kalivas & O'Brien, 2008b) or a hypoactivation of presynaptic inhibitory metabotropic glutamate receptors (mGluR2/3)(Kalivas, 2009; Cannella *et al.*, 2013)."

3.3.3 Effects of cocaine incubation

"Cocaine incubation is associated with a gradual increase in AMPA receptors (AMPARs) in the accumbens (Conrad *et al.*, 2008; Mameli *et al.*, 2009; Loweth *et al.*, 2014; Ma *et al.*, 2014). Extended access (6 h) CSA leads to the insertion of calcium permeable AMPA receptors (CP-AMPARs) into the NAc core after prolonged withdrawal (Ferrario *et al.*, 2011; Wolf & Tseng, 2012).

The here described PFC-evoked extracellular field responses returned to the naïve level during early withdrawal, not showing potentiation when compared to the pre-cocaine condition. However, the earliest time point at which significant CP-AMPARs were detected via an increased rectification index was WD30-35 (Wolf & Tseng, 2012; Wolf, 2016). Therefore, the latest withdrawal time point we assessed electrophysiologically preceded the marked accumulation of CP-AMPARs that persists up to WD90 (Wolf, 2016). On the other hand, the increased ePSP amplitudes at lower stimulation intensities may have already indicated some abstinence-driven postsynaptic adaptations in PFC-NAc synapses at WD28 (Figure 3.6C).

Dissimilar results might also have arisen from technical differences, since whole-cell patch-clamp recordings in medium spiny neurons confirmed elevated levels of AMPARs along dendrites without differentiating between specific synapses (Terrier *et al.*, 2016; Wolf, 2016). Still, a potentiated synaptic state was evident from field recordings, since LTP was occluded three weeks after discontinuing cocaine use (Moussawi *et al.*, 2009). However, this particular study used limited access (2 h) CSA as well as three weeks of extinction training, both of which are known to induce neuroadapations different from long access (6 h) CSA and abstinence. Examples in the NAc core are insertion of calcium-impermeable AMPARs after 45 days of withdrawal from limited access (2 h) CSA (Ma *et al.*, 2014) and adaptations in the Homer-mGluR5 signaling complex following extinction training (Knackstedt *et al.*, 2010)."

"Nonetheless, development of incubation depends upon concerted transmission across the reward processing circuitry, with AMPAR transmission being a consistent and critical mediator. In addition, parallel mechanisms cannot be neglected, specially occurring at earlier withdrawal (Wolf, 2016). At that stage, we describe enduring PPR reduction, which likely reflects persistent strengthening of PFC-NAc core synapses via an alternative mechanism.

Increased glutamate in the PFC-NAc pathway has been implicated as critical mediator for reinstatement of drug-seeking (Kalivas, 2009). Enhanced cue-induced glutamate release was observed in the ventromedial PFC after incubation of cocaine craving (Shin *et al.*, 2016). Consistent with the persistent PPR reduction we observed during withdrawal, glutamate could be released preferably from prelimbic neurons during protracted abstinence, which has been shown to recruit more phasic neurons in the prelimbic than infralimbic cortex (West *et al.*, 2014). Optogenetic inactivation of presynaptic terminals onto the NAc core decreases cocaine-induced reinstatement (Stefanik *et al.*, 2013). Consistently, increased glutamate release has been shown to be involved in reinstatement of cocaine-seeking responses (McFarland *et al.*, 2003). Cue-induced reinstatement of heroin seeking has similarly been shown to be mediated by glutamate release from PFC onto the NAc core (LaLumiere & Kalivas, 2008). DREADD activation of glial glutamate release inhibited extinguished cue-induced reinstatement (Scofield *et al.*, 2015), whereas rescuing PFC hypoactivity prevented compulsive cocaine-seeking (Chen *et al.*, 2013), and systemic delivery of an mGluR2/3 agonist after protracted withdrawal reduced cue-induced cocaine-seeking (Cannella *et al.*, 2013)."

"Growing evidence not only for addictive-like phenotype but also in sensitization has emphasized that drug-driven synaptic adaptations develop differentially across individuals (Borgland *et al.*, 2004; Boudreau & Wolf, 2005; Kasanetz *et al.*, 2010). Importantly, we also found inter-individual differences in cocaine-seeking behavior correlating with the synaptic changes observed. Namely, potentiation of ePSP after chronic cocaine experience positively correlated with the cocaine intake as well as the incubation ratio, respectively. Similarly, a very recent study describes that PFC to NAc core projections are recruited proportionally to cue-induced seeking behavior (McGlinchey *et al.*, 2016). The amount of NAc core neurons that phasically fire during cocaine seeking was also found to correlate with "incubation-like increase" of cocaine seeking (Guillem *et al.*, 2014).

Taken together, these correlations suggest that individuals with increased activation of the PFC-NAc circuit are more likely to engage in augmented drug seeking and taking."

3.4 Concluding remarks

This study demonstrates temporal development of drug-driven neuroadaptations, especially the strengthening of the PFC-NAc synapse after chronic cocaine self-administration. These cocaine-induced synaptic alterations persist into protracted abstinence and may therefore underlie the incubation of drug craving.

3.5 Graphical summary

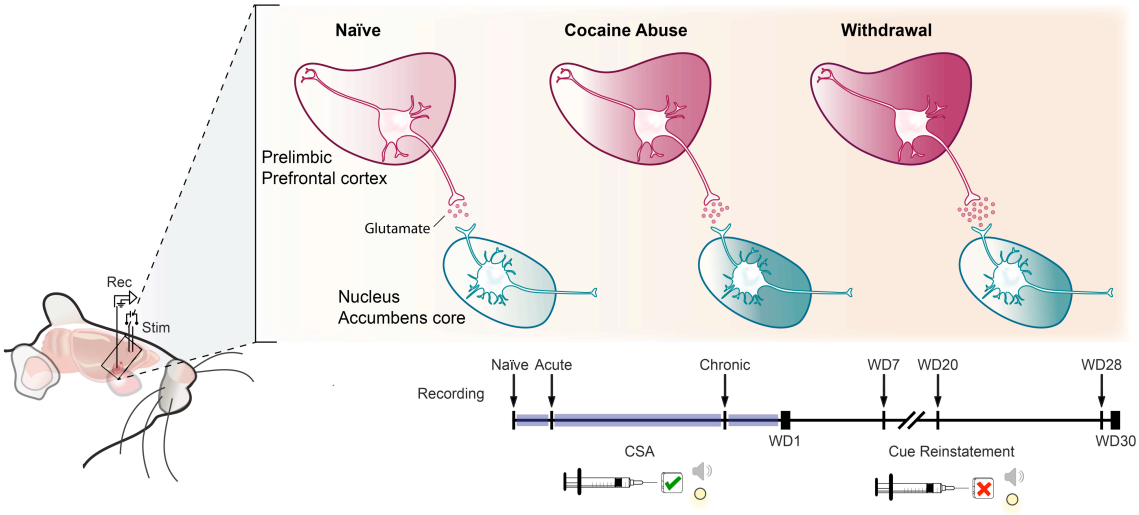

Figure 3.8 | Persistent strengthening of the PFC – NAc pathway during incubation of cocaine-seeking behavior. Longitudinal *in vivo* field potential recordings in awake rats revealed that chronic contingent exposure to cocaine strengthened the prelimbic PFC to NAc core pathway when compared to pre-cocaine condition. This strengthening was associated with decreased paired-pulse ratios (PPR), indicative of presynaptic enhancement of glutamate release, which persisted throughout withdrawal.

4. Results

History of chronic cocaine modifies network activity in the NAc and PFC during spontaneous behavior

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4

History of chronic cocaine modifies network activity in the NAc and PFC during spontaneous behavior

Rationale Neuronal assemblies and networks are formed by synaptically, and thus functionally, connected neurons. As a result, these assemblies exhibit rhythmicity as measured by spontaneous local field potentials. Network oscillations can vary in frequency, ranging from periods of seconds, as in slow wave oscillations, to fast activity in which a cycle can last milliseconds. Conventionally neuronal oscillations are classified into discrete wave bands, namely slow wave oscillations (<1 Hz), delta (1-4 Hz), theta (4-8 Hz), alpha (8-12 Hz), beta (12-30 Hz), and gamma (>30 Hz). Discrete frequency bands change in response to sensory, motor and cognitive events. Moreover, spontaneous local field potentials (LFPs) are thought to modulate regional excitability, and hence affect local computation within a brain region as well as long-range communication across distinct brain areas.

Addictive drugs hijack the mesolimbic circuit, with long term drug abuse leading to reward processing, cognitive and decision-making deficits. Thus, it is reasonable to infer that drug-induced neuroadaptations may include changes in network dynamics.

Previous studies have shown that gamma oscillations in the NAc encode for reward and response vigor (van der Meer *et al.*, 2010). Dopaminergic drugs like amphetamine and apomorphine decrease 'gamma-50' frequency (42-58 Hz) and increase 'gamma-80' (70-90 Hz) in naïve rats (Berke, 2009). Moreover, non-contingent subcutaneous morphine was also reported to transiently decrease delta and increase high gamma in the NAc and PFC (Dejean *et al.*, 2013). Notwithstanding, to date, research focusing on network activity within the cortical-accumbal pathway still remains scarce.

In the current study, we assessed the effects of cocaine intake and withdrawal as well as the effects of a cocaine challenge in both naïve and cocaine experienced subjects on network activity during spontaneous behavior. Studying spectral 'landscape' of NAc and PFC allows for understanding how drug experienced individuals, even after prolonged periods of abstinence, may exhibit altered neural processing when compared to drug naïve subjects.

4.1 METHODS

For detailed description of behavioral paradigms and post mortem processing read Chapter 2 on General Methods. The following experiments were performed with the same cohort of animals described in the previous chapter.

4.1.1 Animal groups

The experimental group consisted of four cocaine self-administration (SA) rats and a control group of four yoked saline rats. Yoked saline controls were paired with a rat that self-administered cocaine, receiving non-contingent saline infusion (0,9% NaCl) in the same temporal manner as self-administered by their matching rat.

In the cocaine challenge experiment a naïve group was also included. The latter did not undergo catheter surgery.

4.1.2 Drugs

Cocaine-HCl (Sigma-Aldrich, Germany) was dissolved in sterile saline and self-administered by the subjects via intravenous (i.v.) route and subcutaneously (10 mg/Kg/ml s.c.).

4.1.3 Electrophysiology: Spontaneous local field potentials (LFPs)

Recordings of spontaneous local field potentials (LFPs) were carried out in the home cage on days without behavioral testing (Figure 3.3, Chapter 3), *i.e.* one day before training started (naïve), the day after the first CSA (acute), the day after the 13th CSA session (chronic) and at withdrawal day (WD) 7, 20 and 28. Before the first recording, rats were left to habituate for 30 min to the recording apparatus.

Approximately 100 days after the last training session rats were recorded again in the home cage one hour before and two hours more following a non-contingent cocaine challenge.

LFPs were recorded using a miniature headstage (1 g, npi electronic GmbH, Tamm, Germany; commutator model SL-12-C, Dragonfly, Ridgeley, West Virginia, USA). Data collection hardware consisted of an EXT-02F amplifier (npi electronic GmbH, Tamm, Germany) and ITC-16 (HEKA Elektronik, Lambrecht, Germany). LFPs (depth EEG) were filtered at 0.3 to 500 Hz, digitized at 2 kHz and stored for subsequent analysis. Noise was

filtered by a Hum Bug Noise Eliminator (AutoMate Scientific, Inc., Berkeley, CA). All recordings were referenced to a stainless-steel skull screw implanted above the cerebellum. Simultaneously 3-dimension accelerometer signals were also acquired.

4.1.4 Spontaneous local field potentials analysis

LFPs were exported from the Fitmaster software (HEKA Elektronik, Lambrecht, Germany), and imported to a customer written Matlab script - EEGProcessing program (Courtesy of Prof. Dr. Andreas Draguhn's lab).

No data staging was performed based on behavioral states. For the longitudinal study across SA training and withdrawal 74 epochs of 25 s (1850 s) were collected for analysis. Regarding the study of non-contingent cocaine, 10 min epochs were selected before, half an hour (25-35 min) and hour (55-65 min) after injection.

Power and coherence spectra were calculated using 4096 point FFT and smoothed with 3-point Gaussian sliding window. Power spectral density histograms were plotted using logarithmic scale for power, and values between 0 and 120 Hz are shown. Based on previous studies (*e.g.*, Dejean et al. 2013) and frequency peaks observed we examined distinct frequency bands (Delta 1-4 Hz; Theta 4-8 Hz; Alpha 8-12 Hz; Low beta 13-20 Hz; Low gamma 30-49 Hz; Gamma 60, 55-70 Hz; Gamma 90, 75-95 Hz and High gamma 100-120 Hz).

Cross frequency coupling analysis measures coupling strength between the phase of a slow oscillation (4-12 Hz) and the amplitude of a fast oscillation (45-65 Hz and 75-100 Hz) through the computation of a modulation index (MI, for detailed description see Tort et al. 2008). Briefly, the MI is a measure to evaluate the divergence of phase-amplitude coupling from a uniform distribution, normalized such that values vary between 0 (no coupling) and 1 (maximal coupling). Next, MI peaks were selected to evaluate the maximum MI values of two distinct gamma bands (45-65 Hz; 65-100 Hz) to delta/theta coupling (1-12 Hz), respectively, for further statistical analyses.

4.1.5 Statistics

To compare sessions (Naïve, Acute, Chronic, WD7, WD20 and WD28) power was averaged across the frequency range of interest and normalized to the whole range of the spectra (0-200 Hz). Repeated measures (RM) two-way factorial ANOVA, followed by Bonferroni multiple comparisons post-hoc test, was used to compare, within subject and

between groups, band power across all time points recorded. The same approach was used to analyze band power and coherence before and after a cocaine challenge. t-student was used to compare between cocaine SA and saline yoked band range spectral power at naïve time-point as well as before non-contingent cocaine for all band ranges analyzed.

Graphs and statistical analysis were performed in Graphpad Prism 5 and Adobe illustrator. All results are shown as mean ± s.e.m ($p<0.05$).

4.2 RESULTS

4.2.1 Chronic CSA increases alpha and decreases high gamma power in the NAc core

We firstly studied how CSA and withdrawal affect baseline LFP processing in the NAc during task-independent spontaneous behavior in the home cageq. Spectral power analysis of longitudinally recorded NAc LFP was performed at six time points: naïve, acute, chronic and withdrawal day (WD) 7, 20 and 28 (see Figure 3.3, Chapter 3).

Spectral analysis of NAc LFP during non-staged spontaneous behavior revealed 4 main frequency bands. As show in Figure 4.1A and B, LFP preferentially oscillate in Delta (1-4 Hz), Theta/Alpha (4-12 Hz) and Gamma rhythms (50-120 Hz), as seen by the presence of peaks at these frequency bands. In the gamma range two peaks can be distinguished, namely Gamma 60 and Gamma 90 (Figure 4.1, inset a2 and b2).

Band power values did not differ at the start of the experiment (naïve) for all frequency bands analyzed (theta, $t_{(6)} = 0.29$, $p = 0.78$; alpha, $t_{(6)} = 0.44$, $p = 0.68$; low beta, $t_{(6)} = 0.11$, $p = 0.91$; gamma 70, $t_{(6)} = 1.16$, $p = 0.16$; gamma 90, $t_{(6)} = 0.94$, $p = 0.38$; high gamma, $t_{(6)} = 0.36$, $p = 0.73$). As such, both cocaine and saline yoked group spectral power was normalized to the naïve condition in a within subject fashion.

Delta oscillation power was not modified by time or drug intake, since no main effect of time or drug was detected for yoke saline and cocaine SA groups, respectively ($F_{(5, 15)} = 2.06$, $p = 0.13$; $F_{(5, 15)} = 1.73$, $p = 0.19$).

On the other hand, theta activity changed over time. Theta power decreased at later withdrawal, WD20 and WD28 (Figure 4.1C, middle panel) in a similar fashion in both groups ($F_{(5, 15)} = 12.78$; $F_{(5, 15)} = 13.17$, $p < 0.0001$).

A similar trend was observed in the alpha band range with band power significantly decreasing towards later time points recorded, namely in yoked saline rats ($F_{(5, 15)} = 13.71$, $p< 0.0001$). More interestingly, in cocaine self-administering rats alpha power specifically but transiently increased following chronic cocaine intake and, alike the yoked saline group, decreased during withdrawal ($F_{(5, 15)} = 14.31$, $p < 0.0001$).

Likewise, low beta increased significantly at the acute and chronic time points in rats that underwent contingent cocaine administration ($F_{(5,15)} = 16.20$, $p < 0.0001$). Within subject comparison in the yoked saline and cocaine SA groups also revealed significant decrease of

beta power at WD28 when compared to naïve condition, as shown in Figure 1D ($F_{(5,3)} = 5.74$, $p < 0.05$).

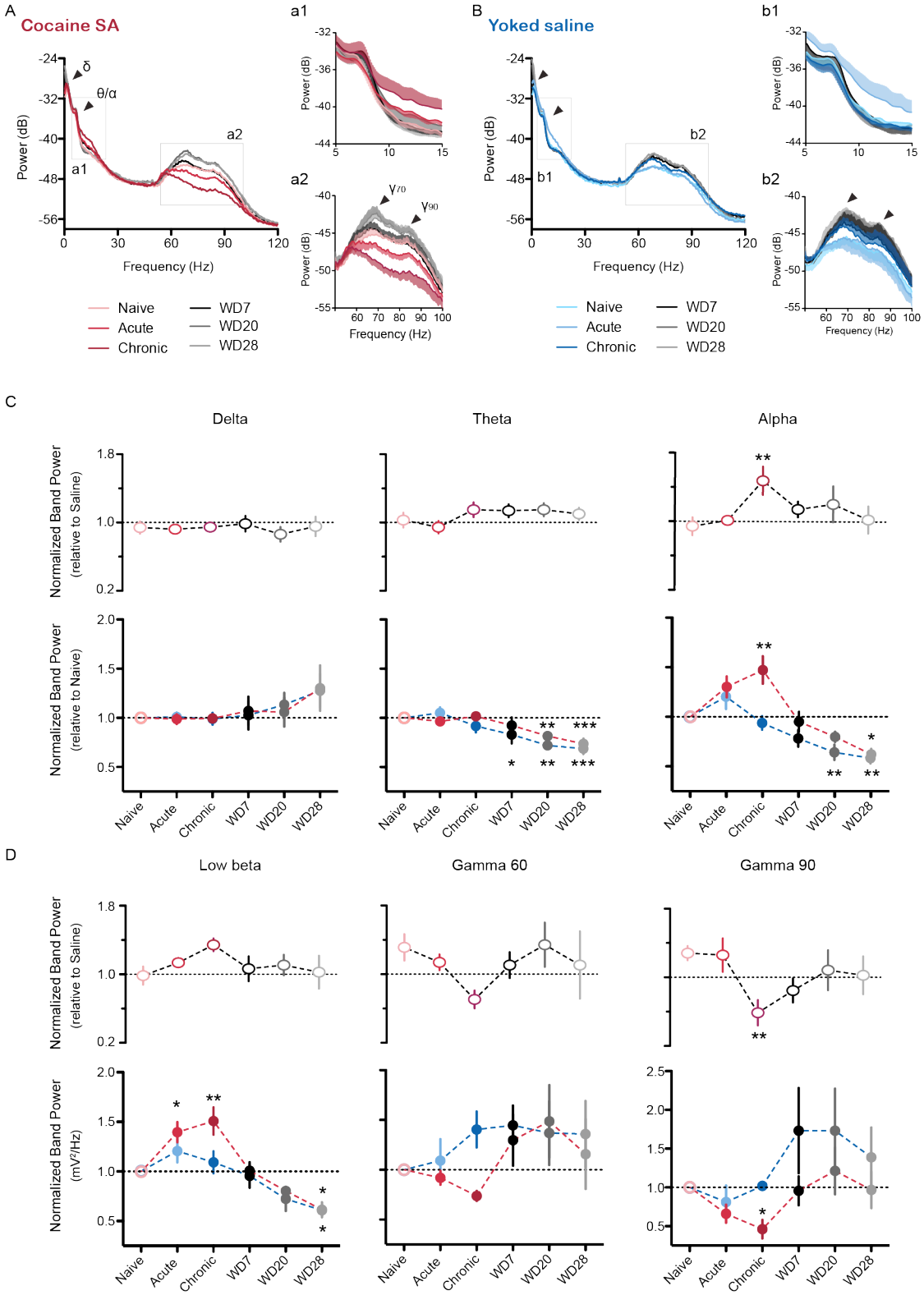

Figure 4.1 Power spectral density across longitudinally recorded sessions. A. NAc spectral density (1-120 Hz) in cocaine self-administering rats. a1. Inset, slow frequencies (5-15 Hz). a2. Inset, gamma bands (50-100 Hz), arrows indicate frequency peaks for gamma 60 and gamma 90 bands. B. Same as in A for

yoked saline rats. C and D. Upper panels: Normalized power of cocaine SA group relative to the same condition in yoked saline group. Lower panels: Normalized NAc spectral density for slower frequencies, Delta (1-4 Hz), Theta (4-8 Hz) and Alpha (8-12 Hz); and for faster frequencies (Low beta (13-30 Hz), Gamma 60 (55-70 Hz) and Gamma 90 (75-95 Hz)) in cocaine self-administering and yoked saline rats. All values presented as mean±s.e.m; n=4. * p<0.05; **p<0.001; ***p<0.0001 compared to naïve condition.

Cocaine modulation of faster frequencies was also assessed, namely in the gamma range (Figure 4.1D). However, no main effects of time or drug were detected regarding gamma 60 in both yoked saline groups and cocaine SA, respectively ($F_{(5,15)} = 0.75$, $p = 0.60$; $F_{(5,3)} = 1.77$, $p = 0.18$).

Conversely, gamma 90 power was significantly affected by cocaine self-administration ($F_{(5,15)} = 4.73$, $p < 0.05$). Decreases in gamma 90 were restricted to chronic CSA, since at WD20 and WD28 band power was significantly distinct from chronic condition. No effect of time was detected in the yoked saline group ($F_{(5,3)} = 2.07$, $p = 0.13$).

To better discern differential effects of cocaine history in NAc oscillatory activity, cocaine group's band power was normalized to the correspondent session of yoked saline mean band power (Figure 4.1C and D, upper panel). No cocaine-related changes were observed for delta band, since both groups exhibit similar trends. Theta is marginally increased following cocaine self-administration ($F_{(5,15)} = 4.91$, $p < 0.05$).

Alpha and low beta were strongly modulated by cocaine experience, since power increased, in particular following 13 days of cocaine self-administration ($F_{(5,3)} = 4.06$; $p < 0.05$). This increase was transient and spectral power returned to naïve levels during withdrawal.

In the gamma 60 range no significant differential effects of cocaine consumption were observed ($F_{(5,3)} = 1.55$, $p = 0.23$). However, a transient but significant decrease relative to yoke saline was observed in the gamma 90 band range ($F_{(5,3)} = 4.23$; $p < 0.05$).

In summary, chronic contingent cocaine exposure transiently increases alpha and decreases high gamma power. Thus, NAc oscillatory state was altered during task-independent spontaneous behavior in the home cage and in the absence of drug.

4.2.2 Chronic cocaine self-administration does not alter phase modulation in the NAc

To determine whether interactions occurred across frequency ranges, we used a cross-frequency measure to assess phase-to-amplitude modulation.

In the nucleus accumbens, theta band oscillations (≈4-8 Hz) modulated a band of higher frequency oscillations in the gamma range (≈80-100 Hz), as shown in Figure 4.2. Theta also modulated, although to a lesser extent, slower gamma oscillations (≈50-70 Hz), as shown by a second peak during withdrawal in Figure 4.2B.

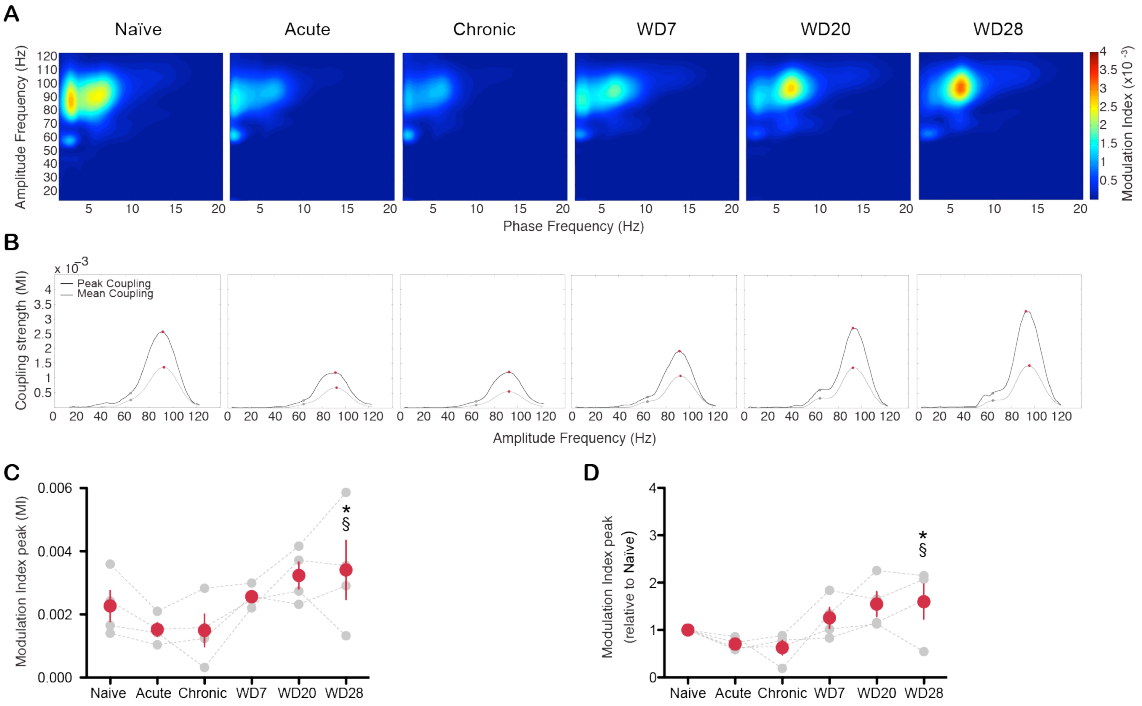

Figure 4.2 Phase to amplitude modulation in the NAc of rats that underwent CSA. A. Phase-to-amplitude comodulograms obtained from LFPs recorded at distinct time points during cocaine self-administration training and withdrawal. Plots show amplitude modulation over a wide oscillatory range (10-120 Hz); B. Phase-to-amplitude peak (black) and mean (grey) across the same frequency range as shown in A. C. Quantification of peak modulation index (MI) across all time points and showing individual MI (n=4) in grey. D. Same as in C, but MI was normalized within subject to the naïve condition. All values presented as mean±s.e.m; n=4, * $p<0.05$ compared to chronic; $^{\S}p<0.05$ compared to acute condition.

When analyzing cross coupling throughout CSA and withdrawal time points a pattern emerged, in which modulation of high frequency gamma increased significantly towards later withdrawal (WD28) when compared to the acute and chronic condition ($F_{(5,3)} = 4.64$, $p = 0.009$, Figure 4.2C). The modulation peak reduction was transient and restricted to SA training, as late withdrawal the modulation index did not differ from the naïve condition (Figure 4.2B and C).

Although a trend was also apparent in the yoked saline group, an evident modulation pattern was not observed, since the modulation index peak did not change significantly across the experimental conditions ($F_{(5,3)} = 0.31$, $p = 0.90$; see Figure 4.3).

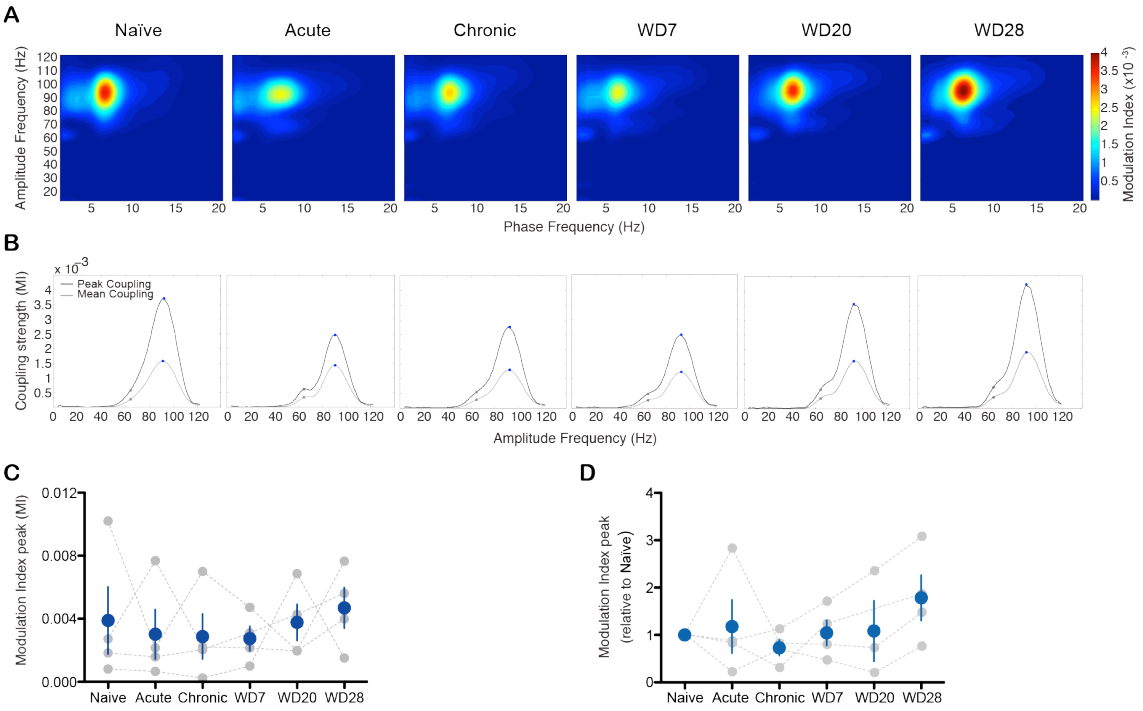

Figure 4.3 Phase to amplitude modulation in the NAc of yoked saline rats. A. Phase-to-amplitude comodulograms obtained from LFPs recorded at distinct time points during yoked saline administration training and withdrawal. Plots show amplitude modulation over a wide oscillatory range (10-120 Hz); B. Phase-to-amplitude peak (black) and mean (grey) across the same frequency range as shown in A. C. Quantification of peak modulation index (MI) across all time points and showing individual MI (n=4) in grey. D. Same as in C, but MI was normalized within subject to the naïve condition. All values presented as mean±s.e.m; n=4.

When comparing both groups, no difference was found, indicating that phase-amplitude modulation might be associated to time in husbandry and handling rather than drug experience ($F_{(11,7)} = 0.73$, $p = 0.70$).

4.2.3 Cocaine challenge differentially affects network activity in rats with history of CSA

To assess how history of cocaine taking affects LFP processing of a non-contingent cocaine challenge in the PFC-NAc, approximately 100 days after the last CSA session, a subcutaneous cocaine injection (10 mg/Kg) was administered in the home cage to both CSA and saline-yoked rats (see Figure 4.4).

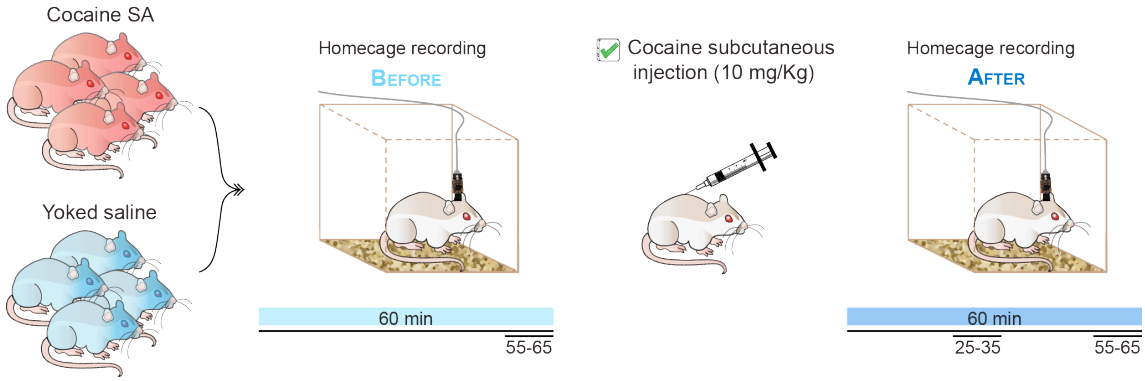

Figure 4.4 Cocaine challenge experimental set up. Rats with history of cocaine SA and yoked saline rats were recorded in the home cage, preceding and following a non-contingent subcutaneous injection. Ten minutes segments of LFP were analyzed for all subjects.

Band power in the NAc did not differ at the start of the experiment (before injection) between groups, for all frequency bands analyzed (Table 4.1; theta, $t_{(6)} = 0.27$, $p = 0.80$; alpha, $t_{(6)} = 0.35$, $p = 0.74$; gamma 60, $t_{(6)} = 0.31$, $p = 0.23$; gamma 90, $t_{(6)} = 0.18$, $p = 0.87$).

Table 4.1 NAc normalized band power before injection in both yoked saline and CSA rats.

	Theta	Alpha	Gamma 60	Gamma 90
Cocaine SA	0.182 ± 0.020	0.058 ± 0.011	0.141 ± 0.015	0.068 ± 0.007
Yoked Saline	0.173 ± 0.025	0.053 ± 0.008	0.110 ± 0.019	0.072 ± 0.022

(Mean ± s.e.m, n=4 per group; Unpaired t-test)

In cocaine experienced rats the main effect was observed at the gamma frequency range. Particularly, gamma60 power significantly decreased 30 minutes after injection (Figure 4.5A/D). This decrease persisted up to one hour following cocaine challenge ($F_{(2,3)} = 22.01$, $p < 0.01$; Figure 4.5A/D). Conversely, gamma90 tended to increase after non-contingent cocaine, although no significant effects were observed ($F_{(2,3)} = 0.80$, $p = 0.49$). Non-contingent cocaine challenge also induced persistent increase after half and one hour post-injection in theta, though not significantly ($F_{(2,3)} = 5.10$, $p = 0.05$, Figure 4.5D).

In contrast, in rats without previous history of cocaine intake a non-contingent cocaine challenge exerted its principal effects at lower frequencies. Namely, a transient increase of alpha power in the NAc, observed 30 minutes after injection ($F_{(2,3)} = 11.91$, $p = 0.008$; Figure 4.5B). Sixty minutes after injection alpha power was restored to pre-injection levels (Figure 4.5D). The power of all other frequencies did not significantly change following injection (theta, $F_{(2,3)} = 1.75$, $p = 0.25$; gamma90, $F_{(2,3)} = 0.57$, $p = 0.59$ and gamma60, $F_{(2,3)} = 0.97$, $p = 0.43$; Figure 4.5D).

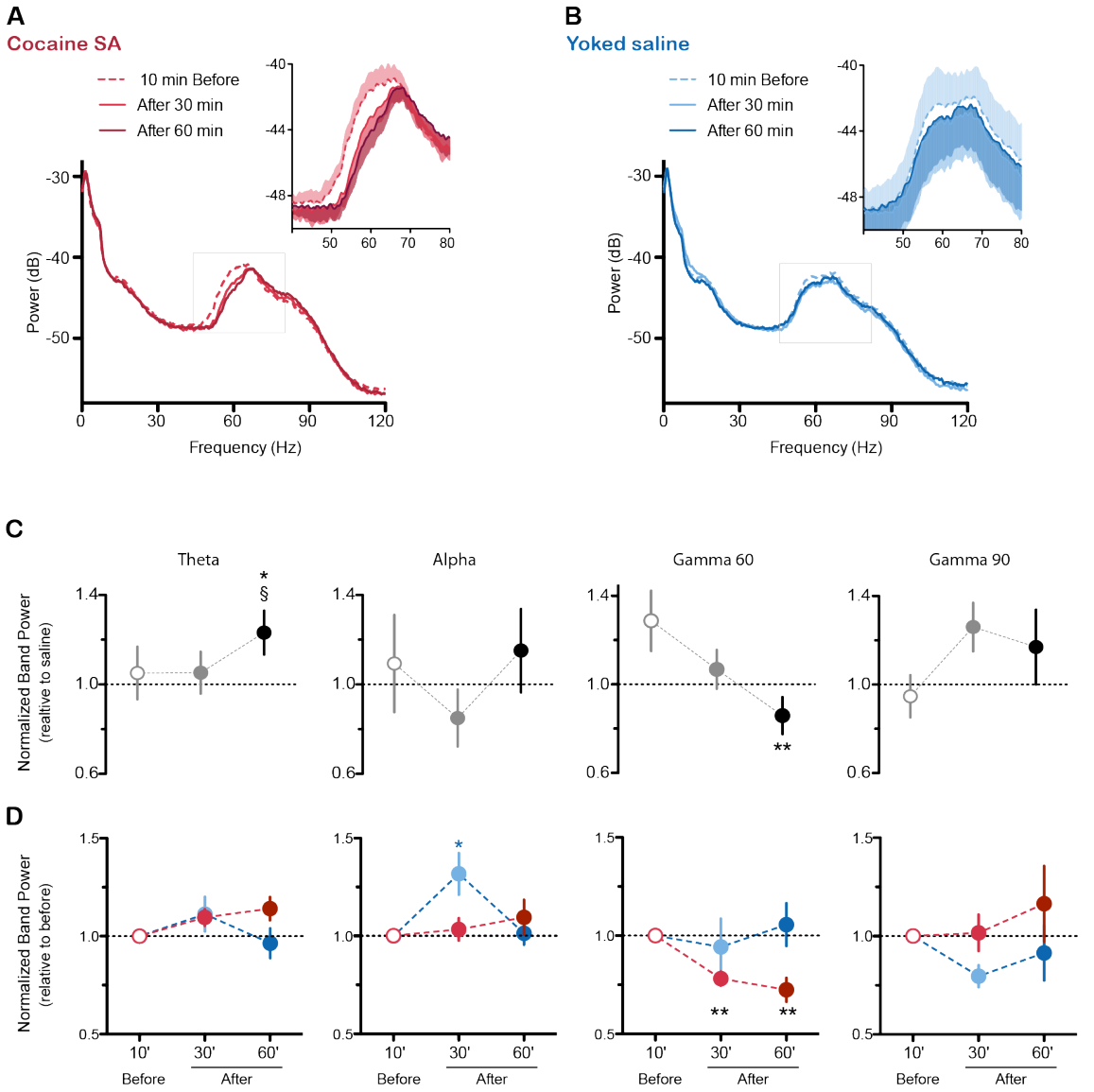

Figure 4.5 Power spectrum density in the NAc of cocaine SA and yoked saline rats before and after non-contingent cocaine. A. NAc power spectrum in CSA rats, inset shows detail between 40-80 Hz. B. NAc power spectrum in yoked saline rats, inset shows detail between 40-80 Hz C. Normalized band power normalized to the mean of the yoked saline group. D. Normalized power for theta (4-8 Hz), alpha (8-13 Hz), gamma60 (55-65 Hz) and gamma90 (75-85 Hz) before, 30 and 60 min after non-contingent cocaine challenge (10 mg/Kg) in cocaine SA and saline yoked rats. All values presented as mean±s.e.m; n=4; *p<0.05, **p<0.001 compared to before injection; § p<0.05 compared to 30 min after injection.

To dissected differential effects of the history of CSA, frequency band power was normalized to the respective condition in the yoked saline group (Figure 4.5C).

It became evident that animals with CSA history predominantly exhibited longer lasting effects when compared to more transient alteration in yoked saline rats. Significant effects were detected at exclusively 60 min after injection: theta band power was increased and

gamma60 was decreased relative to rats without previous drug experience ($F_{(2,3)} = 12.22$, $p < 0.001$; $F_{(2,3)} = 15.78$, $p < 0.01$, respectively).

Relative to yoked saline and 30 min after injection, alpha power seemed to be decreased ($F_{(2,3)} = 4.84$, $p = 0.056$) and gamma90 power to be increased ($F_{(2,3)} = 2.99$, $p = 0.13$). However, both changes were the consequence of exclusive changes in the yoked saline group.

Spectral density was also analyzed in the prefrontal cortex. Similarly to the NAc, no differences were found between cocaine SA and yoked saline rats before injection, with exception of gamma60, which is significantly increased in cocaine SA rats (delta, $t_{(6)} = 0.53$, $p = 0.61$; alpha, $t_{(6)} = 0.45$, $p = 0.67$; low gamma, $t_{(6)} = 0.31$, $p = 0.77$; gamma60, $t_{(6)} = 3.20$, $p < 0.05$), as shown in Table 4.2.

Spectral density was also analyzed in the prefrontal cortex. Similarly to the NAc, no differences were found between cocaine SA and yoked saline rats before injection, with exception of gamma 60 range, which is significantly increased in cocaine SA rats (delta, $t_{(6)} = 0.53$, $p = 0.61$; alpha, $t_{(6)} = 0.45$, $p = 0.67$; gamma 60, $t_{(6)} = 3.20$, $p < 0.05$; high gamma, $t_{(6)} = 0.09$, $p = 0.93$), as shown in Table 4.2.

Table 4.2 PFC normalized band power before injection in both Saline-yoked and CSA rats.

	Delta	Alpha	Gamma 60	High Gamma
Cocaine SA	0.457 ± 0.015	0.063 ± 0.002	0.044 ± 0.002*	0.007±0.0006
Yoked Saline	0.444 ± 0.019	0.067 ± 0.006	0.035 ± 0.002	0.007±0.0006

(Mean ± s.e.m, n=4 per group; Unpaired t-test)

Firstly, in rats that underwent 16-day regime of CSA delta power was increased following 30 minutes, having recovered after one hour after injection ($F_{(2,3)} = 13.27$, $p = 0.006$, Figure 4.6D). An opposing effect was observed in the yoked saline group, in which delta decreased 30 minutes after injection ($F_{(2,3)} = 5.93$, $p = 0.038$, Figure 4.6D).

Low gamma power was significantly reduced half an hour after challenge only in cocaine SA rats ($F_{(2,3)} = 6.57$, $p = 0.03$, Figure 4.6D). No significant changes were observed in this band frequency in the yoked saline group ($F_{(2,3)} = 2.25$, $p = 0.19$, Figure 4.6D).

Similarly to the NAc, non-contingent cocaine reduced gamma 60 power both in cocaine SA and yoked saline groups, although not significantly ($F_{(2,3)} = 4.30$, $p = 0.07$; $F_{(2,3)} = 4.23$, $p = 0.07$).

Delta was oppositely modulated in both groups thus increasing transiently when normalizing the cocaine SA to yoked saline group ($F_{(2,3)} = 66.38$, $p < 0.0001$; Figure 4.6C).

On the other hand, alpha band ($F_{(2,3)} = 8.78$, $p < 0.02$) was transiently decreased relative to saline yoked. Gamma 60 power were also reduced one hour post-injection, indicating longer lasting effects of non-contingent cocaine in cocaine SA compared to yoked saline rats ($F_{(2,3)} = 10.52$, $p < 0.05$). Finally, high gamma, although not significantly, seemed to have decreased more prominently in the cocaine SA relative to yoked saline groups ($F_{(2,3)} = 3.42$, $p = 0.10$).

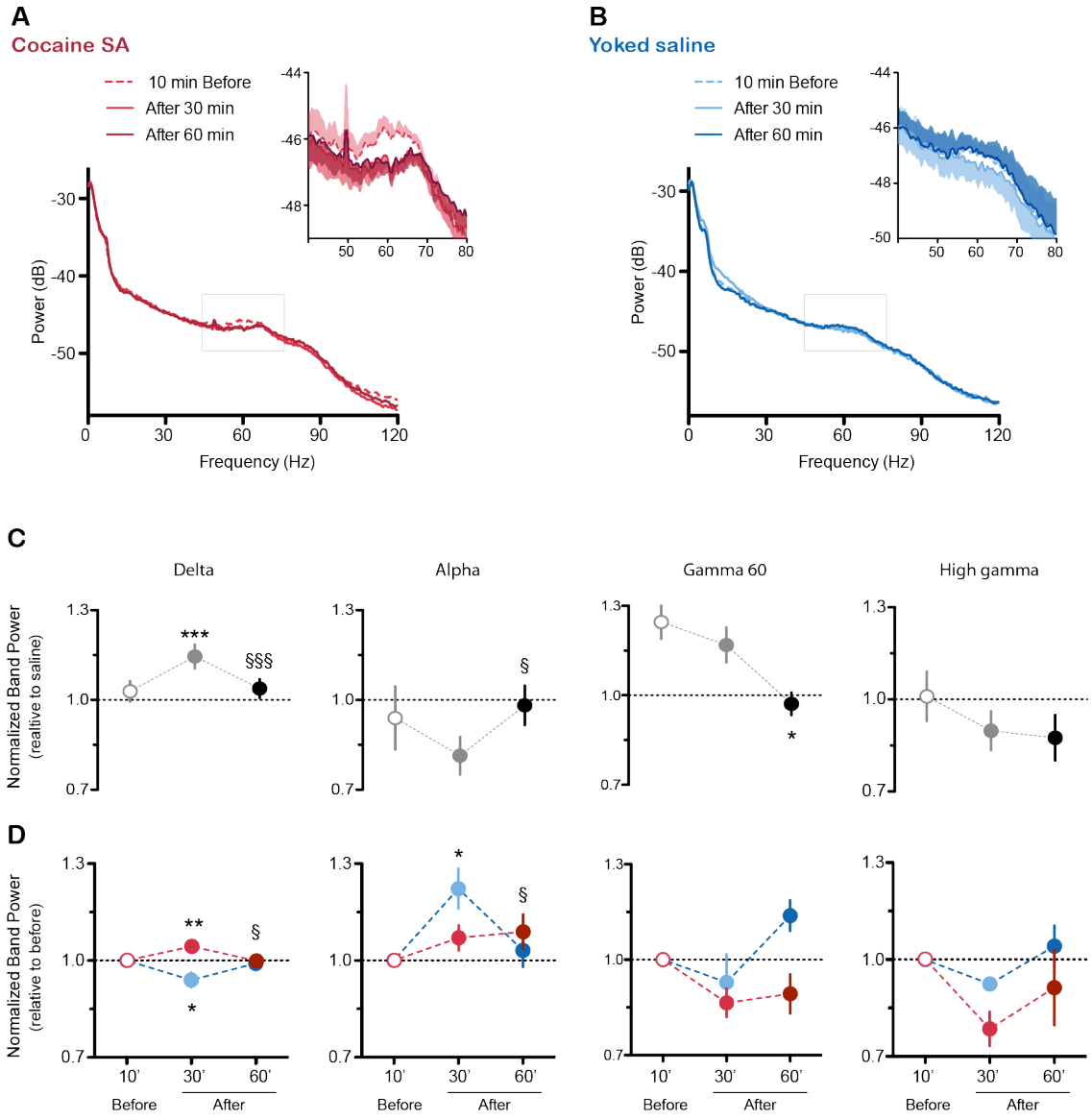

Figure 4.6 Power spectrum density in the PFC of cocaine SA and yoked saline rats before and after non-contingent cocaine. A. PFC power spectrum in CSA rats, inset shows detail between 40-80 Hz. B. Same as in A but in yoked saline rats. C. Normalized band power normalized to the mean of the yoked saline group. D. Normalized power for delta (1-4 Hz), alpha (8-13 Hz), gamma60 (55-70 Hz) and high gamma (100-120 Hz) before, 30 and 60 min after non-contingent cocaine challenge (10 mg/Kg) in cocaine SA and saline yoked rats. All values presented as mean±s.e.m; n=4; *p<0.05, **p<0.001 compared to before injection; § p<0.05, compared to 30 min after injection.

Notwithstanding the specific alterations observed at distinct frequency bands, it became apparent that animals with CSA history exhibited longer lasting effects (30 and 60 min) in opposition to shorter transient effects detected in the yoked saline group, perhaps indicative of altered processing in subjects with previous drug taking history.

4.2.4 Phase-to-amplitude modulation of fast LFP rhythms by theta in the NAc increases following cocaine challenge

Cross-frequency coupling was also quantified pre and post injection in both yoked saline and cocaine self-administration rats.

Phase-to-amplitude modulation was higher in the accumbens when compared to the prefrontal cortex (data not shown). No significant changes were observed in PFC regarding cross-frequency coupling in both cocaine SA and yoked saline groups. Two peaks were observed, one referring to Delta-Gamma60 modulation and a second larger peak mainly due to theta-Gamma90 modulation.

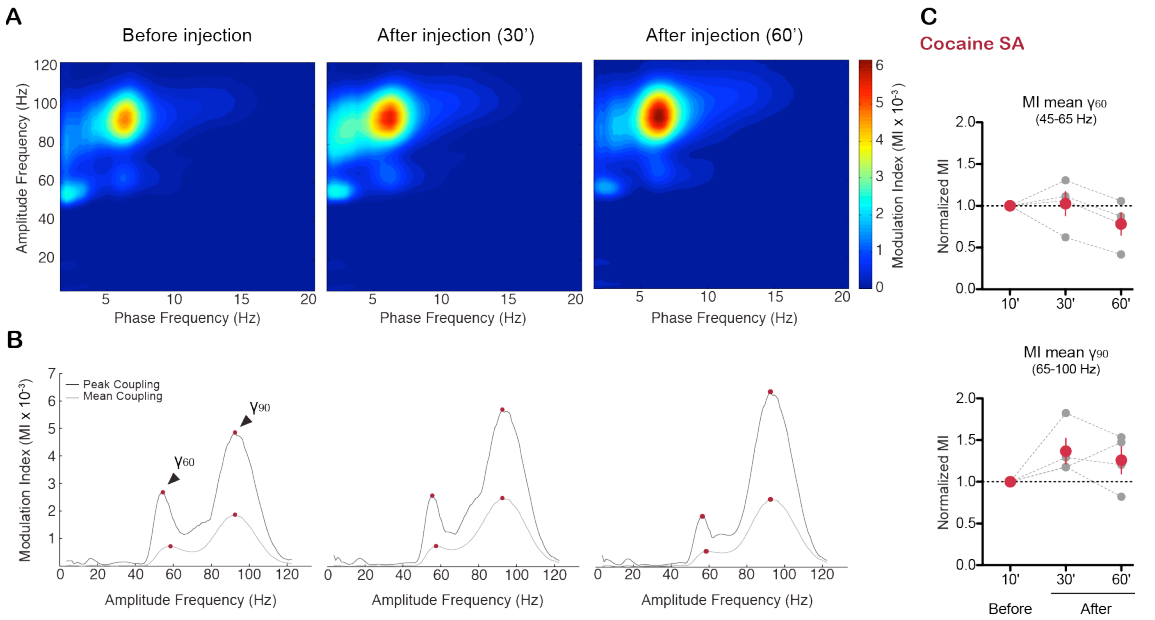

Figure 4.7 Phase to amplitude modulation in the NAc of cocaine SA rats. A. Phase-to-amplitude comodulograms obtained from LFPs recorded before, 30 and 60 minutes after cocaine challenge. Plots show amplitude modulation over a wide oscillatory range (10-120 Hz); B. Phase-to-amplitude peak (black) and mean (grey) across the same frequency range as shown in B. C. Quantification of peak modulation index (MI) across conditions and showing individual MI (n=4) in grey. All values presented as mean±s.e.m; n=4; *p<0.05, compared to before injection.

In the nucleus accumbens the intensity of coupling between the phase of slow oscillation and the amplitude of fast oscillation was not significantly altered in rats that underwent self-

administration (Figure 4.7). Although not significantly, theta-gamma90 modulation index appears to be increased following cocaine challenge, in cocaine SA rats ($F_{(2,3)} = 2.95$, $p = 0.13$; Figure 4.7). No changes were detected in the delta-gamam60 modulation both in cocaine SA (Figure 4.7) and saline yoked animals ($F_{(2,3)} = 2.73$, $p = 0.14$; $F_{(2,3)} = 1.38$, $p = 0.32$, Figure 4.8).

Interestingly, in rats without previous cocaine self-administration history phase-amplitude mean index of theta-gamma90 modulation significantly but transiently decreases following a non-contingent cocaine injection ($F_{(2,3)} = 5.67$, $p < 0.05$, Figure 4.8).

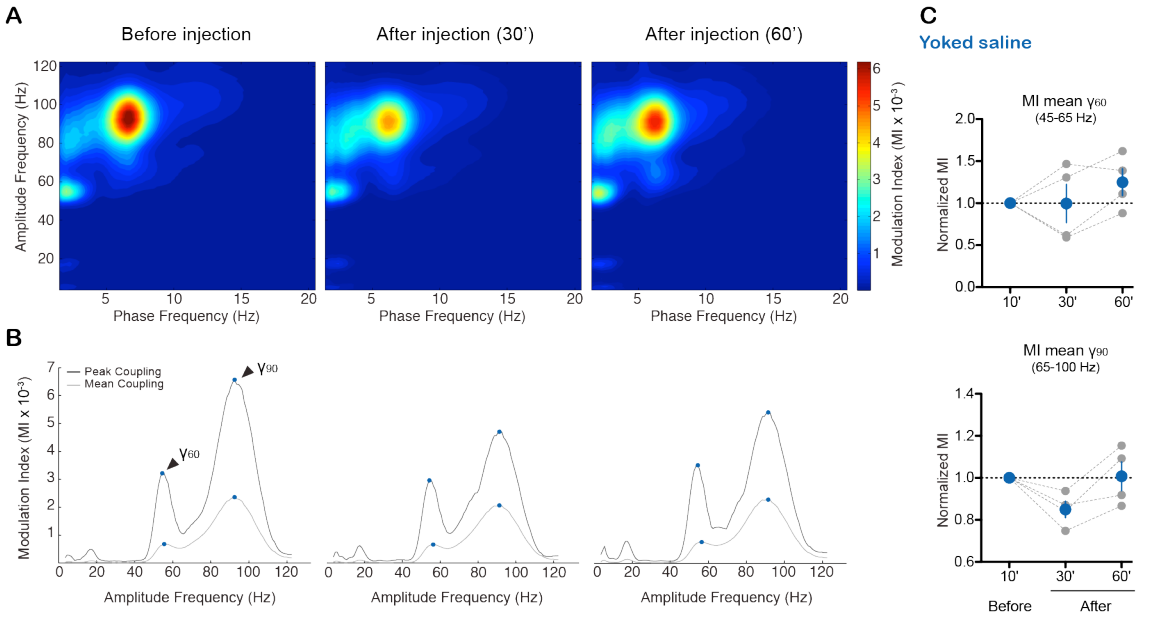

Figure 4.8 Phase to amplitude modulation in the NAc of yoked saline rats. A. Phase-to-amplitude comodulograms obtained from LFPs recorded before, 30 and 60 minutes after cocaine challenge. Plots show amplitude modulation over a wide oscillatory range (10-120 Hz); B. Phase-to-amplitude peak (black) and mean (grey) across the same frequency range as shown in B. C. Quantification of peak modulation index (MI) across conditions and showing individual MI (n=4) in grey. All values presented as mean±s.e.m; n=4; *p<0.05, compared to before injection.

4.2.5 Reduced functional connectivity between PFC and NAc following cocaine challenge in rats that previously self-administered cocaine

To quantify functional connectivity, we measured coherence of local field potentials between prelimbic cortex and the nucleus accumbens core. Coherence spectrogram revealed high coherence in the theta and alpha, as well as gamma range between PFC and NAc core, as shown by the peaks in these bands (Figure 4.9A and B).

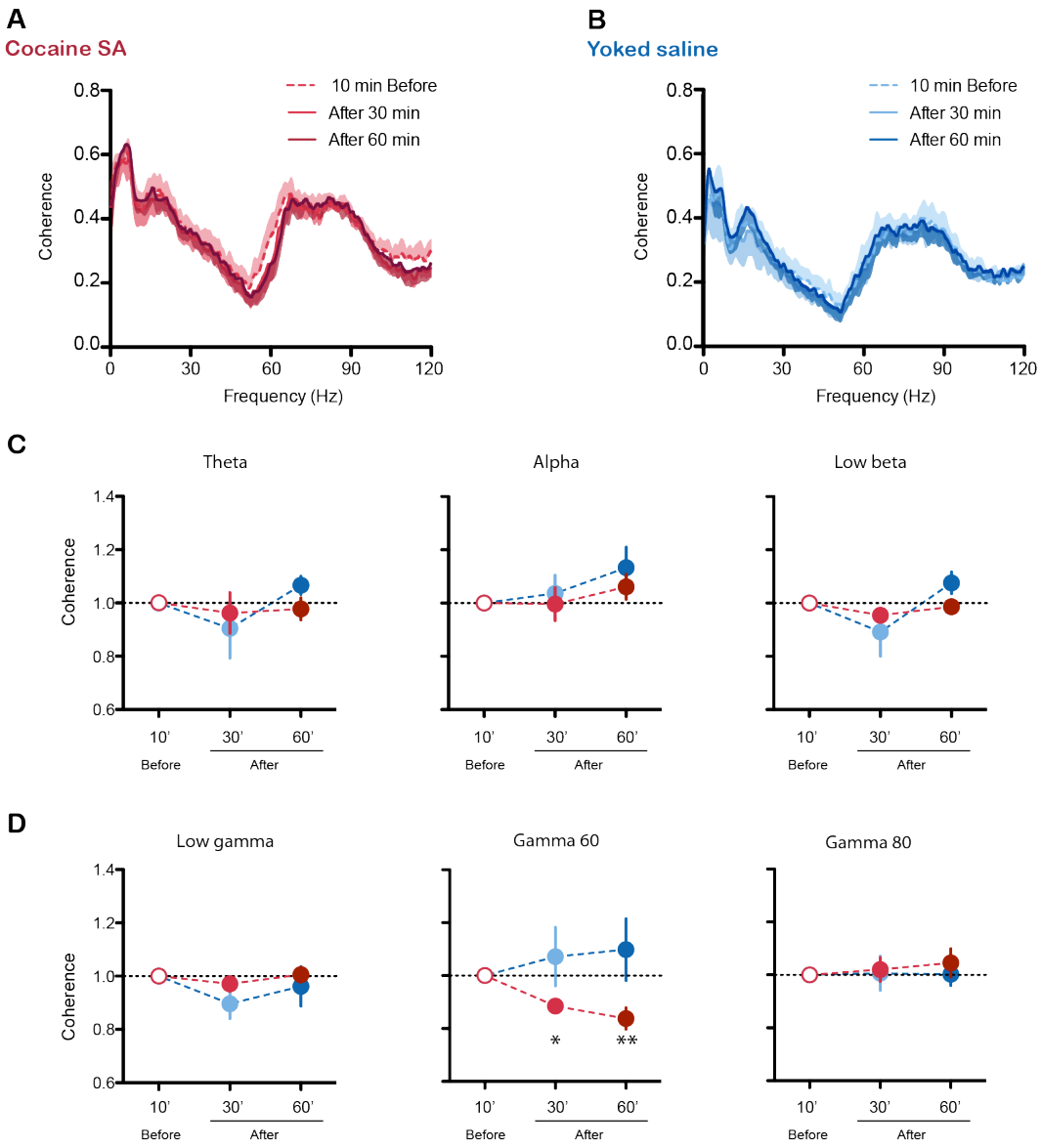

Figure 4.9 Coherence between frequency peaks in LFP obtained simultaneously from PFC and NAc before and after a cocaine challenge in the home cage. A. Mean coherence spectrogram of PFC and NAc core in rats with history of cocaine self-administration (SA) and in yoked saline rats (B). C. Coherence in the theta (4–8 Hz), alpha (8-13 Hz) and low beta (13-20 Hz) bands. D. Coherence in the low gamma (30–49 Hz), gamma60 (55-70 Hz) and gamma90 (75-95 Hz) bands. The curves and shaded areas indicate the mean ± s.e.m. All values presented as mean±s.e.m; n=4; *p<0.05, **p<0.001 compared to before injection;

No significant differences were observed in coherence between cocaine SA and yoked saline before injection (theta, $t_{(6)} = 1.62$, $p = 0.16$; alpha, $t_{(6)} = 1.68$, $p = 0.14$; low beta, $t_{(6)} = 0.1.45$, $p = 0.20$; low gamma, $t_{(6)} = 1.65$, $p = 0.15$; gamma60, $t_{(6)} = 1.29$, $p = 0.25$; gamma90, $t_{(6)} = 0.99$, $p = 0.36$).

A single cocaine challenge did not significantly alter functional connectivity in yoked saline rats for all band ranges analyzed (theta, $F_{(2,3)} = 1.40$, $p = 0.32$; alpha, $F_{(2,3)} = 1.19$, p

=0.37; low beta, $F_{(2,3)} = 2.63$, $p = 0.15$; low gamma, $F_{(2,3)} = 1.66$, $p = 0.27$; gamma60, $F_{(2,3)} = 0.49$, $p = 0.63$; gamma90, $F_{(2,3)} = 0.006$, $p = 0.99$).

In contrast, coherence spectra showed a significant decrease in the gamma 60 band specifically in rats that experienced cocaine self-administration. The low PFC-NAc coherence persisted up to 60 minutes following injection ($F_{(2,3)} = 16.85$, $p < 0.05$, Figure 4.9D).

No further changes in functional connectivity were observed across other frequency bands in cocaine SA group (theta, $F_{(2,3)} = 0.20$, $p = 0.83$; alpha, $F_{(2,3)} = 0.66$, $p = 0.55$; low beta, $F_{(2,3)} = 1.67$, $p = 0.26$; low gamma, $F_{(2,3)} = 1.33$, $p = 0.33$; gamma90, $F_{(2,3)} = 0.47$, $p = 0.65$).

Thus, specific disruption of gamma60 synchrony between PFC-NAc occurs following cocaine only in rats that previously experienced the drug.

4.3 Discussion

Here, for the first time, we analyzed the effects of long-access self-administration on the dynamics of accumbal network in freely moving rats during task-independent behavior.

The present study shows that chronic contingent cocaine transiently modulates alpha and gamma 90 activity in an opposite manner. Thus, history of cocaine intake alters NAc oscillatory state during spontaneous behavior in the home cage and in the absence of drug. Yet these changes proved to be transient as oscillatory activity progressively normalized following abstinence.

Moreover, a single cocaine challenge after long-term abstinence (≈100 days) has differential effects in the prefrontal-accumbal network on subjects with previous history of cocaine intake. This observation demonstrates consequential adaptations to the presence of the drug that depend on past experience.

Therefore, in addition to synaptic adaptations, oscillatory activity adaptations are also likely to contribute to dysregulation of homeostatic processes – allostasis - that underpin persistent vulnerability to relapse in addiction (Koob & Le Moal, 2001; Dejean *et al.*, 2013).

4.3.1 NAc oscillations dynamics following chronic cocaine intake

Here, we recorded simultaneously from the nucleus accumbens core (dorsal-medial portion) and the prelimbic prefrontal cortex. Studies with similar recording sites describe comparable spectral profiles in baseline, awake animals (Berke, 2009; Gruber *et al.*, 2009; Dejean *et al.*, 2013; Catanese *et al.*, 2016). Showing that prefrontal cortex and nucleus accumbens oscillate preferably in delta, theta and gamma rhythms. Interestingly, we as others also report the presence of two gamma peaks in the nucleus accumbens (*i.e.* 'gamma50/60' and 'gamma90'). Although difference in nomenclature, the band ranges across studies are rather comparable (Berke *et al.*, 2004; van der Meer & Redish, 2009; Dejean *et al.*, 2013, 2017; Donnelly *et al.*, 2014; Howe *et al.*, 2017).

As far as it was possible to ascertain, there are no studies assessing the impact of contingent cocaine on network activity. However, a growing body of literature is focusing on network activity associated with natural rewards and drugs of abuse.

Recently, some studies consistently focused on cortical-basal ganglia circuits' oscillations in behaving animals (Berke *et al.*, 2004; Berke, 2009; van der Meer & Redish, 2009; van der Meer *et al.*, 2010). Gamma oscillations are of particular interest since there is a dorsal-ventral power gradient (Berke *et al.*, 2004; Carmichael *et al.*, 2017). Gamma power is more

prominent in the NAc when compared to dorsal striatum, and thus likely has a functional role in ventral striatal processing. As such, accumbal gamma rhythms have been studied in the context of reward and addictive drugs. Here we report significant suppression of gamma90 activity following an extended access CSA regime.

Animals that underwent 14 days of non-contingent cocaine administration, enough to elicit locomotor sensitization, also showed reduced gamma (62-100 Hz) in the nucleus accumbens compared to animals that received saline (McCracken & Grace, 2013).

Interestingly, two days of continuous subcutaneous non-contingent morphine, sufficient to cause dependence, also decrease gamma90 in the NAc. Yet, this effect was accompanied by increased gamma60 and delta in the same region (Dejean *et al.*, 2013). Hence, there seem to be some common and divergent mechanisms relative to stimulants effects on LFP (Badiani *et al.*, 2011).

Gamma 80 increases have been associated with anticipatory and uncertainty aspects of reward (Berke, 2009; van der Meer & Redish, 2009). Gamma oscillations have been implicated in distinct cognitive functions, such as attention and working memory.

Gamma oscillations are proposed to dynamically regulate neural ensemble spike timing synchrony, and thus functionally '*bind*' (van der Meer & Redish, 2009) different regions (Buzsáki & Schomburg, 2015; Fries, 2015). Dysregulation of such synchrony might functionally impact information flow, leading to inability to flexibly update computing and impaired output.

In parallel to gamma suppression, there was an increase in alpha and beta activity selectively in animals that self-administered cocaine.

High beta EEG activity is a hallmark of Parkison's disease (PD), and is strongly correlated with severity of symptoms and response to treatment (reviewed in Little & Brown, 2014). PD stems from degeneration of dopamine producing neurons, leading to a hypodopaminergic state and consequently, motor control impairments - bradykinesia and rigidity. High beta has been reported in the subthalamic nucleus, cortex, and dorsal striatum after dopamine depletion in rodents (Sharott *et al.*, 2005; Mallet *et al.*, 2008). In PD patients, dopaminergic replacement therapy (e.g L-DOPA) suppresses beta oscillations (10-30 Hz, which includes alpha as defined in the present study) and enhances high gamma in the basal ganglia (Brown *et al.*, 2001; Cassidy *et al.*, 2002).

In the current study, high alpha and beta might also reflect a reduced tonic dopaminergic state. In fact, reduced basal dopamine release has been observed following chronic cocaine administration (Chefer & Shippenberg, 2002; Lee *et al.*, 2011). And active cocaine addicts

consistently exhibit blunted stimulant-induced DA responses in striatal regions (Volkow *et al.*, 2014).

Synaptic integration within the corticostriatal pathway is dependent on DA modulation. Enhanced burst firing of VTA neurons enhances D1-receptor activity, thus facilitating limbic inputs to the NAc. Conversely, decreasing and increasing tonic firing of DA neurons, causes inhibition and facilitation of cortical inputs into the NAc, respectively, in a D2-dependent manner and without altering limbic responses. Thus, DA release maintains the balance between limbic and cortical inputs into the NAc (Goto & Grace, 2005a, 2008). Hence, modulation of DA levels regulates the balance between D1 (phasic) and D2 (tonic) driven states and between limbic and cortical inputs to the NAc, respectively.

As such, CSA-induced high beta and alpha may reflect dopamine deletion due to decreased tonic dopamine release. Tonic DA release reduction selectively affects prefrontal but not hippocampal inputs, producing potentiation of cortical inputs mediate by D2R (Goto & Grace, 2005a, 2005b). Moreover, reduced D2 stimulation facilitates LTP in the PFC that is likely dependent on increased glutamate release (Goto & Grace, 2005b).

Potentiation of PFC to NAc evoked-responses was in fact observed in the same rats, as the high beta activity, at the same time point (see Chapter 3), which was accompanied by enhanced prefrontal glutamate release (Luís *et al.*, 2017).

Thus, our findings support a D2-mediated facilitation of prefrontal glutamatergic inputs into the NAc, at the expense of limbic inputs, which might mediate drug seeking behavior (Kalivas, 2009) – Figure 4.10.

This scenario of hypodopaminergic tonus might also partially account for changes observed in the gamma activity. Although gamma oscillations, namely gamma50/60, in the ventral striatum are likely to have a strong contribution from the piriform cortex, through volume conduction (Carmichael *et al.*, 2017), recordings from accumbal activity of fast-spiking interneurons (FSI) show entrainment to gamma oscillations. Additionally, different FSI cohere with gamma50 or gamma80, or can also change their firing rate differentially between the two rhythms (van der Meer & Redish, 2009). This indicates, despite LPF origin, there are regional effects within the NAc (Berke, 2009; van der Meer *et al.*, 2010).

FSI activity can be modulated by dopamine, as shown by decreased striatal FSI firing by D2 antagonist, which mimics DA depletion (Wiltschko *et al.*, 2010). Conversely, *in vitro* dopamine induces FSI depolarization, which shifts the intrinsic gamma-band membrane oscillations to higher frequencies (Bracci *et al.*, 2002).

Decreased gamma power (30–60 Hz) is also observed in the subthalamic nucleus, cortex, and dorsal striatum following dopamine depletion (Kühn *et al.*, 2005; Burkhardt *et al.*, 2009). Accordingly, in PD patients, replacement therapy also increases gamma oscillations (70-85 Hz, Cassidy *et al.*, 2002).

Thus, reduced dopamine release following CSA, could cause suppression of FSI firing that contributes to gamma generation in the NAc. Furthermore, inhibiting FSI firing may also release MSN from normal feedforward inhibition, creating a state of greater NAc excitability, which is corroborated by the LTP-like state reported in Chapter 3 (Luís *et al.*, 2017) – Figure 4.10.

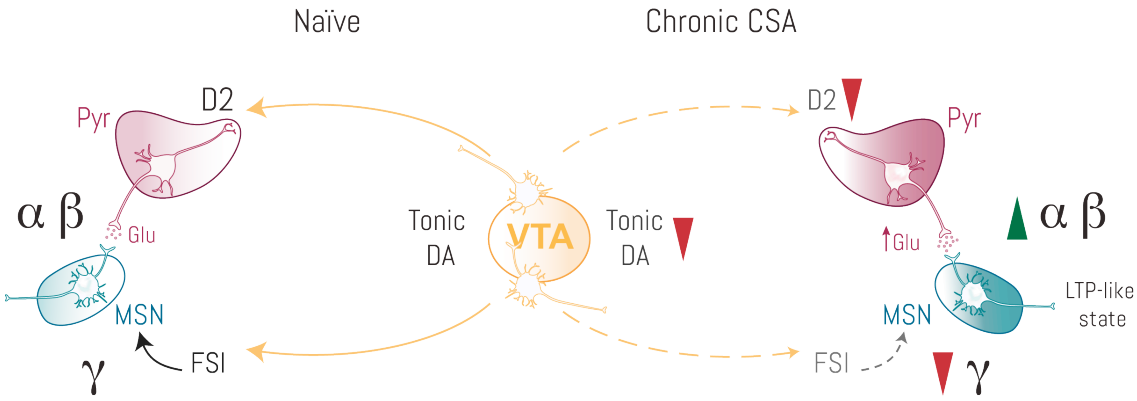

Figure 4.10 Synaptic integration within the corticostriatal pathway is dopamine-dependent. Drug-induced synaptic adaptations may dysregulate DA modulation and alter both synaptic and network activity, via pre-synaptic D2 receptors and by regulating FSI activity.

Although in some cortical areas (*e.g.* occipital) alpha has been related to attentional disengagement, in the basal ganglia, alpha arises as a particularly relevant rhythm. Animals in a resting state, exhibit DA tonic single-spike firing at frequencies <10 Hz, and phasically active DA neurons burst at frequencies >10Hz (Schultz, 2007). Thus, at ≈10 Hz DA concentrations switch from *'steady-state'* action to phasic, and midbrain electric stimulation at 10 Hz is sufficient to elicit DA release in the NAc (Wu *et al.*, 2002).

Furthermore, dorsomedial, but not dorsolateral, striatal FSI and MSN exhibit significant entrainment with LFP at ≈10 Hz (Thorn & Graybiel, 2014). Dorsomedial striatum, is functionally closer to the ventral striatum (Voorn *et al.*, 2004). The findings presently described corroborate this view, as electrodes were located dorsomedial NAc core (see section 3.2.1; Figure 3.2).

Alpha in the corticostriatal pathway has also been shown in humans to allow connectivity between the neocortex and the ventral striatum. Interestingly, alpha was found to

predominantly convey unidirectional communication from the cortex to the nucleus accumbens in the alpha-band during an attention switching task (Horschig *et al.*, 2015). Authors suggested that alpha might mediate inhibitory control over behaviorally irrelevant attentional cues; in the context of drug seeking this could narrow the focus exclusively towards drug cues in detriment of natural rewards. Binge cocaine regime in mice induced enhanced cortical EEG activity at low frequencies, including alpha band (Urbano *et al.*, 2009). And stimulation of PFC afferents to the NAc at 13 Hz abolishes drug seeking, by reversing drug-induced maladaptive plasticity (Pascoli *et al.*, 2014; Creed *et al.*, 2015).

Thus, alpha oscillations are well positioned to be behaviorally relevant in corticostriatal reward processing mechanisms.

4.3.2 PFC-NAc network activity is dependent on previous history for the processing of the same drug stimuli

A cocaine challenge, following 100 days of abstinence, differentially alters oscillatory network activity in animals with previous cocaine experience. In the NAc, non-contingent cocaine selectively decreased gamma60 in CSA rats, which lasted up to 60 min following administration. In contrast, alpha was transiently increased only in rats without history of cocaine intake. Interestingly, these effects implicate the same frequency ranges observed when analyzing changes during CSA, as discussed in section 4.3.1.

In naïve animals, systemic administration of the dopamine agonist apomorphine (2 mg/Kg, i.p) causes a shift in gamma activity, with gamma50 suppression giving place to prominent gamma80 events. Amphetamine (2.5 mg/Kg, i.p) that also increases striatal dopamine, has an identical effect on accumbal gamma (Berke, 2009). Here we did not observe gamma60 suppression in saline animals.

A similar experiment was conducted by McCrcken and Grace (2013), in which following a sensitizing regime of cocaine (14 days, i.p.), a cocaine challenge was administered 30 days later (3 mg/Kg, i.v.). Main effects in the NAc occurred in the saline group with decreases in theta/alpha (4-12 Hz), low (30-58 Hz) and high gamma (62-100 Hz). Accumbal theta activity attenuation was also observed in the cocaine group (McCracken & Grace, 2013). Prefrontal low gamma (30-58 Hz) decreased in rats previously exposed to cocaine. Yet, the same experiment also showed decreased theta/alpha (4-12 Hz) and beta (McCracken & Grace, 2013). One caveat of this study, is the fact that recordings were conducted under anesthesia, which can considerably alter neuronal oscillations (Sloan, 1998).

Here similar effects were observed in the PFC as in the NAc, with gamma60 suppression in CSA group and alpha enhancement in saline rats. However, delta band was inversely modulated by non-contingent cocaine in CSA and saline rats. Thirty minutes following injection, delta power increased in rats with history of CSA and decreased in yoked saline rats.

Decreased prefrontal, but not hippocampal gamma (40–70 Hz) was also found in aged animals when compared to young adults (Insel *et al.*, 2012). Recently, a causal association between prefrontal gamma, FSI activity and top-down control was uncovered. Specifically, inhibiting FSI decreases gamma and weakens cognitive flexibility (Cho *et al.*, 2015).

Another report also shows that history of drug intake causes differential effects on prefrontal LFPs (Lapish *et al.*, 2012). Chronic non-contingent amphetamine (23 days, 1 mg/Kg, i.p.) induced locomotor sensitization and caused general increase in power across the whole frequency spectrum. Delta, was selectively increased in sensitized rats, but decreased slightly following amphetamine injection. Changes in delta have been associated with anticipation and drug craving. Cocaine addicts when exposed to drug-associated discrete and contextual cues elicit enhanced delta activity (Reid *et al.*, 2008).

Discrepancies observed might be related to several aspects. First, due to differences in dose (10 *vs* 2.5 mg/Kg), and administration route (s.c. *vs* i.v.). With intravenous having a faster and stronger effect, when compared to subcutaneous, which has a slower onset and can be dependent on fat content. Age might also impact the LFP effects of cocaine challenge. The rats in this study were approximately 7 months old at the time of the challenge. And without any food restriction, they were likely to have a high fat body percentage, which might change absorption rate of drugs administered, specially compared to young adults often used in other studies.

Despite possible inconsistencies, multiple studies report differential effects of drugs in drug experienced animals, relative to naïve. Moreover, rats with previous history of CSA, generally exhibited longer lasting effects of drug challenge. These observations substantiate the claim that even after long term abstinence (100 days) LFP processing is altered in CSA rats, and that these subjects might be more susceptible to drug effects than rats without any drug intake experience.

Therefore, cocaine history seems to affect how subsequent drug experiences are processed. This might have implications in explaining long lasting vulnerability to relapse in addicted individuals, but also why addicts might be more susceptible to develop concomitant addiction to other substances.

4.3.3 Functional connectivity: Coherence and Cross-frequency coupling

We also measured cross-frequency coupling, both during CSA paradigm as well as before and after a cocaine challenge.

Oscillations might be a suitable biological candidate to regulate "*multi-scale integration*", as information processing within the brain requires integration across temporal and spatial scales in a hierarchically manner (Canolty & Knight, 2010). Cross-frequency coupling (CFC) has been put forward as a possible mediating mechanism.

Both delta-gamma and theta-gamma correlations have been previously described in the striatum and they appear to depend on the animals' behavioral state. Delta-gamma coupling predominantly occurs during instrumental behavior (Donnelly *et al.*, 2014), while theta-gamma prevailed during exploration or during maze runs (Tort *et al.*, 2008; Gruber *et al.*, 2009).

Here we did not observe a significant effect of self-administration nor withdrawal in cross-frequency coupling. Yet, there was a trend, more prominent in CSA rats, in which theta-gamma CFC decreased following chronic cocaine. However, this effect might be related to the reduction in gamma power observed at the same time point.

Furthermore, a similar but less obvious effect is observed in saline animals, which suggests that novelty or handling might also contribute to changes in CFC. During CSA animals were being taken daily to the operant chambers, whereas during withdrawal rats would only be handled on days with recording sessions. Moreover, we did not stage the behavior during home cage recording sessions, hence the 30-min used to analyze LFP might contain different behavioral stages, *i.e.* active, quiet and sleep, which may affect results.

Despite the latter considerations, drugs of abuse have been shown to affect both CFC and coherence in the corticostriatal network. Amphetamine suppressed prefrontal theta-gamma CFC in sensitized animals (Lapish *et al.*, 2012). In contrast, morphine increased both delta-gamma and theta-gamma phase-amplitude coupling in the nucleus accumbens of mice (Reakkamnuan *et al.*, 2017). In the striatum, theta-gamma CFC was maximal in decision making epochs, in a T-maze task (Tort *et al.*, 2008).

Theta-gamma CFC suppression has been implicated in cognitive deficits in several neuropsychiatric diseases (Dzirasa *et al.*, 2010). Conversely, CFC is enhanced during cognitive tasks, and it is proposed to support memory formation and retrieval (Jensen & Colgin, 2007; Canolty & Knight, 2010). Moreover, theta-gamma CFC in human NAc was

correlated with cognitive control needed to monitor actions, in a serial reaction time task (Dürschmid, 2013).

Following a cocaine challenge, yoked saline and CSA rats exhibited opposite, but mild, CFC effects. Interestingly, despite decreased gamma power CSA rats show increased theta-gamma CFC. This differential effect might be due to coupling strength differences at baseline (*i.e.* before injection). In fact, it appears that rats with CSA history have reduce coupling compared to yoked saline. If that is in fact the case, cocaine would reverse withdrawal-induced adaptations, thus increasing coupling (Spencer *et al.*, 2017). However, in subjects with no history of drug use, cocaine decreased CFC, which might be associated in the mild decrease of gamma90 power observed after the challenge.

Prefrontal-accumbal coherence, as a measure of inter-area synchrony, was assessed before and after a cocaine challenge. Coherence peaks were observed at delta, alpha/beta and gamma bands (Berke, 2009; Dejean *et al.*, 2013). A decrease in gamma60 was observed exclusively in rats with previous cocaine intake history. This is not surprising, since both in the PFC and NAc there was a decrease in the same frequency range following non-contingent challenge.

Gamma-band synchrony between PFC and NAc has been described in previous reports. Coherence following a cocaine challenge decreased in saline exposed rats at low gamma range, under anesthesia (McCracken & Grace, 2013).

More recently, Catanese and colleagues have demonstrated not only gamma synchrony between these two structures, but its directionality; with prefrontal gamma90 preceding striatal gamma90, while for gamma60 the NAc leads the PFC (Catanese *et al.*, 2016). Thus, altered gamma coherence may reflect impaired connectivity in the corticostriatal network.

In sum, previous history of cocaine intake, not only alters regional accumbal oscillatory activity but seems to impact inter-regional mechanisms of information processing.

4.4 Concluding remarks

Chronic contingent cocaine administration transiently increased alpha and beta and suppressed high gamma frequency bands in a drug free environment. A cocaine challenge following long-term abstinence exerts differential effects in subjects with history of cocaine taking when compared to naïve subjects. Alterations in the oscillatory baseline state of the nucleus accumbens might be an additional maladaptive process contributing to impaired processing and relapse risk.

4.5 Graphical Summary

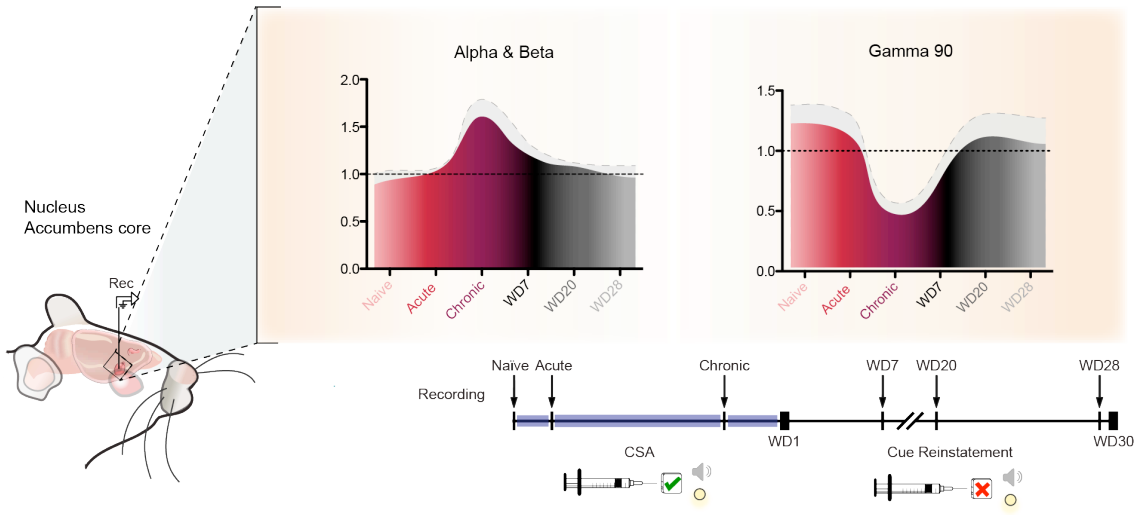

Figure 4.11 Cocaine self-administration transiently augments alpha and beta, and suppresses gamma90 oscillations in the nucleus accumbens. Based on longitudinal *in vivo* spontaneous local field potential recordings, chronic contingent exposure to cocaine increases alpha and decreases gamma90 LFP power in the nucleus accumbens core during task-independent behavior and in the absence of the drug. This concurrent effect was transient, since it normalized during abstinence.

5. Results

Drug seeking modulates LFP processing in the NAc and PFC

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5

Drug seeking modulates LFP processing in the NAc and PFC

Rationale Cue processing and action selection are required to obtain rewards and thus are key in associative learning and survival. Contextual and discrete cues previously paired with addictive drugs can have a subversive effect on behavior, contributing to long lasting risk of relapse (Robinson & Berridge, 1993). Maladaptive processes that linger throughout abstinence in circuits mediating inhibitory control, reward learning and motivation are likely to contribute to such persistent vulnerability (Lüthi & Lüscher, 2014; Pascoli *et al.*, 2014). The prefrontal cortex (PFC) and nucleus accumbens (NAc) are part of the interconnected limbic network and have been implicated in mediating drug cues, selection, initiation and refinement of actions and reward guided learning.

Neuronal oscillations are proposed to be a strong candidate mechanism that allows neuronal coding and information processing across multiple spatial and temporal scales (Canolty & Knight, 2010). Thus, neuronal oscillations represent a fitting tool to study circuit dynamics in a temporal and spatially defined manner. In rodents, oscillatory activity in the cortical-accumbal network has been associated with impulsivity and reward outcome (Donnelly *et al.*, 2014), features known to be implicated in addictive behavior. Interestingly, recordings from deep brain stimulation electrodes in humans, also reveal that accumbal gamma is modulated in a gambling task (Cohen *et al.*, 2009a). Human theta and alpha activity was also reported to facilitate cortical-accumbal communication during an attention visual task (Horschig *et al.*, 2015).

Addictive drugs, such as nicotine, modulate gamma oscillations in the prefrontal cortex during a visual attention task (Bueno-Junior *et al.*, 2017). Morphine withdrawal also differentially modulates gamma bands and correlates with preferred and aversive environments (Dejean *et al.*, 2017).

Yet, there are no studies focusing on oscillatory adaptations associated with relapse-like behavior. Here, we tested whether incubation of drug seeking modulates PFC and NAc processing during extinction and cue-induced reinstatement of drug seeking.

The data analysis described in the following chapter has been performed in collaboration with Dr. Raul C. Muresan, PhD and Harald Bârzan from Coneural at Bucarest University

5.1 METHODS

5.1.1 Animal groups

The experimental group consisted of four cocaine self-administration (SA) rats that underwent 16 days of cocaine self-administration followed by 30 days of abstinence (for details see).

5.1.2 Extinction and Reinstatement

Cue-induced reinstatement of cocaine seeking was tested at withdrawal day (WD) 1 and 30 during a 2 h session in the operant chamber. The first hour consisted of extinction of nose-poking behavior, in which active nose-poking was neither rewarded nor paired with the discrete light/tone cues, and the blue-light was turned off. The second hour comprised cue-induced reinstatement and started with turning on the blue light (session ON) and one non-contingent presentation of the light/tone cues, followed by contingent presentations of the light/tone cues in the absence of cocaine delivery.

5.1.3 Spontaneous *in vivo* field potential recordings during operant behavior

Rats were connected to a miniature headstage (1 g, npi electronic GmbH, Tamm, Germany) and commutator (model SL-12-C, Dragonfly, Ridgeley, West Virginia, USA) mounted in the operant chamber that allowed rats to freely move with the chamber, and thus to normally behave in the presence of drug related stimuli. Nose-poking events were detected both via behavioral software (SK_AA software, Imetronic, France) and via a TTL signal (ITC-16, HEKA Elektronik, Lambrecht, Germany) in order to align the events with local field potentials recorded.

Continuous sLFPs (EEG signals) were filtered at 0.3 to 500 Hz, digitized at 2 kHz. Simultaneously 3-dimension accelerometer signals were also acquired in order to aid sLFP analysis. Noise was filtered by a Hum Bug Noise Eliminator (AutoMate Scientific, Inc., Berkeley, CA). All recordings were referenced to a stainless-steel skull screw implanted above the cerebellum.

5.1.4 Analysis of spontaneous field potential recordings

LFP data was sampled at 1984 samples *per* second and was filtered using two bidirectional Butterworth infinite impulse response (IIR) filters: 1) 3rd order bandpass filter with 0.1 - 300 Hz cutoff frequencies - to get rid of DC and high frequency noise components - and 2) 3rd order bandstop filter with 49 - 51 Hz cutoff frequencies - to extract the 50 Hz AC noise. The Short-Time Fourier Transform (STFT) was used to compute the spectrograms as follows: 500 ms (992 samples) were taken around each event, totaling 1000ms (1984 samples). Across this 1 second frame overlapping 130ms (257 samples) windows were taken with a step size of 10 samples. Each window was multiplied with an equal length Blackman window and zero-padded up to a length of 2048 samples, to increase the frequency resolution. Then the spectrum was computed for each window using FFTW's one-sided real-to-complex Discrete Fourier Transform (DFT) function. The power was computed for each window and laid out in a spectrogram according to their order in the 1 second frame. The spectrograms for each condition are then averaged together in a single, grand average spectrogram.

The grand average spectrogram is normalized by Z-scoring to the period before the event: this means that for each event, a separate spectrogram is computed for the period preceding the event (*i.e.* first 500 ms - 992 samples - of the 1 second frame). The spectrogams were then concatenated and mean and standard deviation was computed across the time-domain of the spectrogram. These were used to Z-score the grand average spectrogram with the following formula: $Sav\_norm(i, j) = (Sav(i, j) - mean(i)) / std(i)$, where i is the index on the frequency axis and j is the index on the time domain axis. The resulting normalized spectrograms were then restricted to a frequency band of 55-110 Hz and 0-30 Hz (Fourier window: 4096).

5.1.5 Statistics

Statistical significance was computed via a paired *t*-test in all frequency bands (Delta 1-4 Hz; Theta 4-8 Hz; Alpha 8-12 Hz; Low beta 13-20 Hz; Gamma 60, 55-70 Hz; Gamma 90, 75-95 Hz), by calculating the average power, as area under the curve, in a period of interest *vs* baseline across all subjects. Baseline period was defined as 200 ms (-400 to -200 ms) or 100 ms (-400 to -300 ms) prior to nose-poke offset. Nose poke and post-nose poke periods were defined as -100 to -100 ms (G90), 0 to 100 ms (G60), 100 to 300 ms (G90), and 0 to 200 ms (low frequencies), and were compared to baseline. Graphs and statistical analysis

were performed in Graphpad Prism 5 and Adobe illustrator. All results are shown as mean ± s.e.m ($p<0.05$).

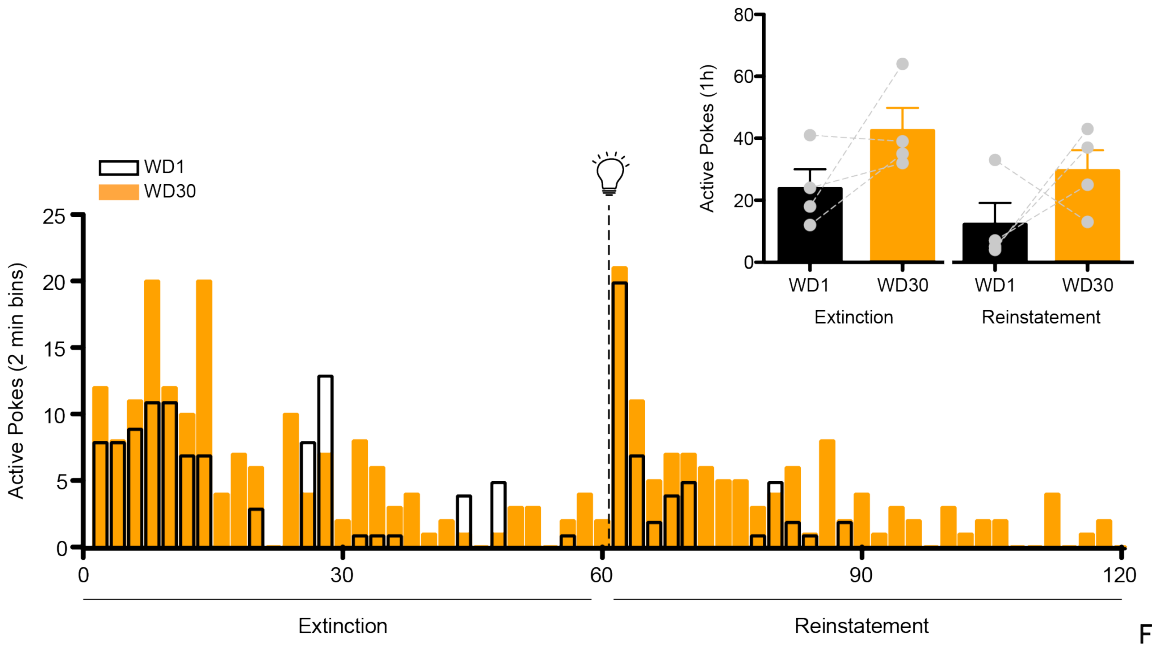
Note: This chapter is especially descriptive, since analytical approaches to examine the data here presented are still being developed and implemented with assistance of Dr. Raul C. Muresan and Harald Bârzan from Coneural, at Bucarest University.

5.2 Results

5.2.1 Biphasic response of accumbal gamma 90 activity at nose poke retrieval

Animals that underwent 16 days of cocaine self-administration in 6 hour daily sessions developed incubation of drug seeking behavior.

The cumulative histogram (Figure 5.1) not only shows that rats on average poked more at WD30 when compared to WD1, but also that they were more persistent and extinction resistant, as shown by the more distributed poking behavior (Figure 5.1). Only three of the four rats here assessed developed incubation of drug seeking (Figure 5.1, inset). However, the rat that did not show an increment in poking over time, poked above average at both WD1, displaying highly motivated behavior during training (not shown) and at early withdrawal (Ext, 77 active pokes *vs* 18 ±3.46; Reinst, 55 *vs* 5.33±0.88).



igure 5.1 Long-access CSA produced incubation of drug seeking. Cumulative histogram of all active nose pokes during extinction and cue-induced reinstatement test at WD1 (black) and WD30 (orange). Light bulb indicates the non-contingent presentation of cue light that marks the start of cue-induced reinstatement. Inset shows active nose pokes mean during one-hour extinction and reinstatement of drug seeking behavior.

Spontaneous LFP were recorded when animals were behaving in the operant chambers, during extinction and reinstatement sessions. LFP power in both the gamma60 and gamma90 frequency bands was influenced by nose poke retrieval (Figure 5.2).

Gamma60 power increased transiently closely after the end of poke both in the NAc and PFC. This increase was more prominent at WD1 when compared to WD30 for all conditions tested (Figure 5.2B). Significant effects were observed during reinstatement at WD1, when compared to before the end of poke ($t_{(3)} = 5.06$, $p < 0.05$). In the PFC, there is an noticeable increase during extinction at WD1 ($t_{(3)} = 2.78$, $p = 0.07$).

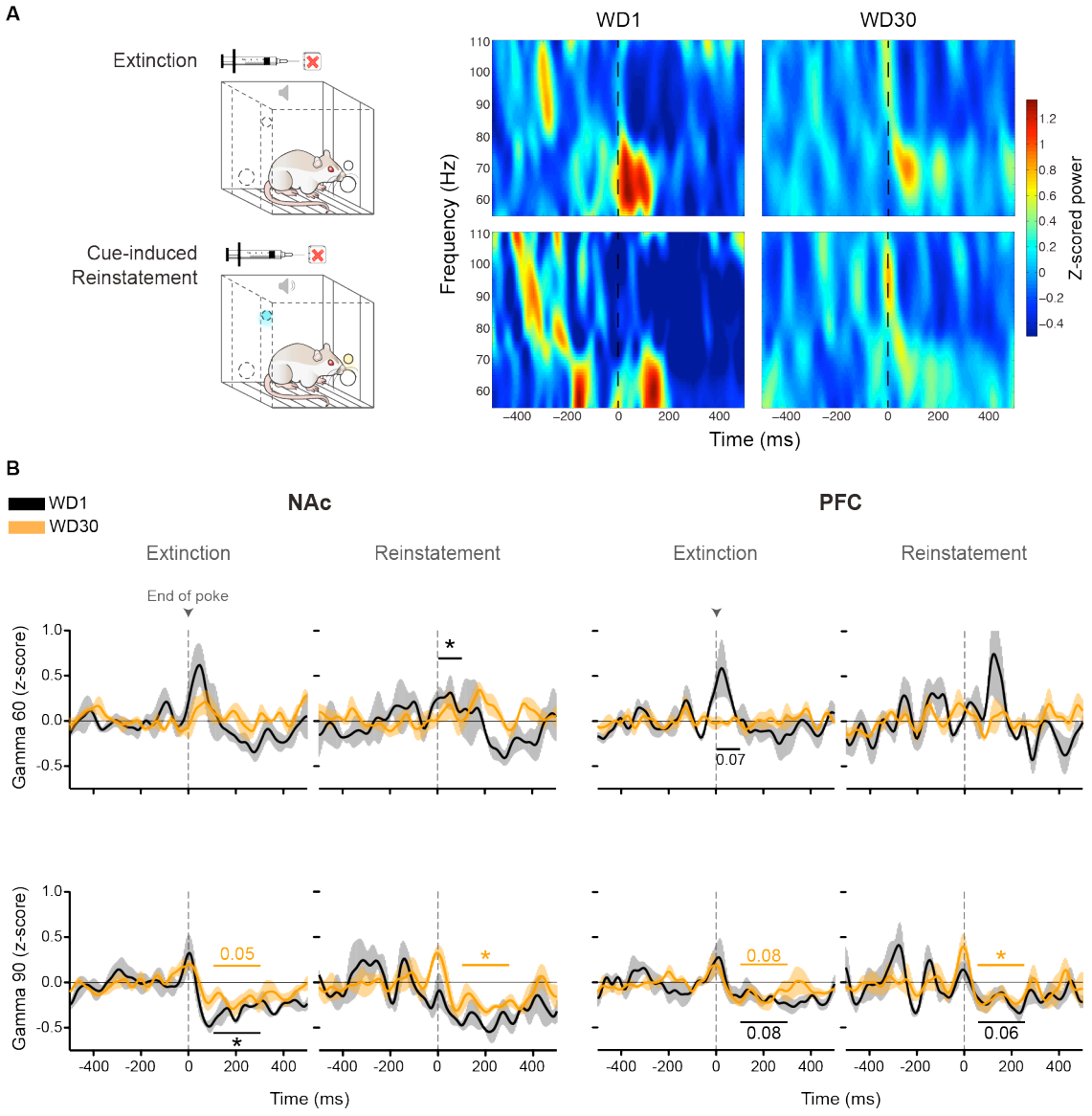


Figure 5.2 Gamma 60 and gamma 90 LFP power at the end of poking. A. Illustrative spectograms of z-score LFP power recorded in the NAc core. It depicts baselined z-power from 500 ms before and after the end of nose poke event, between 50-110 Hz during extinction and reinstatement. B. Z-scored LFP power for distinct gamma bands: gamma60 (55-70 Hz) and gamma90 (75-95 Hz). Solid lines represent the mean power of all pokes and shading the standard error of the mean (SEM). Black line refers to withdrawal day (WD) 1, while orange refers to WD30.

Gamma90 oscillations displayed a biphasic response to end of nose poke, with a fast event-locked increase followed by a more persistent decrease in power, both in extinction and reinstatement (Figure 5.2B, lower panel). The sharp gamma90 increase was observed both in the NAc and PFC and at WD1 and WD30, while the slower but prominent decrease was only present in the NAc. Exceptionally, gamma90 power in the NAc during reinstatement was higher at late withdrawal (WD30) when compared to WD1. Additionally, gamma 90 activity was significantly depressed 100-300 ms after nose poke retrieval during extinction (WD1, $t_{(3)} = 8.85$, $p < 0.05$; WD30, $t_{(3)} = 3.07$, $p = 0.05$). During reinstatement suppression of gamma 90 was only significant at WD30 in the nucleus accumbens (WD1, $t_{(3)} = 1.25$, $p = 0.30$; WD30, $t_{(3)} = 4.90$, $p < 0.05$). The same trend occurred in the PFC, with gamma 90 decreasing 100 ms after nose poke, in all conditions (WD1, $t_{(3)} = 2.63$, $p = 0.08$; $t_{(3)} = 3.05$, $p = 0.06$; WD30, $t_{(3)} = 2.54$, $p = 0.09$; $t_{(3)} = 4.89$, $p < 0.05$).

Gamma60 and gamma90, rather than co-occur seem to be mutually exclusive, with gamma90 LFP power changes preceding gamma60 changes. Modulation of both gamma bands was more evident when aligned to the offset rather than the onset of nose poking (data not shown). Methodologically, this approach might be more suitable due to the fact that nose poke duration was variable and cannot be controlled for.

Conceptually, it is also interesting to address this period since it might be related to reward expectancy, as during training cocaine injection was delivered following nose retrieval from the hole.

Gamma60 showed discrete increase at end of nose poke, while gamma 90 was inhibited following end of poke. Changes were generally more prominent at early withdrawal.

5.2.2 Delta and theta activity increases following nose-poke retrieval

Modulation of low frequency bands at the end of nose poking was also assessed in the NAc and PFC during extinction and reinstatement of drug seeking (Figure 5.3).

Accumbal delta activity significantly increased ≈200 ms post-poke, especially during extinction and reinstatement and at early withdrawal (WD1, $t_{(3)} = 4.14$, $p < 0.05$; $t_{(3)} = 1.87$, $p = 0.16$). At WD30, a smaller but long-lasting increase in delta LFP power was observed, but only during reinstatement, although not significant (Figure 5.3B).

Similarly, in the PFC delta power increased immediately following nose poke offset, both during extinction and reinstatement at WD1. However, this increase was no longer observed following 30 days of abstinence (WD30).

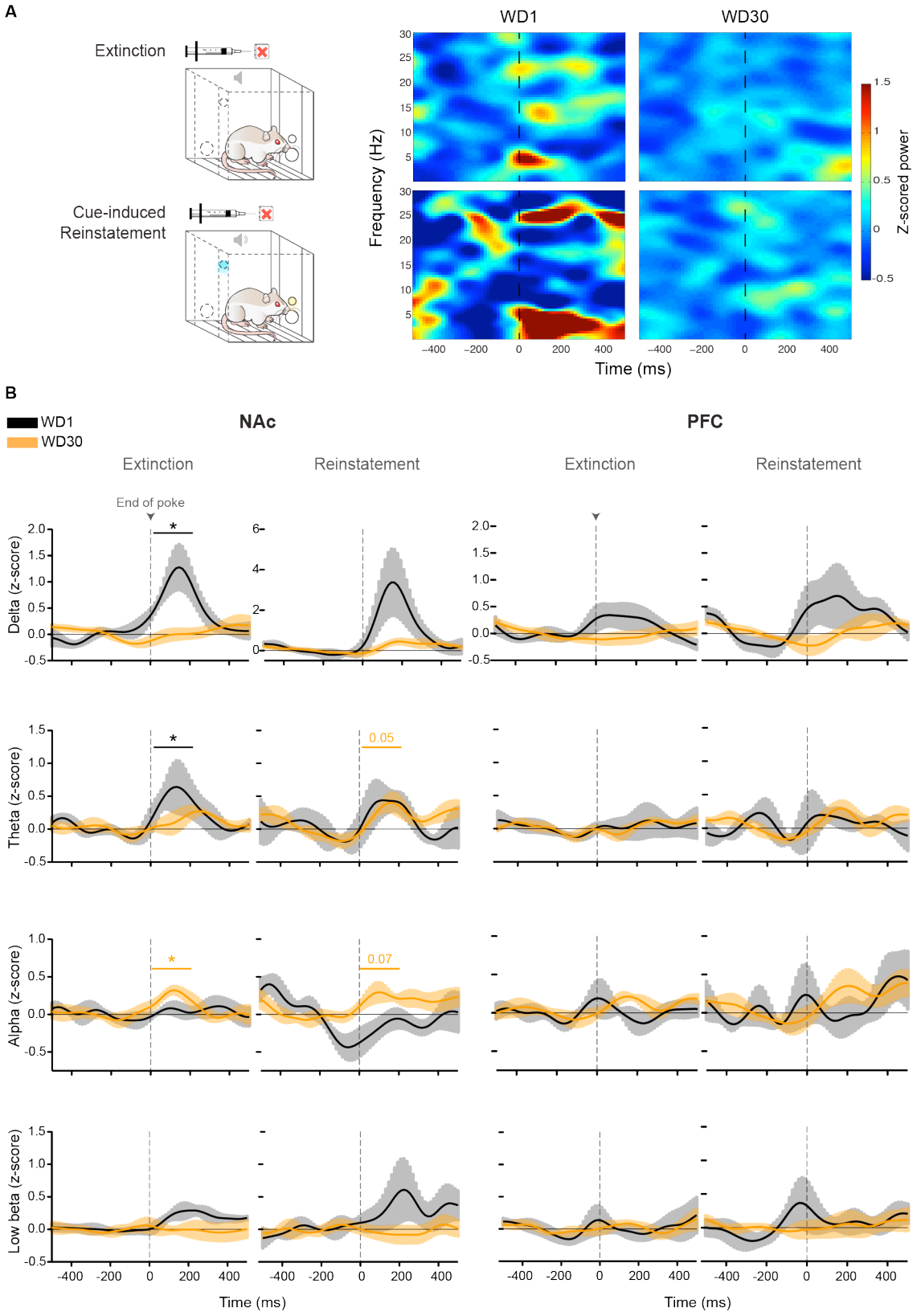


Figure 5.3 Low frequencies LFP power at the end of poking. A. Illustrative spectrograms of z-score LFP power recorded in the NAc core. It depicts baselined z-power from 500 ms before and after the end of nose poke event, between 1-30 Hz during extinction and reinstatement. B. Z-scored LFP power for distinct frequency

bands: delta (1-4 Hz), theta (4-8 Hz), alpha (8-12 Hz) and beta (13-30 Hz). Solid lines represent the mean of all pokes and shading the standard error of the mean (SEM). Black line refers to withdrawal day (WD) 1, while orange refers to WD30.

Accumbal theta power also increased both at WD1 and at WD30 during extinction and reinstatement. With more prominent effects occurring during WD1 extinction and reinstatement a WD30 ($t_{(3)} = 3.56$, $p < 0.05$; $t_{(3)} = 3.13$, $p = 0.05$). Contrarily, no modulation of theta power was detected in the PFC for all conditions studied.

Alpha power, in the NAc, increased following nose poke offset at WD30, but not at WD1, both during extinction and reinstatement ($t_{(3)} = 4.74$, $p < 0.05$; $t_{(3)} = 2.68$, $p = 0.07$). This increase at WD30 was transient during extinction and persistent during reinstatement. PFC alpha LFP power increased transiently at nose poke offset at WD1 and was delayed at WD30 by about 200 ms. Cocaine seeking mildly modulated beta activity in the NAc, exclusively at WD1. However, no significant effects were detected.

5.2.3 Accumbal alpha-modulated gamma points towards gating mechanism

A persistent observation when analyzing spontaneous local field potentials in the nucleus accumbens during operant behavior was the presence of alpha modulated gamma oscillations (Figure 5.4). This consisted of peri-event (poke) gamma 90 oscillations that emerge with a frequency of ≈8.3 Hz, *i.e.* alpha range. Although to different degrees, this phenomenon was detected in all rats analyzed (n=4). Therefore, it seems to be an intrinsic feature of accumbal oscillations during operant behavior.

Alpha-modulated gamma seemed more consistently expressed in NAc, relatively to the PFC (data not shown), and it is aligned to poke offset. That is, when the rat processes the information in relation to the nose withdrawal event, which is likely related to reward expectancy.

As observed in the individual example showed (Figure 5.4), alpha modulated gamma was not constant, but it was rather modulated and modified under distinct behavioral conditions. Specifically, it seemed to heighten with extinction learning, in both extinction and reinstatement (under extinction conditions) sessions; as can be observed by stronger power later in the session (Figure 5.4).

Qualitatively, it also seemed that this pattern could be disturbed by unexpected outcome (reward prediction error), as seen by the first session segment of extinction, which was recapitulated in the first part of reinstatement. That is, both in extinction and reinstatement,

alpha-modulated gamma was more reliably expressed towards the end of the period, as if this rhythmic structure were related to extinction learning and thus the realization that the reward is no longer anticipated.

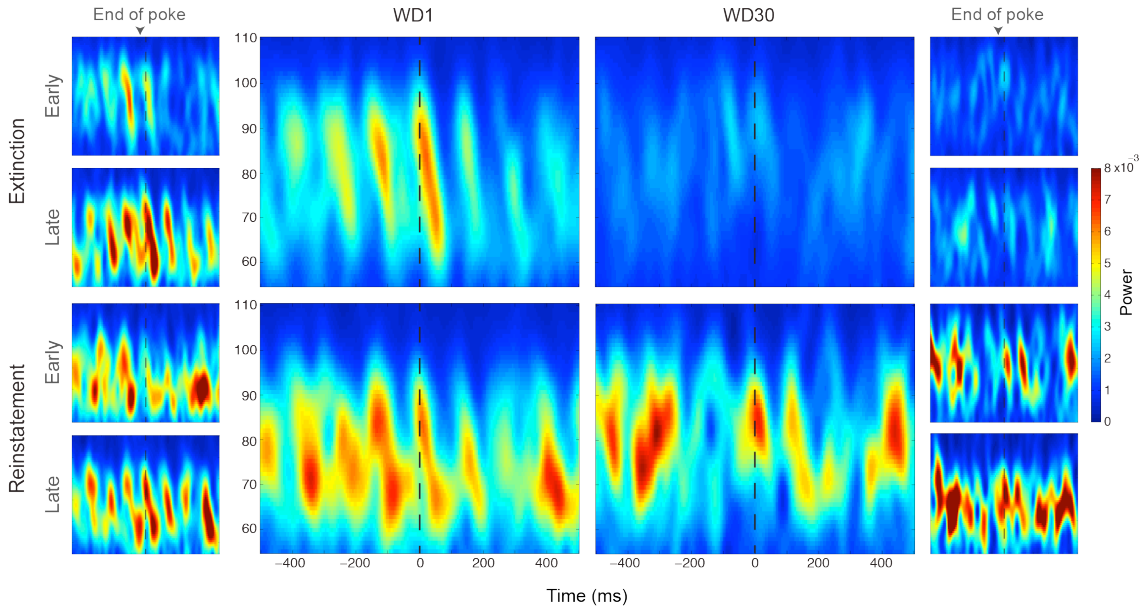


Figure 5.4 Alpha modulated gamma in the NAc. Alpha-modulated gamma in the NAc. Individual example (COC2) of event locked gamma rhythm in the nucleus accumbens. In the outer panels mean z-scored power for early and late poking events is shown, referring to the first third and the last third of total active pokes within each session, respectively. Y axes show high frequency range (50-110 Hz). Traces were aligned to end of poke, when the rat redraws the nose from the bout, as indicated by the arrows. In the inner panels, mean z-scored power for all poking events was averaged.

Due to the continuous and variable nature of the data, it has been so far difficult to develop a way to quantify the phenomenon as well as changes during different conditions. Thus, further quantitative analysis is necessary in order to pinpoint underlying mechanisms.

The observation of alpha-modulated gamma might, however, reflect a NAc gating mechanism in order to regulate multiple inputs (Gruber *et al.*, 2009).

5.2.4 Reduced functional connectivity between PFC and NAc during extinction of drug seeking

Coherence, as a measure of relatedness between two LFP signals, was used to assess functional connectivity between two interconnected brain areas, the NAc and PFC.

Similarly to power analysis, coherence was also aligned to nose poke retrieval and determined for distinct frequency bands.

Gamma90 coherence decreased after the end of the nose-poking event (Figure 5.5B), particularly during WD1 extinction ($t_{(3)} = 3.95$, $p < 0.05$), and also during reinstatement, but nly significantly at WD30 ($t_{(3)} = 3.29$, $p < 0.05$).

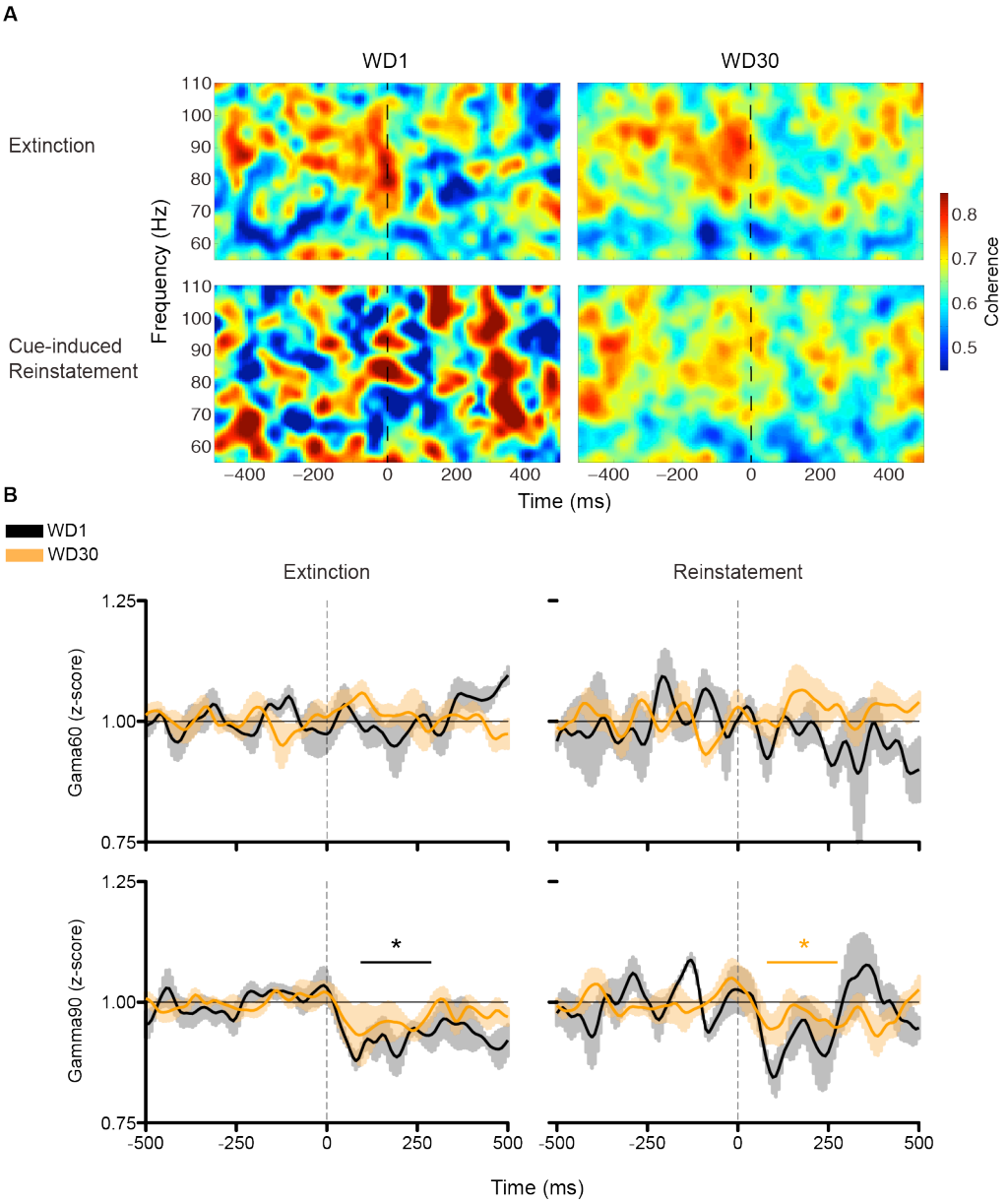


Figure 5.5 Gamma coherence between PFC and NAc during cocaine seeking. A. Representative spectrogram of PFC-NAc coherence from 500 ms before and after the end of nose poke event, between 5-110 Hz, during extinction and cue-induced reinstatement (not normalized). B. Normalized coherence z-scored LFP power for distinct gamma bands: gamma60 (55-70 Hz) and gamma90 (75-95 Hz). Solid lines represent the mean of all pokes and shading the standard error of the mean (SEM). Black line refers to withdrawal day (WD) 1, while orange refers to WD30.

Conversely, no changes are observed in gamma60 range (Figure 5.5B). During cue-induced reinstatement a similar, but less clear, trend was detected; with coherence decreasing exclusively in the gamma 90 band frequency (Figure 5.5B, lower panel).

Development of incubation of drug seeking did not alter coherence between PFC and NAc, since similar changes were observed at WD1 and WD30. However, modulation seemed to be stronger at early withdrawal.

Low frequency coherence was equally assessed (Figure 5.6). Here, the main effects took place at WD1. Increased delta LFP coherence is event-locked to the nose-poke offset during extinction, and was observed exclusively at WD1 ($t_{(3)} = 4.53$, $p < 0.05$; Figure 5.6B). Delta coherence also seemed increased during reinstatement at WD1, although in a delayed fashion, when compared to extinction effect.

Similarly, theta also showed event-related increase at WD1 extinction ($t_{(3)} = 6.65$, $p < 0.05$), and to a lesser extent during reinstatement (Figure 5.6B). No clear modulation is observed at WD1.

Lastly, alpha PFC-NAc coherence was not modulated by extinction of drug seeking behavior (Figure 5.6B, middle panel). Yet, reinstatement at late withdrawal (WD30) appeared to trigger slow increase in alpha coherence after nose retrieval, which may be related to incubation of drug seeking.

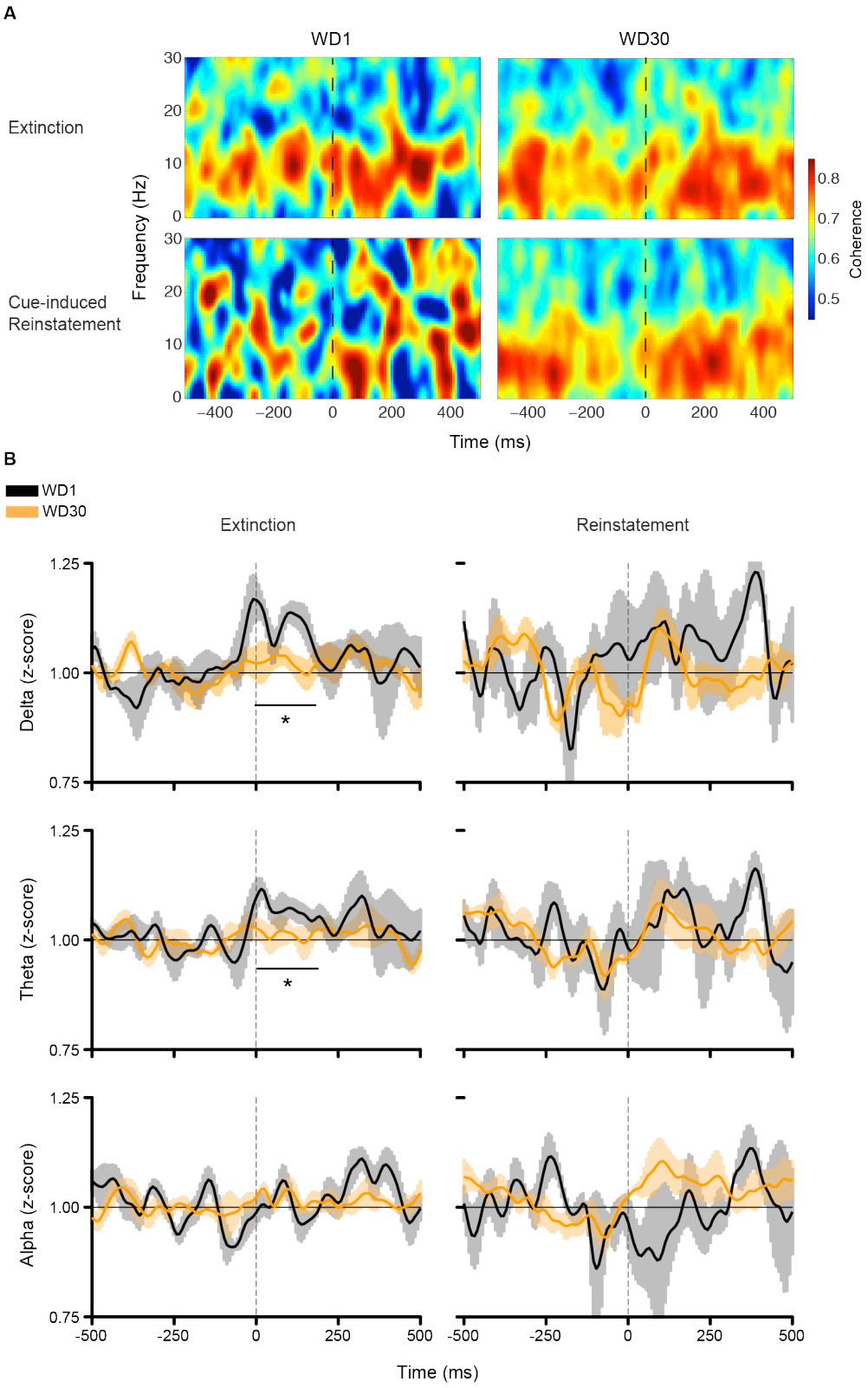


Figure 5.6 Low frequency coherence between PFC and NAc during extinction and reinstatement. A. Illustrative spectrogram of PFC-NAc coherence from 500 ms before and after the end of nose poke event, between 1-30 Hz during extinction and cue-induced reinstatement (not normalized). B. Normalized coherence z-scored LFP power for distinct frequency bands: delta (1-4 Hz), theta (4-8 Hz), alpha (8-12 Hz). Solid lines represent the mean of all pokes and shading the standard error of the mean (SEM). Black line refers to withdrawal day (WD) 1, while orange refers to WD30.

5.3 Discussion

Environmental cues previously associated with drugs strongly shape behavior, contributing to long-lasting risk of relapse after halting drug abuse.

Two-channel recordings from awake, freely moving rats during relapse-like behavior revealed that different LFP frequency components are differentially modulated at the offset of nose-poke events. Moreover, specific LFP activity was differentially altered at early and late withdrawal in rats that exhibit incubation of drug seeking.

5.3.1 Task-related modulation of corticostriatal gamma

We show that both gamma60 and gamma90 are modulated during relapse-like behavior, thus displaying a functional role in reward-related information processing. Namely, sharp increase of gamma60 and gamma90 multiphasic relationship to nose-poke retrieval in the accumbens, with similar, but weaker effects in the PFC. To avoid confusion regarding gamma sub-band denominations of different authors, from here on forward I will refer to bands with comparable range to gamma60 and gamma90 as low and high gamma, respectively.

Previous *in vivo* electrophysiological studies report modulation of the two gamma sub-bands analyzed, namely with respect to reward-related behavior in the ventral striatum.

Low gamma increased sharply at the site of reward, in a T-maze task, whereas high gamma presented a complex pattern, with band power gradually increasing and abruptly decreasing at the time of reward receipt (van der Meer & Redish, 2009).

In a triangular track, low gamma also increased at reward delivery, while high gamma was higher during approach than after arrival at reward sites (Kalenscher *et al.*, 2010). This is in line with the high gamma suppression seen during extinction and reinstatement, as trails were normalized to the time before nose-poke ended. In addition, high gamma decreased when compared to the period of approaching/poking the hole. Conversely, Berke (2009) found that low gamma decreased, while high gamma transiently increased at reward sites.

However, the latter reports focus on maze paradigms, multiple-T, triangular and radial mazes respectively, in which reward was highly associated with immobility.

During operant behavior, which more closely relates to cue-induced reinstatement, modulation of low and high gamma is also observed. Malhotra and colleagues, with very similar recording sites, report an overall increase in gamma power during nose poke, with high gamma preceding low gamma (Malhotra *et al.*, 2015). This sequential pattern has been

observed in several studies (van der Meer & Redish, 2009; Howe *et al.*, 2011) Similarly, here we also report enhancement of high gamma power aligned to nose-poke offset, while low gamma follows and increases about 100 ms after nose-poke end (see Figure 5.2).

Malhotra *et al.* detected no correlation of accumbal gamma with outcome value (size of the reward) or reward sites *per se*. Instead, since similar gamma was recorded during nose-pokes and off-task (no reward) epochs, they suggested gamma activity might be reflective of a brain state, i.e. "*default mode network*" that can only be mildly modified by reward and motivation (Malhotra *et al.*, 2015). Although gamma events in-between trials were variable and stochastic, higher order features, such as cross-frequency coupling, were rather stable (discussed in section 5.2.2).

Another study (Donnelly *et al.*, 2014), with simultaneous recordings from NAc core and shell, prelimbic and infralimbic PFC, reported low gamma modulation during 5-choice serial reaction time task (5-CSRTT), which is widely used to assess impulsive behavior, *i.e.* inhibitory control. Specifically, low gamma (55-60 Hz) in the NAc core and Pr-PFC increased 400 ms after nose-poke. Post-poke low gamma activity supports the timing reported in the present chapter, since Donnelly and colleagues aligned power to onset, thus 0.4s after onset will likely be adjacent to nose retrieval (Figure 5.2).

Interestingly, PFC and NAc low gamma increased in both correct responses as well as following errors (*i.e.* premature or incorrect responses), which also supports the gamma 60 post-poke increase observed during extinction and reinstatement in which the rats' expectations are not met since nose-poking no longer predicts cocaine infusion, thus signaling an "error" as compared to the previously learned association.

Human clinical studies reliably display task-dependent modulation of accumbal gamma. Cohen and colleagues report enhanced gamma (20-80 Hz) following all trials in a probabilistic reversal learning task. Negative feedback trails (*i.e.* expectation of rule did not conform to response) exhibited the higher enhancement (Cohen *et al.*, 2009a, 2009b).

Here, low gamma activity enhancement seemed more prominent at WD1 relative to WD30, although not significantly. This might be due to different number of nose-pokes analyzed in the two conditions, and possible effects of learning on gamma modulation.

Fittingly, some authors report adaptation of oscillatory activity with learning. Specifically, increased gamma was shown to decrease later in the task, when compared to initial (learning) trials (van der Meer & Redish, 2009). Mechanistically similar, Cohen *et al.* also observed that event-related potentials, as an overall measure of NAc activity, were higher at the last 10 trials when compared to 10 initial trials of reward learning task in humans (Cohen

et al., 2009c). Additionally, in the dorsomedial striatum, learning was associated with increased gamma power that decreased once behavior became habitual (Howe *et al.*, 2011).

In contrast to low gamma, high gamma did not obviously differ between WD1 and WD30, which associated with extinction resistance and enhanced drug seeking due to incubation at WD30, might relate to inflexible behavior characteristic of addicts. This feature supports corticostriatal gamma as regulating information coding for dynamically updating behaviorally-relevant strategies, necessary to adapt to new contingencies.

There are a few confounders regarding the current results, as already mentioned, the number of events differs at WD1 and WD30. Thus, in the case of a progressive adaptation of the oscillatory modulation throughout the session, similar number of events should be compared from early and late in the reinstatement session. Since, more prominent effects on WD1 might be the result of fewer events; as such, early nose-pokes at WD30 perhaps show a similar effect magnitude, which evolves with extinction learning as seems to happen with alpha modulated gamma.

Furthermore, analysis of movement needs to be conducted, in order to assess how it correlates with gamma activity. Previous studies have found correlations between accumbal gamma power and movement initiation (van der Meer & Redish, 2009; Kalenscher *et al.*, 2010). These studies, however, involved long running periods in mazes. In studies using operant chambers and instrumental behavior paradigms, correlations between movement initiation and gamma activity were not so convincing, as gamma power changes were better explained by task-related events (Cohen *et al.*, 2009a; Donnelly *et al.*, 2014; Malhotra *et al.*, 2015; Catanese *et al.*, 2016). Thus, during cue-induced reinstatement it is likely that gamma is primarily mediating task-relevant behavior rather than motion initiation.

Finally, analysis of absolute power, not baselined to the time before nose-poke offset, but rather using off-task epochs, should be conducted in order to prevent normalization artifacts.

5.3.2 Alpha modulated gamma and information processing in the NAc

Gamma activity by itself did not correlate with outcome value or losses vs rewards both in animals and humans (Cohen *et al.*, 2009b; Malhotra *et al.*, 2015). Yet, a more stable and behaviorally predictive higher-order LFP feature seems to be cross-frequency coupling, particularly alpha-coupled gamma.

In patients implanted with DBS electrodes, gamma power increased significantly during alpha peaks in all trials. Only when authors accounted for alpha-phase, then it became predictive of trial outcome, allowing to distinguish between loses and rewards. This phenomenon was consistently observed in all patients (Cohen *et al.*, 2009b). In rats, alpha-gamma coupling could also discriminate between one-pellet and 5-pellet conditions (Malhotra *et al.*, 2015).

One confusing aspect when searching literature, is band nomenclature and differences between human and animal reports. A lot of animal studies report theta-gamma coupling, which usually included alpha frequency range (8-12 Hz). Still, Horschig and colleagues found distinct modulation of theta-gamma and alpha-gamma in the human nucleus accumbens. More interestingly, they found directionality associated with each frequency. With theta-gamma occurring during anticipation and processing of a visual stimulus, and mediating NAc to PFC connectivity, while alpha in the PFC predicted activity in the NAc (Horschig *et al.*, 2015).

Other studies also observe delta-gamma coupling, namely during instrumental behavior (Gruber *et al.*, 2009; Donnelly *et al.*, 2014). In humans, PFC-NAc coupling at delta was detected both at rest and during action selection in a decision-making task (Stenner *et al.*, 2015). In an interval-timing task in rodents, delta activity was increased in the dorsomedial striatum of all trials, independent of reward (Emmons *et al.*, 2016). Prefrontal delta is also associated with expectation (Stefanics *et al.*, 2010) and is reliably triggered by cues (Narayanan *et al.*, 2013).

We observed delta increases, both in the PFC and NAc, following nose-poke, during extinction and cue-induced reinstatement. Delta activity was higher at WD1 than WD30, which might reflect changes in expectation of rewarding, since reinstatement was performed under extinction conditions.

Nesting of gamma in lower frequencies are seen in hippocampal and cortical areas and are suggested to be a property of information coding, as to aid timing and afferent connectivity. CFC has been implicated in several cognitive processes, such as memory, attention and perception (Canolty & Knight, 2010; Fell & Axmacher, 2011).

A similar mechanism might be at play in the corticostriatal network. The nucleus accumbens is known to be an interface that allows integration from different cortical and limbic inputs and conveys information to output nuclei, hence functioning as a "functional gatekeeper". Cross-frequency coupling might support gating, through which the NAc dynamically selects task-relevant – salient – and input-dependent information to be

conveyed to the basal ganglia. The presence of multiple low frequency bands might hint to a hierarchical organization of LFP frequencies over faster bands, as seen in the globus pallidus, which is modulated by dopamine (Dejean *et al.*, 2011) .

Therefore, analyzing CFC during the extinction and reinstatement task will possibly yield more clarifying results, regarding how connectivity with the PFC is modulated both during the task stages as well as during early and late withdrawal.

5.3.3 NAc oscillations: Where do they come from?

Our LFP analysis has some limitations, specifically concerning the synaptic mechanisms underlying LFP generation in non-layered areas such as the NAc, since we did not record unit activity nor nearby cortical areas (*e.g.* piriform cortex).

A recent report builds a compelling case for accumbal LFP to originate in the piriform cortex. Ipsilateral, but not contralateral, naris occlusion, which is a manipulation known to block gamma activity in the piriform cortex, strongly reduced gamma power in the nucleus accumbens. This effect was present for both gamma bands, low gamma and high gamma (Carmichael *et al.*, 2017). Thus, it seems, that volume conduction from the piriform cortex seems to account for NAc gamma oscillations. However, this observation does not undermine our findings, nor does it entail that accumbal LFP do not have functional role or are an epiphenomenon. Distinct neuronal populations, within the NAc, cohere with low and gamma, and accumbal neurons can also intrinsically resonate in the gamma frequency (Taverna *et al.*, 2007; Berke, 2009). Neuronal spiking phase-locked to gamma is also synchronized with afferent and efferent areas, as the PFC, amygdala, hippocampus and globus pallidus (Dejean *et al.*, 2011, 2013). Lastly, the human piriform cortex is not as closely located to the accumbens as in rodents, and recordings in patients use local referencing, as such it is unlikely that volume conduction would account for strong gamma activity in the human accumbens.

Although these findings anatomically restrain our interpretation, accumbal oscillations can still assist as "markers of temporal organization at a meso-scale neural activity" and across brain areas (Catanese *et al.*, 2016).

5.4 Concluding remarks

Corticostriatal recordings during extinction and cue-induced reinstatement revealed that distinct LFP frequencies are modulated at the offset of nose-poke events. Specifically, gamma showed a biphasic response while delta and theta increased. Stable alpha-modulated gamma, as an accumbal oscillatory feature, seems to be modulated by extinction learning.

Changes in coherence can suggest alterations in PFC-NAc functional connectivity that may affect processing of drug-associated cues, hence contributing to cue reactivity observed in incubation of drug seeking.

5.5 Graphical Summary

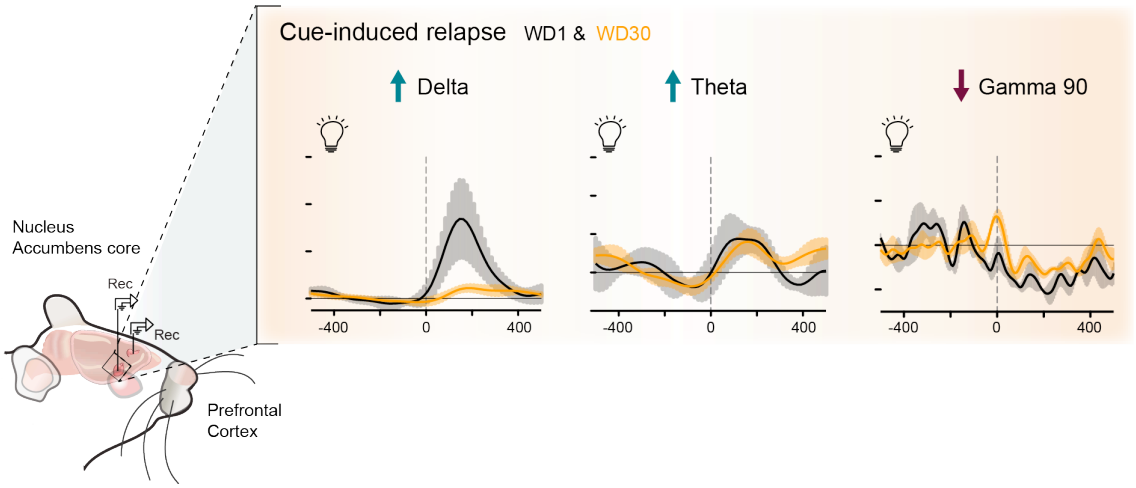


Figure 5.7 Cue-induced drug seeking differentially modulates low and high frequency activity in the corticostriatal pathway. During relapse-like behavior low frequencies, specifically delta and theta tend to increase following nose-poke retrieval, while gamma 90 exhibits a biphasic response, with strong suppression after nose-poke.

6. Conclusions

6 Conclusions

The present dissertation work was set out to identify both synaptic and network neuroadaptations driven by chronic cocaine use, withdrawal and relapse in the prefrontal-accumbal circuit. As such:

i) *In vivo* recordings in freely behaving rats proved to be a suitable tool and successful approach to monitor in a within subject fashion, evoked and spontaneous local field potentials (LFP). By recording distinct 'readouts' of neurophysiological activity, the present study shed light on corticostriatal synaptic adaptations and concomitant network alterations during different stages of addiction-relevant behavior, such as chronic cocaine exposure, abstinence and relapse like behavior (Figure 6.1).

ii) Specifically, chronic cocaine self-administration potentiates the prefrontal (PFC) to nucleus accumbens core (NAc) pathway, involving augmented pre-synaptic glutamate release that persisted throughout withdrawal.

iii) Network activity in the nucleus accumbens following chronic self-administrating of cocaine was characterized by high alpha and beta, as well as suppression of high gamma oscillations. Moreover, non-contingent cocaine challenge differentially modulated corticostriatal oscillatory dynamics in subjects with history of cocaine intake.

iv) Lastly, several frequency bands were modulated during cue-induced reinstatement, particularly locked to end of conditioned response. Incubation of drug seeking modified mainly low frequencies, namely delta, which showed distinct activity at early and late withdrawal.

The present incubation paradigm does not allow to distinguish between drug-driven plasticity and adaptations necessary for the development of compulsive behavior and "full blown" addiction. Transition to addiction only develops with very long self-administration paradigms (>45 days; Deroche-Gamonet, 2004; Chen *et al.*, 2013). Still, the incubation paradigm allowed to uncover neuronal mechanisms underpinning chronic exposure and relapse-related behavior, and even individual differences could be seen that correlated to the degree of neuroadaptations observed (Luís *et al.*, 2017).

Additionally, evoked activity was electrically induced, as such specificity of neuronal population could not be ensured, as it would be with an optogenetical approach. Yet, within subject design ensures that modifications are due to experimental conditions, since electrodes do not change locations throughout the experiment.

Despite experimental limitations, the main findings reported align with the current state of the art. Glutamatergic signaling, namely within corticostriatal circuit, is a well-studied substrate of drug-driven adaptations, specially regarding chronic drug effects (Scofield *et al.*, 2016a). Here for the first time, dynamic changes, assessed both at the synaptic and circuit level, in the excitatory PFC to NAc pathway were monitored throughout different stages of addictive behavior.

An overarching plausible explanation involves dopaminergic regulation of glutamatergic transmission at the PFC to NAc pathway, which seems to account for both levels of evidence, synaptic and oscillatory drug-driven neuroadaptations.

As high alpha and beta in the basal ganglia co-occurs in conditions of dopaminergic depletion (e.g. Parkinson's disease) it is reasonable to conceive that the same observation in a different brain pathology, *i.e.* addiction, can stem from similar underlying causes. Thus, if enhanced alpha and beta activity are reflective of reduced dopaminergic signaling, D2-mediated presynaptic inhibition of glutamate release would be impaired. In this scenario, PFC disinhibition would lead to increased presynaptic release, as corroborated by decreased paired-pulse ratio, following chronic cocaine intake. Concomitantly, reduced dopamine tonus can also affect the nucleus accumbens, particularly by suppressing fast-spiking interneurons (FSI) activity, which is supported by the observation of decreased gamma power at the same time point. FSI inhibition, in turn, would release accumbal medium spiny neurons (MSN) from feedforward inhibition, resulting in enhanced excitability, which is confirmed by postsynaptic potentiation measured, *i.e* LTP-like state (see section 4.3.1; Figure 4.10) Involvement of indirect pathway is further supported, since rescuing D2-mediated transmission confers resilience to compulsive cocaine use (Bock *et al.*, 2013).

Hypofunction of prefrontal cortex is an hallmark of addiction, observed both in rodents and patients (Kalivas & Volkow, 2005; Chen *et al.*, 2013). Although at the first contradictory it ties in with the current observations of increased synaptic glutamatergic transmission (Luís *et al.*, 2017). First, prefrontal hypofunction concerns activity measured at rest in patients and spiking activity in rodents. Patients, however, display weighted reactivity to drug-associated cues (*i.e.* fMRI; BOLD activity; Vollstädt-Klein *et al.*, 2012), which can be predictive of relapse (Grüsser *et al.*, 2004). Accordingly, reinstatement in animals elicits enhanced

glutamate release in the prefrontal-accumbal pathway (McFarland *et al.*, 2003; Stefanik *et al.*, 2013). Thus, evoked activity, specially triggered by drug relevant stimuli, is enhanced in a background of reduced extrasynaptic glutamate (Baker *et al.*, 2003; see Figure 1.5, p21). This setting can have devastating consequences by biasing and narrowing processing of salient stimuli towards drug-associated cues in detriment of natural rewards. This is likely to contribute to impaired decision-making, cue generalization and relapse risk – Figure 6.1.

Indeed, targeting prefrontal glutamate signaling, by pharmacologically restoring glutamate levels with N-acetylcysteine or by optogenetical activation, abolishes reinstatement and prevents compulsive seeking, respectively (Chen *et al.*, 2013; Reissner *et al.*, 2015). Both showed translational validity, and have been tested in humans with promising results. Mechanistic action of N-acetylcysteine on glutamate homeostasis has been confirmed and beneficial in (some) patients (Schmaal *et al.*, 2012); and optogentic approach inspired a transcranial magnetic stimulation pilot study targeting the dlPFC of cocaine patients (Terraneo *et al.*, 2016).

Therefore, identification of addiction-relevant neurophysiological mechanisms can directly impact the development of targeted approaches to treatment.

Oscillatory activity during relapse like behavior might also be a marker for impaired processing. Failing to correctly process stimuli and updating behavioral strategies can lead to behavioral inflexibility and further difficulty to avoiding resumption of drug seeking – Figure 6.1. In fact, addicts do display anomalous EEG activity compared to healthy controls, such as high beta and alpha in cocaine abusers (Costa & Bauer, 1997). Reversing EEG abnormal patterns via neurofeedback protocols is also being perused as a therapeutic strategy in substance use disorders (Sokhadze *et al.*, 2008).

Alike, relapsing, as a re-exposure to the drug (*i.e.* cocaine challenge) may reverse withdrawal-driven adaptations and as such subside the craving and high motivation to obtain the drug, observed during abstinence (Spencer *et al.*, 2017), which could account for differential effects of stimulants in patients when compared to healthy controls even after long periods of abstinence. Atypic oscillatory state in functionally connected areas, such as the PFC and NAc, indicates a dysregulation of the circuit dynamics homeostasis. With the emergence of new, persistent and anaplastic setpoint – *allostasis* – both 'driven by' and 'driving' drug use and the spiraling cycle of addiction (Koob & Le Moal, 2001; Dejean *et al.*, 2013).

In sum, both synaptic and network adaptations were *'linked'* to distinct addiction-related behaviors in awake rats, contributing to better understanding of etiological and pathological mechanisms of neuropsychiatric illness of drug *wanting* – Addiction.

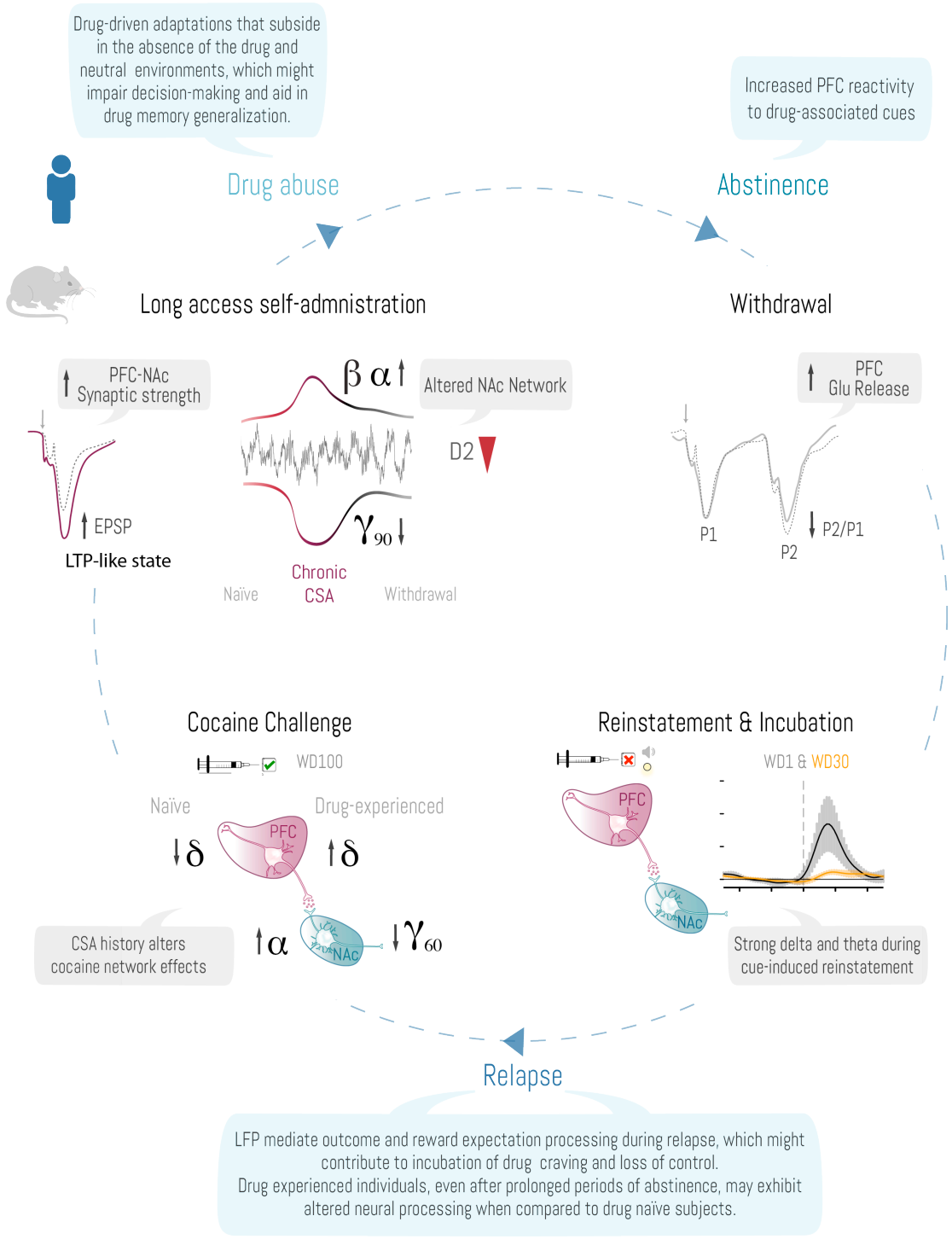


Figure 6.1 Principal findings obtained using animal model and behavioral paradigms, and possible broader implications for understanding the different stages of human addictive behavior – addiction cycle.

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Note: All figures referenced to Catarina Luís were conceptualized and designed by myself, using Adobe Illustrator. Use of those images should be properly authorized and credited to the author.

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