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Role of astroglial glucocorticoid receptor in the mechanism of opioid action

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Kraków, 2018

This doctoral thesis was performed in the Department of Molecular Neuropharmacology, Institute of Pharmacology of the Polish Academy of Sciences. Funding for this work was provided by Polish National Science Centre Grants: 2013/08/A/NZ3/00848, 2012/07/E/NZ3/01785

Pragnę podziękować

mojemu promotorowi, prof. Ryszardowi Przewłockiemu,
za inspirację, dzielenie się wiedzą i doświadczeniem
oraz pomoc w wykonaniu niniejszej pracy.

Wszystkim koleżankom i kolegom z Zakładu Neurofarmakologii Molekularnej za
życzliwość i pomoc okazaną w trakcie powstania tej pracy.
Magdzie, Sławkowi i Justynie za pomoc w przeprowadzeniu badań biochemicznych.
Marysi i Agnieszce za prowadzenie hodowli zwierząt i pomoc w genotypowaniu.
Łucjom, za pomoc w prowadzeniu eksperymentów, wsparcie i przyjaźń.
Michałowi, Marcinowi, Basi, Wiktorowi, Magdzie, Przemkowi, Łukaszowi
i Jankowi za konstruktywną krytykę i cenne uwagi.

Kolegom i koleżankom z Zakładu Fizjologii,
za owocną współpracę oraz przeprowadzenie badań elektrofizjologicznych.

Moim rodzicom, siostrze i braciom, za nieustanną wiarę we mnie
i dzielenie radości z każdego sukcesu.

Abstract

The role of stress and glucocorticoids in the regulation of brain function and pathological behavior, such as addiction, depression or stress-induced disorders, have been primarily studied in neurons. Astrocytes express glucocorticoid receptors (GR), however, to date, there is no direct evidence for functional contribution of astrocytic GR signaling to the central effects of glucocorticoids in addictive behavior.

Primary goal of this study was to identify behavioral patterns and molecular alterations underlying opioid addiction. Analysis of the effects of chronic morphine self-administration in C57BL/6J mice revealed behavioral alterations and long-lasting adaptations in transcriptional profiles in the striatum and frontal cortex of morphine-dependent animals, which pointed out to a potential role of glucocorticoid regulatory network and astrocytes in opioid addiction. We have therefore compared gene expression induced by glucocorticoids in different cellular compartments, astrocytes and neurons *in vivo*. Obtained results show that GR-dependent transcriptional changes in astrocytes are a major site of glucocorticoids action in the nucleus accumbens (NAc).

Further, we have evaluated the functional role of astrocytic GR in animal behavior using selective elimination of the GR from astrocytes in the brain of adult mice by utilizing two transgenic animal models. We have examined new transgenic mouse line, based on CreERT2/loxP technology that allowed for conditional GR knockout in astrocytes in several brain regions, including hippocampus and amygdala. GR knockout resulted in impairment of stress-induced memory expression and extinction, and lead to decreased expression of opioid withdrawal. In the second model, we have selectively targeted astrocytic GRs in the NAc *in vivo* using lentiviral vector harboring Cre-dependent shRNA

expression cassette in mice expressing the Cre recombinase under aldehyde dehydrogenase 1 family promoter (Aldh1L1Cre), a protein that is broadly and specifically expressed by nearly all astrocytes, but not other cell types. GR knockdown in accumbal astrocytes caused increase of sensitivity to opioid reward as well as alterations of morphine-induced synaptic plasticity, including decreased cell excitability of medium spiny neurons and long term potentiation in the NAc. Both astrocytic GR knockout and GR knockdown mice presented unaltered behavioral performance under basal conditions when compared to appropriate controls.

Overall, results in this doctoral thesis reveal a critical role of astrocytic GR in the mediation of opioid- and stress- induced behaviors in mice, which provides a novel insight into the coordinated activity of astrocytes and neurons in the regulation glucocorticoid-mediated effects on opioid reward and addiction.

Streszczenie

Rolę stresu i glukokortykoidów w regulacji funkcji mózgu i patologicznych zachowań, takich jak uzależnienie, depresja lub zaburzenia wywołane stresem, badano do tej pory głównie w kontekście różnych populacji neuronalnych. Receptory glukokortykoidowe (GR) są zlokalizowane także w astrogleju, jednak jak dotąd, nie ma bezpośrednich dowodów na funkcjonalny udział astrocytarnego GR w ośrodkowym wpływie glukokortykoidów na zachowania związane z uzależnieniem.

Celem moich badań była identyfikacja zespołu zachowań oraz zmian molekularnych leżących u podłoża uzależnienia od opioidów. Analiza efektów chronicznego samopodawania morfiny u myszy szczepu C57BL/6J wykazała profil zachowań przypominających uzależnienie oraz trwałe zmiany profilów transkrypcyjnych zwierząt uzależnionych od morfiny. Wyniki te wskazały na potencjalną rolę astrocytów i sieci genów regulowanych przez GR w uzależnieniu od opioidów. Kolejny etap badań objął porównanie zmian transkrypcyjnych zależnych od GR w różnych przedziałach komórkowych- astrocytach i neuronach *in vivo*. Uzyskane wyniki pokazują, że głównym celem działania glukokortykoidów w jądrze półleżącym (NAc) są astrocyty.

Aby zbadać funkcjonalną rolę astrocytarnego GR w zachowaniu, wykorzystałam dwa modele myszy transgenicznych. W pierwszym modelu użyłam nowej linii myszy opartej na technologii CreERT2/loxP, która pozwoliła na indukowalną eliminację GR w astrocytach w kilku obszarach mózgu, w tym w hipokampie i ciele migdałowatym. Zwierzęta pozbawione astrocytarnego receptora GR wykazały osłabienie pamięci związanej ze stresem oraz zmniejszenie natężenia fizycznych objawów odstawienia od opioidów. W drugim modelu selektywnie wyciszyłam astrocytarny GR w NAc przy użyciu wektora

lentiwirusowego z Cre-zależną kasetą shRNA u myszy transgenicznych, które ekspresjonują rekombinazę Cre pod promotorem dehydrogenazy aldehydowej 1 (Aldh1L1Cre), typowej dla astrocytów. Wyciszenie GR w astrocytach w NAc spowodowało wzrost wrażliwości na nagrodę opioidową. Wyniki elektrofizjologiczne wskazują na modulacyjny wpływ astrocytarnego receptora GR na wywołaną morfiną pobudliwość średnich neuronów kolczystych a także długotrwałe wzmocnienie synaptyczne w jądrze półleżącym. W obu modelach transgenicznych zwierzęta nie wykazały różnic w zakresie podstawowego fenotypu w odniesieniu do odpowiednich grup kontrolnych.

Podsumowując, uzyskane wyniki wskazują na kluczową rolę astrocytarnego GR w mediowaniu behawioralnych efektów opioidów i stresu. Badania umożliwiły nowatorski wgląd w skoordynowaną aktywność astrocytów i neuronów regulującą wpływ glukokortykoidów na nagradzające i uzależniające efekty opioidów.

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Chapter 1 Introduction

1.1 Opioids: mechanisms of action

Since the discovery of opioid receptors and their endogenous ligands, it has been established that opiates exert their effects by mimicking effects of three endogenous opioid peptide families. Opioid system is implicated in the regulation of the central and peripheral antinociception, motor activity, regulation of body temperature respiration, as well as cardiovascular and gastrointestinal functions. Opioids are also involved in reward processing, learning and memory, and modulation of the emotional states. Today they remain the most widely used pain killers in medicine, despite an array of adverse side effects, like respiratory depression, constipation, drowsiness, tolerance and dependence. This chapter reviews current knowledge of the role of opioids and opioid system in behavioral control and molecular regulations following drug exposure.

Opioid receptors and their ligands. Over the last two decades, our understanding of the biogenesis, anatomical distribution and characteristics of endogenous opioid system has advanced considerably. The opioid system operates via three G-protein coupled receptors, delta, kappa and mu which are activated by endogenous opioid peptides processed from protein precursors. Proenkephalin is cleaved to form met-enkephalin and leu-enkephalin that are ligands of delta receptor. It is expressed in the striatum, midbrain, hypothalamus and throughout the limbic system. Dynorphin A and B are derived from prodynorphin and bind to kappa receptor. Prodynorphin is moderately expressed in the striatum, ventral tegmental area

(VTA), amygdala and midbrain (Fallon and Leslie 1986). Proopiomelanocortin is the parent compound for β-endorphin, which is primarily mu receptor agonist, and adrenocorticotropin (ACTH), that is involved in the steroidogenic activity of the hypothalamic-pituitary-adrenal (HPA) axis. Expression of proopiomelanocortin and derived form it peptides is high in hypothalamus and anterior and intermediate lobes of pituitary (Cawley, Li, and Loh 2016). The most recently described group of endogenous opioid peptides are endomorphins, which show high selectivity for the mu-opioid peptide receptor (Zadina et al., 1997).

Molecular and cellular mechanisms of opioid action. Opioid receptors belong to the family of seven transmembrane G protein-coupled receptors. Upon ligand binding, they act via G_i/G_o classes of G proteins that inhibit cyclic AMP (cAMP). Furthermore, the activation of opioid receptors leads to inhibition of Ca^{2+} currents and the increase of K^+ conductance, resulting in inhibition of neuronal excitability and synaptic transmission (Nestler, 1994). Alternatively, opioids can exhibit excitatory activity in the brain via both reduction of inhibitory transmission and by direct excitatory activities. In specific subpopulations of cells, opioids stimulate inositol lipid hydrolysis and the production of IP3 and diacylglycerol, which could lead to the mobilization of intracellular Ca^{2+} stores and an increase in intracellular Ca^{2+} concentrations (Przewlocki, 2013). Thus, opioid exposure could further activate the mitogen-activated protein kinase (MAPK). The most abundant members of the MAPK family in neurons are extracellular signal-regulated kinases (ERK1/2). The activation of the ERK1/2/MAPK pathway further activates transcription factors, including the Ca^{2+}/cAMP response element-binding protein (CREB). CREB is a transcription factor that binds to cAMP-responsive elements in the promoter region of several target genes, regulating their expression. Thus, opioids have potent effects on ion channel modulation, signal transduction

pathways, and gene expression and evoke a cascade of genomic changes which affect cellular plasticity.

Behavioral and physiological effects of opioids. In clinical use stimulation of different opioid receptors produces a range of effects often dependent upon location of specific receptor. Opioid receptors are expressed throughout the brain including the reward circuitry, in spinal cord and in peripheral nervous system and are activated in response to both natural rewarding stimuli and drugs of abuse (Merrer et al. 2009). Generally, systemic mu and, to a lesser extent, delta agonists produce positive reinforcement, whereas kappa agonists induce aversive states. Conversely, mu and delta antagonists suppress the positive reinforcing properties of natural rewards and opiate or nonopioid drugs, whereas kappa antagonists facilitate these effects (Shippenberg and Elmer 1998).

Majority of opioids used in clinical practice exert their action, at least in part, through mu receptor. Out of currently used drugs, morphine is generally considered to be the archetypal mu agonist, although it also displays some degree of activity at both delta and kappa receptors. Mu receptor agonists cause analgesic effects mediated by both spinal cord and midbrain by indirectly increasing neuronal trafficking through the descending pathway at the periaqueductal grey (PAG) and nucleus reticularis paragigantocellularis (NRPG) or by directly inhibiting nociceptive afferents in the periphery (Przewłocki and Przewłocka 2001). Activation of delta receptors can cause spinal analgesia and reduce gastric motility, while kappa receptor stimulation may produce spinal analgesia, diuresis and dysphoria (Pathan and Williams 2012).

Opioids induce euphoria and may cause a reduction in conscious level, making them drugs of abuse. The central point of reward and reinforcement circuit comprises of the mesolimbic dopaminergic projections that originate from the VTA and project to various regions of the forebrain with a major input to the

nucleus accumbens (NAc; Everitt and Robbins 2005). Acutely, administration of opiates increase dopaminergic signals to the NAc via activation of VTA dopamine neurons. This activation occurs indirectly through inhibition of GABAergic interneurons in the VTA (Nestler 1996). Opiates also directly affect NAc neurons independently of dopamine via activation of opioid receptors expressed by these neurons (Koob 1992). Early studies demonstrated that rats readily self-administer opioids into the VTA (van Ree and de Wied 1980) and NAc (Olds 1982). This opioid-mediated activation of the VTA-NAc is thought to elicit compensatory adaptations in neuronal networks that underlie motivational dependence by causing deficient VTA–NAc function that underlies aversive states during periods of drug withdrawal, changes alleviated by further drug exposure. However, accumulating experimental evidence argues against dopamine being a mediator of reward processes per se (Kelley 2004; Kelley et al. 2005). As a result of this debate, the concept of the reinforcement system has expanded beyond the VTA-NAc circuitry to include other structures, such as hippocampus, responsible for contextual learning and memory, amygdala, involved in emotional learning and medial prefrontal cortex, which processes decision-making, planning and controls impulses (Koob and Le Moal 2001).

Interestingly, although in humans opioids have sedative effects, morphine administration in rodents induces notable rises in locomotor activity, that seems to be dependent on mesolimbic and hypothalamic dopamine release (Zarrindast and Zarghi 1992; Belknap et al. 1998).

Opioids exert effects on the respiratory system, causing respiratory depression and attenuating airway reflexes, effects that are considered advantageous during anesthesia. Although opioids are generally considered to preserve cardiac stability, histamine release and the associated reductions in systemic vascular resistance and blood pressure are marked after morphine administration. Among many other side-effects, opioids can also cause dysphoria,

nausea, vomiting, urinary retention and reduction in gastric motility (Hutchinson et al. 2011).

Numerous studies support an important contribution of opioid systems in the mediation, modulation, and regulation of stress responses by acting on HPA axis. Acutely administered morphine was presented to stimulate both the synthesis and release of CRF and ACTH in limbic system (Suemaru 1985; Nikolarakis et al. 1989; Maj et al. 2003). Opioids modulate organism's adaptation to stress, balancing out the response that the stressor places on the central nervous system. Chronic stressors induce changes in specific components of the opioid system, including enkephalin, delta and mu opioid receptors (Drolet et al. 2001).

Transcriptional effects of opioids. Research of the last two decades resulted in large progress in our understanding of adaptive changes produced by opiates. Generally, these adaptations include changes in the structure of dopaminergic neurons in the reward system (Georges et al. 1999) and alterations in the activation of G proteins, protein kinases and numerous cellular proteins (Nestler and Aghajanian 1997). They lead to alterations in the expression of transcription factors and target genes, determining the plasticity of neural networks. Long-lasting changes in gene expression are thought to underlie opioid tolerance and dependence. It was therefore proposed that identification of the candidate genes could determine susceptibility to the addiction and recognize targets for future therapy (Przewlocki 2004).

Recent whole-genome expression profiling studies have uncovered several groups of opioid-regulated transcriptional patterns (Korostynski et al. 2007; Piechota et al. 2010; Zhang et al. 2017). Morphine and heroin regulate transcripts connected with protein phosphatase activity, circadian rhythms and early transcriptional activity, that were also shown to be altered by other drugs of abuse, like cocaine and amphetamine (Graybiel et al. 1990; Hope et al. 1992). Another

group of opioid-induced genes is involved in regulation of metabolic processes and the cell cycle (Piechota et al. 2010). Further, opioids regulate genes connected to enzyme inhibitor activity and lipid metabolism, the stress response, glucose transport and cell differentiation. These genes appear to be regulated by steroid hormones that respond to morphine, heroin and other drugs of abuse (George and Way 1955). In fact, GR-dependent signaling system is emerging as a key element of the neuroadaptive changes that are induced by various drugs of abuse (Marinelli and Piazza 2002; de Jong and de Kloet 2004).

Repeated opioid administration was shown to regulate mRNA level of genes related to negative regulation of apoptosis, genes encoding gap junction membrane proteins α12 and β1 and several genes that are involved in nucleosome assembly (Korostynski et al. 2007). Chronic opioids also result in changes of a large number of inflammation and immune-related genes (Zhang et al. 2017).

1.2 Stress hormones and molecular mechanisms of glucocorticoid action

Glucocorticoids are primary stress hormones necessary for life that regulate and influence numerous functions of the central nervous system- cognition, mood, arousal and sleep. They are some of the most widespread hormones in mammalian organisms, their actions affect nearly all tissues, including the brain. Growing knowledge in the field of glucocorticoid systems points out to their important role in the formation of various pathological conditions. In the following subchapters, a review of the selected stress response theories and definitions will be presented, along with the top recent findings about the mechanisms of action, functions and role of glucocorticoids in the brain.

The stress response, glucocorticoid receptors. Stress has apparent relevance to everyday life, however, there is no definition of this phenomenon that is commonly agreed upon. One of the earliest theories of stress was Cannon's concept of homeostasis that he coined to describe the maintenance, within acceptable ranges, of physiological as well as psychosocial features of the individual. In the early 1900s he described for the first time that when an individual perceives a threat, the organism is rapidly alerted and motivated through endocrine and sympathetic nervous systems to either attack the threat or to escape and avoid it, therefore he named this reaction the fight-or flight response (Cannon 1929; Abboud 2010). In the 1950s Seyle defined stress as a state characterized by a uniform response of the body to external factors, regardless of the particular stressor. He proposed 3 stages of stress response pattern: an initial alarm reaction, a stage of adaptation with resistance to the stressor and lastly, a stage of exhaustion when the organism is no longer capable to adapt to persistent aversive stimulus, what may lead to long-term pathological changes (Selye 1950; 1973). Selye's

concept that prolonged stress may lead to both physical disease and mental disorders is now widely accepted, however modern approaches have redefined the term of stress itself. Currently stress is viewed as a consciously or unconsciously sensed threat to the organism's homeostasis (McEwen 2000; Goldstein and McEwen 2002) that is different for each individual, depending on the type of the stressor, one's perception of the stressor and the perceived ability to cope with it (Goldstein and Kopin 2007). Therefore, stress response includes physiological and behavioral responses that attempt to alleviate, limit, or reverse the actual or potential change caused by a given stressor. It involves multiply neurotransmitter and hormonal signals that rapidly respond to stressors in an effort to return to homeostasis.

Stress response is a complex process that includes action of various neurotransmitters, such as noradrenaline and serotonin, peptides, like corticotropin releasing hormone (CRH) and steroid hormones, for example cortisol in humans and corticosterone in rodents. In general, the stress system has two modes of operation (**Figure 1**). Rapid mode involves CRH-induced sympathetic response and behavioral mobilization to deal with the stressor. HPA axis is activated by CRH, which is produced in the parvocellular neurons of the hypothalamic paraventricular nucleus. These neurons also secrete peptides into the portal vessel system to activate the synthesis of proopiomelanocortin (POMC) in the anterior pituitary, which, among others, is further processed to ACTH, opioid and melanocortin peptides. ACTH stimulates the adrenal cortex to secrete corticosterone that elevates blood sugar, and accelerates metabolism (de Kloet, Joëls, and Holsboer 2005). The second, slower mode of stress response promotes adaptation and recovery of the organism and involves action of urocortins, that were shown to have anxiolytic properties (Hsu and Hsueh 2001; Heinrichs and Koob 2004). Corticosteroids operate in both stress response

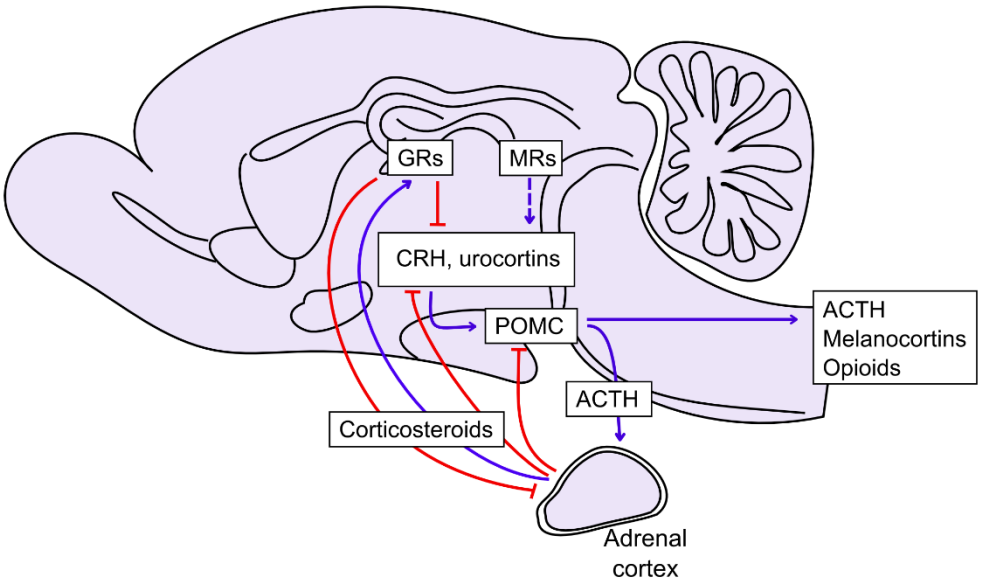

Figure 1. Stress response in the brain. The stressor triggers operation of two stress system modes. The fast mode promotes mobilization of energy and mediates sympathetic arousal to activate 'fight of flight' response. It involves MR that activates the HPA axis through CRH. The other, slower mode mediates suppression of the stress response, facilitates adaptation and recovery of the organism. It involves parasympathetic stimulation and action of urocortins. In negative feedback loop corticosteroids released by adrenal cortex inhibit the production of CRH and ACTH and activate GRs that in turn limit release of these stress hormones into the system.

modes through mineralocorticoid (MR) and glucocorticoid (GR) receptors. MRs take part in the appraisal and onset of the stress response. They have a high affinity for steroid hormones, so they are mostly occupied even when circulating corticosteroid levels are low. GRs have about tenfold lower affinity, therefore they are activated only when corticosteroid levels grow during stress response (Reul and de Kloet 1985). In a classic negative feedback loop they target the hypothalamus and anterior pituitary to terminate the stress reaction by inhibiting the production and release of CRH and ACTH (Herman et al. 2003; Kitchener et al. 2004). Overall, the complex nature of stress signaling at various brain areas and

HPA axis modulation at multiple levels by glucocorticoids allows many neural circuits and signaling cascades to be influenced and regulated by stress.

Mechanisms of glucocorticoid action. Corticosteroids affect in principle all brain cells, including glia, in spatially unrestricted manner. Still, the brain areas where corticosteroids are effective are limited by the distribution of the corticosteroid receptors. MRs are highly expressed in the hippocampus, prefrontal cortex and lateral septum and moderately expressed in hypothalamic paraventricular nucleus, locus coeruleus and amygdala. These regions are involved in the initial emotional, cognitive and neuroendocrine processing of the stressors. GRs are ubiquitously expressed in neurons and glia in the whole central nervous system (Joëls and Baram 2009).

At the cellular level, glucocorticoids act primarily as regulators of gene transcription (Lu et al. 2006). In addition to mediating stress response, glucocorticoids are involved in various physiological processes, including development (Cole et al. 1995), metabolism (Lupien et al. 2009), immune response and cytokine signaling (Liberman et al. 2007), circadian rhythms (Balsalobre et al. 2000), apoptosis and cell cycle progression (Viegas et al. 2008). Most of the physiological and pharmacological actions of glucocorticoids are mediated by the GR, which is a member of the nuclear receptor superfamily of ligand-dependent transcription factors (Evans 1988). General pathway of GR signaling is diagramed in **Figure 2**. In the absence of hormone, GR resides predominantly in the cytoplasm of cells as part of protein complex that includes chaperone proteins (e.g. hsp90) and immunophilins of the *Fk506* family, which maintain the receptor in a conformation that is transcriptionally inactive and favors high affinity ligand binding (Grad and Picard 2007). After binding to the hormone, GR induces or represses the transcription of its target genes which can include up to 20% of the genome (Galon et al. 2002; Ren et al. 2012). GR can regulate genes

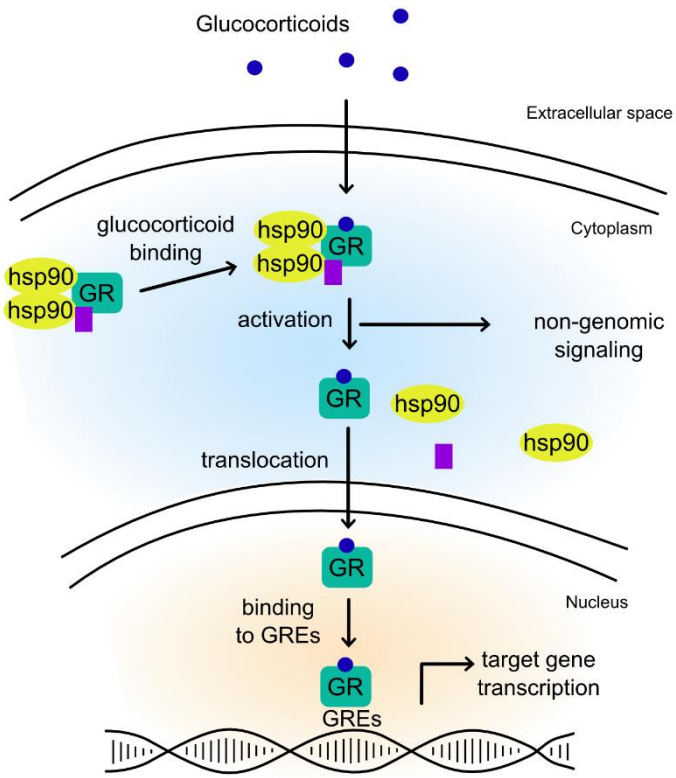

Figure 2. Cellular mechanisms of transcriptional regulation by glucocorticoids. Glucocorticoids pass through the cell membrane and bind inactive receptors that are complexed with chaperone proteins (e.g. hsp90) in the cytoplasm. Binding of glucocorticoid ligand to specific receptors activates the receptor proteins followed by translocation of the activated receptors to the nucleus. In the nucleus, glucocorticoid receptors bind to DNA sequences called glucocorticoid-responsive elements (GREs) that upregulate or downregulate transcription of target genes. Note that receptors can also translocate to the nucleus while in complex with chaperone proteins.

in 3 primary ways: by binding directly to DNA at specific sequences known as glucocorticoid-responsive elements (GREs; Slater et al. 1994), by tethering itself to other DNA-bound transcription factors, or binding directly to DNA and interacting with neighboring DNA-bound transcription factors (Oakley and Cidlowski 2013). GR can also regulate genes through the activity of various kinases without direct DNA binding (Stoecklin et al. 1997; Almawi and

Melemedjian 2002). The process of glucocorticoid-mediated transcriptional regulation is estimated to last minutes to hours.

Recently, large-scale gene expression profiling methods, such as whole-genome microarrays and serial analysis of gene expression, have been applied to identify glucocorticoid responsive genes. In the hippocampus, activation of corticosteroid receptors led to alterations in expression of 70-100 of glucocorticoid-regulated genes (Datson et al. 2001; Morsink et al. 2006), of which about 50% were up-regulated. *In vitro* studies of glucocorticoid action in both neural and glial cells have also identified a number of GR target genes (Bohn et al. 1994; Morsink, Joëls, et al. 2006; Piechota et al. 2017). Some of the most frequently reported genes associated with the transcription of genes regulating growth factors, neurotrophins (Hansson et al. 2000; Suri and Vaidya 2013), mTORC1 signaling and progression of the cell cycle (Sandi 2004). Glucocorticoid-induced regulation of cell function involves changes in the mRNA levels of genes responsible for the regulation of transcription and the selective degradation of mRNA. Other genes regulated by glucocorticoids in the brain are involved in the regulation of metabolism, gap junctions, immune response, signaling mediated by thyroid hormones, calcium, catecholamines and neuropeptides (Sabban et al. 2004). The actions of corticosteroids are restrained by feedback mechanisms depending on the transcription of *Sgk1*, *Fkbp5* and *Nr3c1* (Juszczak and Stankiewicz 2017). These findings indicate that glucocorticoid action involve the expression of high- and medium-abundant genes that underlie aspects of cell metabolism, structure and synaptic transmission.

While the principal effects of glucocorticoids are mediated by transcriptional responses, it is worth noting that GR may also act through non-genomic mechanisms to elicit cellular responses that do not require changes in gene expression (Groeneweg et al. 2012; Samarasinghe et al. 2012). These signaling events seem to involve multiply mechanisms that recruit activity of

various kinases, such as PI3K, AKT and MAPKs, that were reported to influence gap junction intercellular communication and neural progenitor cell proliferation (Solito et al. 2003; Samarasinghe et al. 2011). The existence of non-genomic signaling points out to how complex and diverse are biological actions of glucocorticoids. Although these non-genomic actions are not investigated in this thesis, they have been extensively reviewed in the literature (see: Stellato 2004; Evanson et al. 2010).

Impact of glucocorticoids on brain function and behavior. Most of the functional research on glucocorticoid action in the brain and its behavioral consequences have focused on harmful effects of prolonged corticosteroid exposure. Research from over the past decade have presented strong evidence that alterations in corticosteroid levels strongly affect neuronal activity in limbic areas. Prolonged glucocorticoid exposure results in structural changes of cell dendrites, decreases in cell volume, spine density and dendritic complexity in the hippocampus and prefrontal cortex (Woolley et al. 1990; Magariños and McEwen 1995; McEwen 1999) however, similar paradigms induce opposite effects in the amygdala and orbitofrontal cortex (Popoli et al. 2011). Glucocorticoids also impact cell viability by suppressing neurogenesis, decreasing glial progenitor cells and glial proliferation, as well as promoting neuronal cell death (Sapolsky 1999; Czéh et al. 2006; Banasr et al. 2007). Further, glucocorticoids and stress were shown to modulate long-term potentiation (LTP) properties, generally decreasing LTP dynamic range and modifying learning and memory processes (Maroun and Richter-Levin 2003; Mailliet et al. 2008; Yuen et al. 2012).

Glucocorticoid signaling can also affect neurotransmission in several different ways. First, acute exposure to stress or administration of glucocorticoids rapidly increases glutamate release at excitatory synapses of the hippocampus, prefrontal cortex and amygdala (Moghaddam 1993; Venero and Borrell 1999;

Reznikov et al. 2007). Previous studies found that this increased glutamate concentration may be neuronal in origin (Hascup et al. 2010). However, it has been suggested that a large portion of the amino acid neurotransmitters is of non-neuronal origin, that is, they may result from reverse transporter activity or be derived from glial cells (Timmerman and Westerink 1997). Second, glucocorticoids impact glutamate-based synaptic currents based on postsynaptic glutamate receptors dynamics (AMPAR and NMDAR). Acute stress or corticosterone treatment increases the concentration of both types of glutamate receptors at the postsynaptic membrane, thus resulting in increased AMPA and NMDA currents in the prefrontal cortex (Yuen et al. 2011). Third, glucocorticoids influence glutamate reuptake transporters and glutamine metabolism in astrocytes. Glial glutamate transporter activity was shown to influence stimulation of NMDARs and metabotropic glutamate receptors (mGluRs; Zheng et al. 2008). Glucocorticoids can increase expression of GLT-1/EAAT2 in astrocytes in vitro, one of the two primary glutamate transporters expressed in astrocytes (Zschocke et al. 2005). Finally, glucocorticoids affect neurotransmission through other, indirect mechanisms. Upon stress exposure, membrane-bound GRs can stimulate postsynaptic endocannabinoid production that subsequently results in inhibition of presynaptic neurotransmitter release (Chávez et al. 2010; Hill et al. 2011). Glucocorticoids can also modulate mitochondrial functionality and calcium buffering capacity (Du et al. 2009).

In relation to these effects, stress and glucocorticoids have apparent but often divergent consequences on cognitive and emotional processes, some of which include spatial and declarative memory, the memory of emotionally arousing experiences, such as fear, and executive functions (Lupien et al. 2009). The specific effects of stress and glucocorticoids on behavior and cognition depend on the type of the stressor, timing and the duration of the exposure and also on the interaction between genomic effects and previous experiences. Some

of the early reports on both enhancing and impairing properties of glucocorticoids on memory have showed that the effects of these hormones on cognition are complex and multifaceted (Bohus and Lissák 1968; Luine et al. 1993; Arbel et al. 1994). More recent studies investigated glucocorticoid effects on distinct memory phases and their interaction with emotional arousal. There is now extensive evidence that acute corticosteroid elevation enhances the consolidation of new information connected to emotionally arousing experiences (Roozendaal et al. 2006; Yao et al. 2007; Li et al. 2010), but impairs memory retrieval of already stored information (Roozendaal et al. 1996; de Quervain et al. 1998 ; Roozendaal et al. 2004). Prolonged elevations in glucocorticoid levels are usually associated with impaired cognitive performance and these deficits are thought to result from a cumulative and long-lasting alterations of hippocampal function and morphology (Sapolsky 2000; McEwen 2001). Findings from studies in both rodents and humans show that effects of glucocorticoids on cognition can be illustrated by an inverted U-shaped function between dose and memory performance. Moderate doses enhance memory, whereas lower or higher doses are typically less effective and may even impair memory consolidation (Roozendaal 2002). This differential effects of glucocorticoids on memory performance may be explained by the different functions of the two types of corticosteroid receptors. Animal studies reveal that MRs affect behavioral reactions during various situations (Oitzl et al. 2010), whereas GRs are more likely to be involved in memory consolidation, as immediate post training administration of a GR antagonist, but not MR antagonist resulted in impaired spatial memory (Oitzl and de Kloet 1992). However, recent studies show that not only GRs, but also MRs play a role in establishing emotionally arousing memories, especially in the early phases of memory formation (Zhou et al. 2010; Zhou et al. 2011).

To summarize, extensive evidence shows that corticosteroids play a vital role in the regulation of various brain functions that influence behavior and

cognition, including memory formation, maintenance and recall. HPA axis dysregulation that leads to excessive, prolonged or inadequate release of glucocorticoids impairs adaptation to stress and is considered a hallmark of stress-related disorders, such as major depressive disorder, posttraumatic stress disorder, anxiety disorders and substance abuse.

1.3 Astrocytes in the spotlight: redefining the functional architecture of the brain

For most of the history of modern neuroscience, neurons have received the majority of attention in the field, because of their broadly accepted role in transmitting information, with very little regard for other cell types. While this approach has been and continues to be fruitful in many aspects, it is insufficient in understanding brain function in health and disease, due to the growing recognition of glial cells as active participants in the brain signaling. This thesis section will focus on the most abundant glial and perhaps the most diverse cell type in the brain, astrocytes. Following subsections will discuss how astrocytes influence synapses, neurons and neurotransmission, cell metabolism, neural networks and ultimately, behavior.

Astrocyte function: overview. Astrocytes (literally: ‘star like cells’) are the most numerous cells in the central nervous system that display a remarkable diversity in their morphology and function. Conceptually astroglial cells are as heterogeneous as neurons, therefore astrocytes in different brain regions may have very different physiological properties. Astrocytes develop in concert with neurons (Götz and Huttner 2005), deriving information from neuronal signals and

actively participating in both synaptogenesis (Christopherson et al. 2005) and synapse elimination (Stevens 2008). Early anatomical studies established the distinction between fibrous and protoplasmic astrocytes based on morphological differences and their locations (Miller and Raff 1984). Protoplasmic astrocytes occur mainly in gray matter and have many fine processes, which are extremely elaborate and complex. The processes of protoplasmic astrocytes contact blood vessels and form multiple contacts with neurons. Each astrocyte appears to occupy a unique domain (Halassa et al. 2007). Compared with protoplasmic astrocytes, fibrous astrocytes display fewer processes that are longer but much less complex**.** They send numerous extensions that contact axons at nodes of Ranvier. Fibrous astrocytes are present in white matter (García-Marín et al. 2007).

Schematic representation that summarize astrocyte functions in healthy central nervous system is presented in **Figure 3** and selected topics are reviewed in the following subchapters. Astrocytes are involved in the development of the brain environment, they provide structural and metabolic support, maintain brain homeostasis and blood-brain barrier, control synaptogenesis and synaptic maintenance and mediate inflammatory reaction after injury (Freeman and Rowitch 2013). During development, radial glia produce neuronal precursors that migrate to their destinations using astroglial processes as a guide-line. The radial glia acts also as progenitors for both astrocytes and oligodendrocytes. Some of the astrocytes in the adult brain retain the stem cell properties and can generate additional astrocytes. Indeed, recent studies indicate that astrocytes actively turn over in the brain throughout life (Ge et al. 2012), showing that cell cycle regulation in astrocytes continues to be important beyond development.

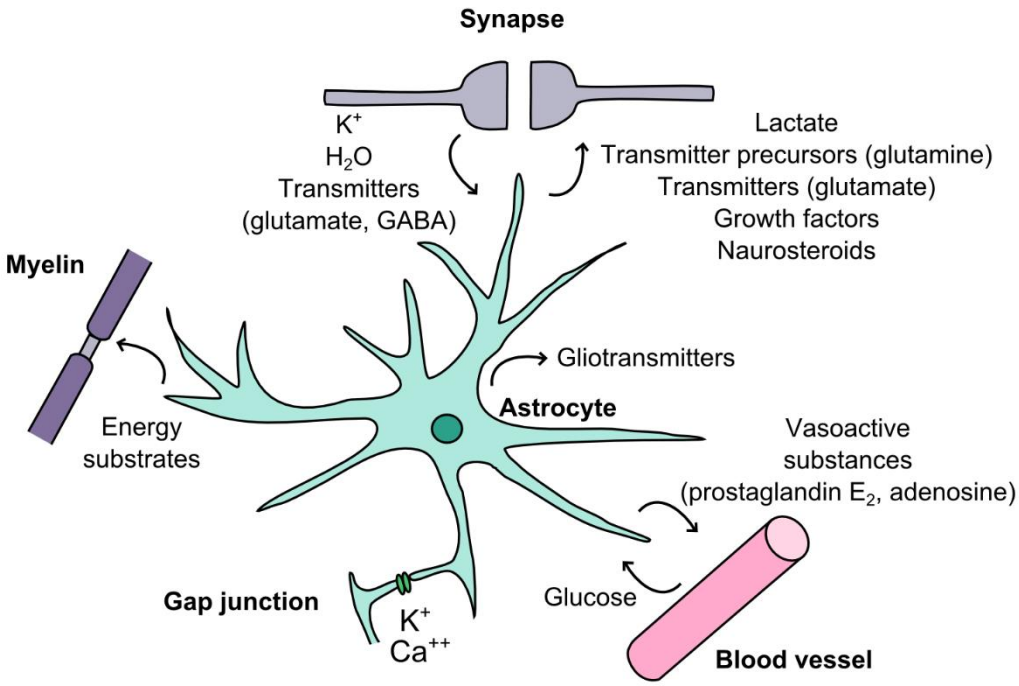

Figure 3. Overview of astrocyte functions. Astrocytes are integral to neurotransmission in multiple ways, including clearance of neurotransmitters from the synapse and cycling neurotransmitters back into neurons. Astrocytes also release their own gliotransmitters, both onto neurons as well as other glial cells. In contrast to chemical coupling at the synapse, astrocytes have extensive electrical coupling via gap junctions, forming dynamic networks that transfer molecules between astrocytes and other cells. This transfer includes interactions with the vasculature at the blood brain barrier and transporting metabolites to supply energy for neurons.

Traditionally astrocytes have been viewed as simple homogenous cells that serve similar supportive role throughout the central nervous system. Recent studies instead indicate that functions of adult astrocytes greatly diversify and vary by brain region. The concept that astrocytes display a range of functional attributes that are brain circuit specific was derived from a number of evidence showing diversity of astrocytic molecules and markers and distinct astrocyte responses in circuits and microcircuits of various brain regions (Khakh and Sofroniew 2015). Glial fibrillary acidic protein (GFAP) is widely regarded as prototypical astroglial

marker (Eng et al. 2000), however many astrocytes do not express GFAP in amounts detectable by immunohistochemistry. Still, the absence of GFAP expression does not imply the absence of astrocytes, that can be detected by other markers such as glutamine synthetase, S100β or Aldh1L1 (Sofroniew and Vinters 2010). Astrocytes from different brain regions can exhibit evident differences in GFAP levels. For example, nearly all astrocytes in the hippocampus express GFAP, whereas very few do so in the thalamus and nucleus accumbens (Middeldorp and Hol 2011). Astrocytes in different brain regions present also differences in glutamate transporters GLT-1 and GLAST (Lehre et al. 1995), expression of gap junction protein connexin 30 (Cx30; Nagy et al. 1999) and Kir4 potassium channels (Poopalasundaram et al. 2000). Recent large-scale transcriptional analyses have also supported the notion of molecular heterogeneity of astrocytes in different brain regions (Bachoo et al. 2004; Cahoy et al. 2008), suggesting that astrocyte functions are tailored to the functions of the neural circuits in which they reside.

Tripartite synapse: astrocytes role in neurotransmission. The term 'tripartite synapse' was coined in the late 1990s with reference to growing evidence in synaptic physiology showing bidirectional communication between astrocytes and neurons (Araque et al. 1999). Early pioneer studies demonstrated for the first time that cultured astrocytes display a form of excitability based on variations of the intracellular Ca^{2+} concentration (Cornell-Bell et al. 1990; Charles et al. 1991). Until then, astrocytes had been considered to be non-excitable cells, because, unlike neurons, they do not show electrical excitability (e.g. Orkand et al. 1966; Sontheimer 1994). More recent research both *in vitro* and *in vivo* have confirmed astrocyte excitability manifested as elevations of cytosolic Ca^{2+} that results from the mobilization of Ca^{2+} stored in the endoplasmic reticulum (Perea and Araque 2005b). Astrocytes respond with Ca^{2+} elevations to synaptic activity (Perea and

Araque 2005a) which indicates the existence of neuron-to-astrocyte communication. It also has to be noted that Ca^{2+} elevations can occur spontaneously and independently from neuronal activity (Aguado et al. 2002; Nett et al. 2002), possibly as a part of astrocyte-to-astrocyte interchange.

Astrocytes contribute to neurotransmission by clearing glutamate and other neurotransmitters from the synapse. Astrocytes account for up to 90% of the glutamate removal from the synaptic cleft (Nadler 2012). Once glutamate is taken up from the synapse, astrocytes can convert glutamate to glutamine via glutamine synthetase, an enzyme uniquely expressed in astrocytes in the brain. The non-neuroactive glutamine is then released by astrocytes and taken up by transporters on neurons (Bröer and Brookes 2001). These processes between neurons and astrocytes allow for high accuracy of excitatory neurotransmission. In response to glutamatergic neurotransmission, astrocytic glutamate receptors (mGluR5) respond to neuronal activity with an elevation of Ca^{2+} concentration, that in turn induces feedback regulation of neuronal activity and synaptic strength through the release of gliotransmitters (Araque et al. 1999; Panatier et al. 2011). Although well-characterized in the literature, this mechanism has been recently questioned due to new evidence that astrocytic expression of mGluR5 is developmentally regulated and is undetectable after postnatal week 3 (Sun et al. 2013). Therefore neuroglial signaling in the adult brain might be fundamentally different from that so extensively described in the literature, as majority of the studies to date have used cultured astrocytes or slices prepared from young rodents. Astrocyte Ca^{2+} signaling can stimulate presynaptic intake of extracellular K^+, resulting in neuronal hyperpolarization that was shown to modulate basal neuronal excitability (Wang et al. 2012).

Astrocytes are also implicated in the modulation of the inhibitory GABAergic neurotransmission (Losi et al. 2014). Astroglia responds to GABA through different mechanisms that include GABA receptors and transporters

(Kettenmann and Schachner 1985; Backus et al. 1988; Steinhäuser et al. 1994). It was found that GABA evokes astrocytic Ca^{2+} events through different intracellular pathways mediated by both ionotropic $GABA_A$ and metabotropic $GABA_B$ receptors (Serrano et al. 2006; Meier et al. 2008). Astrocytes can in turn modulate GABAergic transmission through GABA transporters that set the tone of inhibition in local neural circuits through the control of the extracellular GABA concentration (Semyanov et al. 2004; Farrant and Nusser 2005). Several recent works showed that astrocytes can also directly affect neurotransmission by releasing GABA as a gliotransmitters (Kozlov et al. 2006; Jiménez-González et al. 2011, Le Meur et al. 2012).

Gliotransmission: impact of astrocyte-derived signals. One of the most interesting questions investigated in current neuroscience is what are the functional consequences of the astrocyte Ca^{2+} signal on neuronal physiology. According to the concept of the tripartite synapse, in order to fully comprehend synaptic function, astrocytes must be considered as integral components of synapses where they have crucial roles in synaptic physiology. Variations of intercellular Ca^{2+} can trigger the release of several neuroactive molecules, such as glutamate, d-serine, ATP, adenosine, GABA, prostaglandins, proteins and peptides, the so-called gliotransmitters, that can influence neuronal and synaptic physiology (Bezzi and Volterra 2001; Araque et al. 2014).

Gliotransmitters activate neuronal receptors and modulate synaptic transmission and plasticity (e.g. Sasaki et al. 2011; Schmitt et al. 2012; Fossat et al. 2012). How exactly gliotransmitters mediate synaptic modulation is a subject of ongoing research that already reveals a wide variety of signaling processes and physiological consequences of astrocyte neuromodulation. Just like neurotransmitters, single gliotransmitter can act on different targets depending on brain area, targeted neuronal circuits and location of activated neuronal receptors.

Astrocyte-derived glutamate acts on presynaptic ionotropic NMDA receptors in the hippocampal dentate gyrus (Jourdain et al. 2007), while at hippocampal CA1-CA3 synapses it can activate presynaptic metabotropic mGluRs (Navarrete and Araque 2010; Navarrete et al. 2012). Similarly, adenosine can act on both presynaptic terminals to modulate presynaptic inhibition and postsynaptically to regulate NMDAR trafficking (Martín et al. 2007; Deng et al. 2011; Panatier et al. 2011). Astrocytes that are a part of the same circuit release multiple gliotransmitters which can influence synaptic transmission in different ways. For example, in addition to glutamate, astrocytes in hippocampal CA1 area can release D-serine, which is the NMDA receptor coagonist (Henneberger et al. 2010) and ATP (Zhang et al. 2003), which after conversion to adenosine acts on different receptors (like A1 or A2A) to depress or enhance excitatory synaptic transmission (Pascual et al. 2005; Serrano et al. 2006; Panatier et al. 2011). Further, gliotransmitters can coordinate whole networks of neurons and synapses, operating as bridges for intersynaptic communication. Recent research showed that astrocytic Ca^{2+} signals evoked locally by endocannabinoids enhanced synaptic efficacy at relatively distant synapses from the endocannabinoid source (Navarrete and Araque 2010). Research up to date strongly supports the existence of dynamic, bidirectional regulation of neuronal communication by astroglial cells, however some recent studies have challenged these findings. Debate focuses mainly on possibility that astrocytes may not express the machinery needed to exocytose neurotransmitters *in vivo* (Hamilton and Attwell 2010). Moreover, regardless of the release machinery, activation of exogenous G-protein coupled receptors or knock-out of IP_3R2 that reportedly blocked all astrocytic Ca^{2+} signaling failed to modify synaptic transmission at hippocampal CA1 cells (Fiacco et al. 2007; Petravicz et al. 2008; Agulhon et.al 2010).

Astrocytic gap junctions. In addition to the chemical coupling of neuronal synapses and gliotransmission, astrocytes in the adult brain are connected to each other via gap junction channels, allowing the intercellular exchange of ions, metabolites and amino acids. Gap junctions are composed of connexin proteins and allow for diffusion of ions and small molecules between cells. Astrocytes primarily express 2 types of connexins, connexin-43 (Cx43) and connexin-30 (Cx30; Dermietzel et al. 1989). Connexin proteins form hexameric structures called connexons, which can form either hemichannels on the cell membrane, that allow for diffusion between cytoplasm and extracellular space, or gap junctions with another cells (Goodenough and Paul 2009). Glial connexin gap junctions link astrocytes to other astrocytes and oligodendrocytes in the network that is responsible for removing excitotoxic ions and metabolites (Lapato and Tiwari-Woodruff 2018). Most gap junction signaling in astrocytes has been attributed to Cx43 based on its greater overall expression across the brain (Yamamoto et al. 1990; Nagy and Rash 2003), but astrocyte connectivity may be more complex considering the differential anatomical patterns of Cx43 and Cx30 expression (Griemsmann et al. 2015). Dye transfer studies using Cx30 knockout mice have attributed 25% of astrocyte-astrocyte coupling to Cx30 in the hippocampus (Gosejacob et al. 2011), but recent results indicate that Cx30 accounts for up to 70% of astrocyte-astrocyte coupling in the thalamus (Griemsmann et al. 2015). These anatomical distinctions presumably impact intercellular communication dependent on astrocyte gap junctions.

Gap junctions in astrocytes are mediating the spread of Ca^{2+} waves (Enkvist and McCarthy 1992). As mentioned earlier, local changes in Ca^{2+} concentrations in astrocytes can mobilize intracellular Ca^{2+} wave initiated by ATP release (Charles et al. 1991; Guthrie et al. 1999; Fujii et al. 2017), that diffuses across gap junctions to other astrocytes. These Ca^{2+} changes can act as a signal across a network of astrocytes and are associated with activation of the

metabotropic purine receptors on nearby postsynaptic GABAergic interneurons, leading to increased synaptic inhibition in interneuron networks (Bowser and Khakh 2004; Kawamura et al. 2004). This suggests another form of astrocyte-to-neuron communication in which Ca^{2+} waves may be involved in augmenting feedback inhibition through purinergic signaling, expanding the ways that astrocytes modify both excitatory and inhibitory transmission.

Both astrocyte connexins are also involved in electric coupling with oligodendrocytes, the myelinating glial cell in the brain. Although the content interchanged between astrocytes and oligodendrocytes is currently unknown, the presence of these gap junctions appears critical for myelination, as selective knockout of connexins in mice to remove astrocyte-oligodendrocyte coupling results in progressive neurodegeneration and early mortality (Magnotti et al. 2011; Tress et al. 2012).

Metabolic roles of astrocytes. The energy demands of the brain are estimated to account for about 25% of the body's energy consumption (Bélanger et al. 2011). The brain contains very little energy reserves, therefore it is highly dependent on the supply of energy substrates from the circulation. Astrocytes play important roles in supplying neurons with energetic compounds and neurotrophic molecules derived from the blood. Early studies showed that glucose is the obligatory energy substrate for the brain, where it is almost fully oxidized (Sokoloff 1977). Although neurons can derive glucose directly from the blood supply, they also receive glycolytic compounds from astrocytes. The postulated glucose-lactate shuttle model introduced the concept that astrocytes take up glucose from the blood, convert it to lactate, release it and thus provide an energy substrate to neurons (Chih and Roberts Jr 2003). A large proportion of the glutamate released at the synapse is taken up by astrocytes via glutamatergic transporters (GLT-1 and GLAST). Following its uptake by astrocytes, glutamate is converted to glutamine

by the glutamine synthetase and shuttled to neurons, where it is converted back to glutamate. The metabolic burden created by glutamate uptake causes nonoxidative glucose utilization in astrocytes and glucose uptake from the circulation through the glucose transporter GLUT1 expressed by astrocytes. Glycolytically derived pyruvate is converted to lactate and shuttled to neurons, where it can be used as an energy substrate (Magistretti and Allaman 2015). Simultaneously, astrocytes participate in the recycling of synaptic glutamate via the glutamate-glutamine cycle (Pellerin and Magistretti 2012).

Astroglial cells are also central elements of the neurovascular units that integrate neural circuitry with local blood flow. The basal lamina of blood vessels is almost entirely covered by astrocyte endfeet. Astrocytes are therefore serving as a bridge between the endothelial cells and neurons. The blood-brain barrier regulates the flux of ions, fluid, and metabolites in and out of the brain. Astrocytic endfeet serve as exchange points with the blood supply and contain specific types of transporters, including high concentrations of multiple potassium channels (e.g. Kir4.1) and water channels (AQP4). In response to neural activity, the water produced as a byproduct by glucose metabolism in neurons and accumulated in astrocytes by potassium buffering is spatially limited and needs to be removed, and the high density of water channels in perivascular astrocytic endfeet facilitates redistribution and excretion of the water from the brain into the blood (Bélanger et al. 2011). Astrocytes are also able to modulate the blood-brain barrier structure and function as well as respond to signals from the endothelial cells (Abbott et al. 2006; Moura et al. 2017)

Investigation of astrocyte-specific markers. Research on astrocytic functions have made extensive use of primary astrocyte cell culture models. These models have contributed numerous findings on astrocyte function (e.g. Khelil et al. 1990; McClennen and Seasholtz 1999) and continue to be refined (Foo et al. 2011; Zhang

et al. 2016). Advantages of using cell culture models include selective isolation of the cell of interest, good control of experimental conditions and relatively convenient sample generation. However, astroglial cultures are typically prepared using pups right after birth, therefore they are derived from a small, unidentified population of proliferating glial precursor cells that express several astrocyte markers, but which have an immature or reactive phenotype. Their properties are thus of unclear relationship to those of mature astrocytes in adult brain. Given still very limited knowledge about astrocytic functionality compared to neurons and other brain cell types, most of current cell culture and animal studies require prior knowledge of an astrocyte function to investigate. One way in which the field is seeking to address this shortcoming is by further defining astrocytes by their gene expression patterns relative to other cell types in the brain. The logic of these studies is based on the notion that a cell and corresponding cellular function are defined in part by the set of mRNAs and proteins that are expressed in that cell. Knowing mRNAs expressed by specific cells can implicate the presence of cell-independent mechanisms, common across cell types, as well as mechanisms specific for certain cell types.

Recent whole genome transcription profiling performed on intact brain tissue have produced convincing evidence that there are indeed distinct mRNA expression patterns specific for astrocytes, oligodendrocytes, and neurons (Cahoy et al. 2008). These findings led, among other things, to characterization of new astrocyte-specific marker, aldehyde dehydrogenase 1 family, member L1 (Aldh1L1) that was shown to strongly label more astrocytes than GFAP. What is more, Aldh1L1 mRNA is more widely expressed throughout the brain, whereas GFAP show more predominant expression in white matter (Cahoy et al. 2008). Further analysis of genes expressed by astrocytes provided new insights into astrocyte specification, development, function, and signaling interactions with blood vessels and synapses (Cahoy et al. 2008; Zhang et al. 2016).

1.4 Glucocorticoid receptors, astrocytes and addiction

Developing a better understanding of both stress response mechanisms and astrocyte functionality may have clinical relevance in the field of mental health disorders. Alterations of glucocorticoid signaling and astrocytes have each been individually implicated in the pathogenesis of major depressive disorder, posttraumatic stress disorder, anxiety and substance abuse, but how glucocorticoid signaling and astrocytes influence each other and what is the role of GR in astrocytes is currently not well known, an area this thesis attempts to address. The following section focuses mainly on reviewing the association of the HPA axis alterations with addiction, emerging data indicating changes in astrocytes in drug misuse disorder and the precedence for glucocorticoid regulation in astrocytes.

Associations between stress response systems and addiction. Drug addiction have been conceptualized as a chronically relapsing disorder of compulsive drug seeking. It impacts various motivational mechanisms and progresses from positive reinforcement, connected to rewarding properties of drugs, to negative reinforcement, such as observed in withdrawal state, when drug taking alleviates a negative emotional state (Koob et al. 2014). The central point of reward and reinforcement circuit comprises of the mesolimbic dopaminergic projections that originate from the VTA and project to various regions of the forebrain with a major input to the NAc (Everitt and Robbins 2005). However, accumulating experimental evidence argues against dopamine being a mediator of reward processes per se (Kelley 2004; Kelley et al. 2005). As a result of this debate, the concept of the reinforcement system has expanded beyond the VTA-NAc circuitry to include other structures, such as hippocampus, responsible for contextual learning and memory, amygdala, involved in emotional learning and medial

prefrontal cortex, which processes decision-making, planning and controls impulses (Koob and Le Moal 2001).

Positive reinforcement has been associated with the activation of mesocorticolimbic dopamine system including VTA-NAc projections, whereas negative affect related to withdrawal involves decreased activity of this system and activation of the CRF and dynorphin in the amygdala (**Figure 4**; Koob et al. 2014). These so called 'anti-reward' circuits are recruited as systemic neuroadaptations during the development of addiction and produce aversive or

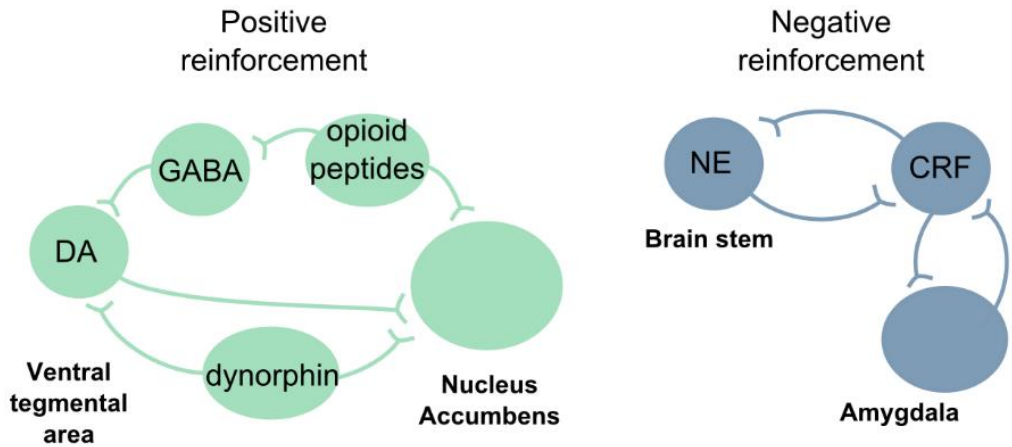

Figure 4. Simplified neurocircuitry framework for the systemic neuroadaptations hypothesized to mediate the transition to addiction. Excessive activation of the brain reward circuitry with major contributions from mesolimbic dopamine and opioid peptides that converge on the nucleus accumbens activates dynorphin in the ventral striatum, that in turn suppresses dopamine release and may lead to negative, dysphoric-like effects of drug withdrawal. Concurrently, activation of the brain stress systems in the amygdala during withdrawal (CRF, norepinephrine and dynorphin) sensitize through feed-forward mechanisms and also contribute to the negative emotional, anxiety-like effects of drug withdrawal. DA- dopaminergic neuron, GABA- VTA GABAergic interneuron, NE- norepinephrine, CRF- corticotropin releasing factor.

stress-like effects through at least two mechanisms: activation of stress response that induces fear-like and anxiety states in the extended amygdala and suppression of dopamine that may result in depressive-like states (Nestler 2001; Koob 2003). Therefore, use of drugs of abuse lead to attempts of the brain stress system to maintain stability, but at a cost. Its prolonged activation produces negative emotional state that represents a combination of recruitment of anti-reward systems and consequent chronic decreased function of reward circuits, both of which lead to the compulsive drug seeking and loss of control over intake.

Among the factors that may influence drug addiction, the stress response is known to potentiate behavioral responses to drugs of abuse in humans (Sinha 2009; Sinha 2013) and in animal models (Lu et al. 2003). Early studies demonstrated the involvement of glucocorticoids in this process. It was presented that corticosterone secretion is one of the mechanisms by which stress amplifies behavioral responses to stimulants and opiates (Deroche et al. 1992; Deroche et al. 1993). Blockade of corticosterone secretion reduces the locomotor activity induced by infusions of morphine and cocaine in the VTA and NAc (Deroche et al. 1995; Marinelli et al. 1998). Inactivation of the GR gene (*Nr3c1*) in the entire brain markedly decreases stress-related behavior, including anxiety and motivation to self- administer cocaine (Tronche et al. 1999; Deroche-Gamonet et al. 2003). It was later proposed that glucocorticoids facilitate behavioral effects of cocaine through mesolimbic dopamine circuit (Piazza and Le Moal 1996). In attempt to investigate the role of GR in addiction, several transgenic mice generations that under-express GR in different types of neurons were generated and described. Genetic ablation of the GR restricted to dopaminergic neurons had no effect on their firing activity and rewarding effects of cocaine (Ambroggi et al. 2009). However, transgenic mice with GR deleted in dopaminoceptive neurons resulted in insensitivity to psychomotor action of cocaine, and diminished cocaine-conditioned behaviors (Ambroggi et al. 2009). It had been later presented, that in

fact GR inactivation in dopaminoceptive neurons partly alters molecular and behavioral responses to psychostimulants, but not opiates (Barik et al. 2010; Parnaudeau et al. 2014). Overexpression of GR in the hippocampus and NAc in young animals led to increased anxiety and cocaine sensitization (Wei et al. 2012). GR brain signaling is also implicated in the modulation of somatic expression of drug withdrawal. $CRF^{-/-}$ mice, that display basal and stress-related plasma corticosterone deficits (Timpl et al. 1998), lack the negative affective states opiate withdrawal (Contarino and Papaleo 2005), but present increased somatic expression of somatic withdrawal (Papaleo et al. 2007). In concordance, adrenalectomy increased somatic signs of morphine withdrawal and impaired naloxone-induced conditioned place aversion (García-Pérez et al. 2016; García-Pérez et al. 2017). On the other hand, mifepristone, a GR antagonist, was shown to alleviate the somatic signs of naloxone-precipitated opiate withdrawal (Navarro-Zaragoza et al. 2012). Altogether, these experiments presented that glucocorticoids mediate physical signs of opiate withdrawal and play an essential role during the formation of aversive memory consolidation that is associated with withdrawal.

Impact of glucocorticoids in pathology: from adaptive to harmful. The primary function of the HPA axis is adaptation to stressors, but glucocorticoid action can change from protective to harmful under conditions of extreme or chronic stress. Inadequate or prolonged HPA axis stimulation associated with persistent glucocorticoids elevation can make the reinstatement of homeostasis unattainable, a condition that is termed 'allostatic load' (McEwen 2000). As a consequence, HPA reactions may become inadequate and coping and adaptation may become less efficient. A number of possible explanations involving stress hormones and receptors have been put forth to answer how this phenomenon could occur and its relevance in the development of pathological behavior (Oitzl et al.

2010). One of the hypotheses points out that chronic stress induces an excess glucocorticoid release and a compensatory downregulation of GRs in the brain. The final outcomes of this mechanism are dramatic physiological changes and cellular damage to the brain, observed, for example, as decreased cell survival, proliferation, and neurogenesis, morphological changes and decreased hippocampal volume (Müller et al. 2005; Joëls et al. 2007).

Another hypothesis proposes that alterations in behavior and HPA axis signaling are connected to dysfunctional signaling in neural circuits caused by an imbalance in the MR:GR ratio in specific brain regions, such as limbic system. The MR:GR balance can be affected by behavioral, genetic, and epigenetic factors. Results from experiments using transgenic mice associate changes in MR and GR levels with anxiety and depressive-like behaviors (Gass et al. 2001; Chourbaji and Gass 2008). Overexpression of GR in the forebrain neurons in mice resulted in increased emotional lability (Wei et al. 2004), while a depressive-like phenotype has been described in mice with a forebrain complete GR knock-out (Boyle et al. 2005). Interestingly, overexpression of MR in forebrain neurons in mice caused decreased anxiety-like behavior and decreased GR expression in the hippocampus (Rozeboom et al. 2007). One interpretation of these data is that increasing steroid receptor availability may increase the magnitude of glucocorticoid-mediated regulation; alternatively, increasing one receptor may disrupt signaling to the other receptor. How is the MR:GR balance associated with vulnerability and resilience to disease? It is proposed that MR and GR gene variants modulate the stress responses (van Rossum et al. 2005; Klok et al. 2011). An example is the 'loss of the function' MR gene variant, which enhances the neuroendocrine response to psychosocial stressors, and is associated with feelings of depression in the elderly (de Rijk et al. 2006; Kuningas et al. 2007). Secondly, stressors during adult life, like traumatic event, or chronic stress serve as triggers for the development of psychopathologies in predisposed individuals (de Quervain 2006; Joëls et al.

2007). Recent evidence also revealed that early-life programming events, such as prenatal stress and mother–infant interactions enhance individual vulnerability to stress through activation of specific epigenetic processes related to GR promoters (Seckl and Holmes 2007; Meaney et al. 2007). In fact, the effects of maternal care on hippocampal GR expression are associated with an epigenetic modification of the neuron-specific GR promoter (Weaver et al. 2004; Weaver 2009). Recent human study reported that hippocampal GR gene expression was decreased in suicide victims with a history of childhood abuse (McGowan et al. 2009). These data translate findings in rats to humans, suggesting a common effect of parental care on the epigenetic regulation of hippocampal GR expression. These hypotheses are not mutually exclusive, and a combination of them likely occurs in conditions of chronic stress and disease involving excesses of glucocorticoids.

Glucocorticoid regulation in astrocytes. Given the independent association of stress signaling and astrocytes with addiction, the interaction of glucocorticoids and astrocytes may be relevant to understanding mechanisms underlying reward-related biology. More generally, astrocytes may also play a role in modulating stress signaling via their response to glucocorticoids. A limited literature on glucocorticoid regulation in astrocytes indicates stress hormones do have specific effects on this cell type. Astrocytes are known to express both MR and GR (Bohn et al. 1991) and thus are sensitive to glucocorticoid regulation.

In terms of transcriptional regulation, multiple mRNAs associated with astrocytes (e.g. GFAP, *Glul*, *Slc1a2*) have been shown to be regulated by glucocorticoids in cultured astrocytes (Rozovsky et al. 1995; Zschocke et al. 2005) and in the brain (O'Callaghan et al. 1989). Recent whole genome microarray study *in vitro* showed that glucocorticoids robustly regulate mRNAs in astrocytes. The study found about 400 genes altered by dexamethasone, a selective GR agonist. Regulated genes are involved in multiple aspects of cell metabolism (e.g. *Slc2a1*,

Pdk4 and *Slc45a3*) and the inflammatory response (e.g. *Il1b*, *Tnf*; Piechota et al. 2017). How alteration of these mRNAs in astrocytes impacts brain physiology is currently not well characterized.

Recent study attempted to investigate glucocorticoid regulation of astrocytic function in the hippocampus (Yu et al. 2011). It presented that, unlike hippocampal neurons, astrocytes are resistant to glucocorticoid-induced apoptosis and that glucocorticoids influence hippocampal cell fate by inducing the expression of astrocyte-derived growth factors implicated in the control of neural precursor cell proliferation. Glucocorticoids have also been shown to decrease glutamate uptake in cultured astrocytes (Virgin et al. 1991) and modulate astrocytic calcium signaling via cytosolic receptors (Simard et al. 1999). Most of the functional impact of glucocorticoids in astrocytes has been demonstrated *in vitro*; the modulation of astrocytes *in vivo* remains to be investigated.

There is strong clinical and preclinical data suggesting that glial cells are involved in stress-related pathologies (Jauregui-Huerta et al. 2010). Recent human postmortem studies strongly suggest that deficits in astrocyte density in the limbic regions of the brain contribute to the pathology of stress and glucocorticoid overproduction (Rajkowska and Miguel-Hidalgo 2007). Parallel to these findings, animal experiments showed that chronic stress results in reduced gliogenesis in the hippocampus and prefrontal cortex (Czéh et al. 2007). Moreover, *in vitro* studies showed that dexamethasone, GR agonist, selectively blocks spontaneous astrogliogenesis from neural precursor cells (Sabolek et al. 2006).

The amount of evidence accumulated over recent years regarding the responses of glial cells to stress and glucocorticoids is likely to change our view on the role of these cells in the neurobiology of the stress response. Accumulated evidence indicates an unknown role of glia in the central response to stress and glucocorticoids and much remains to be done to fully understand how the glia-glucocorticoid interactions produce pathological or neuroprotective effects that

may play an important role in posttraumatic stress disorder, depression, addiction and other stress-related pathologies.

The role of astrocytes in drug abuse. As previously mentioned, recent research presented that astrocytes control synaptic connectivity, neurotransmission, metabolism and release several chemical transmitters that can modulate the activity of neighboring neurons and other glial cells (see: Chapter 1.3). Astrocytes express receptors for most neurotransmitters and peptides (Miguel-Hidalgo 2009), therefore it is not surprising that many drugs can affect astrocytic physiology. Exposure to cocaine and morphine leads to reactive astrocytosis and altered GFAP expression (Bowers and Kalivas 2003; Narita et al. 2006). GFAP is generally upregulated in response to brain injury and neurotoxicity (Hill et al. 1996), although many other plastic changes in the brain also result in increased GFAP expression (Steward et al. 1991; Minn et al. 1998). Chronic treatment with morphine also results in increased GFAP expression or enlarged astrocytes in several brain regions, like VTA, NAc, frontal cortex and locus coeruleus (Beitner-Johnson et al. 1993; Marie-Claire et al. 2004; Garrido et al. 2005). In addition, astrocytes were reported to play a role in the development of tolerance to analgesic effects of morphine. Inactivation of astrocytes by the gliotoxin fluorocitrate attenuates both tolerance to morphine analgesia and morphine-induced increase in GFAP density in the spinal cord and the hippocampus (Song and Zhao 2001). Tolerance to morphine has been also related to downregulation of glial glutamate transporters GLT-1 and GLAST in the spinal cord (Mao et al. 2002).

Another possible contribution of astrocytes to the effects of drugs of abuse might be mediated through water channels composed of the protein aquaporin-4. Aquaporin-4 is mostly localized to the plasma membranes of the astrocytic endfeet that wrap blood vessels, and aquaporin-4 channels are considered a main route for water regulation in the brain (Fan et al. 2005). Aquaporin-4 knockout mice

reportedly presented attenuated locomotor activity caused by either single or repeated administration of cocaine as well as reduction of dopamine and glutamate levels in the NAc was observed (Li et al. 2006).

Recent research on the role of astrocytes in drug addiction has used medium collected from cultured astrocytes to determine whether there are soluble factors released by astrocytes that might alter the rewarding effects of methamphetamine and morphine. These studies showed that the astrocyte conditioned medium infused into the NAc was sufficient to enhance the rewarding effects by induced by both drugs (Narita et al. 2005; Narita et al. 2006), presenting a direct evidence of astrocytic modulation in the development of reward-related behavior.

Another area of considerable interest is the role of astrocytic cystine/glutamate transporters that are altered following cocaine exposure (Baker et al. 2002; Kalivas et al. 2003). Withdrawal from repeated cocaine self-administration causes extracellular glutamate levels fall due to reduced function of the astrocytic cystine/glutamate transporters. This loss of glutamate correlates with the sensitivity to drug relapse following a re-exposure to cocaine. It was shown that exogenous cysteine administration (that stimulates cystine uptake and glutamate release) prevented cocaine-primed reinstatement of drug seeking behavior (Baker et al. 2003). Glutamate transport activator, MS-153, co-administered with morphine, methamphetamine or cocaine significantly reduced conditioned place preference in mice, without altering their locomotor responses (Nakagawa et al. 2005). Thus it seems that drug exposure can change protein expression in astrocytes and the activity of glial transporters which can have critical consequences on drug-seeking behavior (Haydon et al. 2009). Clinical studies proposed that alteration of glutamate transport mediated by astrocytes might be also related to genetically inherited vulnerability to alcoholism (Sander et al. 2000; Foley et al. 2004).

Although it is clear that neurons are the principal players that ultimately integrate and execute behavioral, cognitive and emotional consequences accompanying drug addiction, there is significant evidence demonstrating that astrocytes play active role in the regulation of synaptic transmission and synaptic plasticity. Therefore, the involvement of glial cells must be taken into account to fully understand adaptations of the brain that underlie addictive actions of substances of abuse.

Chapter 2 Research issue and aims

Given the established link between the glucocorticoid system and addiction and the emerging evidence for astrocyte pathology in drug abuse, further comprehension of glucocorticoid action in astrocytes would broaden our understanding of cell type specific contribution to glucocorticoid signaling and may set new directions for research in the field of stress related behaviors. Majority of research on involvement of glucocorticoids in addictive behaviors thus far was focused on neuronal actions of glucocorticoids. Although it is recognized that astrocytes can control synaptic plasticity and express glucocorticoid receptor (GR), the involvement of astrocytic GR in the modulation of animal behavior and synaptic transmission has not been yet explored. The global goal of the present thesis was therefore to investigate the specific function of glucocorticoid receptor in astrocytes in the mechanism of opioid action, as well as development and expression of stress related disorders, such as addiction, posttraumatic stress disorder and depression.

The specific objectives of the present study were:

I. To identify behavioral patterns and molecular markers accompanying lifelong opioid treatment. We aimed to compare the development of opioid dependence with the effects of a natural reward (saccharin) and to analyze behavioral patterns in animals exposed to several months of uninterrupted access to the drug or the sweetener under 24 h monitoring and minimal intervention by the experimenter. Moreover, we sought to determine differences in gene expression profiles between

morphine-dependent and non-dependent animals and to evaluate the influence of long-term voluntary morphine or saccharin intake on drug-induced transcription. To analyze the abovementioned changes, we introduced a new model of chronic morphine self-administration using automated cages. Among other observations, our results pointed out to possible relevance of glucocorticoid action in astrocytes in the development of opioid addiction.

II. To explore the functional significance of GR in astrocytes. Based on previous literature, our hypothesis was that glucocorticoids regulate specific mRNAs in different cell-types *in vivo*, and that perhaps a portion of mRNAs regulated by glucocorticoids in astrocytes are uniquely regulated in this cell type compared to neurons. We have aimed to establish global impact of glucocorticoid regulation on astrocytic and neuronal transcriptome in the NAc with the use of whole-genome microarrays.

III. To evaluate whether astrocytic GR might contribute to the modulation of animal behavior. It is recognized that astrocytes can control synaptic plasticity and express GR, we therefore hypothesized that GR located in astrocytes might influence stress- and opioid- related behaviors. To test this hypothesis, we aimed to specifically eliminate GR from astrocytes in the brain of adult mice. To achieve that, we used two distinct approaches:

- **A.** We have generated new transgenic mouse line, based on CreERT2/loxP technology that allowed for conditional GR knockout in astrocytes.
- **B.** We have selectively targeted astrocytic GRs in the NAc *in vivo* using lentiviral vectors in Aldh1L1Cre transgenic mice

Chapter 3 Materials and methods

3.1 Animals

Mice used for experiments were housed in Plexiglas home cages with 2-5 mice per cage in a humidity- and temperature- (22±1ºC) controlled room, under a 12-h light/dark cycle (lights on at 7:00 am). The animals had free access to water and food (standard diet, Special Food Services, England), unless stated differently in the methods descriptions. For 5 days prior to the start of the experiments, mice were habituated to the experimenter's grasp. In experiments we have used C57BL/6J mice (Jackson Laboratories, USA), and 2 strains of transgenic mice, Aldh1l1Cre and Cx30xGR$^{flox/flox}$. All the experiments were conducted on male mice, with the exception of the experiments conducted in the IntelliCage system, to which we have used female mice. All experimental procedures complied with the requirements of the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the II Local Bioethics Commission in Krakow, Poland (permit numbers: 969/2012, 970/2012, 1152/2015, 1156/2015, 1285/2015, 1305/2016).

Conditional GR knockout mice. Mice for selective, inducible elimination of GR from astrocytes were obtained by breeding a transgenic line carrying tamoxifen (TAM)-inducible version of Cre recombinase driven by astrocyte-specific promoter (Tg(Cx30-CreERT2)T53-33Fwp line, (Slezak et al. 2007)), called Cx30-CreERT2 with the transgenic line carrying critical exons of gene encoding glucocorticoid receptor (*Nr3c1*) flanked by loxP sites (Nr3c1tm2Gsc, (Tronche et

al. 1999), called $GR^{lox/lox}$. Pups originating from the mating were genotyped for the presence of Cre (Slezak et al. 2007) and GRlox locus (Tronche et al. 1999). Bigenic animals of 8-10 weeks were treated once daily for 5 days with 100 mg/kg tamoxifen (ip, Sigma, 1:9 solution of ethanol and sunflower, respectively). Behavioral experiments started at least 3 weeks after TAM injection to ensure the efficient elimination of the GR protein.

Astrocytic GR knockdown mice. To generate selective GR knockdown in astrocytes we have used transgenic mice expressing the Cre recombinase under aldehyde dehydrogenase 1 family promoter (Aldh1l1Cre; Tien et al. 2012). Animals were purchased from Jackson Laboratories (USA), bred congenically to C57BL/6N mice (Charles River, USA) and housed in the animal house of Institute of Pharmacology, PAS, Krakow, Poland. This transgenic strain enables tissue-specific excision of floxed genomic segments in astrocytes. The animals were intracerebrally injected with a lentiviral vector harboring Cre-dependent shRNA expression cassette for silencing astrocytic GR. In this lentiviral vector, DNA stuffer sequence is placed between a U6 pol III promoter and the shRNA sequence. After Cre-mediated excision, the stuffer sequence is removed, and the functionality of the U6 promoter is restored, permitting transcription of shRNA and gene knockdown (Wiznerowicz, Szulc, and Trono 2006). For all experiments, animals were injected with LV-pSico-shGR or LV-pSico-shGFP (control) constructs, and tested at least 3 weeks post surgery. Stereotactic surgery was performed essentially following previously described methods (Tashiro, Zhao, and Gage 2007) and was conducted when the youngest animals in cohort turned 10 weeks old. All animals were injected bilaterally into the nucleus accumbens (anterior +1.0 mm, lateral: ±1.4 mm, ventral: -4.2 mm relative to Bregma), with an injection volume of 1 µl.

3.2 Behavioral procedures

Mice were handled by experimenter for 3 days before any behavioral test. All behavioral tests were analyzed by automated cages or video-recorded and assessed by an experimenter unaware of genotype and/or treatment of the animals.

Locomotor activity. Animals were placed in activity chambers (10 cm × 20 cm × 10 cm) equipped with photocells (custom made for the Institute of Pharmacology PAS). Horizontal (ambulatory counts) activity was measured in 30 min sessions for each animal to assess basal locomotor activity.

Novel object recognition test. The test was performed in an open field arena. The objects to be discriminated were a plastic toy (2- 2.5 cm diameter) and a plastic dice (2 cm). Animals were first habituated to the open-field for 30 min, during locomotor activity test. The next day, they were submitted to a 10-min acquisition trial (first trial), during which they were individually placed in the open field in the presence of an object A (toy or dice) placed in one of the two presentation positions (in the corner, 5 cm from side walls). The time taken by the animal to explore the object A (when the animal's snout was directed toward the object at a distance <1 cm) was manually recorded. Next, animals were tested in the 10-min retention trial (second trial) that occurred 24 h later. A recognition index was defined as [tB/ (tA + tB)] x 100, where tB/tA indicate the times the mouse spent exploring the object B/A, respectively. The type of the objects and their positions of presentation during acquisition and retention phase were counterbalanced across animals.

Y maze. The Y-maze test was performed as previously described (Wietrzych et al., 2005). The apparatus consisted of three identical arms. For each mouse, the

three arms of the Y-maze were randomly pre-assigned as the ‘start’ arm. The mouse was placed in that arm and allowed to freely explore the apparatus for 5 minutes. Mice exploratory behavior was assessed visually by scoring the successive entries into each of the three arms in overlapping triplet sets. Spontaneous alternation performance (SAP) was calculated as the percentage ratio of actual number of three successive different arms entries to possible number of triplet sets (total arm entries minus two). The alternate arm returns (AARs) and same arm returns (SARs) were also scored for each animal.

Automated behavioral procedures in the IntelliCage system. The experiments were conducted in two automated IntelliCages (New Behavior, Switzerland; software version 2.17.1.0, 2013 New Behavior AG), that allows for constant 24-hour monitoring of individual animals behavior with minimal intervention of the experimenter. A diagram of the cage is presented in **Figure 5a**. Each corner of a cage is equipped with presence detectors, an antenna that reads RFID chip signals and two photocell-equipped holes that control access to bottles containing water or another liquid. Instrumental responses toward a hole (nose pokes) opened previously closed gate and allowed the animal to drink from the bottle. The numbers of visits in the corners, nose pokes and licks on the bottle were recorded. Additionally, the nozzles of tubes connected to tanks of compressed air located above each corner could be used to deliver air puffs to the animals' backs. Before the experiments began, animals were implanted with transponders (UNO PICO ID, AnimaLab, Poland) and introduced to IntelliCages in groups of 12. All mice that lost their transponders (which prevented the behavior from being recorded) received replacement transponder chips. The mice were allowed to habituate to the cages for 7 days prior to the initiation of the experiments. Access to the bottles in the corners required an instrumental response of 3 nose pokes (FR3) or more, depending on the experimental series.

Development of opioid dependence. After adaptation period, rewarding substances were introduced. In one of the cages animals could choose between water and saccharin solution, whereas in the other cage mice had a choice between water and sweetened morphine, however sweetener was then gradually withdrawn. In the cages animals had access to two corners containing tap water and two corners containing rewarding substance (as presented in **Figure 5a**). Drugs administration have been carried out in 6 phases: 1) adaptation period: tap water available in all corners of both cages; 2) 2 mg/ml saccharin introduced to both groups of animals; 3) morphine cage: combined 2 mg/ml saccharin and 0.3 mg/ml morphine solution; saccharin cage: 2 mg/ml saccharin; 4) morphine cage: combined 2 mg/ml saccharin and 0.5 mg/ml morphine solution; saccharin cage: 2 mg/ml saccharin; 5) morphine cage: combined 1 mg/ml saccharin and 0.5 mg/ml morphine solution; saccharin cage: 1 mg/ml saccharin; 6) morphine cage: 0.5 mg/ml morphine solution with no saccharin; saccharin cage: 0.2 mg/ml saccharin. The duration of each phase was dictated by the time in which animals reached stable preference of drinking (7- 21 days). Experimental schedule is presented in **Figure 5b**.

Progressive ratio instrumental schedule. Progressive ratio (PR) schedules required the animals to perform increasing numbers of operant responses to obtain access to the bottles containing morphine (0.5 mg/ml) or saccharin (0.2 mg/ml). The number of required responses was increased by 1 (PR1) or 3 (PR3) each time the animal gained access to the bottles. During the subsequent 12 h sessions, the animals had free access to water in two of the corners, and the remaining two corners had bottles with rewarding substance that required PR1 or PR3 responses. There was no time limit to perform the PR task, but the task had to be completed within one visit to a corner. PR1 and PR3 procedures were applied during two independent sessions that were separated by an interval of a few days.

Intermittent reward access procedure. In the intermittent reward access procedure, the access to the bottles containing the rewarding substances was restricted for two hours. During this period, the mice were able to visit the corners and make nose pokes, but the gates that barred the access to the bottles would not open regardless of the number of performed nose pokes. Mice were provided continuous access to the bottles that contained water. The numbers of individual visits, drinking events and bottle licks were calculated as sums for each corner that contained either morphine, saccharin or water.

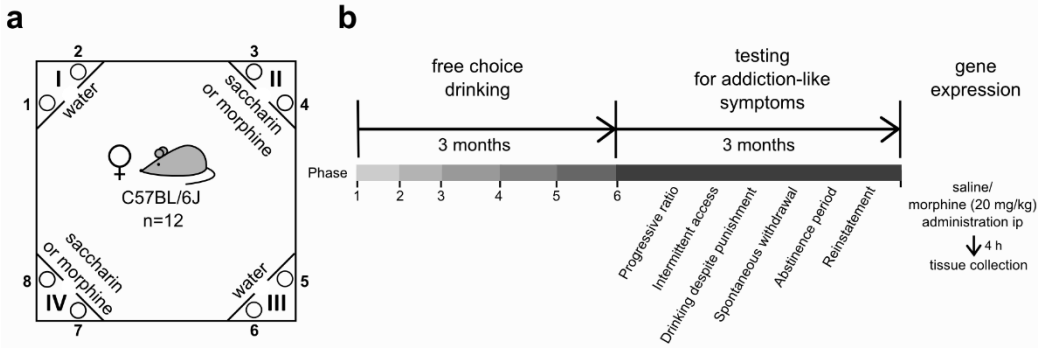

Figure 5. Experimental conditions and design. (**a**) The IntelliCage system. The corners are marked with Roman numerals, and the bottles are numbered clockwise from 1 to 8. (**b**) After an adaptation period (phase 1), 2 mg/ml saccharin was introduced to both cages (phase 2). Afterwards, in one of the cages, increasing concentrations of morphine were introduced (0.3 mg/ml in phase 3 and 0.5 mg/ml in phase 4), and in both of the cages, the saccharin concentration was gradually lowered (1 mg/ml in phase 5; and 0.2 mg/ml in saccharin cage but no saccharin in the morphine cage in phase 6). After phases 1-6 of the experiment, the animals were subjected to behavioral procedures: a progressive ratio procedure on reward-associated and water-control corners, intermittent access to reward, a procedure involving the risk of punishment, a 14 day period of abstinence, a 28 day period of reinstatement followed by the ip administration of either saline (10 ml/kg) or morphine (20 mg/kg), and tissue collection.

Drinking despite the risk of punishment. An experiment modeling the drinking of morphine or saccharin under the risk of punishment was conducted after the completion of PR and the intermittent access to a reward test. During a 12 h testing

session, the animals were provided with FR3 access to morphine or saccharin that was associated with a 25% risk of punishment in two corners and FR3 access to water without punishment in the remaining two corners. The punishment consisted of a 0.5 bar air puff that was delivered to the animal's back 2 s after the FR3 was completed. The punishment intensity was adapted from the work of (Smutek et al. 2014). The air puff was not harmful but aversive.

Opioid withdrawal. Spontaneous signs of opioid withdrawal were measured as part of experiments with the use of the IntelliCage system. Access to morphine or saccharin was restricted for 14 consecutive days after the completion of the automated behavioral procedures. Opioid withdrawal symptoms were measured during first 54 hours of the abstinence period. Mice were individually observed continuously for the occurrence of spontaneous somatic signs of opioid withdrawal for 15 minutes at each time point. Withdrawal behaviors were evaluated, including: teeth chattering, wet-dog shaking, paw tremor, rearing and jumping.

Naloxone-precipitated opioid withdrawal after chronic morphine treatment was evaluated as previously described (Parkitna et al. 2012) by measuring behavioral manifestations of naloxone-precipitated withdrawal in mice treated with chronic morphine in growing doses. Morphine was administered subcutaneously (sc) 3 times daily for 4 days in doses: day 1:10 mg/kg, day 2: 20 mg/kg, days 3-4: 40 mg/kg. On day 5 mice received single injection of 40 mg/kg morphine. Three hours after the last morphine treatment, each mouse was injected with naloxone (4 mg/kg, ip) and placed in a Plexiglas tube for 15 min. Withdrawal behaviors were evaluated as described above.

Tail-flick test for analgesia. Animals were individually placed in a tail-flick apparatus (Analgesia Meter; Ugo Basile, Italy). A focused beam of light projected from light bulb was applied directly to dorsal part of the tail, and a digital timer

measured the latency to raise the tail. To avoid the possibility of tissue injury, the cut-off latency was set to 9 s. Latency to reflexively withdraw the tail was measured twice, with each test separated by a minimum of 15 s. The two test results were later averaged.

Morphine-induced analgesia in morphine-tolerant mice. The pain threshold during the development of tolerance to the analgesic effect of morphine was assessed using the tail-flick test as described above. To induce tolerance, mice received 3 daily injections of morphine in growing doses for 4 days: day 1:10 mg/kg, day 2: 20 mg/kg, days 3-4: 40 mg/kg. Pain sensitivity was measured 1 day prior to and 1 day after the chronic morphine administration. Baseline tail-flick measurements were performed 15 min before morphine injection. Animals were tested for analgesia 30, 90 and 180 min after single ip morphine (2.5 or 5 mg/kg) administration.

Conditioned place preference. Procedure was performed in place-preference boxes with three distinct, neutral compartments (Med Associates, USA). On days 1 and 5, during the pre- and post-conditioning tests, mice were placed in the center compartment allowed to explore the apparatus freely for 20 min. Assignment of mice to the compartments was unbiased. On days 2-4 morphine-paired animals received saline injection in each morning session, and morphine (5 mg/kg, sc) in each afternoon session. Saline-paired animals received saline injections during both sessions. Each conditioning session lasted 40 min. The CPP scores are presented as the increase of time spent in the paired chamber between post-and preconditioning tests.

Morphine-induced locomotor sensitization. For the measurement of morphine-induced locomotor sensitization, animals received daily injections of saline or

morphine (5 mg/kg, sc) in 2 h sessions for 6 days in locomotor activity chambers, described above. Expression of morphine sensitization was tested 7 days following the cessation of morphine administration. All animals were habituated to the locomotor activity chambers for 2 h 1 day before the onset of the experiments.

Tail suspension test. Mice were suspended by the tail with adhesive tape from an aluminum bar that was set at a height of 30 cm from the ground. The total duration of immobility was calculated over the 6 min of the test.

Saccharin preference test. During this test, mice were individually housed and given a free choice between two bottles: one with 0.1% saccharin solution and the other with tap water, for 24 hours. To avoid the possible effects of side preference in drinking behavior, the position of the bottles was changed after 12 hours. No previous food or water deprivation was applied before the test. The consumption of water and saccharin solution was estimated simultaneously in the control and experimental groups by reading the amount of consumed fluid from the scale in the marked bottles. The preference for saccharin was calculated as a percentage of saccharin solution compared to the total amount of liquid consumed.

Light/dark box test. During the light/dark box test, each mouse was placed in the middle of the dark compartment of the apparatus, consisting of two compartments (20 × 20 × 14 cm each), one of which was made of black Plexiglas and was lit by a dim light (50 lux). The other compartment was made of white Plexiglas and was illuminated with a lamp (300 lux). The 5-minute trials were video-recorded and analyzed using ANY-maze software (Stoelting Co., Wood Dale, IL, USA).

Shock application and test of conditioned fear. Procedure was similar to a previously described protocol (Szklarczyk et al. 2016). Briefly, the test comprised of 2 parts: fear conditioning training and a series of memory retrieval tests. The training consisted of a 2 min acclimation to an automated shock chamber (Ugo Basile, Italy), followed by the application of five footshocks (1 mA, 2 s each, separated with 1 min intervals). After the training animals were kept separately until all mice from the home cage had completed training. During intershock intervals, freezing (immobility, except for respiratory movements) was measured as an expression of fear learning. At 24 h, 72 h and 120 h after conditioning, mice were placed in the conditioned fear context, and freezing was measured for 3 min as an expression of contextual fear memory. To maximize contextual conditioning, the external conditions (odor, lighting, time of day) remained unaltered throughout the duration of the tests. The freezing data were recorded, stored, and analyzed using ANY-maze software (Stoelting, USA).

3.3 Biochemical analysis

Cell culture. Brain hemispheres were dissected from 5-6-day-old C57BL/6J mice and dissociated into single-cell suspension using Neural Tissue Dissociation Kit (T) (Miltenyi Biotec, Germany) according to the manufacturer's protocol. Astrocytes were isolated by magnetic sorting using Anti-Glast Microbead Kit and MS Columns (Miltenyi Biotec, Germany). Cells were cultured on polyornithine-coated dishes (Sigma, USA) in DMEM (Life Technologies, USA) supplemented with 10% fetal bovine serum (Life Technologies, USA) and penicillin/streptomycin (Life Technologies, USA) under standard conditions.

Lentiviral vectors. To produce the lentiviral vectors shRNA sequences: control (non-mammalian targeting) and targeting GR (TRC library clone ID NM_008173.3-2119s21c1) were cloned into pSico lentiviral plasmid (a gift from Tyler Jacks; Ventura et al. 2004; Addgene plasmid # 11578). Vectors were produced in the core facility of Nencki Institute of Experimental Biology in Warsaw. Titers of both viruses were comparable and ranged between 1.13×10^8 and 2.19×10^8 transducing U ml-1. Virus suspensions were stored at -70 °C until use, and were kept on ice immediately before injection. For *in vitro* validation of LV-pSico-shGR, astrocytes were co-transduced with lentiviral vectors expressing Cre recombinase (LV-Cre pLKO.1, Addgene plasmid #25997) in the presence of 4 μg/ml polybrene (Sigma, USA). 96h post-infection cells were stimulated with 100 nM dexamethasone for 4h.

Immunofluorescence staining. Mice were perfused with 4% PFA, and brains were immediately transferred to 30% sucrose in PBS and incubated overnight. Coronal sections were cut on a vibratome and incubated, free-floating in PBS overnight. Subsequently, brain slices were blocked in 5% normal goat serum (NGS) and incubated in a mixture of primary antibodies for GR and GFAP, in 0.25% PBST overnight at 4°C. After washes, sections were further incubated in a mixture of secondary antibodies (anti-rabbit Alexa fluor 633 for GR and anti-mouse Alexa fluor 555 for GFAP) made up in PBS for 2 hr at room temperature. Subsequently, sections were washed, mounted, and coverslipped using vectashield mounting medium for fluorescence with DAPI (H-1200). Fluorescent signals were examined using a confocal laser fluorescence microscope system (Nikon, USA).

Tissue collection and RNA preparation. All animals were accustomed to saline injections for 5 days prior to actual drug administration and tissue collection.

For the analysis of the effects of chronic opioid self-administration, mice in each cage were randomly divided into two groups, one receiving saline and the other morphine, therefore we have analyzed 4 groups: animals chronically drinking saccharin with a saline or morphine injection, and mice chronically drinking morphine with saline or morphine injection. Animals were sacrificed 4 hours after receiving either a saline (10 ml/kg, ip) or morphine (20 mg/kg, ip) injection. Brains were removed immediately after decapitation and tissue samples containing the rostral part of the caudate putamen and the nucleus accumbens (referred to hereafter as the striatum) and frontal cortex were collected. Tissue samples were placed in individual tubes with the tissue storage reagent RNAlater (Qiagen Inc., USA) and preserved at -70°C.

GR knockdown animals were sacrificed 4 hours after receiving either saline (10 ml/kg, ip) or dexamethasone (4 mg/kg, ip) injection. Brains were removed immediately after decapitation and stored in RNAlater (Qiagen Inc., USA). The brains were cut into 150 μm slices and tissue samples containing the nucleus accumbens were collected. Tissue samples were verified for lentiviral vector presence under immunofluorescent microscope. Samples, in which nucleus accumbens was verified as sufficiently transduced (≥60%), were placed in individual tubes with the tissue storage reagent RNAlater and preserved at -70°C.

In the experiments with the use of astrocytic GR knockout mice, CNS structures were isolated from experimental mice, immediately immersed in RNAlater solution (Qiagen Inc., USA) and stored in -70°C until isolation.

All collected samples were thawed at room temperature and homogenized in 1 ml Trizol reagent (Invitrogen, USA). RNA was isolated following the manufacturer's protocol and further purified using the RNeasy Mini Kit (Qiagen Inc., USA). The total RNA concentration was measured using a NanoDrop ND-1000 Spectrometer (NanoDrop Technologies Inc., USA). RNA quality was determined using chip-based capillary electrophoresis with an RNA 6000 Nano

LabChip Kit and an Agilent Bioanalyzer 2100 (Agilent, USA) according to the manufacturer's instructions.

Quantitative PCR. The qPCR reactions were performed using Assay-On-Demand TaqMan probes (Applied Biosystems, USA) and run on the CFX96 Touch Real-Time PCR machine (BioRad, USA). cDNAs were diluted 1:10 with H2O, and approximately 50 ng of the cDNA synthesized from the total RNA template from each animal was used for each reaction. To reduce the contribution of contaminating genomic DNA, primers were designed to span exon junctions. In addition, control reactions without the RT enzyme were performed for each assay. The amplification efficiency for each assay was determined by running a standard dilution curve. The expression of the hypoxanthine guanine phosphoribosyltransferase 1 (*Hprt1*) transcript was quantified at a stable level between the experimental groups to control for variations in cDNA amounts. The cycle threshold values were calculated automatically by CFX MANAGER v.2.1 software with default parameters.

Gene expression profiling. A starting amount of 200 ng of high-quality total RNA (samples from individual mice were hybridized to separate microarrays) was used to generate cDNA and cRNA with an Illumina Total Prep RNA Amplification Kit (Illumina Inc., USA). The procedure consisted of reverse transcription using an oligo (dT) primer bearing a T7 promoter with an Array-Script. The obtained cDNA became a template for *in vitro* transcription with T7 RNA polymerase and biotin UTP, which generated multiple copies of biotinylated cRNA. The purity and concentration of the cRNA were checked using an ND-1000 Spectrometer. High-quality cRNA was then hybridized using Illumina's direct hybridization array kit (Illumina). Each cRNA sample (1.5µg) was hybridized overnight to MouseWG-6 v2 BeadChip arrays (Illumina) in a multiple-

step procedure according to the manufacturer's instructions. The chips were washed, dried and scanned on a BeadArray Reader (Illumina). Raw microarray data were generated using BeadStudio v3.0 (Illumina). To provide appropriate balance within the whole dataset, groups were equally divided between the array hybridization batches. Microarray quality control was performed using BeadArray R package v2.14.1. The data were normalized using quantile normalization and then log2-transformed. The obtained signal was taken as the measure of mRNA abundance that was derived from the level of gene expression.

Evaluation of serum corticosterone (CORT) levels. Endogenous CORT blood serum levels were determined using a CORT rat/mouse enzyme linked immunosorbent assay (ELISA) Kit (MyBioSource, USA) according to instructions provided by the manufacturer. Blood samples were collected immediately after decapitation for tissue collection 4 h after administration of dexamethasone or saline. The absorbance was measured at k=450 nm using a spectrophotometer (MultiskanSpectrum, ThermoLabsystems, USA). The concentration of CORT was then calculated from the appropriate standard curve and expressed as ng/ml serum. The sensitivity of the CORT ELISA was 4.1 ng/ml.

3.4 Statistical analysis

Statistical analysis of gene expression profiling was performed by dChip software using compare samples module and followed by correction for multiple testing (estimation of expected false positives). The false discovery rate (FDR) was estimated using Benjamini and Hochberg method. Hierarchical clustering was performed using the measure of Euclidian distance and average distance linkage methods. Cluster visualization was performed using dChip software gene

expression profiling. Gene annotation tool Enrichr (http://amp.pharm.mssm.edu/Enrichr) was used to identify over-represented ontological groups among the gene expression patterns and to group genes into functional classifications (Chen et al. 2013).

Statistical analysis of biochemical and behavioral data was done by GraphPad Prism version 7.0 or R version 3.3.1. Three-way ANOVA was used in morphine-induced locomotor sensitization to analyze three parameters (treatment × genotype × time). Two-way ANOVA was used in biochemical and behavioral studies that analyze two parameters (treatment × genotype; treatment × time), followed by Bonferroni's post hoc tests where appropriate. Presented ANOVA analyses show interaction effect, unless stated differently in the figure caption. Student's t-test was used to compare biochemical and behavioral data from two groups. All Student's t-tests analysis were two-tailed, unless indicated differently in the figure caption. $P < 0.05$ was considered to be statistically significant. Data are presented on graphs as the means of absolute values ± SEMs. All figures captions contain precise descriptions of used statistical analysis, as well as n values used to calculate the statistics. Statistically significant differences between tested groups are marked with the symbols * and # (*,# $p<0.05$; **, ## $p<0.01$; ***, ### $p<0.001$).

Chapter 4 Results

4.1 In search of opioid addiction markers

One of the main goals of the present study was to identify behavioral and molecular patterns accompanying lifelong opioid treatment. We aimed to compare the development of opioid dependence with the effects of a natural reward (saccharin) and to analyze behavioral symptoms of addiction in animals exposed to several months of uninterrupted access to drug or sweetener. Animal models commonly used to test these symptoms typically last less than two weeks, which is presumably too short to observe the alterations in the brain that accompany drug addiction, therefore we introduced a new model of chronic morphine self-administration using group-housed mice. Moreover, we sought to determine differences in gene expression profiles between morphine-dependent and non-dependent animals and to evaluate the influence of long-term voluntary morphine or saccharin intake on drug-induced transcription.

Behavioral profiling in the automated IntelliCages. The basic behavioral profile of the animals during the first three months of free-choice drinking is summarized in **Figure 6**. During that time, we have observed stable, high (>70%) preference to rewarding substances, both saccharin and morphine (**Figure 6a**). The preference was calculated as a percentage of the number of rewarding substance licks (morphine or saccharin) relative to water licks. For the analysis of preference, phase 6 was divided into two sub-phases, what was dictated by the changing pattern of drinking. Statistical analysis indicated that preference was similar in

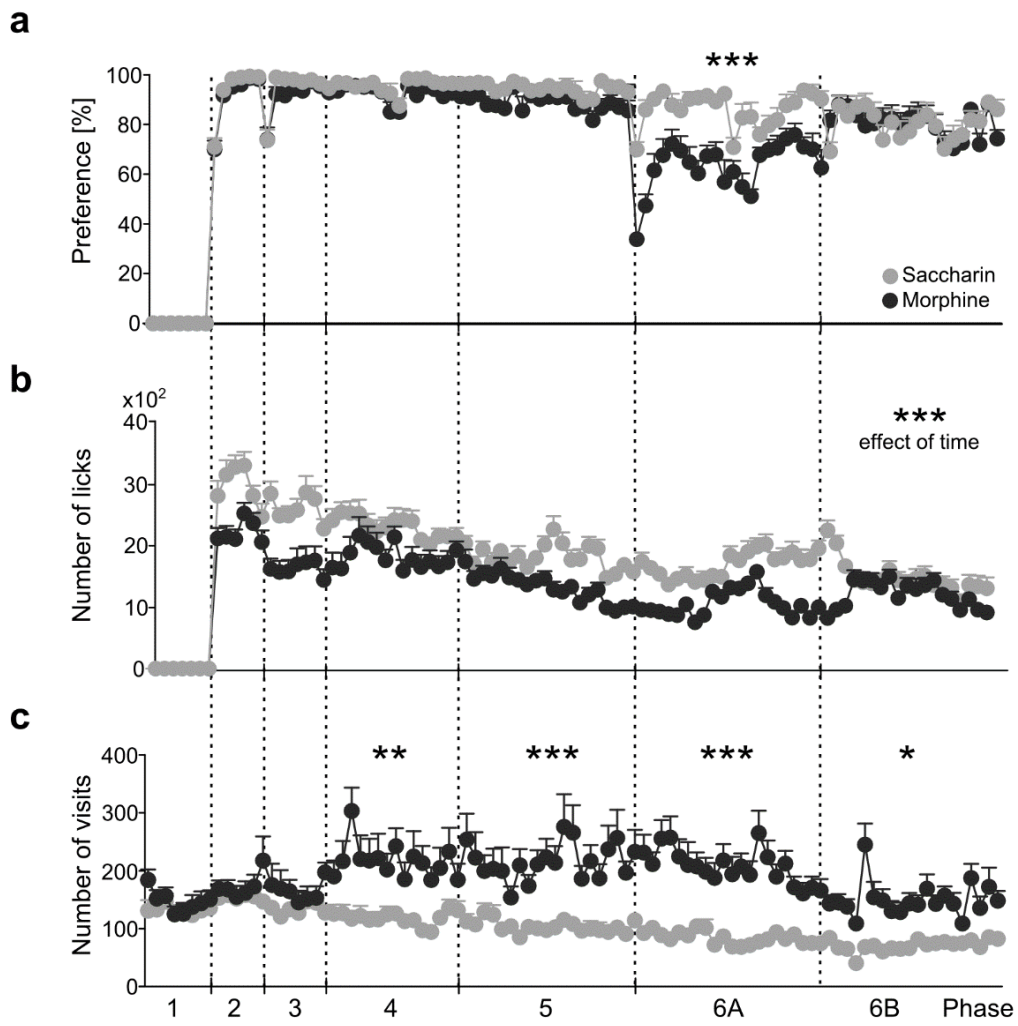

Figure 6. Behavioral activity in the IntelliCages. (**a**) Preference to the rewarding substances was similar in both groups of animals during phases 1-5. After introduction of unsweetened morphine solution (phase 6A) significant decrease in preference was observed in morphine drinking mice. Preference was then restored in morphine drinking group (phase 6B) and remained stable throughout the experiment. (**b**) Number of licks in the corners containing rewarding substances have decreased over time in both groups of animals (**c**) Morphine drinking animals presented increased activity expressed as increase in the total number of visits in corners of the cages. **a-c:** two-way repeated measures ANOVA for each phase **a:** $F_{6,132}$=9.11, p<0.001; **b:** time effect $F_{5,110}$=56.4, p<0.001; **c:** $F_{5,110}$=12.76, p<0.001. Where appropriate, tests were followed by Bonferroni post-hoc analysis. Each circle represents one day. Saccharin group n=12, Morphine group n=12. Data presented as mean ±SEMs. Significant differences between groups marked with *p<0.05, **p<0.01 ***p<0.001

both groups of animals throughout the examined period of time, except phase 6 of the experiment: after unsweetened morphine solution was introduced (for experimental schedule, see **Figure 5**, p. 52), the preference was significantly lower in morphine than in saccharin drinking animals. However, over time, the preference to rewarding substances in both groups equalized and remained stable at about 75%. Interestingly, despite stable high preference to saccharin and morphine we have observed that the total number of licks in the corners containing rewarding substances in both cages decreased from ~3000 licks at the beginning to ~1500 licks after 3 months of the experiment (**Figure 6b**). After phase 6 of the experiment, animals in both cages continued to drink about 1500 licks of rewarding substance per day. We have estimated that a single lick on a bottle corresponds to the consumption of 3 μl of liquid, therefore, animals in morphine cage consumed approximately 90-120 mg/kg of the drug per day. In addition to high preference to the drug, the animals drinking morphine showed an increased number of visits to the corners in phases 4-6 of the experiment, after the morphine concentration had reached 0.5 mg/ml (**Figure 6c**). To summarize, both saccharin and morphine were strongly preferred, even though the saccharin concentration was gradually lowered and unsweetened morphine was introduced. What is more, morphine consumption resulted in a constantly sustained increase in the animals' activity, as measured by the number of entries into the corners.

Changes in the circadian activity during protracted saccharin or morphine self-administration. To examine overall patterns of activity during active (night, 12 h, lights off at 7 p.m.) and inactive (day, 12 h, lights on at 7 a.m.) phases of circadian rhythm of the animals, we counted the total number of visits to all of the corners in each cage for three consecutive days during the adaptation period, as well as during first, second and third month of saccharin or morphine self-

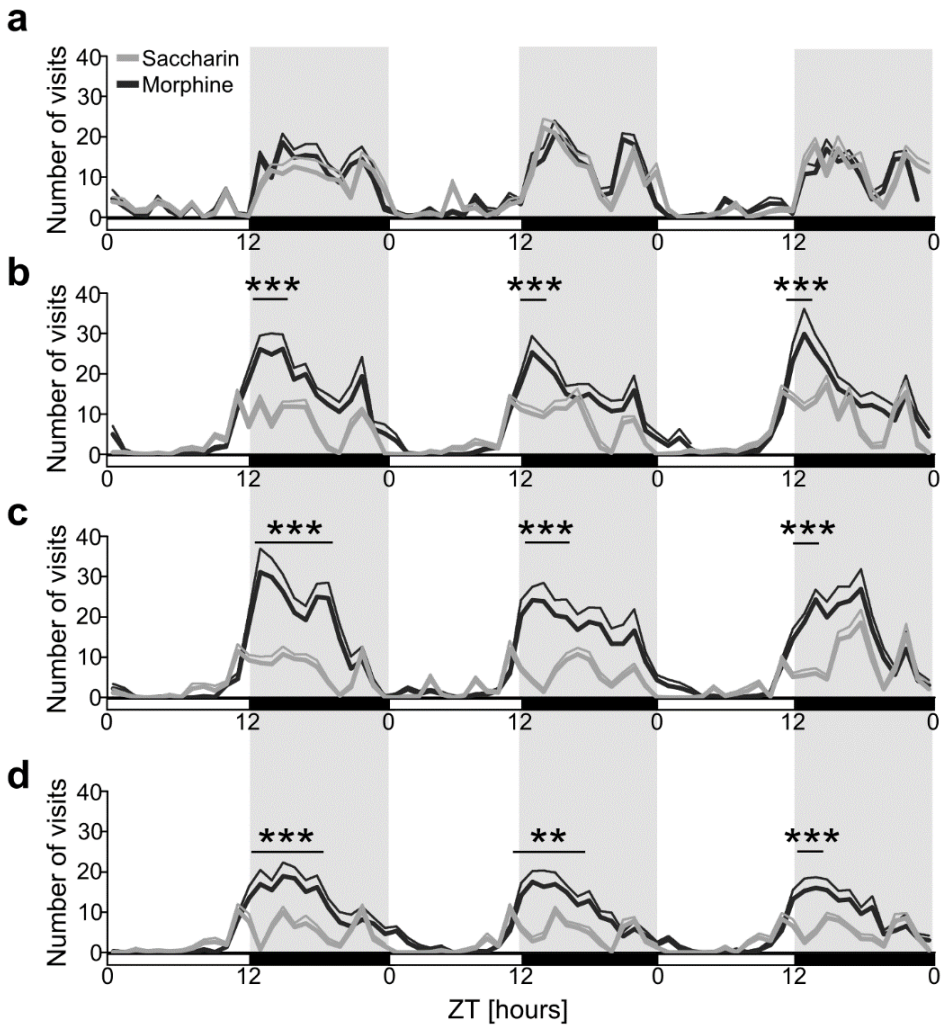

Figure 7. Changed patterns of circadian activity after prolonged morphine self-administration. (**a**) During the adaptation period animals in both groups presented comparable behavior, with 2 characteristic peaks of activity, at the beginning and the end of the dark phase. However, after 1 (**b**), 2 (**c**), or 3 (**d**) months, morphine consumption have caused significant increase of the activity at the beginning of the dark phase of the light/dark cycle, while the second peak of activity gradually diminished. Patterns of circadian activity are shown as the number of visits to all corners of the cages in the light and dark (marked with grey panels) phases of the cycle for 3 consecutive days, representative for each of the presented phases of self-administration. **a-d:** two-way repeated measures ANOVA **a:** $F_{72,1872}=1.2$, $p=0.13$; **b:** $F_{72,1872}=5.3$, $p<0.001$; **c:** $F_{72,1872}=8.36$, $p<0.001$; **d:** $F_{72,1872}=7.22$, $p<0.001$. Tests were followed by Bonferroni post-hoc analysis where appropriate. Saccharin group n=12, Morphine group n=12. Data presented as mean (thick lines) ±SEMs (thin lines). ZT- Zeitgeber time. Significant differences between groups marked with **p<0.01 ***p<0.001

administration. The representative results are presented in **Figure 7**. During the adaptation period, the circadian activity of the animals in both groups was similar (**Figure 7a**). After rewarding substances were introduced, mice drinking morphine were clearly more active than the control group (**Figure 7b-d**) at the beginning of the dark phase of the light/dark cycle. Additional analysis showed, that both groups of animals did not differ in the number of visits with drinking episodes in the corners containing water (**Figure 8a**). However, saccharin group drank more effectively over time in the rewarding corners, unlike the morphine-dependent animals, which made more visits in the reward-paired corners without drinking (**Figure 8b**). It is possible, that this effect is due to the association of the context of the corners with the rewarding effects of morphine, and thus, tendency to spend there more time, similarly to conditioned place preference paradigm.

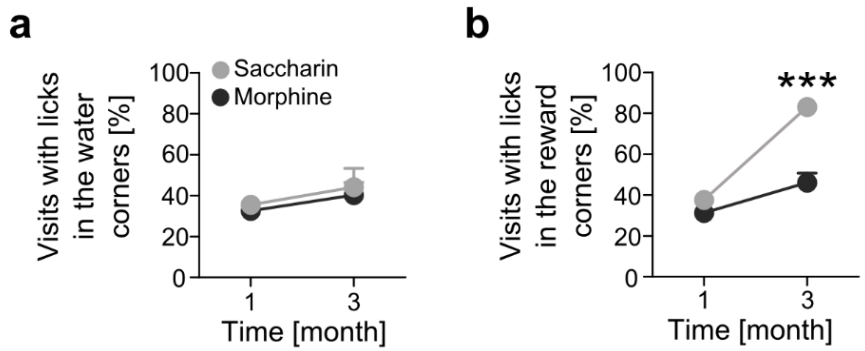

Figure 8. Number of visits with drinking episodes during first and third month of saccharin or morphine self-administration. (**a**) The percentage of visits with licks in the corners containing water was similar in both groups of animals after 1 and 3 months of saccharin or morphine drinking. (**b**) Animals drinking saccharin present significant increase in the number of visits with licks in the corners containing reward, when compared to morphine group. **a-b:** two-way repeated measures ANOVA **a:** $F_{1,22}$=0.1, p=0.95; **b:** $F_{1,22}$=39.17, p<0.001. Tests were followed by Bonferroni post-hoc analysis where appropriate. Saccharin group n=12, Morphine group n=12. Data presented as mean ±SEMs. Significant differences between groups are marked with ***p<0.001

Motivation to obtain morphine or saccharin. After 3 months of free choice self-administration of either saccharin or morphine the animals were tested for addiction-like behavior in automated behavioral procedures in the IntelliCage system (**Figure 9**). PR schedules were used to measure the motivation to obtain a reward. To test the motivation to acquire saccharin or morphine, we used a schedule in which the number of instrumental responses (nose pokes) required was progressively increased until the animal could no longer complete the task and thus reached its 'breakpoint'. The procedure was used twice: first, to obtain the reward, the number of required responses was increased by 1 (PR1), and then a few days later, the procedure was repeated, but the number of instrumental responses required to gain access to the bottles increased by 3 (PR3). As control, we repeated the procedure in the corners containing water. During PR1, the mice in both groups reached their breakpoints at approximately 15 nose pokes. Interestingly, when the procedure required higher effort to obtain the reward, animals drinking morphine reached a significantly higher breakpoint (approximately 19 nose pokes) than the control group (approximately 14 nose pokes, **Figure 9a**). Moreover, when the procedure was repeated in corners that contained bottles with water, both groups of animals reached similar breakpoints (**Figure 9b**).

Craving for morphine or saccharin. Animals were given intermittent access to rewarding substances in both cages for two hours twice over a single active phase (dark phase of the light/dark cycle), during the periods of enhanced activity in the IntelliCages. The animals that had been restricted from access to morphine performed significantly more instrumental responses (nose pokes) in an attempt to obtain the access to a rewarding substance than the animals drinking saccharin (**Figure 9c**). These results show that during the intermittent access schedule, the

morphine-drinking animals put more effort into reward seeking than the control group, which indicates increased craving for morphine.

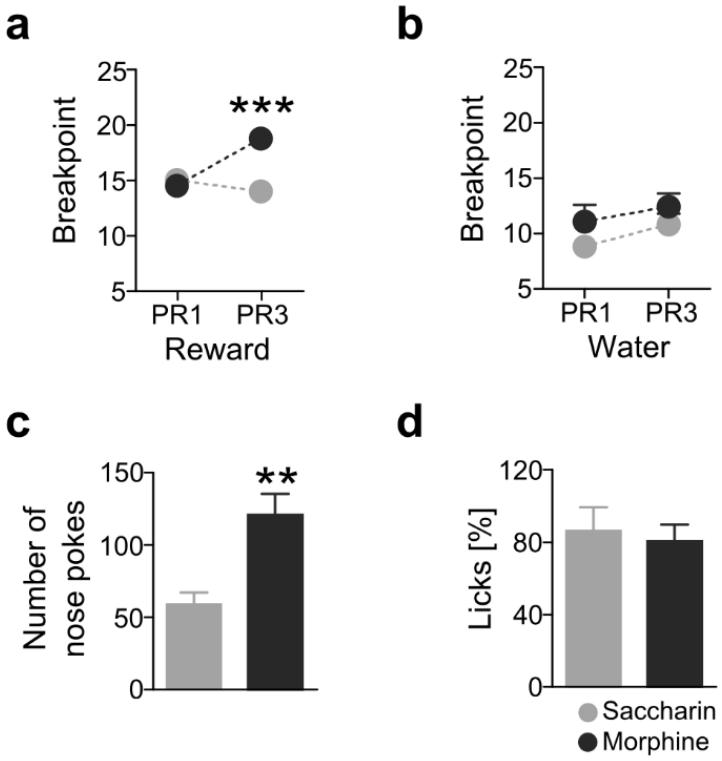

Figure 9. Addiction-like symptoms measured in the IntelliCage system. (**a**) Morphine-dependent animals presented increased motivation to obtain the access to a rewarding substance than saccharin drinking animals. However, (**b**) no such effect was observed in the corners containing water- animals in both groups reached similar breakpoints. (**c**) Morphine group presented drug craving expressed as the increased number of nose pokes made during the intermittent access procedure when compared to saccharin drinking animals. (**d**) Animals in both groups presented comparable propensity to drink despite the risk of punishment (air puff). The result is presented as the percent of licks of the rewarding substances during the procedure relative to average number of licks of those substances over the last 7 days before the test. **a-b:** two-way repeated measures ANOVA **a:** $F_{1,22}$=17.9, p<0.001; **b:** $F_{1,22}$=0.45, p=0.5. Tests were followed by Bonferroni post-hoc analysis where appropriate. **c-d**: Student's t-test **c**: t_{22}=3.6, p=0.002; **d**: t_{22}= 0.36, p=0.72. Saccharin group n=12, Morphine group n=12. PR- progressive ratio. Data presented as mean ±SEMs. Significant differences between groups are marked with **p<0.01 ***p<0.001

Continued drinking despite the risk of punishment. The propensity to drink despite the risk of punishment is presented in **Figure 9d**. The animals underwent a single session that was performed during the dark phase of the light/dark cycle that included a 25% risk of punishment for intake of either morphine or saccharin and FR3 access to water without a risk of punishment. The punishment consisted of a 0.5 bar air puff that lasted 0.2 s and was delivered after the completion of the FR3 task. Both saccharin and morphine-drinking mice drank from the bottles containing rewarding substances despite the risk of punishment at approximately 80% of the average lick number. These results indicate that the animals were willing to risk a punishment to obtain the reward regardless of the type of rewarding substance, however, there is a possibility that 25% probability of punishment was insufficient to observe differences between groups.

Withdrawal and analgesia during abstinence. The effects of morphine self-administration on spontaneous withdrawal symptoms and the duration of analgesic effects were measured during first 54 hours of the abstinence period, when the access to rewarding substances was restricted for 14 days. The animals in both cages were randomly divided into 2 groups of 6 and tail flick or withdrawal tests were carried out in these separate groups. Morphine-dependent animals exhibited a variety of spontaneous withdrawal symptoms compared to the control group (**Figure 10a-e**). The highest intensity of those symptoms appeared to last for the first 16 hours of abstinence.

Simultaneously with the withdrawal tests, 6 mice in each group were submitted to tail flick tests to investigate whether chronic self-administration of morphine changed reactions to painful stimuli. Pain response thresholds were comparable in both groups of animals (**Figure 10f**). These results point out that chronic morphine self-administration did not result in alterations of pain sensitivity levels.

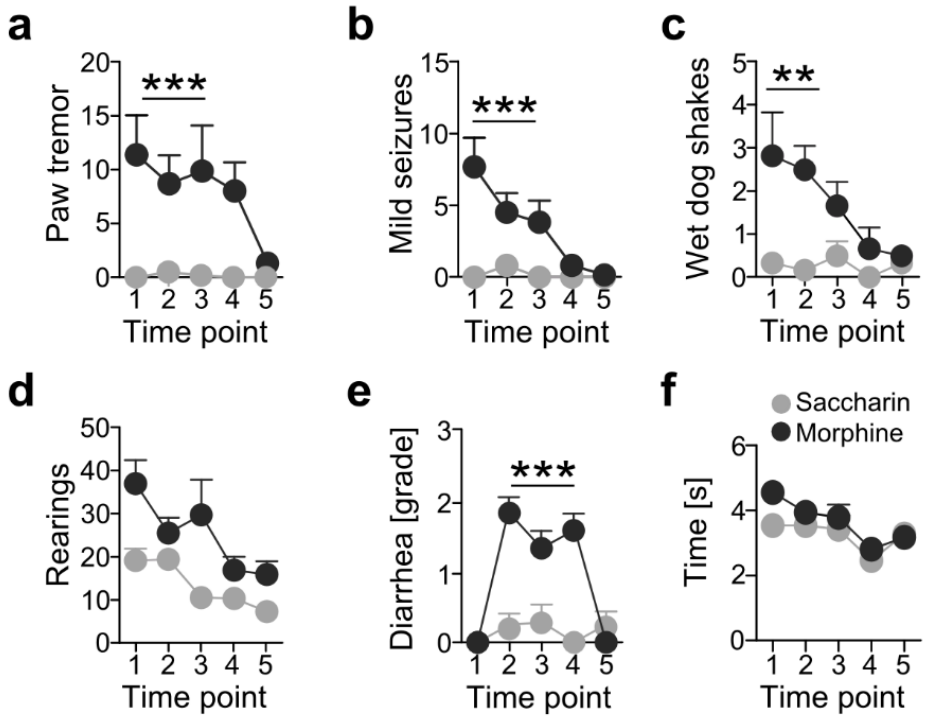

Figure 10. Individual signs of spontaneous morphine withdrawal and pain sensitivity measured during first 54 hours of the abstinence period. Morphine-dependent animals presented a variety of withdrawal symptoms when compared to saccharin group. Observed symptoms included (**a**) paw tremor, (**b**) mild seizures, (**c**) wet-dog shaking, (**d**) rearing (graded signs) and (**e**) diarrhea (observed sign, scoring: 0= no change, 1= mild, 2= moderate, 3= severe). (**f**) Morphine drinking animals did not differ in pain response thresholds from saccharin group. **a-f:** two-way repeated measures ANOVA **a:** $F_{4,40}$=2.98, p=0.03; **b:** $F_{4,40}$=4.4, p=0.004; **c:** $F_{4,40}$=3.23, p=0.02; **d:** $F_{4,40}$=1.87, p=0.13; **e:** $F_{4,40}$=12.53, p<0.001; **f:** $F_{4,40}$=1.07, p=0.38. Tests were followed by Bonferroni post-hoc analysis where appropriate. Time points: 1- 4h, 2- 8h, 3- 16h, 4- 32h, 5- 54h. Saccharin group n=6, Morphine group n=6. Data presented as mean ±SEMs. Significant differences between groups are marked with *p<0.05 **p<0.01 ***p<0.001

Reinstatement of morphine addiction. After 14 days of abstinence, the rewarding substances were reintroduced to both groups of animals (0.2 mg/ml saccharin in saccharin cage, 0.5 mg/ml unsweetened morphine in morphine cage). Morphine- dependent animals presented a considerable increase of preference to drink rewarding solution over water in 3 weeks after reward reintroduction, while

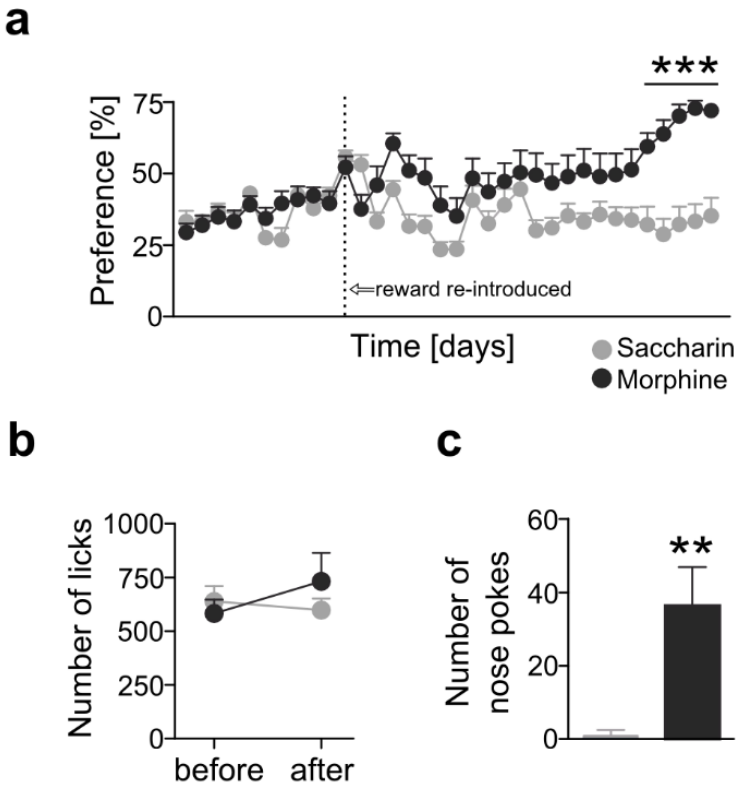

Figure 11. Reinstatement of addiction and morphine-induced craving. (**a**) Morphine-dependent animals presented increasing preference to reward over water during 3 weeks after the access to rewarding substance was restored. Saccharin group did not present such increase. However, (**b**) the mean number of licks on the bottles containing rewarding substances before and after the abstinence period was similar in both groups, therefore, no escalation in consumption of neither saccharin nor morphine was observed. (**c**) After single morphine administration (20 mg/kg, ip), morphine-dependent animals made significantly more instrumental responses than saccharin-drinking animals in order to gain access to the reward. **a-b:** two-way repeated measures ANOVA **a:** $F_{34,782}$=6.82, p<0.001; **b:** $F_{1,22}$=2.32, p=0.14. Tests were followed by Bonferroni post-hoc analysis where appropriate. **c**: Student's t-test, t_{10}=3.33, p=0.008. **a-b** Saccharin group n=12, Morphine group n=12; **c**: Saccharin group n=6, Morphine group n=6. Data presented as mean ±SEMs. Significant differences between groups are marked with **p<0.01 ***p<0.001

saccharin-drinking animals presented preference levels (~50%) that indicated a random choice of the consumed liquid (**Figure 11a**). Therefore, after 3 weeks morphine group have restored high preference level (~70%), that was similar to

preference from before abstinence period. However, although we have observed differences in preference to rewarding substances, the average amount of licks on the bottles containing rewarding solutions from before and after the abstinence periods were comparable in both groups (**Figure 11b**), indicating that there was no increase in actual morphine intake. However, examination of individual animals pointed out that 4 out of 12 of the morphine-dependent animals showed increased morphine consumption after the abstinence period by at least 300 licks, which was not observed in the control group. This observation indicates that only some of the morphine-drinking animals developed an escalation in drug consumption, one of the symptoms of opioid dependence.

Morphine-induced craving. During the last week of the experiment, saccharin and morphine-drinking animals were randomly divided and put into separate IntelliCages (6 animals in each). At 4 hours before tissue collection, 6 mice of both groups received saline injection, while the other 6 animals of both groups received a single morphine injection (20 mg/kg, ip). During that time, access to the rewarding substances was not allowed, but animals were free to explore the cages and enter the corners. Immediately after the injections, mice were put back into the cages, which led to an interesting observation. After morphine injection, morphine-dependent animals made repeated attempts to gain access to the reward, as shown by a striking increase in the number of nose pokes in the corners that was associated with morphine compared to the control group (**Figure 11c**). The groups of animals that received saline injections did not differ in the number of nose pokes (t_{10}=0.6, p=0.56, data not shown). These results indicate that a single morphine injection induced craving behavior in morphine-dependent animals but not in these drinking saccharin.

To summarize, mice voluntary self-administering morphine showed addiction-related behavioral pattern, which included: increased activity in the IntelliCage, changed patterns of circadian activity, higher motivation to work for a drug reward, increased reward seeking and craving. When the access to rewarding substances was restricted, morphine-dependent mice presented spontaneous symptoms of withdrawal, and stable restoration of morphine consumption during reinstatement. What is more, a single high dose of morphine potently increased drug craving.

Gene expression alterations related to morphine dependence. After the evaluation of addiction-like symptoms in both groups, we next aimed to observe the effects of chronic morphine self-administration on basal and morphine-induced gene transcription using whole-transcriptome microarrays 4 h after either saline or single morphine injection (20 mg/kg, ip). We identified 67 transcripts using a 5% FDR statistical threshold (nominal $p<7\times10^{-5}$) for acute administration factors (**Figure 12b**). While acute morphine treatment altered the expression of numerous transcripts, analysis did not indicate any persistent alterations in the striatal transcriptome after long-term morphine administration (**Figure 12a**). A total of 39 substantially altered transcripts exhibited more than 1.3-fold (21 with 1.5-fold) changes after acute morphine exposure compared to the saline-treated groups. Increased expression was observed for 37 transcripts and the abundance of 2 of the transcripts was reduced after the administration of the drug. The greatest induction of gene expression was observed after a single administration in mice that chronically self-administered saccharin (22 genes with 1.5-fold changes). This response appeared to be tolerated in animals that were chronically treated with

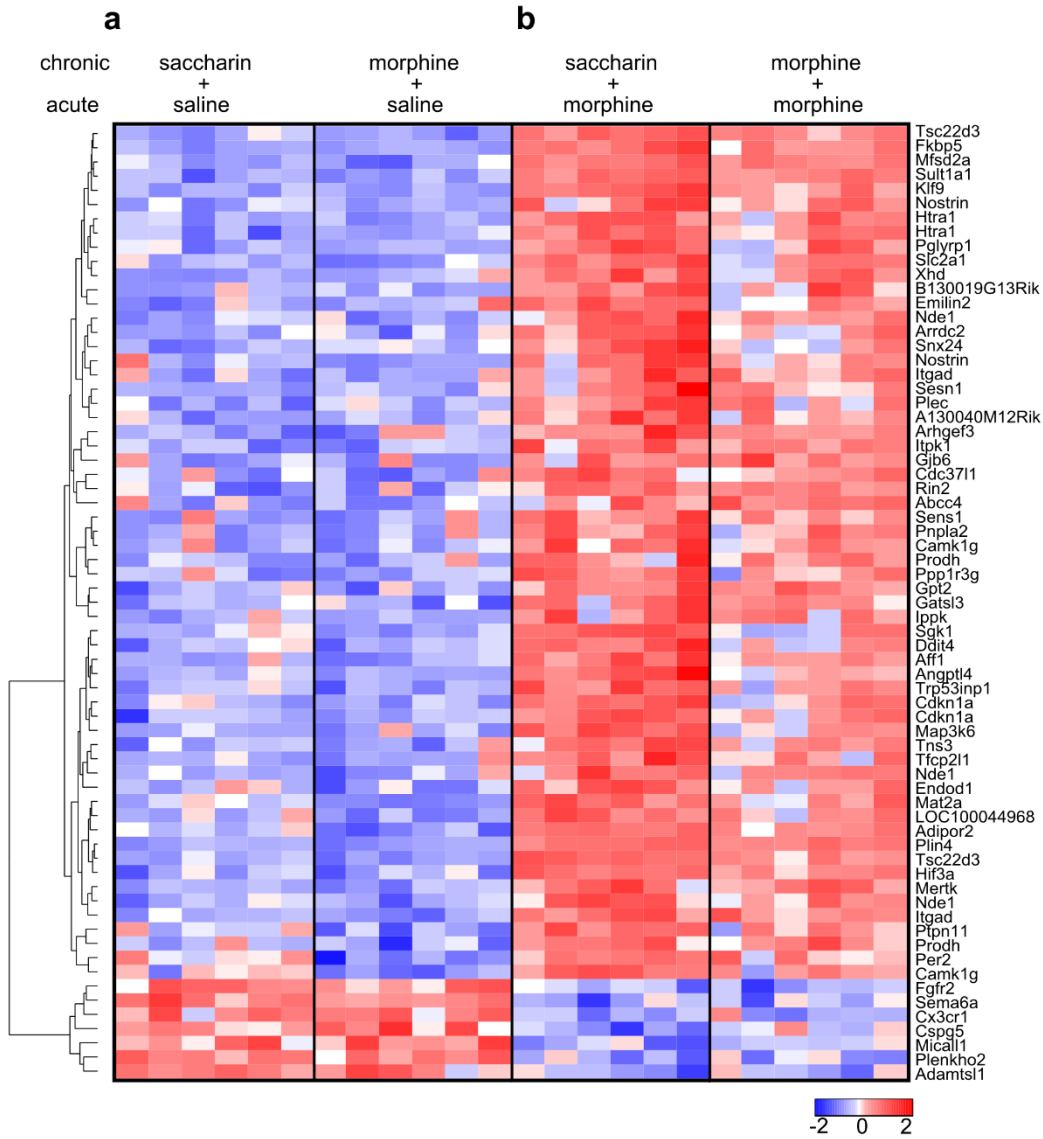

Figure 12. The effects of morphine on gene expression. Hierarchical clustering of drug-dependent alterations in the mouse striatum revealed (**a**) no basal differences in gene expression between analyzed groups measured after saline administration. (**b**) Induction of gene expression was suppressed in morphine-dependent animals when compared to saccharin group after acute morphine injection (20 mg/kg, ip). **a-b**: Microarray results from two-way ANOVA with the chronic and acute administration presented as a heat map, which includes genes with genome-wide significance. The colored rectangles represent transcript abundance at 4 h after exposure to saline or morphine for the gene labelled on the right. The alterations in the expression of the displayed genes were found to be statistically significant for the acute drug administration factor at 5% FDR. The intensity of the color is proportional to the standardized values (between -2 and 2) for each microarray, as indicated in the legend.

Table 1. Gene pathway analysis. The results of functional enrichment analysis performed with Enrichr tool for genes altered by the morphine treatment. The table consists of an enriched term, a number of input genes in the pathway (overlap), p-value, adjusted p-value and the overrepresented genes.

Term	Overlap	P-value	Adjusted P-value	Genes
Insulin Signaling	5/153	0,001	0,125	*Rhoj, Slc2a1, Ptpn11, Map3k6*
Diurnally Regulated Genes	2/48	0,030	0,401	*Per2, Klf9*
Exercise-induced Circadian Regulation	2/52	0,034	0,401	*Per2, Klf9*
miRNA regulation of DNA Damage Response	2/63	0,048	0,401	*Cdkn1a, Sesn1*
Alpha6-Beta4 Integrin Signaling Pathway	2/64	0,049	0,401	*Cdkn1a, Plec*
Methylation	1/8	0,053	0,401	*Mat2a*
Leptin and adiponectin	1/10	0,058	0,401	*Adipor2*
EGFR1 Signaling Pathway	3/172	0,068	0,401	*Cav1, Ptpn11, Plec*
PluriNetWork	4/284	0,068	0,401	*Cdkn1a, Ptpn11, Tfcp2l1, Sgk1*
Folic Acid Network	1/13	0,073	0,401	*Xdh*
TNF-alpha NF-kB Signaling Pathway	3/179	0,075	0,401	*Cav1, Ptpn11, Fkbp5*
Selenium Micronutrient Network	42386	0,093	0,401	*Xdh*
Amino Acid metabolism	2/92	0,092	0,401	*Gpt2, Pdk4*

morphine (14 genes with 1.5-fold changes). The list of investigated genes is available on NCBI gene expression array data repository, Gene Expression Omnibus (GEO, access code: GSE78280). Functional enrichment analysis was

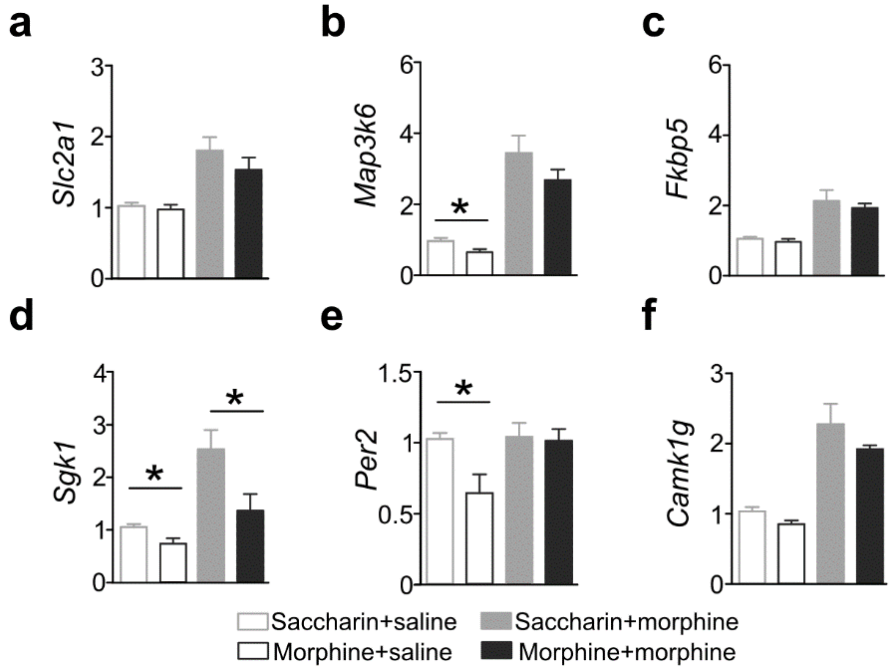

Figure 13. The effects of morphine on selected genes expression in the striatum. Fold change of mRNA abundance levels relative to control group (saccharin+saline) was examined for (**a**) *Slc2a1*, (**b**) *Map3k6*, (**c**) *Fkbp5*, (**d**) *Sgk1*, (**e**) *Per2* and (**f**) *Camk1g* genes. Significant reduction in gene expression was found in the mRNA expression of *Per2*, *Map3k6* and *Sgk1* for chronic treatment (white bars, saline injection) along with *Sgk1* for acute treatment (colored bars, morphine 20 mg/kg, ip). **a-f:** Student's t-test performed between groups of animals for each treatment separately **a:** chronic t_9=0.54, p=0.6; acute t_{10}=1.03, p=0.33; **b:** chronic t_9=2.73, p=0.002; acute t_{10}=1.32, p=0.2; **c**: chronic t_9=0.79, p=0.44; acute t_{10}=0.6, p=0.5; **d**: chronic t_9=2.27, p=0.04; acute t_{10}=2.43, p=0.03; **e**: chronic t_9=2.71, p=0.02; acute t_{10}=0.22, p=0.8; **f**: chronic t_9=2.21, p=0.05; acute t_{10}=1.21, p=0.25. **a-f** n=6 per each group. Data presented as mean ±SEMs. Significant differences between groups are marked with *p<0.05

used to investigate cell signaling pathways related to morphine-induced gene expression alterations (**Table 1**). Enrichr, a gene signature search tool based on the WikiPathways database, indicated overrepresentation of genes involved in insulin signaling pathway (*Slc2a1*, *Ptpn11*, *Rhoj*, *Map3k6*, *Sgk1*) and diurnally

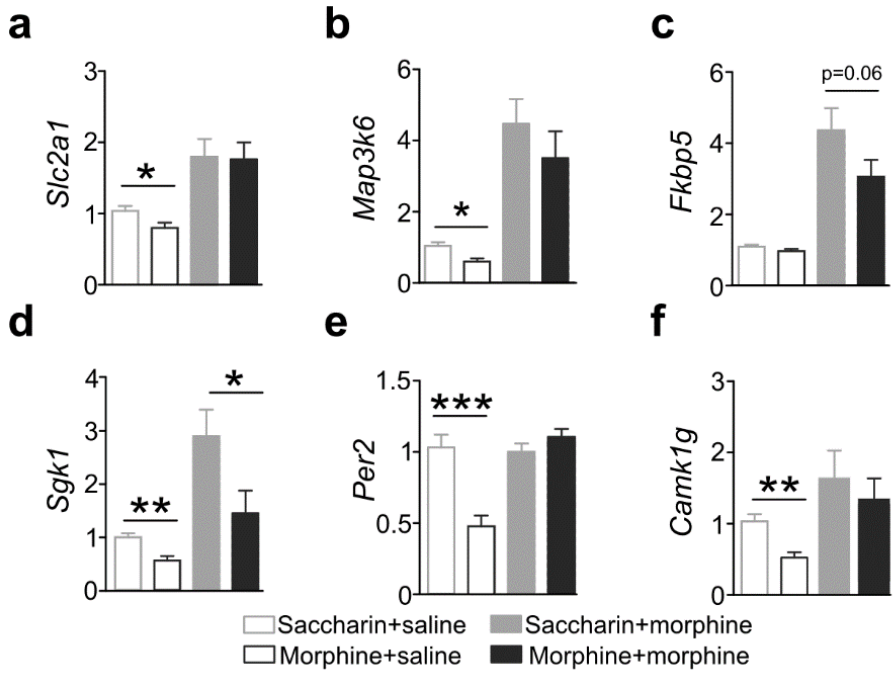

Figure 14. The effects of morphine on selected genes expression in the frontal cortex. Fold change of mRNA abundance levels relative to control group (saccharin+saline) was examined for (**a**) *Slc2a1*, (**b**) *Map3k6*, (**c**) *Fkbp5*, (**d**) *Sgk1*, (**e**) *Per2* and (**f**) *Camk1g* genes. Significant reduction in gene expression was found in the mRNA expression of *Per2*, *Map3k6*, *Sgk1* and *Slc2a1* for chronic treatment (white bars, saline injection) along with *Sgk1* for acute treatment (colored bars, morphine 20 mg/kg, ip). **a-f:** Student's t-test performed between groups of animals for each treatment separately **a:** chronic t_{12}=2.26, p=0.04; acute t_{11}=0.11, p=0.91; **b:** chronic t_{12}=3.05, p=0.01; acute t_{11}=0.93, p=0.37; **c**: chronic t_{12}=1.31, p=0.2; acute t_{11}=1.64, p=0.06; **d**: chronic t_{12}=3.84, p=0.002; acute t_{11}=2.18, p=0.05; **e**: chronic t_{12}=4.8, p<0.001; acute t_{11}=1.26, p=0.23; **f**: chronic t_{12}=3.9, p=0.002; acute t_{11}=0.53, p=0.06. **a-f** n=6 per each group. Data presented as mean ±SEMs. Significant differences between groups are marked with *p<0.05 **p<0.01 ***p<0.001

regulated genes (*Per2, Klf9*). Selected candidate genes from the identified pathways were analyzed in the striatum and the frontal cortex using qPCR (**Figures 13-14)**. Prolonged morphine self-administration have caused long-lasting suppression of gene expression levels in both analyzed structures. Since differences are very subtle, they might not have been detected by whole-transcriptome microarray analysis. Significant reduction in gene expression between compared groups in the striatum was found in the mRNA expression of *Sgk1* for acute treatment along with *Per2*, *Map3k6* and *Sgk1* for chronic treatment (**Figure 13**). In frontal cortex overall pattern of gene expression appeared to be similar to striatum. Significant differences of mRNA expression were found in *Sgk1* and *Fkbp5* for acute treatment along with *Per2*, *Map3k6*, *Sgk1* and *Slc2a1* for chronic treatment (**Figure 14**). We have observed differential transcriptional alterations of *Fkbp5*, *Camk1g* and *Map3k6* to morphine treatment between the striatum and frontal cortex, indicating that some of the changes are region-specific.

Molecular signatures of opioid responsiveness. The clustering of gene expression profiles indicated that among the opioid-dependent animals that were injected with morphine prior to tissue collection, groups of 'high' and 'low' responders to morphine treatment were distinguished (**Figure 15a-b**). Those mice that were classified as high (HRs) or low (LRs) responders due to their transcriptional response to acute morphine administration, were retrospectively analyzed, and behavioral changes were found to accompany those two molecular profiles. The number of animals per group was rather small, nevertheless, some visible differences between the groups can be noted, which may contribute to a better understanding of the factors underlying differences in these profiles during the development of addiction-like behaviors. LRs consumed more morphine than HRs

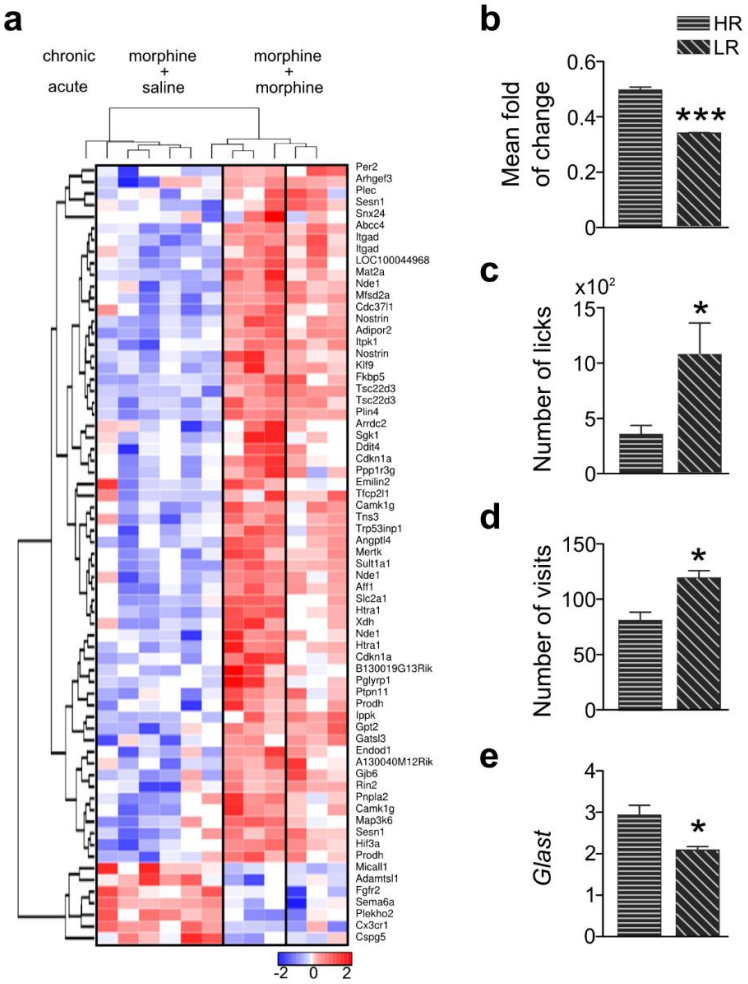

Figure 15. High- and low-responsive morphine-dependent mice. (**a**) The clustering of morphine-induced gene expression alterations in the striatum of individual morphine drinking animals pointed out to high (middle panel, HR) and low (right panel, LR) responders to acute morphine administration. The effect of clustering is summarized and presented as (**b**) mean fold changes in gene expression between the analyzed groups. (**c**) Mean number of daily licks in the reward-related corners, as well as (**d**) mean number of daily visits to all corners was significantly higher in LR animals. (**e**) Animals with more severe dependence (LR mice) showed decreased mRNA expression of astroglial marker, *Glast*. **a:** Microarray results are shown as heat maps that include morphine-responsive genes selected using two-way ANOVA acute drug treatment factor at 5% FDR. Colored rectangles represent transcript abundance after saline or morphine injection. Regulated gene are labeled on the right of the heat map. The intensity of the color is proportional to the standardized values (between -2 and 2). **b-e:** Student's t-test **b:** t_4=12, p<0.001; **c:** t_4=2.3, p=0.04; **d:** t_4=3.5, p=0.013; **e:** t_4=3.14, p=0.03. Data represent mean ± SEMs. HR- high responders n=3, LR- low responders n=3. Significant differences between groups are marked with *p<0.05, ***p<0.001

after the abstinence period (**Figure 15c**), and they also exhibited an increased activity expressed as greater number of visits in the corners of the IntelliCage (**Figure 15d**). Interestingly, we have also found that the mRNA expression of glutamate aspartate transporter (*Glast*, *Slc1a3*), a common marker of astrocytes was decreased in LRs (**Figure 15e**), pointing out to the possible role of astrocytes in mediating severity of opioid dependence.

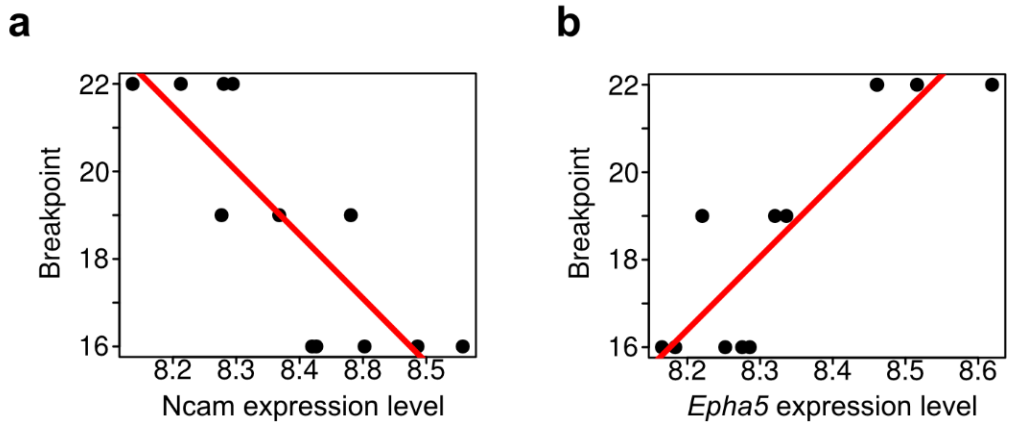

Figure 16. Relationship between the motivation to obtain morphine and gene expression level. Scatter plot shows significant correlation between the abundance level of (**a**) *Epha5* gene and (**b**) *Ncam* gene in the striatum of individual morphine-drinking mice and motivation to work to obtain morphine expressed as breakpoint reached to obtain the access to reward in progressive ratio procedure. **a-b:** Pearson's correlation **a:** r=-0.84, p<0.001 **b:** r=0.88, p<0.001

Moreover, in the search for molecular contributors to addiction-like syndromes, correlation analyses were performed between individual gene expression levels and motivation (progressive ratio results). Using a threshold of r>0.8, there were clearly more genes correlated with motivation to obtain morphine (59 genes) than saccharin (11 genes). Taking into consideration the fact that neither chronic nor acute morphine administration changed the transcription

of the identified genes, this correlation may indicate that developmental changes, such as epigenomic modifications, have contributed to individual predispositions toward drug seeking behavior. The most interesting genes that might point to such a predisposition are the ephrin receptor (*Epha5*), and neural cell adhesion molecule (*Ncam*) the transcription of which was correlated with motivation to drink morphine (**Figure 16a-b**).

To summarize, microarray and qPCR results in both acute and chronic treatments indicate that protracted opioid administration results in the suppression of gene transcription in the striatum and frontal cortex that may affect molecular sensitivity to opioid reward, and thus influence the behavior associated to drug seeking. Functional enrichment analysis of cell signaling pathways related to morphine-induced gene expression alterations indicated overrepresentation of genes involved in insulin signaling pathway and diurnally regulated genes. What is more, we have observed that genes induced by morphine included a large group of transcripts that are associated with the glucocorticoid receptor (GR) regulatory network (e.g. *Fkbp5, Sgk1, Camk1g, Cdkn1a, Gjb6, Slc2a1*). Alterations in expression of *Gjb6, Slc2a1* and *Slc1a3* genes, which are expressed specifically in astrocytes, indicate a potential role for these glial cells in the development of addiction. In search for specific molecular profiles that accompany opioid addiction, we were able to distinguish high and low responders among the morphine drinking animals that presented different patterns of morphine drinking and activity in the cages. Moreover, correlation analyses performed in search of the presence of genetic predispositions to addiction-like behavior pointed out to *Epha5* and *Ncam*, the transcription of which correlated with motivation to drink morphine.

4.2 Cell specific activation of GR-dependent genes in the nucleus accumbens

Our results revealed that large fraction of morphine-induced alterations to the striatal transcriptome included GR-dependent genes, many of which are expressed in astrocytes. Previous report also suggested an overlap between morphine-induced transcripts in striatum and GR-dependent transcripts in cultured astrocytes (Slezak et al. 2013), therefore we aimed to investigate whether GR-dependent transcriptional changes in the striatum are cell-type specific.

To compare GR-dependent gene expression alterations between main cellular compartments of the brain we used whole-transcriptome microarrays. Firstly, we measured the effects of dexamethasone (4 mg/kg, ip), a GR agonist on gene expression in ventral striatum 4 h after acute administration. We identified 47 transcripts regulated by dexamethasone in the striatal tissue, with false discovery rate empirically estimated at 6.4%. Further, identified gene expression changes were analyzed in neurons and astrocytes again using whole-transcriptome microarrays. Different cell types were separated from the ventral striatum using magnetic cell sorting. We have identified 30 dexamethasone-regulated transcripts (**Figure 17**; 17 transcripts that were not regulated in neuronal or astroglial compartments of the brain were filtered out). The treatment caused an increase in the mRNA abundance levels of 27 and decrease of 3 transcripts. According to transcriptional changes in astrocytes and neurons we have identified 3 main gene clusters (labeled as a-c): genes activated in astrocytes (**Fig. 17, cluster a**), down-regulated in astrocytes (**Fig. 17, cluster b**) and induced in neurons (**Fig. 17, cluster c**). We found that GR-dependent transcriptome alterations in the ventral striatum

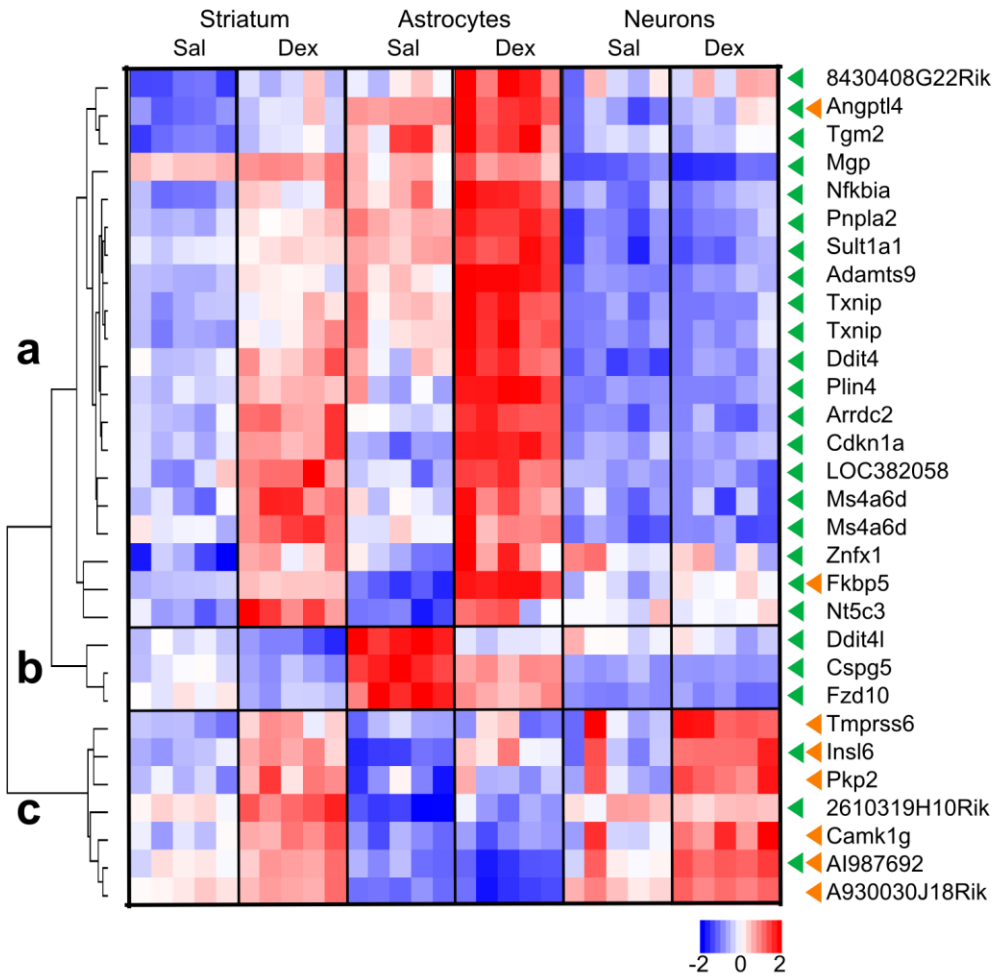

Figure 17. GR-dependent transcriptome alterations *in vivo* are mostly confined to astrocytes. Dexamethasone (Dex) caused an increase in the mRNA abundance levels of 27 and decrease of 3 transcripts in the NAc of mice. Major GR-responsive gene transcription patterns: (**a**) transcripts induced in astrocytes, (**b**) down-regulated in astrocytes and (**c**) induced in neurons. Microarray results include genes with genome-wide significance filtered out using Student's t-test $p<0.05$ and fold change >0.5, presented as a heat map. The colored rectangles represent transcript abundance at 4 hours after saline or dexamethasone (4 mg/kg, ip) administration in different brain compartments (tissue, astrocytes and neurons) for the genes labelled on the right. The intensity of color is proportional to the standardized values for each microarray, as indicated in the legend. Genes regulated in specific cell types are marked with arrows on the right. Green arrows point to genes regulated in astrocytes, orange arrows point to genes regulated in neurons.

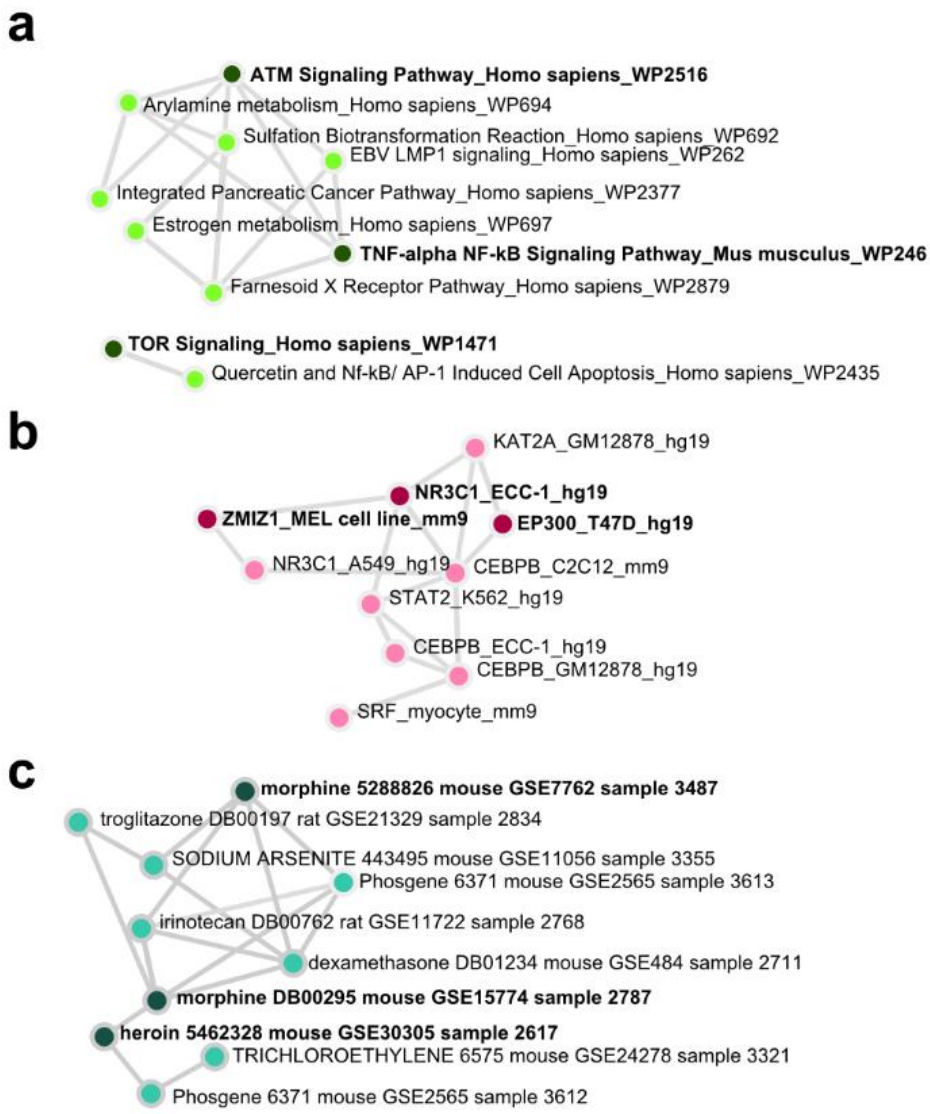

Figure 18. Functional associations between the genes induced by dexamethasone in astrocytes identified using (**a**) WikiPathways 2016 presented significant enrichment of pathways involved in apoptosis, cellular reaction towards nutrient and energy availability and immune response. (**b**) ENCODE TF ChIP-seq 2015 showed significant overexpression of genes regulated by the GR (*Nr3c1*). (**c**) Drug Perturbations from GEO revealed an overlap of genes regulated by dexamethasone with genes regulated by opioids. Functional links were visualized by the Enrichr online application. The top significant results (sorted by $p<0.001$) is indicated in darker color and bold font.

are mostly confined to astrocytes, as about 85% of identified genes were regulated in these cells when compared to neurons. These results show for the first time that glucocorticoid-induced response in the brain might be in fact mostly managed by non-neuronal cells. In search for functional associations between identified transcripts and alterations induced by morphine, we used EnrichR data-mining tool. We exploited available WikiPathways 2016 data which revealed overrepresentation of genes involved in ATM signaling pathway, which takes part in apoptosis (e.g. *Nfkbia*, *Cdkn1a*), mTOR signaling pathway that is involved in cellular reaction towards nutrient and energy availability (e.g. *Ddit4*, *Ddi4l*); and TNF-alpha NF-kB signaling pathway which is involved various biological processes, such as cell proliferation, differentiation, apoptosis, lipid metabolism and immune response (e.g. *Fkbp5*, *Nfkbia*; **Figure 18a**). ChIP-seq data (ENCODE TF 2015) analysis allowed for identification of significant overrepresentation of GR-dependent genes in the cluster of transcripts induced by morphine in astrocytes (e.g. *Map3k6*, *Cdkn1a*, *Ctgf*, *Fkbp5*; **Figure 18b**). Moreover, enrichment analysis of the Drug Perturbations module (based on the GEO database) indicated that genes regulated by dexamethasone in the NAc show significantly overlapping pattern of transcriptional alterations after morphine and heroin treatment (e.g. *Sult1a1*, *Nfkbia*, *Arrdc2*, *Ddit4*, *Cdkn1a*, *Fkbp5*; **Figure 18c**).

4.3 Behavioral phenotyping and opioid effects in conditional astrocytic GR knockout mice

Generation of new transgenic mouse line for conditional GR knockout in astrocytes. To specifically impair GR signaling in astrocytes in vivo, we crossed the transgenic line Cx30-CreERT2 carrying tamoxifen-inducible version of Cre recombinase driven by astrocyte-specific promoter (Slezak et al. 2007) with the transgenic line $GR^{lox/lox}$ carrying critical exons of the *Nr3c1* gene, encoding GR, flanked by loxP sites (Tronche et al. 1999; **Figure 19a**). The offspring of this mating was genotyped for both transgenes. Bigenic, adult mice (8-10 weeks of age) were injected for 5 consecutive days with 100 mg/kg tamoxifen. Control group, monogenic mice, were subjected to identical tamoxifen administration. Behavioral and molecular experiments were performed at least 3 weeks after tamoxifen injection to ensure efficient elimination of the GR protein (**Figure 19b**).

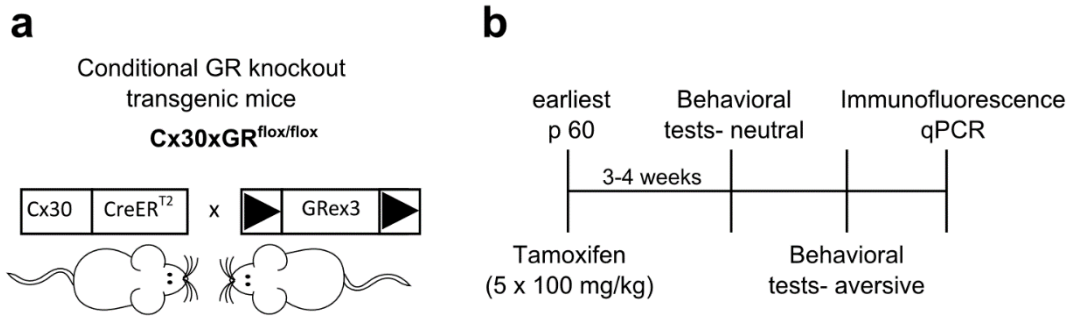

Figure 19. Transgenic model for astrocyte-specific elimination of GR. (**a**) Scheme of breeding of Cx30-CreERT2 line with the line containing exon 3 of GR gene flanked by loxP sites. (**b**) Experimental timeline. Animals older than 8 weeks were injected once daily with 100 mg/kg tamoxifen for 5 consecutive days. 3 to 4 weeks after tamoxifen injection animals were subjected to behavioral procedures. Animals that were not subjected to aversive/pharmacological studies were further used for molecular analysis.

Molecular effects of astrocytic GR knockout *in vivo*. The ablation of GR was verified by several methods. First, we examined mRNA expression levels of the *Nr3c1* (GR) transcript using qPCR in several regions of the central nervous system: prefrontal cortex, hypothalamus, hippocampus, striatum, amygdala and spinal cord. The levels of GR mRNA was significantly decreased in the hippocampus and spinal cord of GR knockout mice in comparison to control mice (**Figure 20**), while no difference was detected in other regions tested. We therefore examined functional effects of GR ablation in these regions. For that purpose, mice from the two groups received single injection of a GR agonist, dexamethasone (4 mg/kg, ip) and were sacrificed after 4 hours. Tissue were collected to analyze the

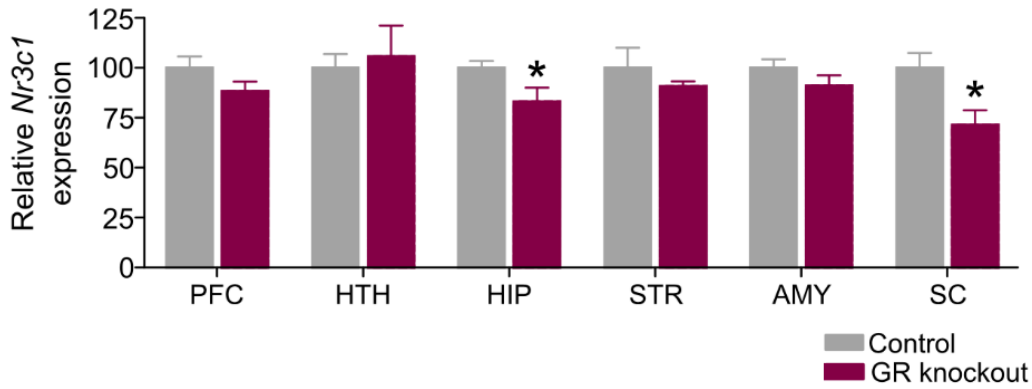

Figure 20. Abundance of Nr3c1 (GR) transcript in different brain areas of astrocytic GR knockout mice. mRNA expression levels of GR was significantly reduced in hippocampus (HIP) and spinal cord (SC) of astrocytic GR knockout mice when compared to control animals. GR expression levels in prefrontal cortex (PFC), striatum (STR), amygdala (AMY) and hypothalamus (HTH) was comparable between groups. Student's t-test, PFC t_6=0.12, p=0.9; HTH t_6=1.55, p=0.17; HIP t_6=2.55, p=0.04; STR t_6=0.88, p=0.41; AMY t_6=1.32, p=0.23; SC t_6=2.72, p=0.03. Control n=4, GR knockout n=4. Significant differences marked with * p<0.05. Data presented as mean ±SEM.

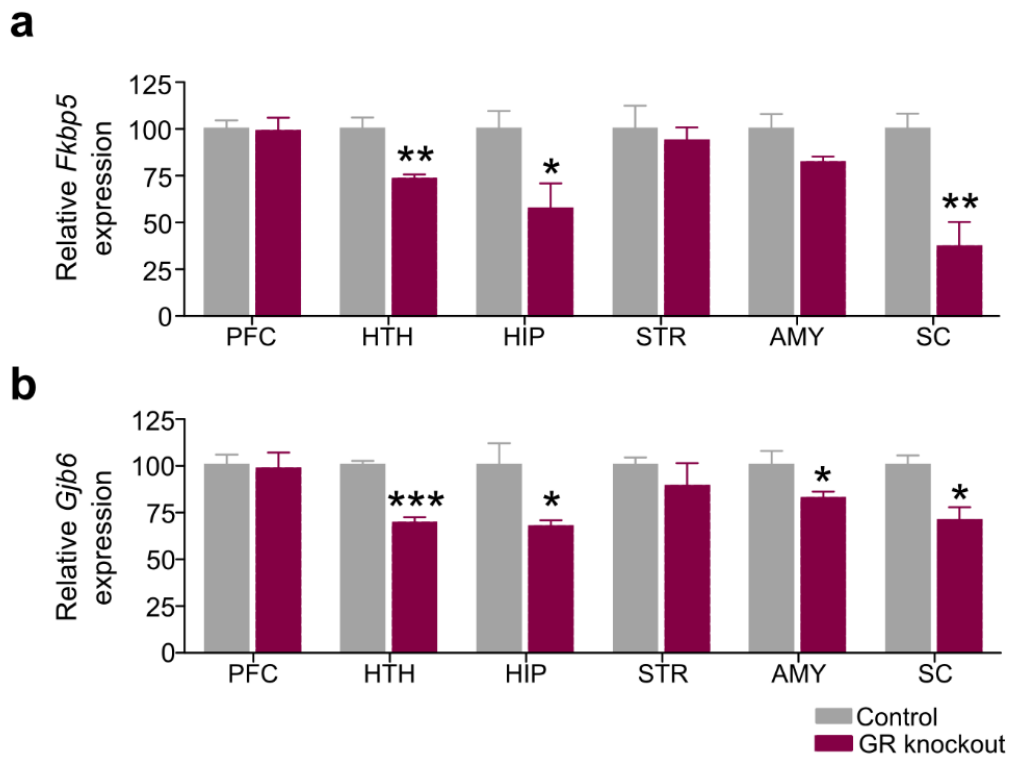

Figure 21. Abundance of *Fkbp5* and *Gjb6* transcripts in different brain areas of astrocytic GR knockout mice. (**a**) mRNA expression levels of *Fkbp5* was significantly reduced in hypothalamus (HTH), hippocampus (HIP) and spinal cord (SC) of astrocytic GR knockout mice. Gene expression levels in prefrontal cortex (PFC), striatum (STR) and amygdala (AMY) and was comparable between groups. (**b**) Similarly, *Gjb6* mRNA expression was decreased in HTH , HIP, AMY and SC of astrocytic GR knockout animals when compared to control mice. *Gjb6* expression in PFC and STR was unaffected by mutation. **a-b:** Student's t-test, **a:** PFC t_6=0.12, p=0.9; HTH t_6=3.98, p=0.007; HIP t_6=2.55, p=0.04; STR t_6=0.43, p=0.68; AMY t_6=2.06, p=0.08; SC t_6=4.07, p=0.006; **b:** PFC t_6=0.17, p=0.86; HTH t_6=8.32, p<0.001; HIP t_6=2.69, p=0.04; STR t_6=0.86, p=0.42; AMY t_6=2.11, p=0.04; SC t_6=3.41, p=0.01. Control n=4, GR knockout n=4. Significant differences marked with * p<0.05, ** p<0.01, *** p<0.001. Data presented as mean ±SEM.

mRNA expression of the GR-dependent genes, *Fkbp5* and *Gjb6* (Cx30). Selective elimination of the GR from astrocytes in GR knockout mice resulted in lowering dexamethasone-induced increase of *Fkbp5* and *Gjb6 in* spinal cord, hypothalamus and hippocampus (**Figure 21**). No significant differences of the GR-induced

transcripts were marked in the prefrontal cortex, striatum and amygdala of astrocytic GR knockout mice, as compared to the control groups. This pattern faithfully recapitulates the expression profile of Cre recombinase in Cx30-CreERT2 line (Slezak et al. 2007). Further, we analyzed GR protein expression in astrocytes of GR knockout and control animals using GFAP/GR immunofluorescent labeling. The GR and glial fibrillary acidic protein (GFAP) were clearly co-expressed in control animals, while no co-expression of the GR with GFAP was observed in GR knockout mice (**Figure 22**). Taken together, our results indicate that our strategy leads to partial blockade of GR signaling in astrocytes in several brain regions, including limbic system.

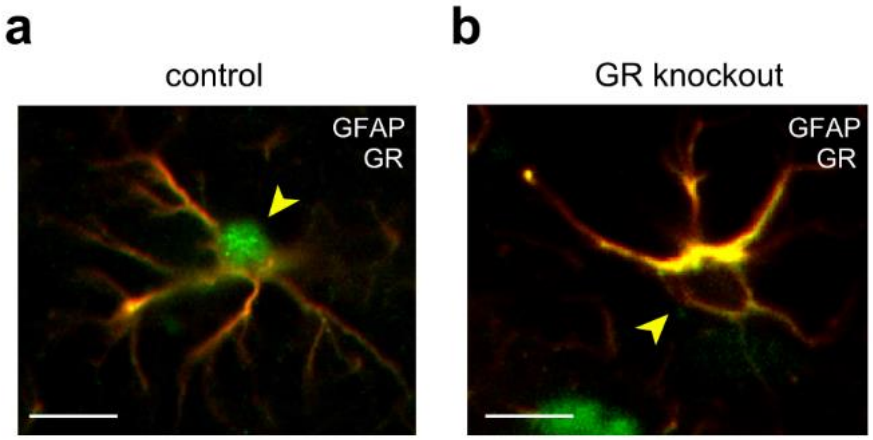

Figure 22. Astrocytic GR knockout immunofluorescence labelling. Labelling for (**a**) GFAP (astrocytic marker) and (**b**) GR confirmed the silencing of GR expression in astrocytes of GR knockout mice when compared to control group. Image shows merged stainings of representative astrocytes. Scale bars: 10 μm.

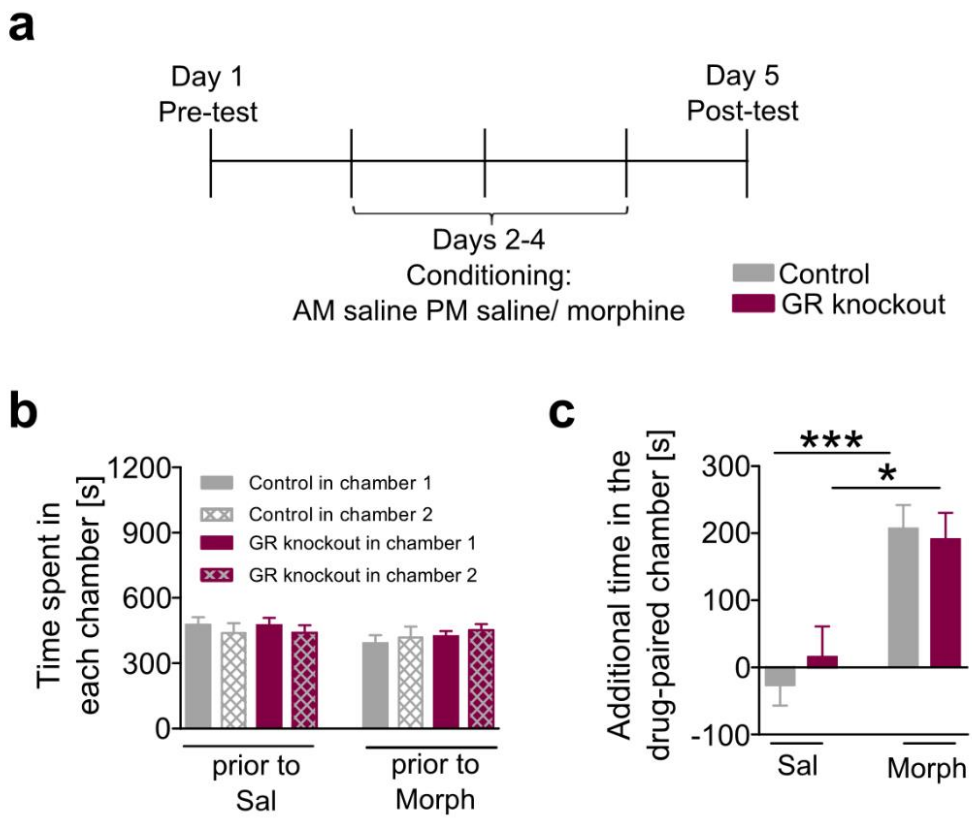

Figure 23. Astrocytic GR knockout does not alter sensitivity to opioid reward. (**a**) Experimental schedule of conditioned place preference (CPP). The experiment consisted of pre-conditioning test on day 1, 6 alternating saline and morphine-paired conditioning sessions on days 2-4 and post-conditioning test on day 5. Morphine paired animals received saline in the morning sessions and morphine (5 mg/kg, sc) in the afternoon sessions. Saline-paired animals received saline injections during both sessions. (**b**) During the pre-conditioning phase, control and GR knockout mice spent similar amount of time in the two conditioning chambers. (**c**) Both groups of animals have acquired comparable CPP to morphine-paired compartment. **b:** One-way ANOVA, $F_{7,110}$=0.64, p=0.72; **c:** Two-way ANOVA, $F_{1,55}$=0.9, p=0.35, treatment effect: $F_{1,55}$=23.17, p<0.001, genotype effect not significant. Control Sal n=14, GR knockout Sal n=15, Control Morph n=12, GR knockout Morph n=17. Where appropriate, tests were followed by Bonferroni post-hoc analysis. Significant differences in treatment (Sal vs Morph) marked with * p<0.05, *** p<0.001. Data presented as mean ±SEM.

Evaluation of opioid reward sensitivity in astrocytic GR knockout mice. We hypothesized that elimination of GR from astrocytes in the brain would affect reward-related behavior. Control and astrocytic knockout mice could freely explore the two-compartment during pretest on day 1 of the procedure. On days 2-4 they received saline in the morning and either saline or morphine (5 mg/kg, ip) in the afternoon. On the posttest day, mice were again allowed to explore the entire apparatus and the time that mice spent in the saline- and drug-associated compartments was measured (**Figure 23a**). During pretest, control and GR knockdown mice spent a similar amount of time in each of the two distinct compartments of the apparatus, indicating that CPP paradigm was unbiased (**Figure 23b**). We found that administration of morphine led to development of similar conditioned place preference in both control and astrocytic GR knockout groups (**Figure 23c**).

Effects of GR knockout in astrocytes on pain sensitivity, morphine analgesia and tolerance. Validation of the transgenic animals revealed a significant decrease of GR mRNA expression in the spinal cord. This structure is known to take part in the processing of pain, therefore we examined astrocytic GR knockout mice in a series of experiments to evaluate basal pain sensitivity, morphine-induced analgesia and tolerance. Firstly, animals were tested for baseline pain sensitivity. To induce tolerance, mice received 3 daily injections of morphine in growing doses for 4 days. Morphine-induced analgesia (2.5 or 5 mg/kg, ip) was measured 1 day prior to and 1 day after the chronic morphine administration **(Figure 24a)**. Both control animals and astrocytic GR knockout mice presented similar pain sensitivity (**Figure 24b**) and analgesic response to different doses of morphine (**Figure 24c-d**, dotted lines). Chronic administration of morphine resulted in tolerance to analgesic effects of this drug in both groups of animals (**Figure 24c-d**, continuous lines).

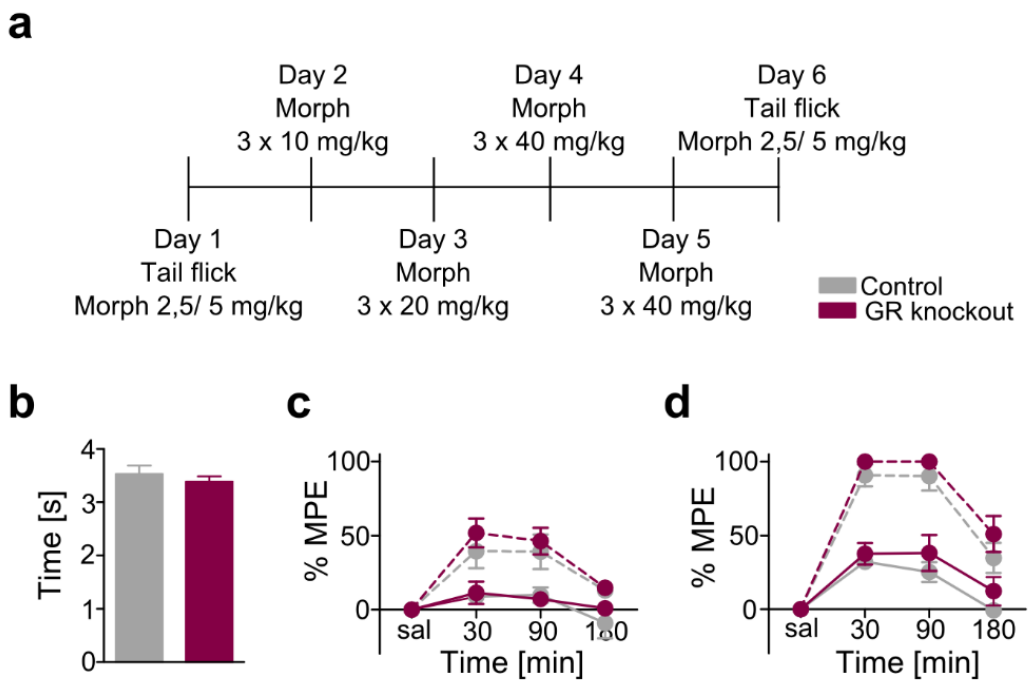

Figure 24. GR knockout in astrocytes does not affect pain sensitivity, opioid-induced analgesia and tolerance. (**a**) Experimental schedule of pain measurements. Tail-flick after single morphine administration (in doses 2.5 or 5 mg/kg) was evaluated before and after morphine was injected 3 times daily for 4 days in growing doses (10-40 mg/kg). (**b**) Basal pain sensitivity was similar in both groups of animals. Morphine in both doses: (**c**) 2.5 mg/kg and (**d**) 5 mg/kg caused similar analgesia in both groups of animals after single administration (dotted lines) as well as after chronic treatment (continuous line). **b:** Student's t-test, t_{18}=0.75, p=0.46; **c-d:** two-way repeated measures ANOVA, **c**: $F_{7,126}$=0.32, p=0.94, time effect: $F_{7,126}$=16.74, p<0.001, genotype effect insignificant. **d**: $F_{7,112}$=0.46, p=0.86, time effect: $F_{7,112}$=75.71, p<0.001, genotype effect insignificant. **c:** Control n=10, GR knockout n=10, **d:** Control n=9, GR knockout n=9. Data presented as mean ±SEMs.

Opioid withdrawal in astrocyte-specific GR knockout mice. As previously described, the procedure consisted of 3 daily morphine injections in growing doses for 4 days. On day 5, 3 hours after the last morphine dose (40 mg/kg), animals

were injected with naloxone (4 mg/kg, ip) and withdrawal symptoms were observed (**Figure 25a**). Number of jumps, rearings, teeth chattering and wet dog

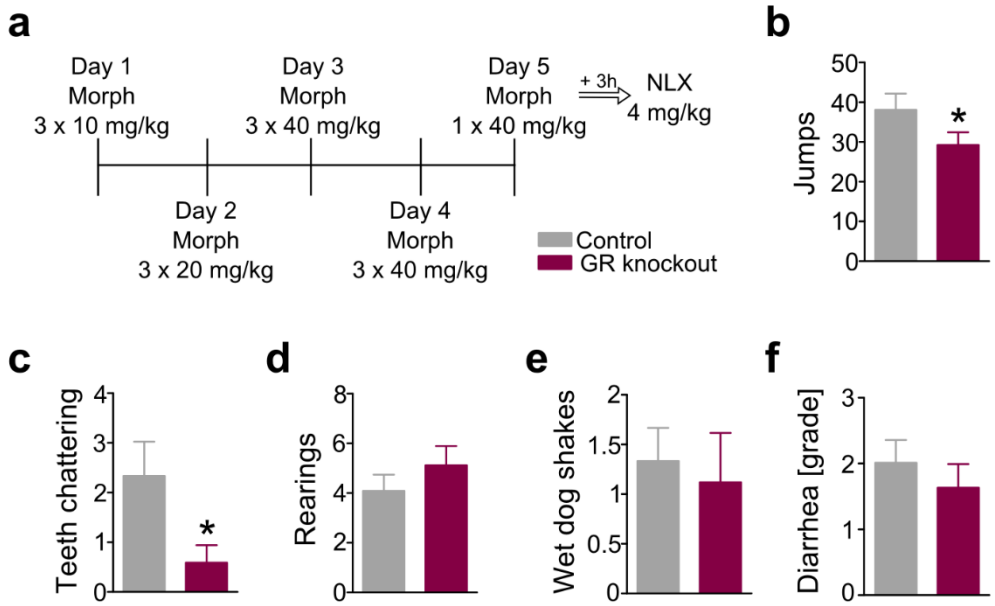

Figure 25. Astrocyte-specific GR knockout decreases the expression of opioid withdrawal. (**a**) Experimental schedule naloxone-precipitated morphine withdrawal. Morphine was injected 3 times daily for 4 days in growing doses (10-40 mg/kg). On day 5, 3 hours after the last morphine treatment, each mouse was injected with naloxone (4 mg/kg, ip). Number of naloxone-precipitated (**b**) jumps and (**c**) teeth chattering were significantly decreased in astrocytic GR knockout mice when compared to control group. Other observed symptoms of withdrawal were comparable between both groups and included (**d**) rearings, (**e**) wet-dog shaking (graded signs) as well as (**f**) diarrhea (observed sign, scoring: 0= no change, 1= mild, 2= moderate, 3= severe). **b-f:** Student's t-test, **b:** t_{27}=1.73, p=0.05 **c:** t_{27}=2.44, p=0.02; **d:** t_{27}=0.96, p=0.34; **e:** t_{27}=0.32 p=0.74; **f:** t_{27}=0.73, p=0.46. Control n=12, GR knockout n=17. Data presented as mean ±SEMs.

shakes were observed and counted and diarrhea was graded as expression of opioid withdrawal. Astrocytic GR knockdown mice displayed significantly less jumping

and teeth chattering when compared to control animals (**Figure 25b-c**). Number of rearings, wet dog shakes and observed diarrhea were comparable between groups (**Figure 25d-f**). Thus, astrocytic GRs act as a modulator of behavioral symptoms of morphine withdrawal.

Stress-induced memory formation and expression in astrocytic GR knockout mice. To evaluate whether astrocyte-specific GR signaling contributes to formation and expression of stress-induced memory, we used fear conditioning paradigm. During training session, mice received 5 consecutive foot shocks (1 mA). The freezing time measured during the training session was similar in both

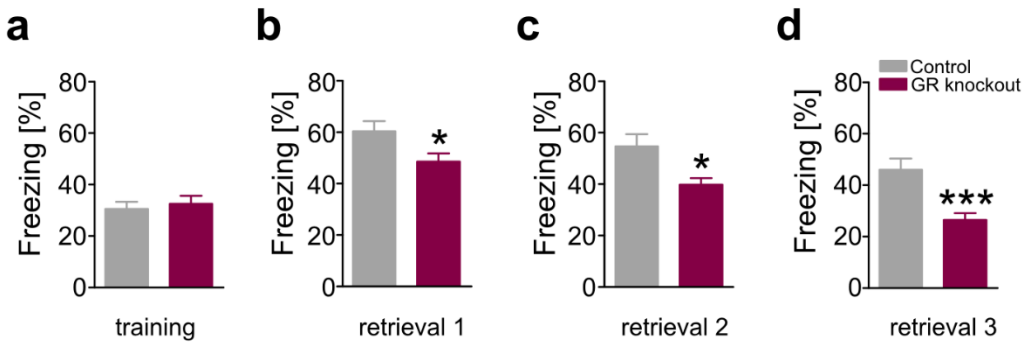

Figure 26. Astrocyte-specific elimination of GR leads to the impairment of fear memory. (**a**) Both control and GR knockout animals presented similar time of freezing during acquisition of conditioned fear. However, astrocytic GR knockout presented significant decrease of fear expression during retrievals (**b**) 24 h, (**c**) 72 h and (**d**) 120 h after training session. **a-d:** Student's t-test, **a:** t_{29}=0.48, p=0.63; **b:** t_{29}=2.2, p=0.03; **c:** t_{29}=2.6, p=0.01; **d:** t_{29}=3.7, p<0.001. Control n=17, GR knockout n=14. Data presented as mean ±SEMs.

groups (**Figure 26a**). Together with the fact that the nociception of GR knockout mice did not differ from the control animals (see: **Figure 24**, p. 93) this data confirms even sensory-activated responses in both groups. We then measured conditioned fear memory in 3 retrieval sessions, 24 h, 72 h and 120 h after the

training. Control mice displayed pronounced freezing response upon exposure to the context during retrieval sessions while this response was significantly diminished in the astrocytic GR knockout mice (**Figure 26b-d**).

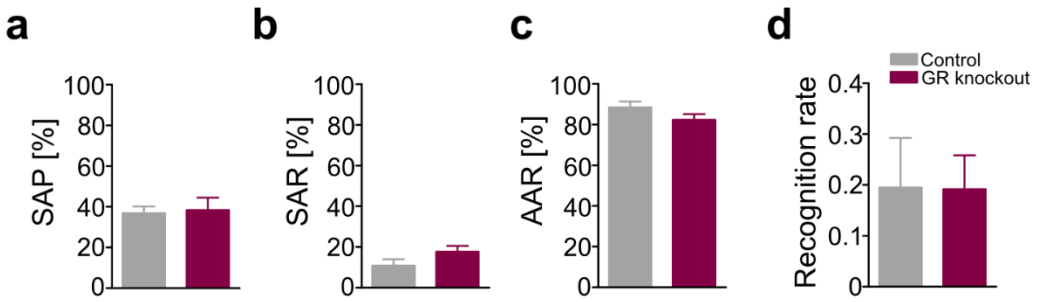

Figure 27. Astrocyte-specific elimination of GR does not alter basal memory. Both control and GR knockout animals presented similar working memory measured as (**a**) spontaneous alternation performance (SAP), (**b**) same arm returns and (**c**) alternate arms returns in the Y maze test. What is more, (**d**) GR knockout did not influence declarative memory measured as recognition rate in the novel object recognition test. **a-d:** Student's t-test, **a:** t_{18}=0.23, p=0.81; **b:** t_{18}=1.6, p=0.12; **c:** t_{18}=1.49, p=0.15; **d:** t_{10}=0.03, p=0.97. **a-c:** Control n=11, GR knockout n=9; **d:** Control n=6, GR knockout n=6. Data presented as mean ±SEMs.

Importantly, in a series of control experiments we excluded the possible effects of the mutation on several behavioral parameters that may confound the interpretation of these results. We did not observe the impact of the mutation on basal memory of the animals, since the working memory, measured as spontaneous alternation performance in the Y-maze test (**Figure 27a-c**), as well as novel object recognition rate in the novel object recognition test (**Figure 27d**) were similar in both groups of animals. What is more, both astrocytic GR knockout mice and control group presented comparable results in basal locomotor (**Figure 28a**) measured in activity arenas. This data reveals that astrocyte-specific GR signaling is involved in the expression of memory induced by stress.

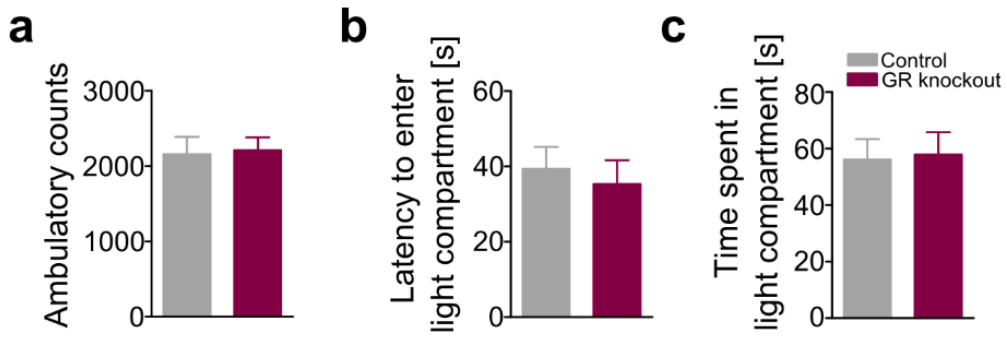

Figure 28. Astrocytic GR knockout does not influence anxiety. (**a**) GR knockout and control mice presented similar basal locomotor activity measured in activity arenas for 30 min. Both groups of animals presented similar anxiety levels measured as (**b**) latency to enter the illuminated compartment of the light-dark box, as well as (**c**) total time spent in this compartment. **a-c:** Student's t-test, **a:** t_{26}=0.18, p=0.85; **b:** t_{18}=0.47, p=0.64; **c:** t_{18}=0.15, p=0.87. **a:** Control n=12, GR knockout n=16; **b-c:** Control n=11, GR knockout n=9. Data presented as mean ±SEMs.

Assessment of anxiety and depression-like behavior in astrocytic GR knockout mice. The two groups did not differ in anxiety, since animals displayed similar latency to enter the anxiolytic, light compartment of the light/dark box (**Figure 28b**), as well as total time spent in that compartment (**Figure 28c**). Furthermore, we evaluated whether astrocytic GR knockout may affect depressive-like symptoms. We have measured learned helplessness in tail suspension test and evaluated anhedonia in saccharin preference test. Latency to immobility (**Figure 29a**) as well as total immobility in the tail suspension test (**Figure 29b**) were similar in both groups of animals. What is more, GR knockout and control mice presented comparable preference to sweetened solution (**Figure 29c**). Therefore, mutation of astrocytic GR did not elicit anxiety nor depressive-like symptoms.

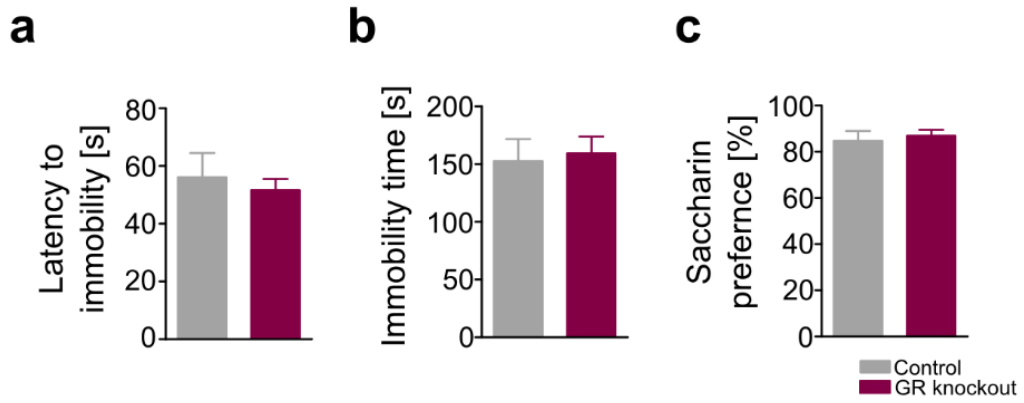

Figure 29. GR knockout in astrocytes does not influence depressive-like behavior. Depressive-like behavior in mice was measured with tail suspension and saccharin preference tests. Both control and GR knockout animals presented similar (**a**) latency to immobility and (**b**) total time spent immobile. Also, (**c**) no significant differences were marked between both groups in saccharin preference. **a-c:** Student's t-test, **a:** t_{16}=0.47, p=0.64; **b:** t_{16}=0.22, p=0.78; **c:** t_{16}=0.44, p=0.66. Control n=9, GR knockdown n=9. Data presented as mean ±SEMs.

4.4 Role of astrocyte-specific GR knockdown in the NAc in opioid action

Cell specific activation of GR-dependent genes in ventral striatum revealed that transcriptional changes in astrocytes rather than in neurons may be a key site of glucocorticoids action (see: **Figure 17,** p. 84). We have therefore targeted GRs, selectively in astrocytes, using viral-mediated RNA interference in the NAc.

Molecular effects of GR knockdown in astrocytes *in vitro* and *in vivo*. To gain a better understanding of the role of astrocytic GR in the NAc we have selectively

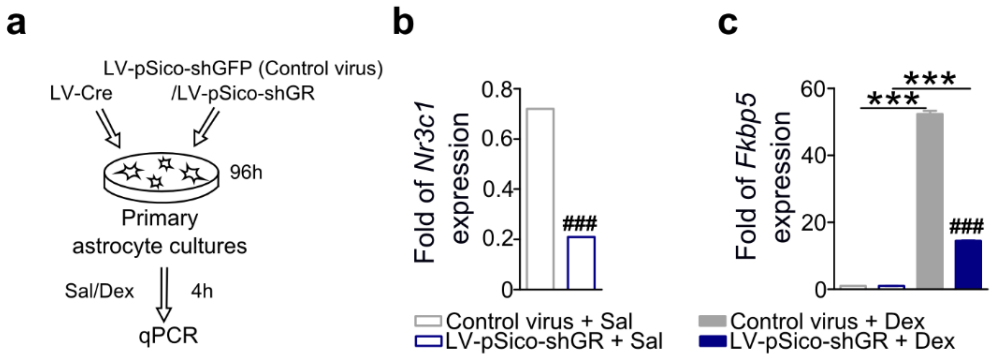

Figure 30. GR knockdown in astrocytes *in vitro*. (**a**) Experimental design for in vitro experiments. For validation of the construct, primary murine astrocytes were sequentially double-transduced with LV-Cre and LV-shGR or LV-shGFP vectors. 96 h post-transduction, cells were incubated with saline or 100 nM dexamethasone (Dex) for 4 h. Results showed suppressed expression of (**b**) *Nr3c1* as well as (**c**) decreased induction of *Fkbp5* gene by dexamethasone in LV-pSico-shGR transduced astrocytes. **b:** Student's t-test, t_2= 72.12, p<0.001; **c:** two-way ANOVA $F_{1,4}$=615.91, p<0.001. Control virus n=2 experiments, LV-pSico-shGR n=2 experiments. Where appropriate, tests were followed by Bonferroni post-hoc analysis. Significant differences in treatment (Sal vs Dex) marked with * p<0.05, ** p<0.01, *** p<0.001, significant differences between genotypes (control virus vs LV-pSico-shGR) marked with # p<0.05, ## p<0.01, ### p<0.001. Data presented as mean ±SEM.

downregulated the expression of GR protein and mRNA levels in astrocytes. For *in vitro* experiments, primary murine astrocytes were sequentially double-transduced with LV-Cre and LV-shGR (vector harboring Cre-dependent shRNA expression cassette for GR silencing) or LV-shGFP (control vector). 96h post-transduction, cells were stimulated with dexamethasone (100 nM) for 4h (**Figure 30a**). Expression of *Nr3c1* in LV-shGR transfected astrocytes was reduced by 70 % (**Figure 30b**). What is more, the induction of expression of *Fkbp5*, a GR-dependent gene, after dexamethasone stimulation was reduced by 75% in LV-shGR astrocytes when compared to LV-shGFP astrocytes, although significant increase of gene expression was observed in both control and LV-shGR transfected cultures when compared to saline treated astrocytes (**Figure 30c**).

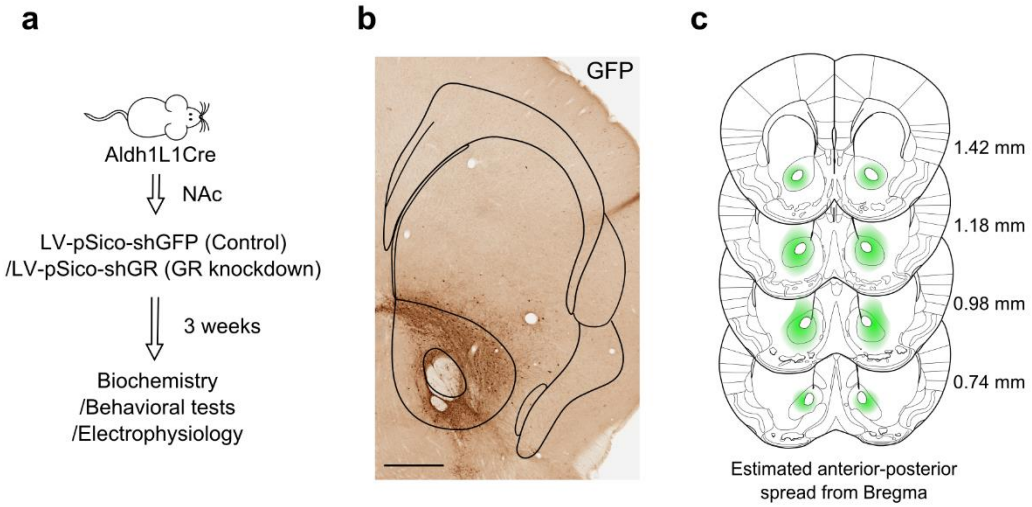

Figure 31. GR knockdown in astrocytes *in vivo*. (**a**) Experimental design for in vivo experiments. To obtain GR knockdown selectively in astrocytes, we have used Aldh1L1Cre mice that express the Cre recombinase under promoter specific for astrocytes. Animals were injected with LV-pSico-shGFP or LV-pSico-shGR vectors bilaterally into NAc. All tests were performed 3 weeks post surgery. (**b**) Image showing lentiviral encoded GFP staining of NAc transfected area. (**c**) Visualization of estimated lentiviral vector spread in the NAc. **b:** Scale bar: 500 µm; **c:** scheme based on n=5 animals

For *in vivo* studies we have used Cre-mediated GR targeting shRNAs in transgenic mice that express the Cre recombinase under aldehyde dehydrogenase 1 family promoter (Aldh1L1Cre), specific for astrocytes. As control vector we have used scrambled shRNA in the same animals (**Figure 31a**). Transfection site in the NAc was verified in brain slices using lentiviral encoded GFP staining (**Figure 31b-c**). Further, we analyzed GR protein expression in astrocytes in the transduction site with GFP/GFAP/DAPI/GR immunofluorescent labeling. The GR, nuclei marker (DAPI) and glial fibrillary acidic protein (GFAP) were clearly co-expressed in control animals, while no co-expression of the GR with aforementioned markers after lentiviral delivery has confirmed GR knockdown in astrocytes (**Figure 32**).

To test for functional molecular effects of astrocytic GR knockdown we have analyzed mRNA expression levels of the selected GR-dependent genes in the NAc after acute saline or dexamethasone (4 mg/kg i.p.) administration *in vivo*. The genes were chosen based on the microarray cluster of genes up-regulated specifically in astrocytes after dexamethasone injection (see: **Figure 17**, p. 84). The results show that induced by dexamethasone mRNA expression of *Cdkn1a*, *Ddit4*, *Fkbp5*, *Nfkbia* and *Arrdc2* was significantly reduced by astrocytic GR knockdown by about 30% (**Figure 33a-e**). No significant differences in mRNA expression of abovementioned genes were noted between groups after saline administration (**Figure 33a-e**), indicating that GR knockdown in astrocytes limits inducible transcriptional changes but does not cause any basal molecular alterations. In addition, we measured animals' blood serum corticosterone levels after saline and dexamethasone administration. Basal, as well as suppressed by dexamethasone corticosterone levels were comparable in both groups of animals (**Figure 33f**), indicating that astrocytic GR knockdown in the NAc does not affect general HPA activity of the animals.

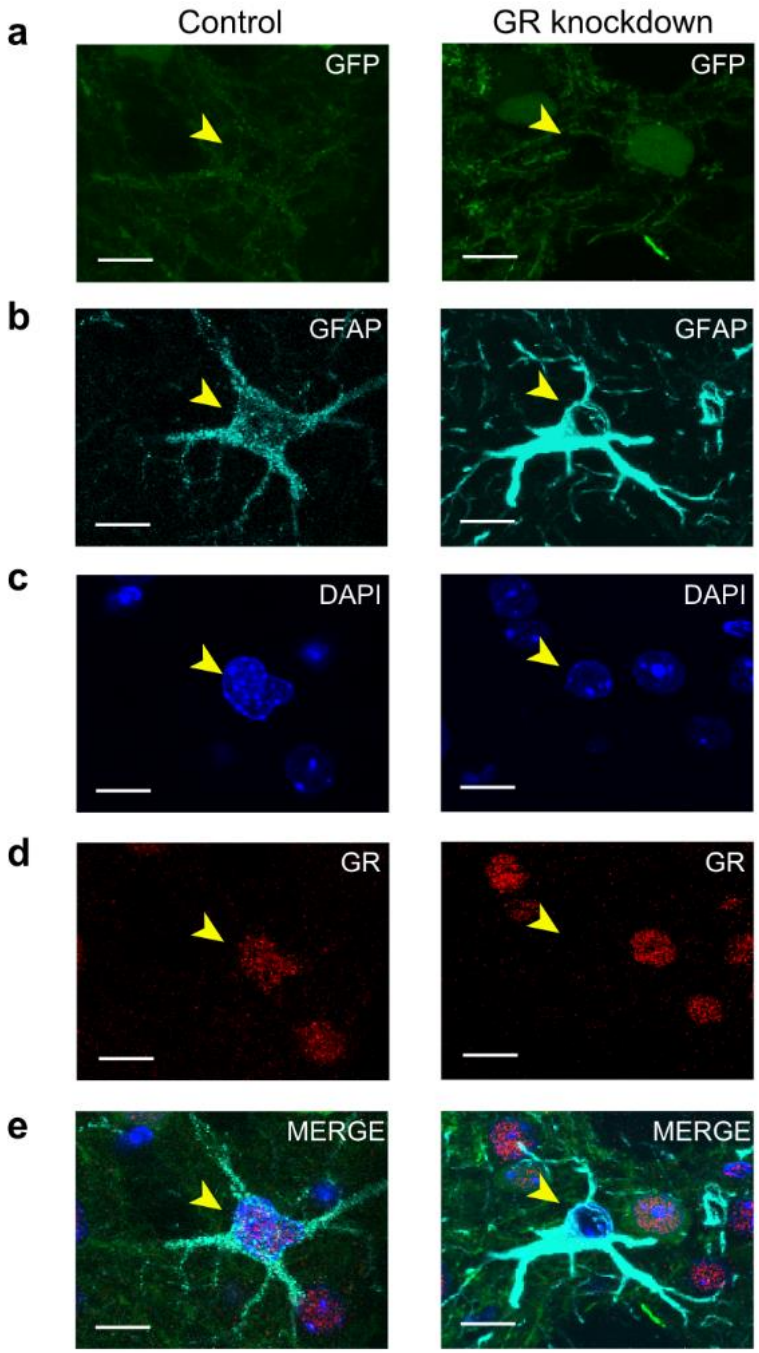

Figure 32. Astrocytic GR knockdown immunofluorescence labelling. Labelling for (**a**) GFP (viral vector), (**b**) GFAP (astrocytic marker), (**c**) DAPI (nuclei) and (**d**) GR confirmed the silencing of GR expression in astrocytes of LV-pSico-shGR transduced mice when compared to control virus. (**e**) Image showing merged stainings. Scale bars: 10 µm.

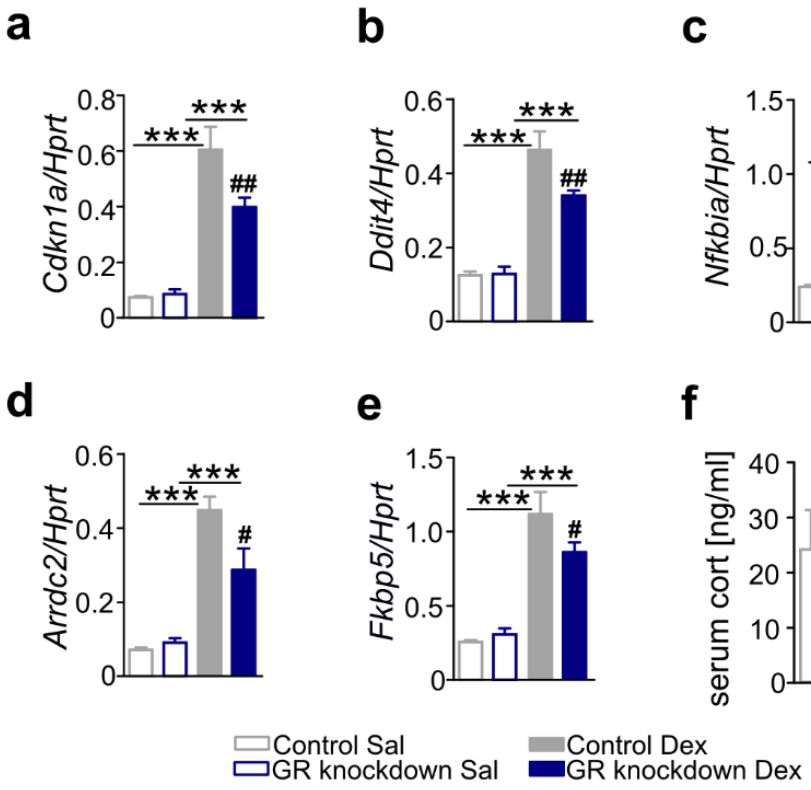

Figure 33. Selected GR-dependent genes expression in the NAc of GR knockdown and control mice. mRNA expression levels of (**a**) *Cdkn1*, (**b**) *Ddit4*, (**c**) *Nfkbia*, (**d**) *Arrdc2* and (**e**) *Fkbp5* in the NAc was comparable between groups after saline injection, but induction of these genes with dexamethasone (4 mg/kg, ip) was suppressed by astrocytic GR knockdown by about 30%. (**f**) Dexamethasone (Dex) administration (4 mg/kg, ip) caused similar suppression of blood serum corticosterone levels in both control and astrocytic GR knockdown mice **a-f:** two-way ANOVA **a:** *Cdkn1a* $F_{1,18}$=8.79, p=0.008; **b:** *Ddit4* $F_{1,18}$=7.41, p=0.01; **c:** *Nfkbia* $F_{1,18}$=7.93, p=0.01; **d:** *Arrdc2* $F_{1,18}$=6.24, p=0.02; **e:** *Fkbp5* $F_{1,18}$=4.85, p=0.04; **f:** $F_{1,18}$=0.29 p=0.59, treatment effect: $F_{1,18}$=18.02 p<0.001, genotype effect insignificant. Sal n=6, Dex n=5 in each group. Where appropriate, tests were followed by Bonferroni post-hoc analysis. Significant differences between treatments (Sal vs Dex) marked with * p<0.05, ** p<0.01, *** p<0.001, significant differences between genotypes (control vs GR knockdown) marked with # p<0.05, ## p<0.01, ### p<0.001. Data presented as mean ±SEM.

Effects of GR knockdown in astrocytes on opioid reward sensitivity. To address the behavioral consequences of the GR knockdown in astrocytes on reward-related behavior, we analyzed morphine-induced conditional place preference (CPP) and locomotor sensitization at least 3 weeks after viral vector administration. CPP paradigm consisted of pretest, 3 consecutive conditioning sessions (saline-paired session each morning and saline or morphine-paired session each afternoon) and posttest (**Figure 34a**). Before conditioning, control and GR knockdown mice spent a similar amount of time in each of the two distinct compartments of the apparatus, indicating that CPP paradigm was unbiased (**Figure 34b**). Morphine administration (5 mg/kg, sc) induced CPP when compared to saline in both groups of animals, however GR knockdown animals presented considerably greater preference to morphine-paired compartment than control mice (**Figure 34c**). This demonstrates, that astrocytes take part in modulation of reward sensitivity and expression of morphine-associated memory through GR-dependent pathway.

Effect of astrocytic GR silencing on morphine-induced locomotor sensitization. Before investigating the effects of astrocytic GR knockdown on morphine-evoked locomotor sensitization, we tested both groups of animals in activity arenas for 30 minutes, to ensure GR knockdown does not influence basal behavior and in fact all animals presented similar levels of locomotor activity (**Figure 35a**). Further, we have studied development of sensitization to morphine-induced hyperlocomotion. After adaptation to locomotor activity chambers, animals received alternate daily injections of saline or morphine (5 mg/kg, sc) for 6 days. Expression of locomotor sensitization was tested 7 days following cessation of morphine administration (**Figure 35b**). Astrocytic GR knockdown did not influence acquisition (**Figure 35c**) nor expression (**Figure 35d**) of morphine-induced locomotor sensitization.

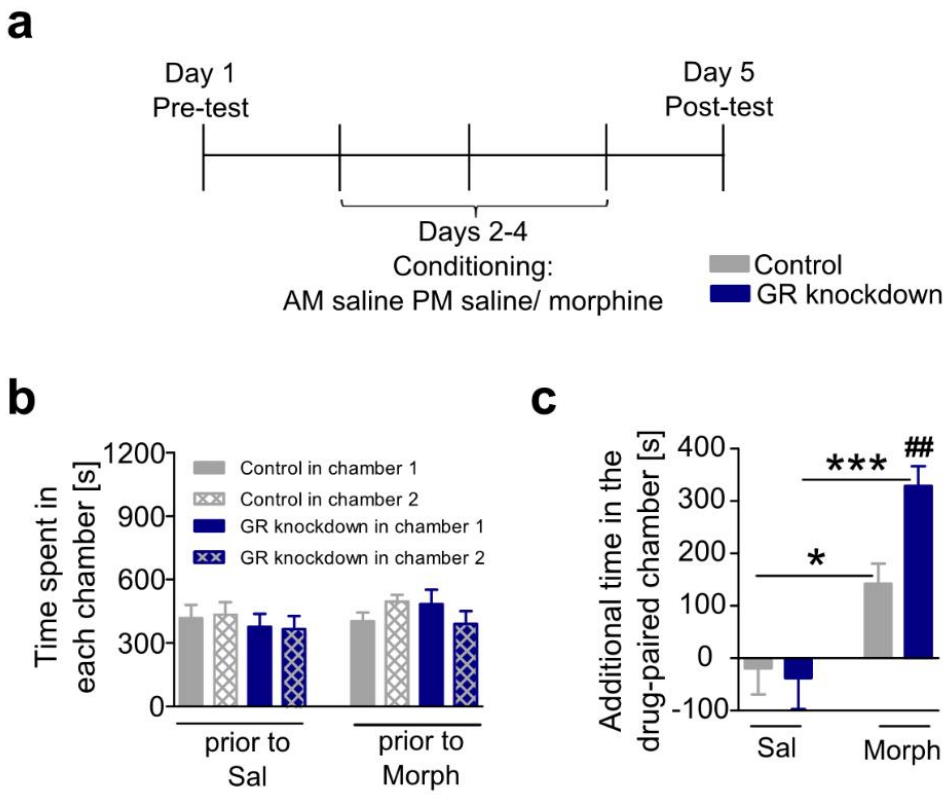

Figure 34. Astrocytic GR in the NAc regulates sensitivity to opioid reward. (**a**) Experimental schedule of conditioned place preference (CPP). The experiment consisted of pre-conditioning test on day 1, 6 alternating saline and morphine-paired conditioning sessions on days 2-4 and post-conditioning test on day 5. Morphine paired animals received saline in the morning sessions and morphine (5 mg/kg, sc) in the afternoon sessions. Saline-paired animals received saline injections during both sessions. (**b**) During the pre-conditioning phase, control and GR knockdown mice spent comparable amount of time in the two conditioning chambers. (**c**) Both groups of animals have acquired CPP, however astrocytic GR knockdown resulted in escalation of morphine-induced effect when compared to control animals. **b:** One-way ANOVA, $F_{7,108}=0.77$, $p=0.61$; **c:** Two-way ANOVA, $F_{1,54}=4.77$, $p=0.03$. Control Sal n=12, GR knockdown Sal n=14, Control Morph n=16, GR knockdown Morph n=16 Where appropriate, tests were followed by Bonferroni post-hoc analysis. Significant differences in treatment (Sal vs Morph) marked with * $p<0.05$, *** $p<0.001$, significant differences between genotypes (control vs GR knockdown) marked with ## $p<0.01$. Data presented as mean ±SEM.

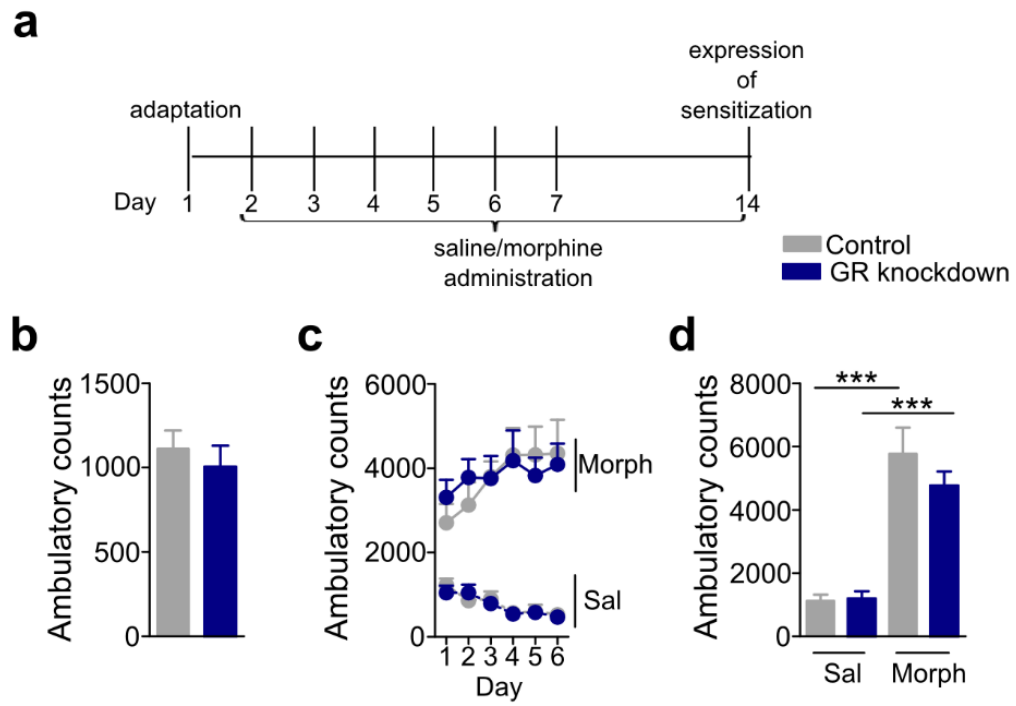

Figure 35. GR knockdown in astrocytes does not cause changes in morphine-induced locomotor sensitization. (**a**) Experimental schedule of morphine-induced locomotor sensitization. After adaptation to activity chambers, animals received daily injections of saline or morphine (5 mg/kg, sc) in 2 h sessions for 6 days. The challenge morphine injection was performed 7 days later. (**b**) Basal locomotor activity measured for 30 minutes was similar in both groups of animals. (**c**) Astrocytic GR knockdown had no effect on acquisition or (**d**) expression of morphine induced sensitization. **b:** Student's t-test, t_{38}=0.63, p=0.53, n=20 per group; **c:** Three-way ANOVA, $F_{1,270}$=175.64, p<0.001 with significant treatment × time interaction: $F_{5,270}$=10.36, p<0.001, other effects insignificant; **d:** Two-way ANOVA, $F_{1,53}$=1.27 p=0.26, treatment effect: $F_{1,53}$=75.98 p<0.001, genotype effect insignificant. **c-d:** Control Sal n=14, GR knockdown Sal n=15, Control Morph n=14, GR knockdown Morph n=15. Where appropriate, tests were followed by Bonferroni post-hoc analysis. Significant differences in treatment (Sal vs Morph) marked with *** p<0.001. Data presented as mean ±SEM.

Opioid withdrawal in astrocytic GR knockdown mice. Taking into consideration previous reports about the participation of the GR in opiate withdrawal (McNally and Akil 2003; Navarro-Zaragoza et al. 2012), we have examined animals for signs of naloxone-precipitated physical dependence after chronic morphine treatment. The procedure consisted of 3 daily morphine

injections in growing doses for 4 days. On day 5 mice were injected with naloxone (4 mg/kg, ip) 3 hours after the last morphine dose (40 mg/kg) and withdrawal symptoms were observed for 15 min (**Figure 36a**). During chronic morphine administration we have observed similar decrease in weight of both GR knockdown and control animals (**Figure 36b**). Number of jumps, rearings and wet

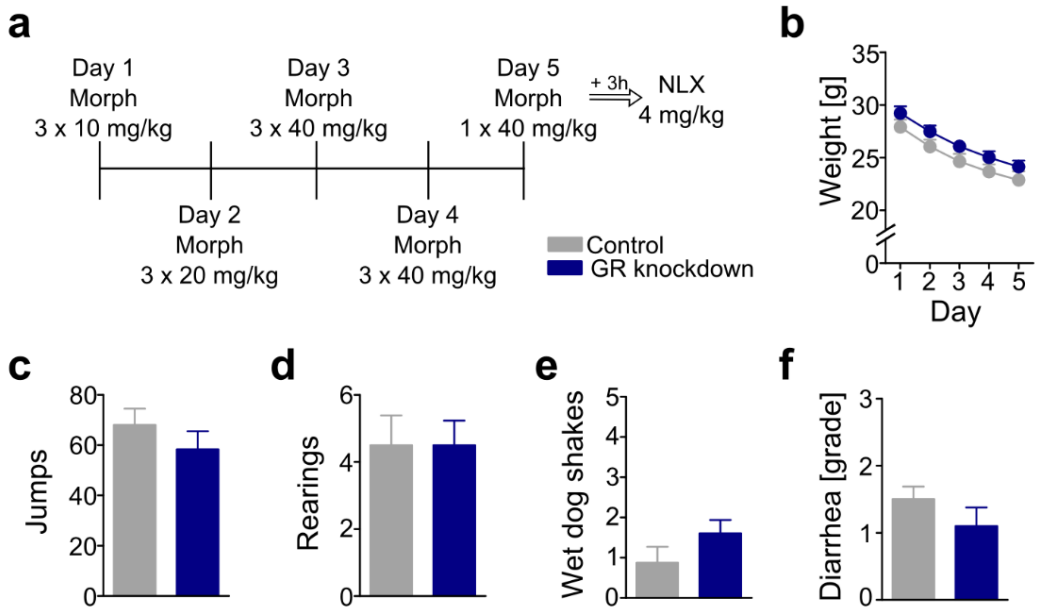

Figure 36. GR knockdown in astrocytes does not affect the expression of opioid withdrawal. (**a**) Experimental schedule naloxone-precipitated morphine withdrawal. Morphine was injected 3 times daily for 4 days in growing doses (10-40 mg/kg). On day 5, 3 hours after the last morphine treatment, each mouse was injected with naloxone (NLX, 4 mg/kg, ip). (**b**) Morphine administration caused similar decrease in weight in both groups of animals. Observed symptoms of withdrawal were comparable between GR knockdown and control animals and included (**c**) jumps, (**d**) rearings, (**e**) wet-dog shaking (graded signs) as well as (**f**) diarrhea (observed sign, scoring: 0= no change, 1= mild, 2= moderate, 3= severe). **b:** two-way repeated measures ANOVA, $F_{4,64}$=0.07, p=0.99, time effect: $F_{4,64}$=177.56, p<0.001, genotype effect insignificant. **c-f:** Student's t-test, **c:** t_{16}=0.98, p=0.34; **d:** t_{16}=0.0, p=1; **e:** t_{16}=1.39, p=0.18; **f:** t_{16}=1.31, p=0.27. Control n=8, GR knockdown n=10. Data presented as mean ±SEMs.

dog shakes were observed and counted and diarrhea was graded as expression of opioid withdrawal. No significant differences were observed in either of the symptoms between astrocytic GR knockdown and control animals, indicating similar withdrawal intensity in both groups of animals (**Figure 36c-f**). Altogether, our results present that astrocytic GR in the nucleus accumbens selectively modulates sensitivity to opioid reward, without changing other opioid-related addictive behaviors, such as locomotor sensitization and withdrawal.

Stress-induced memory formation and expression in astrocytic GR knockdown mice. GR is thought to be an important factor in the development of stress-related disorders, such as posttraumatic stress disorder and depression (Skupio et al. 2014; Miranda et al. 2008). Previous studies in animal models showed the involvement of the ventral striatum in these disorders (Ikegami et al. 2014; Bagot et al. 2015), therefore we have assessed the behavior of astrocytic GR

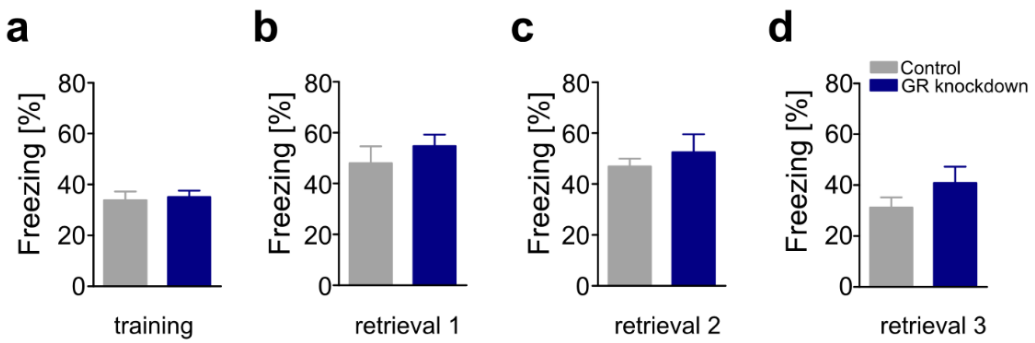

Figure 37. GR knockdown in astrocytes does not change memory induced with stress. Both control and GR knockdown animals presented similar time of freezing during (**a**) acquisition and expression of conditioned fear during retrievals (**b**) 24 h, (**c**) 72 h and (**d**) 120 h after training session. **a-d:** Student's t-test, **a:** t_{18}=0.28, p=0.78; **b:** t_{18}=0.82, p=0.42; **c:** t_{18}=0.71, p=0.48; **d:** t_{18}=1.27, p=0.21. Control n=10, GR knockdown n=10. Data presented as mean ±SEMs.

knockdown animals in a series of tests that measure stress-related memory, anxiety and depression-like behavior. To measure stress-induced memory formation and expression we have behaviorally challenged the animals in fear conditioning paradigm using foot shocks (5 x 1mA) as aversive stimuli. At 24 h and 72 h after training, mice were placed in the conditioned fear context, where freezing was measured as an expression of contextual fear memory. The animals of both groups presented similar acquisition (**Figure 37a**) and expression (**Figure 37b-c**) of conditioned fear, indicating that astrocytic GR knockdown did not influence stress-induced memory formation, consolidation and reconsolidation.

Evaluation of anxiety and depression-like behavior in astrocytic GR knockdown mice. To measure the core symptoms of depressive-like behavior animals were subjected to tail suspension test and saccharin preference test, to evaluate learned helplessness and anhedonia, respectively. No significant differences were marked between both groups of animals in latency to immobility (**Figure 38a**) and time spent immobile in tail suspension test (**Figure 38b**). What is more, both GR knockdown and control groups presented similar preference to saccharin (**Figure 38c**) indicating that basal depressive-like behavior was unaffected by the astrocytic GR knockdown. Similarly, so significant differences were observed in anxiety levels measured as novel object exploration time (**Figure 39a**), latency to enter the anxiolytic, illuminated compartment of the light-dark box (**Figure 39b**), as well as total time spent in this compartment (**Figure 39c**). Behavioral profile of the animals indicates that astrocytic GR knockdown in the ventral striatum does not cause alterations in basal stress-related behavioral traits, such as fear memory, anhedonia, learned helplessness and anxiety.

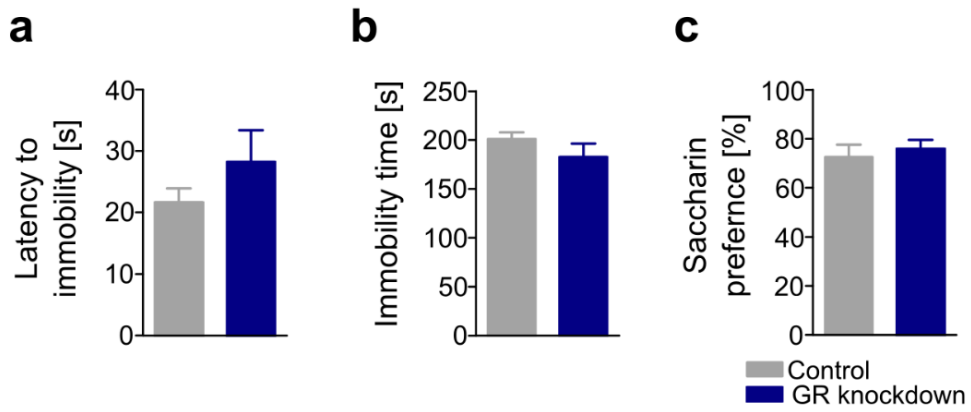

Figure 38. GR knockdown in astrocytes does not influence depressive-like behavior. Depressive-like behavior in mice was measured with tail suspension and saccharin preference tests. Both control and GR knockdown animals presented similar (**a**) latency to immobility as well as (**b**) total time spent immobile. Also, (**c**) no significant differences were marked between both groups in saccharin preference. **a-c:** Student's t-test, **a:** t_{21}=1.21, p=0.23; **b:** t_{21}=1.22, p=0.24; **c:** t_{21}=0.53, p=0.6. Control n=12, GR knockdown n=11. Data presented as mean ±SEMs.

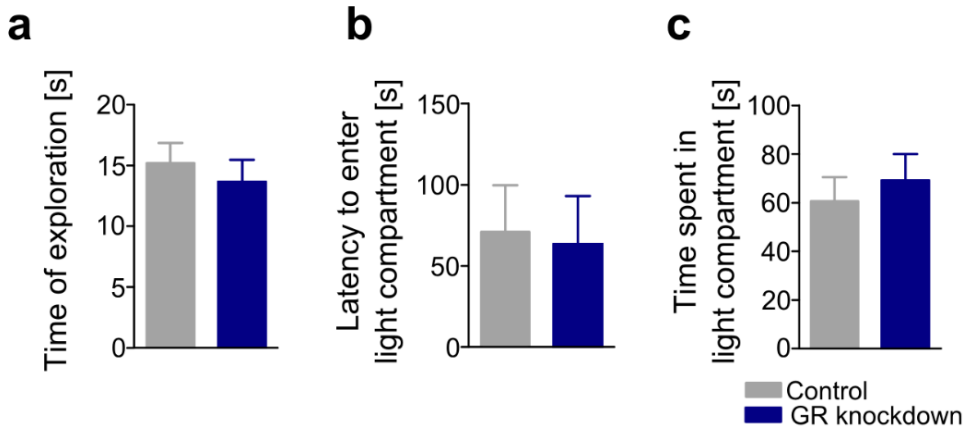

Figure 39. Astrocytic GR knockdown does not influence anxiety. Both groups of animals presented similar anxiety levels measured as (**a**) novel object exploration time, (**b**) latency to enter the illuminated compartment of the light-dark box, as well as (**c**) total time spent in this compartment. **a-c:** Student's t-test, **a:** t_{21}=0.62, p=0.54; **b:** t_{21}=0.18, p=0.86; **c:** t_{21}=0.58, p=0.57. Control n=12, GR knockdown n=11. Data presented as mean ±SEMs.

Astrocytic GR knockdown in nucleus accumbens alternates morphine-induced synaptic plasticity. Silencing of GR in astrocytes in NAc have caused selective increase of sensitivity to opioid reward. We aimed to further examine possible neural correlates of these behavioral changes. The experiments were conducted in collaboration with the Department of Physiology, Polish Academy of Sciences by Joanna Ewa Sowa, Marcin Siwiec and Bartosz Bobula. We have studied the effect of astrocytic GR knockdown on properties of single neurons as well as synaptic plasticity in the NAc after either saline or morphine (5 mg/kg, ip) administration *in vivo*. Single cells in the transfected area of the NAc were studied using whole-cell patch-clamp analysis. Membrane potential and resistance of the recorded neurons was similar in control and astrocytic GR knockdown animals and remained unchanged regardless of treatment (**Figure 40**). After saline administration spontaneous excitatory postsynaptic currents (sEPSC) amplitude and frequency (**Figure 40**) were similar in both groups. Interestingly, astrocytic GR knockdown caused significant decrease of morphine-induced sEPSC amplitude, but not frequency (**Figure 40**). We observed comparable stimulus-induced excitability of NAc neurons between groups after saline administration, however astrocytic GR knockdown decreased neuronal excitability after morphine administration (**Figure 41**). Consistently with other results, we observed no significant differences between both groups in LTP in the NAc after saline injection, but significant alteration of LTP after morphine treatment in astrocytic GR knockdown animals (**Figure 41**). Altogether, electrophysiological results confirmed no effects of astrocytic GR knockdown on basal properties of the medium spiny neurons, however, GR-knockdown permitted a considerable morphine-induced inhibition of neural excitability and plasticity.

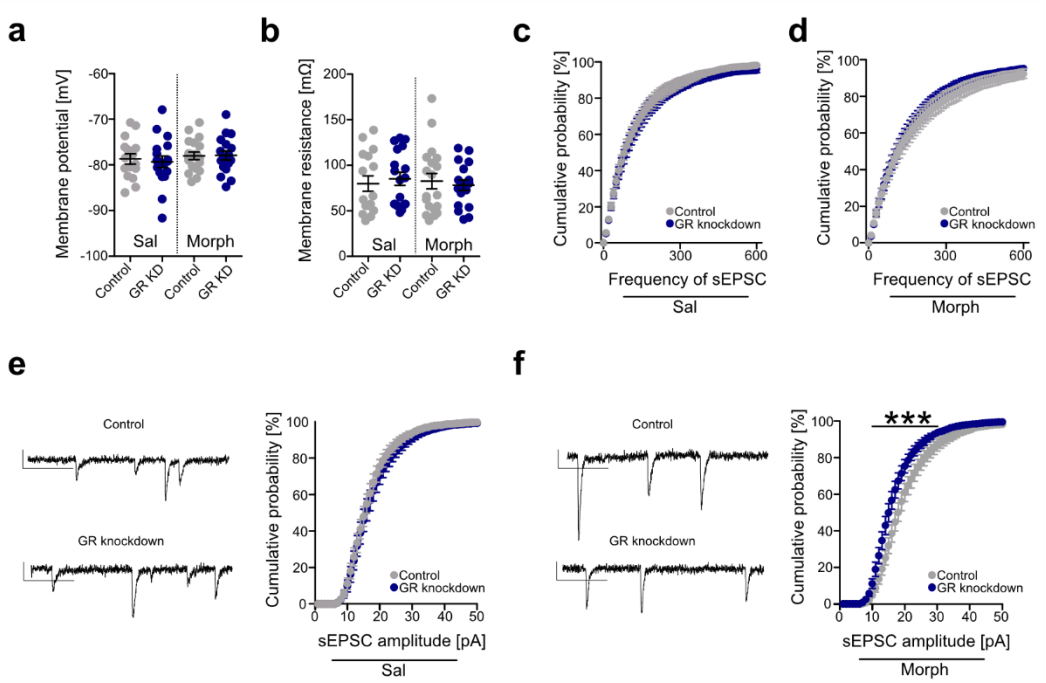

Figure 40. Whole-cell patch-clamp analysis of the NAc cells in GR knockdown and control mice. (**a**) Membrane potential as well as (**b**) membrane resistance of the recorded neurons was similar in both groups of animals after both saline and morphine treatment. sEPSC frequency was comparable in control and astrocytic GR knockdown animals after both (**c**) saline and (**d**) morphine administration. (**c, d**) Graphs present mean cumulative probability of the frequencies of the recorded sEPSC. (**e**) Basal sEPSC amplitude was comparable in both groups. Surprisingly, (**f**) astrocytic GR knockdown induced significant attenuation of morphine-induced neuronal sEPSC amplitude. (**e, f**) Left: representative sEPSC traces. Scale bars: 10 pA/100 ms Right: mean cumulative probability of sEPSC amplitude. **a-f**: Two-way ANOVA; **a-b**: Control Sal n=16, GR knockdown Sal n=18, Control Morph n=19, GR knockdown Morph n=17; a: $F_{1,66}$=0.12, p=0.73; b: $F_{1,66}$=0.39, p=0.53; **c**: Control n=10, GR knockdown n=15, $F_{60,1740}$=0.07, p=1; **d**: Control n=17, GR knockdown n=16, $F_{60,1860}$=0.35, p=1. **e**: Control n=10, GR knockdown n=15, $F_{49,1127}$=0.26, p=1; **f**: Control n=17, GR knockdown n=16, $F_{49,1519}$=5.19, p<0.001. Where appropriate, tests were followed by Bonferroni post-hoc analysis. Significant differences between genotypes (control vs GR knockdown) marked with *** p<0.001. GR KD- GR knockdown. Data presented as mean ±SEM.

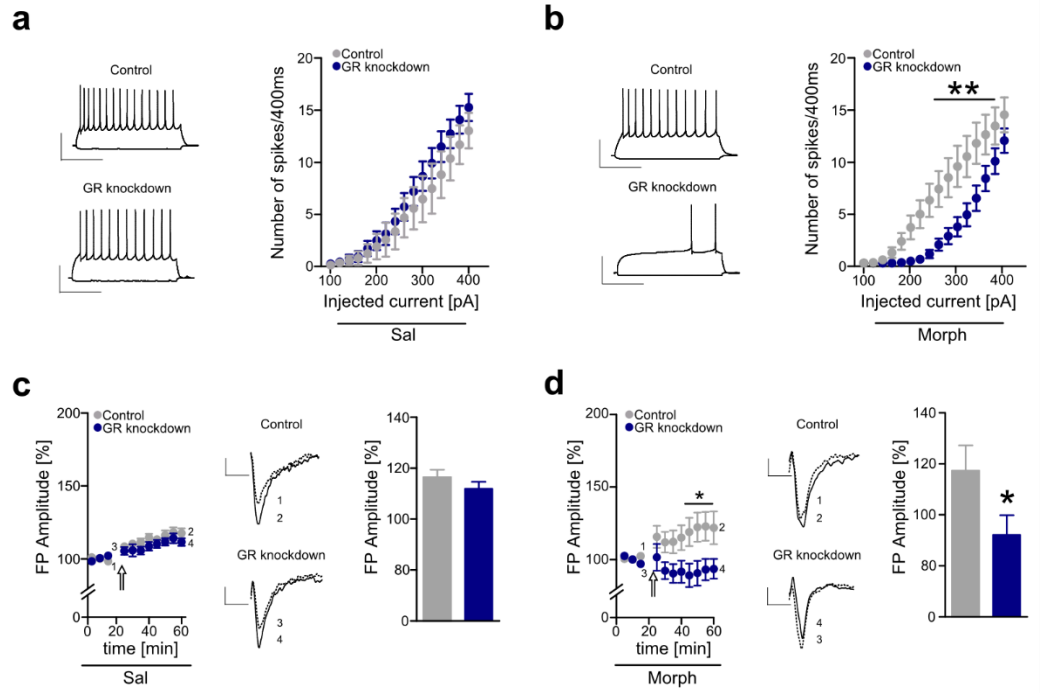

Figure 41. GR knockdown in astrocytes alternates morphine-induced synaptic plasticity. (**a**) No significant differences in the stimulus-induced excitability of medium spiny neurons were observed between groups under basal conditions, but (**b**) astrocytic GR knockdown resulted in reduced neuronal excitability after morphine administration. (**a, b**) Left: representative traces of neuronal action potentials induced by depolarizing current steps. Scale bars: 50 pA/200 ms Right: relation between the stimulus intensity and the number of generated spikes. (**c**) Basal LTP in the NAc was comparable in both groups of animals, however (**d**) astrocytic GR knockdown caused significant alteration of LTP after morphine administration. (**c, d**) Left panels: relative amplitude values of the field potentials (FP). The use of amplitude for NAc was necessitated by the small potential amplitudes and the resulting low signal-to-noise ratio. The arrow indicates the time point at which stimulus was applied. Middle: representative evoked potentials in control and astrocytic knockdown mice before and after stimulation, as indicated by the numbers 1–4. Scale bars: 0,1 mV/5 ms Right: mean averaged values 45–60 min after stimulus. **a-d**: Two-way ANOVA; **a**: Control n=9, GR knockdown n=17, $F_{15,360}=0.73$, p=0.75; **b**: Control n=16, GR knockdown n=16, $F_{15,450}=4.22$, p<0.001; **c**: Control n=8, GR knockdown n=7, $F_{10,130}=1.67$, p=0.1; **d**: Control n=8, GR knockdown n=10, $F_{10,160}=3.8$, p<0.001. Where appropriate, tests were followed by Bonferroni post-hoc analysis. Significant differences between genotypes (control vs GR knockdown) marked with * p<0.05, ** p<0.01, *** p<0.001. Data presented as mean ±SEM.

Chapter 5 Discussion

New model of chronic opioid self-administration. One of the primary goals of the current study was to identify behavioral patterns and accompanying molecular changes that are associated with chronic opioid treatment. The most widely currently used animal paradigms to model opioid addiction involve self-administration and conditioned place preference (Ettenberg et al. 1982; Tzschentke 1998). These approaches allow to reproduce the main features of addiction, but they also have serious limitations, which include: isolation, restricted time of testing sessions and major experimenter's interference with the animals. What is more, these models allow only to study certain symptoms of addiction, such as reward-sensitivity, craving or dependence, separately. Paradigms that involve chronic administration of the drug are conducted within short periods of time, usually 5-14 days, which can be regarded as both an advantage and a drawback. The benefits would include reproducibility and the ability to quickly achieve results, however such an approach does not reflect natural development of addiction, as it occurs in humans, and may not capture significant behavioral and molecular changes that develop over longer periods of time. As a response to this criticism new models are continuously developed using the IntelliCage system (Galsworthy et al. 2005; Radwanska and Kaczmarek 2012). This new, holistic approach allows to test animals' motivation for obtaining addictive substances under various behavioral procedures and assess each animal's behavior and molecular changes in the brain individually, while avoiding the abovementioned limitations of other paradigms.

The model we created used a self-administration paradigm that did not include any prior opioid injections. Earlier publications have shown that at least 3

months of voluntary self-administration is needed to induce alcohol dependence in rats (Wolffgramm and Heyne 1995; Steensland et al. 2012; Jonsson et al. 2014). In the present study, throughout the administration of rewarding substances, both saccharin and morphine were strongly preferred. Interestingly, morphine consumption induced long-lasting elevated activity in the cage. Moreover, after 3 months, the morphine-dependent animals had spent more time in the corners associated with reward than the saccharin-drinking mice. Animals appeared to associate the rewarding effect of morphine with a certain context. In this case, it was a particular corner, and they therefore tended to spend more time there without drinking episodes, whereas the saccharin-treated animals directed most of their behaviors at gaining access to the bottle. This effect might be similar to those observed using conditioned place preference paradigms (Tzschentke, 1998). Morphine administration have also induced changes in circadian activity patterns. In the first phase of the experiment, the animals' circadian activity in both cages was similar. However, after 3 months of saccharin or morphine consumption, activity during the dark phase changed in both groups. Activity in the saccharin-drinking animals decreased: instead of 2 typical 'peaks' of activity during the active phase, we observed 3. Previous studies showed that such fragmentation of sleep episodes and decreased amplitude of rhythmic behaviors may be associated with aging processes in mice (Valentinuzzi et al. 1997; Nakamura et al. 2011). However, morphine-drinking animals seemed to maintain high activity after 3 months. Interestingly, instead of 2 peaks of activity, we observed that these animals were most active during the first hours of the dark phase. These alterations in natural circadian behaviors might possibly be the result of increased drug craving at the beginning of the dark phase, while the lack of a second 'peak' might be the result of the prolonged effects of morphine, which can last up to several hours (Koek et al. 2012).

Long-term self-administration induced a spectrum of behavioral symptoms connected to opioid dependence. Morphine-drinking mice presented increased motivation to obtain a rewarding substance in the progressive ratio procedure compared to the animals drinking saccharin. During the intermittent access test, which took place over a single dark phase, the morphine-dependent animals were also more persistent in their attempts to obtain the substance that was not available. These results, showing that morphine, but not saccharin self-administration resulted in increased motivation and drug seeking, stand in line with previously described models of addiction (Deroche-Gamonet et al. 2004; Kasanetz et al. 2010). We have observed that both groups of animals were willing to risk a punishment to drink the rewarding substances, morphine as well as saccharin. In previously published work, Pelloux, Everitt, and Dickinson (2007) presented that drug and natural reward, namely cocaine and sucrose, may show comparable sensitivity to punishment. Self-administration of cocaine but not sucrose significantly enhanced resistance to punishment when the procedure involving punishment was repeated several times, but seemingly not after the first punishment session. It is then possible, that single punishment risk procedure that we have used was insufficiently long to observe differences in compulsive behavior of the animals drinking natural reward or drug.

In morphine-drinking animals, spontaneous withdrawal symptoms were observed for up to 32 hours, which is similar to the results of Papaleo and Contarino (2006). During withdrawal, we have observed similar pain thresholds in morphine and saccharin drinking animals. Clinical and animal data point out that opioid abstinence may result in hypersensitivity to painful stimuli (Wachholtz and Gonzalez 2014; Craig and Bajic 2015). However, we have measured pain sensitivity during first hours of abstinence which is relatively early stage of abstinence, therefore hypersensitivity to painful stimuli might develop in the later stages of abstinence. When access to the rewarding substances was restored after

a 14 day abstinence period, morphine group have reestablished high preference level (~70%) in contrast to saccharin group. However, although we have observed differences in preference to rewarding substances, the average amount of licks on the bottles containing rewarding solutions before and after the abstinence periods were comparable in both groups, indicating that there was no escalation in actual morphine intake. However, we observed individual differences in morphine consumption: some mice seemed to control and restrain their use of morphine, while others showed a visible escalation. Those results stand in line with previous studies that showed that only a small percentage of the rats subjected to self-administration paradigms are positive for all addiction-like behaviors (Belin et al. 2009; Kasanetz et al. 2010). Interestingly, after a single injection of morphine, the morphine-dependent mice showed aggravated drug seeking behavior, which was observed as an increase in the number of visits and nose pokes made in effort to obtain access to the drug. Previous studies showed that morphine-pretreated animals strongly preferred morphine-associated environments during the abstinence period (Harris and Aston-Jones 1994; 2003), and a single dose of morphine administered after the conditioned place preference extinction paradigm effectively reactivated opioid-seeking behavior (Ribeiro Do Couto et al. 2003). Our results confirm that a high single dose of morphine (20 mg/kg) potently increases craving. To summarize, the novel paradigm we implemented allowed for the observation of the acquisition of various addiction-like behaviors, combined with the ability to observe individual animals that were housed in groups.

Molecular changes accompanying opioid addiction. In the present study, we investigated the effects of chronic morphine self-administration on basal and morphine-induced gene transcription in the striatum by using whole-transcriptome microarray analyses and qPCR. Microarray results showed that basal gene expression profiles were similar in both of these groups, whereas acute morphine

treatment induced lower increase of gene expression levels in the morphine group compared to the saccharin group, indicating a tolerance effect. However, further validation of the selected genes in the striatum and frontal cortex revealed subtle, but significant differences between basal gene expression levels, that is in chronic treatment groups (injected with saline). These changes in both acute and chronic treatments indicate that protracted opioid administration results in the suppression of gene transcription in the striatum and frontal cortex that may affect molecular sensitivity to opioid reward, and thus influence the behavior associated to drug seeking.

Functional enrichment analysis of cell signaling pathways related to morphine-induced gene expression alterations indicated overrepresentation of genes involved in insulin signaling pathway and diurnally regulated genes. Recent study reported that downregulation of insulin signaling in the ventral tegmental area mediates the decrease in dopamine cell size after morphine administration as well as tolerance to morphine reward in rats (Russo et al. 2007). Our findings seem to confirm the importance of this pathway as regulator of opiate reward, as morphine self-administration suppressed the mRNA expression of genes associated with insulin signaling, *Slc2a1*, *Sgk1* and *Map3k6* in the striatum and the frontal cortex. Another group of genes that is potentially crucial to drug dependence is the circadian clock genes. Animals drinking morphine presented altered circadian behaviors compared to the saccharin group. In addition, lower mRNA expression levels of the circadian clock gene *Per2* were observed in both striatum and frontal cortex of morphine dependent animals in the chronic treatment group. Dysfunction in *Per2* expression have been associated with enhanced alcohol intake in mice (Spanagel et al. 2005). Both our transcriptional and behavioral results also support the importance of circadian genes in opioid addiction.

Further, were able to distinguish high and low responders among the morphine drinking animals that were additionally injected with morphine prior to tissue collection. Mice with low expression of the identified genes (low responders) consumed more morphine than the high responders after the abstinence period and more actively explored the corners of their cages. Our results indicate that high-responding mice might have developed a neuroprotective mechanism that preserved their brain systems against the effects of morphine and resulted in reduced craving and increased tolerance to the substance. This mechanism might be connected to the glucocorticoid regulatory network, because glucocorticoids activate *Trk* neurotrophin receptors (Jeanneteau et al. 2008) and modulate mitochondrial functions in cells (Du et al. 2009), but this hypothesis needs to be further studied. Moreover, correlation analyses were performed between individual gene expression levels and motivation (progressive ratio results), which may indicate the presence of genetic predispositions to addiction-like behavior. More genes were clearly correlated with motivation to drink morphine than to drink saccharin. Among genes that might indicate a predisposition to addiction-like behaviors are the ephrin receptor (*Epha5*) and the neural cell adhesion molecule (*Ncam*), the transcription of which correlated with motivation to drink morphine. Previous studies seem to connect the role of *Eph* and *Ncam* with opioid dependence and development of analgesic tolerance to morphine in mice. *Eph* receptor is necessary for development of neuropathic pain, and knock-out of this gene resulted in modifications of anti-nociceptive responses following chronic morphine treatment, opioid tolerance and withdrawal (Han et al. 2008; Liu et al. 2009; Huroy et al. 2015). In our study mice with the lowest expression of *Ncam* exhibited the highest motivation to obtain morphine. Interestingly, decreased expression of *Ncam* was found to be associated with alcohol seeking behavior in rats (Barker et al. 2012), and its deletion induced a

cognitive and behavioral phenotype reflective of impulsivity in mice (Matzel et al. 2008).

The potential role of glucocorticoid regulatory network and astrocytes in opioid addiction. We have observed that genes induced by morphine included group of transcripts that are associated with the GR regulatory network (e.g. *Fkbp5*, *Sgk1*, *Camk1g*, *Cdkn1a*, *Gjb6*, *Slc2a1*). Consistently with our results, previous studies showed that a large fraction of the striatal transcriptome induced by morphine included GR-dependent genes (Korostynski et al. 2007; Piechota et al. 2010). Our data show changes in the expression of genes with functional importance to the effects of prolonged morphine administration, such as FK506-binding protein 5 (*Fkbp5*), the inhibition of which was found to prevent the symptoms of opioid withdrawal syndrome (McClung et al. 2005). Another such gene is already mentioned *Sgk1*. *Sgk1* knock-down resulted in alterations to dendritic spines in mice, possibly reflecting the influence of Sgk1 on neuronal plasticity (Piechota et al. 2010), and increases in its activity may play a role in drug-dependent behaviors (Heller et al. 2015). Moreover, it was previously shown that GR-dependent transmission influences behavioral responses to morphine in rodents (Marinelli et al. 1998; Stöhr et al. 1999; Dong et al. 2006; Attarzadeh-Yazdi et al. 2013). We have observed suppression of the Ca^{2+}/calmodulin-dependent protein kinase γ (*Camk1g*) gene expression in the frontal cortex of long-term morphine drinking animals. Modulation of *Camk1g* activity was reported to alter outgrowth of neuronal processes and lead to changes in neuronal morphology (Wayman et al. 2006). Therefore, we propose that *Camk1g* is involved in neuroplastic alterations in the frontal cortex that might be associated with the development of addiction-like behavior.

Chronic opioid self-administration resulted in altered expression of gap junction protein beta 6 (*Gjb6*) and *Slc2a1*, both of which are expressed in

astrocytes, might indicate a significant role for these cells in the development of addiction. Previous studies showed that astrocytes might be involved in learning processes, which may be implicated in the development of the behavioral symptoms of addiction (Rothstein et al. 1996; Tanaka et al. 1997). The opioid-activated gene transcription in part occurs in glial cells and is mediated and regulated by GR (Slezak et al. 2013). This indicates that morphine administration activates glucocorticoid system and affect astrocytes in the brain reward circuit. What is more, animals with more severe dependence in our study (low responders) showed decreased mRNA expression of astroglial marker, GLAST, pointing out to the possible role of astrocytes in mediating severity of opioid dependence. It was previously presented that tolerance to morphine was associated with downregulation of GLAST (Mao et al. 2002). Thus, our results suggest that transcriptional tolerance might be a consequence of the interplay between opioid and glucocorticoid systems, possibly with the participation of astroglial cells. Blockade of GR with mifepristone was reported to prevent the development of tolerance to analgesic effects of morphine in rats (Lim et al. 2005) as well as to attenuate morphine induced up-regulation of CREB and extracellular-signal regulated kinases, both of which have been shown to be important transcriptional factors in drug addiction (Navarro-Zaragoza et al. 2015). Chronic morphine treatment resulted in up-regulation of GR in distinct brain regions (He et al. 2009). Therefore, we propose that reduced transcriptional responsiveness of GR-dependent genes after long term opioid administration may point out molecular basis for persistence of addiction related neuroadaptations.

Astrocytes are the main target for GR-activated gene expression in the nucleus accumbens. Our results obtained after chronic opioid self-administration indicated that a number of genes induced by morphine were both associated with the GR-dependent gene regulatory network and astrocyte-specific. We therefore

decided to establish global impact of glucocorticoid regulation on astrocytic and neuronal transcriptome in the NAc with the use of whole-genome microarrays. We observed that transcriptional changes in the NAc induced by single administration of dexamethasone are confined mostly to astrocytes, pointing out to these cells as the primary targets of steroid action in the central nervous system. These results are in agreement with other studies, which suggested that the substantial component of transcriptional response to steroids is astroglial (Piechota et al. 2010; Carter et al. 2012; Carter et al. 2013; Piechota et al. 2017). Our results suggest that glucocorticoid-induced response in the brain might be in fact mostly managed by GR expressed by non-neuronal cells and that astrocytes are involved in several processes that can influence neural plasticity, as previously hypothesized (Haydon et al. 2009). These processes include regulation of cellular metabolism (*Sult1a1*, *Angptl4*; Carter et al. 2013), neuroimmune response involving TOR signaling (*Nfkbia;* Hutchinson et al. 2012) and control of negative feedback on the action of steroids (*Fkbp5*; Grad and Picard 2007).

An important question arising from this interesting discovery is why astrocytes are more responsive to glucocorticoid stimulation than neurons? Glucocorticoid availability may vary by cell type due to the expression or absence of different enzymes that act on glucocorticoid ligands. GR binding can also be affected by the specific combinations of coregulators that form complexes with glucocorticoid receptors to influence dynamics and properties of transcriptional regulation (Rosenfeld and Glass 2001). In addition, ligand-activated GRs also differentially bind DNA of target genes based on cellular context. GR binding of GRE sites in a human lung cell line were found to be present near many genes whose mRNA was regulated by glucocorticoids in that cell line, but GR binding was not present near genes known to be regulated by glucocorticoids in other tissues or types of cell lines (So et al. 2007). Another possible reason for differential GR regulation in astrocytes and neurons might be due to differences

in chromatin structure between cell types. Recent study indicated that chromatin accessibility pre-determines up to 95% of GR genomic binding, suggesting that cell-specific differences in GR binding might be primarily based on chromatin accessibility patterns in different cell types (John et al. 2011). In addition, recent experiments using GR ChIP-seq methods suggested that GR-dependent transcription is not established by sequence but is influenced by epigenetic regulators, context, and other unrecognized regulatory determinants (Uhlenhaut et al. 2013).

Similar to our results, previous *in vitro* study showed steroid nuclear receptor repression in neurons, in contrast to astrocytes (Piechota et al. 2017). The authors discussed potential mechanisms of this cell-type specific response. First hypothesis considered occurrence of non-active isoforms of these receptors in neurons. However, neurons and astrocytes express the same transcriptional isoforms of both *Nr3c1* and *Nr3c2*, which disproves this conjecture. Another hypothesis proposed an imbalance between coactivators and corepressors of steroid receptors in neurons which would inhibit their transcriptional activity (Zhang et al. 1998; Kumar et al. 2005) and would allow nongenomic mechanisms of action (Lösel and Wehling 2003). Based on these recent findings, cellular context may thus be a critical factor in determining the character and outcome of glucocorticoid receptor-mediated mRNA regulation. Together, these data suggest that the cellular environment might influence glucocorticoid signaling in different cell types through the cellular expression and relative ratios of coregulators, chaperone proteins and/or chromatin structural arrangements.

Astrocytic GR modulates effects of opioids. To evaluate the functional contribution of GR-dependent signaling in astrocytes, we have used two transgenic animal models.

In the GR knockdown model, we have selectively silenced astrocytic GR in the NAc using Cre-mediated lentiviral vector in mice expressing Cre recombinase in Aldh1L1-positive astrocytes. In vitro validation confirmed about 70% reduction in the basal mRNA expression of GR in astrocytes and significant reduction in glucocorticoid-induced gene expression levels were observed both *in vitro* and *in vivo*. Our results show that animals with selective astrocytic GR knockdown presented striking increase of reward sensitivity and expression of morphine-associated memory in conditioned place preference paradigm. Therefore, it seems that the silencing of astrocytic GR potentiates morphine-induced effects on neurons that mimic the effects of stress-induced increase of addictive behaviors (Ambroggi et al. 2009). Interestingly, we have not observed significant differences between both control and astrocytic GR knockdown animals in the acquisition and expression of locomotor sensitization induced by repeated morphine administration. The apparent enhancement of morphine-induced place preference and lack of effect on morphine locomotor sensitization is most probably due to the proposed association of these behaviors with different neural substrates (Hnasko et al. 2005; Chefer and Shippenberg 2009). The common circuitry for locomotor sensitization includes dopamine projections (Pierce and Kalivas 1997), while it was recently reported that dopamine is not required for morphine-induced reward measured by conditioned place preference (Hnasko et al. 2005; Borgkvist et al. 2007). Therefore, we hypothesize that astrocytic GR knockdown have targeted specifically NAc-mediated effects that are dopamine-independent.

Next we examined the possible neural correlates of increased behavioral sensitivity to morphine reward in the NAc. Electrophysiological results showed that GR-knockdown permitted a considerable morphine-induced inhibition of neural excitability and plasticity. We have observed significant decrease in sEPSC amplitude, but not frequency, what shows that GR-dependent signaling in

astrocytes contributes to the regulation of synaptic plasticity in the NAc through postsynaptic glutamatergic terminals. Furthermore, we observed weakened excitability of GABAergic inhibitory neurons and significant reduction of LTP in the NAc. Consistently with our results, it was previously presented, that enhanced morphine reward is attributed at least in part to the suppression of GABAergic transmission (Koo et al. 2014). We therefore propose that astrocytes modulate morphine-induced synaptic plasticity in the NAc partly through GR-dependent signaling, through which they can influence inhibitory output from the NAc to target structures, that in turn can directly affect animal behavior.

In the GR knockout model we introduced a new transgenic mouse and demonstrated efficient ablation of the receptor in several brain regions, including hippocampus and amygdala, allowing the investigation of the relevance of the GR-dependent signaling in astrocytes in animals' behavior. However, it is important to point out that due to Cx30 expression pattern in the brain, GR was not eliminated from forebrain regions implicated in appetitive memory, NAc and prefrontal cortex. Therefore, as expected, we did not observe an effect of the GR ablation in Cx30-positive astrocytes on morphine-induced CPP. These results may point out to region-specific role of GR in astrocytes. It has been proposed that astrocytes vary in molecular responsiveness and have distinct functions in different brain regions that are tailored to neighboring neural circuits functions (Bachoo et al. 2004; Cahoy et al. 2008), and our results seem to support that concept.

Taking into consideration previous reports about the participation of the GR in opiate withdrawal (García-Pérez et al. 2016, 2017), we have tested animals in both transgenic models for signs of naloxone-precipitated physical dependence after chronic morphine treatment. No significant differences were observed between NAc astrocytic GR knockdown and control animals. Astrocytic GR knockout resulted in decreased expression of opioid withdrawal. These results are

in line with previous reports, which showed that GR blockade with systemic administration of mifepristone attenuated the somatic expression of naloxone-precipitated morphine withdrawal (Navarro-Zaragoza et al. 2012). We therefore propose that these effects are, at least partly, mediated through astrocytic GRs most likely in the amygdala and hippocampus, as we observed efficient elimination of the astrocytic GR in these structures in GR knockout model. Supporting that notion, recent studies report that adrenalectomy, which impairs HPA axis function, resulted in decrease of naloxone-precipitated opiate withdrawal symptoms and alternations of *Arc* (activity-regulated cytoskeletal-associated protein) expression in glutamatergic and GABAergic neurons in dentate gyrus and basolateral amygdala (García-Pérez et al. 2016, 2017).

Validation of the knockout animals revealed a significant decrease of GR mRNA expression in the spinal cord, which is known to take part in the processing of pain. It was previously shown that spinal GR may contribute to the development of opioid tolerance, as anti-nociceptive effect of morphine was substantially attenuated after administration of mifepristone, GR antagonist (Lim et al. 2005). Our results show that GR knockout in astrocytes does not affect pain sensitivity, opioid-induced analgesia and tolerance. Possible interpretation of this data is that neuronal GR-signaling mediates pain processing, while glial GR signaling is dispensable for that aspect.

Altogether, our results present that astrocytic GR in the NAc selectively modulates sensitivity to opioid reward, without changing other opioid-related addictive behaviors, such as locomotor sensitization and withdrawal. On the other hand, knockout of GR in Cx30-positive astrocytes in the central nervous system resulted in attenuated expression of naloxone-precipitated morphine withdrawal, but no significant modulation of morphine reward or nociception was observed. In the GR knockdown model we have targeted selectively NAc, while GR knockout animals present decreased GR expression in hippocampus and

amygdala, what suggests that GR in astrocytes contributes differently to the modulation of opioid effects depending on neural circuitry next to which they reside. To conclude, our results reveal a critical role of astrocytic GR in the mediation of opioid-induced behaviors and synaptic transmission.

Glucocorticoid receptor in astrocytes alters contextual aversive memory. Dysfunctional GR was previously associated with development of stress-related disorders, such as depression and posttraumatic stress disorder (Tronche et al. 1999; Miranda et al. 2008). We have therefore assessed the behavior of transgenic animals in both GR knockdown and GR knockout models in a series of tests that measure depression-like behavior, anxiety as well as stress-related memory formation and expression. GR knockdown in NAc astrocytes did not influence basal nor stress-related behavior of the animals. Locomotor activity, responses to stressful stimuli, anxiety and depression-related behaviors were comparable in both groups of animals. However, utilizing GR knockout model, we discovered that GR signaling in astrocytes contributed to the aversive memory in fear conditioning paradigm. Our data recapitulate the inhibition of conditioned fear memory resulting from lesions of amygdala (Goosens and Maren 2001; Nader et al. 2001) and hippocampus (Wiltgen et al. 2006), as well as pharmacological blockade of GR activation in amygdala and ventral hippocampus (Donley et al. 2005). Our results indicate that, apart from the contribution of neuronal compartment (Kolber et al. 2008), astrocytes also mediate glucocorticoid action in amygdala and hippocampus and modulate fear related behavior. It seems that astrocytic GR did not influence memory acquisition, since we observed identical freezing responses in training phase of the fear conditioning between GR knockout and control animals. Similar results were observed between groups also in the Y-maze and novel object recognition tests indicating well-functioning working and declarative memory formation. Therefore, our results suggest probable

involvement of astrocytic GR signaling in memory consolidation and reconsolidation, occurring hours after exposure to stress. It seems that the effects we have observed are selective for stress-induced memory, as locomotor activity, anxiety and depression-related behaviors were not influenced by astrocytic GR knockout.

Several critical synaptic functions of astrocytes may be affected by stress-induced glucocorticoids. Recent study provided evidence that GR signaling in spinal cord regulates ATP release through activation of *Sgk1* (Koyanagi et al. 2016). Both, ATP and ATP-derived adenosine of astrocytic origin are known to regulate synaptic transmission and plasticity (Panatier et al. 2011), hence this pathway may be involved also in mediating stress-induced plasticity. Our data also suggests the role of astrocytic GR in regulating fear memory extinction, since freezing responses were diminished in late retrieval sessions in GR knockout mice. A similar phenotype was recently described in mice with genetic elimination of astrocytic water channel, *Aqp4* (Wu et al. 2017). *Aqp4* deficiency altered NMDA-dependent long-term potentiation in the hippocampus. Glucocorticoids regulate the expression of glial glutamate transporter GLT-1 (Tian et al. 2007). Synaptic levels of this transporter shape the transmission in glutamatergic synapses, known to be increased upon corticosteroid exposure (Karst and Joëls 2005). Elimination of GR may therefore impair the levels of the GLT-1 and affect the plasticity of glutamatergic synapses.

Chapter 6 Thesis Summary and Conclusions

The initial goal of this dissertation research was to investigate behavioral patterns induced by long-term, voluntary morphine self-administration in mice. We observed that morphine consumption induced a spectrum of addiction-like behaviors, including increased craving, increased drug-seeking behavior, increased motivation to work to obtain the reward and spontaneous symptoms of withdrawal. Further, we have identified molecular alterations accompanying opioid addiction, with a number of transcripts regulated differently in morphine and saccharin groups after both acute and chronic morphine treatment. Functional analysis of these genes pointed out to a crucial role of glucocorticoid-dependent and astrocyte-specific transcripts in the development of opioid dependence. We conclude that prolonged morphine intake caused adaptive processes in the brain that manifested as altered behavior and transcriptional sensitivity to opioids. Furthermore, the suppression of gene expression in morphine dependent animals might be directly related to the long-lasting addiction symptoms as well as persistent drug craving. Lastly, we have identified genes that might potentially contribute to individual predispositions toward drug seeking behavior.

Our results, consistently with previously published observations, pointed out to possible relevance of glucocorticoid action in astrocytes in the development of opioid abuse. We therefore aimed to enhance our understanding of glucocorticoid action in the brain by characterizing glucocorticoid-mediated mRNA regulation in astrocytes and neurons and generating transgenic animal models with astrocyte-specific GR elimination.

Alterations in GR function have been previously hypothesized to underlie many stress-related disorders, such as addiction, depression and posttraumatic stress-disorder, however, majority of research so far have focused on neuronal GR as main contribution to the development of these dysfunctional behaviors. Here, we presented that GR-dependent transcriptional changes in the NAc are confined mostly to astrocytes, pointing out to these cells as the essential targets of steroid action in the central nervous system.

To investigate the role of astrocytic GR in appetitive learning and motivated behaviors, we targeted GR selectively in Aldh1L1-positive astrocytes in the brain reward system, the NAc. Our results show that astrocytic GR knockdown caused enhanced morphine reward sensitivity and altered morphine-induced synaptic plasticity, including decreased neuronal excitability and decreased LTP.

GR knockout in Cx30-positive astrocytes resulted in decreased expression of opioid withdrawal, which possibly involved astrocytic GR in the amygdala. What is more, we observed that GR knockout lead to impairment of stress-induced memory expression and extinction. Interestingly, conditioned responses to morphine were unaltered. This might be due to the fact that efficient elimination of the GR in astrocytes in the conditional GR knockout occurred in several brain regions that mediate stress responses, including hippocampus and amygdala, but not forebrain structures, including NAc, involved in reward processing.

Based on functional analysis of GR-dependent genes altered by morphine and/or dexamethasone administration in astrocytes we propose several potential genes that point out to processes which might underlie observed alterations of reward sensitivity and stress-induced memory expression (**Figure 42**). These genes are involved in metabolic functions (e.g. *Pdk4*, *Sgk1*), glucose transport (e.g. *Slc2a1*), glutamate synthesis (e.g. *Tgm2*), regulation of ion channels (e.g. *Tsc22d3*) and neuronal plasticity (e.g. *Camk1g*). GR-dependent genes in astrocytes may be

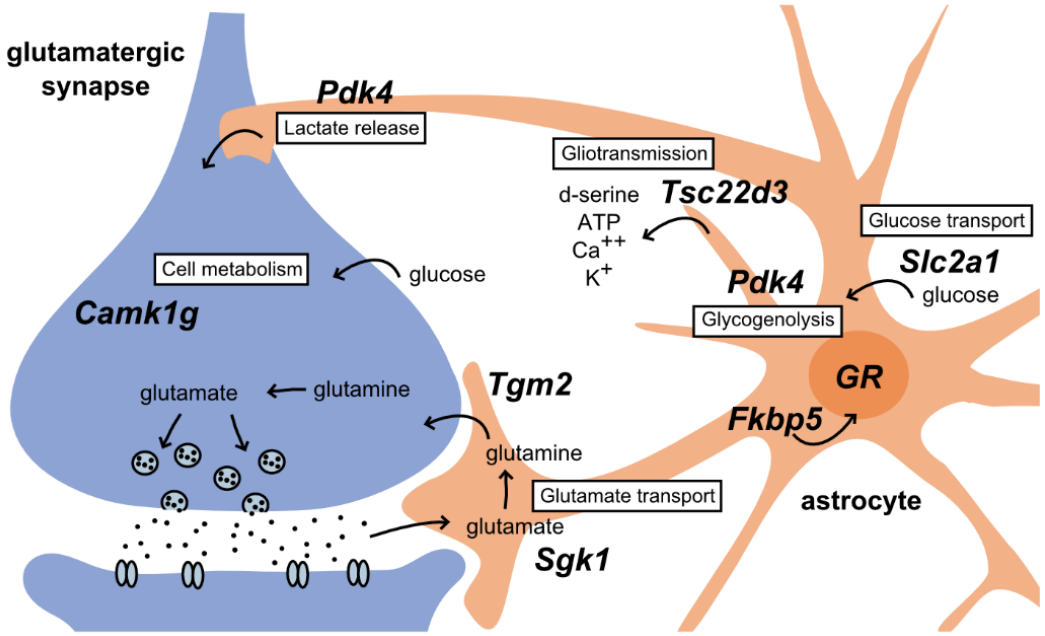

Figure 42. Proposed contribution of GR-dependent signaling on synaptic function. The expression of GR-dependent genes in astrocytes may modulate activity-induced neuronal plasticity through control of cell metabolism, glycogenolysis and lactate release (*Pdk4*, *Sgk1*), glutamate synthesis (*Tgm2*), release of gliotransmitters (*Tsc22d3*), glucose transport (*Slc2a1*) or control of negative feedback on the action of steroids (*Fkbp5*).

involved in modulation of glucocorticoid-induced neuronal plasticity, therefore our further studies will focus on determining GR-mediated changes in astrocytic functions.

Altogether, our results may suggest that astrocytes play a vital role in the mechanism of rewarding action of opioids in the NAc. It was previously shown that opioids exert their rewarding effects via inhibition of the NAc medium spiny neurons (David, Cazala 2000). Furthermore, GR activation is critical for morphine-induced behaviors and gene expression in mice (Marinelli et al. 1998; Slezak et al. 2013). Here, we propose that glucocorticoids act locally in the NAc through astrocytic GR to provide neurons with metabolic support that counteracts inhibitory effects of morphine (**Figure 43**).

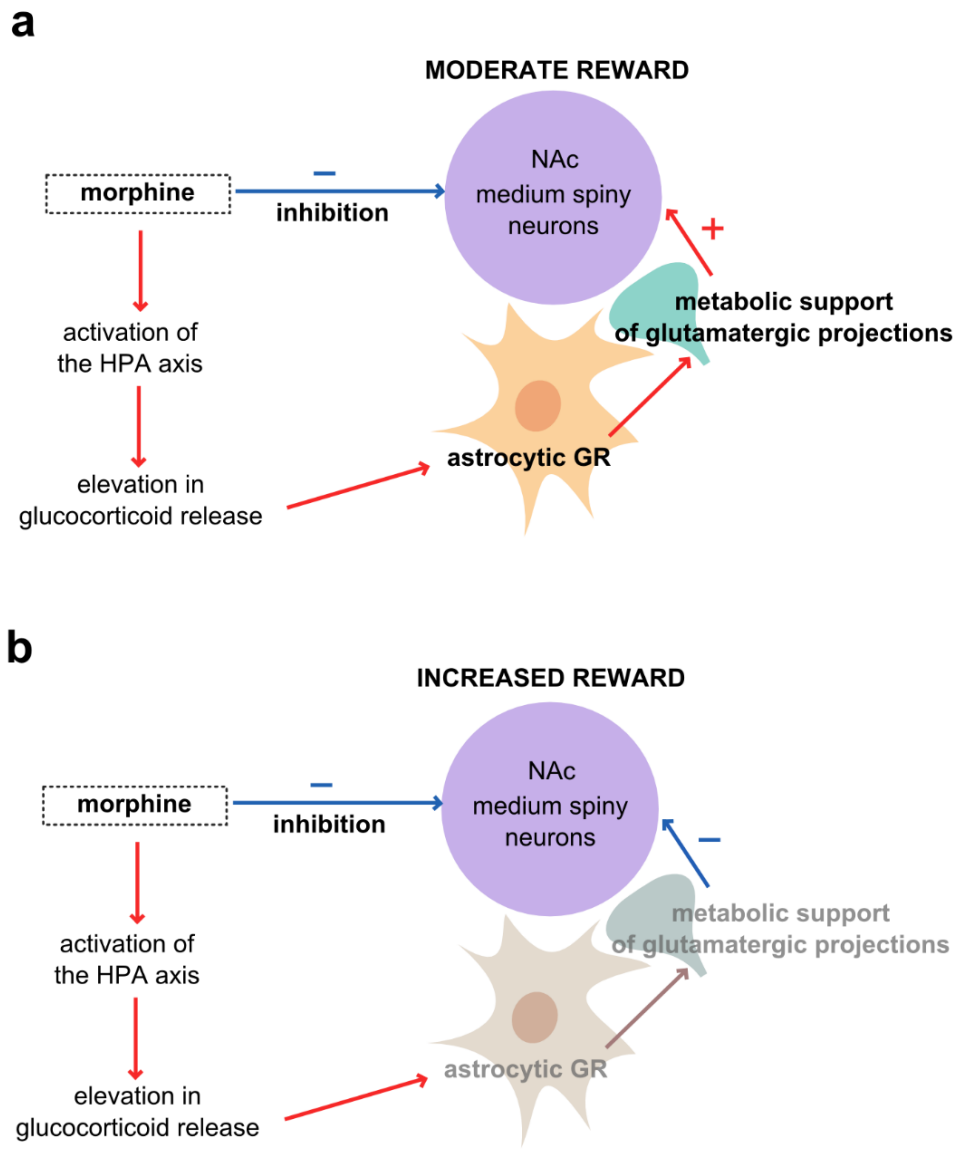

Figure 43. Proposed involvement of astroglial GR in the mechanism of opioid action in the NAc. (**a**) Under control conditions, morphine administration causes inhibition of the NAc medium spiny neurons. It also acts indirectly through the activation of the HPA axis and release of glucocorticoids, which triggers GR-dependent gene transcription in astrocytes. Glial cells than provide metabolic support of glutamatergic projections that counteracts direct inhibitory effects of morphine, resulting in moderate rewarding effect of the drug. (**b**) Astrocytic GR knockdown causes functional alterations and further inhibition of NAc medium spiny neurons, resulting in increased reward.

Overall, our results revealed a critical role of astrocytic GR in the mediation of opioid-induced and stress-related behaviors and synaptic transmission, which provides a novel insight into the coordinated activity of astrocytes and neurons in the regulation of glucocorticoid effects in the brain. Possibly through the regulation of metabolic processes, gliotransmission and/or glutamate uptake, astrocytic GR modulates neuronal responsiveness to opiates and thus, actively shapes animal behavior. Our experiments have implications not only for glucocorticoid-mediated mechanisms of opioid action but potentially broader views on how a cell-type specific responses shapes our perspective of hormonal regulation of stress-associated behaviors, such as drug addiction, posttraumatic stress disorder or depression.

List of Figures and Tables

List of Abbreviations

AARs	alternate arm returns
ACTH	corticotropin
AKT	protein kinase B
Aldh1L1	aldehyde dehydrogenase 1 family, member L1
AQP4	aquaporin 4
Arc	activity-regulated cytoskeletal-associated protein
ATP	adenosine triphosphate
CNS	central nervous system
CORT	corticosterone
CPP	conditioned place preference
CRF	corticotropin releasing factor
CRH	corticotropin releasing hormone
Cx30	gap junction protein connexin 30
Cx43	connexin 43
DA	dopaminergic neuron
FDR	false discovery rate
GABA	gamma-aminobutyric acid
GFAP	glial fibrillary acidic protein
GLAST	glutamate aspartate transporter,
GLUT1	glucose transporter
GR	glucocorticoid receptor
GREs	glucocorticoid-responsive elements
HPA	Hypothalamic-pituitary-adrenal
Hprt1	hypoxanthine guanine phosphoribosyltransferase 1
HRs	high responders

LRs	low responders
LTP	long-term potentiation
MAPK	mitogen-activated protein kinase
MR	mineralocorticoid receptor
NAc	nucleus accumbens
NE	norepinephrine
NGS	normal goat serum
NRPG	nucleus reticularis paragigantocellularis
PAG	periaqueductal grey
PI3K	phosphoinositide 3-kinase
POMC	proopiomelanocortin
PR	progressive ratio
SAP	spontaneous alternation performance
SARs	same arm returns
sEPSC	spontaneous excitatory postsynaptic currents
VTA	ventral tegmental area

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