UNIVERSITA' DEGLI STUDI DI PARMA

CORSO DI LAUREA IN PSICOBIOLOGIA E NEUROSCIENZE COGNITIVE

The role of the glucocorticoid receptor from the NAcc in stress-related behaviours.
Genetic dissection by viral transduction.

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Abastract: Glucocorticoid Receptor gene inactivation in dopaminoceptive neurons (GR^{D1aCre} mice) deeply affects some stress-related behaviors, including behavioral responses to psychostimulants and social withdrawal acquired after repeated social stress. At the physiological level, the mutation of GR in post-synaptic neurons deeply reduces the electrical activity of presynaptic DA neurons of the VTA, showing a role for GR in the modulation of the feedback exerted by dopaminoceptive neurons on DA neurons. In GR^{D1aCre} mice, GR gene is recombined in several structures including the NAcc, the dorsal striatum, the amygdala and the basal layers of the cortex, among which the NAcc and the mPFC are known to control VTA DA neurons activity. It is therefore essential to refine the identification of the relevant brain structures.

My objective was to identify whether the GR in the NAcc is important for the observed phenotypes. We therefore specifically inactivated GR, by stereotactic injection of AAV2 expressing the Cre recombinase and the GFP in $GR^{loxP/loxP}$ mice. Control animals were injecting in parallel with an AAV2 expressing only the GFP. We performed locomotor sensitization to cocaine in mutant and control animals. We observed a marked sensitization but no differences between genotypes.

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I. The stress response

1) History

To overcome the environmental variations, living organisms developed, throughout the evolution, increasingly complex mechanisms. In this way, they acquired a relative independence from the surrounding environment by constituting an own "inner environment". Following the description of the "stability of their inner environment" by the physiologist Claude Bernard, the dynamic mechanisms that maintain this stability were studied in the early twentieth century by Walter B. Cannon that forged the word homeostasis (1929). In his famous "*fight or flight*" paradigm, he then characterized the physiological responses activated by threats, and showed that they involve an activation of the sympathetic nervous system which leads to the release, by the adrenal glands, of adrenaline in the blood. Cannon introduced also the word stress in biology, as an analogy from the physic of beams, it describes the forces exerted on a beam that can lead to a strain, as the environment can impact the physiology of an animal organism (Canon, 1934).

Building on those works, Selye devised in 1936 a more complex physiological model, initially called the "*general adaptation syndrome*" and that he defined as the non-specific response of an organism to any stressor (Selye,1936). Selye observed that stressed rats (cold, surgical injury, production of spinal shock - transcision of the cord -, excessive muscular exercise, or intoxications with sublethal doses of diverse drugs such as adrenaline, atropine, morphine, formaldehyde, etc.), were characterized by lymph node and thymic atrophy, gastric erosions/ulcers, and enlarged adrenal glands. These anatomical changes were later found to be associated with increased glucocorticoid circulating levels (named by Selye). This experience led him to the concept of a stereotypic/generic response to stress. Besides, he hypothesized that a severe, prolonged and uncontrollable stress determined an excessive, and so non adaptive response that led to serious pathologies. We now know that the adaptive response is specific for the type of stressor met and that this specificity is gradually lost when the intensity of the stress increases (Tsigos, 2002).

2) Neuroendocrine response: Hypothalamic–pituitary–adrenal axis

a. An overview

During the perception of a stressful event, the central nervous system integrates all the sensory data and activates specific structures to prepare a response. Particularly relevant for this purpose is the activation of the autonomic nervous system and of the endocrine response through the activation of the hypothalamic–pituitary–adrenal axis (HPA). Under stress, the autonomic nervous system, activated by the hypothalamus, controls autonomic functions such as gastrointestinal and kidney functions. Activation of adrenal medulla, leads to the secretion of adrenaline by chromaffin cells and modulates cardiac and respiratory rhythms. During stress, activation of the HPA axis results in the release of corticotropin releasing hormone (CRH) from hypothalamic neurons (Tsigos, 2002), that reache, through the hypophyseal portal system, the anterior pituitary gland to induce the synthesis and the release of adrenocorticotropin (ACTH). ACTH, released into blood circulation, stimulates the secretion of glucocorticoids hormones (GC) by the adrenal cortex (Fig.1). GC circulating levels are maintained through a double negative feedback exerted by GC on the synthesis and release of both ACTH and CRH (McEwen, 1979; Kellendonk et al., 2002).

b. The glucocorticoids: a hormone to cope with stress

Glucocorticoids, the main representative of which is cortisol in men, and corticosterone in rodents, are steroid hormones produced from plasmatic cholesterol in the adrenal cortex. Thanks to their wide spectre of action, the GCs allow the coordination of the metabolic, tissular, immune and behavioural responses of the organism in order to cope with stress, and to allow recovering and adaptation (Fig.1).

Total glucocorticoid hormone levels in plasma of various species, including rodents and humans, follow a circadian rhythm, characterized by a peak at the beginning of the activity period and a minimum at the beginning of the rest period (McEwen, 1986). Furthermore, it has been shown that such a discrete pattern of release follows significant ultradian rythms (oscillatory period ~1hr) (Qian, 2012).

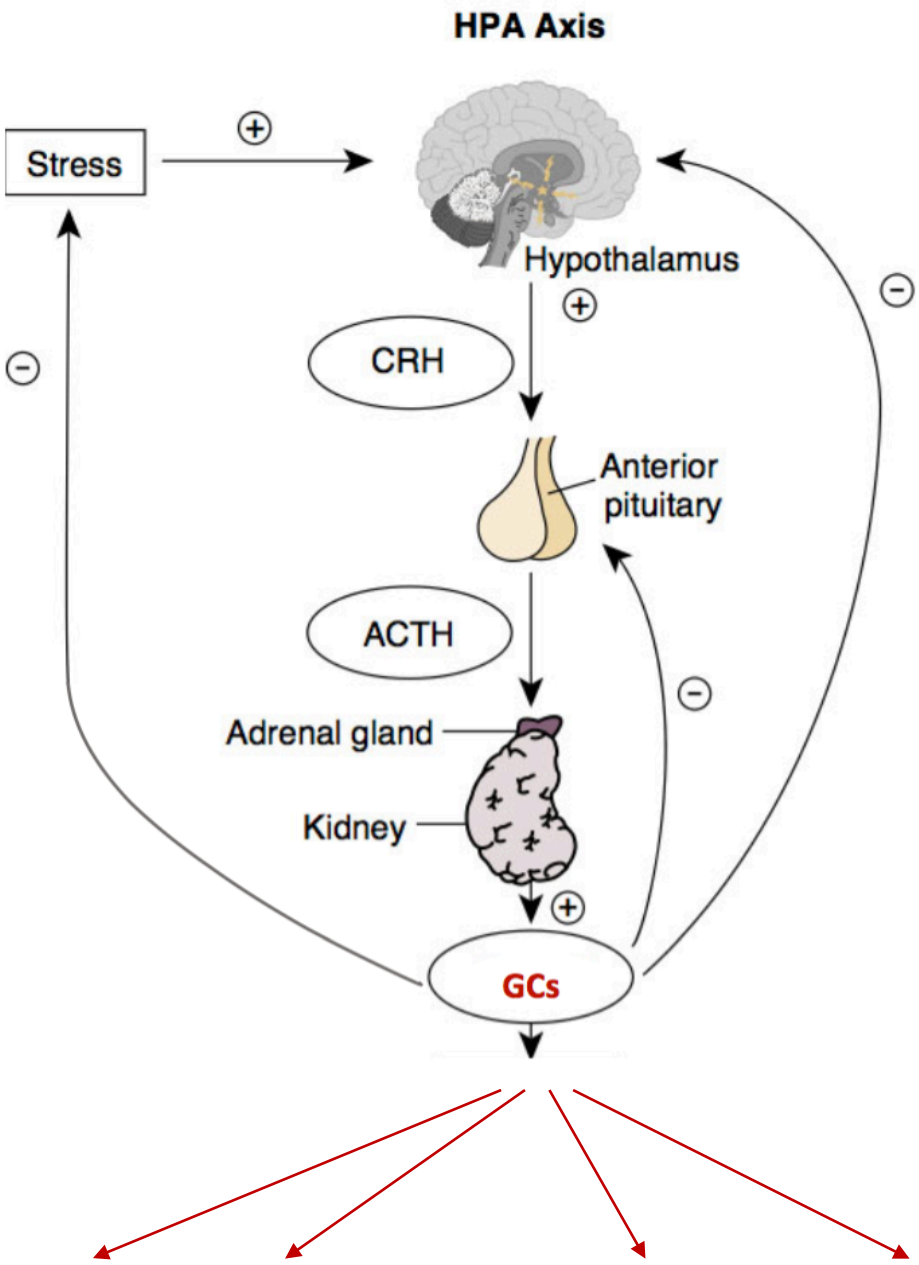

Fig.1. The physiologic effects of glucocorticoids.

Stress response induces endocrine cascade involving the hypothalamus, pituitary and adrenal gland. Glucocorticoids released into the systemic circulation exert a negative feedback on the synthesis and the release of adrenocorticotropin and corticotropin releasing hormone. Glucocorticoids act on numerous functions such as metabolism, inflammatory response, cardiovascular function, circadian rhythm and behaviour to cope with stress, to recover and to adapt. Adapted from: (Ballantyne, 2014).

Increased plasma levels of GCs are also met with almost all events that could disrupt the physiological or psychological homeostasis. In this way, the maximum values are reached 15 to 30 minutes after the stressful event, and the baseline level restored after 90 minutes, through the negative feedback system (Kitchener,2004). The level of physiological activation of the stress response depends on the type and intensity of stress, genetic features, as well as on individual history including the influence of perinatal environment (Chrousos, 1992 Stratakis, 1995). For instance, rats who underwent prenatal stress or maternal separation stress in the first days of life present in adulthood, hypersensitivity of the HPA axis, metabolic alterations, cognitive deficits (Nelson, 1998, Meaney, 2001) and greater vulnerability to addiction (Anand, 1999, Kosten, 2000).

II. Glucocorticoid receptor and behavioural adaptation

The GR belongs to the superfamily of nuclear receptors, that includes the receptors for the other steroid hormones (mineralocorticoids, androgens, progesterone and estrogens) (Thornton, 2003). Nuclear receptors are transcription factors characterized by the presence of a DNA binding domain, a ligand binding domain and protein interfaces that control gene transcription.

GCs have two distinct receptors, the GR that is widely expressed and the MR that have a 10-fold higher affinity but that is expressed in a restricted number of cell types. Due to their respective affinities fo GCs, it is considered that the MR is already fully activated by low circulating GCs levels whereas the GR is activated when higher circulating levels are reached, in response to stress (Tronche, 2007).

The MR acts as a glucocorticoid receptor in a few cell types, such as hippocampal neurons, but is mostly expressed in cells involved in salt-balance control, in which it serves as a mineralocorticoids (aldosterone) receptor. In these cells, the MR is indeed protected form GCs by 11 β Hydroxysteroid Dehydrogenase (11ß-HSD2), an enzyme that converts GCs into inactive forms.

1) The Glucocorticoid Receptor

a. The protein

The expression of the GR protein (91 kDa) is detected in all central nervous system cells, with higher expression in the limbic system structures. The GR gene contains 9 exons which represent only 2% of the 140kb of the transcribed portion. Like the other members of the nuclear receptor superfamily, each domain of the GR gene carries distinct functions (Giguere, 1986). In the N-terminal part, the first 421 amino acids of the GR have a transcriptional activation (AF1). This domain allows interaction of GR with cofactors and other transcription factors to modulate transcription of genes. The following 65 amino acids contain two zinc-fingers that form the DNA Binding Domain and a homodimerization interface. The C-terminal part contains the ligand-binding domain (LBD) and the second domain of interaction with other proteins (AF2). The C-terminal portion is also involved in the interaction with cytosolic chaperone or co-regulatory proteins (Tang, 1998).

b. Transcriptional functional effects

As previously evoked, GCs can act via rapid, non-genomic mechanisms but the transcriptional effects of GR have been much more widely studied. The effects of GR are categorized according to the stage at which they act on transcription.

GR controls the expression of target genes via very diverse strategies. For some target genes, GR binds to specific response elements in the DNA to activate or to repress transcription. For others, it indirectly anchors onto the promoter or enhancer by interacting with other transcription factors and inhibits (e.g. AP-1 and NFkB) or potentiates STAT5, their activities. Once bound to a gene, GR regulates its expression by recruiting basal transcriptional machinery and chromatin and DNA modificators and remodeling complexes.

In some promoters, GR binds as homodimer or heterodimers (with MR) on Glucocorticoid Responsive DNA Elements (GREs) to activate the transcription or on negatives GREs (nGREs) to repress gene expression (Fig.2) (Tronche, 2007).

An important part of the action of the GR on transcription is also due to direct interaction with other transcription factors, independently of binding to GREs sites (Fig.2). Thus, repression by the GR of the transcriptional activity of AP1 and NFkB is responsible for the main anti-inflammatory effect of the glucocorticoids. GR is also capable of interacting physically with the transcription factor STAT5 (Signal Transducer and Activator of Transcription 5) at regulatory regions of IGF-1 (Insulin-like Growth Factor-1). In hepatocytes, the GR acts as co-activator of the STAT5- dependent transcription during the stimulation by growth hormone (GH). The absence of this interaction in mice with an invalidation of the GR in hepatocytes, is responsible for a sharp decrease in their size (Tronche, 2004). Some interactions with transcription factors such as CREB (Imai, 1993, Focking, 2003) could explain the harmful consequences of prolonged exposure to glucocorticoids on some cognitive functions (De Kloet, 1999). As previously evoked, GCs can act via rapid, non-genomic mechanisms but the transcriptional effects of GR have been much more widely studied. The effects of GR are categorized according to the stage at which they act on transcription.

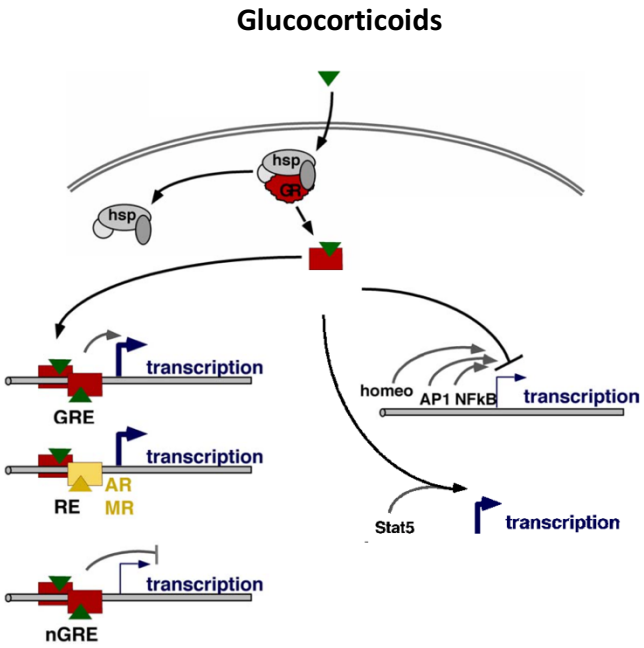

Fig.2. Mechanisms of action of the glucocorticoid receptor.

The inactive GR is cytoplasmic and binds to a complex containing in particular heat shock proteins (hsps). The binding of GC to GR allows its translocation to the nucleus. The GR can then inhibit or activate the transcription of genes via two mechanisms: (i) interaction of GR as a dimer with the target sequences GREs or (ii) protein-protein interaction of the GR with other factors such as AP1, NFkB or Stat5 transcription complexes. Note that heterodimers with the mineralocorticoids or androgens receptors may also activate transcription at GREs sites. Adapted from: (Tronche, 2007).

2) Mesocorticolimbic reward pathway, HPA axis and GR: from adaptation to addiction and depression

a. The reward system

For the survival of species, it is crucial that basic behaviours such as feeding, sexual activity or the ability to respond to aggression/harmful variations of the environment, are maintained. To this end, every disturbance of the homeostatic balance leads to the acquisition of a behaviour which, if effective, is reinforced and so conserved by a reward process. The reward system is therefore essential for this adaptive process (Ledoux, 1992) and is characterized by interconnected neural networks including the ventral tegmental area (VTA), the nucleus accumbens (NAcc), the prefrontal cortex (PFC), the ventral pallidum, the hippocampus and the amygdala (Fig.3).

It comprises a main axis of dopaminergic projections from neurons of the VTA to limbic structures such as the NAcc (the mesolimbic axis) and to the PFC (mesocortical axis). Briefly, the VTA is known to alert the organism when gratifying stimuli appear or are anticipated (Schultz, 1998) while the NAcc is involved in the onset of reward-oriented behaviours (Cardinal, 2002, Everitt, 2002) and the PFC participates in motivational process (Schoenbaum, 2005).

Also, the mesocorticolimbic pathway modulates activity of structures that regulate the integration of environmental signals (hippocampus and amygdala) and the planning of motor responses (striatum, thalamus and pallidum).

Due to such properties, the reward system is the primary target of addictive drugs. The latters chemically mimics the natural activation of the reward system leading to a gratifying feeling, normally proper to the adoption of an effective adaptive behaviour. In that way, drugs "trick the brain" and produce a very effective reinforcement of the behaviour that leads to their repeated consumption. The increase in dopamine release in the NAcc constitutes the hallmark of this reinforcement.

Indeed, all addictive drugs have the feature to interact with the mesolimbic system (Olds, 1954; Di Chiara, 1986; Imperato, 1986).

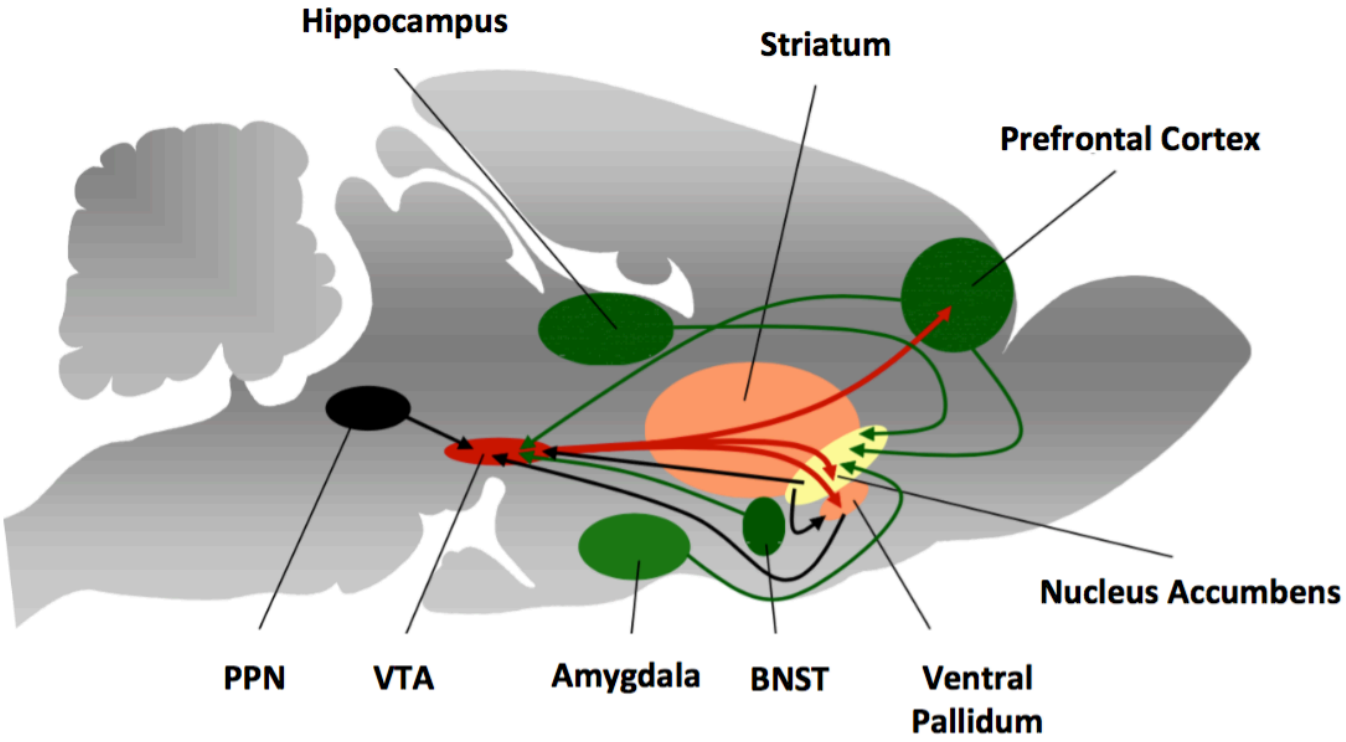

Fig.3. Anatomical presentation of key structures involved in the reward system.

The reward system is characterized by interconnected neural networks including in particular the ventral tegmental area (VTA), the nucleus accumbens, the prefrontal cortex, the ventral pallidum, the bed nucleus of the stria terminalis (BNST), the amygdala, the pedunculopontine nucleus (PPN), and the hippocampus. The nucleus accumbens, located in the ventral striatum is a key structure of the reward system. With its main afferents from the VTA, the hippocampus, the prefrontal cortex and the amygdala, it contributes to the associative learning of "responses-reward" and is involved in the onset of motor responses to stimuli with emotional value, such as cocaine. Adapted from: (Turiault, 2006).

Striatum and dopaminergic system: the VTA – NAcc projections

The Striatum is the main input structure of the basal ganglia, a group of subcortical nuclei that integrate information about context, actions and outcomes to shape adaptive behaviour (Ena, 2011, Macpherson, 2014). It receives glutamatergic projections from the cortex, hippocampus and thalamus and projects to output basal ganglia nuclei that regulate thalamo-cortical projections.

Modulatory dopaminergic neurons from the VTA and substancia nigra *pars compacta* (SNc) projects to the striatum which in turn regulates these midbrain nuclei activity through feedback projections (Heimer, 2003a, Heimer, 2003b, Devan, 2011, Liljeholm and O'Doherty, 2012, Gerfen, 2011, Do, 2012, Voorn, 2004).

This structure has been shown to control cognitive functions such as learning and memory, attention and motivation, and is particularly known to regulate reward processing.

Based on its afferent and efferent cortical projections, the striatum was initially divided into dorsal (caudate-putamen in rodents) and ventral (NAcc and olfactory tubercles) striatum (DS and VS respectively) sharing the same cytoarchitecture and biochemical composition (Heimer, 2003a, Heimer, 2003b, Devan, 2011). Furthermore, the NAcc can be segregated into a central and dorsal part, called the *Core*, and a surrounding lateral, ventral and medial part, labelled the *shell* regions due to their neurochemical and anatomical specificities (Groenewegen, 1999). The VS is thought to be involved in motivational processes and has been shown to encode the prediction of rewarding stimuli. For instance, lesions of the NAcc impair previously acquired conditional response to food-paired conditional stimuli (Liljeholm, 2012, Blaiss, 2009, Kelley, 2004). On the other hand, the dorsal region of the striatum is mainly known for its role in motor and instrumental learning (Balleine, 2009, Jankowski, 2009, Atallah, 2007).

In rodents, the striatum is composed of about 90-95% of medium sized perikaryon densily spined cells called Medium spiny neurons (MSNs) and 5-10% of medium to large aspiny GABAergic and cholinergic interneurons. According to their projection sites and the proteins they express, neurons projecting from NAcc to VTA form different inibitor pathways. A first (mediated by D1-type MSNs) comprises direct GABAergic projections between the two structures while another (mediated by D2-type MSNs) inervates indirectly the VTA via GABAergic neurons of the ventral pallidum (Fig.4).

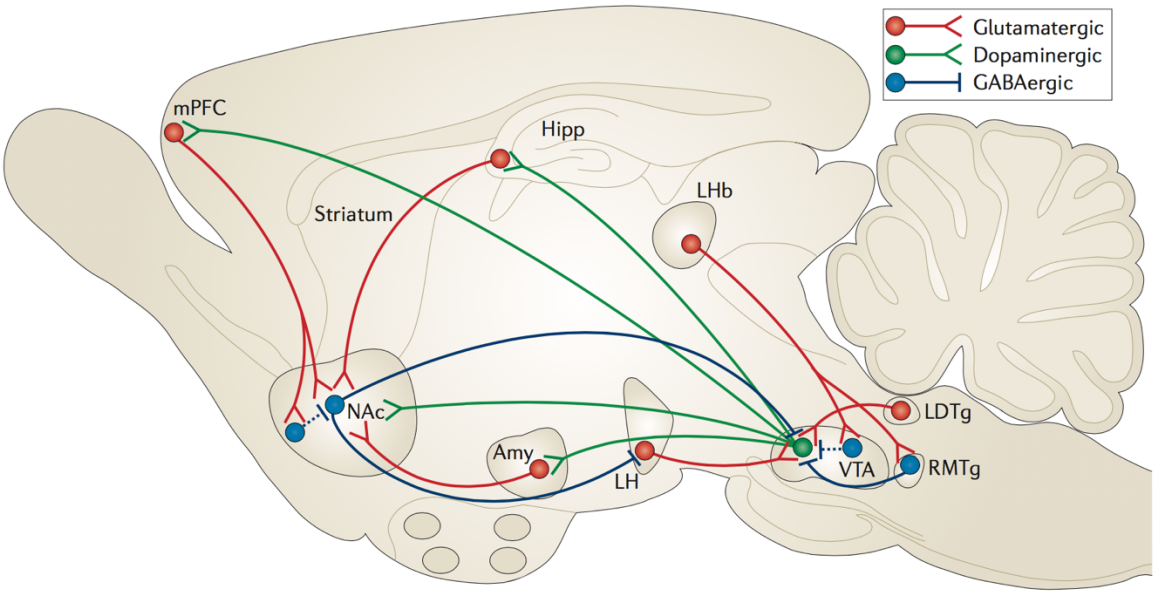

Fig 4. VTA–NAcc reward circuit.

A simplified schematic of the major dopaminergic, glutamatergic and GABAergic connections to and from the ventral tegmental area (VTA) and nucleus accumbens (NAc) in the rodent brain. The primary reward circuit includes dopaminergic projections from the VTA to the NAc, which release dopamine in response to reward-related stimuli (and in some cases, aversion-related stimuli). There are also GABAergic projections from the NAc to the VTA; projections through the direct pathway (mediated by D1-type medium spiny neurons (MSNs)) directly innervate the VTA, whereas projections through the indirect pathway (mediated by D2-type MSNs) innervate the VTA via intervening GABAergic neurons in the ventral pallidum (not shown). Adapted from: Scott J, et al. (2013).

Dopamine is thought to oppositely regulate glutamatergic transmission in distinct MSNs subtypes based on their differential enrichment in excitatory D1R or inhibitory D2R. Briefly, activation of D1R (acting through G_s and G_{olf}) facilitates glutamatergic transmission by somatic depolarization through increased L-type Ca2+ channels flow and decreased K+ currents. Oppositely, D2R stimulation increases outward hyperpolarizing K+ channels and decreases dendritic Ca2+ currents reducing the excitability of neurons.

Also, dopamine receptors are believed to differentially respond to phasic or tonic dopamine neurons firing. In fact, DA phasic firing modulates low-affinity D1R and is assumed to be crucial in reward related behavior. Conversely, tonic DA firing stimulation of high-affinity D2R has been shown to be suppressed by aversive stimuli (Grace, 2007, Mirenowicz, 1994, Mirenowicz, 1996, Ungless, 2004).

Interestingly, it was also shown that D1R inhibition (D1R antagonist SCH23390) and D2R activation (D2R agonist quinpirole) in the NAcc mimics the deficit in reinforcement and punishment (respectively) caused by neurons transmission blocking (Hikida, 2013).

Recent technological advances in mouse genetics, virus-mediated gene transfer and optogenetics have made it possible to determine the roles of specific neuronal cell types in reward-related behaviour. In an initial study, the use of a light-activated cation channel, channelrhodopsin 2 (ChR2), has shown that stimulation of VTA dopaminergic neurons was sufficient to drive intracranial self-stimulation in rats (Witten, 2011). Optogenetic stimulation of these neurons also promoted a conditioned place preference, confirming their role in inducing reward, whereas stimulation of GABAergic interneurons in the VTA disrupted reward and promoted conditioned place aversion (Tan, 2012, Tsai, 2009, van Zessen, 2012). In the NAcc, optogenetic stimulation of D1-type MSNs enhanced cocaine-induced place conditioning and locomotor activation, whereas stimulation of D2-type MSNs had the opposite effect (Lobo, 2010).

Interestingly, a distinct role of accumbens projection neurons was also demonstrated in social interaction behavior also considered as a form of reward. Recent studies in mice demonstrate that intra-NAcc infusion of D1R but not D2R antagonist blocked the pro-social effect elicit by increased DA levels in the NAcc.

b. Vulnerability to drugs of abuse and stress

The reinforcement mechanisms barely described constitute the final stage of a wider adaptive process. As anticipated, it is first of all the perception of a stressful event (homeostatic imbalance) which pushes an organism to resort to new behavioural strategies, and this, in order to restore a state of well-being (homeostatic balance). It thus appears that the stress response (through HPA activity), by preparing the organism to act, and the reward system (depending on the mesocorticolimbic pathway), by potentially conferring a hedonic value to the adopted behaviours, are closely linked.

In that way, they constitute the foundations that underlie the trial-and-error learning that can drive to efficient adaptation or to addiction. Studies based on animal models had allowed to better understand the interaction between the stress response and drug addiction revealing that HPA and especially GCs, are decisive factors in the appearance of vulnerability to addictive drugs. In addition, GCs have been clearly shown to be strongly interacting with the activity of the mesolimbic dopaminergic pathway.

In stressful situations, large amounts of GCs are released in the brain. These hormones increase the brain sensitivity to psychotropic and encourage the emergence of addictive behaviours in repeatedly stressed animals (Piazza, 1989, Marinelli, 2002). Meanwhile, experiments show that in addictive rats, the administration of competitive antagonists for the GCs have the effect of reducing their drug intake (Richardson, 2008, Shalev, 2006, Specio, 2008, Zislis, 2007). Importantly, each organism has a proper threshold of tolerance to stressful situations. Therefore, GCs secretions are higher or lower depending on the individual.

In the clinical literature, a growing body of evidences suggests that there is a strong link between stress response and addiction. One of the most studied cases concerns veteran soldiers suffering from post-traumatic stress disorder (PTSD) (Penk, 1988, Zaslav, 1994, Brady, 2001). Indeed, many studies show a comorbidity between PTSD and addiction. Although a causal relationship between exposure to combat and substance abuse has not been clearly established, veterans with PTSD typically report a higher consumption of alcohol, cocaine and heroin that other veterans (Saxon, 2001). The addiction was also associated with exposure to other types of stress such as an unhappy marriage or a depressing job (Jose, 2000). Even smoking (including cannabis use) has been linked to stress in family or in social life and to the incapacity to face them (Siqueira, 2001, Butters, 2002).

In addition, stress or the presentation of a stimulus associated with a known stress, has also been identified in humans as a powerful event to cause a relapse of drug use (Lamon, 1997, Sinha, 2001).

Finally, in the same way that susceptibility to stress influences the vulnerability to addiction, the reverse has also been recently demonstrated. Indeed, individuals addicted to cocaine exhibit a heightened sensitivity to stressful events (Fox, 2008). Stress becomes thus a risk factor of great importance in the relapse phenomenon.

Most of the effects of the stress response on the response to drugs are due to the release of glucocorticoid hormones. The inhibition of the HPA axis or suppression of circulating GCs by adrenalectomy blocks the stress-induced sensitization to cocaine (Cole, 1990, Deroche, 1992, Rouge-Pont, 1995, Prasad, 1998). On one side, blocking the synthesis or the release of GCs brings down self-administration in rodents for low doses of cocaine, and greatly decreases the amount of cocaine consumed as well as the number of animals that meet the criteria acquisition (Goeders, 1996, Goeders, 1998, Campbell, 2001). On the other, chronic stress or chronic administration of high doses of corticosterone in rodents, induces a propensity to self-administer psychostimulants at doses that leave indifferent the control animals, together with a hyperresponsiveness to these products both at the behavioral (locomotor activity) and neurochemical (dopamine release in the nucleus accumbens) levels (Piazza, 1990, Pauly, 1993). These results clearly establish that release of GCs is a fundamental element of the influence of the stress response on the consumption of addictive drugs.

c. Depression and GR

Dysregulation of the HPA axis is associated with major health problems and is thought to intervening in the etiology of psychiatric disorders such as anxiety and depression (de Kloet, 2005). These disturbances can result in depressed subjects by a defect of negative feedback on the GCs secretion. The dexamethasone suppression test is commonly used in medicine to study these disturbances of the dynamic response of the HPA axis. Dexamethasone (a synthetic GC), crosses the blood-brain barrier but is expelled actively and penetrates little in the brain (Meijer, 1998). It acts on the feedback mechanism of the HPA axis and leads to the suppression of the secretion of corticosterone in providing negative feedback directly at the pituitary (McEwen, 1986).

For a large number of depressive patients, a strong alteration of the suppressive effect of dexamethasone (Carroll, 1980) is observed. Treatment of these patients with antidepressants leads to a normalization of the HPA axis, which precedes the improvement of clinical signs of depression. Since the 2000s, pioneer works based on the mutation of the GR revealed its role in pathologies associated with anxiety or depression, through the use of tests designed as behavioural models.
Indeed, in mice, the absence of GR in the brain is accompanied by a decrease of behaviours associated with anxiety and depression (Tronche, 1999). Conversely, overexpression of the GR in the forebrain (telencephalon and diencephalon) leads to an opposite phenotype (Wei, 2004). Finally, the absence of GR in the forebrain also results in a decrease of behaviours associated with anxiety (Boyle, 2006).

Understand how the stress response and the reward system interact leading to depression, represents a methodological challenge due to the complexity of the circuitry involved. However, one view is that the reduced responses to rewarding experiences and exaggerated responses to aversive ones that characterize depression might — together with the associated maladaptive cognitive style — reflect abnormalities in the perception and interpretation of reward valence, in the motivation for rewards and in subsequent decision-making (Koob, 2008, Ikemoto, 2002, Schultz, 2011, Blackemore, 2012).

d. Animal models: conditional mutagenesis as a tool to uncover the specific roles of GR

In the early 90s, the development of new genetic tools has revolutionized the study of gene function. The ability to specifically modify a gene by homologous recombination (HR) in embryonic stem cells (ES cells) of mice allowed to observe the impact of this modification on living animals (Gu, 2004).
With the advent of transgenesis, "targeting" the expression of an exogenous gene has become possible. This technique allows to build transgenes which contain the regulatory sequences of a gene A and the coding sequences of a gene B. The transgenic mice produced with such a transgene express the protein encoded by gene B in tissues or cell types where the gene A is normally expressed.
The gene B is then called a "reporter" of the activity of the regulatory sequences of the gene A. However, the study of these first mutants was limited by several technical problems.

First, the targeted mutation of a gene in ES cells requires the use of selection markers such as the open reading frame of neomycin under the control of a strong promoter. The presence of the promoter influences the expression levels of neighbouring genes and the gene of interest itself. Modifying the expression level of the gene of interest can be a problem particularly in the case of subtle genetic modifications such as introduction of point mutations.

Secondly, in many cases, inactivation of a gene in all body cells is lethal during early embryonic or postnatal development, preventing thus the study of its function in adults. This is the case of the GR gene (Tronche, 2007). Third, if a gene is expressed in many different cell types, its inactivation can lead to a complex phenotype that is hard, if not impossible to interpret.

To solve those problems and allow the survival of the mice in adulthood, it was necessary to restrict the inactivation of the gene to only one tissue/cell type of the animal. This specificity has been made possible through the development of the Cre/loxP system which allows, in mice, the invalidation of the desired gene in a given cell type, without jeopardizing its activity in other body cells (Lasko, 1992, Orban 1992, Gu 1994, Tronche, 2002). In addition, this system allows the elimination of the selection marker and its strong promoter.

The Cre recombinase (Cyclization recombination) is a 38 kDa protein encoded by the bacteriophage P1 that recognizes a 34 bp DNA target on the P1 genome called loxP (locus of X-over of P1). It catalyzes reciprocal DNA recombination between two loxP sites. LoxP sites are composed of two inverted DNA segments of 13 bp Cre monomer binding sites and a spacer of 8 bp. When two loxP sites are oriented in the same direction, Cre recombinase induces by intrachromosomal recombination (called "site specific") the deletion of the DNA sequence between the two sites, allowing the specific invalidation of the desired gene (Fig.5).

The principle is to develop initially mice in which two loxP sites flank an essential part of the gene of interest without disrupting its functionality. These mice are then bred with a transgenic mouse that expresses the Cre in one cell type, through the use of appropriate regulatory sequences. In the double transgenic mice obtained from this crossing, the recombinase causes the excision of sequences located between the loxP sites and therefore induces an inactivation in the cell type in which the Cre is expressed. Despite the relative simplicity of this technique, the transgene integration site is not controlled.

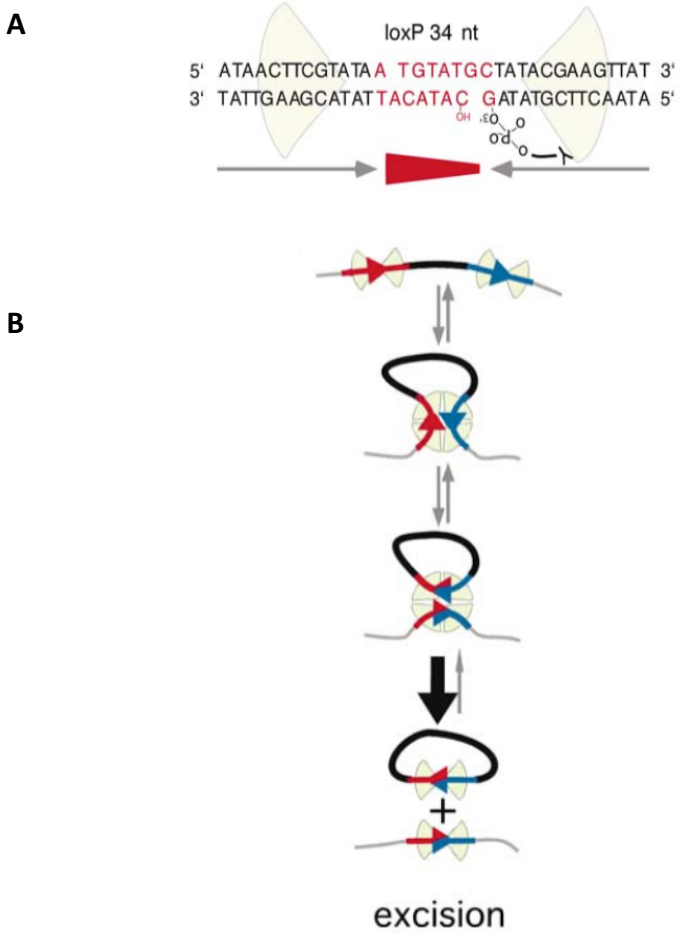

Fig 5. Mechanism of site-specific Cre recombination.

A. The loxP site consists of two inverted 13 bp Cre binding sites that surround a central 8 bp spacer. A red arrow indicates the orientation of the loxP site. Cre recombinase is pictured in green. **B**. If the two recombination sites are in the same orientation the strand exchange leads to excision. Adapted from: (Tronche, 2002).

These techniques, by mutating the Nr3c1 (the glucocorticoid receptor gene) in targeted cell populations of the brain, have helped to clarify, in the mouse, the role of the GR in the modulation of depression and anxiety-like behaviours (Tronche, 1999) and in the behavioural responses to cocaine (Deroche, 2003).

The precision of gene inactivation using the Cre/LoxP system relies on the existence of specific promoters. However, some subregions of the brain lack of specific promoters precluding the study of precise neuronal subcircuits. Over the past 10 years, the development of virally-mediated gene transfer using adeno-associated viruses (AAV), have made it possible to refine the subregions in which we can inactivate a gene of interest and to determine the roles of specific neuronal cell types in reward and anxiety related behaviours to a degree that was not possible in earlier investigations. AAVs are nonenveloped, single-stranded DNA viruses that replicate only in the presence of helper virus, such as adenovirus or herpes simplex virus, and are deprived of pathogenic sequence.

In our case, we used AAV2-CMV-Cre-IRES-GFP that allows co-expression of Cre and GFP, and a control virus AAV2-CMV-GFP. Stereotactic injection of AAV2-CMV-Cre-IRES-GFP in GRlox/lox mice enables the expression of Cre and the inactivation of GR gene in the targeted brain structure. The serotype 2 has been chosen for its natural tropism for neurons over other cell types (Bartlett, 1998, Parnaudeau, 2013). The cytomegalovirus promoter (CMV) allows high expression in any cell type. It is noteworthy that while transgenic based Cre/loxP system induces recombination during development, the use of virally-mediated gene transfer enables recombination during adulthood, precluding any potential compensatory mechanisms. The drowback of this method however is the fact that it implies anesthesia and surgery. In addition, the efficacy and reliability of this method depends largely on the experimenter's ability to consistently target the structure of interest. This is however systematically controlled histologically after the experiment and all mis-targeted animals are excluded from the cohort.

To validate the hypothesis that the interplay of GC-mediated stress response and dopaminergic neurotransmission modulate behaviours related to addiction (Ambroggi, 2009, Barik, 2010) and to the social aversion (Barik, 2013), animals deprived of GR in either dopaminergic (DA) neurons (GR^{DATCre}) or dopaminoceptive neurons (GR^{D1aCre}) have been obtained using a Cre/loxP approach. GR^{DATCre} mice were generated crossing GR^{loxP} mice, in which the third exon of the GR gene is flanked by two loxP sites, with a mouse line carrying a BAC encompassing the dopamine transporter gene and ensuring GR gene inactivation in all dopaminergic neurons (Fig.6). GR^{D1aCre} mice were obtained by using a mouse line expressing the Cre recombinase in dopaminoceptive neurons, under the control of the D1a receptor gene regulatory elements.

These studies have revealed that GR expressed in the dopaminoceptive neurons, but not in dopamine-releasing neurons, is a key element in the reinforcing effects of the cocaine. Indeed, GR^{D1aCre}, but not GR^{DATCre} mice, show a markedly decreased motivation to self-administer cocaine (Ambroggi, 2009). These behavioural changes in GR^{D1aCre} mice have been shown to be specific to cocaine reinforcement, since behavioural morphine responses are not altered by GR gene mutation in either dopaminoceptive or dopamine neurons. Besides, although the invalidation of the GR in the whole brain have highlighted its involvement in the regulation of anxiety-like behaviours (Boyle, 2006), neither GR^{DATCre} nor GR^{D1aCre} mice show a specific genotype in the elevated plus maze and open-field tests. These results demonstrate that, although the comorbidity between addiction and anxiety can be explained by their common dependence of a GR regulation, these two behaviours are independent and dissociable, as they are mediated by GR in different neuronal populations.

It has also been demonstrated that GR in dopaminoceptive neurons participates in the hedonic values of cocaine, as well as in the locomotor sensitization resulting from its consumption (Barik, 2010). Indeed, GR^{D1aCre} mice, but not GR^{DATCre} ones, present a reduced cocaine-induced conditioned place preference, as well as a diminished increase in locomotor activity after cocaine intake. Finally, GR^{D1aCre} mice, but not GR^{DATCre} ones, are more resilient than control littermates, in a chronic social defeat stress paradigm, demonstrating the role of GR in dopaminoceptive neurons, in the appearance of depression-like behaviours (Barik, 2013).

Importantly, all these results have shown that the phenotype of GR^{D1aCre} mice is associated with a reduced activity of the VTA for this population only. This effect is not a result of a change in HPA axis regulation, as circulating GCs levels were systematically checked, and result normal in both GR^{D1aCre} and GR^{DATCre} mice under basal conditions or in response to stress.

Rather, this suggests that the GR in dopaminoceptive neurons act mainly by influencing the dopamine release through the modulation of the feedback exerted on the VTA.

Thus, GR expressed in dopaminoceptive neurons appears to play a key role in regulating the activity of dopamine neurons and in the modulation of stress-related behaviours such as behavioural responses to cocaine and to social aversion induced by repeated aggressions.

The absence of glucocorticoid receptor in D1R–expressing neurons probably changes the control that they exert on VTA dopaminergic neurons. Such changes of dopaminergic neurotransmission could form the basis of the observed modulations in the behavioural responses to cocaine consumption and chronic social stress. Importantly, in GR^{D1aCre} mice, GR gene recombination is restricted to dopaminoceptive neurons but is larger than the expected population of D1R-expressing neurons in adults and affects a large proportion of D2R expressing ones. Both nucleus accumbens GABAergic and prefrontal cortex glutamatergic neurons express the dopamine receptor 1a gene, Drd1a, and are known to strongly regulate the activity of dopamine neurons. Either one or both of these structures could then mediate the reduced dopamine neuron activity.

Measurements of the dopaminergic activity in the VTA have allowed to gain insight into the putative circuitry underpinning the failure of social avoidance that characterizes GR^{D1aCre} mice. Thereby, the measurement of DA release in the NAcc after aggression by *in vivo* microdialysis in freely moving mice has revealed that, while social defeat triggered a robust release of DA in control animals, this effect was markedly diminished in GR^{D1aCre} littermates, suggesting that DA neurotransmission is a primary component of social stress–induced neuroadaptations. In the same way, the examination *in vivo* of the electrophysiological properties of VTA DA neurons, shows that only in the control populations the frequency and the number of bursting events of DA neurons from defeated mice was

significantly increased relative to those of undefeated ones, whereas GR^{D1aCre} failed to demonstrate any significant electrophysiological adaptations, hence mirroring the lack of social avoidance. Finally, the injection in control mice of quinpirole, a DA D2-R agonist that leads to the suppression of DA neurons activity, by restoring social interaction after repeated aggressions suggests that this increase in DA neurons firing is a prerequisite for social avoidance or a subsequent neuroadaptation to stress (Barik, 2013).

III. Aims of the research

The hosting group have shown that GR gene inactivation in dopaminoceptive neurons (GR^{D1aCre} mice) deeply affects some stress-related behaviors, including behavioral responses to psychostimulants and social withdrawal acquired after repeated social stress (Ambroggi 2009, Barik 2010, Parnaudeau 2014). At the physiological level, the mutation of GR in post-synaptic neurons deeply reduces the electrical activity of presynaptic DA neurons of the VTA, showing a role for GR in the modulation of the feedback exerted by dopaminoceptive neurons on DA neurons.

In GR^{D1aCre} mice, GR gene is recombined in several structures including the NAcc, the dorsal striatum, the amygdala and the basal layers of the cortex, among which the NAcc and the mPFC are known to control VTA DA neurons activity It is therefore essential to refine the identification of the relevant brain structures.

The main aim of my project was to identify whether the GR in the NAcc is important for the observed phenotypes. We therefore specifically inactivated GR, by viral transduction of the cre recombinase in this structure and studied a behavioural response (locomotor sensitization to cocaine, affected in GR^{D1aCre} mice) in mutant and control animals. It corresponds to the "experiment 1" presented below.

The second aim was to further investigate, in GR^{D1aCre} mice, the activity of DA neurons by measuring the DA release in the NAcc of mutant and control animals under basal conditions and following repeated social defeats. It corresponds to the "experiment 2" presented below.

IV. EXPERIMENT 1: locomotor sensitization to cocaine in transgenic mice selectively deprived of GR in the nucleus accumbens.

1) Material and Methods

Animals: 24 $GR^{loxP/loxP}$ male mice 2 to 4 months old and backcrossed for more than 10 generations on C57BL/6 genetic background have been used for this experiment. Animals were maintained as $GR^{loxP/loxP}$ homozygous by crossing males and females of the same genotype.

Mice were genotyped for GR^{loxP} alleles by PCR on DNA extracted from 0,1cm of mouse tail recovered at weaning. Tails were digested overnight adding 20µl of proteinase K in 750 µl of "tail buffer" (NaCl 100 mM, Tris-HCl 50 mM pH 8, EDTA 100 mM pH 8, SDS 1%). The day after, proteins were precipitated following the addition of 250µl of saturated NaCl and centrifugation (5 min 13000 rpm). DNA in the supernatant was precipitated by adding 500µl of isopropanil. DNA pellets were washed with 500µl EtOH (70%) and resuspended in 100µl of TE (Tris-HCl 10 mM, EDTA 1 mM, pH 7,5). For PCR, 2µl of DNA solution (diluted 50X) was added to 23µl of PCR mix (dNTP 0,4 mM, $MgCl_2$ 1 mM, primers 0,4 mM, Taq polymerase 0,5 U Promega, reaction buffer 1X Promega). Amplification was obtained by 35 cycles (45 s at 95°C, 45 s at 60°C and 2 min at 72°C) using 5' CAT-GCT-GCT-AGG-CAA-ATG-ATC-TTA-AC 3' and 5' CTT-CCA-CTG-CTC-TTT-TAA-AGA-AGA-C 3' primers. Amplicons were analysed on 2% agarose gels migrated in TBE 0,5X. GR^{wt} allele gave a 400 bp band and GR^{loxP} a 460 bp band.

Mice were bred and raised under standard animal housing conditions, at 22°C, 55% to 65% humidity, with a 12-hour light/dark cycle (7 AM/7 PM) and free access to water and a rodent diet. All of these studies were conducted in compliance with national and European directives relating to animal testing. They were performed by minimising as much as possible, the number of animals as well as potential sources of suffering.

GR gene inactivation by viral transduction of Cre: AAV2-CMV-GFP and AAV2-CMV Cre IRES-GFP viruses were obtained from University of North Carolina vector core facility (NC, USA). The procedure was as followed: 4 weeks old $GR^{loxP/loxP}$ male mice were anesthetized with an

intraperitoneal (10 ml/kg) injection of ketamine (Virbac, 100mg/kg) and xylazine (Bayer, 10 mg/kg) dissolved in sterile isotonic saline solution (NaCl 0.9%). Once anesthetized mice were mounted onto a stereotaxic frame (David Kopf Instruments, CA, USA). The scalp was shaved and the local anesthetic « lidocaine » was applied. Holes were drilled bilaterally at the appropriate coordinates from Bregma: -1,5 mm anteroposterior and +/-0,8 mm lateral median. Hamilton seringe of injection was introduced at 4,5 mm depth for the injection (Fig.7). A Volume of 0.5 µl of AAV2 vectors per site of injection was delivered bilaterally with a slow injection rate (0,1 µl/min). After the injection was completed, the injector was left in place for 5 min before placing the cannula at the contralateral point of injection to minimize backflow while withdrawing the injector. Animals were under daily surveillance for a week after injection procedure. The experimental group of mice was injected with AAV2-CMV-Cre-IRES-GFP. These animals expressed in the NAcc the Cre recombinase gene leading to selective GR gene inactivation ($GR^{AAV\text{-}Cre}$ mice; n = 11). The control group was injected with AAV2-CMV-GFP only ($GR^{AAV\text{-}GFP}$ mice; n = 12).

Experimental design and locomotor sensitization to cocaine. Behavioural experiments were started 4 weeks after AAV vectors injections. Previous experiences showed that this time is sufficient to allow GR gene recombination and GR protein disappearance (S Parnaudeau unpublished). Locomotor activity was assessed in circular chamber (4.5-cm width, 17-cm external diameter) crossed by four infrared captors (1.5 cm above the base) placed at every 90° (Imetronic, Bordeaux, France). Circular chambers are connected to a computer for automatic recording of locomotor activity of each individual. The locomotor activity was counted when animals interrupted two successive beams and thus had travelled a quarter of the circular corridor. Mice were habituated to the apparatus for 3 hours for 3 consecutive days and received a saline injection on days 2 and 3 (NaCl 0.9% saline solution, 10 ml/kg, *i.p.*). Then, both $GR^{AAV\text{-}Cre}$ and $GR^{AAV\text{-}GFP}$ mice were repeatedly treated with cocaine hydrochloride (Sigma-Aldrich, 10mg/kg *i.p.*) for 5 consecutive days. For each session, animals were placed in the apparatus 90 min before injection, and let inside 120 min after injection. Following 7 days of withdrawal, all mice received a challenge injection of cocaine (10mg/kg *i.p.*).

Histology : Following behavioural experiments, animals received a lethal dose of pentobarbital (30 mg/kg, i.p.; Centravet) and were intracardiacally perfused with phosphate buffer (PB, 0,1M; Na_2HPO_4/NaH_2PO_4, pH 7,2) then with fixation solution (PFA 4% in PB 0,1 M). Brain were then removed and post-fixed overnight in the same solution (4°C). Coronal brain sections (30 µm thick) were prepared with a vibratome. For immunostaining, sections encompassing the NAcc were rinsed three time, for10 min, in PBS. Then incubated for 30 min in a blocking solution (PBS, BSA 0,5%, Triton X-100 0,1% (PBS-BT), NGS 10%). They were then incubated over-night in PBS-BT with 1% NGS and either a rabbit polyclonal anti-Cre antibody (1:3,000 dilution, Kellendonk et al., 1999), or a rabbit polyclonal anti- GR antibody (Santa Cruz, cat. SC1004, M20, 1:1000 dilution) as primary antibody. The day after, sections were rinsed for 30 min in PBS and then incubated with a mouse polyclonal GFP antibody (2h; 1:300, in PBS-BT with 1% NGS) for double immunostaining. After 30 min rinsing (PBS) sections were incubated with *ad hoc* secondary antibodies (Alexa-488 coupled anti-mouse antibody, Molecular Probes, and Cy3-coupled anti-rabbit antibodies, Jackson Immunoresearch (2h; 1:100, in PBS-BT with 1% NGS). Sections were rinsed 30 min in PBS, then incubated for 5 min in DAPI (Hoechst) (0,1 µg/ml) to stain the nuclei. Sections were finally rinsed twice for 10 min in PBS and once for 10 min in Tris 50 mM pH 7.2 and mounted using Vectashield (Abcys). Images were obtained on a Zeiss epifluorescent microscope (X10) with AxioVision 4.7 software (fig 6).

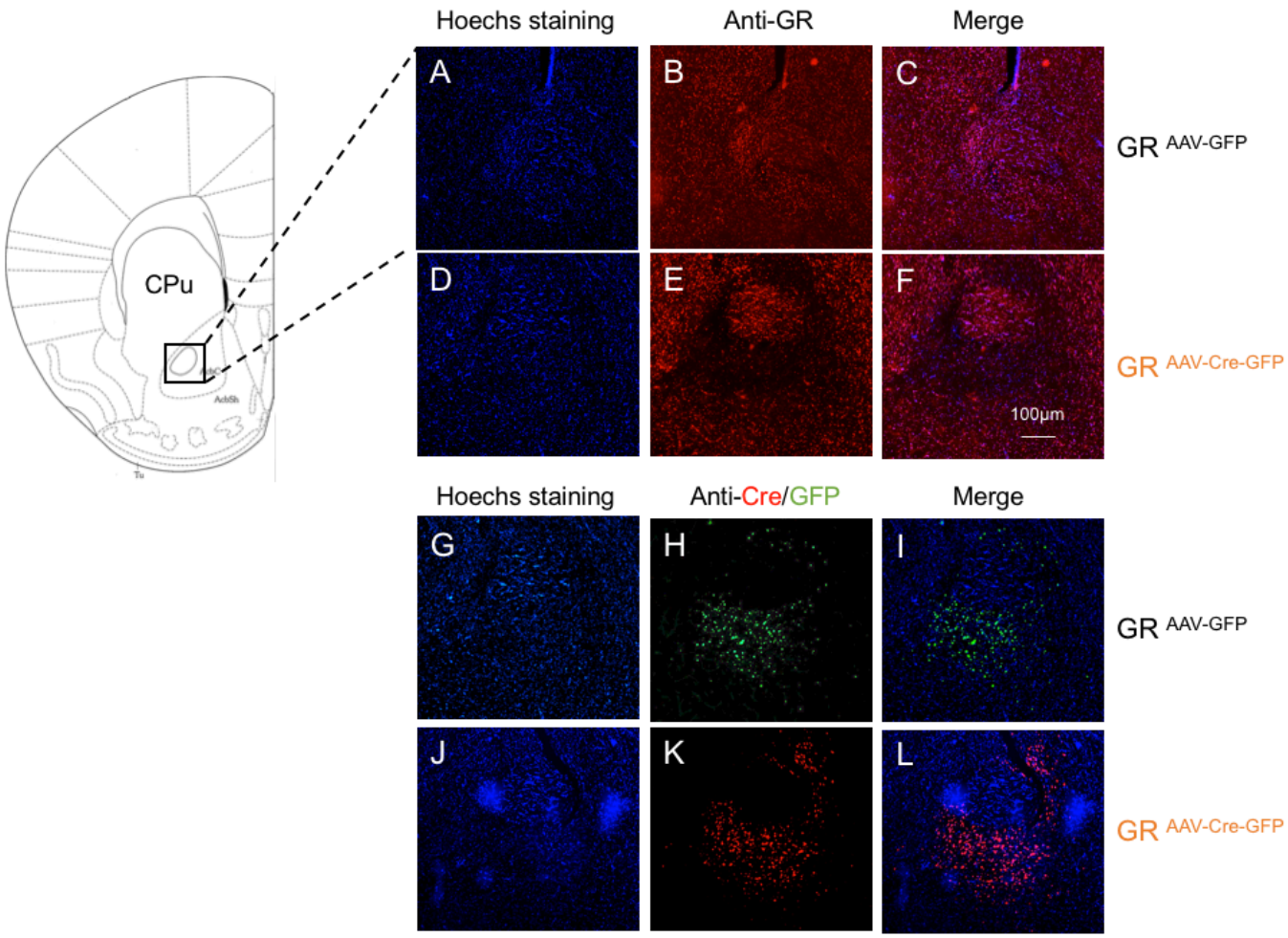

Fig 6. Immunofluorescent staining of GR, Cre and GFP proteins in the left NAcc of $GR^{AAV\text{-}GFP}$ and $GR^{AAV\text{-}Cre}$ mice (magnification: X 10).

All fields correspond to similar brain regions, from the different mouse models. The position of the fields is indicated on the left scheme, t**he animal models on the right. A, D, G, J.** DAPI staining (blue) were used to stain all cells nuclei. **B, E, H, K**. Immunofluorescent staining probing GR (red: **B. E**), Cre (red: **K**) and GFP (green: **H**) in the left NAcc. Merged pictures: **C, F, I, L**.

Statistics: Data are presented as means ± S.E.M. Statistical analysis was carried out using mixed two-way ANOVA for cocaine sensitization.

A $GR^{loxp/loxp}$ mouse was removed from the experimental protocol owing to under-nutrition due to abnormal tooth morphology. This animal, unable to nourish normally, presented a low weight which could constitute a bias for our experiment. Consequently, the experiment was conducted on 23 mice.

2) Results

Generation of animals recombined for GR gene in the Nucleus accumbens.

To obtain selective GR gene inactivation in the Nucleus Accumbens, we locally injected AAV2 viruses expressing the Cre recombinase and the GFP under the control of the CMV promoter into the brain of $GR^{loxP/loxP}$ male mice, to obtain $GR^{AAV\text{-}Cre}$ mice (n=11). The control animals were $GR^{loxP/loxP}$ mice injected with a similar virus that expresses only the Cre recombinase (AAV2-CMV-GFP, $GR^{AAV\text{-}GFP}$ mice, n=12). The efficiency of viral transduction was assessed by immunofluorescence 6 weeks after injection of the viruses, in the NAcc region (Fig. 6). In the NAcc of $GR^{AAV\text{-}GFP}$ mice, GFP protein was detected (Fig. 6H) and the presence of Cre recombinase protein was restricted to the NAcc of $GR^{AAV\text{-}Cre}$ mice (Fig. 6K). Disappearance of GR protein in $GR^{AAV\text{-}Cre}$ mice was compared to GR expression in $GR^{AAV\text{-}GFP}$ mice. As shown in Fig. 8B, GR is widely expressed in the NAcc of $GR^{AAV\text{-}GFP}$ mice but disappeared in a large fraction of the core of the NAcc of $GR^{AAV\text{-}Cre}$ mice (Fig. 6E). In addition to this restricted recombination of GR gene, the most anterior parts of the NAcc did not display efficient recombination.

Effect of GR gene inactivation in the NAcc on cocaine induced locomotor sensitization

Repeated exposure to drugs of abuse promotes gradual and enduring increase of locomotor activity termed behavioural sensitization believed to reflect the reinforcing effects of abused drugs (Robinson, 1993). This phenomenon became a cardinal behavioral test for research on addiction since it appears to be specific for drugs that are addictive and since it is maintained even over long periods of abstinence (Paulson, 1991). The group previously reported that the absence of GR gene from dopaminoceptive neurons (GR^{D1aCre} mice) does not significantly affect behavioral responses to a single cocaine injection but blocks behavioral sensitization to psychostimulants (Barik, 2010; Parnaudeau, 2014). The recombination was however affecting different structures, including the NAcc and the PFC. To see wether GR in the NAcc could be responsible for this behavioral response, we compared cocaine induced locomotor sensitization in $GR^{AAV\text{-}GFP}$ and $GR^{AAV\text{-}Cre}$ mice. Animals were placed in a circular actimeter (Fig. 7A) daily for 3 h.

The three first days (Fig. 7A, D-3, D-2 and D-1), animals were habituated to the actimeter. They received a saline injection 90 min after the beginning of the session on the second and third days (Fig. 7A, D-2 and D-1).

Mutant male $GR^{AAV\text{-}Cre}$ mice displayed normal spontaneous locomotor response and habituation to a novel environment when compared to control $GR^{AAV\text{-}GFP}$ animals (Fig. 7B and 7C, Day-1). Shortly after placing the animal in the actimeter, both control and mutant mice had moderated locomotor activity, due to exploration of the environment, that decreased rapidly upon habituation. Just after the saline injection, we observed for both groups a slight increase in locomotion that reduced rapidly. Animals received then, for 5 consecutive days, an injection of cocaine (10 mg/kg).

In response to a first injection of cocaine, control mice had a significant increase in locomotion when compared to either locomotion after a saline injection on day -1. Injections on following 4 days led to further increase of total locomotion during the session. After a withdrawal of six days, on the challenge day, both groups displayed similar retention of the sensitization (Fig. 7D).

Sensitization developped in $GR^{AAV\text{-}GFP}$ animals as previously observed for control animals (Barik et al. 2010) and localized GR inactivation in the core of the NAcc did not affected differently the locomotor sensitization of $GR^{AAV\text{-}Cre}$ animals.

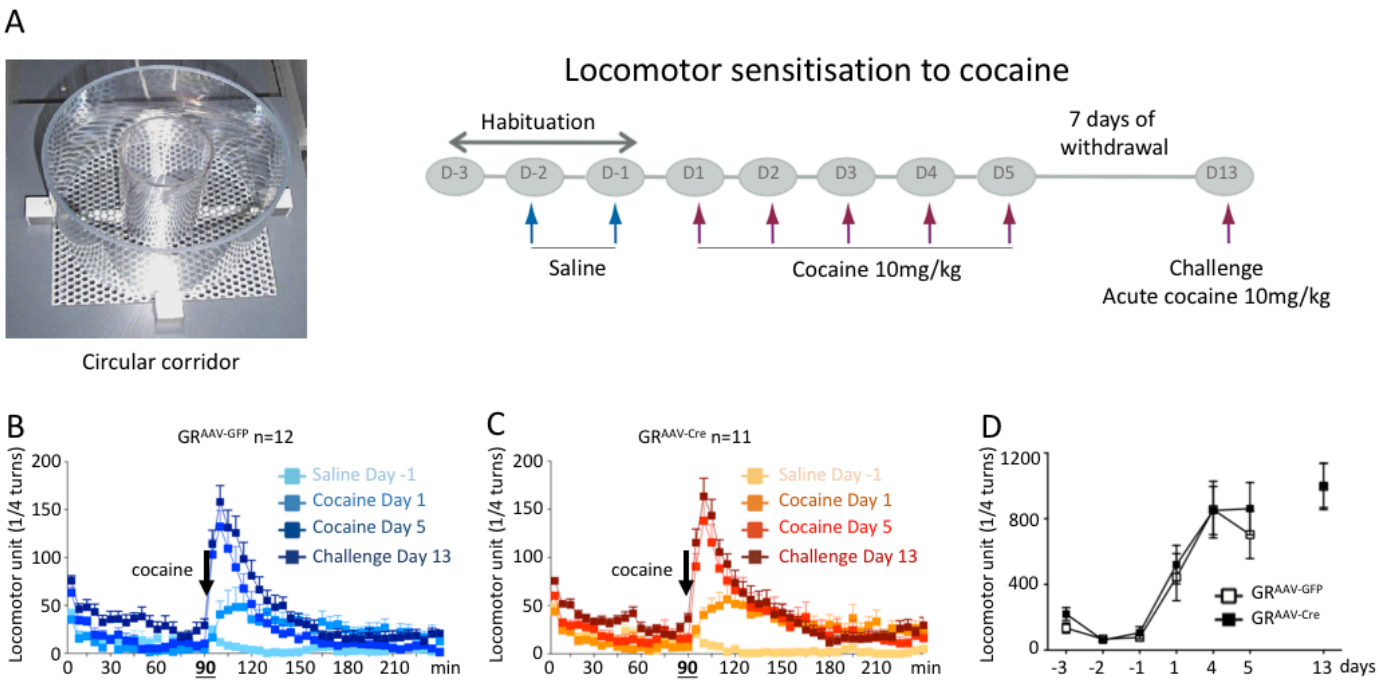

Fig 7. Locomotor sensitization to cocaine in GR$^{AAV\text{-}Cre}$ and GR$^{AAV\text{-}GFP}$ mice.

A) Photo of the actimeter (left) and experimental scheme. D: day. **B)** Time course of locomotor activity of GR$^{AAV\text{-}GFP}$ mice measured on days -1, 1, 5, 13. **C)** Time course of locomotor activity of GR$^{AAV\text{-}Cre}$ mice measured on days -1, 1, 5, 13. **D)** Development of locomotor sensitisation to cocaine as the comulative locomotor activity for the two mouse models pictured for each day. Data are presented as means ± SEM. Locomotor activity is expressed in units of turn quarters, for time brackets of 5 minutes. Locomotor sensitization was analysed with a mixed analysis of variance (ANOVA with one repeated factor – Days – and one "between" factor - genotype). The treatment (day factor) has a significant effect on the locomotor activity [F (6, 126) = 67,87; p***<0.001 - Greenhouse Geisser correction] revealing a marked increase in locomotor activity under cocaine treatment compared to habituation and saline processing. In contrast, neither genotype [F (1, 21) = 0,17] nor the interaction between these two factors [F (6, 126) = 0,29] show significant effects.

3) Discussion

This experiment aimed at identifying the region in which the GR modulates the locomotor sensitization to cocaine. Given the expoloratory nature of this experiment and the number of mice needed, the saline treated groups have not been included. However, the results show a clear locomotor sensitization in both control and GR$^{AAV\text{-}Cre}$ mice. No difference was found between the responses of GR$^{AAV\text{-}Cre}$ and the one of GR$^{AAV\text{-}GFP}$ control mice.

This lack of specific phenotype in GR$^{AAV\text{-}Cre}$ mice, in sharp contrast with the one observed in GR$^{D1a\text{-}Cre}$, may indicate that the locomotor response to cocaine mostly depends on dopaminoceptive neurons located in another or other structure(s).

Nevertheless, the histological analysis of AAV-Cre injected mice brains has revealed that, if the injection sites have well targeted the NAcc, the amount of injected virus has allowed only a partial deletion of the gene encoding the GR in this structure. It is therefore possible that the lack of phenotype in $GR^{AAV\text{-}Cre}$ mice is due to insufficient invalidation of GR.

More specifically the recombination occurred mainly within the core part of the NAcc. NAcc core has been shown to be part of a distinct loop of the meso-striato-cortical system than the medial part of the shell. Indeed, the core of the striatum receives input from the more lateral part of the VTA and its function has been mainly related to the function of the dorsal part of the striatum (Voorn, 2004, Cardinal, 2002). On the other hand, the shell is anatomically linked to the medial part of the VTA. Our group previously showed that dopamine neurons in the medial part of the VTA have a decrease of spontaneous firing activity in GR^{D1aCre} mice (Ambroggi, 2009). In addition, a decrease of dopamine release within the shell has been observed in these mice (Barik, 2010) suggesting that GR function may be more important in the shell subregion for the regulation of cocaine related behaviors. These points are currently being addressed in a new cohort of mice in which we injected a larger volum of AAV in two sites per emisphere in order to recombine the GR in the whole NAcc both core and shell. Another structure, which may be involved in the phenotype observed in GR^{D1aCre} mice, is the PFC that also receives projections from the VTA dopamine neurons. A recent study using optogenetics have shown that decreased PFC activity reduces cocaine self-administration behavior (Chen, 2013). In addition, previous work from our group showed the importance of monoamine signalling within the medial part of the PFC in the development of cocaine sensitization (Lanteri 2008, Tassin 2008). Therefore, the GR within the PFC may contribute to the regulation of behavioural responses to cocaine. We are currently planning to inactivate the GR in the medial PFC (prelimbic and infralimbic cortices) and test this cohort of mice in locomotor sensitization.

So far our behavioural characterization is focused on locomotor sensitization since it is the less time and mice consuming behavioural experiment. The identification of a subregion in which the GR modulates cocaine sensitization will be an incentive to deepen the physiological and behavioural characterization of the relevant model. In terms of behaviors, it would be onteresting to see whether

GR in the same region also modulates conditioned place preference (CPP) and self-administration to cocaine. All these behaviors have been shown to be impaired in GR^{D1aCre} mice, however it is easy to imagine that these tasks may involve very different brain regions. In the case of CPP, the GR within the dorsal medial part of the striatum may be important given the role of this subregion in associative learning.

Finally, it is important here to remind that we used virally-mediated gene transfer in order to inactivate GR in the brain of adult mice. In GR^{D1aCre} mice, GR gene is inactivated during development from E12.5 (Lemberger, 2007). Thus the phenotypes observed in these mice may be due to early recombination of the GR, which is not the case here when using viruses.

V) EXPERIMENT 2: social avoidance consecutive to repeated social defeat in GR^{D1aCre} transgenic mice

1) Material and Methods

Animals: 8 $GR^{loxP/loxP}$ (control) and 7 $GR^{loxP/loxP}$; Tg:YAC-D1aCre (thereafter denominated GR^{D1aCre}) mice 5 months old and backcrossed for more than 10 generations on C57BL/6 genetic background have been used for this experiment (Ambroggi et al. 2009). In GR^{D1aCre} mice, the GR gene is selectively inactivated in dopaminoceptive neurons using a transgene that expresses the Cre recombinase under the control of the D1a receptor gene. In this model, recombination occurs in the expected brain structures, including the striatum and the PFC but GR gene recombination is wider than expected (in 88% of neurons of the striatum and deep cortical layers) and occurs in D1R and a fraction of D2R expressing neurons (Barik et al. 2013).

Mice were genotyped for GR^{loxP} alleles as described above (experiment 1). To detect the presence of D1aCre transgene, amplification was obtained by 35 cycles (45 s at 95°C, 45 s at 60°C and 2 min at 72°C) using 5' GCC-TGC-ATT-ACC-GGT-CGA-TGC-AAC-GA 3' and 5' GTG-GCA-GAT-GGC-GCG-GCA-ACA-CCA-TT 3' primers. The size of the amplicons was 350 bp band.

Social defeat and interaction paradigms: Social defeat was performed as described (Berton, 2006). Six month-old CD1 breeder male mice were screened for their aggressiveness. GR mutants and their respective control littermates were subjected to 10 consecutive days of social defeat. Each defeat consisted of 5 min of physical interactions between the resident mouse (CD1) and the intruder (experimental mouse). The rotation schedule was set to exclude a repeated defeat by an already-encountered resident. Following this, the intruder remained for another 24 h in the resident's home cage that was partitioned in half by a perforated transparent polycarbonate, which allowed visual, auditory, and olfactory communication whilst preventing tactile contact. Undefeated mice were handled and rotated daily, and housed 2 per cage with the polycarbonate partition separating the cage in 2 halves.

Social interaction was performed 24 h after the last defeat (day 11). Undefeated and defeated mice were introduced for 150 s in an open-field (40cmx40cmx25cm) containing an empty perforated polycarbonate box ('no target' condition). Immediately after this, mice were rapidly removed and an unfamiliar CD1 mouse was placed in the box ('target' condition) and mice were re-exposed to the open-field for another 150 s. The time spent in the interaction zone surrounding the polycarbonate box was recorded and used as an index of social interaction.

Quantification of dopamine and DOPAC: Quantification of dopamine and DOPAC.

The procedure was performed by JP Tassin (GRAB team, NPS, IBPS). Briefly, animals were sacrificed by decapitation. Brains were rapidly dissected from the skull and frozen at - 12°C on the stage of a Leitz-Wetzlar microtome. Coronal brain sections (300µm thick) were cut and placed onto the refrigerated stage. Two DA terminal fields were assayed: the mPFC and the NAcc. For each structure, two or four tissue punches (1 mm diameter) from two consecutive sections were taken bilaterally and each side analysed separately for the NAcc. and mPFC, respectively. Tissue punches were immersed into a solution (50 µl/ punch) of 0.1 N $HClO_4$ containing $Na_2S_2O_5$ (0,5%), disrupted by sonication and centrifuged at 15000 g for 20 min. Aliquots (10µl) of supernatant were diluted with mobile phase and injected into a reverse-phase high pressure liquid chromatography system

consisting of a C18 column (HR-80 Catecholamine 80 x 4.6 mm, Thermo Scientific, USA) and a 0.1 M NaH_2PO_4 mobile phase containing 1-octanesulfonic acid (2.75 mM), triethylamine (0.25 mM), EDTA (0.1 mM), methanol (6 %) and adjusted to pH = 2.9 with phosphoric acid. Flow rate was set at 0.6/min by an ESA-580 pump. Electrochemical detection was performed with an ESA coulometric detector (Coulochem II 5100A, with a 5014B analytical cell; Eurosep, Cergy, France). The conditioning electrode was set at – 0.175 mV and the detecting electrode at + 0.175 mV, allowing a good signal-to-noise ratio. External standards were regularly injected to determine the elution times (9.8 and 27.2 min.) and the sensitivity (0.3 and 0.4 pg), for DOPAC and DA respectively.

Statistics: Data are presented as means ± S.E.M. Statistical analysis was carried out using three-way ANOVA followed by a Bonferroni's post hoc test when appropriate.

2) Results

Mice are territorial animals aggressing any conspecific intruder. Repeated social defeat, obtained by exposing an experimental animal to an aggressive and stronger resident, daily for ten days, leads to distinct persistent behavioural changes including increased anxiety and social aversion (Berton, 2006). Our research group, previously reported that the absence of GR in dopaminoceptive neurons selectively abolished the induced social aversion but not the induced anxiety. This change in behaviour was associated with a blockade of induced firing and bursting DA activity upon repeated social defeat and a blockade of induce DA release in the NAcc upon a unique aggression.

My aim was, in this experiment to measure whether changes in DA release could be assessed by measuring DA release in dopaminoceptive through the ratio of DOPAC/DA contents in tissue punches (Vezina,1992). This will be important to analyse, during my PhD thesis the consequences of GR gene inactivation, mediated by viral transduction, on dopaminergic tone.

Mice selectively deprived of GR in dopamine neurons (GR^{D1aCre} mice) and control littermates were subjected to daily social defeats by a new dominant encounter, during 5 min, for 10 consecutive days

(see Material and Methods section). The animals spent then the rest of the day in the cage of their aggressor, separated by a polycarbonate wall but exposed to olfactory, visual and auditive cues of the aggressor (Fig. 8A). Defeated animals were compared to undefeated littermates that followed a similar procedure, being placed in the cage of a new conspecific everyday but never exposed to physical aggression. On the day following the last aggression, we assessed social behaviour in undefeated and defeated animals. We recorded the time spent establishing social contacts with an unfamiliar mouse placed in a small polycarbonate cage, within an openfield. This interaction session lasted 150 sec. Interaction time was defined as the time spent in the interaction zone pictured in red in Fig. 8B. Both undefeated control and GR^{D1aCre} mice displayed social behaviour and spent more time in the interaction zone when an unknown mouse was placed in the polycarbonate box cage ("Target" in Fig. 8C). Both genotypes spent comparable amount of time performing social behaviour. It is to note that statistic analysis could not be performed due to the small number of animals but the differences observed were similar to previous results of our research group. Surprisingly, repeated defeat did not trigger any social avoidance in control littermates, in sharp contrast with what has been repeatedly observed by our team. Even more surprisingly, whereas a clear reduction in social avoidance has been repeatedly observed with GR^{D1aCre} mice, we observed in this experiment a social withdrawal in these mutant mice.

Assessment of dopamine release in the NAcc of GR^{D1aCre} and control littermates

Faced with the surprising results obtained concerning the induction of social avoidance by repeated social defeat in mutant and control animals, and before to repeat later this experiment when animals will be available, we decided to nevertheless measure dihydroxyphenyl-acetic acid (DOPAC) in the NAcc of undefeated animals. DOPAC is the first metabolite obtained from DA recaptured by dopamine neurons. Its amounts are therefore indicative of the amount of functional DA that hes been previously released. The measured quantity of DOPAC was normalized by the amount of DA contained in DA-cells to normalize for variations in the number of DA-cells present in the punches. The total amounts and DOPAC, DA and the ratio DOPAC/DA were similar in both GR^{D1aCre} mice and control littermates, suggesting that under basal conditions the DA neurons of both genotypes

release similar amounts of DA.

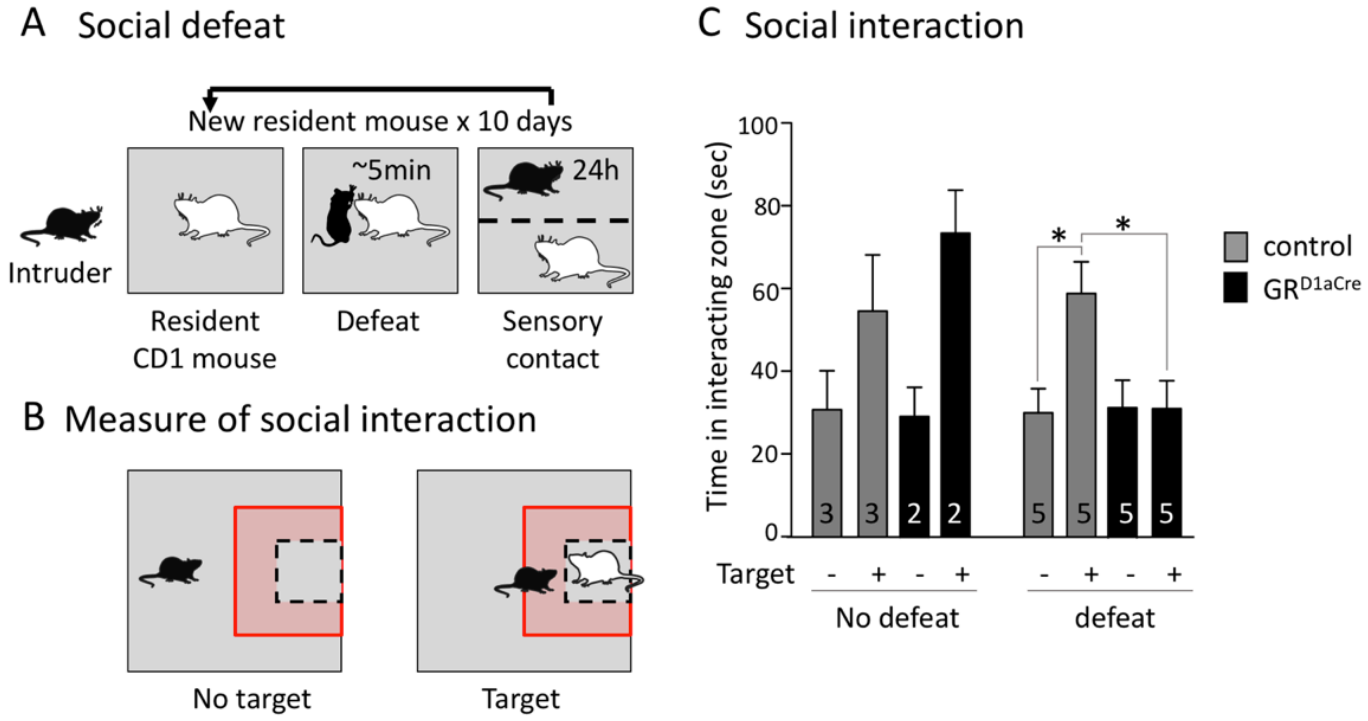

Fig 8. Repeated social defeat in GR^{D1aCre} mice and control littermates.

A) Procedure followed fo the repeated social defeat paradigm. The challenged mice (intruder) is pictured in black, the aggressor mice (resident), in white. Every day challenged mice are defeated by a new aggressor for 5 min and spend the rest of the day in the aggressor's cage, separated by a plastic wall. **B)** Measure of social interaction. Scheme of the experiment. Challenged mice are placed in an openfield containing an empty plastic box (left) for 150 Sec and then in the same arena with a new encounter in the plastic box (right) for 150 sec. The time spent in the interaction zone (red is quantified. **C)** Social interaction is expressed as time in the interaction zone. The treatment (defeated or no defeated), the presence of a target and the genotypes are indicated. (Three ways ANOVA: interaction defeat × target × group, $F_{1,11} = 11,14$ $P < 0.01$).

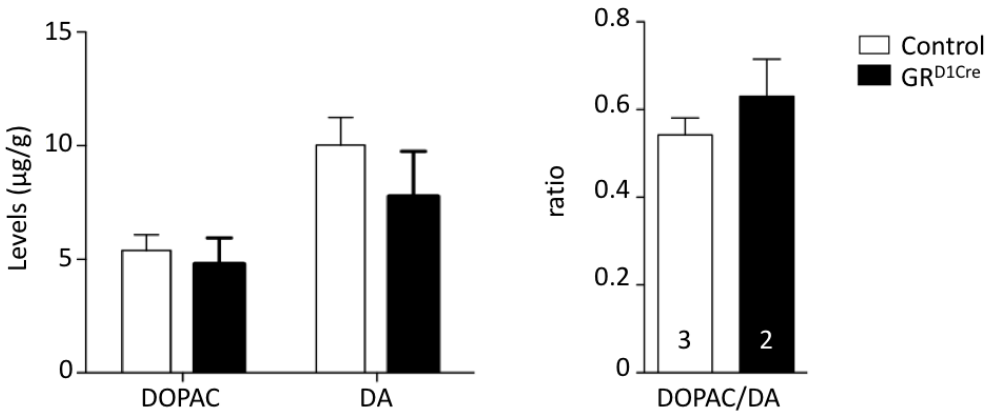

Fig 9. Measurement of DA release in the NAcc of GR^{D1aCre} mice and control littermates.

The total amounts of DOPAC and DA and the ration DOPAC/DA are presented.

Counting of the time spent in the interaction zone and the control corners

In order to count up the time spent in either the interaction area or in the control corners, we screened the video recordings on a 13,3-inch screen (28,6 x 17,9 cm), affixing to the latter, a plastic film that allowed us to draw the interest areas. All dimensions indicated below relate to measurements made on the basis of this construct. The interaction zone corresponds to the rectangle (2x4) immediately surrounding the polycarbonate box (1x2) in which is placed the social stimulus, the former exceeding the dimensions of the latter by 1 cm on of both sides in the direction of its width, and by 1,5 cm on the left side in the direction of its length. To define the two control corners, we split of the total area of the interaction zone ($8cm^2$), in order to obtain two squares of (2x2) dimensions each, and for which the cumulated areas ($2 \times 4cm^2$) amount to the area of the interaction zone.

3) Discussion

The results obtained are inconsistent with the data reported previously by the group (Barik, 2013). Although the results obtained with undefeated animals are in the range of the results repeatedly observed, we observed in defeated animals the opposite results than the ones usually obtained. Control mice did not developped social aversion, whereas mutant mice may have developed un unexpected social aversion. These results have first to be moderated by the fact that we used a very small number of animals compared to previous experiments. Several explanations could be hypothetized. First, the aggression could have been too mild in this set of experiments not allowing the appearance of enduring social withdrawal in the control. Second, these mice have been previously used for a set of behavioural tasks that needed a period of food restriction. Food deprivation constitutes a particularly strong stress. It may have had an impact on the subsequent behaviours observed during the tests. In coherence with this hypothesis, mice submitted to food deprivation showed a reversed phenotype in chronic social stress paradigm, switching from social aversion to social preference (Cabib 2000).

Since we observed an absence of social aversion in control defeated mice, we decided to restrict the analysis of DA release to the undefeated group. We did not observed difference in the DOPAC/DA

ratio between control and mutant animals, a result that again is limited by the very small number of animals.

V. Conclusion and perspectives

Histological examinations of $GR^{AAV\text{-}Cre}$ mice allow to confirm the viability of the protocol since we observe the disappearance of GR protein in targeted neurons of the NAcc. However, getting a larger deletion of GR is a crutial prerequisite to establish weither the GR in MSNs of the NAcc constitues a key mediator element for the manifestation of stress-related behaviours. So, the experiment will be done again, increasing the doses of injected virus (see above).

Meanwhile, the validation of an induced conditional mutant constitutes an advance to better understand the role of the GR relative to psychiatric pathologies mainly encountered in adulthood. Indeed, beyond the fact that we can target the GR gene invalidation more accurately, the constitutive mutation that characterizes GR^{D1aCre} model, may induces compensative mechanisms (vicariance) which potentialy limits the interpretation of resulting phenotypes. In such a case, behaviroual differences in adults can not be attributed exclusively to the disabled gene (to the lack of GR in our case).

Once identified the precise relevant structures in which the GR determines a significant modulation of the dopaminergic neurons of the VTA, leading to the specific phenotype found with GR^{D1aCre} mice relative to addictive and depression-like behaviours, it will be pertinent to study the underlying molecular mechanisms involved in such processes. As mentioned earlier, we know that GR recruits the chromatin remodeler BRG1 for almost the half of the genes whose it regulates the expression. This enzyme is a crutial element of the chromatine remodeling complex (CRC), whose activity allows to make accessible some ADN segments, leading thus to the regulation of genes expression. To study the role of the interaction between GR and BRG1, mice lacking of BRG1 in dopaminoceptive neurons ($BRG1^{D1aCre}$ mouse) have already been generated. Invalidation of BRG1 gene does not affect neuronal survival (Baranovski, unpublished). However, the absence of BRG1 abolishes the behavioural sensitization to cocaine, affects the conditioned place preference and blocks the

emergence of a social aversion after repeated attacks. This similarity of the phenotypes of $BRG1^{D1aCre}$ and GR^{D1aCre} mice suggests that the GR in dopaminoceptive neurons recruits BRG1 to control the expression of genes involved in addictive and depressive-like behaviours. As before, we cannot distinguish between the specific role of BRG1 in eather the nucleus accumbens or the prefrontal cortex. The results on the GR mutant mice, would allow a more precise targeting of BRG1 invalidation, permitting to validate this mechanistic hypothesis.

Finaly, if the behavioural responses to cocaine consumption and chronic social stress is mediated by the stress response, several studies pointed out the existence of a correlation between social rank in various species and differences in stress response, a main mediator of a wide range of health problems (Sapolsky, 2004, Sapolsky, 2005).

Mice are social animals living in structures of 4 to 15 adult members that share territorial defense and exhibit behaviors (interactions, vocal communication, cooperation) that are the hallmarks of sociability (Berry, 1992, Berry, 2015). House mouse populations are capable of assuming a variety of social organizations (ranging from exclusive male territoriality to hierarchical groups) depending on different socio-ecological conditions (Berry, 1981). The actual social organization employed has been traditionally thought to reflect variations in the degrees of inter-male aggressiveness; male mice compete for establishing and holding a territory and/or establishing social rank, in order to mate with females, since reproduction is largely confined to dominant or exclusively territorial males. Such groups of mice establish dominance hierarchies, resulting from aggressive contests and social rank strongly conditions a wide range of behaviors.

Hierarchy is therefore an essential organizing principle for understanding social behavior and individuation. The physiological and molecular mechanisms underlying the establishment of a hierarchy remain however poorly known. Agonistic interactions in mice evoke a dopaminergic response, usually associated with an increase in aggression, and induced glucocorticoids release. Also, lower glucocorticoid levels are observed in dominants of many species, including rodents (Gesquiere, 2011). Stress response could therefore shape the behavioral trajectories of individuals, through social and environmental challenges, during the establishment of social ranking in a colony.

Alternatively, social status in an established colony could condition stress coping and exposure to stress leading to distinct sensitivity to the development of stress-related brain pathologies.

Futur research will thus have to further investigate the role of GR in dopaminoceptive neurons in shaping social behaviors and in the establishment of social ranking, in order to better understand the interaction of these processes in determining pathological or efficient adaptive behavioural responses.

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