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Role of G protein coupled inwardly rectifier K^+ channels (GIRK) on the neurobiology of depression.

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ESKERRAK

Gurasoei, ikastea formatzea eta aldi berean nire bizitzako beste zenbait arlotan aurrera egitea posible egin dutelako. MILA ESKER

Luisari, ikerkuntza zer den ulertarazteagatik, eta ikerkuntzatik aparte erakutsitako beste zenbait gauzengatik.

Mariari, nahiz eta ikuspuntu ezberdinak izan, zuk uste baino gehiago irakatsi didazulako. Nirekin izan duzun pazientziagatik, elkarrekin ikasi eta hobetu dugunagatik.

Asierri, lagun isilari, esperimentu egun luze horietan hitz egiten utzi izanagatik, aguantatzeagatik.

Teresa eta Cristinari, tesian ondo pasarazi didazuelako, gauzak ondo atera edo ez norbaitekin hitz egitea behar izan dudanean hor egon zaretelako. Irakatsi eta lagundutako guztiagatik.

Rosiri

Isabel eta JA-ri.

Laborategira etorri berri diren Cristina, Ainhoa eta Nereari.

Mariri suizak hitz egiten ez duena zuk hitz egiteagatik, beti ere hitz egiteko prest egoteagatik.

Departamentuko beste kideei.

Departamentuko idazkariari, Itziarri.

Douglas-i.

Familiari, ezagutza zientifikoan laguntza handikoak izan ez badira ere beste gauza askotan laguntzeagatik.

Ziharari oporrak opor ez zirenean aurpegi ona jartzeagatik. Nahiz eta protestaka, teknologiarekin lagundu izanagatik. Enara zaintzeagatik tesia egin behar nuenean. Zuk dakizun guztiagatik (+GD). ESKERRIK ASKO.

“En todas las cosas humanas, cuando se examinan de cerca, se demuestra que no pueden apartarse los obstáculos sin que de ellos surjan otros”

(Maquiavelo)

“La verdadera sabiduría está en reconocer la propia ignorancia”

(Socrates)

“El mayor de todos los misterios es el hombre”

(Socrates)

Enarari

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INDEX

ABBREVIATION LIST

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5-HIIA	5-hydroxyindolacetic acid
5HT	serotonine
5-HTTLPR	5HTT gene linked polimorphic region
ANOVA	analysis of the variance
BDNF	brain derived neurotrophic fator
CNS	central nervous systems
COMT	catechol-O-methyl transferasa
DA	dopamine
DMI	desipramine
DRN	dorsal raphe nucleus
ED_{50}	effective dose 50
E_{max}	maximum effect
GABA	gamma-aminobutyric acid
GIRK	G- protein coupled inwardly rectifying potassium channels
$GIRK2^{-/-}$	homozygous animal
$GIRK2^{+/-}$	heterozygous animal
GPCR	G- protein coupled receptor
HPLC	high performance liquid chromatography
ICV	intracerebroventricular
KIR	inwardly rectifying potassium channels
LC	locus coeruleus
MAO-A	monoamine oxidase A

mRNA	messenger ribonucleic acid
NE	noradrenaline
NMDA	N- metil D- aspartato
PFC	prefrontal cortex
TST	tail suspension test
WT	wild type animal

1. INTRODUCTION

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1.1 . DEPRESSION

Major depression is a recurrent or chronic mood disorder with impaired psychosocial function, and an increased tendency of relapse for patients who fail to reach remission. In industrialized societies, it affects 15% of the population being the age range of affected people between 18 – 44 years (Kessler *et al*., 2007). The World Health Organitation has predicted that major depression will become a key cause of illness-induced disability by the year 2010, followed only by ischemic heart disease (Murray and Lopez, 1997). Major depression symptomatology is known as "depressive syndrome" and includes a long-lasting depressed mood and loss of interest and/or pleasure -the core symptoms-, feelings of guilt, anxiety, and recurrent thoughts of death and suicide. This symtomatology is associated with marked personal social and economic morbidity, and creates significant demands on service providers in terms of workload, resulting in an enormous social cost. Depression is particularly common in women, in patients with chronic medical disorders such as persistent insomnia, and patients who have experienced stressful life events, functional decline, or social isolation.

The essential feature of major depression is depressed mood and anhedonia (loss of interest and pleasure) which is associated with changes in quotidian activities such as working, studying, sleeping or

eating. The criteria for major depression diagnosis defined by the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV) of the American Psychiatric Association establishes the presence of five (or more) of the following nine symptoms nearly every day during the same 2 week period and represent a change from previous functioning. These symptoms are not due to direct physiological effects of a substance or a general medical condition.

- Depressed mood most of the day, nearly every day, as indicated by either subjective report (e.g., feels sad or empty) or observation made by others (e.g., appears tearful).

- Markedly diminished interest or pleasure in all, or almost all, activities most of the day, nearly every day (as indicated by either subjective account or observation made by others).

- Significant weight loss when not dieting or weight gain (e.g., a change of more than 5% of body weight in a month), or decrease or increase in appetite nearly every day.

- Insomnia or hypersomnia nearly every day.

- Psychomotor agitation or retardation nearly every day (observable by others, not merely subjective feelings of restlessness or being slowed down).

- Fatigue or loss of energy nearly every day.

- Feelings of worthlessness or excessive or inappropriate guilt (which may be delusional) nearly every day (not merely self-reproach or guilt about being sick).

- Diminished ability to think or concentrate, or indecisiveness, nearly every day (either by subjective account or as observed by others).

- Recurrent thoughts of death (not just fear of dying), recurrent suicidal ideation without a specific plan, or a suicide attempt or a specific plan for committing suicide.

1.1.1. Major depression: Ethiopathology

Although the disorder is thought to be the outcome of gene-environmental interactions, the causative genes and environmental factors underlying depression remain to be identified (Lee *et al*., 2010). On the other hand, there are many hypotheses to explain the molecular mechanism of depression. Many of them are related, but a definite ethiopathogenic mechanism is still not identified. Over the last fifty years, many attempts to understand the ethiopathology of depression and the mechanisms responsible for the treatment effect have been focused on brain monoamines. The action of reserpine in causing depression by depleting catecholamines and the reversal of these effects by triciclic

antidepressants formed the basis of the catecholamine hypothesis of depression (Schildkraut, 1965). The monoamine hypothesis proposes that the underlying biological or neuroanatomical basis of depression is a deficiency or imbalance of central monoamine neurotransmission (Schildkraut, 1965; Van Praag, 1982). This hypothesis was supported by the effects of the antidepressant drugs on these neurotransmitter systems. In fact, the first two kinds of antidepressants monoamine oxidase inhibitors and triciclic antidepressants enhance the synaptic availability of monoamines. In addition, several data from plasma, cerebro spinal fluid and brain of patients affected with depression have revealed an inappropriate function of noradrenaline (NE), serotonin (5-HT) and dopamine (DA) in depressive patients.

Thus, 3-methoxy-4-hydroxyphenylglycol (MHPG), the major metabolite of NE is decreased in plasma and urine of patients affected of depression (Schatzberg *et al*., 1995; Lambert *et al*., 2000). Studies in postmorten brain tissue have shown that in depressive subjects the level of NE transporter is reduced (Klimek *et al*., 1997) and the tyrosine hydroxylase and the α_2-adrenoceptors are elevated in the Locus coeruleus (LC) (Meng-Yang *et al*., 1999; Ordway *et al*., 2003). In addition, β-adrenergic receptor binding is increased in the frontal cortex of suicide victims (Mann *et al*., 1986).

In the case of 5-HT, low concentrations of its major metabolite, 5-hydroxyindoleacetic acid (5-HIIA), in the cerebrospinal fluid of suicide victims have been found (Asberg *et al*., 1976). Platelet 5-HT and plasma

tryptophan levels are reduced in unmedical depressed patients but reach control levels after two months of imipramine treatment (Quintana 1992). However, the levels of 5-HIIA in cerebrospinal fluid do not correlate with the severity of depression, and remain decreased after clinical recovery (Coppen *et al*., 1972). A reduction of 5-HT transporter sites has been found in several areas of postmorten brain of suicide victims (Mann *et al*., 2000). 5-HT receptors are also altered in depression, for example, 5-HT_2 receptors are upregulated in the frontal cortex of suicide victims (Arora and Meltzer 1989; Leonar *et al*., 2000; Mann *et al*., 1986). 5-HT_{1A} receptor is abnormally decreased in the depressive phase of familiar mood disorders in multiple brain regions being the magnitude of the reduction most prominent in the midbrain raphe (Wayne *et al*., 1999).

The DA hypothesis of depression (Reul *et al*., 1993) emerged third among the monoamine hypothesis. It was shown that the DA turnover is decreased, as indicated by lower levels of homovanillic acid, a mayor DA metabolite, in the cerebrospinal fluid (Kapur *et al*., 1992) and plasma in depressed patients when compare to undepreassed patients (Lambert *et al*., 2000). Moreover, there is a negative correlation between the levels of this metabolite and the severity of depression (Lambert, 2000). However, homovanillic acid levels in the cerebrospinal fluid are not affected by antidepressant treatment (Bowden *et al*., 1985). Plasma DA levels also correlates with the score of the severity of depression (Hammer and Diamond, 1996). D_2/D_3 receptors are increased in basal ganglia and striatum (D'Haenen and Bossuyt., 1994; Shah *et al*., 1997) and dopaminergic transporter is decreased in depressed patients (Klimek

et al 2002; Meyer *et al*., 2001). Consistently, it has been shown that a deregulation of dopamine D_2 receptor signalling results in depression-like behaviors in experimental animals (Park *et al*., 2000), and that neuronal nitric oxide synthase knockout mice with altered dopamine D_1 receptor signalling exhibit decreased depression-related behaviors (Tanda *et al*., 2009). Depression and related mood disorders are accompanied by abnormalities in dopaminergic transmission in the nucleus accumbens and ventral tegmental area, regions that are core parts of the brain reward circuit (Nestler *et al*., 2006). It is well established that depressed patients have difficulties in the expression of pleasure and acquisition of motivation, which are mainly governed by a normal accumbens and ventral tegmental area DA circuit.

Finally, clinical studies have been performed to test the monoaminergic hypothesis. Thus, clinical studies have been conducted in subjects diagnosed of depression and treated with alpha-methylparatyrosine which causes rapid depletion of brain catecholamines, tryptophan depletion or para-chlorophenylalanine which depletes 5-HT (Charney, 1998). A recent meta-analysis of these monoamine depletion studies by Ruhé *et al*., (2007) concludes that, monoamine depletion depreases mood in subjects with familiar history of major depressive disorder and in drug-free patients with major depressive disorder in remission but mood was not modified in healthy people. Authors conclude that depletion studies do not give us the cause of depression. However they link NE, DA and 5HT with depression and may be useful to identify vulnerable subjects (Riedel *et al*., 2007).

In its original form, the monoamine hypothesis does not provide a complete explanation for the actions of antidepressants, and the etiopathology of depression itself remains unknown (Racagni *et al*., 2010). Thus, although nowadays most available antidepressant marked drugs, both acutely and chronically, increase levels of NE, DA or/and 5-HT by inhibiting their degradation or their uptake or acting as a monoamine receptor agonists. They need as long as 4-8 weeks to develop their therapeutic effects and each drug is efficacious in only 60-70 % of patients (Lanni *et al*., 2009). The hypothesis neither accounts for issues, such as why antidepressants are effective in anxiety disorders like phobia (Sheehan *et al*., 1993) or why drugs that enhance 5-HT or NE transmission are not necessarily effective in depression. On the other hand, 5-HT reuptake inhibitors are effective antidepressants (Lanni *et al*., 2009) and drugs such tianeptine that increases 5-HT uptake in the brain have an antidepressant effect (Loo *et al*., 1999). Therefore, other neurotransmitters, neuropeptides and intracellular signal transduction have been implicated in depression.

Also, abnormal regulation of sleep/wake cycles, body temperature, blood pressure, and various endocrine functions under the control of circadian clock are prominent symptoms of mood disorders (Bell-Pedersen *et al*., 2005; Wasielewski 2001). However, molecular mechanisms underlying the link are still largely unknown. Recently, interesting observations have been made in the mutant mouse that has a deletion of 19th exon of Clock gene, a core component of molecular

clock. The mouse exhibits hyperactive ventral tegmental area dopaminergic neurons and behavioral phenotypes that are considered a reminiscence of behavior observed in bipolar disorder patients (McClung *et al*., 2005; Roybal *et al*., 2007).

Potential links between circadian rhythm and the monoamine system are also reported. The synthesis and/or secretion of monoamine neurotransmitters and the function of their receptors are under the influence of circadian rhythms. The circadian rhythmicity of DA transporter and tyrosine hydroxylase expression in dopaminergic neurons is also disrupted when the suprachiasmatic nucleus of the hypothalamus, the central part of endogenous clock, is damaged (Sleipness *et al*., 2007).

The cholinergic hypothesis of depression rests on the evidence that cholinomimetics provoke a depressive mood state in patients with affective disorder or in subjects with a family history of affective illness (Sitaram, 1982). Increased cholinergic activity decreases rapid eye movement sleep latency and increases adrenocorticotropic hormone and cortisol levels in depressed patients (Riemann and Berger 1992). As proposed by Janowsky *et al*., (1972) depression could be related to a functional insufficient noradrenergic transmission in combination with cholinergic dominance. The existence of an imbalance of the colinergic-adrenergic systems was confirmed by several binding and functional studies which showed that prolonged activation of central and peripheral cholinergic pathways reduced noradrenergic function (Hollingswoth 1988; Hollingswoth and Smith 1989; Olmos *et al*., 1993). Nevertheless,

even though many antidepressants block muscarinic receptors, the majority of the newer drugs do not, and there is no consistent evidence for biochemical alterations in the acetylcholine system in depression (Harrot *et al*., 2001).

Given the prominence of anxiety symptoms in depression and the fact that anxiety is related with the gamma-aminobutyric acid (GABA) system some studies have been carried out to assess its dysfunction in depression. It has been found that the levels of GABA are significantly decreased in cerebrospinal fluid and plasma of depressed patients while GABA uptake binding site, and GABA receptors were unaltered (see Schechter *et al*., 2005). On the other hand, the excitatory amino acid system has also been implicated in depression. Excitatory amino acid receptor antagonists are active in "behavioral despair" procedures, such as the forced swim (Porsolt *et al*., 1977) and tail suspension test (TST) (Steru *et al*., 1985). Following chronic administration of imipramine (15 mg/kg, i.p. for two weeks) to mice, significant changes were detected in radioligand binding to N-meti D- aspartato (NMDA) receptors in cerebral cortex (but not in other brain regions such as the hippocampus and striatum) as well as producing regional alterations in mRNA expression that encodes multiple NMDA receptor subunits (Skolnick *et al*., 1996, Boyer *et al*., 1998).

Chronic stress is an important component in depression even though it does not seem to function as a necessary or sufficient factor. From this point of view, the hypothalamic-pituitary-adrenal axis, a core

neuroendocrine circuit for managing stress in the body, has been a topic of interest in depression research (De Kloet *et al*., 2005). A marked hyperactivity of hypothalamic-pituitary-adrenal axis is evident in approximately half of depressed patients and chronic treatment with antidepressants often reverses this phenomenon (Parker *et al*., 2003; Raison *et al*., 2003). A reduced number of corticotropin releasing factor receptors in the frontal cortex has been found in depression (Nemeroff *et al*., 1988). Animal studies suggest that chronic treatment with antidepressants contribute to the recovery of the abnormal function of the hippocampus by increasing neurogenesis (Scaccianoce *et al*., 2006; Tsankova *et al*., 2006). Chronic stress augmentes mRNA expression that encodes α-adrenoceptors (Flugge *et al*, 2003). Studies using corticotropin releasing factor receptor antisense administration, non-peptide corticotropin releasing factor receptor antagonists and genetically manipulated animals have shown that these manipulations have anxiolytic-like effects (Habib *et al*., 2000; Keck *et al*., 2001; Liebsch *et al*., 1999; Timpl *et al*., 1998). Moreover antidepressants may, functionally antagonize corticotropin releasing factor neuronal effects in the LC (Valentino *et al*., 1990).

Long-term stress appears to reduce the expression level of brain derived neurotrophic factor (BDNF) in the hippocampus (Monteggia *et al*., 2007). Also, in a post-mortem study of depressed patients, a reduction in BDNF expression was reported (Karege *et al*., 2005). In addition, polymorphisms of BDNF gene are associated with neuroticism, a personality trait linked to increased susceptibility to depression (Sen *et*

al., 2003). A family-based association study showed that polymorphisms in BDNF genes are related to bipolar disorders (Neves-Pereira *et al*., 2002). Conversly, a chronic treatment with antidepressants not only enhances the BDNF levels but also increases the stress resistance in animals (Duman *et al*., 2006; Shirayama *et al*., 2002). On the other hand, neuropeptide Y one of the most abundant peptides found within the mammalian brain must be considered in the pathophysiology of depression. There is no clear clinical evidence for a role of neuropeptide Y in depression, but this peptide co-localizes within monoamine systems (Everitt *et al*., 1984; Pau *et al*., 1998). Stress and desipramine affect neuropeptide Y mRNA levels and these effects depend upon corticosterone fluctuations (Makino *et al*., 2000). Furthermore, several alterations on the postsynaptic level, in signal transduction and in gene transcription, have been implicated in depression (Duman *et al*., 1997; Holsboer *et al*., 2000). Evidence is accumulating on the effect of antidepressants on protein phosphorylation, and on neurotrophic factors, which play a critical role in the survival and function of mature neurons in adult animals. However, it has to be emphasized that several neurotrophic factors and other target genes are likely to be involved in the ethiology and treatment of affective illness.

Functional changes in brain regions such as prefrontal cortex (PFC) and cingulate cortex, hippocampus, striatum, amygdala, and thalamus are correlated with depression (Drevets *et al*., 2001). The neocortex and hippocampus also appear to play critical roles in the symptoms related to the cognitive deficits that are prevalent in depressed

patients (Dranovsky *et al*., 2006), and the nucleus accumbens and amygdala seem to be core regions for anhedonia and emotional memory-related symptoms (Schlaepfer *et al*., 2008; Phelps *et al*., 2005). Thus, extensive studies have led to a variety of hypotheses for the molecular mechanism of depression, but a definite pathogenic mechanism has yet to be defined.

The genetic contribution to the manifestation of depression has been estimated as 40-50% (Fava and Kendler, 2000) However, combinations of multiple genetic factors may be involved in the development of depression, because a defect in a single gene usually fails to induce the expression of multifaceted symptoms of depression (Burmeister, 1999). Also, various non-genetic factors such as stress, affective trauma, viral infection, and neuronal developmental abnormalities increase the complexity of the pathogenesis of the disease. As a way to discovering genes predisposing depression, geneticists have long been searching for gene variants that play a role in the response to life stresses. Stress is a critical environmental factor for the onset of depression, which would be an example of 'gene-environment interaction' whereby an environmental factor is filtered through the activity of a gene to confer differential susceptibility to depression among individuals. In particular, a short variant of 5- HTTLPR (5-HTT gene-linked polymorphic region), appears to be associated with repressed transcriptional activity of the promoter, decreased 5-HT transporter expression, and decreased 5-HT uptake when compared with a long variant of 5-HTTLPR (Lesch *et al*., 1996). Significantly, genetic studies

have shown that these polymorphisms are associated with major depressive disorder in humans (Ogilvie *et al*., 1996).

1.1.2. Treatment

All antidepressant drugs now in use modulate monoamine neurotransmission and are similar in terms of efficacy and delay in the onset of the antidepressant effect. They take four to eight weeks to exert their effects and each drug is efficacious in only 60-70% of patients. Compounds that exert antidepressant action enhance the synaptic availability of monoamines by mechanisms that include: inhibition of enzymes that break down synaptic 5-HT, NE and DA or blockage of monoamine uptake. In addition some new antidepressants also block monoamine receptors such as α_2-adrenoceptors and 5-HT_{2C} receptors.

Monoamine oxidase inhibitors were the first group of antidepressant agents introduced 50 years ago (Lopez-Munoz *et al*., 2007). However, monoamine oxidase inhibitors have limitations related to the risk for hypertensive crisis and toxicity in overdose (Baumbacher and Hansen, 1992). Triciclic antidepressants are efficient in encouraging depressive symptoms but the anticholinergic effects and the toxicity in overdose make them not safe for the treatment of depression (Nutt, 2005; Thanacoody and Thomas, 2005). Due to the adverse effects of triciclic antidepressant and monoamine oxidase inhibitors, several drugs with safer profile and similar efficacy have been developed. Most of them

block the monoamine reuptake sites increasing noradrenergic, serotonergic or dopaminergic transmission:

- Selective 5-HT reuptake inhibitors such as fluvoxamine, fluoxetine, citalopram, paroxetine or sertraline.

- 5-HT-NE reuptake inhibitors such as venlafaxine and duloxetine.

- NE reuptake inhibitors such as reboxetine.

- NE-DA reuptake inhibitors. So far, only bupropion has been demonstrated to be effective in depression (Meyer *et al*., 2002).

- Recently a new antidepressant wich is a melatonergic receptor agonist and 5-HT_{2C} receptor antagonist has been comercialized. Agomelatine.

Although selective 5-HT, NE or DA agents can produce an antidepressant action on their own, it is generally accepted that the combination of at least two effects can produce greater therapeutic results. Moreover, to improve the efficacy of antidepressant treatment, triple reuptake inhibitors (5-HT, NE and DA) are currently being developed (Guiard *et al*, 2008). Approximately, 70-80% of monoamine oxidase needs to be inhibited, before an antidepressant action can occur with inhibition of this enzyme (Zimmer 1990). Selective 5-HT, NE and DA agents need an 80% occupancy of uptake transporters before an

antidepressant action (Blier, 2009). For example, it has been shown that the minimal effective dose of venlafaxine inhibits 80% of 5-HT transporters (Meyer *et al*., 2004).

Binding assays show that most antidepressant blocks preferentially 5-HT, or NE transporter. As 5-HT, NE and DA systems are interconnected to each other the three systems may be directly or indirectly modulated by antidepressants. Selective 5-HT reuptake inhibitors such as fluoxetine increases not only 5-HT but also NE and DA extracellular levels (Bymaster *et al*., 2002; Gobert *et al*., 1997; Jordan et al., 1994). Fluoxetine also inhibits the firing rate of NE neurons (Miguelez *et al*., 2009). Moreover other studies also show that neuron sensitivity to antidepressant is also altered after dopaminergic degeneration; fluoxetine was less effective on noradrenergic neurons (Miguelez *et al*., 2010). However, although this interaction may help to understand the effects of antidepressants, the mechanisms underlying the action of antidepressant drugs are not completely understood. It is now believed that an adaptation of downstream events, including lasting changes in gene expression by chronic treatment underline the antidepressant efficacy.

From the clinical point of view antidepressant medications represent the best established treatment for major depressive disorder and are among the most widely prescribed medications worldwide (Ioannidis, 2008). However, several problems remain unsolved as the delayed onset of antidepressant action, effectiveness in refractory patients, treatment

remission (recovery, relapse and recurrence) and side effect profiles. In addition, recently the efficacy of the antidepressant treatment has been a matter of controversy in two meta-analysis. The first one concludes that the difference in antidepressant efficacy between antidepressants and placebo is only evidenced in severe depression and that it is relatively small (Kirsch *et al*., 2008). The second one concludes that the efficacy in severe depression is substantial (Fournier *et al*., 2010). A Systematic review of published and unpublished clinical studies of antidepressants showed that antidepressant efficacy was less than previously published (Turner *et al*., 2008).

1.2. MONOAMINERGIC SYSTEMS

1.2.1. Monoamine turnover, synthesis, release, reuptake and metabolism

Monoamines are neurotransmitters that contain one amino group that is connected to an aromatic ring by a two-carbon chain (-CH_2-CH_2-). All monoamines are derived from aromatic amino acids such as tyrosine and tryptophan. The former gives rise to NE, adrenaline and DA, known as catecholamines (Fig. 1).

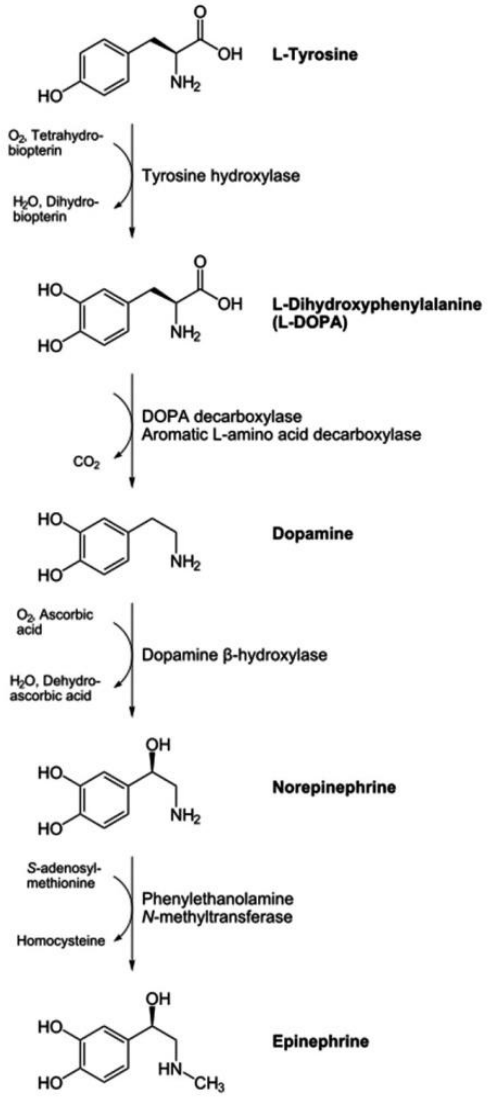

Figure 1. Noradrenaline synthesis. L-tyrosine is the precursor of all catecholamines .

After the synthesis, monoamines are stored in vesicles inside the synaptic buttom. When a nerve impulse arrives at a serotonergic or noradrenaline nerve terminal the neurotransmitter is released from the synaptic vesicle into the synaptic cleft. Neurotransmitter molecules bind to their specific receptors on the post-synaptic membrane and the nerve impulse is propagated or inhibited, depending on the receptor type. 5-HT and NE molecules are then released from their receptors and taken back into the nerve terminal via either the serotonin or NE transporter. The neurotransmitters are degraded by the monoamine oxidase A and B (MAO-A; MAO-B) or catechol-O-methyltransferase (COMT), these enzymes are found in both the synaptic cleft and the nerve terminal (Fig. 2).

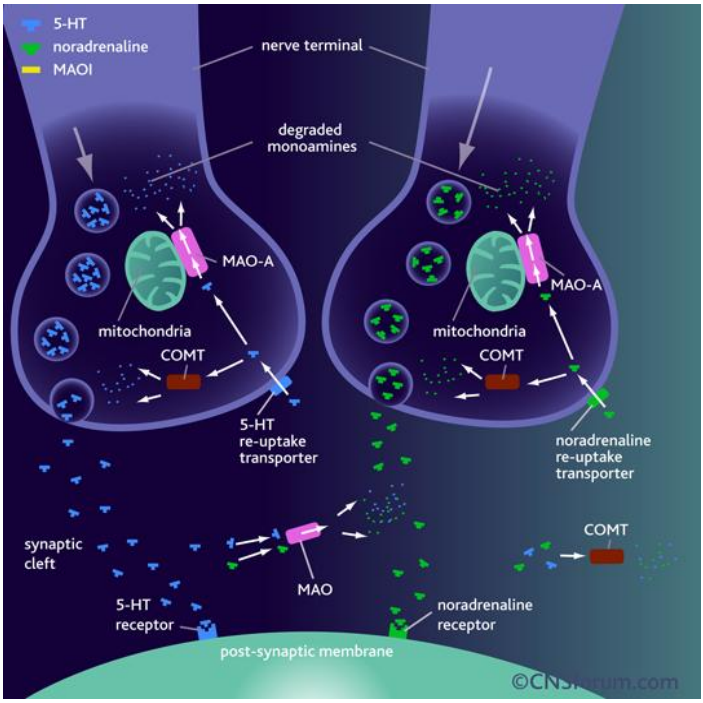

Figure 2. Normal processes of noradrenergic neurotransmission. Briefly, monoamines are synthesized and stored in vesicles in the synaptic buttom. Together with the nerve impulse, neurotransmitters are released in the synaptic cleft were interact with postsynaptic receptors. Then, monoamines are metabolized or reuptake by the transporter. Taken from CNSForum.com.

Release, reuptake, and recycling of neurotransmitters at the synapse are regulated by neurotransmitter transport systems. The monoamine transporters are proteins with 12 transmembrane alpha-helices with intracellularly oriented amino and carboxy termini (Fig. 3). These proteins act also as co-transporters of sodium and chloride ions (Kavanaugh, 1998). Transporters use the cellular sodium gradient that is maintained by Na^+/K^+-ATPase. Two sodium ions and one chloride ion are co-transported with each positively charged monoamine molecule (Sonders and Amara, 1996).

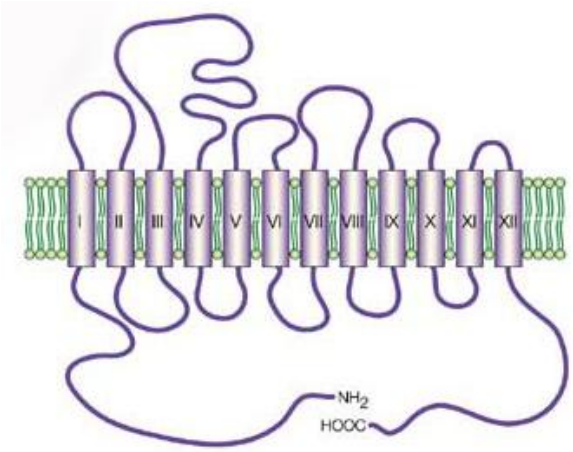

Figure 3. Monoamine transporter. Proposed topology of monoamine transporter depicting 12 transmembrane domains connected by intracellular and extracellular loops. Taken from CNSForum.com.

1.2.2. Adrenergic Receptors

Adrenoceptors can be defined as the cell membrane receptors, belonging to the seven transmembrane spanning families of G-protein coupled receptors, which respond to the physiological agonist NE and adrenaline by producing a response in the cell. Adrenoceptors can be divided into three broad categories: α_1, α_2 and β (Docherty, 1998). Currently, three subtypes have been described for each receptor: α_{1A}, α_{1B}, α_{1D}, α_{2A}, α_{2B}, α_{2C}, β_1, β_2 and β_3 (Bylund *et al*., 1994). The α_1 and β-receptors are thought to exist primarily at postsynaptic sites, whereas α_2-receptors exist both pre- and postsinaptically.

In vitro autoradiographic techniques have demonstrated wide distribution of α_1- adrenoceptors not only in the thalamus, cortex and hippocampus (Palacios *et al*., 1987) but also in the LC and dorsal raphe nucleus (DRN) (Chamba *et al*., 1991; Gallager and Aghajanian, 1976; Stone *et al*., 2004). *In situ* hybridation studies have reported high levels of α_1 mRNA in regions of the olfactory system, hypothalamus, thalamus, amygdala, dorsal and medial raphe nuclei, cerebral cortex, hippocampus, motor nuclei of the brainstem, inferior olivary complex and spinal cord (Day *et al*., 1997). In the LC, α_1-adrenoceptor and its mRNA are present and may be involved in the regulation of neuron activity (Osborne *et al*., 2002; Pudovkina and Westerink, 2005). In addition to the activation of α_1-adrenoceptor by NE, a recent study has provided evidence that DA may also play a role in the activation of LC neurons (Lin *et al*., 2008). It has been proposed that this receptor may be located presynaptically on

noradrenergic terminal/dendrites in the LC (Pudovkina *et al*., 2001), but more recent studies have also documented presynaptic effects of α_1-adrenoceptor stimulation on the release of both NE and DA (Pudovkina and Westerink, 2005).

The α_2-adrenoceptor shows heterogeneous anatomical distribution with high levels in the neocortex, ventral hypothalamus, hippocampus and some thalamic nuclei, LC, basal ganglia, cerebellum and amygdala, between others (Pascual *et al*., 1992; Pazos *et al*., 1988). The distribution of mRNA α_2-receptors is pronounced in the LC, brain stem, cerebral cortex, septum, hypothalamus, hippocampus and amygdale (Scheinin *et al.*, 1994). In the LC, α_2-adrenoceptors are mainly α_{2A} and α_{2C} (Osborne *et al*., 2002; Sastre and Garcia-Sevilla, 1994a) and are located pre or postsinaptically in noradrenergic neurons (Cedarbaum and Aghajanian, 1976) or in presinaptically on non-noradrenergic terminals (Anden and Grabowska, 1976; Feuerstein *et al*., 1993).

1.2.3. Noradrenergic System: The Locus Coeruleus

Neuromodulators are released by neurons to modify the cellular properties of target neurons and the efficacy of their synaptic transmission. The main neuromodulators in the brain include 5-HT and acetylcholine, as well as the catecholamines, DA and NE. The differential impacts of these systems on cognitive function results largely from the differential innervations of the forebrain and from the afferent control of activity in the respective nuclei. However, any attempt to delineate the relative or specific roles of these systems in behavior and cognition, must take into account the reciprocal connections among all of these nucleus, as well as their common regulation through a descending projection from the PFC (See table 1 to observe timeline of LC).

Table 1. Milestones in research on the LC noradrenergic system, take from Susan J. Sara , (2009*)*

YEAR	DISCOVERY	REFERENCE
1812	Wenzel and Wenzel provide an anatomical description of the LC.	Wenzel, J. & Wenzel, K. De Penitiori Structura Cerebri Hominis et Brutorum (Cotta,Tübingen,Germany,1812)
1949	Moruzzi and Magoun describe the ascending reticular activating system.	Moruzzi, G. & Magoun, H. W. Brain stem reticular formation and activation of the EEG. Electroencephalogr. Clin. Neurophysiol. 1, 455–473 (1949).
1964	Brainstem catecholamine nuclei are chemically identified.	Dahlstom, A. & Fuxe, L. Evidence for the existence of monoamine-containing neurons in the central nervous system.Demonstration of monoamines in the cell bodies of brain stem neurons. Acta Physiol. Scand.Suppl. 232, 1–55 (1964).
1969	The LC is shown to have a role in the regulation of sleep and vigilance.	Roussel, B., Buguet, A., Bobillier, P. & Jouvet, M. [Locus ceruleus, paradoxal sleep, and cerebral noradrenaline]. C. R. Seances Soc. Biol. Fil. 161, 2537–2541 (1967).
1972	Kety publishes his hyphotesis concerning the role of the noradrenergic system in learning and memory.	Kety, S. S. The possible role of the adrenergic systems of the cortex in learning. Res. Publ. Assoc. Res. Nerv. Ment. Dis. 50, 376–389 (1972).
1975	NE is shown to modulate the signal/ noise ratio in the	Foote, S. L., Freedman, R. & Oliver, A. P. Effects of putative neurotransmitters on neuronal activity

	monkey auditory system. The LC is shown to have widespread efferent projections.	in monkey auditory cortex. Brain Res. 86, 229–242. (1975). Swanson, L. & Hartmann, B. The central adrenergic system. An immunofluorescence study of the location of cell bodies and their efferent connections in the rat utilizing dopamine-beta-hydroxylase as a marker. J. Comp. Neurol. 163, 467–505 (1975).
1978	The afferent projections to the LC are found. The role of the LC noradrenaline system in attention and reward is described. The role of the LC in morphine addiction is described.	Mason, S. & Iversen, S. Reward, attention and the dorsal noradrenergic bundle. Brain Res. 150, 135–148 (1978). Aghajanian, G. V. Tolerance of locus coeruleus neurones to morphine and suppression of withdrawal response by clonidine. Nature 276, 186–188 (1978).
1981	In vivo recordings of LC neurons in behaving rats are made and the role of these neurons in vigilance is described.	Aston-Jones, G. & Bloom, F. Activity of norepinephrine containing locus coeruleus neurons in behaving rats anticipates fluctuations in the sleep-waking cycle. J. Neurosci. 1, 876–886 (1981).
1983	The first description of frequency-independent long-lasting potentiation by NE is made.	Neuman, R. S. & Harley, C. W. Long-lasting potentiation of the dentate gyrus population spike by norepinephrine. Brain Res. 273, 162–165 (1983).
1985	The LC is shown to have a role in memory retrieval.	Sara, S. J. Noradrenergic modulation of selective attention: its role in memory retrieval. Ann. NY Acad. Sci.

		444, 178–193 (1985). Sara, S. J. The locus coeruleus and cognitive function: attempts to relate noradrenergic enhancement of signal/noise in the brain to behavior.Physiol. Psychol. 13, 151–162 (1985).
1991	LC neurons are recorded during learning and their role in attention and behavioral adaptation is described.	Sara, S. J. & Segal, M. Plasticity of sensory responses of locus coeruleus neurons in the behaving rat: implications for cognition. Prog. Brain Res. 88, 571–585 (1991).
1999	The different cognitive roles of the tonic and phasic modes of LC activity are described.	Aston-Jones, G., Rajkowski, J. & Cohen, J. Role of locus coeruleus in attention and behavioral flexibility.Biol. Psychiatry 46, 1309–1320 (1999).
2004	LC responses to reward are describe	Bouret, S. & Sara, S. J. Reward expectation, orientation of attention and locus coeruleus-medial frontal cortex interplay during learning. Eur. J. Neurosci. 20, 791–802 (2004).
2005	New theories of LC function are proposed: network reset adaptive gain and optimal performance and unexpected uncertainty.	Bouret, S. & Sara, S. J. Network reset: a simplified overarching theory of locus coeruleus noradrenaline function. Trends Neurosci. 28, 574–582 (2005). Aston-Jones, G. & Cohen, J. D. An integrative theory of locus coeruleus-norepinephrine function: adaptive gain and optimal performance. Annu. Rev. Neurosci. 28, 403–450 (2005). Yu, A. J. & Dayan, P. Uncertainty, neuromodulation, and attention. Neuron 46, 681–692 (2005).

1.2.3.1. Anatomy

The first anatomical description of the LC was published in 1812. The noradrenergic nuclei in the brain comprise mainly the LC and the tegmental lateral cell groups. The LC represents the most rostral noradrenergic complex, being located in the pons, in the upper part of the floor of the fourth ventricle. The LC, which means "blue dot" in Latin, was previously known as *Nucleus Pigmentosus Pontis* due to the melanine bodies that are into neurons. Also known as *Nuclei Celestial* because of its blue colour in fresh human tissues.

It is present in all the mammalian species, contains the 43% of total noradrenergic cells representing the major source of NE for the central nervous systems (CNS). The LC is composed of a small number of neurons. The principal neurotransmitter in these neurons is NE which is stored in neuron soma, dendrites and in axon terminals (Grazna and Molier., 1980). However, in some neurons there are others neurotransmitters such as vasopresine, somatostatine or encephaline.

LC nuclei neurons possess immensely ramified axons through the nucleus projecting broadly throughout the neuroxis, from spinal cord to neocortex. This nucleus is densely innervated by fibres that contain opiates, glutamate, GABA, 5-HT, adrenaline, and the peptide orexin/hypocretin. The two major afferences to the LC include the lateral paragigantocellularis nucleus and the nucleus prepositus hypoglosi (Aston-Jones *et al*., 1986). The former provides strong excitatory amino

acid and the latter GABAergic inhibitory input to the LC (Aston-Jones *et al*., 1991). However, the LC receives additional inputs: inhibitory GABAergic afferences from the periaqueductal gray and preoptic/anterior hypothalamic area (Steininger *et al*., 2001), serotonergic afferences from the DRN, (Kim *et al*., 2004), enkephalinergic projections from the paragigantocellulareis nucleus and the nucleus prepositus hypoglosi (Drolet *et al*., 1992), so LC is an important nucleus for endogenous opiate. Dopaminergic inputs from the hypothalamus and the ventral tegmental area (Maeda *et al*., 1994) and noradrenergic afferences from the bulbar/pontine nuclei and axon collaterals of LC neurons (Singewald and Philippu., 1998). Indeed, the extensive shell of dendrites that surrounds the LC offers additional extensive targets for afferent termination. Importantly, this pericoerulear region is the target for a large number of presynaptic fibres from a variety of sources including medial PFC, amygdala, lateral hypothalamus, bed nucleus of the stria terminalis and DRN (Berridge and Waterhouse, 2003). The connection between the DRN and LC is particularly intriguing since efferent projections of these two monoamine systems demonstrate considerable overlap in forebrain circuits.

As for the efferent pathway, two different types of LC axonal terminals have been described, conventional synaptic structures, and varicosities that are believed to result in the extra-synaptic release of NE which may diffuse over some distance by so-called volume transmission (Marien *et al*., 2004). In the rat, the LC extensively innervates different regions of the CNS including cerebellum, thalamus and hypothalamus,

septum, basal ganglia, cerebral cortex, hippocampus, brainstem and dorsal and ventral horns of the spinal cord (Berridge and Waterhouse, 2003). The vast majority (95%) of LC efferent projections to the cortex remains ipsilateral (Waterhouse *et al*., 1983). In contrast to what is observed in the cortex, subcortical structures receive a bilateral LC innervation. These projections can exhibit an ipsilateral or contralateral bias, depending on the targeted structure (Simpson *et al*., 1997) (Fig. 4).

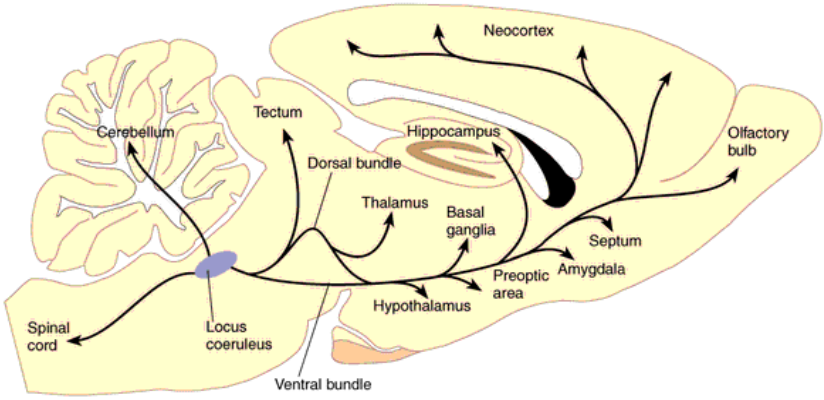

Figure 4. LC and its major ascending and descending projections. Schematic midsagittal section of a mouse brain, showing the locations of the most important groups of noradrenergic neurons and the distribution of their axons and terminal button. Moreover LC received projections from two principal areas, including the lateral paragigantocellularis nucleus and the nucleus prepositus hypoglosi. However, the LC receives additional inputs: inhibitory GABAergic afferences from the periaqueductal gray and preoptic/anterior hypothalamic, serotonergic afferences from the DRN, enkephalinergic projections from the paragigantocellulareis nucleus and the prepositus hypoglosi nucleus. Dopaminergic inputs from the hypothalamus and the ventral tegmental area and noradrenergic afferences from the bulbar/pontine nuclei and axon collaterals of LC neurons. Taken from CNSForum.com.

1.2.3.2. Physiology of the Locus Coeruleus

In anesthetized rats and mice, LC neurons are characterized electrophysiologically by slow, spontaneous discharge rates (0-5 Hz), broad action potential waveforms (1-2 ms) and burst discharges followed by a prolonged period of inactivity or decreased firing (Cedarbaum and Aghajanian, 1976; Gobbi *et al*., 2007). The LC has a homogeneous response and synchronous patterns of discharge all over the nucleus (Christie *et al*., 1989). Electrical interactions between dendrites outside the cell body region are responsible for this synchronous activity within the nucleus (Ishimatsu and Williams, 1996). Some authors suggest that LC neurons are locked on electrically by *gap* unions (Ishimatsu and Willyams., 1996). Firing rate of LC neurons depend to a large degree, of K^+ ion flux, through cell membrane. These fluxes can use K^+ channels depending Ca^{2+} ion or G protein-coupled inwardly rectifier K^+ channels (GIRK) channels which are activated by opiates μ receptors or α_2-adrenoceptors (Williams *et al.*, 1982). On the other hand, activation of AMP_C and the phosphorilation depending of AMP_c increases the firing rate in LC neurons (Wang and Aghajanian, 1987).

LC neurons discharge in a phasic and tonic mode. In the phasic mode, LC cells exhibit phasic activation in response to the processing of task-relevant stimuli but display only a moderate level of tonic discharge. This mode of function is consistently associated with high levels of task performance. In the tonic mode, LC cells fail to respond phasically to task events but exhibit higher levels of ongoing tonic activity. This mode

is associated with poor performance on tasks that require focused attention and corresponds to apparent increases in distractibility (Aston-Jones and Cohen, 2005). Moreover, tonic activity is characterized by relatively low-frequency and highly regular discharge patterns depending on the state:

- LC neurons display highest discharge rates during waking, lower rates during the slow-wave sleep and are virtually silent during REM or paradoxical sleep (Hobson *et al*., 1975).

- In alert situations firing rate of LC in rats awake is higher. (Aston-Jones, 1999).

- LC is involved in learning situations. Moreover, the LC stimulations in early age produce an increase in learning capacity (Velley *et al*., 1991).

- LC stimulation produces a decrease of arterial pressure and bradicardia (Sved y Felsten, 1987; Yao *et al*., 1999) and reciprocally the decrease of blood pressure increases the firing rate of LC neurons.

- Although role of LC in the control of respiratory function is not well-known, it has been observed that external modifications of

CO_2 concentrations regulate firing rate of LC neurons in vitro recording (Pineda and Aghajanian, 1997).

In the LC, even though the majority of neurons are noradrenergic, there is also a limited population of small, round GABAergic neurons (Iijima and Ohtomo, 1988). Within the nucleus, in addition to NE, other neurotransmitters are present such as, galanin, neuropeptide Y, vasopressin, neurophysin, neurotensin, vasoactive intestinal peptide, atrial natriuretic factor, enkephalin, N-acetylaspartylglutamate and 5-HT (Aston-Jones, 2004). As for the receptors, opiate, serotonergic, dopaminergic and α_1 and α_2-adrenoceptors have been described in the LC (Suzuki *et al*., 1998; Yokoyama *et al*., 1994). Calcitonin gene-related peptide and muscarinic cholinergic receptors as well as biding sites for glutamate, purines, hypocretin/orexin, neurokinins and corticotrophin-releasing hormone have also been reported (Aston-Jones, 2004). Although other receptors can modulate the LC electrical activity, it is mainly regulated by α_2-adrenoceptors and the activation of these receptors leads to a progressive reduction in the firing activity (Elam *et al*., 1986; Freedman and Aghajanian, 1984; Ruiz-Ortega and Ugedo, 1997). Thus, LC neurons can release NE onto the somadendritic membrane of other LC neurons and thereby provide local feed-back inhibition (Egan *et al*., 1983). In addition, it is well-known that stimulation of α_2-adrenoceptors by systemic or iontophoretic administration of clonidine, an α_2-adrenoceptor agonist, inhibits LC neuronal activity in a dose-dependent manner (Svensson *et al*., 1975). Iontophoretic application of NE and adrenaline also inhibits LC neuron

activity and this effect is blocked by pretreatment with α_2-adrenoceptor antagonists (Cedarbaum and Aghajanian, 1976). In LC of mice α_2-adrenoceptors are coupled to GIRK channels. These channels are formed by GIRK2 and GIRK3 subunits and alteration of these subunits, double knockout mice for GIRK2 and GIRK3 subunit, implicated alteration in the hyperpolarization induced by the α_2-adrenoceptors agonist UK-14304, as compared to WT animals (Torrecilla et al., 2008).

1.2.2.3. Pathophysiological aspects of the noradrenergic system

In the CNS, the LC is involved in several physiological functions such as sleep-wake cycle (Aston-Jones *et al*., 2000; Berridge and Waterhouse, 2003), novel environmental stimuli (Mansour *et al*., 2003; Vankov *et al*., 1995), cognition and memory (Bouret and Sara, 2005; Clayton and Williams., 2000; Khakpour-Taleghani *et al*., 2008); pain (Jones, 1991; Stamford, 1995; West *et al*., 1993), cardiovascular or respiratory control (Singewald and Philippu., 1998; Svensson., 1987) and rewarding behavior. Indeed, it has been shown that destruction of LC projections to the nucleus acumbens reduces synaptically-evoked DA release in this nucleus (Haidkind *et al*., 2002).

Therefore, several pathophysiological processes have been related to the alteration in the LC system including stress (Sved *et al*., 2002), anxiety (Tanaka *et al*., 2000), depression (Brady, 1994; Brunello *et al*.,

2002), drug addiction (Nestler and Aghajanian, 1997) and Alzheimer disease (Weinshenker, 2008).

- **Stress:** LC can be activated by certain stressant stimulates like an electric shock, air current, confinement or immobility, thus an increase in the activity of noradrenergic neurons can observed.

- **Anxiety:** LC participation in anxiety was postulated on the bases of results observed in primates. Thus, electric stimulations of LC and LC destruction produce anxiety related behaviors.

- **Depressions:** LC inhibitions induced by α_2-adrenergic drugs produce changes in electroencephalograph activity (Berridge *et al*., 1993). Moreover destruction of LC in experimental animals produces antidepressant effects (Cervo *et al* 1990).

A) Role of the noradrenergic system in depression

Many studies on human subjects implicate the noradrenergic system in the pathophysiology of depression. To date, the most compelling evidence is mainly associated with the LC and its projection areas. There is no consistent evidence for the implication of specific mechanisms but, on the other hand, it is clear that changes in the activity of noradrenergic neurons are involved in the antidepressant action of many drugs.

The findings in *postmortem* examinations are not always consistent but seem to indicate an increased α_2-adrenoceptor agonist binding in brains of depressed patients. Significantly higher densities of α_{2A}-adrenoceptors have been found in the frontal cortex (Callado *et al*., 1998; Gonzalez *et al*., 1994), hippocampus (Gonzalez *et al*., 1994), temporal cortex (De Paermentier *et al*., 1997), hypothalamus (De Paermentier *et al*., 1997) and LC in depressed suicide victims (Ordway *et al*., 1994). Apart from a selective increase of α_{2A}-adrenoceptors, decreased binding to NE transporters in the LC of depressed patients has also been reported (Klimek *et al*., 1997). This finding was interpreted as a compensatory down-regulation of this transporter protein in response to an insufficient availability of NE at the synaptic level. Studies on brain α_1-adrenoceptors have revealed an even less consistent pattern of change; both an increase and a decrease in binding being reported (Gross-Isseroff *et al*., 1998). The number of neurons in the LC of depressed patients has been a matter of discussion. Some authors have reported lower number of cells in the LC (Arango *et al*., 1996) while others have observed no change in depressed suicide victims (Klimek *et al*., 1997).

Animal models of depression are based on stressful conditions and depending on the model, different results have been observed. The large variety of models is probably the source of the generally inconsistent findings, but as far as there is any consistency, the evidence points to disturbances in noradrenergic neurotransmission. Thus, up-regulation of α_2-adrenoceptors have been reported in most cases of stress in animals (Fulford *et al*., 1994; Flugge *et al*., 1997).

1.3. G PROTEIN-COUPLED INWARDLY RECTIFIER K^+ CHANNELS (GIRK)

The inwardly rectifier potassium channels (Kir) play pivotal roles in controlling insulin release, vascular tone, heart rate, neuronal signaling, and membrane excitability (Hille, 1992). There are seven Kir subfamilies (Kir1-Kir7) (Doring et al., 1998; Isomoto et al., 1997; Krapivinsky et al., 1998; Partiseti et al., 1998). The Kir3 subfamily, also known as G protein-coupled inwardly rectifier potassium channels (GIRK) are downstream effectors of G protein-coupled receptors (GPCRs), and they can be activated by direct binding of the βγ subunit of G protein to the channels, an interaction with important physiological consequences (Wickman and Clapham, 1995; Yamada et al., 1998).

GIRK channels hyperpolarize neurons in response to activation of $G_{i/o}$ protein coupled receptors and decrease the excitability of neurons, slow synaptic potentials and volume transmission (Krapivinsky *et al*., 1995; Wickman *et al*., 1998; Lüscher *et al*., 1997; Signorini *et al*., 1997; Wickman and Clapham, 1995; Yamada *et al*., 1998). Therefore abnormal GIRK function can lead to excessive or deficient neuronal excitability which is related to several pathologies such as epilepsy or Down's syndrome. Thus, GIRK channels are proposed as new pharmacological targets for the treatment of a plethora of illnesses.

1.3.1. Structure, composition and localization of neuronal GIRK channels.

GIRK channel function and trafficking are highly dependent on the channel subunit composition. The basic building block of a GIRK channel is a subunit which is made up of two transmenbrane α-helix called M_1 and M_2 and an extracellular loop which folds back to form the pore-lining selective filter for the potassium ions, the P re-entrant helix (Fig. 5 A and B).

In mammalian four subunits (GIRK 1-4) have been cloned which assemble into homo and heterotetrameric channels (Wickman *et al*., 2002). GIRK1, GIRK2 and GIRK3 are broadly expressed in many areas of the CNS whereas GIRK4 expression is restricted to a small number of neuron populations including some restricted areas of the neocortex, ventromedial hypothalamic nucleus, laterodorsal and lateral posterior thalamic nuclei and insular cortex, and Golgi cells from the cerebellum (Karschin *et al*., 1996; Wickman *et al*., 2000; Aguado *et al*., 2008). This narrow distribution effectively reduces its contribution to neuronal GIRK channel formation.

GIRK2 and GIRK4 subunits can form homomeric channels (Inanobe *et al*., 1999; Kobayashi *et al*., 1999). Although homomeric GIRK2 and GIRK4 channels have been found in native tissues, exogenously expressed homomers of these channels give very brief and poorly resolved open-time kinetics that are different from native channels

(Chan *et al*., 1996; Corey and Clapham, 1998). Recently, crystal structure of mamalian GIRK2 channels has been presented (Whorton and Mackinnon, 2011). Both GIRK2 and GIRK4 can recruit GIRK1, greatly increasing its surface expression and resulting in highly active channels that are indistinguishable from native channels (Krapivinsky *et al*., 1995; Chan *et al*., 1997; Woodward *et al*., 1997; Kennedy *et al*., 1999).

GIRK1 and GIRK3 subunits are unable to independently form functional channels (Chan *et al*., 1996; Woodward *et al*., 1997; Jelacic *et al*., 1999; Mirshahi and Logothetis, 2004). Indeed, GIRK1 requires co-expression with another GIRK subunit to achieve membrane distribution (Krapivinsky et al. 1995a; Hedin *et al*., 1996; Kennedy *et al*., 1996; Ma *et al*., 2002). GIRK1 and GIRK2 subunits can co-assemble with GIRK3 to form GIRK1/3 and GIRK2/3. Interestingly, it has been showed that GIRK2/GIRK3 heteromers have some activity, but reduced Gβγ sensitivity compared with other heteromers containing GIRK1 subunits (Jelacic *et al*., 2000).

Most neuronal GIRK channels are thought to be heteromeric containing GIRK1 and GIRK2 subunits (Luscher *et al*., 1997; Signorini *et al*., 1997). Indeed, GIRK1 and GIRK2 knockout mice display similar behavioral phenotypes (Pravetoni and Wickman, 2008). In addition, levels of GIRK1 subunit are decreased in the brains of $GIRK2^{-/-}$ mice (Signorini *et al*., 1997; Torrecilla *et al*., 2002). However, several findings indicate that a wide array of GIRK subunit combinations may be relevant and exhibit specific functional properties. Thus, it has been showed that

GIRK2/GIRK3 heterotetramers, which exhibit reduced Gβγ sensitivity, have been isolated from mouse brains (Jelacic *et al*., 2000) and could account for the GIRK currents in GABAergic neurons from ventral tegmental area (Cruz *et al*., 2004). Also in the noradrenergic neurons from the LC GIRK currents activated by opioids are mediated by GIRK channels containing GIRK1, GIRK2 and GIRK3 subunits (Torrecilla *et al*., 2002). In the cerebellum, for example, there is a cell type-specific expression of GIRK channels, since there are at least seven distinct GIRK subunit expression patterns in several cell types (Aguado *et al*., 2008). In neurons from the hippocampus and spinal cord ultrastructural studies show that GIRK2 but not GIRK1, is present at post-synaptic specialization (Koyrakh *et al*., 2005; Marker *et al*., 2005). In dopaminergic neurons from the substantia nigra GIRK channels are formed by homomeric combination of splice variants of GIRK2 subunits (Inanobe *et al*., 1999) and in the ventral tegmental area assembly of distinct GIRK2 alternatively spliced isoforms with GIRK3 is the most habitual combination (Cruz *et al*., 2004, Laouebe *et al*., 2007). Three splice variants of GIRK2 (GIRK2a-c) exist in the brain, and splice variants of GIRK1 have also been reported, although its relevance is not well understood (Lesage *et al*., 1995; Isomoto *et al*., 1996; Nelson *et al*., 1997; Inanobe *et al*., 1999; Iwanir and Reuveny, 2008). No splice variants of GIRK3 and GIRK4 have been reported.

Despite its broad distribution in the CNS, the functional relevance of GIRK3 is uncertain. GIRK3 knockout mice are indistinguishable from WT mice in some behavioral tests (Pravenoti and Wickman, 2008) and

have similar agonist-induced GIRK currents, but exhibit less severe withdrawal from sedatives (Kozell *et al*., 2009) and reduced cocaine-self-administration (Morgan *et al*., 2003). Recently, GIRK3 subunits together with the major splice variant of GIRK2, GIRK2c have been proposed to play a key role in regulating endosomal trafficking of GIRK channels (Ma *et al*., 2002; Lunn *et al*., 2007).

GIRK channels are expressed in dendritic spines in the postsynaptic density as well as extrasynaptic sites. In CA_1 hippocampal pyramidal neurons GIRK are in the soma, along the dendrites and in the dendritic spines (Koyrakh *et al*., 2005; Cena and Johnston, 2005; Kulik *et al*., 2006). Interestingly, differences in the subunit composition of GIRK channels have been detected between synaptic and extrasynaptic plasma membrane (Koyrakh *et al*., 2005), suggesting that there is compartmental molecular diversity. Only GIRK2 is detected in the spines in de postsynaptic density, whereas both GIRK1 and GIRK2 are located in the extrasynaptic membranes of the spine head, as well as in the dendritic shafts (Koyrakh *et al*., 2005). GIRK channels in the cortex and cerebellum are also present at presynaptic sites in a subpopulation of excitatory axon terminals, close to $GABA_B$ receptors. Thus, GIRK channels are perfectly positioned to influence synaptic responses and plasticity, as described in the next chapter.

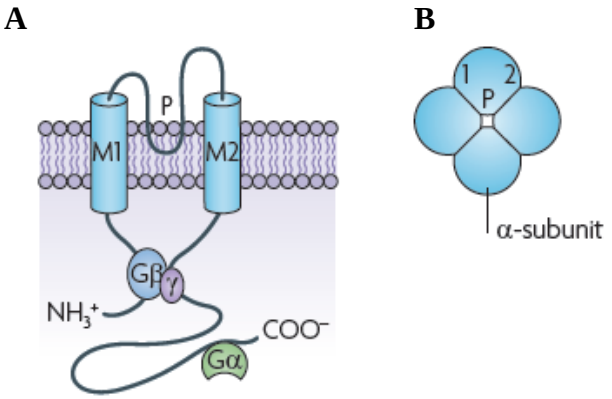

Figure 5. G protein coupled inwardly rectifier K^+ channel structures. **A)** Each subunit in the middle of transmenbrane dominium have two segments of α-helix (M_1 y M_2), and one P re-entrant helix which formed a selective filter for potassium ions. The fattest lines in intracellular segment indicated the approximate union place for βγ complex. The Gα and Gβγ subunits of G proteins regulate channel opening. The segment unions for βγ complex in N-terminal extreme and distal extreme in C-terminal segment were present in all GIRK subunits. While the union at βγ complex in proximal C-terminal was in GIRK4 but not in GIRK1. **B)** GIRK channels are tetrameric assemblies of four α-subunits, and each α-subunit has a pore-forming motif comprising the P loop. Taken from Luján *et al*., 2009.

1.3.2. Physiology, function and dysfunction of GIRK channels in the brain.

1.3.2.1. Physiology of GIRK channels.

As mentioned earlier, GIRK channels are members of the inwardly rectifying K^+ channel family. The term "inward rectification" refers to a change in the slope of the current-voltage relationship of the channel at the reversal potential. At membrane potentials well above the equilibrium potential for K^+, the channel permits the efflux of K^+, causing a very small outward current compared to the inward current caused at potentials well below equilibrium potential for K^+. This rectification is due to the occlusion of the central pore by intracellular Mg^{2+} and polyamines at potential above equilibrium potential for K^+ (Yamada *et al*., 1998). Under physiological conditions, the resting membrane potential of a typical neuron is positive to equilibrium potential for K^+, and the small outward current through the channels decreases the excitability of the neuron.

Postsynaptic inhibitory effects induced by activation of a wide range of pertussis toxin-sensitive, G_i or G_o type couple-receptors are mediated by direct binding of the G protein subunits Gβγ to the GIRK channel (Logothetis *et al*., 1987) (Fig. 6). Thus, activation of GIRK channel by the binding of Gβγ complex permits the efflux of K^+ ions and leads the cell to more hiperpolarized membrane potentials (Stefano *et al*., 1996). The role of G protein subunit Gα in regulating the channel has

been a matter of debate. In fact in Xenopus oocytes, Gα subunit probably in its $G\alpha^{GDP}$ form (classically considered as the inactive form of Gα) or as a part of the Gαβγ heterotrimer, is not only a donor of Gβγ but also controls GIRK gating, being crucial to keep the channel shut in absence of G protein-coupled receptor ligands (Peleg *et al*., 2002). Therefore, this interaction might determine the basal activity levels of the channel and primes it for activation by Gβγ complex. GIRK1 containing channels are regulated by both Gα and Gβγ, while GIRK2 is a Gβγ-effector insensitive to Gα (Rubistein *et al*., 2009). Also in neurons from hippocampus and LC nucleus, GIRK channels display a substantial basal conductance (Luscher *et al.* 1997; Torrecilla *et al.* 2002; Chen and Johnston, 2005; Wiser *et al.,* 2006).

Several modulators have been described that alter GIRK channel activity, including cytosolic Na^+, intracelular magnesio and polyamines, ethanol and phosphorilation by protein kinase A and protein kinase C. Both Na^+ and ethanol seem to stimulate GIRK channels through a specific binding site. PKC-dependent phosphorylation decreases while protein kinase A dependent phosphorylation enhances channel activity. In addition, membrane phosphatidylinositol-4,5-bisphosphate levels are essential for proper GIRK gating by both Na^+ and Gβγ (Sui *et al*., 1998; Huang *et al*., 1998; Ho and Murrell- Lagnado, 1999; Logothetis and Zhang, 1999). Moreover, the functional dependence of Gβγ signalling on phosphatidylinositol-4,5-bisphosphate predicts the possible cross-talk of different metabotropic receptors. In fact, activation of metabotropic receptors coupled to $G_{q/11}$ proteins stimulates fosfolipase C-β, which

leads to activation of protein kinase C and phosphatidylinositol-4,5-bisphosphate depletion, both of which reduce GIRK channels activity (Kobrinsky *et al*., 2000; Cho *et al*., 2001; Lei *et al*., 2001). Also tyrosine kinase, Ca^{2+} calmodulin-dependent protein kinase 2 and protein phosphatase 1 (Huang *et al*., 2005; Ippolito *et al*., 2005; Chung *et al*., 2009) modulates GIRK channel activity (Fig. 7).

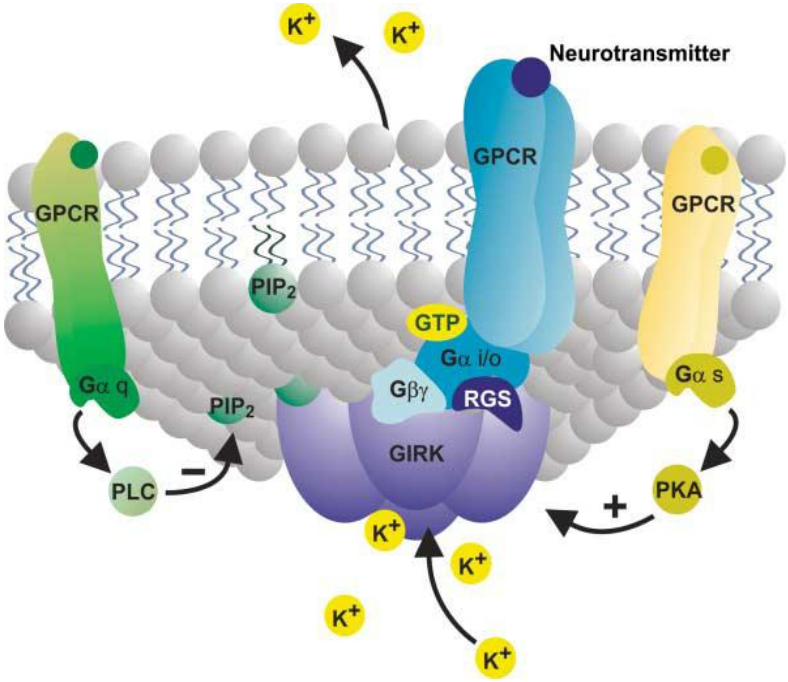

Figure 6. Activation of GIRK channel by the βγ complex. Activation of GIRK channel by the binding of Gβγ complex permits the efflux of K^+ ions and leads the cell to more hiperpolarized membrane potentials. Taken from CNSForum.com.

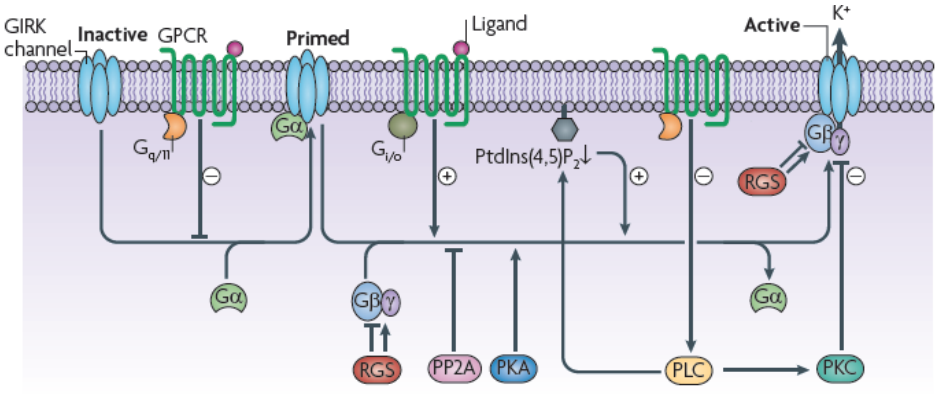

Figure 7. Gating of GIRK channels. G protein-coupled inwardly rectifying K^+ (GIRK) channel signalling pathways. GIRK channels are inactive in the absence of bound G protein subunits. Binding of Gα primes GIRK channels, and activation of G_i or G_o ($G_{i/o}$)- coupled G protein-coupled receptors (GPCRs) results in activation of GIRK channels by Gβγ. GIRK channel activity is increased by protein kinase A (PKA) and decreased by protein phosphatase 2A (PP2A). Activation of G_q and G_{11} ($G_{q/11}$)-coupled G protein-coupled receptors (GPCRs) decreases GIRK channel activity, through activation of protein kinase C, whereas a phospholipase C-mediated decrease in phosphatidylinositol-4,5-bisphosphate (Ptdlns(4,5)P_2) has the opposite effect. Various regulators of G protein signalling (RGS) proteins also modulate GIRK channel activity. Taken from Luján *et al*., 2009.

1.3.2.2. GIRK channel trafficking.

Recently, dynamic regulation of GIRK channel number has been proposed to afford a powerful way to modulate neuronal activity. Today a number of intrinsic amino-acid sequences have been identified that control the intracellular trafficking of GIRK channels. GIRK2 contains a strong endoplasmic reticulum export signal (acidic residues) as well as an internalization (Val-Leu or "VL") motif (Ma *et al*., 2002), enabling this subunit to form homotetramers or heterotetramers (Lesage *et al*.,1995; Kofuji *et al*.,1995; Jelacic *et al*., 1999; Jelacic *et al*., 2000). GIRK1 and GIRK3 subunits, by contrast, are retained in the endoplasmic reticulum, because they lack an endoplasmic reticulum export signal, but they are trafficked to the plasma membrane when they assemble with GIRK2 or GIRK4 subunits as these have various trafficking signals in their intracellular N- and C-terminal domains that regulate endoplasmic reticulum export and post-endoplasmic reticulum trafficking, as well as a VL motif that promotes endocytosis. By contrast, the GIRK3 subunit contains a lysosomal-targeting motif (Tyr-Trp-Ser-Ile or 'YWSI') that promotes degradation of GIRK channels or also reduces its surface expression and accumulation, even in channels formed by assembly with GIRK2 or GIRK4 (Ma *et al*., 2002). Among the GIRK subunits, GIRK2c, a major splice variant of GIRK2, and GIRK3 possess C-terminal PDZ-binding motifs that mediate interactions with sorting nexin 27 (SNX27) and regulate endosomal trafficking of GIRK channels (Lunn *et al*., 2007). The interplay of these trafficking motifs suggests that GIRK2 has a primary role in forming native GIRK currents, whereas

GIRK3 may regulate the availability of GIRK channels on the plasma membrane (Fig. 8).

In this context, excitatory synapses in the brain undergo activity-dependent changes in the strength of synaptic transmission. This phenomenon is named synaptic plasticity which is exemplified by long-term-potentiation, a cellular correlate of learning and memory. The presence of GIRK channels near excitatory synapses on dendritic spines suggests the possible role on synsaptic plasticity. In fact, long-term-potentiation of the slow inhibitory postsynaptic current mediated by $GABA_B$ receptors and GIRK channels due to activation of NMDA receptors as the coincidence detector has been already reported in hippocampal neurons (Huang *et al*., 2005). Thus, depolarization of the postsynaptic neurons to bring the membrane potential close to 0 mV increases the $GABA_B$ receptor-mediated slow inhibitory postsynaptic current several fold for the duration of the experiment. This increased slow inhibitory postsynaptic current is independent of the activation of $GABA_B$ receptors but dependent on ionotropic glutamate receptors such as NMDA receptor activation, similar to hippocampal NMDA receptor dependent long-term potentiation of AMPA receptors (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors) mediated excitatory postsynaptic current. Interestingly, it has been recently shown that GIRK currents become larger because additional channels are inserted in the plasma membrane which reveals a novel mechanism for dynamic modulation of neuronal excitability (Chung *et al*., 2009a). Indeed, the activation of NMDA receptors in cultured dissociated

hippocampal neurons increases the surface expression of GIRK1 and GIRK2 subunit-containing channels in the soma, dendrites and dendritic spines within minutes. This insertion requires dephosphorylation mediated by protein phosphatase 1 of a GIRK2 serine residue (Ser9) that promotes channel recycling from endosomes (Chung *et al*., 2009a). Remarkably, this NMDA receptor-dependent increase in the surface expression of GIRK channels is mediated by adenosine A1 receptors, but not $GABA_B$ receptors; this has important meaning in synaptic plasticity since another form of synaptic plasticity described in the hippocampus called depotentiation, the ability of low frequency stimulus to abort long-term-potentiation, is thought to be a homeostatic mechanism that prevents saturation of long-term-potentiation to increase the storage capacity of neural circuits. Interestingly, depotentiation requires adenosine A1 receptors, which are coupled to GIRK channels, as well as protein phosphatase 1 (Chung *et al*., 2009b). Therefore, the proteins that are involved in the depotentiaction pathways are the same as those that are implicated in regulating NMDA receptor-dependent GIRK channel trafficking, suggesting that depotentiation is tightly linked to increased numbers of channels at the plasma membrane. In strong support of this contention, $GIRK2^{-/-}$ mice lack depotentiation (Chung *et al*., 2009b). In this sense, the possibility cannot be excluded that the lack of effects of NMDA receptors on GIRK channel activation by $GABA_B$ receptors could be caused by an insufficient amount of Gβγ available for full activation of GIRK channels, which requires the interaction of one GIRK channel with multiple Gβγ subunits (Sadja, 2001), given that activation

of $GABA_B$ receptors produces a large and saturating current in hipocampal neurons (Sodickson, 1998).

Because activation of GIRK channels hyperpolarizes neuronal membranes, increased expression of GIRK channels accounts for a net inhibitory contribution to cell excitability. Therefore, GIRK channels represent a key part in many types of neuronal communication and, as outlined in the following sections, modification of GIRK channel function and number could affect CNS function in health and disease.

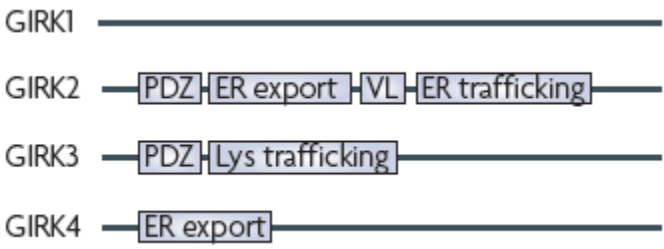

Figure 8. The four GIRK channel subunits, with subunit-specific motifs indicated. The GIRK2 (also known as KCNJ3) subunit contains a PDZ, an endoplasmic reticulum (ER) export, a VL and an ER trafficking motif; the GIRK3 (also known as KCNJ9) subunit contains a PDZ and a lysosomal (lys) trafficking motif; the GIRK4 (also known as KCNJ5) subunit contains an ER export motif. Taken from Luján *et al*., 2009.

1.3.2.3. Role of GIRK channels in animal physiology and pathophysiology.

The physiological activation of GIRK channel can shape the neuronal network behavior at different levels. The basal activity of GIRK channels contributes to the resting membrane potential of neurons, shifting the membrane voltage by approximately -8 mV (Lüscher *et al*., 1997, Torrecilla *et al*., 2002). In addition, receptor activation of GIRK channels provides another level of inhibition, to which three different changes in signalling can be generally ascribed: neuronal self-inhibition, neuron to neuron inhibition and network level inhibition.

As for neuronal self-inhibition or autaptic transmission some neurons release a neurotransmitter that GPCR and, in turn, GIRK channels on their own dendrites causing self-inhibition (Bacci *et al*., 2004). In the second case, the activation of post synaptic $GABA_B$ receptors, D_2 receptors and group II metabotropic glutamate receptors by transmitters released from neighbouring neurons (synaptic transmission) may also activate GIRK channels (Newberry and Nicoll., 1985; Beckstead and Williams., 2007; Dutar and Perkel., 1999). While GABA spontaneously released produces a fast inhibitory postsynaptic potential, mediated by $GABA_A$ receptors, strong or repetitive stimulation is required to elicit the slow inhibitory potential, suggesting that GABA released into the synaptic cleft diffuses and activates perisynaptic $GABA_B$ receptors coupled to GIRK channels. Thus, $GABA_B$ receptors coupled to GIRK channels could be positioned adjacent to synaptically localized $GABA_A$ receptors or in a neighbouring dendritic spine. In the

hippocampus generation of the slow inhibitory postsynaptic current is important for regulating the rhythmic activity of the network (Scanziani, 2000). In addition to postsynaptic activation, recent characterization of $GABA_B$ receptor-mediated presynaptic inhibition suggests a presynaptic role for these channels in inhibiting GABA release (Ladera *et al*., 2008; Michaeli *et al*., 2010).

In the last case the regulation of ambient levels of several endogenous G protein-coupled receptor agonists (for example, adenosine and somatostatin) may have a modulatory effect on large-scale neuronal networks through GIRK channel activation. This large-scale effect of neuromodulators is known as volume transmission. Moderate activation of GIRK channels in a population of neurons would be expected to reduce membrane excitability. For example, somatostatin, acting onto somatostatine receptor subtype 5, might alter the oscillation behavior of thalamic networks through postsynaptic GIRK channel activation, together with presynaptic inhibition (Sun *et al*., 2002). Similarly, endogenous adenosine may suppress gamma oscillations in the hippocampus (Pietersen *et al*., 2009), possibly owing to the selective expression of adenosine A1 receptors on pyramidal neurons, which also activate GIRK channels. All these findings suggest a role for GIRK channels in signalling pathways beyond the direct membrane hyperpolarization.

In this context, the use of GIRK mutant mice have been a key tool of insight into the role of specific GIRK channel composition on

neuronal physiology and animal behavior. Thus, GIRK channels functional relevance has been linked to susceptibility to seizures (Signorini, 1997), hyperalgesia and analgesia (Blednov *et al*, 2003; Marker *et al*., 2004, 2005; Smith *et al*., 2008), drug addiction and alcohol related behavioral effects (Blednov *et al*., 2001b; Morgan *et al* 2003; Kobayashi *et al*., 1999; Cruz *et al*., 2008), motor activity and co-ordination and reward and anxiety-related behaviors (Pravetonti and Wickman, 2008).

The first mutant mice regarding GIRK channels were *weaver* mice ($GIRK^{wv}$). These mice carry an spontaneous mutation in the pore region of GIRK2 subunit ($GIRK2^{wv}$) (Patil et al.,1995), leading to a loss of K^+ selectivity and G protein insensitivity (Slesinger *et al*., 1997; Kofuji *et al*., 1996; Navarro *et al*., 1996). Upon receptor activation, $GIRK2^{wv}$ depolarizes rather than hyperpolarizes neurons. Homozygous mice are characteristic because have an abnormal walk like a consequence of the lost of granular neurons in cerebellum (Lane, 1964; Rakic and Sidman, 1973). They also show motor hyperactivity, spontaneous temblor, ataxia and male sterility by the degeneration of dopaminergic neurons of the substantia nigra (Lane *et al*., 1977; Schmidt *et al*., 1982; Roffler-Tarlov, 1996; Liao *et al*., 1996). In *weaver* mice it is showed an important decrease of GIRK2 subunit as well as GIRK1 subunit (Liao *et al*., 1996). Moreover this mutation could have different qualitative effects in different neuronal populations depending of GIRK2 and GIRK1 expression levels. In neurons which express high levels of GIRK2 but do not GIRK1 like dopaminergic neurons in substantia nigra,

weaver mutation induces chronic depolarization and cell death (Liao *et al*., 1996) (See table 2 to observe the implication of mutation in physiology).

One of the aspects in which that might be more important in the physiology is analgesic or pain perception. GIRK channels have been implicated in pain perception. This notion is based on studies in mice carrying mutations in GIRK channels, and the activation of GIRK channels by analgesic drugs and endogenous pain modulators such as endorphins and endocannabinoids.

GIRK1 knockout and GIRK2 knockout mice, as well as WT mice given intrathecal injection of the GIRK channel blocker tertiapine, exhibited thermal hyperalgesia and blunted analgesic responses to intrathecal morphine and the μ-opioid receptors selective agonist (Marker *et al*., 2004,2005). Interesting GIRK channels were found to make a dose-depedent contribution to the analgesic effect of μ-opioid receptors agonist, such that the effect to high but not lower doses of opioid agonist was selectively blunted after the pharmacologic or genetic ablation of spinal GIRK channels. Morphine and the selective κ-opioid agonist U-50488, which are known to activate GIRK2 containing channels through opioid receptors, reduce pain perception in WT mice but not in the *weaver* mice (Ikeda *et al*., 2000). However, the interpretation of the opioid-dependent behaviors is confounded by the loss of neurons in the substantia nigra and cerebellum. Moreover, in GIRK2$^{-/-}$ mice, for example, the dose–response curve for analgesia produced by morphine

and clonidine (an α_2-adrenergic receptor agonist) is shifted to higher concentrations, but the maximal effect is preserved (Mitrovic *et al*., 2003). This finding was confirmed in a recent study using double knockout mice for GIRK2 and GIRK3 (Cruz *et al*., 2008); in these mice, the potency (coupling efficiency) of opioid-induced analgesia was reduced, but their efficacy (maximal response) was intact. Furthermore GIRK2 channels abolished the antinociceptive effects of ethanol, oxotremorine, baclofen and clonidine in males but not in females (Blednov *et al*., 2003).

GIRK channels have also been implicated in opioid tolerance and dependence, as the channels undergo adaptive changes during chronic exposure to morphine, such as desensitization, this phenomenon seems to involve additional mechanisms. In neurons of the LC (an adrenergic nucleus in the pons that lowers pain threshold when activated) inhibiting signalling by both G protein-coupled receptor kinase 2 and extracellular signal-regulated kinase significantly reduces the met-enkephalin-induced GIRK current desensitization that normally occurs within minutes of drug application (Dang *et al*.,2009). However, GIRK current desensitization may not occur when μ-opioid receptors are activated by morphine. Morphine causes less GIRK channel desensitization *in vitro* and *in vivo* than met-enkephalin (Whistler *et al*., 1999). So alternative role of GIRK channels in opioid dependence are implicated because GIRK2 and GIRK3 lacking mice have strongly reduced withdrawal signs that are normally induced in WT controls by the opioid receptor antagonist naloxone after chronic exposure to morphine. GIRK2 and

GIRK3 double knockout mice also failed to display an increase in LC neuron firing rate, an established electrophysiological hallmark of withdrawal. Furthermore, the GIRK-dependent component of the morphine-induced postsynaptic current was absent in LC neurons from GIRK2 and GIRK3 double knockout mice (Cruz *et al*., 2008).

Regarding drug dependence, GIRK channels are thought to have a role in the acute rewarding effects and/or the adaptation that occurs with chronic exposure to addictive drugs that work through GPCR, such as opioids, the 'club drug' γ-hydroxybutyrate and cannabinoids. Addictive drugs are known to strongly increase DA levels in the mesocorticolimbic system. The initial target for addictive drugs in reward pathway is the ventral tegmental area. Based on *in vivo* and *in vitro* experiments, distinct cellular mechanisms have been proposed for different classes of drugs (Luscher *et al*., 2006). In fact opioids and γ-hydroxybutyrate activate GIRK channels, leading to disinhibition of dopaminergic neurons. Morphine stimulates μ-opioid receptors that are selectively expressed on GABAergic interneurons of the ventral tegmental area and activate GIRK channels, reducing the firing of these cells and eventually leading to disinhibition of dopaminergic neurons (Johnson *et al*., 1992). However, γ-hydroxybutyrate effect is more complex, since $GABA_B$ are expressed in both interneurons and dopaminergic neurons from the ventral tegmental area. Interestingly, $GABA_B$ receptors from DA neurons are less efficaciously coupled to GIRK channels than those from GABAergic interneurons, probably owing to selective expression of GIRK2c and GIRK3 and the lack of the GIRK1 subunit (Cruz *et al*., 2004).

Accordingly, low concentrations of $GABA_B$ receptor agonist, γ-hidroxibutyrate selectively activate GIRK currents from GABAergic interneurons, leading to desinhibition of dopaminergic neurons. In addition, these neurons in the ventral tegmental area also selectively express regulator of G protein signalling, which contributes to the low sensitivity of $GABA_B$ receptors for GIRK channels (Laouebe *et al*., 2007). Furthermore, chronic drug exposure can dynamically regulate the expression levels of regulator of G protein signalling. For example, chronic morphine or γ-hydroxybutyrate enhances the coupling efficiency of $GABA_B$ receptors and GIRK channels by decreasesing regulator of G protein signalling levels in dopaminergic neurons from ventral tegmental area and therefore low drug concentrations are sufficient to inhibit dopaminergic neurons and losing the rewarding properties and becoming aversive (Lomazzi *et al*., 2008). Thus the selective combination of GIRK subunits determines the signalling properties of neuronal circuits in the drug addiction process.

Moreover, GIRK channels are implicated in the response to psychostimulants. GIRK2 and GIRK3 knockout mice exhibit dramatically reduced intravenous self-administration of cocaine relative to WT mice whereas, surprisingly, GIRK2 and GIRK3 double-knockout mice self-administer more cocaine than WT mice, suggesting compensation in the single-knockout mice that is no longer possible in the double-knockout mice (Morgan *et al*., 2003).

GIRK channels are also involved in alcohol-induced behavior and addiction, as alcohol directly binds and activates GIRK channels (Kobayashi *et al*., 1999; Mullner *et al*., 2000; Aryal *et al.*, 2009). Indeed, *weaver* mice lack ethanol analgesia although there were no differences between GIRK2$^{-/-}$ mice and WT mice for ethanol-induced sleep time, acute functional tolerance, or handling-induced convulsions (Kobayashi *et al*., 1999). In GIRK2$^{-/-}$ some effects induced by ethanol, as stimulation of home cage motor activity, anxiety and handling-induced convulsions were absent (Blenov *et al*., 2001a). GIRK3 knockout mice display less severe withdrawal from ethanol and barbiturates (Pravenoti and Wickman, 2008). Recently, it has been demonstrated that ethanol increases $GABA_B$ receptor-induced GIRK currents in ventral tegmental area neurons, decreasing excitability of dopaminergic neurons (Federici *et al*., 2009).

Memory and learning proceses are also altered in mutant mice. So mice with low levels of GIRK1 have difficulty to learn and with memory (Kourrich *et al*., 2003). GIRK4 knockout mice have difficulty with spatial learning too (Wickman *et al*., 2000).

In regard to implication of GIRK channels in the pathophysiology of several diseases, two broad principals can be taken into account: first, loss of GIRK function can lead to excessive neuronal excitability, such as in epilepsy, whereas a gain of GIRK function can considerably reduce neural activity, such as is postulated to occur in Down's syndrome. Second, loss of selectivity can cause aberrant ion fluxes across GIRK

channels, such as aberrant Na^+ influx that triggers cell death, which is exemplified in a model of Parkinson's disease. In epilepsy $GIRK2^{-/-}$ mice develop spontaneous convulsions and show a propensity for generalized seizures when injected with a pro-convulsive $GABA_A$ receptor antagonist (Signorini *et al*., 1997). In Down's syndrome two mouse models have been generated that carry either a partial or full segment duplication of the mouse chromosome 16, the orthologue of human chromosome 21 (Reeves *et al*., 1995; Sago *et al*., 1998) and in both cases GIRK2 protein is upregulated, resulting in a larger slow inhibitory postsynaptic current mediated by $GABA_B$ receptors (Siarey *et al*., 1999). Moreover, hippocampal long term potentiation is reduced and long-term depression is enhanced in both models, and therefore synaptic plasticity of glutamatergic transmission is altered.

On the other hand cell death is implicated with Parkinson's disease. The involvement of GIRK channels in Parkinson's disease was first inferred from the *weaver* mouse (Patil *et al.*, 1995) in which constitutively active $GIRK2^{wv}$ channels produce chronic depolarization and cell death in a subset of neurons in the brain (Slesinger *et al*., 1996; Kofuji *et al*., 1996; Navarro *et al*., 1996), mimicking the neuronal degeneration observed in Parkinson's disease (Schein *et al*., 2005). The gain-of-function phenotype in dopaminergic neurons of *weaver* mice is of clinical interest owing to the progressive degeneration of dopaminergic neurons in the substantia nigra. By contrast, dopaminergic neurons in the ventral tegmental area are spared in the early stages of the disease (Harkins *et al*., 2002). However, other mechanisms are also

implicated in Parkinson's cell death as well as the nerve growth factor. This factor mediated programmed cell death in dorsal root ganglion neurons, which is an important step in the development of the nervous system. This study shows that activation of nerve growth factor receptor (also known as $p75^{NTR}$) increases plasma membrane levels of phosphatidylinositol-4,5-bisphosphate, activating GIRK channels and creating a sustained K^+ efflux that stimulates programmed cell death (Coulson *et al*., 2008). It has also been shown that this mechanism requires functional GIRK channels and that coexpression of $p75^{NTR}$ activates GIRK1/2 channels (Coulson *et al*., 2008). Therefore potassium efflux through activated GIRK channels and the resultant reduction in cytosolic potassium promotes formation of the apoptosome and activation of caspases, because maintenance of physiological levels of potassium, either exogenously *in vitro* or through use of the GIRK channel inhibitor tertiapin *in vivo*, prevented caspase activity (Coulson *et al*., 2008).

Moreover although GIRK channels have a relatively well characterized role in controlling neuronal excitability by mediating the actions of inhibitory neurotransmitters in adult neurons, the role for GIRK channels before the development of mature neuronal circuitry has not been investigated widely. It has been suggested that GIRK channel action during early postnatal development regulates maturation of functional circuits at a time when activity is primarily excitatory (Karschin and Karschin, 1996; Sickmann and Alzheimer, 2002). It has been proposed that GIRK channels participate in the fundamental process

of neuronal selection that occurs both in development and in neurodegenerative disease, as a checkpoint in the $p75^{NTR}$ mediated death pathway (Coulson *et al*., 2008) (Table 2).

Table 2. Physiological roles of GIRK subunits: effects of Girk gene mutation.

GIRK	CHANGE	PHENOTYPE	REFERENCE
GIRK1	Knockout	Hiperalgesia, morphine analgesia is reduced. Light tachycardia, loss of cardiac frequency regulation.	(Bettahi *et al*., 2002; Marker et al., 2004)
GIRK1	Knockout	Learning process alteration.	(Kourrich *et al*., 2003)
GIRK2	Knockout	Spontaneous convulsions, hiperalgesia, analgesia produced by morphine, ethanol and by agonist receptors of $GABA_B$, M_2, α_2 and cannabinoid are reduced. Hiperactivity. Decrease of cocaine auto-administration. Loss of slow inhibitory post-sinaptic potentials in hippocampus and	(Signorini *et al*., 1997; Luscher *et al*., 1997; Slesinger *et al*., 1997; Blednov *et al*., 2001a,b; Mitrovic *et al*., 2003; Morgan *et al*., 2003; Blednov *et al*., 2003; Khorodova *et al*., 2003; Hills

		cerebellum.	et al., 2003; Marker et al., 2004)
GIRK2	*Weaver* spontaneous mutation	Spontaneous tonic-clonal convulsions. Ataxia, hyperactivity, shaking movement, male sterility. Neuronal death in cerebellum cortex and sustantia nigra. Analgesia produced by opioid and ethanol are reduced. K^+ and Na^+ ions selectivity are lost in channel.	(Patil *et al*., 1995; Kofuji *et al*., 1996; Kobayashi *et al*., 1999; Ikeda *et al*., 2002)
GIRK3	Knockout	Hiperalgesia. Morphine analgesia is decreased. Decrease of cocaine auto-administration.	(Morgan *et al*., 2003; Marker et al., 2004)

GIRK4	Knockout	Light tachycardia, loss of cardiac frequency regulation. Resistance in auricular fibrillation. Spatial learning process and memory alteration.	(Wickman *et al*., 2000; Kovoor *et al*., 2001; Bettahi *et al*., 2002)
GIRK2/3	Knockout	GIRK2–GIRK3 double knockout mice self-administer more cocaine. Reduced opioid withdrawal. Met-encefaline induced current in LC neurons was significantly smaller. Hyperpolarization induced by α_2 adrenoreceptor agonist UK-14304 was significantly smaller in LC neurons.	(Torrecilla et al., 2002; Cruz et al., 2008; Torrecilla et al., 2008)

1.3.3. Pharmacology of GIRK channels.

Modulation of GIRK channels can be useful for treatment of different diseases. The utility of GIRK channel modulators should have selectivity by one or more GIRK subunits. The activity of GIRK channels can be regulated by various binds of diverse pharmacological families. Many of these binds are drugs that are used in therapies, and hence, the actions over GIRK channels can be important for explaining some therapeutic and secondary effects (Table 3).

One of the most used drugs in therapy today are antipsicotic drugs like tiorizadine, clozapine, pimozide and haloperidol that inhibit GIRK channels to different levels of efficacy. Risperidone has lower inhibitory effect onto GIRK channels (Kobayashi *et al*., 2000; Kobayashi *et al*., 2004b). For treatment of depression and other psychiatric disorders like anxiety, panic disorder or alcoholism antipsicotic drugs are used as antidepressants (Kobayashi *et al*., 2003; Kobayashi *et al*., 2004). Inhibition of NE or 5-HT transporter in the brain by antidepressants has important implications on the efficacy of these. However it has been shown that the antidepressants also inhibit other receptors and channel functions like muscarinic, α adrenoreceptor, H_1 histaminic, NMDA or nicotinic receptors and Ca^{2+}, Na^{+} and K^{+} channels regulated by voltage. Moreover among the selective 5-HT reuptake inhibitors, fluoxetine can inhibit GIRK channels whereas other 5-HT reuptake inhibitors, like citalopram or fluvoxamine have no effect over this channel overexpressed on *xenopus oocytes* (Kobayashi *et al*., 2003). GIRK

channels are also inhibited by triciclic antidepressants like imipramine, desipramine, amitriptiline, nortriptiline, clomipramine, and maprotiline (Kobayashi *et al*., 2004a). Moreover, fluoxetine and other triciclic antidepressants are able to inhibit GIRK current induced by ethanol at clinical concentrations (Kobayashi *et al*., 2003; Kobayashi *et al*., 2004a). Fluoxetine can also to produce GIRK2 *weaver* aberrant channel inhibition which has less selectivity for K^+ ions (Takahashi *et al*., 2006). Moreover fluoxetine derivates have been also identified as selective agonist and antagonist of GIRK channels (Nishizawa *et al*., 2001). All of these investigations suggest that the inhibition of GIRK channels by antidepressants could contribute to some therapeutic and secondary effects.

Ethanol and methanol increase GIRK channel function. The region which is responsible for GIRK channels activation is situated on C-terminal region between 331 and 337 aminoacids. Ethanol effect is independent of G protein activation and secondary to cytolitic messengers (Kobayashi *et al*., 1999; Lewohl *et al*., 1999). Now it is well known that activation GIRK2 subunits-containing GIRK channels mediates behavioral effects of the ethanol (Blednov et al., 2001b)

Nitric oxide for example increases GIRK channel function mildly (Milovic *et al*., 2004). On the other hand the effect of other anesthetics such as halothane, isofluorane, enfluorane or F3 (1-cloro-1,2,2-trifluorociclobutano) are able to inhibit GIRK1/2 and GIRK2/2 channels but only F3 inhibits GIRK1/4 channels (Yamakura *et al*., 2001). Local

anesthetics such as bupivacaina inhibit GIRK channels but only at high doses (Zhou *et al*., 2001) and lidocaine only inhibits GIRK channels at toxic doses.

Some other drugs have been also described as GIRK channel inhibitors, such as verapamilo, a calcium channel blocker, that is used in the treatment of angina pectoris, hypertension and in arrhythmia (Lesage *et al*., 1995). However, the most selective GIRK channel blocker is tertiapin-Q, a peptide obtained from the honey bee venom (Jin and Lu, 1998; 1999). Recently, a bioflavonoid found in the grapefruit, naringin has been characterized as a direct GIRK channels activator, which overlaps with the tertiapin-Q binding site (Yow et al., 2011).

Table 3. Pharmacology of GIRK channels. Adapted from Kobayashi and Ikeda (2006)

ACTIVATORS	**REFERENCE**
Ethanol	(Kobayashi *et al*., 1999; Lewhol *et al*., 1999; Blednov et al., 2001b)
Flupirtine (K^+ channel activator)	(Jacob and Kriegistein., 1997)
Nitric Oxide, halothane (anesthetic)	(Milovic *et al*., 2004)
Naringin (bioflavonoid)	(Yow *et al*., 2011)
INHIBITORS	**REFERENCE**
Antipsicotics: Tioridazine, Clozapine, Pimozide, Haloperidol.	(Kobayashi *et al.*, 2000; Kobayashi *et al*., 2004b)
Antidepressant: Triciclic, Fluoxetine	(Kobayashi *et al*., 2000; Kobayashi *et al*., 2004a)
Channel blockers: Quinidine, Verapamilo	(Lesage *et al*., 1995; Yamada *et al*., 1998)
Anesthetic: Enfluorane, Isoflurane, Bupivacaine.	(Yamakura *et al*., 2001; Zhou et al., 2001; Milovic *et al*., 2004)
17β-estradiol, ifenprodil	(Jin and Lu *et al*., 1998; Choi *et al*., 2003; Kobayashi *et al*., 2006)
Tertiapin-Q (Selective blocker of inward-rectifier K^+ channels)	(Jin and Lu.,1998; 1999)

2. HYPOTESIS AND OBJETIVES

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It is well known that drugs used to treat depression act by increasing the synaptic concentration of NE and/or 5-HT in a relatively selective manner. However, the mechanism of action of antidepressant drugs which act as selective monoamine reuptake inhibitors, has not yet been fully characterized. Thus, bearing in mind the papers mentioned previously in the introduction, we hypothesized that the mechanism of action of antidepressant drugs involves GIRK channels and that modification of these channels would eventually lead to the malfunctioning of these antidepressant drugs.

The main aim of this work was to investigate the possible implication of GIRK channels in the mechanism of action of antidepressant drugs and their impact on central noradrenergic neurotransmission using GIRK2 mutant mice. To this end, we established the following concrete, specific objectives:

- To determine the contribution of GIRK2 subunit-containing GIRK channels to the bioelectric properties of noradrenergic neurons in the mouse LC *in vivo* and to quantify the levels of monoaminergic neurotransmitters in WT and GIRK2 mutant mice.

- To characterize the effects induced by the administration of the α_2-adrenoceptor agonist clonidine, and the μ opioid receptor agonist morphine, on the electric activity of LC neurons in GIRK2 mutant mice *in vivo.*

- To study the role of GIRK channles on the effects induced by antidepressant drugs on noradrenergic neurotransmission by measuring the electrical activity of the LC neurons in WT and GIRK2 mutant mice *in vivo* and *in vitro*. Additionally, the effect of antidepresants on GIRK currents was examinated in LC slices from WT mice.

- To study the possible implication of GIRK channels on the behavioral response to stress and to the antidepressant-induced effect, by means of behavioral tests such as tail suspension test and activity box, using WT, GIRK2$^{+/-}$ and GIRK2$^{-/-}$ mice.

3. MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1. MATERIALS

3.1.1. Animals

C57bj6 mice (males and females) 3 months of age at the time of the experiment were used for the *in vivo* single-unit extracellular recordings and behavioral tests and 3 weeks of age for the *in vitro*. Animals were provided by Harlan Interfauna Iberica (Barcelona, Spain).

Wild-type (WT) heterozygous ($GIRK2^{+/-}$) and homozygous ($GIRK2^{-/-}$) mice were also used for electrophysiological and behavioral experiments. Initials breading pairs consisting in $GIRK2^{+/-}$ mice were kindly provided by Dr. Kevin Wickman from the University of Minesota (Mineapolis, EEUU). From this initial souce a stable colonie of GIRK2 mutant mice was grown in the animal house of the University of the Basque Country. Mice were genotyped by PCR screening as previously was describated by Torrecilla *et al*., (2002) at the Service of Genomics Bank of DNA of the Faculty of Pharmacy of UPV/EHU (Vitoria-Gasteiz).

Animals were housed under 12:12-h light: dark cycle with food and water provided *ad libitum*. Every effort was made to minimize animal suffering and to use the minimum possible number of animals.

The experimental protocols were reviewed and approved by the Local Committee for Animal Experimentation at the University of the Basque Country. All the experiments were carried out in compliance with the European Community Council Directive on "The Protection of Animals Used for Experimental and Other Scientific Purposes" (86/609/EEC) Spanish Law (RD 1201/2005) and for the care and use of laboratory animals.

3.1.2. Drugs

The following drugs were used in this study:

Drug	Activity	Purchased from
Clonidine Hydrochloride	α_2-adrenoceptor agonist	Sigma-Aldrich Química
Chloral hydrate	Anesthetic	Sigma-Aldrich Química
Desipramine Hydrochloride (DMI)	NE reuptaker inhibitor	Sigma-Aldrich Química
Fluoxetine Hydrochloride	5-HT reuptaker inhibitor	Sigma-Aldrich Química
Noradrenaline (NE) L-(−)-Noradrenaline (+)-bitartrate salt monohydrate	Adrenergic neurotransmitter	Sigma-Aldrich Química
Reboxetine mesylate	NE reuptaker inhibitor	Tocris Cookson Ltd.
RX 821002 Hydrochloride 2-(2,3-Dihydro-2-methoxy-1,4-benzodioxin-2-yl)-4,5-dihydro-1H-imidazole hydrochloride	α_2-adrenoceptor antagonist	Tocris Cookson Ltd.
Morphine	μ receptor agonist	Sigma-Aldrich, Quimica
Tertiapin-Q	Selective blocker of inward-rectifier K^+ channels	Tocris Cookson Ltd
Flupirtine	K^+ channels activator	Tocris Cookson Ltd
Naloxone	Opioid antagonist	Sigma-Aldrich Química
Bupropion	NE-DA reuptaker inhibitor	Sigma-Aldrich Química

Chloral hydrate, desipramine, clonidine, RX 821002, reboxetine and morphine were prepared in physiological saline solution (NaCl 0.9%) for *in vivo* recordings. For *in vitro* recordings, fluoxetine, desipramine, reboxetine, tertiapin-Q, flupirtine and NE were dissolved in artificial cerebrospinal fluid. With the exception of chloral hydrate, drugs were freshly prepared immediately prior to use.

The composition of artificial cerebrospinal fluid used in extracellular recording studies in brain slices was (in mM): NaCl 126; KCl 2.5; NaH_2PO_4 1.2; $MgCl.6H_2O$ 1.2; $CaCl_2.2H_2O$ 2.4; $NaHCO_3$ 21.4 and D-glucose 11 bubbled with 95% O_2/5% CO_2 for a final pH of 7.3-7.4.

3.2. METHODS

3.2.1. Intracerebroventricular drug administration

Injections into lateral ventricle were made by a 10 µl Hamilton syringe with a 26-gauge needle. The tip of the needle was inserted at a depth of 2 mm from the head surface into the right lateral ventricle, 1.0 mm caudal and 1.0 mm lateral to the intersection between the line that pases thought the eyes and its perpendicular bisector. The final volume injected was 4 µl (Sánchez-Blázquez *et al*., 1995).

All substances that were given by the intracerebroventricular (ICV) route to lightly ether anesthetized mice in.

3.2.2. Behavioral studies

All behavioral studies were conducted between 9.00 and 14.00 hours

3.2.2.1. Tail Suspension Test

Depression is a heterogeneous disorder with symptoms manifested at the psychological, behavioral and psychological level, which leads to additional difficulty in attempting to mimic the disorder in the laboratory. In clinical practice many tools have been developed and validated to better diagnose depression and the efficacy of treatment strategies in human.

Since its introduction almost 20 years ago, the tail suspension test (TST) has become one of the most widely used models for assessing antidepressant- like activity in mice. The test is based on the fact that animals subjected to the short-term, inescapable stress of being suspended by the tail, will developed an immobile posture (Cryan *et al*., 2005). The haemodynamic stress of being hung in an uncontrollable fashion by the tail causes the animal to engage in three types of escape-oriented movements: (1) running movements forward or backwards; (2) body torsions with attempts to catch the suspended body; and (3) body jerks followed temporally by bouts of immobility. A mouse is considered motionless, when it does not move its paws, or there is an absence of initiated movements. The immobility is due to inability or reluctance to maintain effort rather than a generalized hypoactivity. If antidepressant treatments are given prior the test, the amount of time animals spend immobile will be shorter than after vehicle treatment.

On the test day mice were suspended 60 cm above the floor, secured with an adhesive tape placed approximately 1cm from the tip of the tail. Animals both acoustically and visually isolated were monitored by video camera for subsequent analysis. The total duration of immobility time was scored during 10 min (Fig. 9). Before the test animals remained in the experimental room for 1h for environment habituation. For intraperitoneal administration drug were injected 15 minutes before the test. For ICV administration tertiapin-Q or vehicle was done 15 minutes before intraperitoneal injection. Results were expressed as Mean ± SEM of n experiments.

Figure 9. Tail Suspension Test. Mice were suspense 60cm above the floor and, both a coustically and visually isolated, were monitored by video camera for subsequent analysis.

.3.2.2.2. Spontaneous locomotor activity measure

The study of locomotor activation in rodents does not involved the extensive learning or conditioning required by other behavioral task. And so this dependent measure is often used as the initial screen for pharmacological effects. (Brooks. *et al*., 2009; Short protocols in Neurocience, systems and behavioral methods, Wiley, Unit 3.2 page 3-8.)

On the experiments day, mice were habituated to testing room environment for 1 hour. Then mice were placed in the activity cage. Locomotor activity was assessed in an open-field (20 x 30 x 30 mm) bar system provided by LETICA Science Instruments (LSI Letica®, LE 886, Panlab S.L., Cornellá, Barcelona). The floor consisted of a stainless steel grid, made of 28 bars of 3mm each associated to a detector of changes in electric resistance.

When the animal was placed into the chamber and it was allowed to explore freely. The activity was monitored for 30 minutes and data was collected for horizontal activity. Results were expressed as Mean ± SEM of n experiments (Fig. 10).

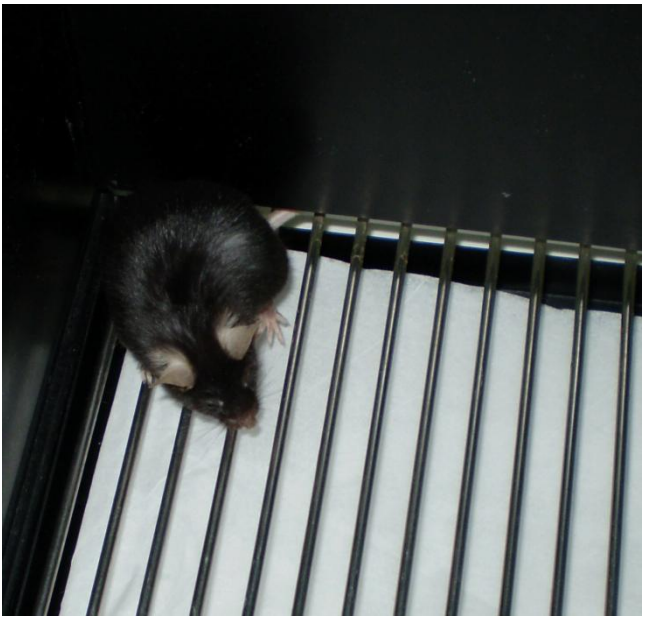


Figure 10. Locomotor activity test. Animals were placed individually in the activity chamber and were allowed to explore freely. The activity was monitored during 30 minutes.

3.2.3. Electrophysiological procedures

3.2.3.1. *In vivo* single-unit extracellular recordings of the LC nucleus neuron activity in anaesthetized mice.

A) Animal preparation and surgery

Mice were anaesthetized with chloral hydrate (400 mg/kg, i.p.). After cannulating the trachea (Clay Adams, Becton Dickinson and Company) a catheter (Terumo Surflo®) was inserted in the peritoneo for additional administrations of anaesthetic and other systemic drugs. Choral hydrate is an anaesthetic which has not marked effects over LC nucleus firing activity so is a perfect anaesthetic to use in this electrophysiological study (Korf, *et al*., 1974). The mouse was placed in stereotaxic frame using Kopf® mouse adaptor with the skull positioned horizontally. The body temperature was maintained at 37ºC for the entire experiments by means with heating pad. A bur hole was drilled and for LC recording an electrode was placed in the following coordinates 1.5mm posterior to lambda and 0.2-1.2mm form the midline and lowered into the LC usually encountered at a depth of range between 2.7 and 4.0mm from the brain surface (Gobbi *et al*., 2007).

B) Neuronal identification and recording

Single-unit extracellular recordings of LC neurons were performed as described previously (Gobbi *et al.*, 2007 ; Miguelez *et al*., 2009.). The recording electrode, consisting of an Omegadot single-barrel glass micropipette was filled with 2% solution of Pontamine Sky Blue in 0.5% sodium acetate and broken back to a tip diameter of 1-2 µm. The electrode was lowered into the brain by means of a hydraulic microdrive (David Kopf® Instruments, Tujunga, California, EEUU, model 640). LC neurons were identified by spontaneous activity displaying a regular rhythm and firing rate between 0.5-5 Hz, characteristic spikes with a long-lasting (2-2.5ms), positive-negative waveform action potentials and the biphasic excitation-inhibition response onto pressure applied to the contralateral hind paw (paw pinch), as previously observed in mice (Gobbi *et al.*, 2007) and (Cedarbaum and Aghajanian, 1976) (Fig. 11).

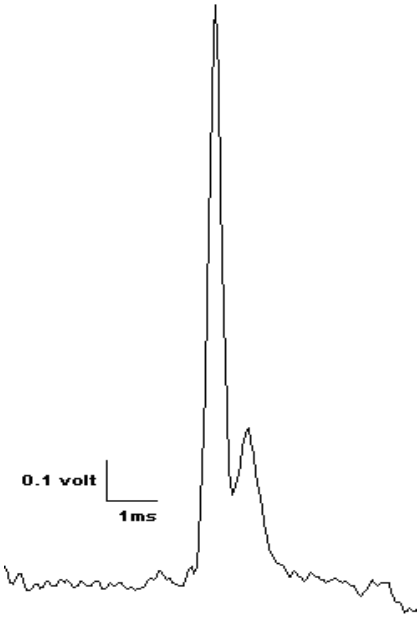


Figure 11. Representative example of a spike from the mouse LC nucleus neurons recorded *in vivo*. Pictures were taken from *Spike2* recordings. The abscise and ordinate axes represent duration (ms) and amplitude (volt) of the action potential signal, respectively.

The extracellular signal from the electrode was preamplified and amplified later with a high-input impedance amplifier (Cibertec S.A., model amplifier 88), and then monitored on an oscilloscope (Tektronix® 5111A) and on an audio monitor (Cibertec S.A., model AN-10) (Fig. 12). This activity was processed using computer software (CED micro 1401 interface and Spike2 software, Cambrigde Electronic Design, UK). Firing patterns were determined by analysing the interspike interval histogram: firing rate, the coefficient of variation (percentage ratio of standard deviation to the mean interval value of an interspike time-interval histogram), percentage of spikes in burst, mean spikes/burst, percentage of cells exhibiting burst firing and response to drug administration. Changes in firing rate were expressed as percentages of the baseline firing rate (mean firing rate during 3 min prior drug injection) and were measured after each dose of drug. Only one cell was studied in each animal when any drug was administered.

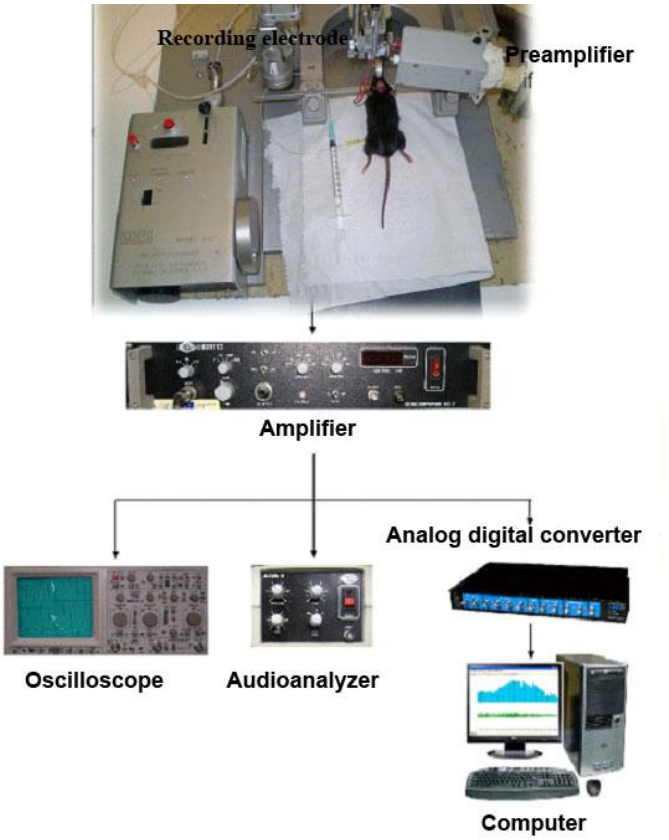


Figure 12. Schematic illustration of extracellular electrophysiological procedure in anaesthetized mice. Briefly, the extracellular signal recorded was passed through a high-input impedance amplifier, displayed in an oscilloscope and monitored with an audioanalyzer. Individual (single-unit) neuronal spikes were isolated and analyzed by means of PC-based software *Spike2*.

C) Histological verification procedures

At the end of each experiment, a Pontamina Sky Blue mark was deposited in the recording site for posterior verification. Five μA cathodic current was constantly applied for 10 minutes through the recording electrode (FICTE 10, Cibertec®, Spain). The brains were carefully dissected and subsequently sliced with a Vibratome. The location of the Pontamin Sky Blue mark was visually examined. Only measurements from cell within the LC were included in the analysis. LC was identified as a dark oval area in the upper pons on the lateral borders of the central gray.

3.2.3.2. Cell-attached and patch-clamp recordings of the LC neuron activity in mouse brain slices

A) Brain slice preparation

Male C57bj6 mice (3 weeks old) were anaesthetized with chloral hydrate (400 mg/kg, i.p) and decapitated. The brain was immediately extracted after death and placed in cooled artificial cerebrospinal fluid. Coronal brainstem sections of 200 μm thickness containing the LC were cut using a microtome with vibrating blade (Microm Model HM 650 V, Barcelona Spain). LC slices were incubated for at least 1h and them transferred to a recording chamber that was contently superfused with warmed (35ºC) physiological saline solution containing (in mM): 125 NaCl, 2.5 KCl, 1 $MgCl_2$, 26 NaH_2CO_3, 1.25 NaH_2PO_4, 11 glucose, 2.4 $CaCl_2$, and bubbled with 95% O_2 and 5% CO_2 (pH 7.3-7.4) at a flow rate of 1.5 ml/min.

B) Neuronal identification and recording

Patch-clamp procedure was used to measure spontaneous action potential signal from mice LC slices using the cell-attached configuration as previously described (Paladini et al., 2007). For that purpose glass pipettes were filled with the above mentioned external solutions. Single units were amplified with an Axoclamp 2B amplifier (Axon Instruments, Foster City, CA) and post-amplified and filtered with a Cibertec (Madrid, Spain) amplifier. The computer softwear that used for analysis was

Axograph 4.9 (Axon Instruments, Foster City, CA). Noradrenergic cells were physiologically identified by the regular spontaneous discharging activity, the slow firing rate (0.5-1 Hz) and the long-lasting biphasic positive-negative waveform (2-4 ms) (Andrade *et al*., 1983).

Resting and drug-induced potassium currents were measured in voltage-clamp mode (whole-cell configuration) with the membrane potential held at -60mV. For that purpose glass pipettes were filled with internal solutions (in mM): HEPES 10; K-Gluconate ($C_6H_{11}O_7K$) 130; NaCl 5; $MgCl_2$ 1; Na_2-ATP 2; Tris-GTP 0.5 and EGTA 0.02. Current–voltage (I-V) relationships were obtained by stepping the membrane potential from -50mV to -130mV in -10 mV increments (100 msec per step). Patch pipettes had a tip resistance of 2-4 MΩ (Torrecilla *et al* 2002) and recordings were detected with an Axopatch-200B (Axon Instruments®), Foster City, CA), filtered at 5 kHz and digitized with a Digidata 1322A (Axon Instruments®), Foster City, CA). Data were sampled at 10 kHz and analyzed with Axograph 4.9 software (Axon Instruments).

LC neurons were visualized using an upright microscope with infrared optics (Eclipse E600FN, Nikon®) as a dense and compact group of cells located on the lateral border of the central gray and the fourth ventricle, just anterior to the genu of the facial nucleus (Fig. 13). Schematic illustratrion of cell-attached and patch-clamp electrophysiological procedure is shown in figure 14.

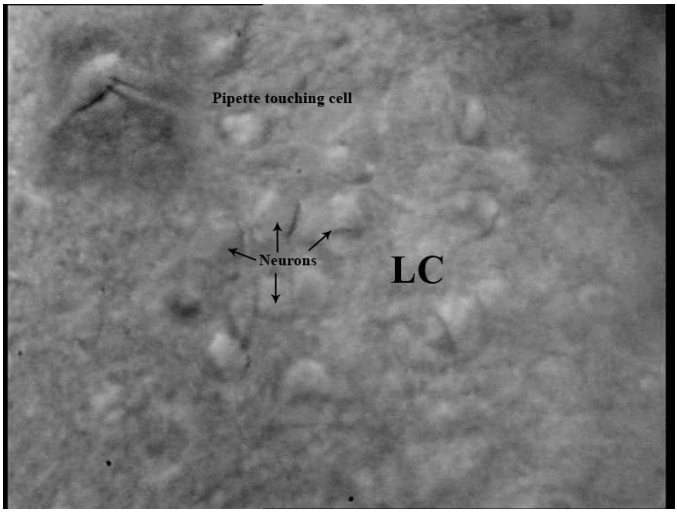


Figure 13. LC neurons were visualized using an upright microscope with infrared optics. LC nucleus is a dense and compact group of cells located on the lateral border of the central gray and the fourth ventricle. The top left shows the recording the pipette patching the neuron (top left).

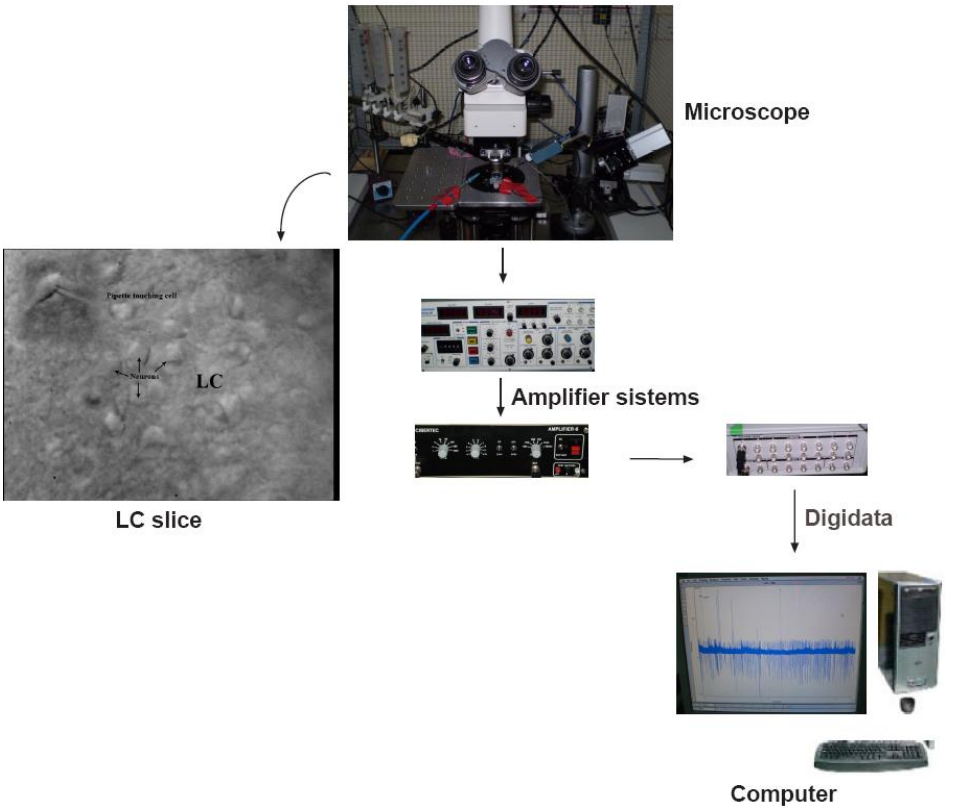


Figure 14. Schematic illustration of the electrophysiological procedure carried out in mouse LC slice. Briefly, the cell signal was amplified and filtered at 5 kHz (Axopatch-200B), and digitized with a Digidata 1322A (all from Axon Instruments, Foster City, CA). Data were sampled at 10 kHz and later analyzed with Axograph 4.9 software (Axon Instruments).

3.2.4. High Performance Liquid Chromatography

The High Performance Liquid Chromatography **(**HPLC) assay using a carbon-based electrochemical detector electrode measures catechol and indole compounds with high sensitivity and selectivity. It is currently used as the methods of choice for the routine analysis of monoamines of brain.

The chromatographic conditions were optimized to allow the simultaneous separation of 5-HT, 5-HIIA and NE in mice brain regions. Representative chromatograms are presented in (Fig. 15)

The HPLC equipment was formed by following list:

- Waters 6000 Solvent Delivery System.
- Waters 717 refrigerated automatic injector set at 4ºC.
- Waters 2465 Electrochemical Detector set at 0.6V vs a ISAAC reference electrode.
- Waters Empower program for the treatment of chromatographic data.

We have utilized a Waters Symetry C18 HPLC column (250 x 4.6 mm) 5μm particle size; and a C18 5 μm particle size precolumn. The

separation was performed at room temperature at a flow - rate of 1 ml/min.

The mobile phase was prepared with purified water and filtered and degassed before use through a 0.45 μm membrane (Millipore).

The mobile phase consisted of:

- 1 litre of 0.02M citric acid containing 1,204 gr of heptanosulfonic acid.
- 0.5 litre of 0.02M dinatriumhidrogenphosphate containing 22mg of EDTA.
- 15% Methanol (vol:vol).

3.2.4.1. Sample preparation

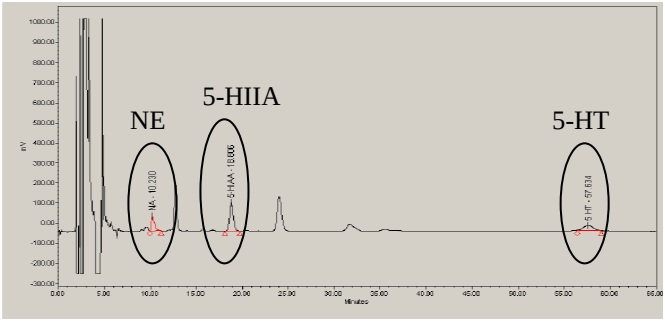
Mice were anesthetized with chloral hydrate (400 mg/kg, i.p.) before sacrifice. The dissection of the LC, DRN and PFC was performed on an ice-cooled plate; the tissue was weighted and stoned at - 40ºC. The day of the assay the tissue was homogenized in 0.1 M perchloric acid, centrifuged (30 min, 12,500 x g) and the supernatant was spin-filtered. Finally, one aliquot, equivalent to 2 mg of tissue, was assessed by HPLC with electrochemical detection (Andia *et al*., 1994).

3.2.4.1. Analysis of 5-HT, 5-HIIA and NE

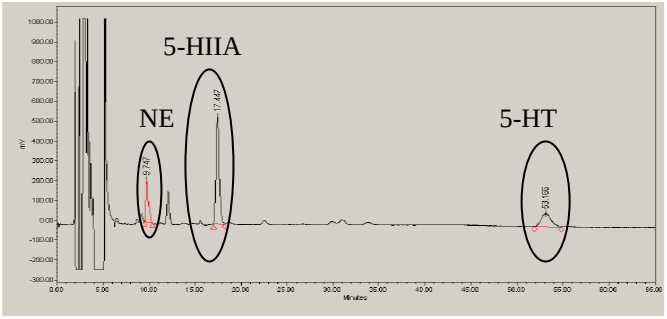
Concentrations of monoamines in the samples were determined by calculating peak height compared to a calibration curve performed with known amount of standards.

All the standards were prepared in 0.1M perchloric acid at a concentration of 1 mg/ml, and stored at 4ºC where they are stable, at least, one month. A calibration curve was prepared daily in 0.1M perchloric acid containing: NE (0.15 to 1.2 ng/10 µl), 5-HIIA (0.3 to 2.4 ng/10 µl) and 5-HT (0.25 to 2 ng/10 µl).

PFC



LC



DRN

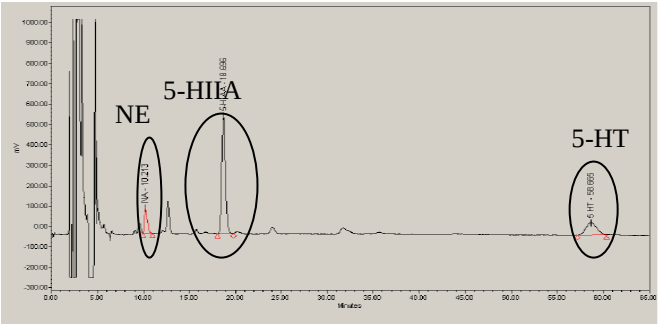


Figure 15. Different chromatograms in three brain areas: LC, PFC and DRN. Concentrations of monoamines in the samples were determined by calculating peak height compared to a calibration curve performed with known amount of standards.

3.2.5. Data and statistical analysis

3.2.5.1. Data analysis

Behavioral experiments were analyzed as previously described for each test.

The analysis of electrophysiological recordings was carried out by means of the software *Spike2* (*in vivo* experiments) and *Axograph 4.9* (*in vitro* experiments).

The following parameters were estimated in experiments done *in vivo*:

A. Firing rate: Defined as the number of neuronal discharges per second. Data were represented in a bar histogram that showed the mean firing rate each 10 seconds. Basal firing rate was recorded for 180 seconds and mean firing rate after drug administration for 40-60 seconds (depending on *plateau* effect for each drug).

B. Coefficient of variation: This parameter is related to the interval between consecutive discharges (inter-spike interval) and gives an idea of the regularity of the firing. The representation of the inter-spike interval histogram followed by the analysis ran by *Spike 2* (script Meaninx.s2s) lead to the numerical value. Data was represented in percentage, as the division between standard deviation and mean value. The recording period analyzed was the same used for the basal firing rate.

C. Burst spikes: Neurons from the LC show burst firing activity. In those neurons the percentage of spikes in burst as well as the mean spikes per burst was analyzed. The beginning of a burst was defined as an inter-spike interval of less than 20 ms and the end of a burst corresponded to an interval of more greater than 160 ms. The analysis of the bust firing was performed by *Spike 2* (script w_burst.s2s) in the same period as the previous parameters.

D. Dose-response curve: Dose-response curves were analyzed for the best non-linear fit to a logistic three-parameter equation (Parker and Waud, 1971):

$$E = \frac{E_{max}\,[A]^n}{(ED_{50}^{\,n} + [A]^n)}$$

[*A*] is the i.p. dose of the used drug

E is the effect on the firing rate induced by *A*

E_{max} is the maximal percentage change at "infinite" dose (100 %)

ED_{50} is the effective dose for eliciting 50 % of E_{max}

n is the slope factor of the dose-response curve.

3.2.5.2. Statistical analysis

Graph Prism (v.5.01; GraphPad Software, Inc.) were used for statistical evaluation. The level of significance was considered as $p < 0.05$.

Statistical significance of neurochemical data, behavioral results and electrophysiological parameters such as basal firing rate and coefficient of variation, was assessed by means of Student's t test and *One-way* analysis of variance (*ANOVA*) followed by *Newman-Keuls post hoc test*, for pair and multiple comparison, respectively. Analysis of burst patter was carried out by the *Fisher´s exact test* and *Kruskal-Wallis test followed by Dunn´s post hoc test*.

Dose-response curves for the inhibitory effect of agonist were constructed by systematic administration of the drug at acumulative doses until a maximal response was reached. *Nonlin-fit* analysis of dose-response curves and fit comparison were done using *extra sum-of-squares F test* (Graph Prism 5.01, GraphPad Software, Inc.)

4. RESULTS

4.1. STUDY I

Characterization of electrophysiological properties of LC neurons from GIRK2 mutant mice *in vivo*.

STUDY I

4.1. Characterization of electrophysiological properties of LC neurons from GIRK2 mutant mice *in vivo.*

Several *in vitro* studies suggest that GIRK channels contribute to neuronal intrinsic excitability (Luscher *et al*., 1997; Torrecilla *et al*., 2002; Chen and Johnson, 2005; Koyrakh *et al*., 2005; Luscher *et al*., 2007 and Cruz *et al*., 2008). Here, we investigated the role of these channels on the *in vivo* spontaneous activity of LC neurons using GIRK2 mutant mice.

As previously described in the rat, LC neurons recorded from WT and GIRK2 mutant mice also showed a characteristic spike with a long-lasting 2-2.5 ms and positive-negative waveform (Fig. 16). LC neurons from GIRK2 mutant mice also displayed a biphasic excitation-inhibition response to a pinch of the contralateral paw (Fig. 17) as previously reported in rats (Cedarbaum and Aghajanian, 1976) and more recently in mice (Gobbi *et al*., 2007).

Regarding basic electrophysiological properties (Table 4), the firing frequency of LC neurons from GIRK2$^{-/-}$ mice was not significantly different from that measured in WT or GIRK2$^{+/-}$ mice (WT: 2.60 ± 0.16 Hz, n = 109; GIRK2$^{+/-}$: 2.23 ± 0.14 Hz, n = 91; GIRK2$^{-/-}$: 2.08 ± 0.16 Hz, n = 47, $p > 0.05$) (Fig. 18A). Similarly, the coefficient of variation did

not differ among genotypes (WT: 50.77 ± 1.41 %, n = 109; GIRK2$^{+/-}$: 45.82 ± 2.09 %, n = 91; GIRK2$^{-/-}$: 50.79 ± 2.06%, n = 47, $p > 0.05$) (Fig. 18B). Nevertheless, the number of neurons discharging in burst was significantly higher in GIRK2$^{-/-}$ mice (55% of total recorded cells, $p < 0.001$) as well as in GIRK2$^{+/-}$ mice (26% of total recorded cells, $p < 0.01$) compared to WT animals (10% of the total recorded neurons). Statistical differences were also found between mutant genotypes ($p < 0.001$) (Fig. 19).

A more extended study of the burst pattern (Fig. 20) was done by analyzing several parameters such as the mean firing rate, the number of burst episodes, the mean spikes per burst, the percentage of spikes firing in burst and the mean inter-spike interval from burst-firing neurons (Table 5). Thus, the basal firing rate of mutant mice was significantly reduced compared to that recorded in the WT group (WT: 3.18 ± 0.60 Hz, n = 11; GIRK2$^{+/-}$: 1.93 ± 0.18 Hz, n = 25; GIRK2$^{-/-}$: 2.43 ± 0.20 Hz, n = 26, $p < 0.01$). Although, the rest of analyzed parameters did not show significant differences as comparing WT and GIRK2$^{-/-}$ genotypes, the burst pattern obtained from GIRK2$^{+/-}$ mice was significantly altered. Thus, the mean spikes per burst ($p < 0.05$) as well as the mean inter-spike interval ($p < 0.01$) were reduced compared to those recorded from WT mice. Also LC neurons from GIRK2$^{+/-}$ mice displayed a reduced number of burst episode ($p < 0.01$) and percentage of spikes in burst firing ($p < 0.01$) than those obtained from the GIRK2$^{-/-}$ mice. Overall, these results suggest that the lack of GIRK2 subunits alters the firing pattern of

LC neurons. Moreover, it seems that this pattern is specifically modified in burst-firing neurons from GIRK2$^{+/-}$ mice.

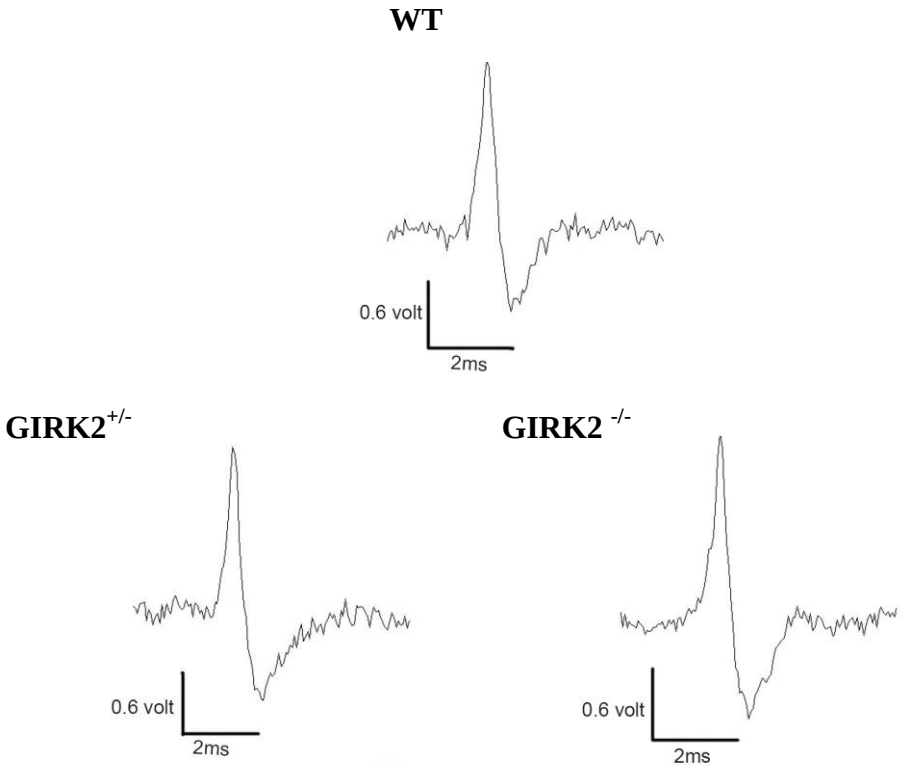


Figure 16. *In vivo* spontaneous activity of LC neurons from WT and GIRK2 mutant mice. Characteristic waveform of an action potential recorded from a mouse LC neuron *in vivo*. The shape of an action potential recorded *in vivo* in from GIRK2 mutant mouse LC displayed those characteristics previously reported in the rat and WT mouse (Cerdarbaum and Aghajanian., 1976; Gobbi *et al*., 2007).

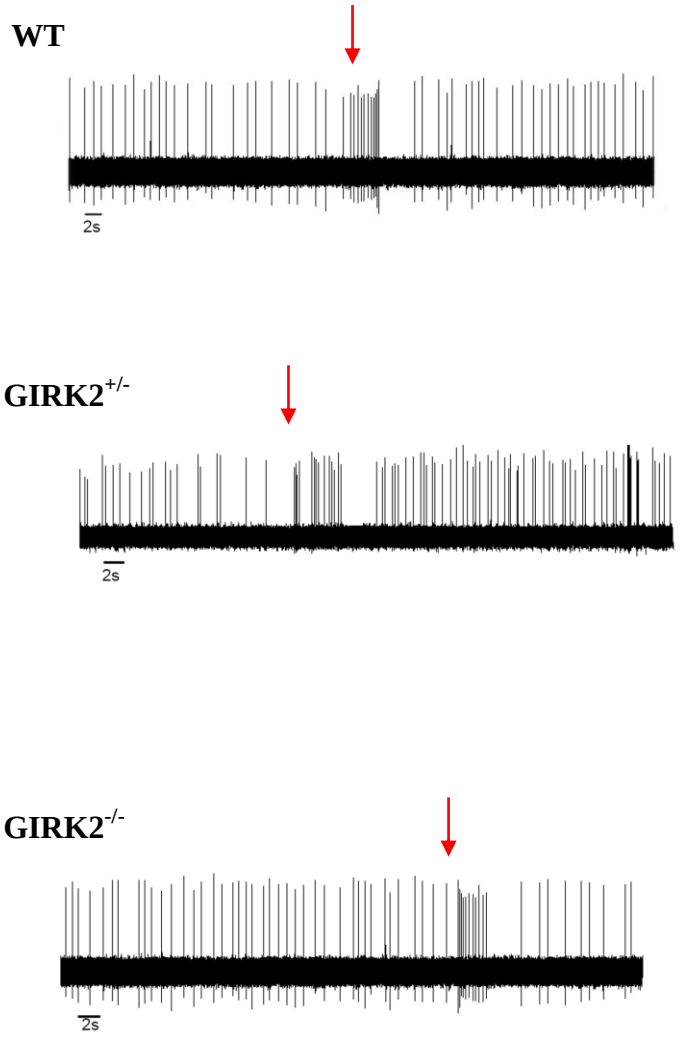


Figure 17. *In vivo* spontaneous activity of LC neurons from WT and GIRK2 mutant mice. LC neurons from GIRK2 mutant mice also responded to a pinch of the contralateral paw with a brisk increase in firing rate followed by a short pause (what so-called Korf's response). Arrow indicates pinch of paw.

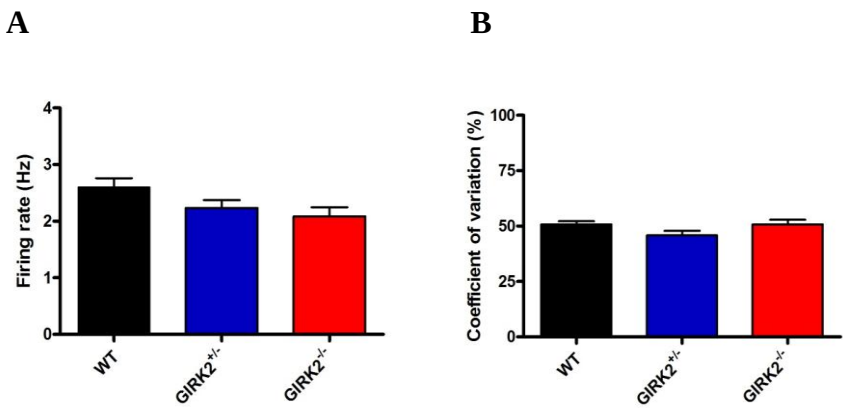


Figure 18. LC neurons from GIRK2 mutant mice showed similar basal firing rate and regularity than those from WT mice *in vivo*. (A) The elimination of either one ($GIRK2^{+/-}$) or both **(**$GIRK2^{-/-}$) alleles for GIRK2 subunit did not alter the firing rate **(B)** nor the coefficient of variation of LC neurons recorded *in vivo*. All the values represented are the mean ± S.E.M. of n experiments (n = 109, n = 91, n = 47 of WT, $GIRK2^{+/-}$ and $GIRK2^{-/-}$ mice, respectively).

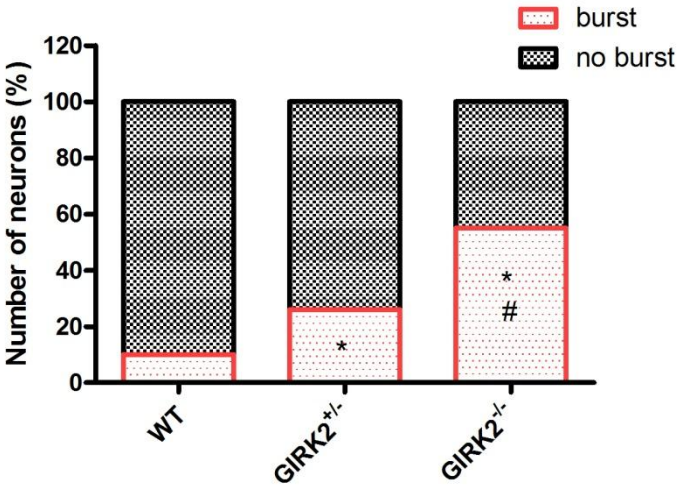


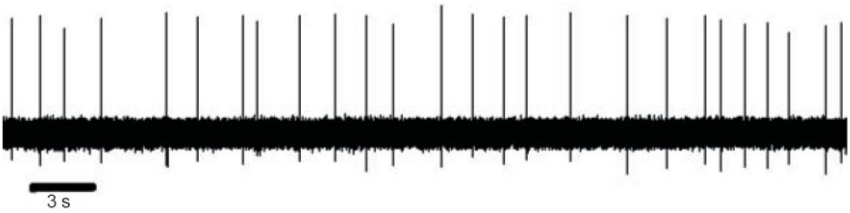
Figure 19. GIRK2 mutant mice showed a greater percentage of neurons discharging in burst than WT mice *in vivo*. The relative amount of LC neurons recorded exhibiting burst firing was increased in the GIRK2$^{+/-}$ genotype and even more in the GIRK2$^{-/-}$ group. Significant differences were also found between mutant groups (*$p < 0.01$ and ** $p < 0.001$ *vs* WT and $^{\#}p < 0.001$ *vs* GIRK2$^{+/-}$, *Fisher's exact test*).

Table 4: Electrophysiological properties of LC neurons from mouse *in vivo*: role of GIRK channels.

	WT (n = 109)	GIRK2$^{+/-}$ (n = 91)	GIRK2$^{-/-}$ (n = 47)
Firing rate (Hz)	2.60 ± 0.16	2.23 ± 0.14	2.08 ± 0.16
Coefficient of variationt (%)	50.77 ± 1.42	45.82 ± 2.09	50.79 ± 2.06
Burst (% of neurons)	10	26 *	55 $^{**\ \#}$

Each value represents the mean ± S.E.M. of n experiments (*p < 0.01 and **p < 0.001 *vs* WT mice; $^{\#}$p < 0.001 *vs* GIRK2$^{+/-}$ mice, *Fisher´s test).*

A Firing pattern of a neuron without burst



B Firing pattern of a neuron with burst

Figure 20. Role of GIRK2 channels on the burst pattern of LC neurons *in vivo*. (**A)** Representative recording of a LC neuron from WT mouse without burst firing and (**B)** from GIRK2$^{-/-}$ mouse discharging several burst episodes.

Table 5: Electrophysiological properties of LC neurons with burst activity *in vivo*: role of GIRK channels

	WT (n = 11)	GIRK2$^{+/-}$ (n = 25)	GIRK2$^{-/-}$ (n = 26)
Firing rate (Hz)	3.18 ± 0.60	1.93 ± 0.18**	2.43 ± 0.20*
Number of burst	3.72 ± 0.91	2.04 ± 0.27$^{\#}$	6.07 ± 1.30
Spikes in burst (%)	3.23 ± 0.99	0.87 ± 0.09$^{\#}$	4.43 ± 0.87
Mean spikes/burst	4.18 ± 0.65	2.72 ± 0.27*	3.03 ± 0.19
Mean interspike (ms)	39.53 ± 4.41	20.02 ± 3.20**$^{\#}$	34.71 ± 2.98

Each value represents the mean ± S.E.M. of n experiments (*$p < 0.05$ and **$p < 0.001$ *vs* WT; $^{\#}p < 0.01$ *vs* GIRK2$^{-/-}$ mice; for firing rate analysis, *One-way ANOVA following Newman-Keuls test*, otherwise for burst pattern analysis, *Kruskal-Wallis test followed Dunn's test*).

4.2. STUDY II

The inhibitory effect induced by clonidine and morphine on LC neurons from GIRK2 mutant mice *in vivo*.

STUDY II

4.2. The inhibitory effect induced by clonidine and morphine on LC neurons from GIRK2 mutant mice *in vivo.*

In LC neurons α_2-adrenoceptors and μ opioid receptors are coupled to GIRK channels formed by GIRK2 and GIRK3 subunits (Torrecilla *et al*., 2002; Cruz *et al*., 2008; Torrecilla *et al*., 2008). Here, we investigated the role of GIRK2 subunit on the effect induced by the activation of these receptors on the basal firing rate of LC neurons *in vivo.*

Thus, increasing doses of the α_2 adrenoceptor agonist, clonidine (2-130 μg/Kg, i.p.) caused a progressive and complete inhibition of the firing rate in all genotype tested. However, significant differences were detected regarding the ED_{50} parameter. The dose-response curve for clonidine was shifted to the right on the $GIRK2^{+/-}$ mice (ED_{50}: 73.26 ± 9.06 μg/Kg, n = 5, $p < 0.0001$) and further more on the $GIRK2^{-/-}$ animals (ED_{50}: 109.60 ± 12.78 μg/Kg, n = 5, $p < 0.0001$) compared to that obtained from WT mice (ED_{50}: 35.89 ± 3.02 μg/Kg, n = 10) (Fig. 21 and 23).

Similarly, the administration of the μ opioid receptor agonist, morphine (0.5-64 mg/Kg, i.p.) reduced the firing rate progressively reaching a complete inhibition on WT mice (ED_{50}: 4.35 ± 0.45 mg/Kg, n = 6). In the $GIRK2^{+/-}$ mice morphine showed a reduced potency (ED_{50}: 6.15 ± 0.26 mg/Kg, n = 4, $p < 0.0001$) which was even weaker on the $GIRK2^{-/-}$ group (ED_{50}: 36.30 ± 1.40 mg/Kg, n = 5, $p < 0.0001$). However, the efficacy of morphine inhibiting LC firing rate in either genotype remained unaltered (Fig. 22 and 24).

Moreover, in each case the inhibition produced by the agonist was similarly reverted in all genotypes with the corresponding antagonist: the α_2-adrenoceptor antagonist RX 821002 (5-15 mg/Kg, i.p.) (WT: 94.01 ± 16.84%, n = 4; $GIRK2^{+/-}$: 78.61 ± 14.87%, n = 8; $GIRK2^{-/-}$: 73.75 ± 17.91%, n = 4) (Fig. 25 A) and μ opioid receptor antagonist naloxone (5-12 mg/Kg, i.p.), (WT: 69.60 ± 3.73%, n = 4; $GIRK2^{+/-}$: 72.04 ± 21.05%, n = 3; $GIRK2^{-/-}$: 84.06 ± 16.46%, n = 4) (Fig. 25 B).

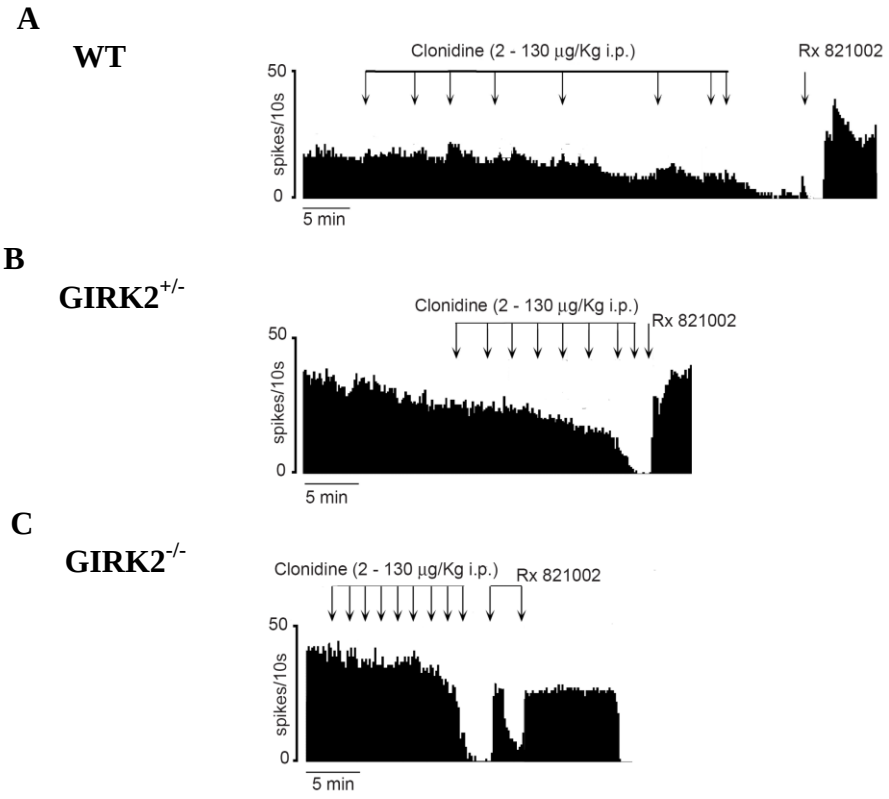


Figure 21. Representative firing rate histograms illustrate the inhibitory effects of clonidine on LC activity from WT and GIRK2 mutant mice. In all genotypes increasing doses of clonidine (2-130 µg/Kg, i.p.) induced a maximal reduction of the firing rate which was reverted by the administration of the α_2 adrenoceptor antagonist RX 821002 (5-15 mg/Kg) However, the potency of clonidine inhibiting LC activity was decreased in the GIRK2$^{+/-}$ mice and furthermore in the GIRK2$^{+/-}$ animals. Vertical arrows indicate the drug administration events.

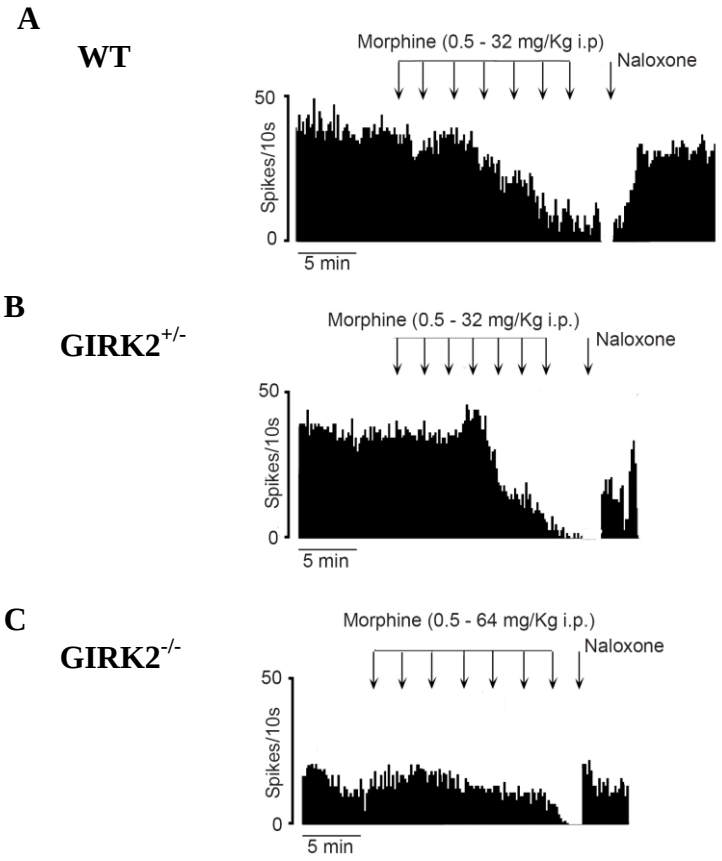


Figure 22. Representative firing rate histograms illustrate the effects of increasing doses of morphine on LC neuronal activity from WT and GIRK2 mutant mice. In all genotypes increasing doses of morphine (0.5-64 mg/Kg, i.p.) induced a maximal reduction of the firing rate which was reverted by the administration of antagonist naloxone (5-12 mg/Kg). However, the potency of morphine inhibiting LC activity was decreased in the GIRK2$^{+/-}$ mice and furthermore in the GIRK2$^{-/-}$ animals. Vertical arrows indicate the drug administration events.

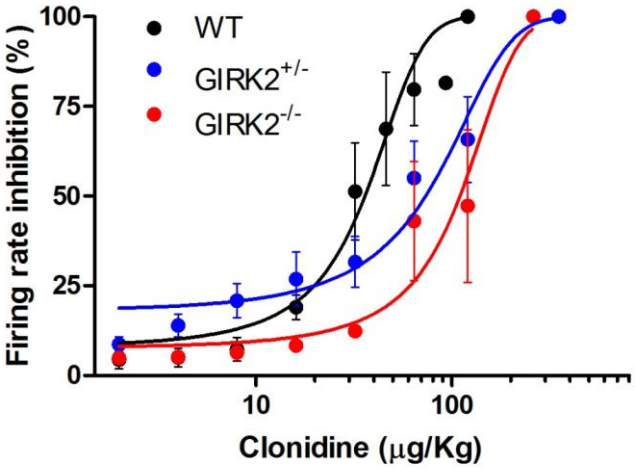


Figure 23. Effect of clonidine on the basal firing rate of the LC neurons from WT and GIRK2 mutant mice. Increasing doses of clonidine (2-130 μg/Kg, i.p.) caused a progressive and complete inhibition of the firing rate in all genotype tested. However, the potency exhibited by clonidine inhibiting the firing rate was reduced in the GIRK2$^{+/-}$ mice (ED_{50}: 73.26 ± 9.06 μg/Kg, n = 5, $p < 0.0001$) and even more in the GIRK2$^{-/-}$ animals (ED_{50}: 109.60 ± 12.78 μg/Kg, n = 5, $p < 0.0001$) compared to that in WT mice (ED_{50}: 35.89 ± 3.02 μg/Kg, n = 10). Each point of the curve represents the mean ± S.E.M. of n experiments. *Nonlin-fit analysis.*

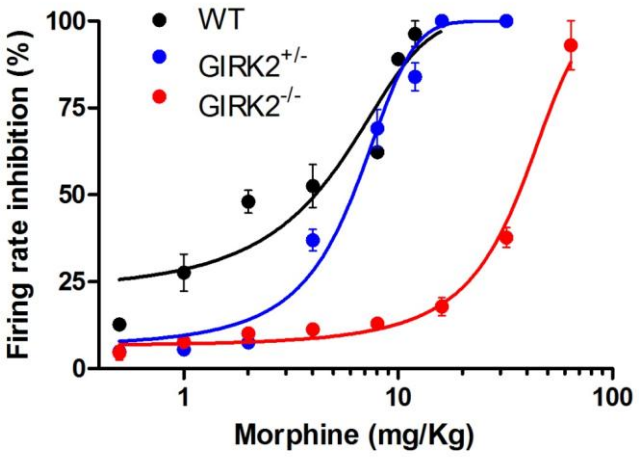
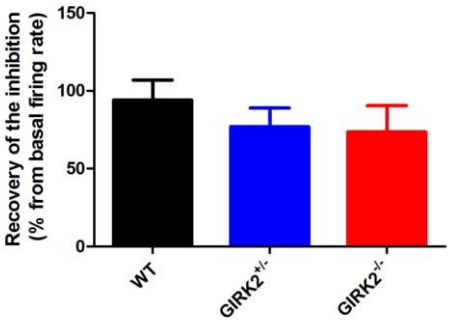


Figure 24. Effect of morphine on the basal firing rate of the LC neurons from WT and GIRK2 mutant mice. Increasing doses of morphine (0.5-64 μg/Kg, i.p.) caused a progressive and complete inhibition of the firing rate in all genotype tested. However, the potency exhibited by morphine inhibiting the firing rate was reduced in the GIRK2$^{+/-}$ mice (ED_{50}: 6.15 ± 0.26 mg/Kg, n = 4, $p < 0.0001$) and even more in the GIRK2$^{-/-}$ animals (ED_{50}: 36.30 ± 1.40 mg/Kg, n = 5, $p < 0.0001$) compared to that in WT mice (ED_{50}: 4.35 ± 0.45 mg/kg, n = 6). Each point of the curve represents the mean ± S.E.M. of n experiments. *Nonlin-fit analysis*.

A



B

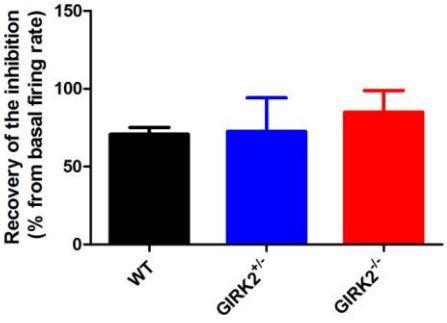


Fig 25. Similar recovery of the inhibitory effect induced by clonidine and morphine in LC neurons from WT and GIRK2 mutant mice. A) The α_2 antagonist RX 821002 (5-15 mg/Kg, n = 4-8) completely recovered the inhibition caused by clonidine in all genotypes. **B)** Similarly, naloxone (5-12 mg/Kg, n = 3-4) reverted the inhibition produced by morphine in WT and GIRK2 mutant mice. All values were expressed as mean ± S.E.M. of n experiments.

4.3. STUDY III

Role of GIRK channels on the mechanism of action of antidepressants: *in vivo* and *in vitro* study.

STUDY III

4.3. Role of GIRK channels on the mechanism of action of antidepressants: *in vivo* and *in vitro* study.

4.3.1. *In vivo* study.

The impact of GIRK2 subunit ablation on the inhibitory effect induced by NE reuptaker inhibitory such as DMI and reboxetine on the spontaneous activity of LC neurons was next evaluated *in vivo*.

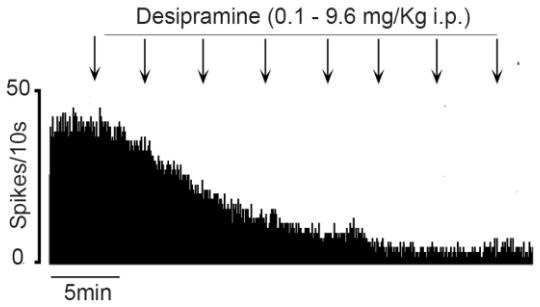
We first administrated DMI (0.2-9.6 mg/Kg, i.p.) to WT and GIRK2$^{+/-}$ animals. In both groups DMI produced a progressive inhibition of the spontaneous firing rate, reaching a maximal effect of 86.81 ± 4.51% in the WT and 97.34 ± 4.42% in the GIRK2$^{+/-}$ group. Significant differences were observed regarding DMI potency inhibiting LC firing activity. Thus, the dose-response curve for DMI showed a greater ED_{50} value in the mutant mice (GIRK2$^{+/-}$: 1.03 ± 0.10 mg/Kg, n = 5) compared to that obtained from the WT group (WT: 0.42 ± 0.07 mg/Kg, n = 6, $p < 0.0001$) (Fig. 26 and 28). The drug efficacy was similar in both genotypes since the maximal effect in the WT and GIRK2$^{+/-}$ group was not different.

Systemic administration of reboxetine (0.05-3.2 mg/Kg, i.p.) also caused a progressive inhibition of LC firing in WT mice, reaching a

maximal decrease of 91 ± 3.39% from the basal frequency and the value of ED_{50} was 0.20 ± 0.04 mg/Kg (n = 5). In $GIRK2^{+/-}$ mice, however, the drug did not only show a weaker potency but also a reduced efficacy inhibiting LC activity (ED_{50}: 0.50 ± 0104 mg/Kg, E_{max}: 33.30 ± 3.33%, n = 8, $p < 0.05$). Furthermore, reboxetine failed to modify LC spontaneous frequency in the $GIRK2^{-/-}$ mice. In this group the maximal inhibitory effect was a 9.38 ± 1.28% of the basal firing rate (n = 5) (Fig. 27 and 29).

A

WT



B

GIRK2$^{+/-}$

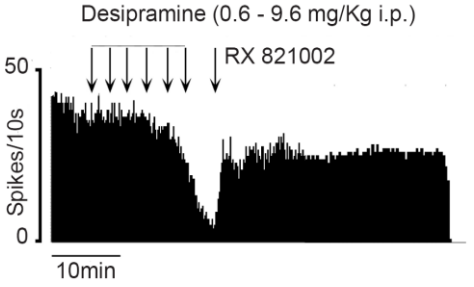


Figure 26. Representative firing rate histograms illustrate the effects of increasing doses of DMI on LC neuronal activity from WT and GIRK2 mutant mice. In both genotypes increasing doses of DMI (0.1-9.6 mg/Kg, i.p.) induced a reduction of the firing rate. The effect was reverted by the administration of the antagonist RX 821002 (10-20 mg/Kg). However, the potency of DMI inhibiting LC activity was decreased in the GIRK2$^{+/-}$ mice. Vertical arrows indicate drug administration events.

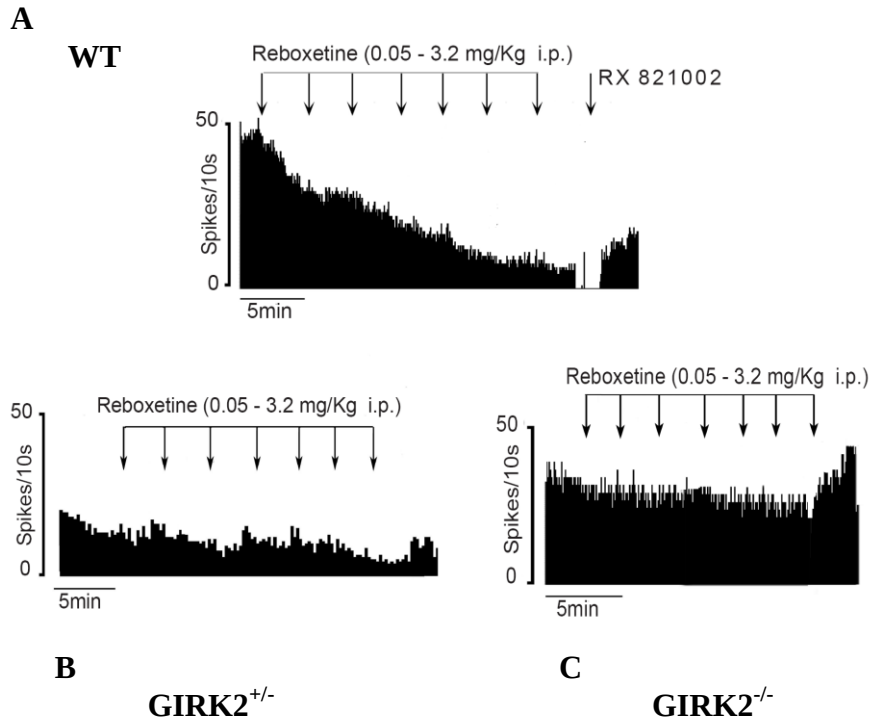


Figure 27. Representative firing rate histograms illustrate the effects of increasing doses of reboxetine on LC neuronal activity from WT and GIRK2 mutant mice. Increasing doses of reboxetine (0.05–3.2 mg/Kg, i.p.) induced a reduction of the firing rate in WT and GIRK2$^{-/-}$ mice, which was reverted by the administration of the antagonist RX 821002 (10-20 mg/Kg). In WT mice firing rate inhibiton was 100%. However, the highest dose in GIRK2$^{+/-}$ failed to obtain a complete inhibition. So, the potency of reboxetine inhibiting LC activity was decreased in the GIRK2$^{+/-}$ mice and furthermore in the GIRK2$^{-/-}$ animals, in which reboxetine did not produce any effects. Vertical arrows indicate drug administration events.

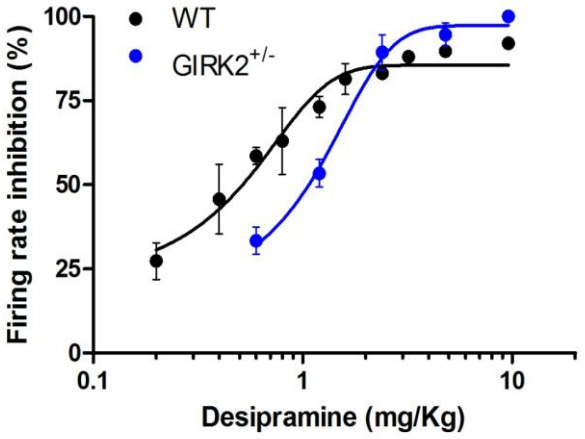


Figure 28. Effect of DMI on the basal firing rate of the LC neurons from WT and GIRK2 mutant mice. Increasing doses of DMI (0.2- 9.6 mg/Kg, i.p.) caused a progressive inhibition of the firing rate in both genotype tested. In the WT mice DMI caused a maximal inhibition of 86.81 ± 4.51% and the ED_{50} value was 0.42 ± 0.07 mg/Kg (n = 6). The potency exhibited by DMI in the $GIRK2^{+/-}$ mice was reduced (ED_{50}: 1.03 ± 0.10 mg/Kg, n = 5, $p < 0.0001$) compared to that obtained in WT although the maximal effect was similar (97.34 ± 4.42%, n = 5) *Nonlin-fit analysis*. Each point of the curve represents the mean ± S.E.M. of n experiments.

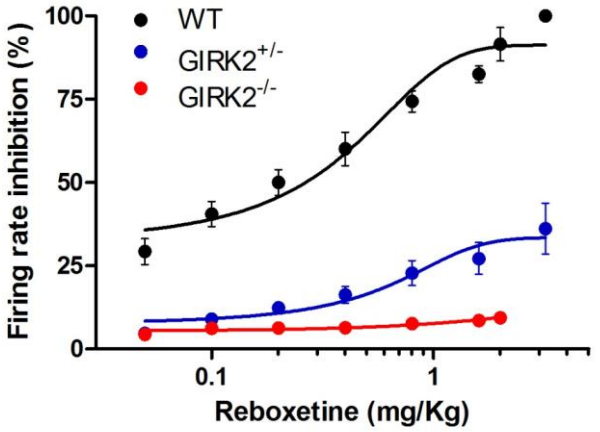


Figure 29. Effect of reboxetine on the basal firing rate of the LC neurons from WT and GIRK2 mutant mice. Increasing doses of reboxetine (0.05-3.2 mg/Kg, i.p.) caused a progressive and complete inhibition of the firing rate in WT genotype tested (91 ± 3.39%; ED_{50}: 0.20 ± 0.04 mg/Kg, n = 5). However, reboxetine showed a reduced potency as well as efficacy inhibiting the LC activity in the $GIRK2^{+/-}$ mice (ED_{50}: 0.5 ± 0.114 mg/Kg, E_{max}: 33.30 ± 3.33%, n = 8, $p < 0.05$). These differences were even greater in the $GIRK2^{-/-}$ animals, since reboxetine failed to modify LC activity in this mice genotype (E_{max}: 9.38 ± 1.28%, n = 5, $p < 0.05$) *Nonlin-fit analysis.* Each point of the curve represents the mean ± S.E.M. of n experiments.

4.3.2. *In vitro* study.

Previous results included in section 4.1 have revealed that GIRK channels contribute to control the basal activity of LC neurons as well as antidepressants-induced effects onto noradrenergic transmission *in vivo*. The next aim was to investigate the role of these channels on the basal activity of LC neuron as well as on the mechanism of action of antidepressant drugs *in vitro.* Besides GIRK2 mutant mice, the specific blocker GIRK 1/4 channels, tertiapin-Q and the potassium channels activator flupirtine, were used to undertake this investigation.

The *in vitro* basal firing rate of LC neurons recorded from WT slices was 0.64 ± 0.10 Hz (n = 6), similar to that recorded from GIRK2$^{+/-}$ mice (0.70 ± 0.07 Hz, n = 7, p = 0.13) (Fig. 30). To completely block GIRK channel activity a pharmacological approach was carried out using tertiapin-Q (250 nM). Thus, perfusion of tertiapin-Q for 5 min increased by 64.33 ± 10% the basal firing rate of WT mice (from 0.64 ± 0.10 to 1.08 ± 0.24 Hz, n = 6, $p < 0.001$) and longer administration of this drug (10 min) did not produce a further augmentation (from 1.08 ± 0.24 to 1.18 ± 0.26 Hz, n = 6, p = 0.52) (Fig. 31). These results, in agreement with previous studies (Torrecilla *et al*., 2002, Cruz *et al*., 2008), indicate that GIRK channels participate in the control of resting properties of LC neurons by reducing the intrinsic activity.

In this tissue preparation, perfusion of DMI (10-100 μM for 5 min) produced a concentration-dependent and complete inhibition of the LC firing rate in WT mice slices (n = 6, $p < 0.0001$). Administration

of tertiapin-Q (250 nM) did not only revert DMI effect but also increased spontaneous firing frequency above basal values (2.22 ± 0.29 Hz, n = 4, $p < 0.05$) (Fig. 32). Similarly, reboxetine (0.3-10 μM for 5 min) completely inhibited LC basal frequency in a concentration-dependent manner (n = 5, $p < 0.05$) and tertiapin-Q recovered spontaneous firing rate toward basal values (n = 5, $p > 0.05$) (Fig. 33 and 34).

Since in GIRK2$^{+/-}$ mice the efficacy of reboxetine inhibiting LC neurons *in vivo* was significantly reduced, here we tested its effect on LC brain slices from GIRK2$^{+/-}$ mice. Unlike in the WT genotype, a maximal concentration of reboxetine (10 μM) did not completely reduce the spontaneous firing rate (41.53 ± 3.87%, n = 7, $p < 0.0001$) (Fig. 35). However, after reboxetine washed-out NE perfusion, which was used as an internal control, produced a total inhibition of firing rate that returned to basal values within 10 min after wash (0.58 ± 0.052 Hz, n = 6, $p = 0.098$) (Fig. 36).

Flupirtine, a potassium channels activator (Jacob and Krieglstein, 1997), also was tested *in vitro*. In these experiments however, flupirtine (100 nM for 5 min) failed to modify LC neurons activity (Fig. 37)

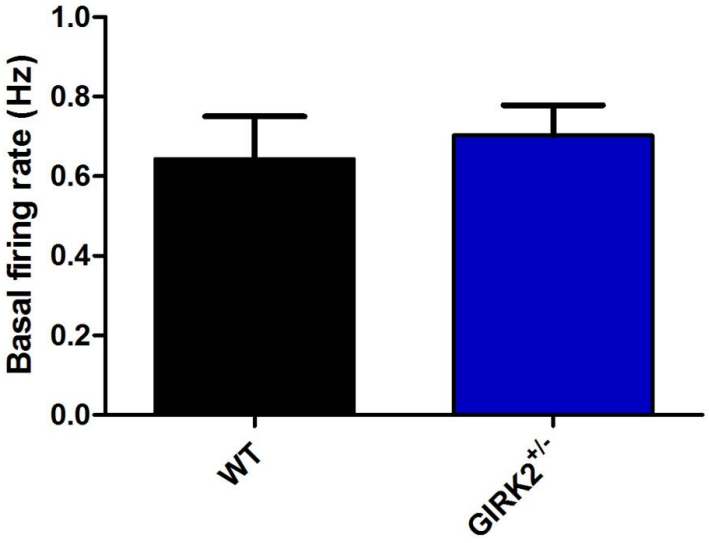


Figure 30. Basal firing rate in WT and GIRK2$^{+/-}$ mice was not different. The *in vitro* basal firing rate of LC neurons recorded from slices of WT mice, was similar to that recorded from GIRK2$^{+/-}$ mice. Data are expressed as mean ± S.E.M of n (6-7) experiments.

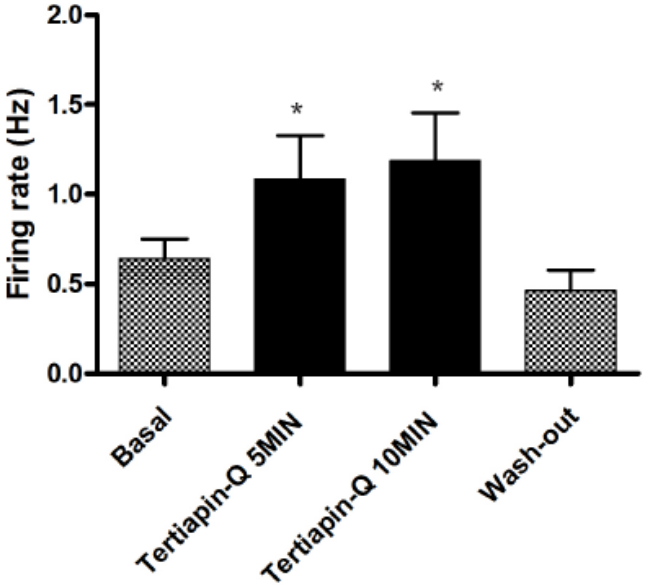


Figure 31. Perfusion of tertiapin-Q (250 nM) produced an increament of firing rate of LC neurons from WT mice. There were not differences between to perfuse tertipin-Q for 5 minutes or 10 minutes. However there were differences when comparing a basal values those obtained after perfuse tertiapin-Q (250 nM) in both groups (5 and 10 minutes perfusion) (*$p < 0.05$ *vs* basal, *One-way ANOVA, followed Newman Keuls test*). Data are expressed as mean ± S.E.M of n (6) experiments.

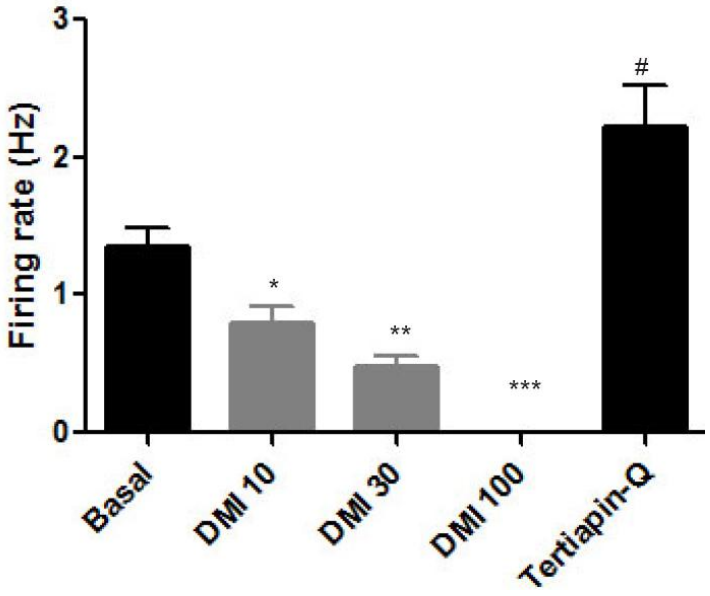


Figure 32. Tertiapin-Q (250 nM) reverted DMI induced inhibition on LC neurons *in vitro* in WT mice. There was concentration dependent inhibition of firing rate. All concentration were statistic different *versus* basal. DMI 100 μM produced the total inhibition of firing rate. The inhibition was reverted with tertiapin-Q and firing rate after reversion was higher than basal value. (***$p < 0.0001$ *vs* basal, *One-way ANOVA, followed Newman-Keuls test*; $^{\#}p < 0.014$ *vs* basal, *t test*). Data are expressed as mean ± S.E.M of n (4-6) experiments.

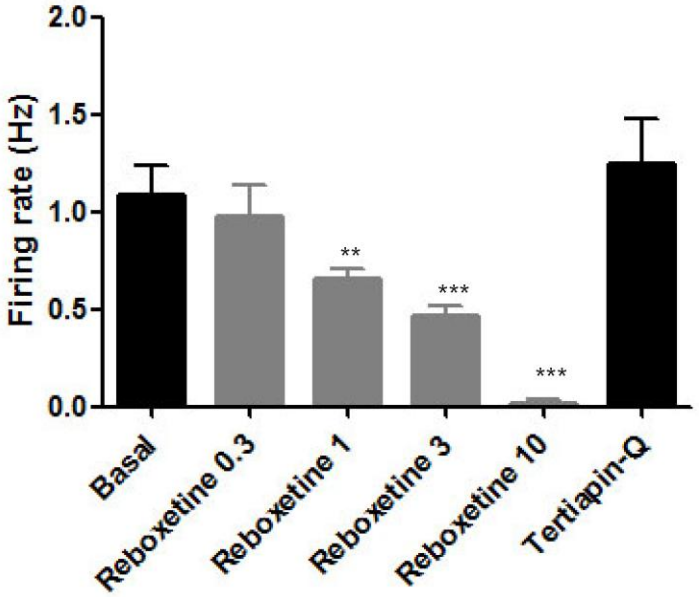


Figure 33. Tertiapin-Q reverted reboxetine induced inhibition of LC neurons rom WT mice slices. Reboxetine (0.3 - 10µM) induced a concentration dependent and total inhibition of LC activity. Reboxetine 10 µM produced the highest inhibition of firing rate wich was reverted with tertiapin-Q (***p < 0.0001 *vs* basal, *One-way ANOVA, followed Newman-Keuls test*). Data are expressed as mean ± S.E.M of n (4-5) experiments.

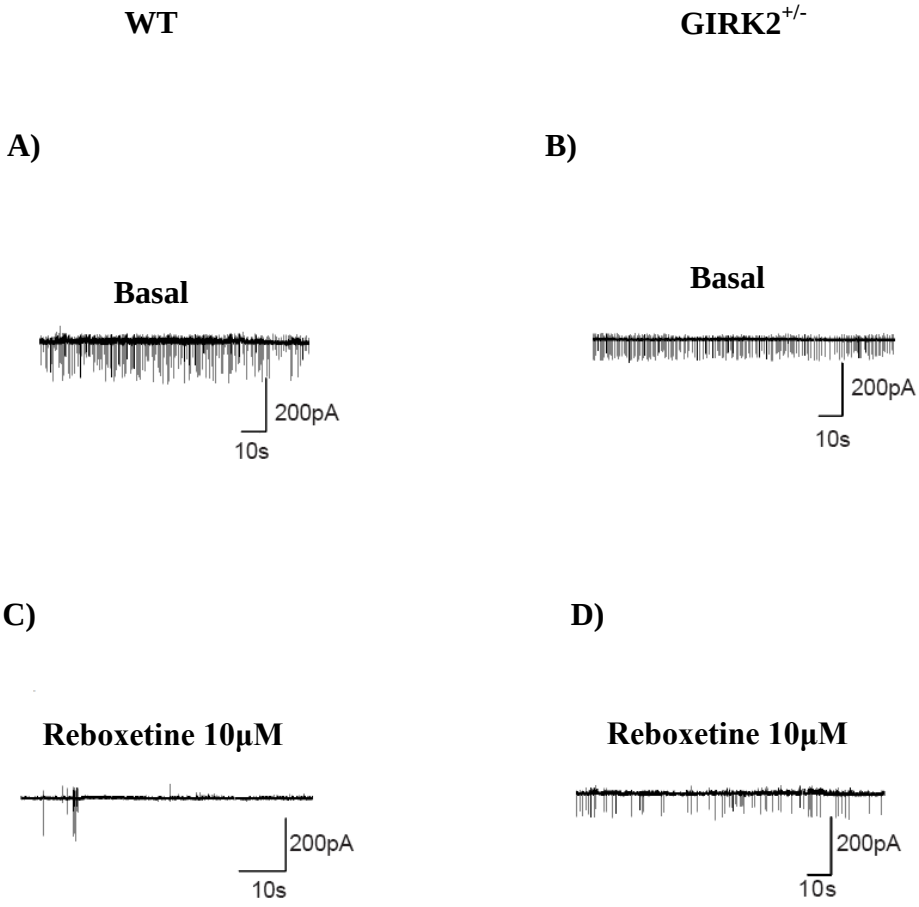


Figure 34. The *in vitro* recording of LC activity from WT mice and GIRK2$^{+/-}$ mice. Basal activity of LC neurons from WT mice **(A)** and GIRK2$^{+/-}$ (**B)** mice. Reboxetine 10µM completely inhibited firing rate in WT mice **(C)** but not in GIRK2$^{+/-}$ mice slices.

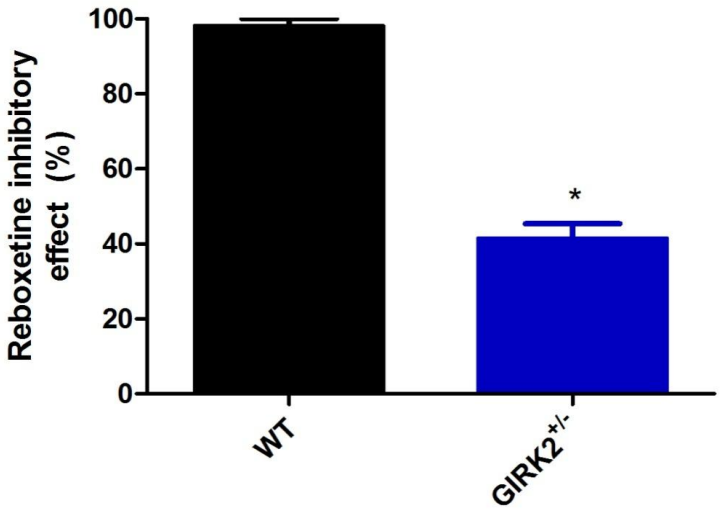


Figure 35. Reduced inhibitory effect of reboxetine in LC slices from GIRK2$^{-/-}$ mice. The inhibition of firing rate with reboxetine in LC neurons from GIRK2$^{+/-}$ mice was smaller than that observed in WT mice. Thus reboxetine 10 μM induced a maximal inhibiton of 41.53% ± 3.87% in GIRK2$^{+/-}$ mice slices (*p < 0.0001 *vs* WT, *t test*). Data are expressed as mean ± S.E.M of n (5-7) experiments.

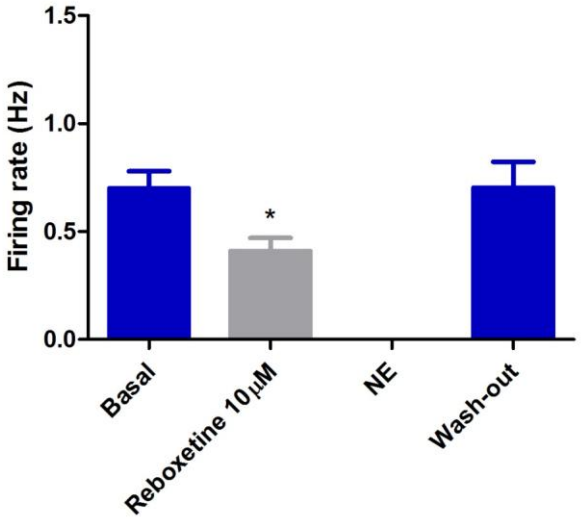


Figure 36. Effect of reboxetine 10 µM and NE 10 µM in the basal firing rate of the LC neurons from of GIRK2$^{+/-}$ mice slices *in vitro*. Reboxetine did not produce a total inhibition of firing rate. However NE perfusion completely inhibited firing activity, and recorvered to basal values after wash-out (*p < 0.01 *vs* basal, *t test)*. Data are expressed as mean ± S.E.M of n (6-7) experiments.

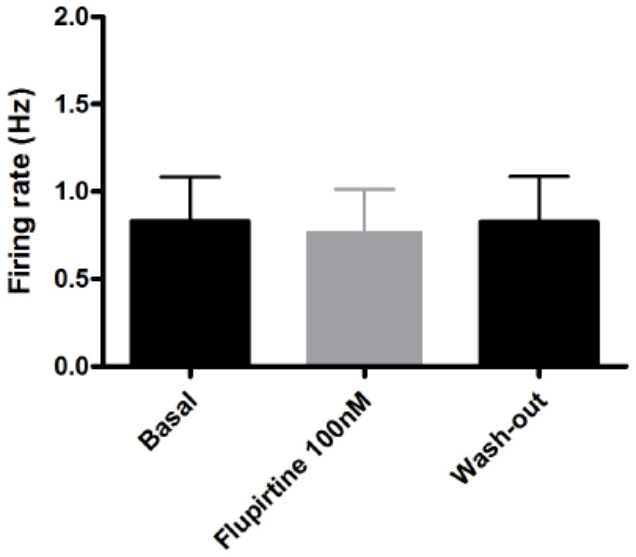


Figure 37. Flupirtine 100 nM perfusion for 5 minutes, did not produce any change in the firing rate of LC neurons from WT mice slices. Firing rate values before and after flupirtine bath perfusion was not different. Data are expressed as mean ± S.E.M of n (5) experiments.

4.4. STUDY IV

Effect of antidepressants on GIRK currents from LC neurons: a patch-clamp study.

STUDY IV

4.4. Effect of antidepressants on GIRK currents from LC neurons: a patch-clamp study.

The molecular mechanisms underlying the inhibitory effects of antidepressants on noradrenergic transmission are not completely clear. It is generally accepted that antidepressants reduce LC firing rate by GIRK channel activation subsequent to an increase of noradrenergic transmission. However, some authors suggest that antidepressants could directly inhibit GIRK channels (Kobayashi *et al*., 2000, 2003, 2004, 2010). In order to clarify the role of GIRK channels on the mechanisms of action of antidepressant drugs, patch-clamp was used in LC brain slices. Thus, whole-cell configuration experiments were carried out to record GIRK currents (voltage-clamp mode).

To identify LC neurons in the *in vitro* preparation current–voltage relationships (I/V) were done by stepping the membrane potential from -50 to -130 mV in -10 mV increments (100 msec per step). This protocol is useful to examine the activation of inwardly rectifying potassium channels, which are located on LC neurons. Thus, all the tested neurons expressed such a rectification (Fig.38). Perfusion of several antidepressants (fluoxetine, reboxetine and bupropion) at different concentrations failed to change basal current of LC neurons recorded at the resting potential (-60 mV). Thus, administration of fluoxetine

(10 - 100 μM for 5-7 min) induced a non significant increase on the amplitude of the basal current (5.46 ± 0.89 pA, n = 7 and 1.93 ± 0.18 pA, n = 3, respectively). Similarly, reboxetine (1 μM and 10 μM for 5-7 min) did not induce significant changes on the amplitude of basal current (3.72 ± 1.03 pA, n = 5 and 4.49 ± 1.86 pA, n = 4, respectively). Neither different concentrations of bupropion for 7 minutes produced changes on basal current of LC neurons. Thus 0.1 μM, 1 μM and 10 μM concentrations produced the similar resultant current (2.56 ± 0.84 pA, n = 6; 3.51 ± 0.81 pA, n = 4; 1.66 ± 0.40 pA, n = 4 respectively).

In addition, we also studied the potential impact of antidepressants on GIRK currents activated at more hiperpolarized membrane potential, in fact at -120 mV. As previously observed at resting potential (-60 mV), antidepressants did not significantly modify the amplitude of the inward current recorded at -120 mV (Fig. 39 and 40). These results indicate that antidepressants-induced effects on GIRK currents from LC neurons are minimal (Table 6).

We next tested the effect of the noradrenergic antidepressants reboxetine and bupropion on the GIRK current induced by NE. For that purpose NE was first perfused alone and next with the antidepressant reboxetine or bupropion. As it was expected NE 3μM increased the amplitude of the resting current of LC neurons by 19.46 ± 1 pA (n = 7). This effect was mediated by the opening of GIRK channels since NE increased I/V relationship which showed inward rectification and reverted at expected potential (Fig. 41, 42A and 43). In addition,

co-administration of NE with reboxetine (10 μM) significantly increased the amplitude of the NE-induced current (32.60 ± 3.7 pA, n = 5, $p < 0.05$) indicating that the antidepressant required exogenous NE for activating GIRK channels in LC slices (Fig. 42B and 43). Conversely, co-administration of bupropion (10 μM) did not significantly augmented the amplitude of the NE-induced current (control: 13.33 ± 3.77 pA, n = 4; co-administration: 18.75 ± 3.77 pA, n = 4) (Fig. 44 and 45).

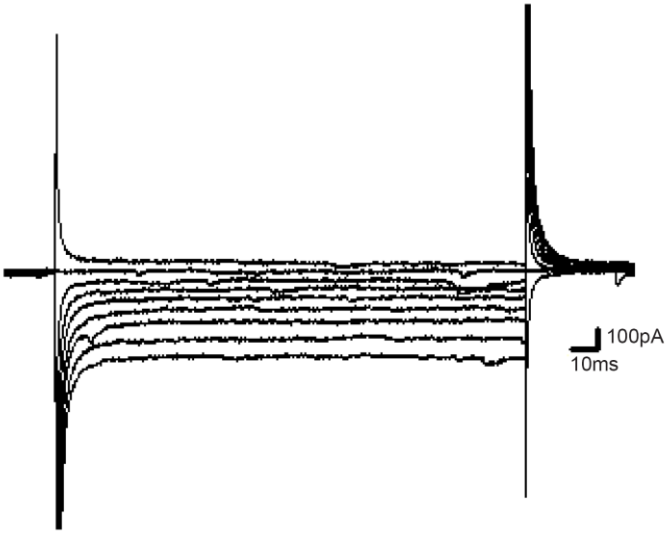
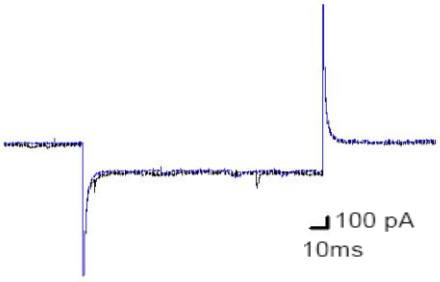
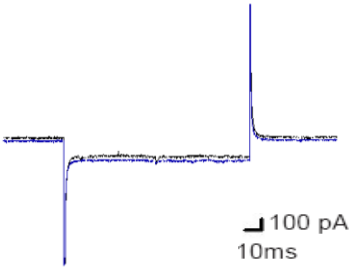


Figure 38. Whole-cell current-voltage relationship (I-V) recorded from an LC neuron. This is a representative recording of inwardly rectifying K^+ current from LC neuron obtained by stepping the membrane potential from -50 mV to -130 mV in -10 mV increments (100 msec per step). The term 'inward rectification' refers to a change in slope of the I-V near the reversal potential. Inwardly rectification occurs in LC neurons around -110 mV as the conductance of the current becomes bigger.

A) Basal + Fluoxetine (100 μM)



B) Basal + Reboxetine (1 μM)



Basal + Reboxetine (10 μM)

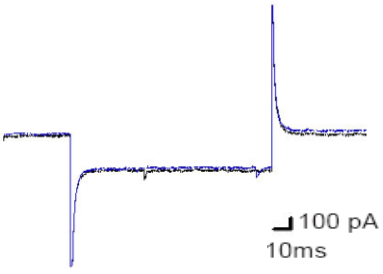
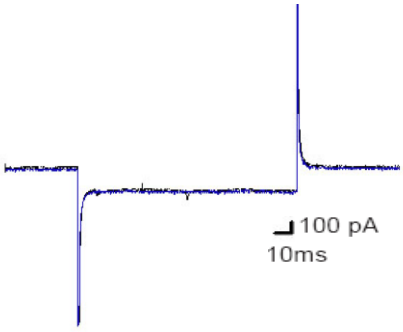
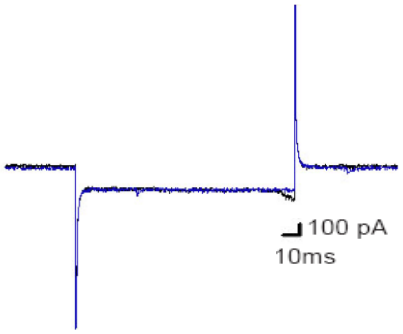


Figure 39. Fluoxetine and reboxetine did not modify basal current from LC neurons measured at -120 mV. A) Fluoxetine (100 μM) and B) and reboxetine (left, 1 μM and right, 10 μM) failed to change potassium currents at more negative potentials. Basal currents are shown with black traces and blue traces represent recorded currents after antidepressants perfusion onto the preparation.

A) Basal + Bupropion (0.3 μM)



B) Basal + Bupropion (1 μM)



C) Basal + Bupropion (10 μM)

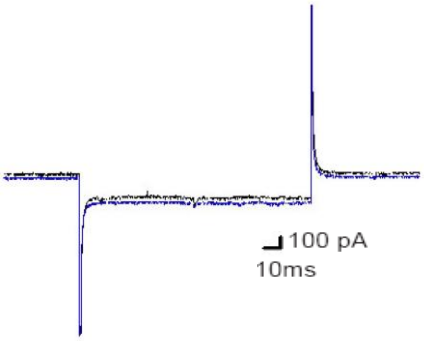


Figure 40. Bupropion did not changed basal current from LC neurons measured at -120 mV. A) Bupropion (0.3 μM) and **B)** and **C)** bupropion (1 μM and 10 μM) failed to change currents at -120 mv. Basal currents are shown with black traces and blue traces represent currents recorded when antidepressants were perfused onto the preparation.

Table 6. Antidepressants did not modify potassium currents measured at -120 mV in LC neurons.

Antidepressant	n values	Basal at -120 mV	Effect of antidepressant at -120 mV
Fluoxetine 10 µM	(n = 7)	-202 ± 36.6 pA	-224 ± 26.7 pA
Fluoxetine 100 µM	(n = 3)	-203 ± 36.1 pA	-233 ± 21.9 pA
Reboxetine 1 µM	(n = 5)	-271 ± 36.6 pA	-261 ± 52.3 pA
Reboxetine 10 µM	(n = 4)	-278 ± 43.1 pA	-238 ± 32.7 pA
Bupropion 0.1 µM	(n = 6)	-174 ± 15.8 pA	-159 ± 31.5 pA
Bupropion 1 µM	(n = 4)	-173 ± 20.5 pA	-167 ± 18.3 pA
Bupropion 10 µM	(n = 4)	-174 ± 55.2 pA	-202 ± 25.9 pA

Fluoxetine, reboxetine and bupropion antidepressant failed to alter potassium currents. Data are expressed as mean ± S.E.M of n experiments ($p > 0.05$, paired *t test*). Data are expressed as mean ± S.E.M of n experiments.

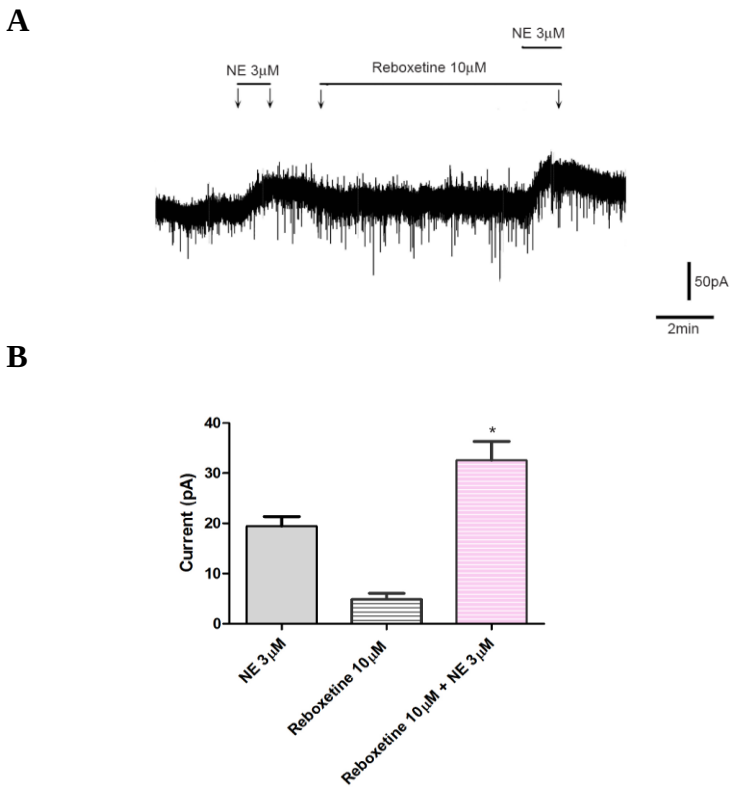


Figure 41. Reboxetine increased the amplitude of the current induced by NE in LC neurons. A) Representative recording of outward current induced by NE and co-administration with reboxetine in LC neurons of brain slices. Reboxetine 10μM increased the amplitude of the outward current induced by NE 3μM. Notice that reboxetine alone failed to alter basal current. **B)** Summary of the effect of reboxetine onto NE-induced current. Data are expressed as mean ± S.E.M. of n (5-7) experiments (*p < 0.05 *vs* NE 3 μM, *One-way ANOVA, followed Newman-Keuls test*).

A) NE 3 μM **B)** Reboxetine 10 μM + NE 3 μM

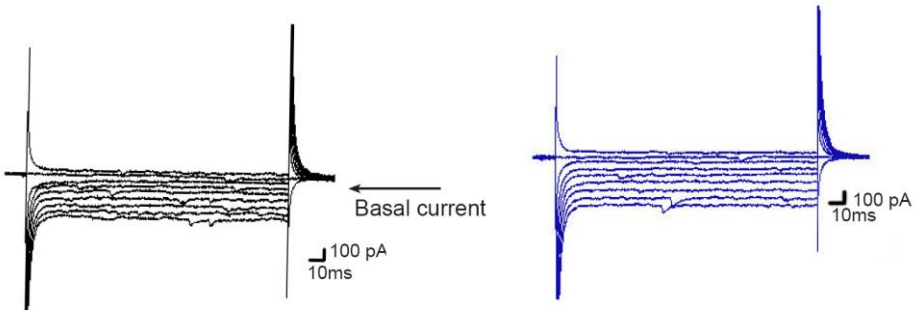


Figure 42. Representative recordings of I/V relationship obtained from LC neurons in mice brain slices in presence of NE (A) and when reboxetine was co-administrated (B). Reboxetine increased the NE-induced current at all potential tested. Notice that inward rectification is also greater when reboxetine is applied.

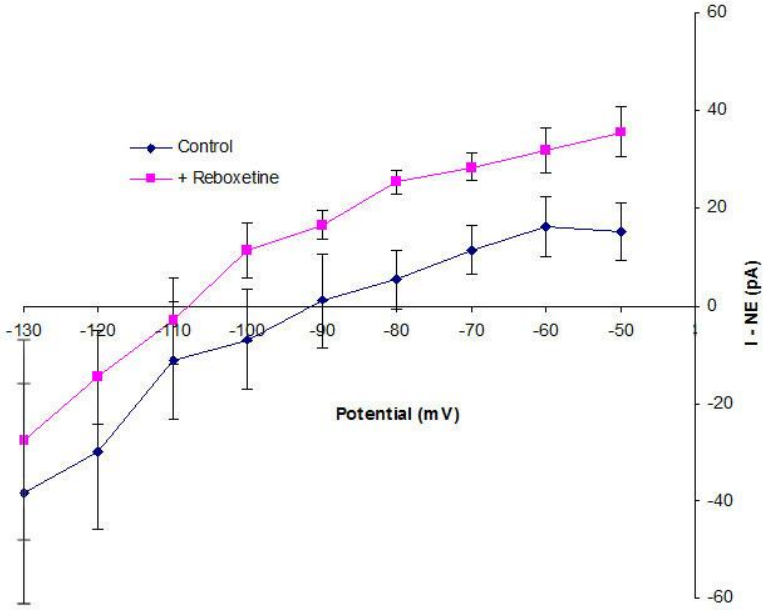


Figure 43. I-V relationship of NE-induced current alone and during reboxetine co-administration. Reboxetine increased the NE-induced current in LC neurons. NE-induced currents (I-NE) were calculated by subtracting the current obtained before NE application from the current obtained in the presence of NE. The holding potential was -60mV. Data are expressed as mean ± S.E.M. of n (5-7) experiments.

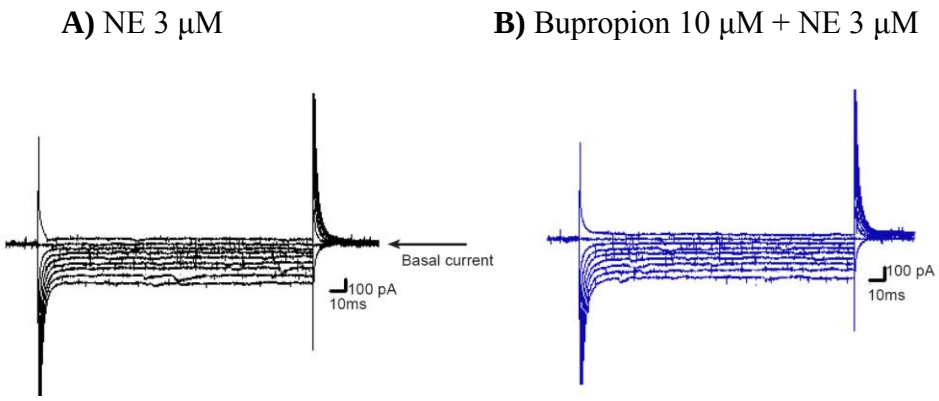


Figure 44. Representative recordings of I/V relationships obtained from LC neurons in brain slices of WT mice in presence of NE (A) and when bupropion was co-administrated (B). In presence of NE 3 μM, bupropion 10 μM, did not alter the NE-current.

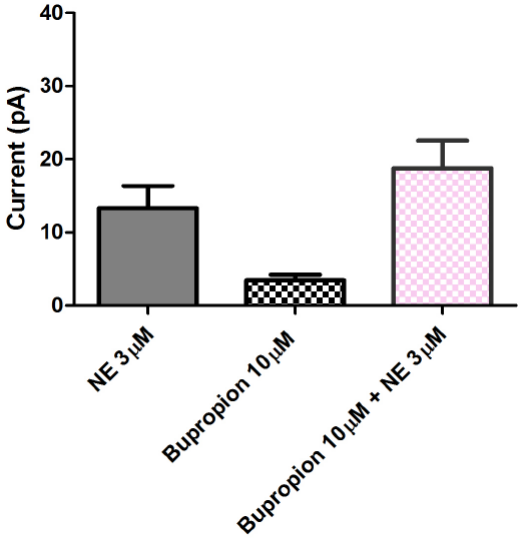


Figure 45. Bupropion failed to activate GIRK currents in brain slices in WT mice. Bupropion (10 μM) did not significantly increase the NE-induced current. Data are expressed as mean ± S.E.M. of n (4-6) experiments.

4.5. STUDY V

Basal levels of monoamines in WT and GIRK2 mutant mice: a HPLC study.

STUDY V

4.5. Basal levels of monoamines in WT and GIRK2 mutant mice: a HPLC study.

We studied whether GIRK2 mutation affects monoamines basal levels in different brain areas which are related with the depression. Thus NA, 5-HT and 5-HIIA levels were evaluated in LC, DRN and PFC from WT, GIRK2$^{+/-}$ and GIRK2$^{-/-}$ mice. Results are summarized in Table 7.

Regarding NE levels GIRK2 subunit mutation did not alter NE levels in LC (WT: 0.43 ± 0.03 ng/mg, n = 10; GIRK2$^{+/-}$: 0.60 ± 0.09 ng/mg, n = 10; GIRK2$^{-/-}$: 0.54 ± 0.04 ng/mg, n = 11) neither in the projecting area PFC (WT: 0.19 ± 0.01 ng/mg, n = 10; GIRK2$^{+/-}$: 0.19 ± 0.01 ng/mg, n = 10; GIRK2$^{-/-}$: 0.21 ± 0.02 ng/mg, n = 10). However, in the DRN NE levels were significantly increased in the GIRK2$^{+/-}$ genotype (WT: 0.32 ± 0.03 ng/mg, n = 9; GIRK2$^{+/-}$: 0.42 ± 0.02 ng/mg, n = 10; GIRK2$^{-/-}$: 0.32 ± 0.03 ng/mg, n = 10; $p < 0.05$) (Fig. 46).

No differences were observed among genotypes regarding basal 5-HT levels in LC (WT: 0.40 ± 0.04 ng/mg, n = 10; GIRK2$^{+/-}$: 0.56 ± 0.09 ng/mg, n = 10; GIRK2$^{-/-}$: 0.37 ± 0.05 ng/mg, n = 9, p = 0.109) neither in PFC (WT: 0.28 ± 0.014 ng/mg, n = 9; GIRK2$^{+/-}$: 0.29 ± 0.038 ng/mg, n = 10; GIRK2$^{-/-}$: 0.20 ± 0.037 ng/mg, n = 10, p = 0.15). However, in the DRN 5-HT levels were significantly reduced in the

GIRK2$^{-/-}$ group compared to that measured from GIRK2$^{+/-}$ mice (GIRK2$^{-/-}$: 0.32 ± 0.05 ng/mg, n = 9; GIRK2$^{+/-}$: 0.71 ± 0.12 ng/mg, n = 10; WT: 0.54 ± 0.03 ng/mg, n = 9, $p < 0.01$). Therefore, these results suggest that GIRK2 subunit ablation affects mainly serotoninergic neurotransmission (Fig. 47).

Finally, the levels of the 5-HT metabolite, 5-HIIA were evaluated in the brain areas already mentioned. Thus, in the LC 5-HIIA levels were significantly greater in the GIRK2$^{-/-}$ mice compared to those from the other genotypes (WT: 0.90 ± 0.07 ng/mg, n = 10; GIRK2$^{+/-}$: 1.10 ± 0.13 ng/mg, n = 10; GIRK2$^{-/-}$: 1.66 ± 0.19 ng/mg, n = 11, $p < 0.01$). In the DRN from GIRK2$^{-/-}$ mice basal levels of 5-HIIA showed a slight although no significant increase (WT: 1.12 ± 0.063 ng/mg, n = 9; GIRK2$^{+/-}$: 1.23 ± 0.12 ng/mg n = 10; GIRK2$^{-/-}$: 1.54 ± 0.15 ng/mg, n = 10, $p = 0.06$). Similar levels of the metabolite were measured in the PFC of all genotype tested, and once more there were not observe a statically significant differences (WT: 0.28 ± 0.01 ng/mg, n = 9; GIRK2$^{+/-}$: 0.26 ± 0.01 ng/mg, n = 10; GIRK2$^{-/-}$: 0.32 ± 0.03 ng/mg, n = 10, $p = 0.20$) (Fig. 48).

Table 7. Basal levels of amines in different brain areas in all three genotypes mice.

Brain area	Genotype	NE (ng/mg)	5-HT(ng/mg)	5-HIIA (ng/mg)
LC	**WT** (n = 10)	0.43 ± 0.03	0.40 ± 0.04	0.90 ± 0.07
	GIRK2$^{+/-}$ (n = 10)	0.60 ± 0.09	0.56 ± 0.09	1.10 ± 0.13
	GIRK2$^{-/-}$ (n = 9-11)	0.54 ± 0.04	0.37 ± 0.05	1.66 ± 0.19 *
DRN	**WT** (n = 9)	0.32 ± 0.03	0.54 ± 0.03	1.12 ± 0.063
	GIRK2$^{+/-}$ (n = 10)	0.42± 0.02*	0.71 ± 0.12	1.23 ± 0.12
	GIRK2$^{-/-}$ (n = 9-10)	0.32 ± 0.03	0.32 ± 0.05*	1.54 ± 0.15
PFC	**WT** (n = 9-10)	0.19 ± 0.01	0.28 ± 0.014	0.28 ± 0.01
	GIRK2$^{+/-}$ (n = 10)	0.19 ± 0.01	0.29 ± 0.038	0.26 ± 0.01
	GIRK2$^{-/-}$ (n = 10)	0.21 ± 0.02	0.20 ± 0.037	0.32 ± 0.03

There were not differences on the NE levels in LC nor in the projecting area of the PFC. However, in the DRN, NE level was significantly increased in the GIRK2$^{+/-}$ genotype (*p < 0.05 *vs* WT). 5-HT levels were not altered in WT and GIRK2$^{-/-}$ although in GIRK2$^{+/-}$ there was slight increase in the LC. In the DRN from GIRK2$^{-/-}$ mice there was a significant decrease on 5-HT level (*p < 0.05 *vs* WT). The 5-HIIA metabolite in LC of the GIRK2$^{-/-}$ mice group, was significantly increased *versus* two other genotypes in LC (*p < 0.01 *vs* WT). *One-way ANOVA, followed Newman-Keuls test*. Data are expressed as mean ± S.E.M of n experiments.

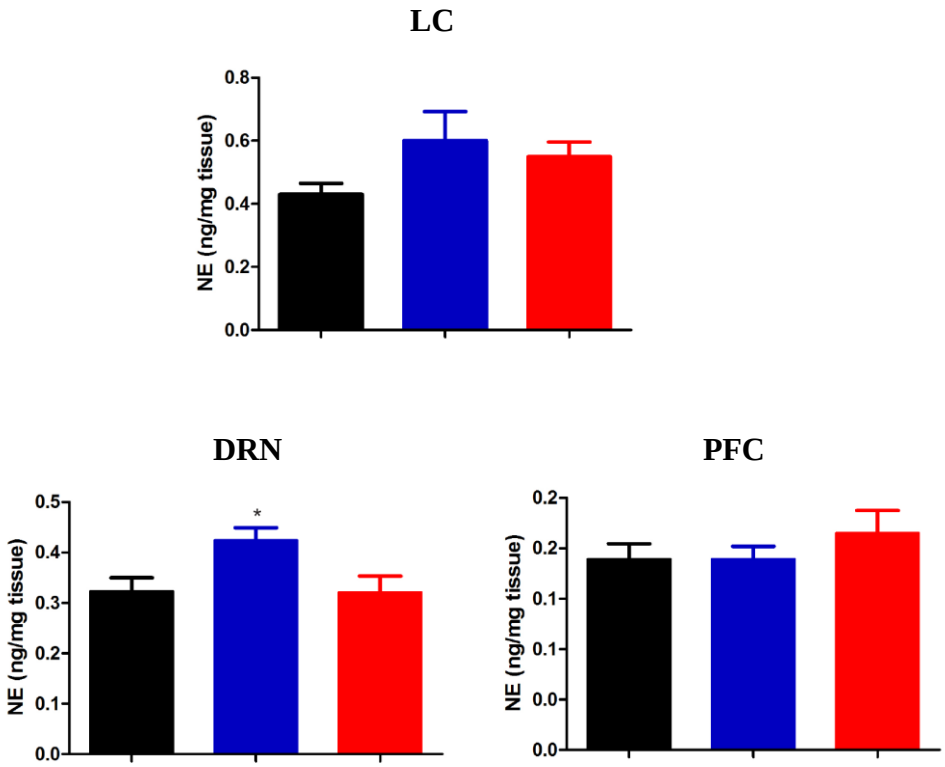


Figure 46. Role of GIRK2 channels on NE basal levels from LC, DRN nucleus and PFC. GIRK2 subunit mutation did not alter NE levels in LC nor in the PFC. However, in the DRN NE levels were significantly increased in the GIRK2$^{+/-}$ genotype (*p < 0.05 *vs* WT, *One-way ANOVA, followed Newman-Keuls test*). Data are expressed as mean ± S.E.M of n (9-10) experiments. In black WT mice, in blue GIRK2$^{+/-}$ mice and in red GIRK2$^{-/-}$ mice.

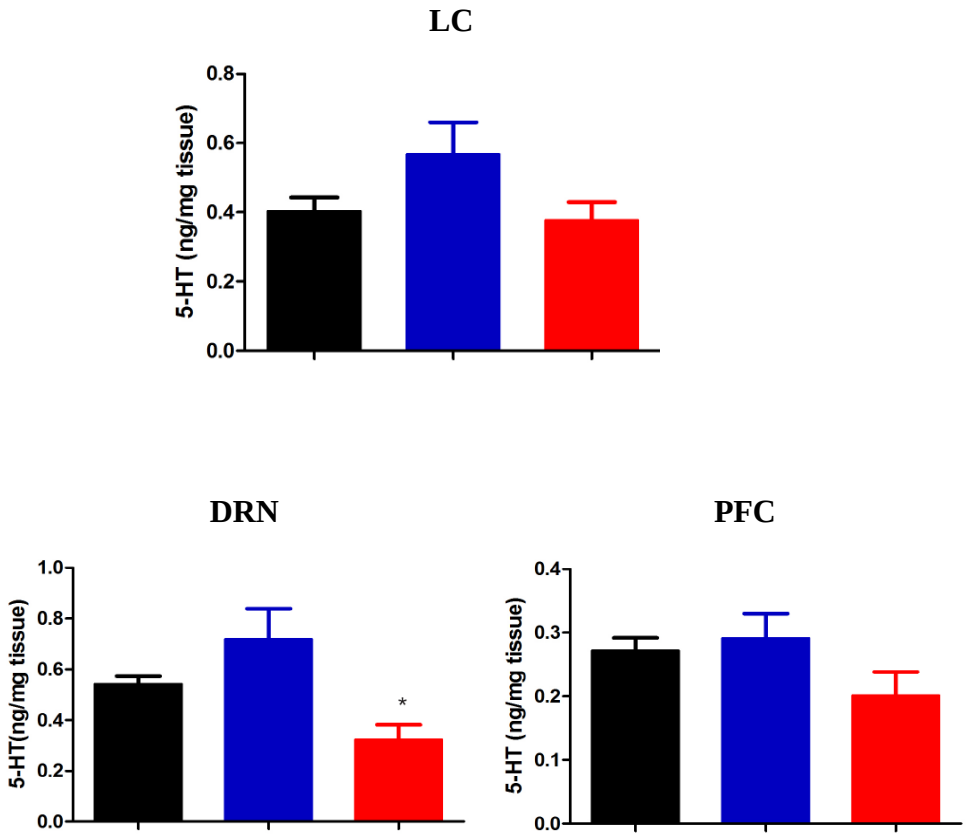


Figure 47. Role of GIRK2 channels on 5-HT basal levels from LC, DRN nucleus and PFC. In LC and PFC 5-HT levels were not altered. However in the DRN from GIRK2$^{-/-}$ mice 5-HT levels decreased (*p < 0.01 *vs* WT *One-way ANOVA, followed Newman-Keuls test*). Data are expressed as mean ± S.E.M of n (9-10) experiments. In black WT mice, in blue GIRK2$^{+/-}$ mice and in red GIRK2$^{-/-}$ mice.

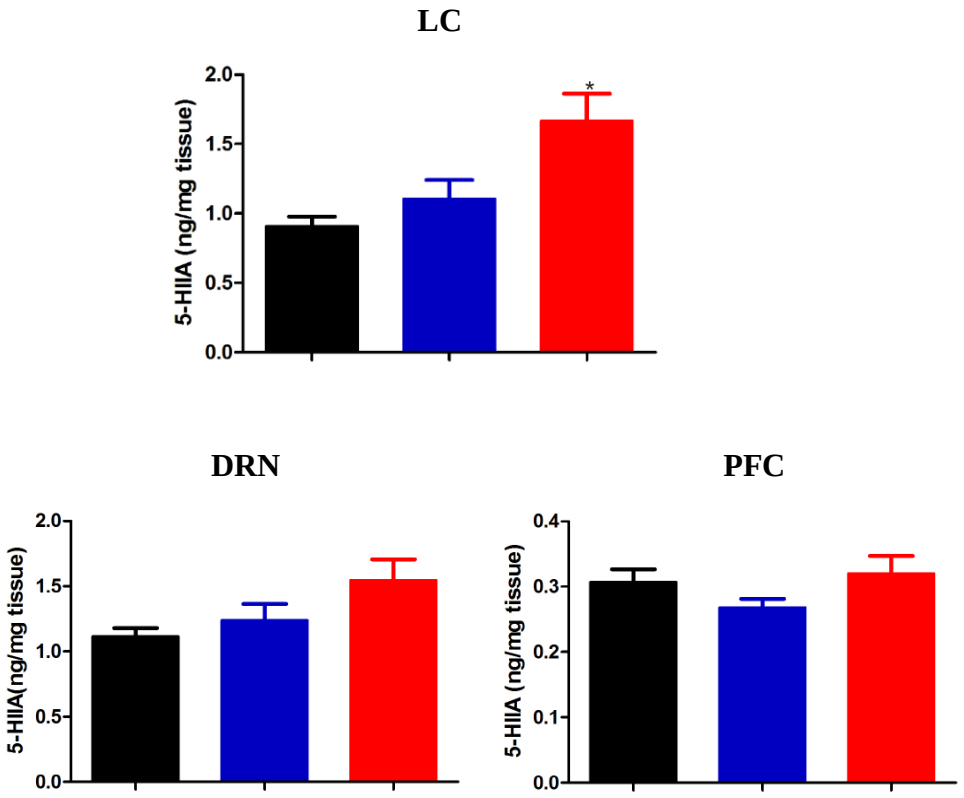


Figure 48. Role of GIRK2 channels on 5-HIIA basal levels from LC, DRN nucleus and PFC. In LC 5-HIIA metabolite level from GIRK2$^{-/-}$ mice was significantly increased. In the DRN and PFC no statistical differences were detected among genotupe (*p < 0.01 *vs* WT, *One-way ANOVA, followed Newman-Keuls test*). Data are expressed as mean ± S.E.M of n (9-11) experiments. In black WT mice, in blue GIRK2$^{+/-}$ mice and in red GIRK2$^{-/-}$ mice.

4.6. STUDY VI

Behavioral study in GIRK mutant mice: tail suspension test and motor activity test.

STUDY VI

4.6. Behavioral study in GIRK mutant mice: tail suspension test and motor activity test.

GIRK mutant mice have been a key tool to insight into the role of specific GIRK channels composition on neuronal physiology and animal behavior (Pravetoni and Wickman, 2008). Here, the role of GIRK2 subunit-containing channels on depression-related behaviors has been examined using the TST, one of the most widely used paradigms for assessing antidepressant-like activity of drugs and depression-related behaviors in normal and genetically modified mice (Cryan *et al*., 2005).

4.6.1. Tail Suspension Test

We first validated the TST by evaluating the acute effect of several antidepressant drugs on the immobility time in WT mice. The baseline behavior in the TST was examined after injections of physiological saline (NaCl 0.9%, i.p.), DMI (5 mg/Kg, i.p.), reboxetine (20 mg/Kg, i.p.) and fluoxetine (20 mg/Kg, i.p.) in WT mice 30 min before testing (Fig. 49). Saline injection had not effect on the global immobility time therefore these experiments were included in the control group. Thus, the basal immobility time mean value was 213 ± 3.49 s in the control group (n = 15). All the antidepressant tested, DMI (100.6 ± 4.05 s, n = 8), fluoxetine (124.8 ± 4.97 s, n = 8) and reboxetine (29.75 ±

1.22 s, n = 8) significantly decreased the immobility time compared to control group ($p < 0.0001$). Reboxetine, in fact, was the most effective drug reducing immobility (Fig. 50).

We next examined the impact of GIRK2 subunit mutation on immobility time in the TST. $GIRK2^{+/-}$ mice as well as $GIRK2^{-/-}$ mice showed reduced immobility time in the TST compared to WT group ($GIRK2^{+/-}$: 148.5 ± 3.40 s, n = 11; $GIRK2^{-/-}$: 130.1 ± 1.40 s, n = 11, $p < 0.0001$) (Fig 51). Furthermore, statistically significant differences were observed between mutant genotypes ($p < 0.0001$). Also, the behavioral effects of reboxetine were studied in GIRK2 mutant mice. Reboxetine did not alter significantly immobility time in $GIRK2^{-/-}$ mice (122.9 ± 3.08 s, n = 7) although it induced a slight but significant increment of immobility time in $GIRK2^{+/-}$ mice (165.4 ± 2.78 s, n = 9, $p < 0.0001$) (Fig. 52).

Also a pharmacological approach was done by using ICV injections of the GIRK channel blocker, tertiapin-Q. In WT animals tertiapin-Q (30 and 100 pmol) dose-dependently decreased the immobility time in the TST (low dose: 161.7 ± 2.57 s, n = 10; high dose: 124.9 ± 1.73 s, n = 10, *vs* values from the control group, $p < 0.0001$). Moreover, the immobility time level induced by the high dose of tertiapin-Q was not different to that displayed by the $GIRK2^{-/-}$ mice ($GIRK2^{-/-}$: 130.1 ± 1.40 s, n = 11) (Table 8).

Tertiapin-Q injection (100 pmol, ICV) 30 minutes before reboxetine administration (20 mg/Kg, ip) completely prevented the effect of the antidepressant (115 ± 2.708 s, n = 10, *vs* tertiapin-Q group) (Fig. 53). Furthermore, in this group the immobility time values were not different from those obtained in the GIRK2$^{-/-}$ mice who had also received reboxetine at the same dose (122.9 ± 3.807 s, n = 7).

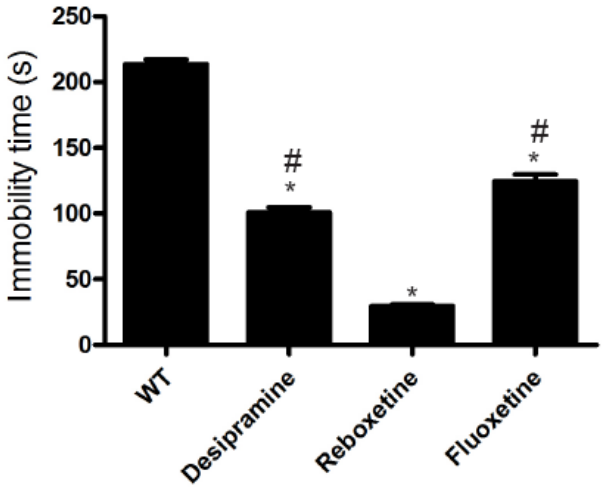


Figure 49. The effects of antidepressants on immobility in TST in WT mice. All the antidepressants tested (DMI (5 mg/Kg, i.p.), reboxetine (20 mg/Kg, i.p.) and fluoxetine (20 mg/Kg, i.p.) reduced the immobility time in WT mice. Reboxetine displayed the most important effect in the WT mice (*$p < 0.0001$ *vs* control, *One-way ANOVA followed Newman-Keuls test*; $^{\#}p < 0.0001$ *vs* reboxetine, *t test*). Data are expressed as mean ± S.E.M of n (8-15) experiments.

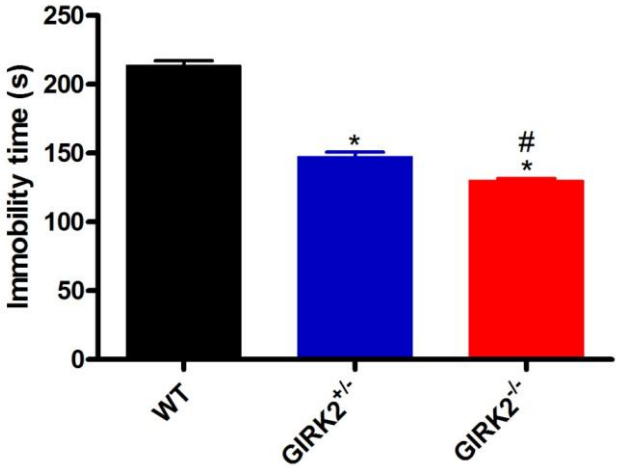


Figure 50. GIRK2 mutant mice showed a reduced immobility time in the TST. In GIRK2$^{+/-}$ and GIRK2$^{-/-}$ mice the immobility time was reduced when comparing to values obtained in WT group. Statistic significant differences between mutant mice were also observed, showing less immobility time GIRK2$^{-/-}$ mice (*$p < 0.0001$ *vs* WT; $^{\#}p < 0.0001$ *vs* GIRK2$^{+/-}$ *One-way ANOVA followed Newman-Keuls test*). Data are expressed as mean ± S.E.M of n (7-15) experiments.

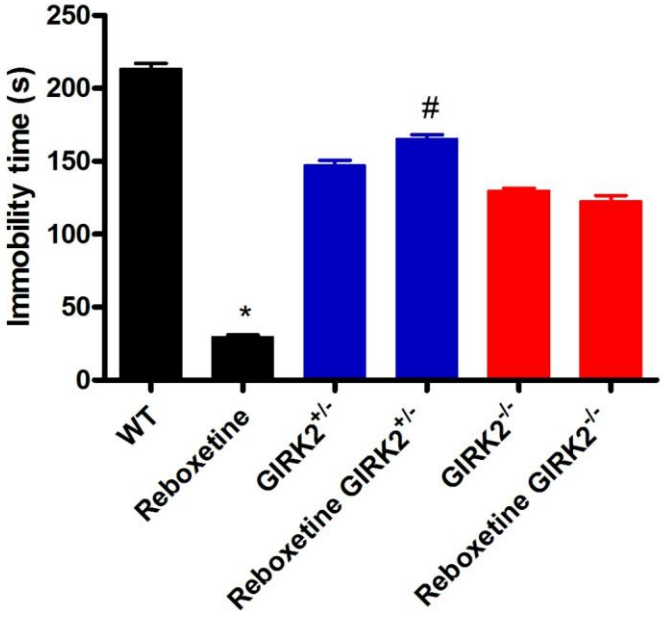


Figure 51. Reboxetine failed to reduce immobility in TST in GIRK2 mutant mice. Reboxetine (20 mg/Kg, i.p.) reduced immobility time in WT mice but the same dose of reboxetine did not reduce the immobility time in the GIRK2$^{-/-}$ mice group. Neither after ICV application of GIRK channels blocker tertiapin-Q (100 pmol), reboxetine (20 mg/Kg, i.p.) reduced the immobility time. Moreover reboxetine (20 mg/Kg, i.p.) induced a slight increase in of immobility time values in GIRK2$^{+/-}$ group (*$p < 0.0001$ *vs* WT, *t test*; $^{\#}p < 0.001$ *vs* GIRK2$^{+/-}$, *t test*). Data are expressed as mean ± S.E.M of n (7-15) experiments.

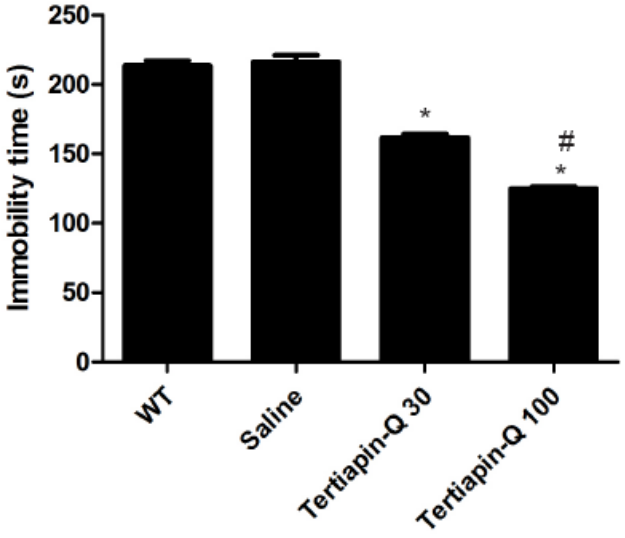
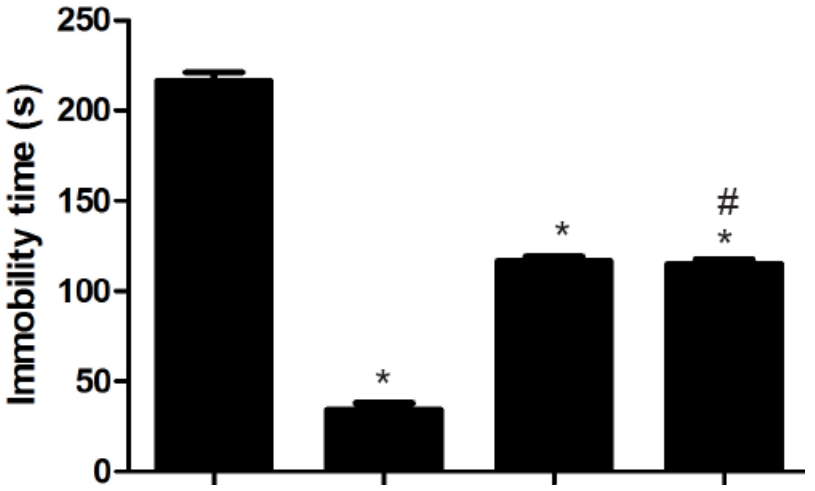


Figure 52. Tertiapin-Q dose-dependently reduced the immobility time in TST in WT mice. The GIRK channels blocker tertiapin-Q reduced immobility time at both doses (30 and 100 pmol, ICV) in WT mice, showing a greater effect with the highest dose (*$p < 0.0001$ *vs* saline; $^{\#}p < 0.0001$ *vs* tertiapin-Q 30, *One-way ANOVA followed Newman-Keuls test*). Data are expressed as mean ± S.E.M of n (7-15) experiments.



Tertiapin-Q (100 pmol)	-	-	+	+
Reboxetine (20 mg/Kg, i.p.)	-	+	-	+

Figure 53. Tertiapin-Q prevented reboxetine-induced behavioral effect on TST in WT mice. The highest dose of tertiapin-Q (100 pmol) reduced immobility time in WT mice. Reboxetine (20 mg/Kg, i.p.) failed to reduce the immobility time after tertiapine-Q (100 pmol) administration (*$p < 0.0001$ *vs* tertiapin-Q + reboxetine (ICV); $^{\#}p < 0.0001$ WT-saline (ICV) + reboxetine i.p, *One-way ANOVA followed Newman-Keuls test*). Data are expressed as mean ± S.E.M of n (8-10) experiments.

Table 8. Mean values immobility time in WT mice and GIRK2$^{-/-}$ mutant mice in control situation and after reboxetine or tertiapin-Q administration.

	Immobility time (s)
WT	213.7 ± 3.49 (n = 15)
WT-Saline (ICV)	216.4 ± 4.7 (n = 10)
WT + Reboxetine (20 mg/Kg, i.p.)	29.75 ± 1.22 (n = 8)*
GIRK2$^{-/-}$	130.1 ± 1.40 (n = 11)
GIRK2$^{-/-}$ + Reboxetine (20 mg/Kg, i.p.)	122.9 ± 3.80 (n = 7)
Tertiapin-Q (100 pmol)	124.9 ± 1.73 (n = 10)
Tertiapin-Q (100 pmol) + Reboxetine (20 mg/Kg, i.p.)	115 ± 2.70 (n = 10)

Reboxetine (20 mg/Kg, i.p.) reduced the immobility time in WT mice. However, reboxetine did not modify the immobility time in GIRK2$^{-/-}$ mice or after application of tertiapin-Q (100 pmol) **(***p < 0.0001 *vs* WT, *t test*). Data are expressed as mean ± S.E.M of n experiments.

4.6.2. Motor activity test

We next tested whether the reduction of immobility time observed in the GIRK2 mutant mice as well as that induced by blocking GIRK channels were due to an alteration on the motor activity of the mice. For that purpose we used the activity chamber and measured the motor activity during 30 min. As previously described, (Blenov et al., 2001b; Blenov et al., 2002; Pravetoni and Wickman, 2008) $GIRK2^{-/-}$ mice (3429 ± 43.65 arbitrary units, n = 14) showed an elevated motor activity when compared to WT (3105 ± 25.39 arbitrary units, n = 15, $p < 0.0001$). $GIRK2^{+/-}$ mice, however, did not showed differences in motor activity as compared to WT. However $GIRK2^{+/-}$ (2962 ± 104.9 arbitrary units n = 14) showed statistical different when compared with $GIRK2^{-/-}$ mice ($p < 0.0001$) in a 30 minutes session (Fig. 54). Tertiapin-Q injections of a dose of 30 pmol and 100 pmol significantly reduced motor activity in WT mice (2567 ± 98.89 arbitrary units, n = 9; 2589 ± 91.8 arbitrary units, n = 9, $p < 0.0001$, for the low and high dose respectively). This effect was not dose-dependent (Fig. 55).

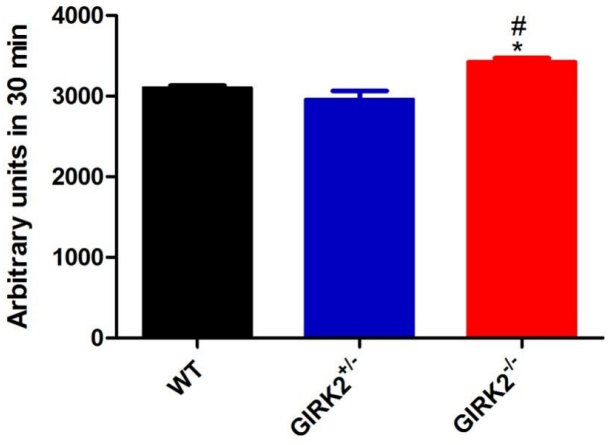


Figure 54. GIRK2$^{-/-}$ mice showed an elevated motor activity. GIRK2$^{-/-}$ mice showed greater locomotor activity than WT and GIRK2$^{+/-}$ mice. Motor activity of GIRK2$^{+/-}$ mice however was not statically different from tham recorded in WT mice (*$p < 0.0001$ *vs* control; $^{\#}p < 0.0001$ *vs* GIRK2$^{-/-}$, *One-way ANOVA followed Newman-Keuls test*). Data are expressed as mean ± S.E.M of n (14-15) experiments.

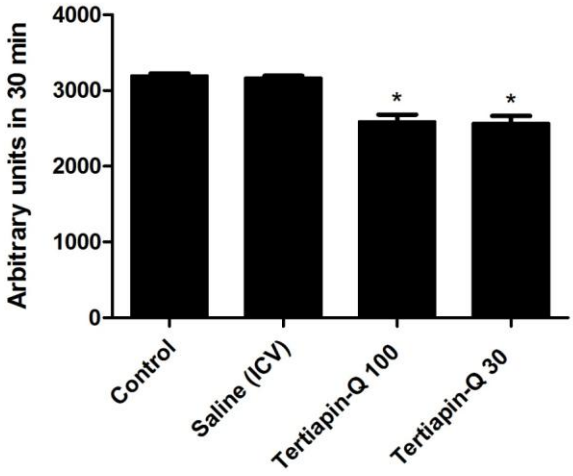


Figure 55. Tertiapin-Q reduced motor activity in control mice. Tertiapin-Q, (30 pmol and 100 pmol (ICV)), reduced the activity in WT mice (*p < 0.0001 *vs* control *One- way ANOVA followed Newman-Keuls test*). Data are expressed as mean ± S.E.M of n (5-10) experiments.

5. DISCUSSION

5. DISCUSSION

Although the most media-oriented side of genetic manipulation finds itself enmeshed in a number of controversies, it is also fair to point out the benefits that this technology has brought and can bring to the human race. Animals models generated through the modification of the mouse genome are important, without which the knowledge on the genetics of many diseases and their treatments would not have been able to advance in the way they have done in the last decades.

In 2002 the first draft of the genome of mouse was published. This first sketch enabled the establishing of the existing homology between the rodent and the human genome, detecting an equivalence of up to 90%. In 2009 it was established that the genomic homology between both species was of 75 per cent. Be it as it may, the genomic proximity with human makes it a good model to study different diseases such as depression. Furthermore, the mouse is a small sized mammal, manageable, prolific and easy to breed in captivity. These characteristics have made it one of the most frequently used models in laboratories across the world. Even if it might seem strange, from 1981 till 2004 in addition to mouse stem cell cultures, only human and some closely related primates had been established, which has made the mouse into the most versatile species when talking of genetic modification for decades. In 2008, reports came out of the first rat embryonic cell cultures, and the production of the first knockout rat.

One of the most important advantages to the understanding of psychiatric illnesses was the development of genetically modified mice in which the alteration of a protein can help in the understanding of those diseases. For this reason there are currently around 40 different genotypes developed to study more closely the disease of depression, and also the molecular mechanisms of antidepressant medication (Cryan and Mombereau, 2005). It is in this way that the GIRK2$^{-/-}$ mice are an adequate tool for the study of psychiatric diseases like depression and the action mechanism of antidepressant medication. These subunits are implicated with neuronal excitability *in vivo,* since GIRK2$^{-/-}$ mice show increased susceptibility to spontaneous seisures (Signorini *et al*., 1997). Thus the differences that could be found on the effects induced by different genotypes could be due to the mutation of the GIRK2 subunit.

On the other hand, electrophysilogical studies *in vivo* and *in vitro* of the LC neurons have provided valuable information concerning the etiopathogeny of many diseases related to alterations of central noradrenergic transmission, like for example drug addiction, anxiety or depression.

The electrophysiology of the LC neurons from mouse *in vivo* is not a widely used technique, in fact to date only one article has been published in which data related to the activity of neurons in the LC of an anaesthetized mice was presented (Gobbi *et al*., 2007). In line with that published data, our results indicate that the electric characteristics of

mice LC neurons are similar to those described in rats presenting a firing reate of 0.5-5 Hz *in vivo*, bursts firing and the biphasic excitation-inhibition response to presure applied onto the contralateral hind paw, as previously described in the rat (Cerbadaum and Aghajanian., 1976) and more recently in mice (Gobbi et al., 2007). In studies carried out in LC brain slices from mice basal frecuency of LC was lower that recorder which oscillates between 0.5-2.5 Hz. *In vitro* results from mice are agrement with those reported from rat previously (Torrecilla *et al*., 2002; Miguelez *et al*., 2009). The difference in the basal frequency of *in vivo* and *in vitro* is due to brain slices excitatory projections to the LC are cutted of.

5.1. STUDY I

Characterization of electrophysiological properties of LC neurons from GIRK2 mutant mice *in vivo.*

STUDY I

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It has been described how the constitutive activity of the GIRK channels can contribute to the stabilisation of the resting membrane of LC neurons *in vitro* (Velimirovic *et al*., 1995). In this respect, electrophysiological studies done on brain slices of LC mutated animals in one or various GIRK subunits, confirm the important role those channels have in the control of the basal electrical activity of the above mentioned nucleus (Torrecilla *et al*., 2002; Cruz *et al*., 2008). However the function of those channels in controlling the noradrenergic transmission *in vivo* has not been explored. In this way, it is widely known that the channels composed by GIRK2 and GIRK1 subunits are important mediators in the neuronal excitability *in vivo* (Signorini *et al*., 1997) and that in subunit for GIRK2 knockout mice there also exists a reduction in the levels of GIRK1 (Signorini *et al*., 1997; Torrecilla *et al*., 2002). Therefore, the use of mice lacking GIRK2 subunit could help to establish the function of those channels in the central noradrenergic transmission *in vivo.*

The first objective of this paper was to study the participation of GIRK2 subunit-containing GIRK channels in the bioelectric properties of the neurons of the LC *in vivo*. In this way the total or partial deletion of

the gene that codifies for the GIRK2 subunit did not modify the basal frequency nor the coeficient of variation of the LC neurons. Our results show that the firing rate of LC neurons of GIRK2$^{+/-}$ and GIRK2$^{-/-}$ mice were not different from those registered in WT mice. The results obtained for the basal frequency in our WT mice are similar to those obtained by Gobbi *et al* (2007), which oscillated between 0.5-5 Hz with a similar duration in all three genotypes (0.8 - 1.2 ms). And also, the basal frequency described in the mouse *in vivo* was in agrement with the values described for rats *in vivo* by Ruiz-Ortega *et al*., (1997). The extra-celullar signal of the action potentials recorded from all genotypes (WT, GIRK2$^{+/-}$ and GIRK2$^{-/-}$**)** presented the typical bi-phasic morphology, with a larger positive part that has notch and another final negative part, similar to the previously described in the rat and mouse (Cedarbaum y Aghajanian, 1976; Gobbi *et al*., 2007). The coefficient of variation of the LC neurons of both GIRK2$^{+/-}$ and GIRK2$^{-/-}$ mice were not different to those registered in WT mice, which indicates the mutation of the GIRK2 subunit does not affect the firing pattern regularity of the LC neurons.

Conversly, the partial or total deletion of the GIRK2 gene subunit did significantly modify the burst activity of the LC neurons. In the present study, and in concordance with that described by Gobbi *et al*., (2007) where the proportion of neurons with burst patterm was of 14%, the percentage of neurons with bursts firing was of 10% in the WT animals. This percentage was significantly greater in both GIRK2$^{+/-}$ and in GIRK2$^{-/-}$ animals, which presented mean values of 26% and 55%, respectively. These findings are similar to those published by Gobbi *et*

al., (2007) in knockout mice for neurokinin 1 receptors, in which the percentage of burst firing of LC neurons is increased without this modifying the basal frequency or the coefficient variation of the discharge pattern of the LC. These results also indicate that the study of the burst discharge pattern is an efficient method to evaluate the changes in neuronal electric activity.

It has been demonstrated that the resting membrane potential of the LC cells *in vitro* is depolarized not only in GIRK2$^{-/-}$ animals but also in GIRK2$^{+/-}$ (Torrecilla *et al*., 2002). Furthermore, the mutation of the GIRK2 subunit produces a reduction in the levels of GIRK1 in both genotypes, so that those modifications in the levels of both GIRK1 and GIRK2 subunits could explain the changes observed in the burst pattern in GIRK2 mutant animals. In the current project the extracellular registers performed on GIRK2$^{+/-}$ animals indicate that the LC neuron firing rate *in vitro* is similar to that of WT animals. On the one hand, in knockout with the double mutation to GIRK2 and GIRK3 subunits the firing rate was significantly increased in animals when the synaptic activity was blocked (Cruz *et al*., 2008). On the other hand, while the GIRK currents induced by the opioid agonist met-encefaline are reduced by half in the LC of GIRK2$^{-/-}$ mice, in GIRK2$^{+/-}$ mice this reduction is not observed (Torrecilla *et al*., 2002). All this supports the idea that a sub-population of GIRK channels, composed of by at least GIRK2 and GIRK1 subunits control specific electrophysiological aspects of the LC neurons. For example their resting membrane potential, which itself could be the cause of the increase in the percentage of neurons that

present a burst discharge pattern in GIRK2$^{-/-}$ and also in GIRK2$^{+/-}$ animals.

Neuronal burst firing pattern has been linked to a greater release of transmitter in projecting areas (Florin-Lechner *et al*., 1996; Berridge and Abercrombie, 1999; Gartside *et al.*, 2000; Devoto *et al*., 2003), which increases the probability of action potential propagation (Luschër and Shiner, 1990), facilitates co-transmitter release (Vilim *et al*., 1996; Devoto *et al*., 2003) and enhances postsynaptic responses (Gartside *et al*., 2000). Altered burst firing pattern is linked to depression-like behaviors and beneficial response to antidepressants (Dremencov *et al*., 2008; Convington *et al*., 2010). Sustained administration of 5-HT selective reuptake inhibitors decreases burst activity from noradrenergic and dopaminergic neurons, which may account for antidepressant-resistant response in some patients (Dremencov *et al*., 2007; 2009). In contrast, chronic administration of imipramine increases burst firing in LC slices (Maubach *et al*., 2002). Atypical antipsychotics, which increase burst activity of LC neurons, are effective adjuncts in antidepressant-resistant patients (Rapaport *et al*., 2006). In mice lacking neurokinin 1 receptors, which display a greater percentage of LC neurons discharging in burst, rewarding effects of opioids are absent, physical response to withdrawal is reduced and anxiety-related behaviors are decreased (Murtra *et al*., 2000; Santarelli *et al*., 2001). Therefore, medications that selectively increase burst activity of monoaminergic neurons, such as drugs that target GIRK channels, might be a more efficacious treatment of depressive behavior and other psychiatric diseases. In this sense,

GIRK2$^{-/-}$ mice show reduced anxiety-related behavior (Pravenoti and Wickman, 2008) and diminished cocaine self-administration (Morgan *et al*., 2003). In addition, the morphine withdrawal syndrome is strongly attenuated in double GIRK2 and GIRK3 knockout mice (Cruz *et al*., 2008).

5.2. STUDY II

The inhibitory effect induced by clonidine and morphine on LC neurons from GIRK2 mutant mice *in vivo*.

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The LC neurons possess a high density of opioid μ receptors (Arvidsson *et al*., 1995; Van Bockstaele *et al*., 1996) and α_2-adrenoreceptors (Pascual et al., 1992; Scheinin et al., 1994) which control to a great extent the firing rate of the LC neurons. In this way, the activation of the α_2 adrenoreceptors and the μ opioid receptors, gives rise to an increase in the potassium current through the GIRK channels (Williams *et al*., 1988) so that this produces a hyperpolarization of the membrane potential which translates finally into the inhibition of the spontaneous discharge frequency of the LC neurons (Aghajanian and Wang, 1978; Williams *et al*., 1982). Today it is known that the GIRK channels located in the LC are made up of subunits GIRK1, GIRK2 and GIRK3 (Torrecilla *et al*., 2002; Cruz *et al.*, 2008; Torrecilla *et al.*, 2008). Therefore, the second objective of this study consisted in studying the effects induced by clonidine and morphine on the electric activity of LC neurons in the three mice genotypes *in vivo*, to determine the impact of GIRK2 subunit mutation over the inhibitory effect induced by previously mentined drugs.

The intraperitoneal administration of accumulated doses of the agonist of the α_2 adrenoreceptor clonidine produced a dose dependent and

complete inhibitory effect of the basal frequency of the LC neurons in WT mice. Those results are in concordance with studies made on the LC of rat *in vivo* (Pineda *et al*., 1997; Ruiz-Ortega *et al*, 1997), although the range on dose employed in the present study was wider since after the intraperitoneal administration the bioavailability of the drug can be diminished. Those differences could also be related to the use of different species of rodents. In this sense there are numerous studies done on *in vivo* mice that concur both with the dose range and method of delivery used in our current study (Lakhlani *et al*., 1997; Blednov *et al*., 2003; Mitrovic *et al*., 2003).

In the GIRK2$^{-/-}$ mice clonidine showed better inhibitory potency with respect to the WT group though it maintained the maximum inhibitory efficacy described in the WT group. That is to say, their dose-response curve found itself moved to the right in relation to the curve obtained with the WT animals but reaching the maximum inhibitory effect. In any case, the maximum inhibition induced by clonidine was reverted by the selective agonist RX 821002 in both genotypes. Similar results were obtained with the opioid agonist morphine. The intraperitoneal administration of morphine produced a complete and dose-dependent inhibition of the electrical activity of the LC *in vivo*. The range of dose used was larger to the on described in studies carried out on rats (Pineda *et al*., 1998). This difference could be due to the fact that intraperitoneal administration significantly reduces the bioavailability of the medication. Numerous studies carried out on mice with some mutation of the GIRK channels which manifest the importance of those

in the analgesia measured by morphine have used intraperitoneal or subcutaneous administration of similar doses to those used in our study (Ikeda *et al*., 2000; Mitrovic, et al., 2003; Cruz *et al*., 2008). In the present study morphine showed a smaller potency to inhibit the electrical activity of the LC neurons of the GIRK2$^{-/-}$ animals in relation to the WT animals, nevertheless the maximum efficacy was maintained. In both groups the maximum analgesic effect was reversed with the opioid antagonist naloxone. These results concur with those described earlier by Cruz *et al*., (2008) where it is shown that in the GIRK2$^{-/-}$ animals the analgesic efficacy of morphine maintains itself while its potency is significantly reduced in relation to the WT group. That is to say, the GIRK channels contribute to the analgesic effect induced by morphine although they are not necessary to achieve their maximum effect (Cruz *et al*, 2008).

This study indicates that the channels formed by the GIRK2 subunits contribute to the inhibitory effect produced by the activation of the α_2 and μ opioid adrenoreceptors, but are not required to obtain a maximum inhibition of the discharge frequency of the LC *in vivo*. In this way, the amplitude of the GIRK current induced by the activation of the μ opioid receptor in the LC *in vitro* is reduced by 50% in GIRK2$^{-/-}$ animals and by 80% in animals knockout with the double mutation for GIRK2 and GIRK3 (Torrecilla *et al*., 2002; Cruz *et al*., 2008). However, these currents are enough to produce a noticeable hiperpolarisation of the potential difference of the neuron membrane of the LC in GIRK2$^{-/-}$ neurons but not in the double knockout for GIRK2 and GIRK3$^{-/-}$

genotype (Torrecilla *et al*., 2002). Equally, the administration by the agonist of the α_2 adrenoreceptors, UK-14303 in slices of the LC of the double knockout for GIRK2 and GIRK3$^{-/-}$ animals produces a hiperpolarisation of the potential difference of the membrane smaller than that observed in the WT group (Torrecilla *et al*., 2008). For this reason, it is probable that the maximum inhibition of LC activity *in vivo* is related with the activation of channels that contain in addition to the GIRK2 subunit, the GIRK3 subunit. Furthermore, other different factors of the GIRK channels, like the voltage-dependent calcium channels might be implicated in this effect, since the former are fully active both in GIRK2$^{-/-}$ animals, as well as in double knockout animals for GIRK2 and GIRK3 animals (Torrecilla *et al*., 2002). So, we see that the presynaptic inhibitory effect of morphine in LC neurons *in vitro* is not affected by the mutation of the gene that codifies for the GIRK2, GIRK3 channels, or both (Cruz *et al.*, 2008).

A surprising result was that referring to the group of GIRK2$^{+/-}$ animals, since in those the dose-response curves of morphine and clonidine were moved to the right in comparison to those obtained with the WT group. This means that even in heterozygotic animals there is a lower potency of the agonists attached to GIRK channels that contain the GIRK2 subunit *in vivo* to inhibit the basal discharge frequency of the LC, while the efficacy does not change. Since the GIRK current induced by the opiates is not found to be modified in the LC of GIRK2$^{+/-}$ animals *in vitro* (Torrecilla *et al*., 2002). It is possible that those differences are due to compensating changes which could be more evident *in vivo*. In fact,

said mutation gives rise to a significant reduction in the levels of the GIRK1 subunit in GIRK2$^{+/-}$ mice (Signorini *et al*., 1997).

This study indicates that the GIRK channels containing GIRK2 subunits are important cellular effectors of the inhibitory effects mediated by the stimulation of the α_2 adrenoreceptors and μ opioids on noradrenergic transmission *in vivo*. So, drugs that act by diminishing cellular signalling due to a total or partial inactivation of the GIRK channels could result in advances in therapies for pathologies like opioid dependence and depression. And so, it has been suggested that the inhibition of the noradrenergic tone measures by the GIRK channels is necessary for the development of opioid dependence, so that double knockout for GIRK2 and GIRK3$^{-/-}$ mice have greatly a reduced withdrawal syndrome to morphine (Cruz *et al*., 2008). On the other hand, the clinical antidepressant effect has been connected to the desensitization of α_2 adrenoreceptors (Szabo *et al, 2000;* Grant and Weiss, 2001) and 5-HT$_{1A}$ receptors (Invernizzi *et al*., 1994; Artigas *et al*., 1996), which are coupled to GIRK channels. In this way, the lesser functional affinity shown by the α_2-adrenoceptors in animals with the mutation in the GIRK2 gene could be an interesting aspect in the study of the efficacy of drugs with the potential of antidepressant activity.

5.3. STUDY III

Role of GIRK channels on the mechanism of action of antidepressants.

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The mechanism of action of antidepressant drugs continues to be a theme of controversy, because of that the third objective of this study was investigated the role of GIRK channels on the effects induced by antidepressant drugs on noradrenergic neurotrasmission by measuring the electrical activity of the LC neurons in WT and GIRK2 mutant mice *in vivo* and *in vitro*. The drugs used were those that had a noradrenergic profile, specifically DMI and reboxetine. Although DMI is a triciclic antidepressant that fundamentally blocks NE reuptake, it also shows selectivity for the 5-HT transporter and moderate affinity for α_1 adrenergic, H_1 histaminergic and muscarinic receptors (Wong *et al*., 2000). Reboxetine, however, is a selective inhibitor of NE transporters. In both cases, antidepressant activity is related to the increase in central noradrenergic tone. Nevertheless, recently a new mechanism of action has been proposed for drugs which would include the blocking of the GRIK channel (Kobayashi et al., 2004; 2010)

The intraperitoneal administration of the antidepressant DMI produced an inhibition in the basal frequency of the LC neurons in WT mice close to 100%. This data concurs with that obtained by other authors in the LC of rats where the administration of DMI produced an

inhibition of 70-80% of the frequency of the neurons (Mateo *et al*., 1998; Béïque *et al*., 1998; Szabo *et al*., 2001). On the other hand, the intraperitoneal administration of reboxetine produced a complete of LC neurons inhibition of 100% as was previously described in rats (Szabo *et al*., 2001; Miguélez *et al*., 2010). The doses used to obtain the maximum effect were slightly greater to those published for rats. This discrepancy can be due to the use of the intraperitoneal route instead of the intravenous one. However, the range of doses used was the same as those used in the behavior studies carried out on mice where the route of administration was intraperitoneal (Ripoll *et al*., 2003).

In GIRK2$^{+/-}$ mice the dose-response curve for DMI showed that the drug was less potent though equally effective inhibiting the basal activity of the LC neurons as compared to the WT group. On the other hand, the dose-response curve for reboxetine showed how this antidepressant was not only less potent but also less effective inhibiting the firing rate of the LC neurons in GIRK2$^{+/-}$ animals. Furthermore, this drug was completely reverted in GIRK2$^{-/-}$ mice. In all cases the antagonist of the α_2 adrenoreceptors RX 821002 reverted in the inhibitory effect induced by the antidepressants on the three genotypes studied. This indicates that the inhibitory effect induced by those drugs in LC neurons is mediated by the stimulation of the α_2 adrenoreceptors. More importantly, those results indicate that the GIRK2 channels play an important though different role of the two *in vivo*. Since reboxetine, unlike DMI, shows smaller efficacy inhibiting the firing rate of the LC

neurons in GIRK2$^{+/-}$ animals it seems that the degree of involvement of GIRK2 channels in this effect is greater for reboxetine than for DMI.

As was indicated in the previous chapter the stimulus dependent signalling of the α_2-adrenoceptor is reduced in GIRK2$^{+/-}$ mice. For this reason it is probable that the differences observed between the inhibitory effects induced by DMI and reboxetine in those animals are related to other receptors/effects different to the α_2 adrenoreceptors stimiulation. In fact, it has been suggested that the effects induced by DMI on the LC *in vitro* can also be due to its interaction with the α_1 adrenoreceptors (Grandoso *et al.*, 2004) since this drug shows moderate affinity for sthose receptors (U´Prichard *et al*., 1978). Furthermore it has been shown using techniques of hybridization and polymerase transcription reaction that α_1-adrenoceptors are situated presynaptically in the dendrites and terminals of the LC (Pudovkina *et al*., 2001; Osborne *et al*., 2002). So, although evidence indicates that α_1 adrenoreceptors do not influence the basal frequency of the LC neurons (Marwada and Aghajanian, 1982; Willians *et al*., 1985; Illes and Norënberg, 1990), it has been observed that the α_1 suppress the GIRK current induced by stimulation of the opioid receptors and α_2-adrenoceptors (Osborne *et al*., 2002). It is possible that the blocking of the α_1 adrenoreceptor with DMI facilitates GIRK currents so that in GIRK2$^{+/-}$ animals efficacy is maximum, which does not happen with reboxetine.

Another difference in the mechanism of action of those drugs is their selectivity in relation to the NE transporter. So, while reboxetine

blocks selectively the transporter (Wong *et al*., 2000), DMI shows certain affinity for the 5-HT transporter (Tatsumi *et al*., 1997). Recently, it has been shown that the selective 5-HT reuptake inhibitor, fluoxetine inhibits *in vivo* the discharge frequency of the rats LC through a mechanism dependent on the stimulation of the α_2-adrenoceptor (Miguelez *et al*., 2009). On the other hand, changes in the levels of NE transporter secondary to the mutation of the GIRK2 gene could explain the decreased efficacy of reboxetine inhibiting the firing rate of the LC neurons in GIRK2$^{+/-}$ and GIRK2$^{-/-}$ animals. Additional research is necessary to assess these hypotheses.

On the other hand, experiments carried out on *Xenopus* oocytes into which mRNA of GIRK1/GIRK2 subunits was injected indicate that the acute administration of several antidepressant drugs, amongst which DMI and reboxetine are found, reduces in a reversible manner, but voltage and time-independently, GIRK currents through the direct blockage of the channel (Kobayashi *et al*., 2003; 2004; 2006; 2010). However, the results of our study are in disagreement with this hypothesis since the inhibitory effect observed in the LC is measured by the activation of the α_2-adrenoceptor, which opens the channel inducing hyperpolarization and activity inhibition of LC neurons. Those discrepancies can be due to the use of different experimental systems, since contrary to what happens in noradrenergic neurons of the LC, in the *Xenopus* oocytes the signalling pathway of the α_2 adrenoreceptor-GIRK channels is incomplete.

In this way, the results obtained from our *in vitro* experiments support the hypothesis that suggest that GIRK channels play a relevant role in the control of the basal electric activity of the LC neurons (Torrecilla *et al*., 2002; Cruz *et al*., 2008). So, the administration of the peptide that blocks the GIRK channels, tetiapin-Q (Jin and Lu, 1999) increased the basal frequency of LC neurons in WT mice (Torrecilla *et al*., 2002; Cruz *et al*., 2008). Specifically, the simultaneous blockage of at least the GIRK2 and GIRK3 subunits seems necessary since the in LC neurons from double knockout mice for GIRK2 and GIRK3 an increased basal frequency is observed *in vitro* compared to WT animals (Cruz *et al*., 2008). As observed in the *in vivo* experiments, the basal frequency of LC neurons from GIRK2$^{+/-}$ mice *in vitro* LC was not different from that obtained from WT mice.

Likewise, our results support the hypothesis that indicates that acute administration of antidepressant drugs activates GIRK channels and it is the opposed that proposed by Kobayashi et al., (2004),where it was proposed that antidepressants drugs acts blocking the GIRK channels. Consistent with the previous research carried out (Egan *et al*., 1983; Williams *et al*., 1985; Illes and Norënberg, 1990; Grandoso *et al*., 2004), the application of the DMI and reboxetine resulted in a concentration-dependent inhibition of the basal frequency of the LC neurons from the WT group slices. This effect was reverted by the tertiapin-Q administration, which induced an increase of the firing rate over the basal value. This work confirms on the one had that the inhibitory effect induced by the NE reuptake inhibitors on LC cells

depends on the activation of GIRK channels and on the other hand, that those channels have a constitutive activity at rest that contribute to the intrinsic electric properties of LC neurons.

Unlike that observed in WT animals, the administration of reboxetine in slices that contain the LC of GIRK2$^{+/-}$ animals did not result in a complete inhibition of the basal firing rate. As it was seen *in vitro*, reboxetine also show a reducer *in vivo* efficacy inhibiting the activity of the LC in the GIRK2$^{+/-}$ genotype. Those differences could be due to a disfunction of the α_2 adrenoreceptor-GIRK the signalling pathway described in this current study *in vivo*. Nevertheless, the administration of NE completely inhibited the basal frequency in both WT and GIRK2$^{+/-}$ neurons *in vitro*. For all those reasons, the reduced efficiency of reboxetine inhibiting the LC neurons in animals with a mutation in the Girk2 gene could be due to compensating changes concerning the NE transporter specific to this genotype. Other studies indicate that the inhibitory effects of the NE on the LC can be mediated by other receptors different to α_2-adrenoceptors (Grandoso *et al*., 2004).

5.4. STUDY IV

Effect of antidepressants on GIRK currents from LC neurons.

5.4. STUDY IV

5.4. Effect of antidepressant on LC current.

It is widely accepted that the inhibitory effect that induces the acute administration of antidepressant drugs in the LC is due to the activation of a GIRK current (Egan *et al*., 1983). However, recently it has been postulated a direct blocking action of numerous antidepressant drugs on the activity of the GIRK channels after acute (Kobayashi *et al*., 2000; 2003; 2004; 2010) and chronic (Cornelisse *et al*., 2007) application. For that reason, the objective of the current chapter was to investigate the role of GIRK channels on the electrical effects induced by some antidepressants in LC neurons. For this, GIRK current recordings were carried out on LC neurons of WT animals by means of the electrophysiological patch-clamp technique in wholle-cell configuration.

The selective 5-HT reuptake inhibitors, show different selectivity on overexpresed GIRK channels in *Xenopus* oocytes since fluoxetine inhibits the current measured through the activation of the GIRK1/GIRK2, GIRK2 or GIRK1/GIRK4 channels (Kobayashi *et al*., 2003) other antidepressants of the same group such as fluoxamine or citalopram do not have any effect on said current (Kobayashi *et al*., 2004). In any case, our results do not support with this hypothesis since fluoxetine did not produce any significant effect on the GIRK currents from LC channels in *in vitro*. According to those results acute

administration of fluoxetine in rat LC slices did not modify the basal firing rate, although it prolonges the recovery of the inhibitory effect induced by NE (Miguelez *et al*., 2009). On the other hand, the acute application (Miguelez *et al*., 2009) and chronic application (Szabo *et al*., 2000) of fluoxetine *in vivo* produced an inhibitory effect on the basal frequency of the LC neurons. Our results support the idea of the participation of the afferents that arrive at the LC in fluoxetine mechanism of action and role as GIRK channels blockers proposed by Kobayashi *et al*., (2004). In this direction, citalopram, another selective 5-HT reuptake inhibitor reduces the firing rate of LC neurons *in vivo* (Béïque *et al*., 1998; Mateo *et al*., 2000) and *in vitro* through a mechanism dependent on α_2-adrenoceptors activation (Grandoso *et al*., 2005). This drug shows little affinity for GIRK1/GIRK2 neuron type channels overexpressed in *Xenopus* oocytes (Kobayashi *et al.,* 2004). Those results together with those published previously indicate that the acute administration of fluoxetine, unlike that observed in *Xenopus* oocytes, does not sufficiently modify the activity of the endogenous GIRK channels located in LC noradrenergic neurons *in vitro*. Similarly, the acute administration of fluoxetine in slices that include the DRN does not produce an inhibition of the GIRK currents as measured by the activation of the serotonergic receptor 5-HT_{1A} (Cornelisse *et al*., 2007).

Chronic and acute administration of bupropion inhibits the basal frequency of rat LC neuron *in vivo* through a mechanism dependent on the activation of the α_2-adrenoceptors subsequent to the increase in

presynaptic levels of NE (Cooper *et al*., 1994; Dong and Blier, 2001; El Mansari *et al*., 2008). However, the acute administration of this drug was neither capable of activating the GIRK channels nor increase the amplitude of the GIRK currents induced by NE in LC neurons *in vitro*. Thus, similar to fluoxetine, the inhibition induced by bupropion can be due to its action on the afferents in the LC. In this way, several evidence indicate that high doses of bupropion are necessary to see the inhibitory effect on both noradrenergic and dopaminergic neurons, increase DA levels in projection areas and block the noradrenergic reuptake system (Ferris *et al*., 1981; Nomikos *et al*., 1989; Cooper *et al*., 1994).

Finally, the acute administration of NE reuptake inhibitor reboxetine did not activate the GIRK conductance but it did increase the amplitude of the GIRK currents induced by NE in LC neurons *in vitro*. Those results were surprising since in the previous chapter it was shown that reboxetine inhibits the discharge frequency of the LC *in vitro* and this effect has reversed with the administration of the GIRK channel blocker tertiapin-Q. Also in the rat LC desipramine produces a complete inhibition of the firing rate that is dependent on the activation of α_2-adrenoceptors and promotes the NE inhibitory effect (Grandoso *et al*., 2004). This indicates that small change in the current was sufficient to inhibit the firing rate. In fact, the correlation between changes in the firing rate and menbrane potential difference is not simple. In line with those results, the application of DMI does not modify the firing rate of the LC neurons *in vitro* despite of a clear hyperpolarization effect on membrane potential (Egan *et al*., 1983). Reboxetine also inhibits the

basal frequency of rat LC neurons after a chronic treatment of at least two days (Szabo *et al*., 2001). However, the lack of effect of reboxetine onto DRN neurons (Szabo *et al*., 2001) indicates that it selectivily acts on noradrenergic neurons. On the other hand investigation research carried out by Kobayashi *et al*., (2010) on *Xenopus oocytes* indicate that reboxetine blocks GIRK2 containing channels. In any case, our results do not support this hypothesis since if reboxetine directly acts by inhibiting GIRK channels of LC neurons an increase in the basal frequency would have been observed, as it was seen with tertiapin-Q in WT mice. In the same way, the firing rate of LC neurons is increased in mice that do not express the GIRK2 and GIRK3 subunits (Cruz *et al*., 2008).

For all this, this study shows that GIRK2 subunits containing channels play a role in the inhibitory effect of noradrenergic antidepressants in LC *in vivo. In vitro*, this participation is less evident due to the need of afferents that reach the nucleus and assure an increase of the noradrenergic tone. On the other hand, this paper has not found evidence that support up the hypothesis that proposes antidepressant drugs as blocking agents of GIRK channels. Finally, the present study indicates the prole of those channels in the spontaneous control of electrical activity of the LC *in vitro*.

5.5. STUDY V

Basal levels of amines in WT and GIRK2 mutant mice.

5.5. STUDY V

5.5. Basal levels of monoamines in WT and GIRK2 mutant mice.

After studying the impact of the GIRK2 gene mutation on the activity *in vivo* and *in vitro* of LC neurons, the following aims was to quantify the levels of monoaminergic neurotrasmotters in WT and GIRK2 mutant mice. For that HPLC experiments were carried out on different brain nucleus such as the LC and projecting areas like DRN and the PFC where levels of NE, 5-HT were measured, 5-HT and its metabolite 5-HIIA were measured.

Our results showed that the GIRK channels containing GIRK2 subunits had greater relevance in the regulation of amine levels related to serotonergic transmission. So, the NE levels were significantly higher in the DRN of GIRK2$^{+/-}$ animals and the levels of 5-HT were significantly diminished in the GIRK2$^{-/-}$ animals. In this group of animals the serotonergic metabolite 5-HIIA levels were increased in the LC. On the contrary, no significant differences were observed in PFC monoamine levels among gemotypes. It is likely that to the mutation of GIRK2 subunits, GIRK3 subunit must also mutated to trigger significant neurochemical modifications in noradrenergic transmission. It has been described that the NE levels are increased in the PFC of animals with double mutation of GIRK2 and GIRK3 subunits, which show an increase in spontaneous electrical activity of the LC *in vitro.* Also a significant

reduction of the severity of morphine withdrawal syndrome is observed in this genotype (Cruz *et al*., 2008). Due to the important reciprocal interaction between the LC and the DRN, alterations on a seratonergic transmission level could participate in said attenuation (Akaoka and Aston-Jones, 1993).

An increase in the number of neurons that discharge in bursts is related with a greater release of neurotransmitter terminal areas (Florin-Lechner *et al*., 1996; Gartside *et al*., 2000). For that reason, the discrepancies in changing levels of neurotransmitters observed in mutated animals were unexpected, since a greater proportion of neurons with burst firing were observed in both groups. Differences in the burst firing pattern or diverse compensatory changes in mutated genotypes could explain these discrepancies.

Thus, in the GIRK2$^{+/-}$ genotype modified burst pattern might be sufficient to specifically increase the levels of NE in the DRN. In the GIRK2$^{-/-}$ mice, however, the greater population of bursty neurons, which showed a similar firing patter to WT mice, could be linked to pronounced changes on serotonergic transmission. Thus, decreased 5-HT levels in DRN from GIRK2$^{-/-}$ mice and increased levels of the serotonergic metabolite 5-HIIA in the LC might support the idea of an accelerated metabolism of 5-HT on these mice. Given that 5-HT exerts an inhibitory control onto LC activity (Szabo and Blier, 2001), reduced 5-HT levels measured on GIRK2$^{-/-}$ mice is consistent with the greater burst activity recorded from LC of this genotype. Genetic and pharmacological

disruption of neurokinin 1 receptor function augments DRN 5-HT neuronal firing by increasing burst firing activity of LC neurons, but does not alter 5-HT basal levels (Santarelli *et al*., 2001; Gobbi *et al*., 2007). Recently, it has been shown that 5-HT_{1A} autoreceptor levels determine vulnerability to stress and response to antidepressants (Richardson-Jones *et al*., 2010). In fact, lower levels of 5-HT_{1A} autoreceptors greatly affects DRN firing rates, but has no effect on basal forebrain 5-HT levels. Since 5-HT_{1A} receptors are coupled to GIRK channels, the contribution of these cellular effectors to DRN basal activity warrants further investigation.

5.6. STUDY VI

Behavioral study in GIRK2 mutant mice.

5.6. STUDY VI

5.6. Behavioral study in GIRK mutant mice: Tail Suspension Test and motor activity test.

Tail suspension test

Numerous studies indicate that exposure to stress could interact with genetic risk factors indicating susceptibility to depression (Caspi *et al*., 2003; Kaufman *et al*., 2006). For this reason, many animal depression models are based on the evaluation of the response of the animal in stress-related tests. More precisely the TST consists of suspending the mouse by the tail during a defined period of time and evaluating its period of immobility (Steru *et al*., 1985). This test is useful to evaluate acute antidepressant activity of drugs, even if methodologically it is not very sophisticated it is easy to carry out, and furthermore economically accessible (Cryan and Mombereau, 2004). Since the GIRK channels participate in the mechanism of action of antidepressant drugs and make important contribution to a wide range of mice behavior (Pravenoti and Wickman, 2008), the next objective was to determine the possible implication of GIRK channles on the behavioral response to stress and to the antidepressant-like effect by drugs induced. For that purpose we used behavioral tests such as TST and activity box, using WT, wich received the GIRK channel blocking agent tertiapin-Q, $GIRK2^{+/-}$ and $GIRK2^{-/-}$ mice.

Our results indicate that both genetic and pharmacological inactivation of GIRK channels cause a better response of the animal in the TST when confronted with stress. Thus GIRK2 mutant mice as well as those who received tertiapin-Q showed less immobility time in the TST. Anxiety and other disorders related to stress, like depression, are frequently co-morbid in humans (Kessler *et al*., 1992). In this sense, it has been described that GIRK2$^{-/-}$ mice show less anxious behavior (decreased anxiety-related behavior) (Blednov *et al*., 2001b; Pravenoti and Wickman, 2008). The phenotype of the knockout mice for the 5-HT$_{1A}$ receptor, which is coupled to GIRK channels, is contradictory since they show a notable anxious behavior and a better response to stress (Heisler *et al*., 1998; Parks *et al*., 1998; Ramboz *et al*., 1998). Recently, it has been published that 5-HT$_{1A}$ autoreceptor levels, which control the serotonergic tone of the DRN and projection areas through feedback inhibition dependent on GIRK channel activation (Hamon *et al*., 1990; Riad *et al*., 2000), determine the vulnerability to stress and antidepression treatment response (Richardson-Jones *et al*., 2010). In this way, mice that have low levels of 5-HT$_{1A}$ receptor in the DRN do not show any alteration in their anxiety behavior, they have a greater electrical activity in the DRN, they can handle better chronic and acute stress and they respond better to antidepression treatment. This phenotype is in part similar to that observed in GIRK2 mutant animals. For this reason the increased mobility observed in TST in either GIRK2$^{-/-}$ or GIRK2$^{+/-}$ animals could be related with a greater monoaminergic activity has been described in (chapter 4.1). It has been proposed that GIRK channels containing GIRK2 subunits possess basal inhibitory

activity that occurs in the absence of receptor stimulation (Lüscher *et al*., 1997). Our results suggest that the blockade of constitutive activity of GIRK channels by tertiapin-Q induces a better response of the animals in the TST. In this sense, neither GIRK2$^{-/-}$ or GIRK2$^{+/-}$ mice responded to acute administration of the noradrenergic antidepressive reboxetine. This suggests that the reduction of inhibitory action of the GIRK channels improves the response in stress-related test and determine the efficacy of the treatment. Further investigations would be needed to evaluate the behavioral response of these animals to chronic treatment with antidepressants of GIRK channels.

In same way, the pharmacological blockage with tertiapin-Q, prevented the antidepressant response of reboxetine. In previous chapters it has been described how of tertiapin-Q increases the basal firing rate of LC neurons *in vitro*. Furthermore, the neurochemical study included in this work shows a significant increase of NE levels in the DRN of GIRK2$^{+/-}$ animals. Since the stimulation of α_1 adrenoreceptors of the DRN produce an increase in its electrical activity (Baraban *et al*., 1978; Millan *et al*., 1994; Svensson *et al*., 1975), it is probable that the antidepressive-like effect observed in those animals is related to greater central monoaminergic activity. Furthermore, the GIRK2$^{+/-}$ animals, unlike the GIRK2$^{-/-}$ animals (Blednov *et al*, 2001b; Blednov *et al*, 2002; Pravenoti and Wickman, 2008), do not show greater locomotive activity. This suggests that the antidepressant-like effect observed in the TST does not have any relation with altered motor activity of the animal. For this reason the GIRK2$^{+/-}$ genotype might be of greater interest than the

GIRK2$^{-/-}$ to study pathological situations related to stress/depression and its treatment.

Recently it has been published that the acute administration of drugs that block GIRK channels have antidepressant activity in the forced swimming test without modifying the locomotive activity in rats (Kawaura *et al*., 2010). In this sense, many studies carried out in GIRK2$^{-/-}$ mice support the role of GIRK channels in many psychiatrics pathologies. In this way, GIRK2$^{-/-}$ mice do not show typical behavioral responses induced by alcohol (Blednov *et al*., 2001a) and they exhibit decreased cocaine self-administration (Morgan *et al*., 2003). For all those reasons, and taken and with the results presented in this work, the compounds that block GIRK channels could be useful in therapies of a number of neuropsychiatric disorders, including depression.

Activity box

The reduction of immobility time observed in TST in GIRK2 mutant mice could be due to an alteration in motor activity. So the next objective consisted in evaluating motor activity, by use of the activity box, in GIRK2 mutant mice who received tertiapin-Q through ICV administration of tertiapin-Q.

In line with previous publications (Blednov *et al*, 2001b; Blednov *et al*., 2002; Pravenoti and Wickman, 2008) the results obtained confirm

that complete delection of the GIRK2 subunit results in an increase of locomotive activity since this hyperactivity is not observed in the group of GIRK2$^{+/-}$ animals (Blednov *et al*., 2001b). So, the increased mobility in the GIRK2$^{+/-}$ group in TST is not due to an increase in locomotive activity of the animal to reduced depression-related behavior.

On the other hand, administration of tertiapin-Q resulted in a mild but significant decrease of locomotive activity in WT animals. So, the increase in mobility observed in TST is produced despite the reduction in motor activity. Those results contradict to some extent that observed in the GIRK2$^{-/-}$ group, nevertheless those differences can be due to the fact that tertiapin-Q produces an acute blockage of all GIRK channels while in GIRK2$^{-/-}$ animals the mutation of the GIRK2 gene affects the channels containing GIRK1 and GIRK2 subunits since birth (Signorini *et al*., 1997).

Due to the importance of dopaminergic transmission in movement control, the changes described in motor activity after the inhibition of GIRK activity could be related to changes in that transmission. In this sense, the increase in motor activity that the GIRK2$^{-/-}$ genotype presents was related to an increase in the D_1 function which occurs through functional blockage of dopamine D_3 receptors. So, the D_3 knockout animals present a similar phenotype to that observed in GIRK2$^{-/-}$ mice (Accili *et al*., 1996). D_3 agonists and antagonists induced hypoactivity and hyperactivity respectively (Svensson *et al*., 1994a, Svensson et al., 1994b; Waters *et al*., 1993) and the administration of antagonists to the

D_1 receptors block the hyperactivity described in GIRK2$^{-/-}$ animals (Blednov *et al*., 2002). On the contrary, dopamine D_2 receptor-deficient mice show hypoactivity and, importantly the expression of other members of DA receptor family is not affected in these knockout mice (Baik *et al*., 1995). Similarly, D_2 receptor antagonist treatment reduces motor activity (Casey *et al*., 1992; Jackson *et al*., 1994), resembling tertiapin-Q effect.

Like D_2 and D_3 receptors, many other receptors including M_2 muscarinic, α_2 adrenergic, $5HT_{1A}$, A_1 adenosine, $GABA_B$, μ, κ and δ opioid and somatostatin receptors may activate GIRK channels (Spauschus *et al*. 1996; Dascal 1997). Therefore, signalling by many of these receptors might be impaired in GIRK2$^{-/-}$ mice. However, available studies suggest that decreased signalling by these receptors (as would be expected in mice lacking GIRK2) decrease motor activity. For example, 5-HT_{1A} receptor knockout mice display decreased exploratory activity (Heisler *et al*. 1998) and elevated levels of anxiety-related behavior (Heisler *et al*. 1998; Parks *et al*. 1998). Mice lacking μ opioid receptors are hypoactive in the open field (Matthes *et al*. 1996). The deletion of m_2 muscarinic receptor did not change the locomotion of null mutant mice in comparison with WT littermates (Gomeza *et al*. 1999). Somatostatin receptor-2 knockout mice demonstrated an increased anxiety-related behavior in a number of behavioral paradigms, while locomotor and exploratory activity was decreased in stress-inducing situations (Viollet *et al*. 2000). Thus, the hypoactivity induced by tertiapin-Q may be related to the acute and complete blockade of GIRK signalling.

6. CONCLUSIONS

6. CONCLUSIONS

On the basis of the aforementioned results, we draw the following conclusions:

- The GIRK2 subunit of GIRK channels plays a relevant role in controlling the basal electrical activity of mouse LC neurons, since in both GIRK2$^{+/-}$ and GIRK2$^{-/-}$ animals, a higher percentage of neurons fires in bursts *in vivo.* Furthermore, the pharmacological blockade of the GIRK channel with tertiapin-Q increases the firing frequency of LC neurons *in vitro*, indicating that GIRK channels control LC activity in a constitutive manner.

- GIRK2 subunit-containing GIRK channels are implicated in the inhibitory effect mediated by the stimulation of α_2-adrenoceptors and μ opioid receptors, since in GIRK2$^{+/-}$ and GIRK2$^{-/-}$ animals the potency of the corresponding receptor agonists to inhibit the firing frequency of LC neurons was diminished. However, their efficacy remained unchanged, suggesting that other subunits than the GIRK2 participate in the effect induced by the activation of α_2-adrenoceptors and μ opioid receptors on noradrenergic transmission *in vivo*.

- The GIRK2 subunit of GIRK channels is implicated in the effect of the NE reuptake inhibitors, DMI and reboxetine. Although, its participation is significantly different for both antidepressants, since in GIRK2$^{+/-}$ mice, DMI results in a total inhibition, whereas reboxetine fails to induce the maximum effect in GIRK2$^{+/-}$ mice and in GIRK2$^{-/-}$ mice no effect was noticed. This efficacy loss was also observed *in vitro*. Since reboxetine selectively inhibits NE reuptake, while DMI also acts on other receptors, reboxetine reduced efficacy could be due to the presence of adaptive changes in the NE transporter derived from the GIRK2 mutation.

- Monoamine reuptake inhibitors do not block GIRK channels. In fact, fluoxetine, reboxetine and bupropion did not modify significantly the potassium currents registered at rest (-60 mV) or after the application of hyperpolarizing potentials (-120 mV) in LC neurons *in vitro.* However, reboxetine-induced small currents were sufficient to inhibit the firing rate. Nevertheless, reboxetine increased the amplitude of the GIRK current induced by NE, probably as a consequence of increased NE levels in the synaptic cleft.

- GIRK2 subunit-containing GIRK channels are implicated in the regulation of amine levels in the CNS. This regulation affects predominantly serotonergic neurotransmission. Thus, 5-HT levels are altered in GIRK2$^{-/-}$ mice in the DRN and its metabolite is

altered in the LC. In contrast, NE levels are only elevated in DRN from GIRK2$^{+/-}$ animals. Significant changes in the PFC were not observed.

- Blocking of the inhibitory effect of GIRK channels could alter behavior related to depression and the efficacy of the treatment. Both the genetic blocking of the GIRK2 subunit and the pharmacological blocking of the GIRK channels results in an improvement in response to stress in mice. So, GIRK2$^{+/-}$ and GIRK2$^{-/-}$ animals, as well as WT animals treated with the channel blocking agent tertiapin-Q, showed reduced immobility in the TST, a similar effect to that observed with antidepressant drugs. Furthermore, the response to reboxetine was blocked.

- The GIRK2$^{+/-}$ genotype may be of greater interest than the GIRK2$^{-/-}$ genotype for studies of behavior related to stress, depression and its treatment. GIRK2$^{+/-}$ animals, unlike GIRK2$^{-/-}$ animals, do not show greater locomotive activity, and so the reduced immobility of the GIRK2$^{+/-}$ mice observed in the TST does not seem to be related to an alteration of motor activity, but rather seems to reflect a better response to stress.

In summary, the present results demonstrate that GIRK2 subunit-containing GIRK channels participate in the tonic control of LC electric activity *in vivo* and *in vitro* and that they are implicated in the acute

effects of several noradrenergic drugs onto noradrenergic transmission and *antidepressant-like* animal behavior. One of the classical problems associated with the pharmacological treatment of depression is the delay of the onset of clinical improvement, which is thought to be due to the desensitization of receptors coupled to GIRK channels. Therefore, given the role of the LC in depression and the fact that the reduction of the inhibitory role of GIRK channels triggers an increase in noradrenergic burst activity, the pharmacological blockage of GIRK channels constitutes a promising therapeutic candidate for faster onset of antidepressant response.

7. REFERENCES

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8. SUMMARY

SUMMARY

Major depression is a recurrent or chronic mood disorder with impaired psychosocial function, and an increased tendency of relapse for patients who fail to reach remission. In industrialized societies, it affects 15% of the population being the age range of affected people between 18 – 44 years (Kessler *et al*., 2007). The World Health Organization has predicted that major depression will become a key cause of illness-induced disability by the year 2010, followed only by ischemic heart disease (Murray and Lopez, 1997).

Although the disorder is thought to be the outcome of gene-environmental interactions, the causative genes and environmental factors underlying depression remain to be identified (Lee *et al*., 2010). On the other hand, there are many hypotheses to explain the molecular mechanism of depression. Many of them are related, but a definite ethiopathogenic mechanism is still not identified. The action of reserpine in causing depression by depleting catecholamines and the reversal of these effects by triciclic antidepressants formed the basis of the catecholamine hypothesis of depression (Schildkraut, 1965). The monoamine hypothesis proposes that the underlying biological or neuroanatomical basis of depression is a deficiency or imbalance of central monoamine neurotransmission (Schildkraut, 1965; Van Praag, 1982). This hypothesis was supported by the effects of the antidepressant drugs on these neurotransmitter systems.

However this hypothesis does not explain why the clinical effects of antidepressants appear at least four weeks after the beginning of the treatment, while the antidepressants increase the monoamine levels immediately.

It is now believed that an adaptation of downstream events, including lasting changes in gene expression by chronic treatment underlay the antidepressant efficacy. However, other molecular mechanisms could also be implicated since the desensitization of the α_2-adrenoceptors seems to be necessary for observing the antidepressant effect. These receptors are coupled to G proteins, which activate the inwardly rectifier potassium channels (GIRK; also known as Kir3). Therefore, the action on the GIRK channel could be a new pharmacological target in the treatment of depression.

GIRK channels are downstream effectors of G protein-coupled receptors (GPCRs), and they can be activated by direct binding of the βγ subunit of G protein, an interaction with important physiological consequences. In fact, GIRK channels hyperpolarize neurons in response to activation of GPCRs which leads to decrease the excitability of neurons, slow synaptic potentials and reduce volume transmission (Krapivinsky *et al*., 1995; Lüscher *et al*., 1997; Signorini *et al*., 1997; Wickman and Clapham, 1995; Wickman *et al*., 1998; Yamada *et al*., 1998). In mammalian four subunits (GIRK 1-4) have been cloned which assemble into homo and heterotetrameric channels (Wickman *et al*., 2002). GIRK1, GIRK2 and GIRK3 are broadly expressed in many areas of the central

nervous systems whereas GIRK4 expression is restricted to a small number of neuron populations (Aguado *et al*., 2008; Karschin *et al*., 1996; Wickman *et al*., 2000). Most neuronal GIRK channels are thought to be heteromeric containing GIRK1 and GIRK2 subunits (Luscher *et al*., 1997; Signorini *et al*., 1997, Torrecilla et al., 2002). Each GIRK subunit has a specific role in the activity of the channel. While GIRK3 subunits may regulate the availability of the GIRK channels on the plasma membrane, GIRK1 subunits are related to the time that channels remain open. However, GIRK2 subunits have a primary role controlling channel conductance. Indeed, the absence of GIRK2 subunit makes the channel become dysfunctional since mice lacking GIRK2 channels exhibit little or no GIRK current in a number of brain regions (Labouèbe *et al*., 2007; Slesinger *et al*., 1997).

Up to date the availability of pharmacological agents that act on the GIRK channels has been poor. In this context, the use of GIRK2 knockout ($GIRK2^{-/-}$) mice has been a key tool of insight into the role of specific GIRK channel composition on neuronal physiology, pathology and animal behaviour. Thus, GIRK channels functional relevance has been linked to susceptibility to seizures (Signorini, 1997), hyperalgesia and analgesia (Blednov *et al*, 2003; Marker *et al*., 2004, 2005; Smith *et al*., 2008), drug addiction and alcohol related behavioural effects (Blednov *et al*., 2001b; Morgan *et al* 2003; Kobayashi *et al*., 1999; Cruz *et al*., 2008), motor activity and co-ordination and reward and anxiety-related behaviours (Pravetonti and Wickman, 2008). Abnormal GIRK function can lead to excessive or deficient neuronal excitability which is related to several

pathologies such as epilepsy or Down's syndrome (Lüscher and Slesinger, 2010). Therefore, GIRK channels represent a key part in many types of neuronal communication and, as outlined in the following sections, modification of GIRK channel function and number could affect central nervous systems function in health and disease.

Studies of locus coeruleus (LC) noradrenergic neurons have had a key role to understand the etiology of some psychiatric illnesses related to dysfunction of this neuromodulatory system (Itoi and Sugimoto, 2010). This nucleus contains the largest population of central noradrenergic neurons and innervates almost the entire neuroaxis (Dahlstrom and Fuxe, 1964; Swanson and Hartman, 1975). In LC neurons, activation of μ opioid receptors and α_2-adrenoceptors leads to a decreased excitability (North and Williams, 1983; Pepper and Henderson, 1980; Williams et al., 1982), mainly though activation of GIRK channels (Aghajanian and Wang, 1986; Travagli et al., 1995; 1996; Williams et al., 1988), and in particular, those formed by GIRK2 and GIRK3 subunits (Cruz et al., 2008; Torrecilla et al., 2002; 2008). Additionally, constitutive GIRK channel activity contributes to the resting membrane potential of LC neurons *in vitro* (Torrecilla et al., 2002; Velimirovic et al., 1995). Moreover, in mice lacking both GIRK2 and GIRK3 subunits the spontaneous firing rate of LC neurons from brain slices and cortical NA concentrations are augmented (Cruz et al., 2008). However, the role of GIRK channels controlling LC-noradrenergic function *in vivo* has not been investigated yet.

In this line, electrophysiological studies using *Xenopus* oocyte expression assays have suggested that GIRK channel modulators might have therapeutic benefits in the treatment of several neurologic disorders and cardiac arrhythmias (Hashimoto et al., 2006, Kobayashi et al, 2004; Kobayashi et al., 2010). Thus, GIRK channels have been proposed as new pharmacological targets that could be effective for the treatment of several illnesses related to an altered central neurotransmission.

Taken all together, the main aim of this work was to investigate the involvement of GIRK2 subunit-containing GIRK channels on the noradrenergic transmission *in vivo* and *in vitro* as well as on the mechanism of action of antidepressant drugs. To this end, we established the following concrete objectives:

- To determine the contribution of GIRK2 subunit-containing GIRK channels to the bioelectric properties of noradrenergic neurons in the mouse LC *in vivo* and to quantify the levels of monoaminergic neurotransmitters in wild type (WT) and GIRK2 mutant mice.

- To characterize the effects induced by the administration of the α_2-adrenoceptor agonist clonidine, and the μ opioid receptor agonist morphine, on the electric activity of LC neurons in GIRK2 mutant mice *in vivo.*

- To study the role of GIRK2 subunits-containing GIRK channels on the inhibitory effects induced by antidepressant drugs on noradrenergic neurotransmission by measuring the electrical activity of the LC neurons in WT and GIRK2 mutant mice *in vivo* and *in vitro*. Additionally, the effect of antidepresants on GIRK currents were examinated in LC slices from WT mice.

- To study the possible implication of GIRK channels on the behavioral response to stress and to the antidepressant-induced effect, by means of behavioral tests such as tail suspension test and activity box, using WT and GIRK2 mutant mice.

For that, we used C57bj6 WT, heterozygous ($GIRK2^{+/-}$) and homozygous ($GIRK2^{-/-}$) mice (males and females) and performed electrophysiological recordings (*in vivo* and *in vitro*), neurochemical determinations by HPLC technique and behavioral experiments.

Our results indicated that the electric characteristics of mice LC neurons were similar to those previously described in rats (Cerbadaum and Aghajanian., 1976) and mice (Gobbi et al., 2007). First, recorded cells presented biphasic excitation-inhibition response to pressure applied onto the contralateral hind paw. Second, the extra-cellular signal of the action potentials recorded from all genotypes (WT, $GIRK2^{+/-}$ and $GIRK2^{-/-}$**)** displayed the typical bi-phasic morphology, with a large positive part that has notch and another final negative part. Third, the firing rate oscillated

between 0.5-5 Hz, and the mutation of the Girk2 gene did not alter the firing rate nor the coefficient of variation of LC neurons in anesthetized mice. However, both GIRK2$^{+/-}$ and GIRK2$^{-/-}$ animals had a higher percentage of LC neurons with burst firing pattern than WT mice. Furthermore, GIRK2$^{-/-}$ animals showed a greater percentage of burst firing neurons as compared with the GIRK2$^{+/-}$ mice. A more extended study of the burst pattern showed that in the latter genotype the mean spikes per burst as well as the mean inter-spike interval were reduced compared to those recorded from WT mice. Therefore, burst firing pattern was specifically modified in GIRK2$^{+/-}$ mice.

Studies carried out in LC brain slices from WT mice showed that the mean basal frequency was lower than that recorded *in vivo*, which was in agreement with previous reports (Torrecilla *et al*., 2002; Miguelez *et al*., 2009). The application of the peptide that blocks the GIRK channels, tertiapin-Q (Jin and Lu, 1999,) increased the basal frequency of LC neurons in brain slice from WT mice indicating that GIRK channels control LC activity in a constitutive manner. In addition, as observed in the *in vivo* experiments, the basal frequency of LC neurons from GIRK2$^{+/-}$ mice *in vitro* was not different from that obtained from WT slices. Therefore, our *in vitro* experiments support the hypothesis that GIRK channels play a relevant role in the control of the basal electric activity of the LC neurons (Cruz *et al*., 2008; Torrecilla *et al*., 2002). However, simultaneous blockage of at least GIRK2 and GIRK3 subunits seems to be necessary to induce relevant changes in basal firing rate. In this sense, in

LC neurons from double GIRK2 and GIRK3 knockout mice increased basal frequency is observed *in vitro* (Cruz *et al*., 2008).

We next evaluated the role of GIRK2 subunits containing GIRK channels on the inhibitory effect induced by α_2-adrenoceptor agonist, clonidine and μ opioid agonist, morphine in LC neurons *in vivo*. As expected, both agonists induced a dose-dependent inhibitory effect onto LC neurons firing rate in all genotypes. However, in mutant mice dose-response curves for clonidine and morphine were shifted to right as compared to those obtained in WT genotype. Statistical differences were also observed between GIRK2$^{-/-}$ and GIRK2$^{+/-}$ mice. In all cases, the maximal inhibitory effect was reached by agonist application. These results suggest that GIRK2 subunits participate on the inhibitory effect induced by α_2 and μ agonist on LC activity although other subunits, likely GIRK3 subunits, may be necessary to reach maximal efficacy of the drug.

When we tested the effect of the antidepressants reboxetine and desipramine (DMI) on LC basal activity we observed that in GIRK2$^{+/-}$ mice DMI was less potent though equally effective inhibiting electric activity compared to the WT group. On the other hand, the dose-response curve for reboxetine showed that this antidepressant was not only less potent but also less effective inhibiting LC neurons in GIRK2$^{+/-}$ animals. Furthermore, reboxetine did not have any effect onto LC neurons from GIRK2$^{-/-}$ mice. The antagonist of the α_2-adrenoceptors RX821002 recovered to the same extent the inhibitory effect induced by the antidepressants in all tested genotypes.

The reduced efficacy of reboxetine inhibiting LC activity from GIRK2 mutant mice was also observed *in vitro.* Thus, in LC slices from GIRK2$^{+/-}$ mice, unlike in the WT LC slices, a maximal concentration of reboxetine did not completely reduce the spontaneous firing rate. However, after reboxetine washed out noradrenaline (NE) perfusion, which was used as an internal control, produced a total inhibition of the firing rate that returned to basal values within 10 min after wash, in both WT and GIRK2$^{+/-}$ mice. On the other hand, perfusion of DMI onto WT LC slices produced a concentration-dependent and complete inhibition of firing rate. Tertiapin-Q not only recovered the inhibitory effect of DMI, but it also increased spontaneous firing frequency above basal values. Similarly, reboxetine completely inhibited LC firing rate in a concentration-dependent manner and tertiapin-Q recovered spontaneous firing rate toward basal values. Since reboxetine, unlike DMI, showed smaller efficacy inhibiting the firing rate of the LC neurons in GIRK2$^{+/-}$ animals *in vivo* and *in vitro*, it seems reasonable to argue that role of GIRK2 channels on the mechanism of action of these two antidepressants may be different. Taken into account that reboxetine selectively inhibits NE reuptake, its reduced efficacy could be due to adaptive changes in the NE transporter derived from the Girk2 gene mutation.

On the other hand, our results obtained from LC WT slices using patch-clamp recordings were in disagreement with the hypothesis that suggests that antidepressant drugs elicit a direct blocking action on GIRK channels (Kobayashi *et al*., 2000; 2003; 2004; 2010): First, acute perfusion of fluoxetine, reboxetine and bupropion did not modify the LC potassium

currents registered at rest (-60 mV) or at more hyperpolarized potentials (-120 mV). Second, NE produced an increase of the resting potassium current which reverted at the potassium equilibrium potential and this NE-current was greater when reboxetine was co-perfused. These results indicated that reboxetine required exogenous NE for activating GIRK. Conversely, co-administration of bupropion did not significantly increase the amplitude of the NE-current.

Our results also showed that the mutation of Girk2 gene had greater relevance in the regulation of basal amine levels related to serotonergic transmission. Thus, 5-HT levels were significantly reduced in the dorsal raphe nucleus of GIRK2$^{-/-}$ mice and also levels of its metabolite 5-HIIA was increased in the LC of this genotype. NE levels were only elevated in dorsal raphe nucleus of GIRK2$^{+/-}$ animals. GIRK2 mutation had no impact on monoamine basal levels of the prefrontal cortex.

After the electrophysiological characterization, we performed behavioural experiments to study the impact of GIRK2 subunit mutation on the depression-related behaviors as well was on the antidepressant-like activity of drugs. For that, we have first validated the tail suspension test (TST) by evaluating the acute effect of several antidepressants on the immobility time in WT mice. The baseline behavior in the TST was examined after injections of physiological saline, DMI, reboxetine and fluoxetine. All antidepressant tested, significantly decreased the immobility time compared to control group. Reboxetine, in fact, was the most effective drug reducing immobility. We next examined the impact of GIRK2 subunit

mutation on immobility time in the TST using WT and GIRK2 mutant mice. GIRK2$^{+/-}$ mice as well as GIRK2$^{-/-}$ mice showed reduced immobility time in the TST compared to WT group. Also, the behavioral effects of reboxetine were studied in GIRK2 mutant mice. Reboxetine did not alter significantly basal immobility time in GIRK2$^{-/-}$ mice although it induced a slight but significant increment of immobility time in GIRK2$^{+/-}$ mice. Also a pharmacological approach was done in WT mice by using intracerebroventricular injections of the GIRK channel blocker, tertiapin-Q. Thus, tertiapin-Q (30 and 100 pmol) dose-dependently decreased the immobility time in the TST. It should be pointed out that the magnitude of the response obtained from these experiments were not different to that observed in the GIRK2$^{-/-}$ genotype. Both the genetic blocking of the GIRK2 subunit and the pharmacological blocking of the GIRK channels improved behavioral response to stress. Moreover, tertiapin-Q injection 30 minutes before reboxetine administration completely prevented the effect of the antidepressant drug in WT mice. In fact, the immobility time values in this group were not different from those obtained in the GIRK2$^{-/-}$ mice who had also received reboxetine at the same dose. Thus, we can conclude that blocking the inhibitory effect of GIRK channels could alter depression-related behaviors and more importantly, the efficacy of the treatment.

We next tested whether the reduction of immobility time observed in the GIRK2 mutant mice as well as that induced by blocking GIRK channels were due to an alteration of animal motor activity. For that purpose we used the activity chamber to measure mice locomotion during

30 min. As previously described, the results obtained confirm that complete delection of the GIRK2 subunit results in an increase of locomotive activity since this hyperactivity is not observed in the group of GIRK2$^{+/-}$ animals (Blenov et al., 2001b; Blenov et al., 2002; Pravetoni and Wickman, 2008). Thus, tertiapin-Q (30 pmol and 100 pmol) significantly reduced motor activity in WT mice. It is probable that the antidepressive-like effect observed in those animals is related to greater central monoaminergic activity. For this reason the GIRK2$^{+/-}$ genotype might be of greater interest than the GIRK2$^{-/-}$ to study pathological situations related to stress/depression and its treatment.

On the basis of the aforementioned results, we draw the following conclusions:

- The GIRK2 subunit of GIRK channels plays a relevant role in controlling the basal electrical activity of mouse LC neurons *in vivo*, since in GIRK2$^{+/-}$ as well as in GIRK2$^{-/-}$ animals, a higher percentage of neurons fires in burst. Furthermore, the pharmacological blockade of the GIRK channel with tertiapin-Q increases the firing frequency of LC neurons *in vitro*, indicating that GIRK channels control LC activity in a constitutive manner.

- GIRK2 subunit-containing GIRK channels are implicated in the inhibitory effect mediated by the stimulation of α_2-adrenoceptors and μ opioid receptors, since in GIRK2$^{+/-}$ and GIRK2$^{-/-}$ animals the inhibitory potency of both agonists was diminished. However, their efficacy remained unchanged suggesting that other subunits than the GIRK2 participate in the effect induced by the activation of α_2-adrenoceptors and μ opioid receptors *in vivo*.

- The GIRK2 subunit of GIRK channels is implicated in the effect of the NE reuptake inhibitors, DMI and reboxetine. Although, its participation is significantly different for both antidepressants, since in GIRK2$^{+/-}$ mice DMI results in a total inhibition of firing rate whereas reboxetine fails to induce the maximal inhibition in this genotype and had no effect in GIRK2$^{-/-}$ mice. This efficacy loss was also observed *in vitro*. Since reboxetine selectively inhibits NE reuptake, while DMI also acts on other receptors, reboxetine reduced efficacy could be due to the presence of adaptive changes in the NE transporter derived from the GIRK2 mutation.

- Monoamine reuptake inhibitors do not block GIRK channels. In fact, fluoxetine, reboxetine and bupropion did not modify significantly the potassium currents registered at rest (-60 mV) or after the application of hyperpolarizing potentials (-120 mV) in LC neurons *in vitro.* However, reboxetine-induced small currents

were sufficient to inhibit the firing rate. Nevertheless, reboxetine increased the amplitude of the GIRK current induced by NE, probably as a consequence of increased NE levels in the synaptic cleft.

- GIRK2 subunit-containing GIRK channels are implicated in the regulation of amine levels in the central nervous systems. This regulation affects predominantly serotonergic neurotransmission. Thus, 5-HT levels are altered in GIRK2$^{-/-}$ mice in the dorsal raphe nucleus and its metabolite is altered in the LC. In contrast, NE levels are only elevated in dorsal raphe nucleus from GIRK2$^{+/-}$ animals. Significant changes in the prefrontal cortex were not observed.

- Blocking the inhibitory effect of GIRK channels could alter behavior related to depression and the efficacy of the treatment. Both the genetic blocking of the GIRK2 subunit and the pharmacological blocking of the GIRK channels results in an improvement in response to stress in mice. So, GIRK2$^{+/-}$ and GIRK2$^{-/-}$ animals, as well as WT animals treated with the channel blocking agent tertiapin-Q, showed reduced immobility in the TST, a similar effect to that observed with antidepressant drugs. Furthermore, the response to reboxetine was blocked under these experimental conditions.

- The GIRK2$^{+/-}$ genotype may be of greater interest than the GIRK2$^{-/-}$ genotype for studies of behavior related to stress, depression and its treatment. GIRK2$^{+/-}$ animals, unlike GIRK2$^{-/-}$ animals, do not show greater locomotor activity, and so the reduced immobility of the GIRK2$^{+/-}$ mice observed in the TST does not seem to be related to an alteration of locomotion, but rather seems to reflect a better response to stress.

In summary, the present results demonstrate that GIRK2 subunit-containing GIRK channels participate in the tonic control of LC electric activity *in vivo* and *in vitro* and that they are implicated in the acute effects of several noradrenergic drugs onto noradrenergic transmission and *antidepressant-like* animal behavior. One of the classical problems associated with the pharmacological treatment of depression is the delay of the onset of clinical improvement, which is thought to be due to the desensitization of receptors coupled to GIRK channels. Therefore, given the role of the LC in depression and the fact that the reduction of the inhibitory role of GIRK channels triggers an increase in noradrenergic burst activity, the pharmacological blockage of GIRK channels constitutes a promising therapeutic candidate for faster onset of antidepressant response.