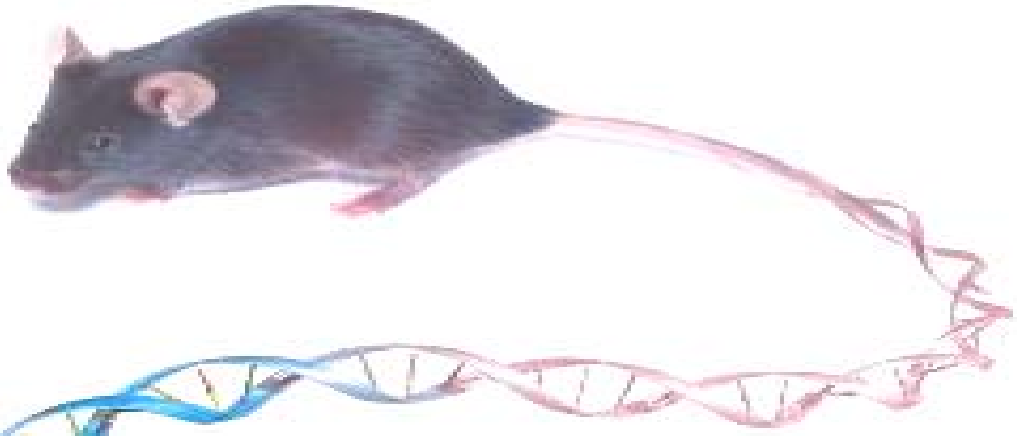
UNIVERSITY OF CRETE
SCHOOL OF HEALTH SCIENCES

GRADUATE PROGRAM IN
THE MOLECULAR BASIS OF HUMAN DISEASE

Division of Basic Sciences

Department of Pharmacology

Supervisor: Venetia Zachariou, PhD, Assistant Professor



The role of RGSz1 protein in depression and the action of antidepressant pharmacological agents

Dimitra D. Terzi

Irakleio, November 2008

Table of Contents

Περίληψη

Η κατάθλιψη είναι μία μείζονα συναισθηματική διαταραχή, που προκαλείται από συνδυασμό γενετικών και περιβαλλοντικών παραγόντων. Η υποκείμενη παθοφυσιολογία της διαταραχής είναι αρκετά περίπλοκη, περιλαμβάνοντας διάφορες περιοχές του εγκεφάλου, που αλληλεπιδρούν μεταξύ τους για την πρόκληση του καταθλιπτικού φαινοτύπου. Φαρμακολογικές ουσίες, που είναι αποτελεσματικές στην αναστροφή του φαινοτύπου, βασίζονται κυρίως στην εμπλοκή τους στο σύστημα των μονοαμινών, παρόλο που περισσότερο στοχευμένα φάρμακα αποτελούν ήδη αντικείμενο εκτεταμένης έρευνας. Οι RGS πρωτεΐνες (ρυθμιστές της σηματοδότησης μέσω των G πρωτεϊνών) είναι μία οικογένεια πρωτεϊνών, που εμπλέκεται στη μεταγωγή σημάτων μέσω υποδοχέων που είναι συζευγμένοι με G πρωτεΐνες, μέσω πρόσδεσης σε G υπομονάδες και επιταχύνοντας την επαναφορά τους στην GDP ανενεργή κατάσταση. Η RGSz είναι μία μικρού μοριακού βάρους πρωτεΐνη, μέλος αυτής της οικογένειας, με σημαντικό ρόλο στη ρύθμιση της σηματοδότησης μέσω των μ-οπιοειδών υποδοχέων. Με βάση προηγούμενες μελέτες που δείχνουν ότι η Gαz υπομονάδα, που είναι εκλεκτικά συζευγμένη με την RGSz, είναι ένα σημαντικό μόριο στην κατάθλιψη, αφού η έλλειψη προκαλεί έναν αξιοσημείωτο καταθλιπτικό και αγχώδη φαινότυπο, ερευνήσαμε το συμπεριφορικό φαινότυπο επίμυων στους οποίους έχει απαλειφθεί το γονίδιο της RGSz (KO επίμυες). Βρέθηκε ότι οι RGSz KO επίμυες είναι περισσότερο καταθλιπτικοί κια αγχώδεις σε σχέση με την ομάδα ελέγχου των αγρίου τύπου επίμυων, και περισσότερο ευαίσθητοι στις αντικαταθλιπτικές δράσεις τις φλουοξετίνης. Επιπλέον προσδιορίστηκε η κατανομή έκφρασης της RGSz σε πρωτεϊνικό επίπεδο σε διάφορες περιοχές του εγκεφάλου και έγιναν προσπάθειες να αναπτυχθεί ένα μοντέλο κατάθλιψης σχετιζόμενο με εθισμό σε φάρμακα και χρόνιο πόνο για την περαιτέρω μελέτη του ρόλου της RGSz σε αυτές τις συναισθηματικές διαταραχές. Τα δεδομένα της μελέτης αυτής υποδεικνύουν για πρώτη φορά το ρόλο της RGSz στη ρύθμιση της διάθεσης και στη δράση των αντικαταθλιπτικών φαρμάκων.

Abstract

Depression is a major mood disorder caused by a combination of genetic and environmental factors. The underlying pathophysiology of the disorder is rather complicating, including various brain regions, which interact with each other for the induction of a depressive phenotype. Pharmacological agents that are effective in reversing this phenotype are primarily based on their intervention in the monoamine system, though more targeted drugs are also under investigation. RGS (Regulators of G-protein Signaling) proteins are a family of proteins, interfering in the signal transduction of G-protein coupled receptors, by binding to G subunits and accelerating their return to the GDP inactive state. RGSz is a small RGS protein with a rather important role in the regulation of mu- opioid receptors signaling. Based on data showing that the Gαz subunit, which is specifically coupled to RGSz, is an important molecule for depression, since its absence leads to increased depression and anxiety-like phenotype, we investigated the behavioral phenotype of mice lacking the RGSz gene (knockouts-KO). We found that RGSz KO mice show an anxiety and depression-like phenotype and are more sensitive to the antidepressant effect of fluoxetine. Moreover, we identified the expression pattern of RGSz at protein level in various brain regions and we tried to establish a model of depression related to addiction and chronic pain, and investigate the role of RGSz in such mood disorders. Our data provide the first evidence for a role of RGSz in mood modulation and antidepressant drug action.

INTRODUCTION

An overview on depression

Depression, a serious mental disorder, is among the greatest health problems and a major cause of morbidity worldwide. 2-5% of the US population is suffering from severe depression, while 20% from milder forms, more frequently women and elderly people. Although depression has been known as a pathological entity since 400 B.C. it remains a rather blurry pathological condition, regarding not only its pathogenesis and pathophysiology, but also its diagnosis and treatment. It is now believed that genetic factors, accompanied by a diverse range of non-genetic, environmental factors, are the main causes of depression, in an unknown mechanism of synergic action. On the other hand, the diagnosis of depression, mainly based on a highly variable set of symptoms, rather than on objective diagnostic criteria, has led to the symptomatic-based identification of various subtypes, which, indeed, may reflect different underlying pathologies. For this reason, though many and generally effective therapeutic approaches (antidepressant agents and psychotherapy), the causative treatment of patients is rather impossible. Concerning the therapeutic effect of the antidepressant drugs, only 50% of the patients under treatment experience complete remission, and 80% partial response [1, 2]. Therefore, much interest has been paid for the last decades on the investigation of the pathophysiological mechanisms underlying depression, as well as the mechanisms of antidepressants' action, in order to fully understand the nature of the disorder that will lead to a more objective classification and a more targeted and effective pharmacological treatment.

Genetic and non-genetic causes of depression

Major depressive disorder (MDD) is a common pathological entity, with lifetime prevalence at least 10% and high risk in women (twice the risk in men), and it seems to have moderate genetic background. For MDD, twin studies have revealed 40-50% heritability, the relative risk (RR-ration of risks to first-degree relatives of MDD probands vs. the general population) is around 2 to 3, while the RR for recurrent and early-onset (in the 30s or earlier) MDD is at least 4 to 5. Additionally neuroticism, a high-order factor in analyses of self-rated or observer-rated measures of personality, characterized by dysphoria, anxiety, tension and emotional reactivity, also has 40-50% heritability, suggesting that there may be common genetic factors that predispose

to both pathological conditions, as well as to generalized anxiety disorder [3]. Based on DSM classification, depressive states are familial and genetic, but the genes for depression are likely the same genes for generalized anxiety and for negative affect personality trails, often called neuroticism or harm avoidance. On the other hand, depressive states coexist with anxious or agitated states [4, 5]. For these reasons, some genetic studies incorporate depressive and anxiety symptoms into a single phenotype.

Apart from the genetic factors, non-genetic factors, such as stress, emotional trauma, social factors (early experiences of parental care or neglect, quality of core intimate relationship), viral infections (e.g. Borna virus) are also very important. According to DSM IV classification, depressive syndromes are observed in the context of various pathological conditions such as endocrine disturbances, Parkinson's disease, cancers, traumatic head injury, collagen vascular diseases, asthma, diabetes and stroke. Moreover, certain drugs and alcohol may induce depressive episodes. Notably, stress seems to play a key role in depression, and it is often referred to as a stress-related disorder, the condition in which episodes of depression occur in the context of some form of stress. However, stress per se in not sufficient to cause depression, and while some people do not become depressed after serious stressful experiences, in others mild stressful experiences are adequate to provoke depression. These observations suggest that there must be some sort of interactions between genetic predisposition and environmental factors that acting additively lead to the depressive phenotype. Here, it should be noted that the post-traumatic stress disorder (PTSD) is distinct from depression based on symptomatology, treatment and longitudinal course of the illness [2].

The investigation of the gene-environment interactions in depression require firstly the identification of candidate genes, mainly based on association studies. The research for the candidate genes has been focused on three categories: (1) genes coding molecules of the monoaminergic pathway, such as the serotonin transporter, serotonin 2A receptor, tyrosine hydroxylase, tryptophan hydroxylase 1,2 (THP1, 2) and catechol-o-methyltransferase, (2) genes of the hypothalamic-pituitary-adrenal axis (HPA), such as the gene coding for the corticotrophin releasing hormone receptor (CRHR1) and (3) gene coding the brain-derived neurotrophic factor (BDNF). The most significant findings involve:

1) The well-known serotonin transporter gene linked polymorphic regions (5-HTTLPR) 44-bp insertion/deletion polymorphism: childhood abuse and stressful life events are associated with high risk of depression in individuals with 5-HTTLPR "short" allele, but has little effect on depression among "long" allele homozygotes. However, 5-HTTLPR short allele carriers have no higher risk for depression, and also derive more benefit from positive environmental influences, such as social support. On the other hand, subjects with the 5-HTTLPR long allele become depressed as often, but for reasons relatively independent of environmental stress. These findings suggest that the 5-HTTLPR short allele conveys sensitivity to both positive and negative life experiences.
2) The BDNF: BDNF *66met* allele interacts with the 5-HTTLPR short allele, thus conferring sensitivity to positive and negative environmental factors.
3) The CRHR1: interacts with child abuse causing adult depression. Unlike 5-HTTLPR, neither BDNF nor CRHR1 are directly associated with depression.
4) The TPH gene: MDD is associated with 10 SNP haplotypes, and a loss of function polymorphism in TPH2. Tryptophan depletion leads to depressed mood only in predisposed individuals.

However, the exact mechanism of the gene-environment interactions remains hardly understood. Three possible mechanisms have been proposed:

1) Early adverse experiences epigenetically modify the polymorphic gene
2) A double-hit mechanism, in which early adverse experiences epigenetically modify one gene and the impact of this modification in adult life is moderated by sequence polymorphism in another functionally related gene.
3) A gene polymorphism modulates the effect of early environmental experiences on the programming of other functionally related genes. For this hypothetical mechanism there is also some empirical support regarding the serotonin transporter: the lack of functional serotonin transporter at plastic early developmental stages leads to increased extracellular serotonin and subsequent downregulation and desensitisation of the inhibitory 5-HT1A receptors, phenotypically observed as life-long anxiety and depression-like behaviors in novel environments [3, 6].

Undoudtedly, genetic and non-genetic factors act synergically to cause depression, with genetic and social studies suggesting that the genetic component is stronger in episodic depression, and psychosocial contribution is more profound in chronic depression. However, the exact mechanisms of these interactions need further investigation and will provide new insights in the effective treatment of the disorder.

Pathophysiology of depression

One of the major questions concerning every pathological condition is how a physiologically functioning part of the human body becomes pathological. In the case of depression, this question should be focused on two parts: which are the brain regions involved in depression, and what is their mode of involvement, which also suggests the mechanisms of the pharmacological treatments. So, much attention has been paid on understanding the neural circuitry of mood and consequently the pathophysiology of depression. Apart from hippocampus and frontal regions of the cerebral cortex, which have been strongly associated with depression and antidepressant medications, mediating mainly cognitive aspects of depression, research has revealed that the striatum (especially nucleus accumbens-NAc) and amygdala, critical for emotional memory, may mediate depression-related anhedonia, anxiety and reduced motivation, while hypothalamus, may be involved in mediating neurovegetative symptoms of depression. The distinct role of each region is not yet clear, and it is believed that all these brain regions operate in a highly interacting circuit to regulate depressive phenotype. This complex neuronal network is summarized in fig. 1: the ventral tegmental area (VTA) provides dopaminergic input to NAc and other brain regions, a pathway that is strongly connected to hypothalamus, while norepinephrine-noradrenaline, from the locus coeruleus (LC) and serotonin, from the dorsal raphe (DR) innervate almost all of the involved brain regions.

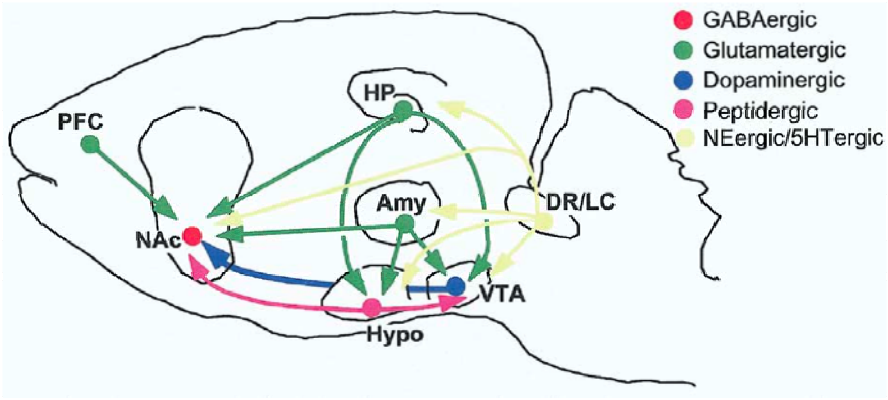


Fig. 1 Neural circuitry of depression [7]

Based on the neuronal circuits described above, many hypotheses have been proposed for explaining how depression occurs and how antidepressant agents work. Much attention has been paid on the following three hypotheses:

1) *Dysregulation of the hippocampus and hypothalamic-pituitary-adrenal (HPA) axis:* The activity of the HPA axis [secretion of CRF-corticotropin releasing factor from hypothalamus ➔ synthesis and release of ACTH-adrenocorticotropin from pituitary ➔ synthesis and release of glucocorticoids from the adrenal cortex ➔ feedback effect on HPA axis] is controlled by the hippocampus (inhibitory effect) and amygdala (excitatory effect). Glycocorticoids enhance hippocampal inhibition of HPA activity, and hippocampal function in general, including cognitive abilities, while sustained elevated levels of glycocorticoids cause hippocampal neuronal damage, reduced inhibition of HPA axis, increased levels of glycocorticoids, in a vicious cycle. Abnormal excessive activation of HPA axis, increased cortisol production, hypersecretion of CRF, increased CRF levels in celebrospinal fluid are usual findings in individuals with depression, abnormalities that are reversed by antidepressant treatment.
2) *Impairment of neurotrophic mechanisms:* Deficiency in neurotrophic support contributes to the hippocampal pathology of depression, while antidepressant treatment reverses this deficiency and the respective symptomatology. Research on the association of BDNF (brain-derived neurotrophic factor) to depression has revealed decreased BDNF expression in hippocampus, neocortex and amygdala of animals upon acute and chronic stress, and vice

versa, antidepressants increase hippocampal BDNF expression, not only in animals but also in humans, partly via the transcription factor CREB (c-AMP response element binding protein). It is also possible that other neurotrophic factors may be involved in the pathology of depression and action of antidepressants but not much is known on this field.

3) *Impairment of brain reward pathways:* Brain imaging and autopsy studies have implicated the presence of abnormalities in brain regions other than the hippocampus, suggesting their involvement in depression, although there is not yet solid and direct association of these regions to depression. More specifically, the **nucleus accumbens (NAc) - ventral tegmental area (VTA)** dopaminergic pathway, mainly studied for its role in reward, seems to mediate depression-like behavior based on experiments on animal models. There is evidence suggesting that stress activates VTA neurons and induces dopamine transmission to NAc, while antidepressant agents alter dopaminergic activity in the VTA and intervention in the dopaminergic transmission in the VTA-NAc pathway regulates depression-like behavior in animals. The involvement of **hypothalamus** to depression, apart form the HPA axis, is implied by the fact that hypothalamic nuclei and their peptide transmitters play an important role in appetite, sleep, circadian rhythms and interest in sex, which are impaired in depressed patients. Hypothalamic mechanisms may also explain the increased risk of depression among females. Finally, **amygdala** may also be involved in depression, since symptoms of anxiety and fear, and abnormal responses to pleasurable stimuli, prominent in depressed patients, are mainly mediated by amygdala [2, 7]. Moreover, the neuropeptide vasopressin, which is synthesized in the hypothalamus, is also found in the amygdala and bed nucleus of the stria terminalis (BNST), where via activation of the V1α, β receptors exerts effects through the limbic system. Vasopressin levels have been found increased in patients with depression, a phenotype that is reversed upon antidepressant treatment, while animal studies have shown that non-peptide antagonists of vasopressin receptor V1β exert an antidepressant like effect in an amygdala-dependent manner [1].

Although there is much experimental evidence in accordance with these hypotheses, there are many other controversial findings. So, the pathophysiological mechanisms

of depression remain still elusive, and much research needs to be done towards this direction.

Treatment of depression: the pharmacological agents

Concerning the treatment of depression, nowadays there are five classes of antidepressant agents, tricyclic antidepressants (TCAs), monoamine oxidase inhibitors (MOIs), selective serotonin reuptake inhibitors (SSRIs), serotonin and norepinephrine reuptake inhibitors (SNRIs) and norepinephrine reuptake inhibitors (NRIs). The mechanism of acute action of all these pharmacological agents involve the inhibition of serotonin and/or norepinephrine reuptake transporters, in the case of TCAs, SSRIs, SNRIs and NRIs, or the inhibition of monoamine oxidase, a major catabolic enzyme for monoamine neurotransmitters, in the case of MOIs. However, the fact that the antidepressant effect of these agents needs prolonged administration to be clinically obvious, suggests that the enhanced serotonergic or noradrenergic neurotransmission is not the main mediating mechanism for drug action, but rather the gradually developing adaptations to this enhanced neurotransmission. Though effective and safe, antidepressant treatment is not ideal, since side effects are still an important limitation and fewer than 50% of all depressed patient exhibit full remission.

Notably all clinically used antidepressant agents are monoanime-based, even the atypical antidepressants, although this may not be the mechanism of their clinical efficacy. This is rather controversial to the accumulated knowledge about non-monoamine systems involved in the pathophysiology of depression, suggesting that drug discovery should also be focused on non-monoamine-based agents [1, 2]. Therefore, research has been focused on the identification of other agents, with antidepressant properties. Some of these agents are mentioned below, which target:

1) The normalization of the dysregulation of the HPA axis (CRF antagonists, glucocorticoid agonists or antagonists)
2) Neurotrophic factors-BDNF
3) Hypothalamic feeding peptides (melatonin receptor agonists)
4) The neurokinin system: substance P, the endogenous ligand for neurokinin (NK1) receptors, is strongly co-localized with serotonin and noradrenalline or their receptors in the human brain, while in animals substance P is released in response to fearful stimuli and both substance P and its receptors are

expressed in fear- and anxiety-related circuits. Based on the above, NK1 receptor antagonists are tested for their antidepressant properties.

5) Intracellular mechanisms, such as phosphodiesterases (PDEs), enzymes catalyzing the degradation of cAMP and cGMP: PDE inhibitors (especially PDE4 inhibitor) are under investigation for their antidepressant effects, since they not only promote the actions of noradrenaline at β-adrenergic receptors, but also induce BDNF expression in the hippocampus, via the cAMP – CREB – *Bdnf* pathway [1].

The experiments for almost all of the non-monoamine-based agents are still limited in animal models, sometimes, with controversial findings. Only a few of them have been tested in clinical trials (CRF antagonists, NK1 receptor antagonists) with unconvincing results, suggesting that the up-to-date available monoamine-based antidepressant drugs remain the unique pharmacological intervention, and that novel drugs are far from clinical application.

RGS proteins

Regulators of G-protein signaling (RGS proteins) are a large family of highly diverse, multifunctional proteins, which differ in their overall size, amino acid identity, structure and expression pattern. All RGS proteins share the conserved RGS domain, responsible for modulating G protein coupled receptors (GPCR) signaling in two different mechanisms: 1) GAP (GTPase accelerating protein) activity: they bind to activated Gα subunits, thus accelerating GTP hydrolysis and termination of G protein signaling (Fig. 1) and 2) effector antagonism: via binding to G proteins, they inhibit G proteins' activity.

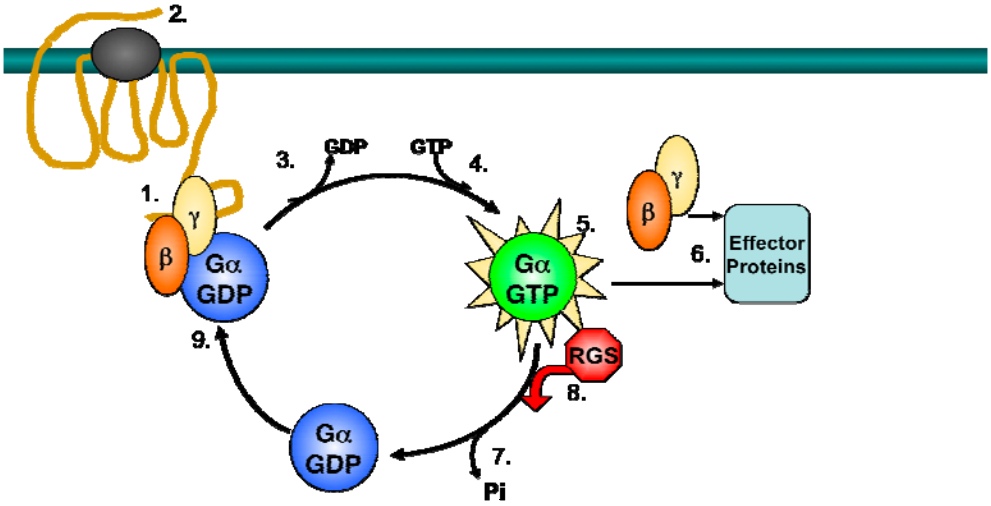


Fig. 1 GAP activity of RGS proteins [8]

Notably, concerning the GAP activity of RGS proteins, there is selectivity for Gα subunits. For example, RGS4 and RGS3 interact with both Gαi and Gαq family proteins, but not with Gαs or Gα12 [9, 10, 11], while RGS6 and RGS7 exhibit relative selectivity for Gαo family members [12].

Moreover, other functional domains, present in many RGS proteins provide unique properties to these proteins, such as specificity, stability, sub-cellular targeting and interaction with other cellular signaling molecules, and are the basis for the classification of RGS proteins in six subfamilies (Fig. 2) [8, 13].

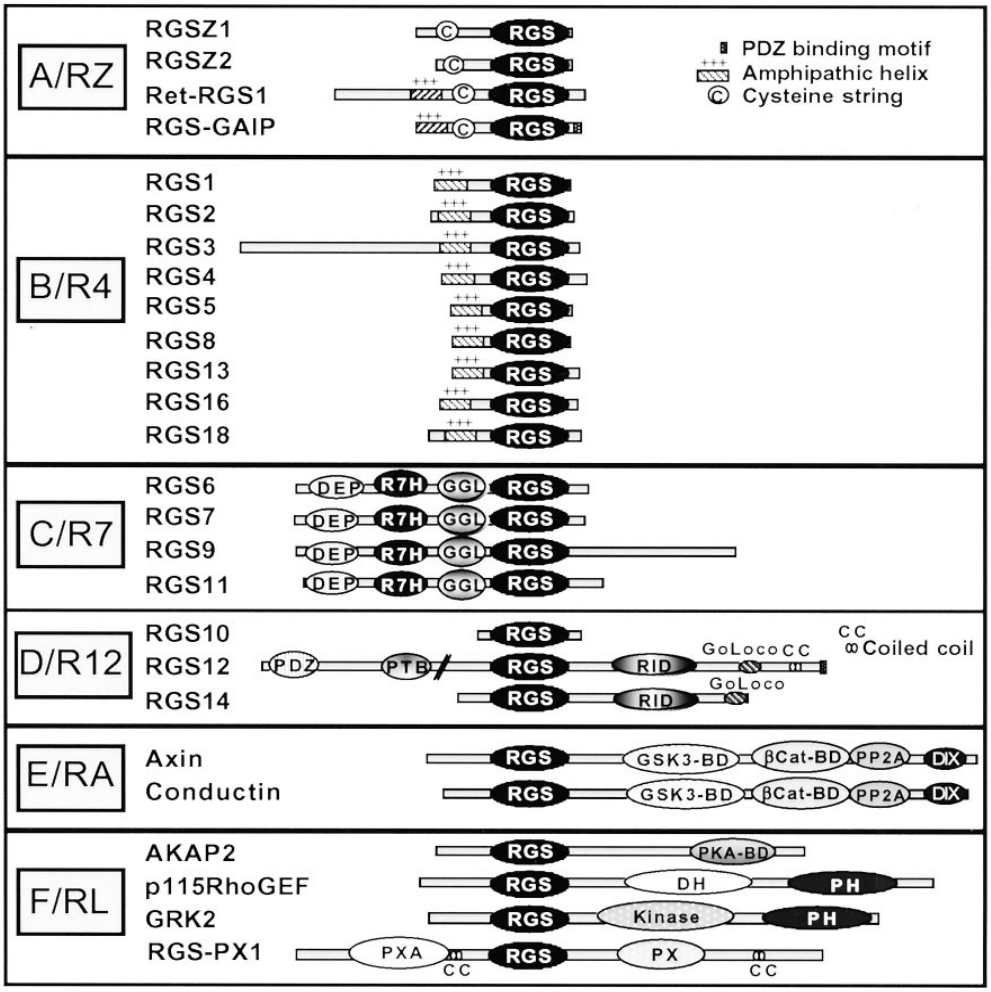


Fig. 2 Classification of RGS proteins [13]

In situ hybridization studies have provided evidence for the presence of RGS at mRNA level in brain and spinal cord regions [14], thus raising the possibility for the involvement of RGS proteins in neuropsychiatric disorders. So, genetic studies have revealed association of RGS4 with schizophrenia, but not with major depressive disorder [15], RGS9-2 with Parkinson's disease [16], while animal studies have shown the role of RGS proteins (RGS4, RGS2, RGS9-2) in drug addiction via the dopaminergic and/or opioidergic system, as well as in opiate analgesia for RGS2, RGS4, RGS9-2, RGS7, RGS8, RGSz [8].

RGSz subfamily

RGSz1 is a member of the RGSz subfamily, which consists of small 20-30-kDa RGS proteins containing short N- and C-terminal regions flanking the RGS domain. These small simple RGS proteins act exclusively as negative regulators of G protein signaling [13]. All members of RGSz subfamily (RGSz1, RGSz2, RET-RGS1, GAIP)

are distinguished by a functionally hydrophobic domain at the N-terminus, they act as tightly bound integral membrane proteins and their binding to phospholipid bilayers depends on the N-terminal domain, although this property has not been directly attributed to the N-terminal hydrophobic domain [17, 18, 19]. Moreover, all RGSz subfamily members also share a cysteine string, a site of probable multiple palmitoylation, responsible for membrane attachment [17, 20].

RGSz1

RGSz1 has been identified as the major GAP for Gαz by two independent groups: Wang et al (1998) purified RGSz1 protein from bovine brain tissue lysate [17], while Glick et al. identified RGSz1 using a yeast two-hybrid screening of a human brain library for proteins that directly interact with a mutationally activated form of Gαz [20]. The sequence of the human RGSz1 protein is 83% identical to bovine RET-RGS1 and 56% identical to human GAIP. Analysis of human genomic databases revealed that RGSz1 is one of six different splice forms of the RGS20 gene product, including the RET-RGS1 isoform. The RGS20 gene products differ in size and sequence of the N terminus and may have different tissue distributions and functions [21]. RGSz1 is expressed exclusively in brain, at high levels in the caudate nucleus and the temporal lobe, at lower levels at other brain regions such as the hippocampus, striatum, substantia nigra, amygdala, and at undetectable levels at the thalamus or the subthalamic nucleus, as this was assessed by Northern blotting [17, 20], implying an important role for RGSz1 in signal regulation in these brain regions. Concerning the intracellular localization of RGSz, it has been found that RGSz is predominantly present in the cytoplasm and in particulat in the Golgi and the trans-Golgi complex. However, a mutant form of RGSz lacking the cysteine-rich motif and the sequences N-terminal to this motif was predominantly localized in the nucleus suggesting that the cysteine string is important in the cytoplasmic retention and targeting of RGSz protein to the Golgi complex [22].

RGSz1, unlike other RGS proteins, has unusually high specificity for Gαz as assessed by an in vitro GTPase assay, but no GAP activity for Gαq or Gαs [17, 18]. However, additional in vitro binding assays and functional studies revealed that RGSz1 also interacts with Gαi subunits. More specifically, RGSz1 protein binds Gαi1 protein in an AlF-4 dependent manner, assessed in protein poll-down, co-immunoprecipitation assays and yeast two-hybrid experiments. Yeast two-hybrid test also reveals strong

interaction of RGSz1 with Gαi3 but weak with Gαi2. Functional studies in cell cultures revealed that RGSz1 accelerated endogenous GTPase activity of Gαi1 and modulated Gαi-mediated cellular responses upon stimulation of Gαi-protein coupled receptors [23].

The yeast two-hybrid system has been also applied for the identification of other interacting partner of RGSz1, apart from the Gα subunits, thus providing insights into the biological role of RGSz1 protein. One such molecule is a microtubule-destabilizing protein, the superior cervical ganglia neural-specific 10 protein (SCG10), a finding that suggests the modulatory role of RGSz1 in cytoskeleton organization [24]. Another interacting partner of RGSz1 is protein kinase C interacting 1 (PKCI-1), which interacts not only with RGSz1, via its cysteine string motif [25], but also with mu-opioid receptor (MOR) leading to the suppression of MOR desensitization [26], suggesting the potential role of RGSz1 in MOR signaling.

Gz protein

Gz is a member of the Gi family, since Gαz shares ~66% homology to Gαi1, Gαi2 and Gαi3, acting an inhibitory effect on adenyl cyclase [27, 28]. However, unlike Gi proteins, Gz is a pertussis toxin-resistant G protein, since Gαz lacks the cysteine residue near the C terminus, which is the site of pertussis toxin-catalyzed ADP-ribosylation, and has a limited expression pattern, predominantly expressed in brain, retina, the adrenal medulla and platelets. Gαz, like all Gα subunits, hydrolyses GTP, but the intrinsic GTPase activity of Gαz is considerably slower than the other Gα subunits [29, 30, 31]. So, in this case GAPs are essential for Gαz to function as a physiological signaling molecule with reasonable temporal acuity. However, Gz was considered an “orphan” protein for years, until RGSz1 was firstly identified.

Concerning the expression of Gαz in the CNS, its mRNA expression is distributed in nearly all regions of the brain with similar abundance [17]. However, as mentioned above, the expression of RGSz1 is limited to specific brain regions, and there is not good correlation between the expression pattern of these two proteins, suggesting that other homologs are represented in areas where Gαz, but not RGSz1, is expressed. For example, in retinal interneurons, where RGSz1 is undetectable, the increased expression of RET-RGS1 functions as the GAP for Gαz [32].

Gαz is an excellent substrate for protein kinase C (PKC). Phosphorylation of Gαz occurs both in vitro and in platelets treated with PKC activators, and phosphorylated Gαz has a greatly reduced affinity for the βγ subunits, thus leading to a downregulation of the Gαz activity [33, 34, 35]. Gαz signaling is further regulated by RGSz1 via reduced ability of the latter to accelerate GTPase activity of phosphorylated Gαz [20]. Another crucial modulator of Gαz signaling is its palmitoylation status, since Gz GAP activity is completely blocked upon Gαz palmiloylation. Thus, this is a protective mechanism for the prevention of GAP-mediated deactivation of Gαz and consequently its signaling [36].

Animal studies

In vivo studies in either naïve, or genetically manipulated animals (animals in which a proteins has been depleted or knocked down) are a very useful tool for the identification of the biological role of a protein. So, in the case of RGSz1 protein much attention has been paid on its role in opiodergic system. More specifically, knockdown of RGSz1 expression with antisense oligodeoxynucleotides in mice resulted in increased supraspinal antinociception in response to morphine administration, development of tolerance to a single dose of morphine and accelerated tolerance to continuous delivery of the opioid. However, delta opioid receptor agonists had no such effects. These results indicate, that mu but not delta opioid receptors are regulated by RGSz1 [37]. At cellular level, administration of morphine increased the association of the mu opioid receptors with both RGSz1 and RGSz2, but decreased its association with Gαz [38], while morphine doses that produced acute tolerance maintained the association of Gα subunits with RGSz1 and RGSz2 proteins, even after the analgesic effects had ceased [39]. The above data are indicative of the important role of RGSz1 as a modulator of morphine actions.

Experiments on mice lacking the Gαz gene (knockout KO mice) cover a broad field of research. Loss of signaling through Gαz resulted not only in abnormal platelet activation, leading to impaired platelet aggregation but also in altered responses to psychoactive drugs in Gαz KO mice. More specifically, Gαz KO mice exhibited greatly exaggerated responses to cocaine and reduced analgesic effects of morphine [40]. A more detailed investigation on the role of Gαz in the dopaminergic system revealed that Gαz KO mice exhibited enhanced sensitivity to the disruption of prepulse inhibition of acoustic startle induced by treatment with amphetamine and

apomorphine, as well as enhanced sensitivity to the locomotor activation effects of amphetamine [41]. Moreover, while D1-like receptor agonists induced comparable locomotor activity in both KO and wild-type (WT) mice, the suppressive phenotype upon D2-like receptor agonist administration was less prominent in KO mice. Similarly, hypothermia and adrenocorticotropic hormone release, resulting from activation of D2-like receptors, were significantly reduced in Gαz KO mice. These data indicate the functional coupling of Gαz to D2-like receptors in vivo [42]. Finally, a behavioral analysis of Gαz KO mice showed heightened anxiety and depression-like behavior compared to their WT littermates an effect that may be mediated via the serotonin-IA receptors [43], which are known to be coupled to Gαz subunits based on studies in cell cultures [44].

Although there is no direct evidence for the involvement of RGSz1 in mood curcuits, the fact that Gαz is coupled to serotonin receptors and its absence is adequate to provoke impaired mood phenotype in animals, it can be hypothesized that RGSz1 is a key player in mood disorders.

AIM OF THE STUDY

The aim of this study is to investigate the role of RGSz protein in:

1) Drug addiction and chronic pain-related depression and anxiety.
2) The mechanism of action of two antidepressant agents, desipramine (TCA) and fluoxetine (SSRI).

For this reason we used genetically manipulated mice in which the RGSz gene was deleted (knock-out mice KO) and their wild type (WT) littermates. The basal behavioral phenotype of these mice was assessed, concerning their anxiety and depression levels.

The antidepressant properties of acute desipramine and fluoxetine administration under basal conditions were assessed in both genotypes and both sexes. The WT vs. KO mice comparison was performed to reveal the potential role of RGSz in TCA's or SSRI's effectiveness, while the female vs. male comparison was performed for the identification of sex differences in responsiveness to these agents.

Drugs of abuse, such as morphine and cocaine were used to induce an addictive phenotype, and a model of neuropathic pain was applied to induce chronic pain conditions. Addicted mice, as well as mice under chronic pain were assessed for their anxiety and depressive phenotype, in order to identify the role of RGSz in addiction and chronic pain-induced depression and anxiety (WT vs. KO mice), as well as possible sex differences (female vs. male mice), since as mentioned above depression is much more frequent in females.

Mechanistically, the first step in understanding the role of RGSz in depression is the identification of the brain regions, where the protein is present or absent, and of particular interest are these regions, mentioned above, that are involved in the mood neuronal circuit.

MATERIALS AND METHODS

Animals

All experiments were performed on male and female adult mice. Mice were kept on a 12h light/dark cycle, were group-housed (4-5 per cage) with food and water available ad libitum. Homozygous null mice for the RGSz gene and their wild-type littermates were used in all experiments in groups of 8-15 mice per genotype. Animal handling and experiments were in accordance to the guidelines of the Institutional Animal Care and Use Committee of the University of Crete.

Drugs

Morphine (morphine sulfate, National Institute on Drug Abuse) and cocaine (National Institute on Drug Abuse) were used for inducing addiction. Mice were treated with freshly prepared morphine for 6 days (10mg/kg), or cocaine for either 6 or 10 days (10mg/kg). Both drugs were dissolved in 0.9% NaCl and injected intraperitoneally. Desipramine (desipramine hydrochloride, Sigma) and fluoxetine (fluoxetine hydrochloride, Sigma) were used as antidepressant agents. Desipramine was dissolved in 0.9% NaCl (50mg/ml), while fluoxetine in ultra pure water. All drugs, including saline, were freshly prepared before use and injected in a volume of 0.1ml.

Surgical procedure

The spared nerve injury (SNI) model of neuropathic pain was performed under 2,2,2-tribromoethanol (Aldrich) general anesthesia. Skin and muscle incision of the left hind-paw at mid-thigh level revealed the sciatic nerve and its three branches, with the help of a stereomicroscope. The common peroneal and the sural nerves were carefully ligated with 6.0 silk suture (Ethicon, Johnson & Johnson Intl.), transected and a 1-2mm section of these nerves was removed, while the tibia nerve was left intact. Muscle and skin were then closed separately with silk 4.0 suture [45, 46].

Behavioral tests

Marble hiding test: Mice were transported in the experiment room 1hr before testing. Each mouse was placed in a plastic cage (33 x 20 x 18cm) with a 5cm high bedding on which ten colorful marbles were evenly placed. Mice remained in this environment for 30 min and afterwards the number of hidden marbles was measured, as a marker of animal anxiety. Marbles were considered hidden if two-thirds, or more, of their surface were buried in the bedding [47].

Open field test: Mice were transported in the experiment room 1hr before testing. Each mouse was placed near the walls of an open field area for 5min. The open field area was an empty square arena (25 x 25cm) surrounded by walls to prevent mice from falling off. The arena was divided in three zones, the center (5 x 5cm), the periphery (10 x 10cm) and the borders (20 x 20cm). This 5-min session was videotaped and mice were then scored for the following three parameters: "latency", the time the mouse needed to enter the center for the first time, the time mice spent near the borders, both measures of animal anxiety, and the time mice spent in the periphery, indicative of less anxiety-related behavior.

Forced swim test: Mice were transported one by one to the testing room, just before the experiment. Each mouse was placed individually in glass cylinders (46cm tall x 18cm diameter) containing water at room temperature at a depth of 15cm. A 6-min test duration was used and water was changes between subjects. All test sessions were recorded by a video camera. The behavioral measures scored, based on the videotapes, were the "latency", that is the time needed until the mouse remained still for the first time for at least 5 sec, as well as the duration of "immobility", that is the state in which mice made only movements necessary for remaining afloat. Mice that were unable to keep their head above water were excluded. Saline, desipramine and fluoxetine acute treatment were used. Desipramine doses were administered at 23.5, 5.0 and 1.0hr (10mg/kg i.p., 10mg/kg i.p. and 20mg/kg s.c. respectively) before test session, while fluoxetine was administered 30min (30mg/kg i.p.) before testing [48].

Locomotor activity assay: Locomotor activity of the mice was determined using an automated system, as previously described. Briefly, mice were single-placed in plastric cages (33 x 18 x 12cm) and 10 pairs of photocell beams, dividing the activity chamber into 11 rectangular fields, measured the ambulatory locomotor activity of the animals for 30min [49].

Nociceptive test: The mechanical threshold for hind-paw withdrawal was determined using von Frey hairs with ascending forces expressed in grams (0.1 - 3.6gr) (Electronic von Frey Anesthesiometer, IITC). Mice were single-placed in Plexiglas boxes on an elevated mesh screen. Each von Frey hair was applies on the plantar surface of mice hind-paws, 5 times per paw, and the mechanical threshold was defined as 3 or more withdrawals out of 5 trials [47].

Immunohistochemistry

Mice were injected with an overdose of pentobarbiral and perfused transcardially with 1x PBS followed by 4% paraformaldehyde. The brains were post-fixed for 12hr in 4% paraformaldehyde and then cryoprotected in 20% glycerol for 6hr. Brains were coronally sectioned at 40 mcm and collected in 1x PBS plus 0.5% sodium azide. Double-labeling immunohistochemistry was performed, using rabbit polyclonal anti-RGSz antibody (1:600, Eliot Ross UT Southwester medical center), and mouse polyclonal anti-tyrosine hydroxylase-TH antibody (1:2000, Sigma) [50]. Briefly, slices of all brain regions were selected and rinsed in PBS. 3% normal donkey serum (Sigma) was used to block nonspecific binding. After the addition of the anti-RGSz and anti-TH antibodies, the slices were incubated overnight. The next day, the slices were washed in PBS and incubated with secondary antibodies conjugated to Cy2 and Cy3 (1:300). After washing in PBS, the slices were mounted on slides, dehydrated and coverslipped using DPX mounting media. Images were obtained by using confocal microscope.

RESULTS

Behavioral analysis of the RGSz knockout mice

The basal anxiety and depression levels of RGSz WT and KO mice were measured, in order to be used as reference values for evaluating the drug addiction- or chronic pain-induced behavioral phenotype of these mice (Fig. 1).

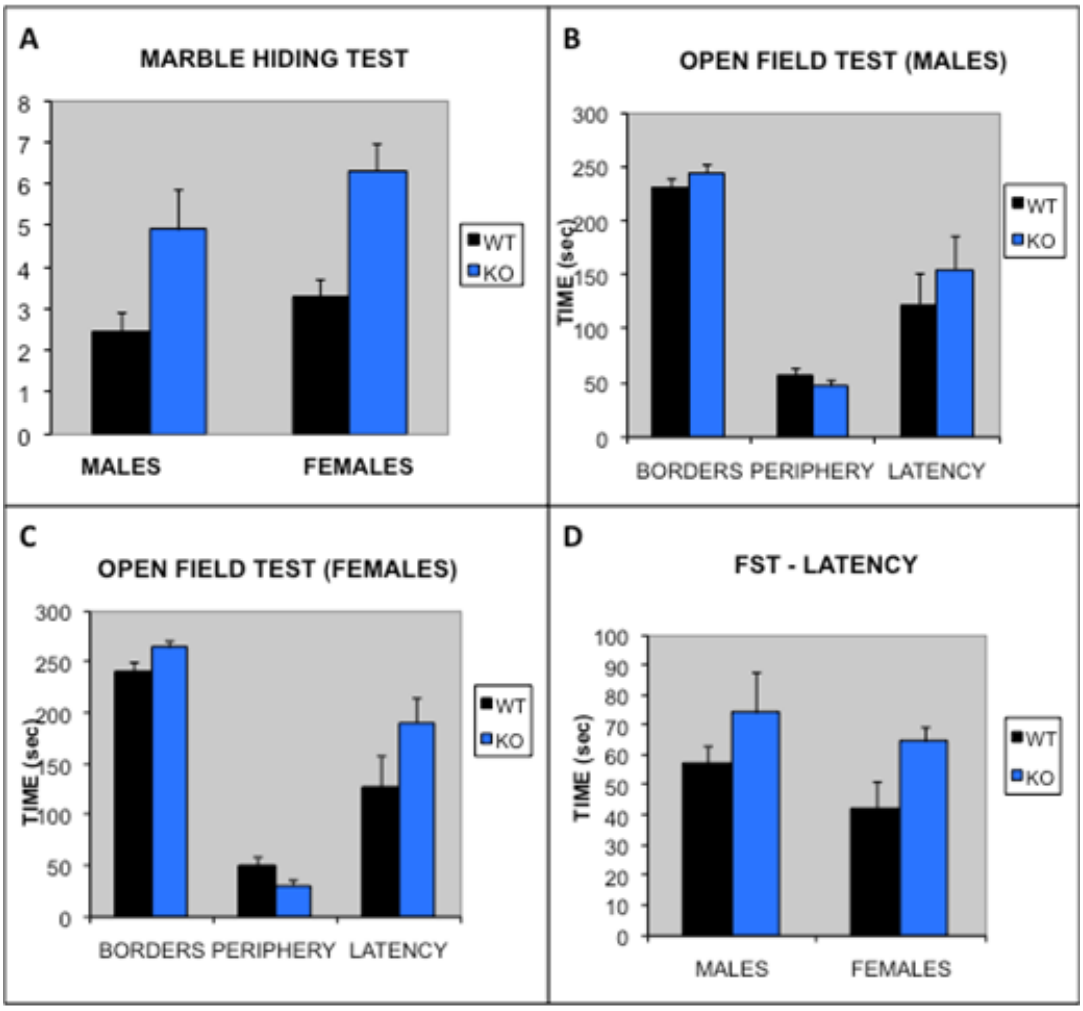


Fig.1 Basal anxiety and depression levels of RGSz WT and KO male and female mice. (A) marble hiding test. (B, C) open field test. (D) forced swim test, latency.

Surprisingly, in both anxiety tests used, marble hiding and open-field test, mice lacking the RGSz gene (KO) exhibited a more anxious phenotype, compared to their wild-type (WT) littermates. This difference was more obvious in the marble hiding test, but open field test also showed such a trend, although the difference was not statistically significant. It should be noted that a profound difference is highly difficult to be observed in this test. Moreover, RGSz KO mice exhibited a less depressive

phenotype, as this was assessed in the forced swim test. These findings were observed in both male and female mice, suggesting significant behavioral deficits in RGSz KO mice.

Differential effect of antidepressants in RGSz WT and KO mice

Since the RGSz KO mice were more anxious and less depressed compared with their WT littermates, we hypothesized that RGSz might also play a role in the effectiveness of antidepressant agents. So, we tested the antidepressant properties of acute desipramine and fluoxetine treatment in RGSz WT and KO mice. We used these two distinct classes of antidepressants, desipramine, a tricyclic antidepressant (TCA) and fluoxetine, a selective serotonin reuptake inhibitor (SSRI), because they have different mechanisms of action, and it is possible that RGSz might be involved in one pathway and not the other. The forced swim test is the most commonly used test for assessment of the acute therapeutic effect of antidepressants [51]. We verified the antidepressant properties of both drugs, upon acute administration, in all mouse groups tested.

More specifically desipramine had similar antidepressant effect on WT and KO mice in both sexes, a phenotype that was more obvious in females than in males, suggesting increased sensitivity of female mice to the effectiveness of antidepressants. Concerning fluoxetine, male mice responded better to fluoxetine than desipramine, and both WT and KO mice showed similar antidepressant phenotype. On the contrary, fluoxetine had no effect on female WT mice, but an obvious antidepressant effect on female KO mice. These findings suggest that either RGSz alone plays a role in the SSRIs mechanism of action, or it is an effect based on sunergic action of RGSz and sex hormones.

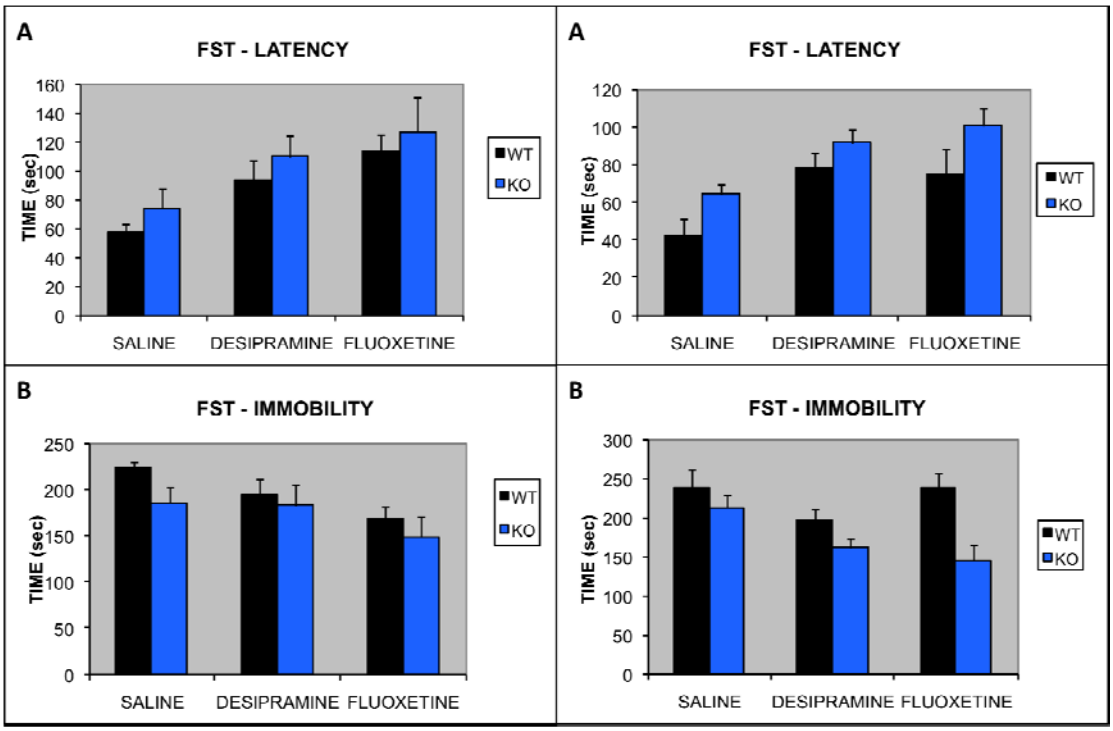


Fig.2 Antidepressant effect of desipramine and fluoxetine in RGSz WT and KO male mice.

Fig.3 Antidepressant effect of desipramine and fluoxetine in RGSz WT and KO female mice.

Expression pattern of RGSz in the central nervous system (CNS)

In order to better understand the underlying mechanisms of 1) the basal behavioral deficits of the RGSz KO mice and 2) differential effectiveness of fluoxetine in the female RGSz KO mice, we performed immunohistochemical studies in brain slices. We found that brain regions where RGSz is highly expressed include the red nucleus, the trigeminal nerve nucleus (Fig. 4). Using an antibody for tyrosine hydroxylase (TH), an enzyme involved in the metabolic pathway of dopamine, we could identify brain regions rich in dopaminergic neurons, such as the locus coeruleus and the ventral tegmental area, traditional dopaminergic regions. Apart from these regions, dopaminergic neurons have been reported to be also present in rostral linear nucleus (RLi), periaqueductal gray (PAG), dorsal raphe (DR) [52]. Notably, PAG and DR are also involved in the serotoninergic pathway, with DR containing the 70% of the serotoninergic neurons in the brain.

Previous immunohistochemical studies in the mesencephalon have shown TH-immunoreactivity not only in VTA, but also in ventrolateral PAG and RLi – Rli/vPAG [53]. As shown in figure 4 we found no RGSz expression in LC, VTA, and

RLi/vPAG, but high expression levels in the red nucleus and the nucleus of the trigeminal nerve.

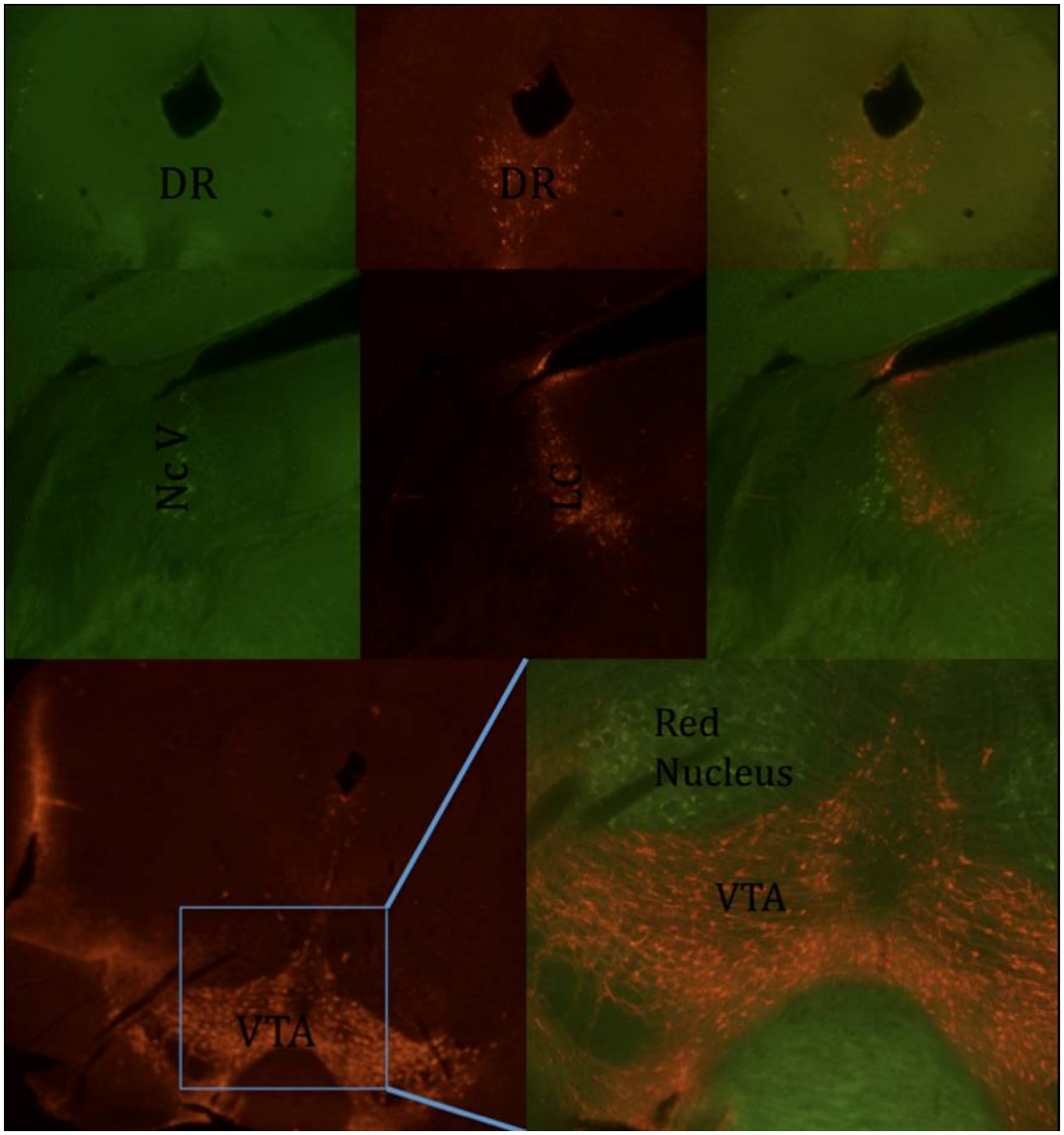


Fig.4 Expression pattern of RGSz1 in murine brain tissue: double immunohistochemistry for RGSz1 (green) and tyrosine hydroxylase (red). Nc V: nucleus of the trigeminal nerve, DR: dorsal raphe, LC: locus coeruleus.

Drug addiction and neuropathic pain-induced anxiety and depression

It is well established that depression and anxiety co-exist in many pathological conditions, and that conditions that induce anxiety and/or depression in humans involve drug addiction in the rewarding phase, chronic pain etc. [54-57]. So, we tried to make a mouse model of depression.

For induction of addiction-related depression, mice were treated with morphine or cocaine for 6 days. Both treatments were effective in inducing addiction, as this was verified by the increased locomotor sensitization in all mice groups (Table 1).

		Beam breaks		
		Baseline	First day of treatment	Last day of treatment
6-d.morphine	WT	814.6	1195.5	3774.5
	KO	546.75	2089.5	5348.5
6-d.cocaine	WT	226.3	664.5	1190.67
	KO	414.5	1336	2164.5

Table 1. Morphine or cocaine induces increased locomotor activity in RGSz mice assessed in the locomotor activity assay.

3-4 days post 6-day drug treatment, mice being in withdrawal were tested for their anxiety and depression levels. Unlike morphine treatment, which did not induce an anxiety phenotype in both sexes, cocaine had a statistically significant anxiolytic effect in female WT mice, but no effect on their KO littermates (Fig. 5A, B).

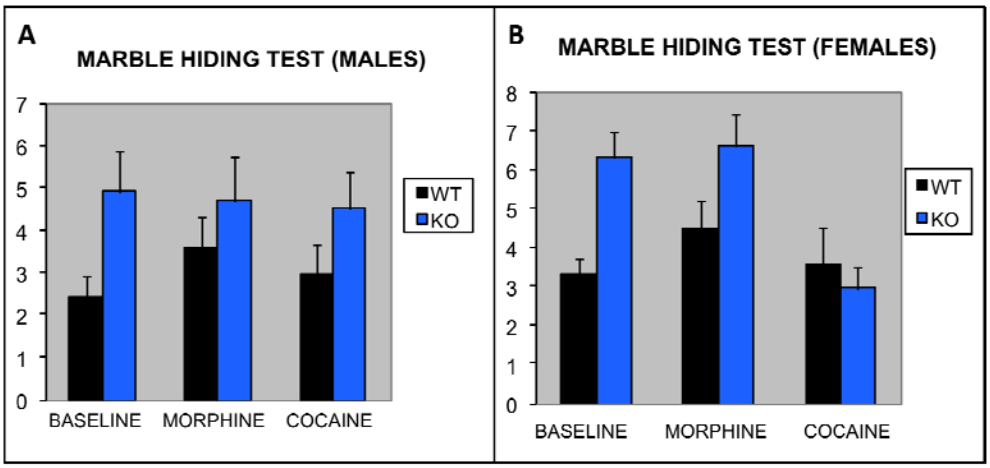


Fig. 5 Morphine and cocaine induced anxiety-like phenotype, assessed in the marble hiding test, in males (A) and females (B).

Given that cocaine, but not morphine, caused increased locomotor sensitization (Table 2) and prominent anxiolytic effect (Fig. 5) on RGSz KO mice, suggests that RGSz is somehow involved in the cocaine-induced neuronal adaptations. The observed sex difference raises the possibility that sex hormones are also involved in this mechanism.

Unfortunately, addiction-related depression could not be induced in any of the groups tested (Fig. 6). Tests were again performed in both male and female mice, in order to identify the presence of sex difference.

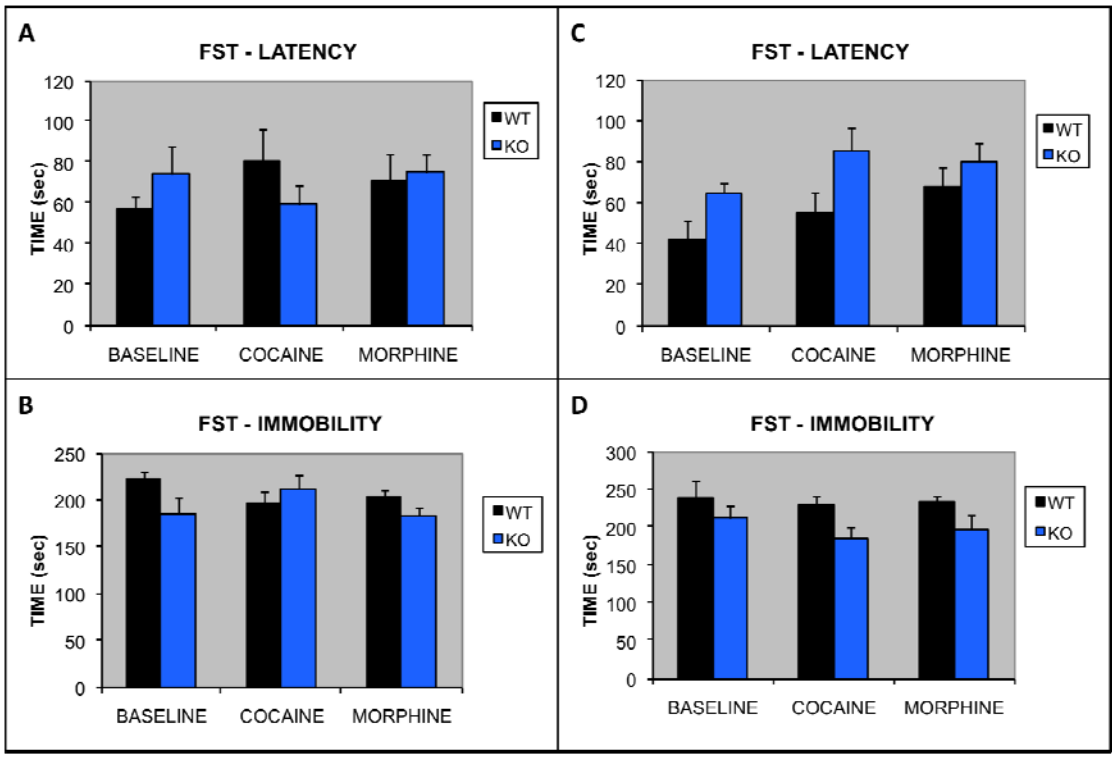


Fig. 6 Morphine and cocaine treatment induced depression-like phenotype, assessed in the forced swim test. Both latency and immobility are measured in males (A, B) and females (C,D), respectively.

Another treatment session was also performed in an attempt to induce addiction-related anxiety and depression, in which mice were treated with cocaine for 10 days and their anxiety and depression phenotype was assessed 6-7 days post treatment. The results were similar to the 6-day cocaine treatment (data not shown).

Concerning the chronic pain-related anxiety and depression, we used the spared nerve injury (SNI) model of neuropathic pain, which was able to cause a stable allodynic phenotype for more than a month, as this was assessed in the von Frey test (Table 2).

		Von Frey hairs, pressure (gr)			
		Baseline	Day 10	Day 20	Day 30
Operated mice	WT	1.9	0.425	0.5	0.6
	KO	1.9	0.475	0.45	0.65
Sham-operated mice	WT	2.05	2.03	2	2.175

	KO	2.225	2	2	1.925

Table 2. SNI model induces stable allodynia in RGSz mice.

These male mice being on chronic pain for a month underwent anxiety and depression tests. Chronic pain did not increase the anxiety levels of the operated mice compared both to sham operated mice, that is mice that were not on chronic pain, and to non-operated mice (Fig. 7A), but induced a pronounce depression phenotype compared to their controls (Fig. 7B, C).

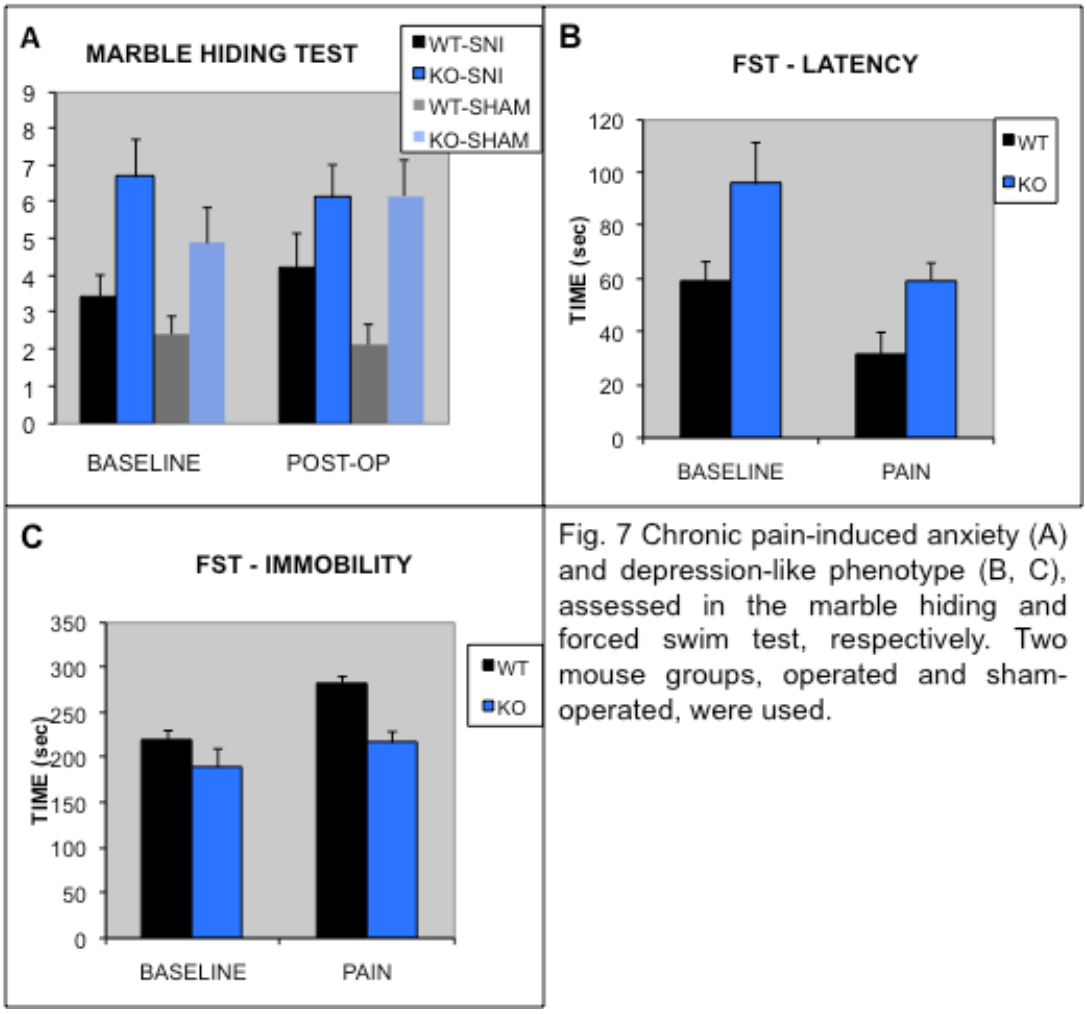


Fig. 7 Chronic pain-induced anxiety (A) and depression-like phenotype (B, C), assessed in the marble hiding and forced swim test, respectively. Two mouse groups, operated and sham-operated, were used.

DISCUSSION

The findings of this study could be summarized in three major points:

1. The behavioral analysis of RGSz1 KO mice reveals significant behavior deficits of these mice, concerning their anxiety and depression phenotype.

In consistent with behavioral experiments on Gαz KO mice, which exhibited increased anxiety and depression-like phenotype, the similar phenotype of RGSz KO mice suggests that Gz signaling is rather crucial in mood regulation.

2. It provides a first indication of the expression pattern of RGSz1 protein in murine brain tissues.

The so far provided evidence for the expression pattern of RGSz1 at mRNA level, provide an indication for the brain regions and subsequent physiological functions in which RGSz1 interferes. However, the presence of mRNA does not necessarily lead to the presence of functional protein. The lack of an available antibody that specifically binds to RGSz1 but not to the other RGSz members was the main limitation for this. So, the availability of such an antibody made possible the identification of the brain regions where RGSz1 is expressed. Our study reveals for the first time the presence of RGSz1 protein in the red nucleus and the nucleus of the trigeminal nerve. Our focus on brain regions of the dopaminergic brain network (such the VTA, LC and RLi/vPAG) showed no detectable RGSz1 expression. However, a more detailed expression analysis in necessary.

3. RGSz1 seems to play a role in the antidepressant action of SSRIs (Fluoxetine) either per se or in combination with sex hormones.

It is well established that the serotonin IA receptor signaling is Gz-mediated. Since the antidepressant action of fluoxetine is mediated by serotonin receptors, it would be of particular interest the investigation of potential co-localization of this receptor with RGSz1 in brain regions outside the dopaminergic network, e.g. in the hippocampus. On the other hand, the observed sex difference requires further investigation, in order to identify whether there is direct on indirect interaction of RGSz1 with receptors of sec hormones.

And one minor point:

We managed to establish a chronic pain-related mouse model of depression, but it was not possible to establish an addiction-related mouse model of depression.

This could be attributed to a variety of reasons, such as the strain or the time-point at which the assessment of the depression-like phenotype took place. It is well established that both conditions are strongly associated with increased depression, so it could be hypothesized that addicted mice were not on the phase of withdrawal. On the other hand, this was not an issue in mice under chronic pain, since the depression-like phenotype was rather obvious. Given that sufficient depression is a long-term effect of various triggers, and the withdrawal phase is not so long-lasting, this could be the reason for the ineffective induction of addiction-related depression.

So, the chronic pain-related model of depression could be used for further investigations on the action of antidepressant agents, under chronic depressive conditions. This study, along with the basal depressive phenotype of RGSz1 KO mice, would be of particular interest, for providing stable evidence on the role of RGSz1 in depression and antidepressants' mechanism of action.

Summarizing, the present study is the first study that provides evidence for a role of RGSz1 in mood disorders, suggesting that, apart from addiction-related behaviors, which have been the main focus on RGSz1 research, RGSz1 is also involved in other neuronal circuits and consequently in other pathological conditions.

REFERENCES

1. Berton O, Nestler EJ. *New approaches to antidepressant drug discovery: beyond monoamines.* Nat Rev Neurosci. 2006 Feb;7(2):137-51.

2. Nestler EJ, Barrot M, DiLeone RJ, Eisch AJ, Gold SJ, Monteggia LM. *Neurobiology of depression.* Neuron. 2002 Mar 28;34(1):13-25.

3. Levinson DF. *The genetics of depression: a review.* Biol Psychiatry. 2006 Jul 15;60(2):84-92.

4. Keller J, Schatzberg AF, Maj M. *Current issues in the classification of psychotic major depression.* Schizophr Bull. 2007 Jul;33(4):877-85.

5. Joyce PR. *Classification of mood disorders in DSM-V and DSM-VI.* Aust N Z J Psychiatry. 2008 Oct;42(10):851-62.

6. Uher R. *The implications of gene-environment interactions in depression: will cause inform cure?* Mol Psychiatry. 2008 Dec;13(12):1070-8.

7. Nestler EJ, Carlezon WA Jr. *The mesolimbic dopamine reward circuit in depression.* Biol Psychiatry. 2006 Jun 15;59(12):1151-9.

8. Hooks SB, Martemyanov K, Zachariou V. *A role of RGS proteins in drug addiction.* Biochem Pharmacol. 2008 Jan 1;75(1):76-84.

9. Berman DM, Wilkie TM, Gilman AG. *GAIP and RGS4 are GTPase-activating proteins for the Gi subfamily of G protein alpha subunits.* Cell. 1996 Aug 9;86(3):445-52.

10. Hepler JR, Berman DM, Gilman AG, Kozasa T. *RGS4 and GAIP are GTPase-activating proteins for Gq alpha and block activation of phospholipase C beta by gamma-thio-GTP-Gq alpha.* Proc Natl Acad Sci U S A. 1997 Jan 21;94(2):428-32.

11. Scheschonka A, Dessauer CW, Sinnarajah S, Chidiac P, Shi CS, Kehrl JH. *RGS3 is a GTPase-activating protein for g(ialpha) and g(qalpha) and a potent inhibitor of signaling by GTPase-deficient forms of g(qalpha) and g(11alpha).* Mol Pharmacol. 2000 Oct;58(4):719-28.

12. Posner BA, Gilman AG, Harris BA. *Regulators of G protein signaling 6 and 7. Purification of complexes with gbeta5 and assessment of their effects on g protein-mediated signaling pathways.* J Biol Chem. 1999 Oct 22;274(43):31087-93.

13. Hollinger S, Hepler JR. *Cellular regulation of RGS proteins: modulators and integrators of G protein signaling.* Pharmacol Rev. 2002 Sep;54(3):527-59.

14. Gold SJ, Ni YG, Dohlman HG, Nestler EJ. *Regulators of G-protein signaling (RGS) proteins: region-specific expression of nine subtypes in rat brain.* J Neurosci. 1997 Oct 15;17(20):8024-37.

15. Mirnics K, Middleton FA, Stanwood GD, Lewis DA, Levitt P. *Disease-specific changes in regulator of G-protein signaling 4 (RGS4) expression in schizophrenia.* Mol Psychiatry. 2001 May;6(3):293-301.

16. Tekumalla PK, Calon F, Rahman Z, Birdi S, Rajput AH, Hornykiewicz O, Di Paolo T, Bédard PJ, Nestler EJ. *Elevated levels of DeltaFosB and RGS9 in striatum in Parkinson's disease.* Biol Psychiatry. 2001 Nov 15;50(10):813-6.

17. Wang J, Ducret A, Tu Y, Kozasa T, Aebersold R, Ross EM. *RGSZ1, a Gz-selective RGS protein in brain. Structure, membrane association, regulation by Galphaz phosphorylation, and relationship to a Gz gtpase-activating protein subfamily.* J Biol Chem. 1998 Oct 2;273(40):26014-25.

18. Wang J, Tu Y, Woodson J, Song X, Ross EM. *A GTPase-activating protein for the G protein Galphaz. Identification, purification, and mechanism of action.* J Biol Chem. 1997 Feb 28;272(9):5732-40.

19. Tu Y, Woodson J, Ross EM. *Binding of regulator of G protein signaling (RGS) proteins to phospholipid bilayers. Contribution of location and/or orientation to Gtpase-activating protein activity.* J Biol Chem. 2001 Jun 8;276(23):20160-6.

20. Glick JL, Meigs TE, Miron A, Casey PJ. *RGSZ1, a Gz-selective regulator of G protein signaling whose action is sensitive to the phosphorylation state of Gzalpha.* J Biol Chem. 1998 Oct 2;273(40):26008-13.

21. Barker SA, Wang J, Sierra DA, Ross EM. *RGSZ1 and Ret RGS: two of several splice variants from the gene RGS20.* Genomics. 2001 Dec;78(3):223-9.

22. Chatterjee TK, Fisher RA. *Cytoplasmic, nuclear, and golgi localization of RGS proteins. Evidence for N-terminal and RGS domain sequences as intracellular targeting motifs.* J Biol Chem. 2000 Aug 4;275(31):24013-21.

23. Wang Y, Ho G, Zhang JJ, Nieuwenhuijsen B, Edris W, Chanda PK, Young KH. *Regulator of G protein signaling Z1 (RGSZ1) interacts with Galpha i subunits and regulates Galpha i-mediated cell signaling.* J Biol Chem. 2002 Dec 13;277(50):48325-32.

24. Nixon AB, Casey PJ. *Analysis of the regulation of microtubule dynamics by interaction of RGSZ1 (RGS20) with the neuronal stathmin, SCG10.* Methods Enzymol. 2004;390:53-64.

25. Ajit SK, Ramineni S, Edris W, Hunt RA, Hum WT, Hepler JR, Young KH. *RGSZ1 interacts with protein kinase C interacting protein PKCI-1 and modulates mu opioid receptor signaling.* Cell Signal. 2007 Apr;19(4):723-30.

26. Guang W, Wang H, Su T, Weinstein IB, Wang JB. *Role of mPKCI, a novel mu-opioid receptor interactive protein, in receptor desensitization, phosphorylation, and morphine-induced analgesia.* Mol Pharmacol. 2004 Nov;66(5):1285-92.

27. Wong YH, Conklin BR, Bourne HR. *Gz-mediated hormonal inhibition of cyclic AMP accumulation.* Science. 1992 Jan 17;255(5042):339-42.

28. Kozasa T, Gilman AG. *Purification of recombinant G proteins from Sf9 cells by hexahistidine tagging of associated subunits. Characterization of alpha 12 and inhibition of adenylyl cyclase by alpha z.* J Biol Chem. 1995 Jan 27;270(4):1734-41.

29. Fields TA, Casey PJ. *Signalling functions and biochemical properties of pertussis toxin-resistant G-proteins.* Biochem J. 1997 Feb 1;321 (Pt 3):561-71.

30. Casey PJ, Fong HK, Simon MI, Gilman AG. *Gz, a guanine nucleotide-binding protein*

with unique biochemical properties. J Biol Chem. 1990 Feb 5;265(4):2383-90.

31. Gagnon AW, Manning DR, Catani L, Gewirtz A, Poncz M, Brass LF. *Identification of Gz alpha as a pertussis toxin-insensitive G protein in human platelets and megakaryocytes.* Blood. 1991 Sep 1;78(5):1247-53.

32. Faurobert E, Hurley JB. *The core domain of a new retina specific RGS protein stimulates the GTPase activity of transducin in vitro.* Proc Natl Acad Sci U S A. 1997 Apr 1;94(7):2945-50.

33. Lounsbury KM, Casey PJ, Brass LF, Manning DR. *Phosphorylation of Gz in human platelets. Selectivity and site of modification.* J Biol Chem. 1991 Nov 15;266(32):22051-6.

34. Carlson KE, Brass LF, Manning DR. *Thrombin and phorbol esters cause the selective phosphorylation of a G protein other than Gi in human platelets.* J Biol Chem. 1989 Aug 5;264(22):13298-305.

35. Fields TA, Casey PJ. *Phosphorylation of Gz alpha by protein kinase C blocks interaction with the beta gamma complex.* J Biol Chem. 1995 Sep 29;270(39):23119-25.

36. Tu Y, Wang J, Ross EM. *Inhibition of brain Gz GAP and other RGS proteins by palmitoylation of G protein alpha subunits.* Science. 1997 Nov 7;278(5340):1132-5.

37. Garzón J, Rodríguez-Muñoz M, López-Fando A, García-España A, Sánchez-Blázquez P. *RGSZ1 and GAIP regulate mu- but not delta-opioid receptors in mouse CNS: role in tachyphylaxis and acute tolerance.* Neuropsychopharmacology. 2004 Jun;29(6):1091-104.

38. Garzón J, Rodríguez-Muñoz M, Sánchez-Blázquez P. *Morphine alters the selective association between mu-opioid receptors and specific RGS proteins in mouse periaqueductal gray matter.* Neuropharmacology. 2005 May;48(6):853-68.

39. Garzón J, Rodríguez-Muñoz M, López-Fando A, Sánchez-Blázquez P. *The RGSZ2 protein exists in a complex with mu-opioid receptors and regulates the desensitizing capacity of Gz proteins.* Neuropsychopharmacology. 2005 Sep;30(9):1632-48.

40. Yang J, Wu J, Kowalska MA, Dalvi A, Prevost N, O'Brien PJ, Manning D, Poncz M, Lucki I, Blendy JA, Brass LF. *Loss of signaling through the G protein, Gz, results in abnormal platelet activation and altered responses to psychoactive drugs.*
Proc Natl Acad Sci U S A. 2000 Aug 29;97(18):9984-9.

41. van den Buuse M, Martin S, Brosda J, Leck KJ, Matthaei KI, Hendry I. *Enhanced effect of dopaminergic stimulation on prepulse inhibition in mice deficient in the alpha subunit of G(z).* Psychopharmacology (Berl). 2005 Dec;183(3):358-67.

42. Leck KJ, Blaha CD, Matthaei KI, Forster GL, Holgate J, Hendry IA. *Gz proteins are functionally coupled to dopamine D2-like receptors in vivo.* Neuropharmacology. 2006 Sep;51(3):597-605.

43. Oleskevich S, Leck KJ, Matthaei K, Hendry IA. *Enhanced serotonin response in the hippocampus of Galphaz protein knock-out mice.* Neuroreport. 2005 Jun 21;16(9):921-5.

44. Raymond JR, Mukhin YV, Gettys TW, Garnovskaya MN. *The recombinant 5-HT1A receptor: G protein coupling and signalling pathways.* Br J Pharmacol. 1999 Aug;127(8):1751-64.

45. Shields SD, Eckert WA 3rd, Basbaum AI. *Spared nerve injury model of neuropathic pain in the mouse: a behavioral and anatomic analysis.* J Pain. 2003 Oct;4(8):465-70.

46. Bourquin AF, Süveges M, Pertin M, Gilliard N, Sardy S, Davison AC, Spahn DR, Decosterd I. *Assessment and analysis of mechanical allodynia-like behavior induced by spared nerve injury (SNI) in the mouse.* Pain. 2006 May;122(1-2):14.e1-14.

47. Benbouzid M, Pallage V, Rajalu M, Waltisperger E, Doridot S, Poisbeau P, Freund-Mercier MJ, Barrot M. *Sciatic nerve cuffing in mice: a model of sustained neuropathic pain.* Eur J Pain. 2008 Jul;12(5):591-9.

48. Conti AC, Kuo YC, Valentino RJ, Blendy JA. *Inducible cAMP early repressor regulates corticosterone suppression after tricyclic antidepressant treatment.* J Neurosci. 2004 Feb 25;24(8):1967-75.

49. Rahman Z, Schwarz J, Gold SJ, Zachariou V, Wein MN, Choi KH, Kovoor A, Chen CK, DiLeone RJ, Schwarz SC, Selley DE, Sim-Selley LJ, Barrot M, Luedtke RR, Self D, Neve RL, Lester HA, Simon MI, Nestler EJ. *RGS9 modulates dopamine signaling in the basal ganglia.* Neuron. 2003 Jun 19;38(6):941-52.

50. Georgescu D, Zachariou V, Barrot M, Mieda M, Willie JT, Eisch AJ, Yanagisawa M, Nestler EJ, DiLeone RJ. *Involvement of the lateral hypothalamic peptide orexin in morphine dependence and withdrawal.* J Neurosci. 2003 Apr 15;23(8):3106-11.

51. Porsolt RD, Le Pichon M, Jalfre M. *Depression: a new animal model sensitive to antidepressant treatments.* Nature. 1977 Apr 21;266(5604):730-2.

52. Hasue RH, Shammah-Lagnado SJ. *Origin of the dopaminergic innervation of the central extended amygdala and accumbens shell: a combined retrograde tracing and immunohistochemical study in the rat.* J Comp Neurol. 2002 Dec 2;454(1):15-33.

53. Flores JA, Galan-Rodriguez B, Ramiro-Fuentes S, Fernandez-Espejo E. *Role for dopamine neurons of the rostral linear nucleus and periaqueductal gray in the rewarding and sensitizing properties of heroin.* Neuropsychopharmacology. 2006 Jul;31(7):1475-88. Epub 2005 Nov 9.

54. Goldstein RZ, Volkow ND. *Drug addiction and its underlying neurobiological basis: neuroimaging evidence for the involvement of the frontal cortex.* Am J Psychiatry. 2002 Oct;159(10):1642-52.

55. Grant BF, Stinson FS, Dawson DA, Chou SP, Dufour MC, Compton W, Pickering RP, Kaplan K. *Prevalence and co-occurrence of substance use disorders and independent mood and anxiety disorders: results from the National Epidemiologic Survey on Alcohol and Related Conditions.* Arch Gen Psychiatry. 2004 Aug;61(8):807-16.

56. Fishbain DA, Cutler R, Rosomoff HL, Rosomoff RS. *Chronic pain-associated depression: antecedent or consequence of chronic pain? A review.* Clin J Pain. 1997 Jun;13(2):116-37.

57. McCracken LM, Spertus IL, Janeck AS, Sinclair D, Wetzel FT. *Behavioral dimensions of adjustment in persons with chronic pain: pain-related anxiety and acceptance.* Pain. 1999 Mar;80(1-2):283-9.